Mode of action of the phenylpyrrole fungicide fenpiclonil in *Fusarium sulphureum*

Promotor: dr. ir. P.J.G.M. de Wit
Hoogleraar in de moleculaire fytopathologie.

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

Ad B.K. Jespers

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Stellingen

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1. Fenpiclonil beïnvloedt de fosforylering van glucose.
   Dit proefschrift.

2. De ontwikkeling van nieuwe fungiciden door middel van 'random' synthese is verre van 'random'.
   Dit proefschrift.

3. Voor de opheldering van het biochemisch werkingsmechanisme van een fungicide dient een gevoelige schimmel gebruikt te worden.
   Edlich and Lyr (1987). Modern selective fungicides. p. 107-118.
   Orth *et al.* (1993). Pestic. Biochem. Physiol. 44: 91-100.

4. Het is onjuist op basis van kruisresistentie tussen verschillende typen fungiciden een uitspraak te doen over hun werkingsmechanisme.
   Leroux *et al.* (1992). Pesticide Science 36: 255-261

5. De eindige voorraad fossiele brandstof is op zich al een voldoende argument om de huidige emissie van kooldioxide te verminderen.

6. Het feit dat een verbinding interfereert met specifieke 'targets' in een schimmel maakt deze verbinding nog geen fungicide.

7. Bestrijdingsmethoden, waarbij natuurlijke stoffen in onnatuurlijke concentraties worden toegepast, zijn niet natuurlijk.

8. Een aanzienlijke reductie van de hoeveelheid verpakkingsmateriaal kan worden bereikt door over te schakelen op kubusvormige verpakkingen.

9. Wetenschappers klagen vaker over gebrek aan geld dan boeren over slecht weer.

10. De drang tot publiceren heeft vooral geleid tot betere jaarcijfers van wetenschappelijke uitgeverijen.

11. Vet geeft smaak.

Stellingen behorend bij het proefschrift:

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Wageningen, 23 maart 1994 Ad Jespers

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Contents

List of abbreviations

2-DG	2-deoxyglucose
2-DG6P	2-deoxyglucose-6-phosphate
2-DGdiP	2-deoxyglucose-1,6-diphosphate
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CPB	cetylpyridinium bromide
DOC	dioleoylphosphatidylcholine
DPH	1,6-diphenyl-1,3,5-hexatriene
GC	gas chromatography
HPLC	high performance liquid chromatography
HRLC	high resolution liquid chromatography
Low Mr	water soluble metabolites of low molecular weight
PAW	phenol/acetic acid/water extract
PDA	potato dextrose agar
PMS	phenazine methosulphate
PPA	propionic acid
RRT	relative retention time
SLS	sodium lauryl sulphate
TCA	trichloroacetic acid
TFP	trifluoperazine
TLC	thin-layer chromatography
TPP^+	tetraphenylphosphonium bromide.
UDPGlc	uridinediphosphoglucose
UDPGlcNAc	uridinediphospho-N-acetylglucosamine.

Chapter 1

Outline of the present thesis

OUTLINE OF THE PRESENT THESIS

The importance of mode of action studies of fungicides.

Crops and natural vegetations are under continuous attack by numerous fungal pathogens. Natural resistance prevents the establishment of most of them. However, a limited number of pathogens still causes considerable yield losses in certain crops. In modern agriculture these yield losses are due to the use of monocultures of genetically similar crop plants, the use of plant cultivars susceptible to pathogens and the use of nitrogenous fertilization at levels which enhance disease susceptibility. Therefore, plant disease control has now become heavily dependent of fungicides (Anonymous, 1991). A variety of other disease control methods is available, but these methods are often not satisfactory and it is therefore anticipated that chemical control will remain an integral component in integrated disease management (Edwards and Stinner, 1990).

Several classes of fungicides with specific modes of action have been developed in the last decades. Benzimidazoles, phenylamides, and the large group of sterol biosynthesis inhibitors are examples of compounds used on a wide scale. Most fungicides were discovered via random synthesis, biological screening, and empirical optimization of lead structures (Cremlyn, 1991). Natural products can also be used as source of leads for the discovery of new classes of fungicides (Chapter 2). Fenpiclonil resulted from such a programme at CIBA-GEIGY AG, Basel, Switzerland. Studies on the mode of action of this fungicide are the subject of the present thesis.

In the initial stages of fungicide discovery, understanding of its mode of action is not regarded important, since optimization of chemical analogues of lead compounds by biological screening has proved to be relatively quick. For reasons of time and money, it is generally not feasible to obtain detailed information on the mode of action of candidate fungicides (Köller, 1992). However, it becomes important in later stages of development. Firstly, to ascertain the novelty of the site of action, since the attractiveness of a new compound is considerably reduced if it has the same mode of action as an existent commercial product. Secondly, to develop an *in vitro* bioassay to get detailed information on the intrinsic activity of the compound. In this way, data are generated for use in quantitative structure activity

relationships (QSAR) and computer modelling studies. Such data are of importance in the rational design of new inhibitors (Huxley-Tencer *et al.*, 1992).

Strategies in mode of action studies.

In general, there are two approaches to establish the mode of action of a new compound. The molecular biological approach involves the selection of resistant strains with a mutation in the gene which codes for the target protein. A genomic library of such a resistant strain in a vector is used to transform a sensitive strain. Transformants resistant to the compound, may contain the gene of interest and give a clue to the mode of action. This procedure is laborious and has many pitfalls, since it is not sure whether resistance is necessarily related to changes in the target protein. Moreover, this procedure is likely to result in a DNA-sequence with an unknown function. This was experienced with the experimental fungicide LY214352, the only case where this approach was applied (Gustafson *et al.*, 1990). Therefore, in this study the

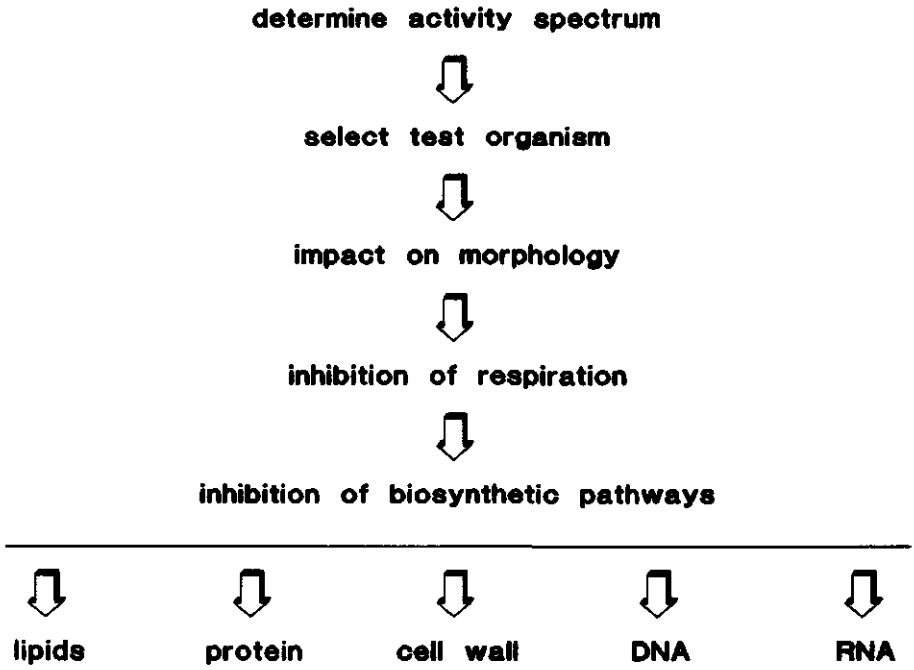

Fig. 1 Steps in studies on mode of actions of fungicides (after Köller, 1992).

conventional biochemical approach was adopted. This approach involves the analysis of possible modes of action in a systematic step-by-step procedure as employed for numerous fungicide classes (Davidse *et al.*, 1983 and Nakanishi and Sisler, 1983). The scheme for the initial steps is depicted in Fig. 1.

The present study

In the present study on the mode of action of fenpiclonil, the plant pathogen *Fusarium sulphureum* (Schlecht) was selected as the test organism as this fungus is sensitive to the fungicide and easy to handle in biochemical studies. In addition, fenpiclonil is recommended as a seed treatment of cereals for control of this pathogen (Nevill *et al.*, 1988). Results of the initial steps of this study are presented in chapter 3. The fate of fenpiclonil after uptake and its effect on membrane function is discussed in chapter 4. It appeared that the fungicide strongly affected incorporation of glucose in fungal macromolecules. Various aspects of its effect on glucose metabolism are presented in chapters 5 and 6. In chapter 7, the results obtained on studies on the mode of action of fenpiclonil sofar are generally discussed.

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Chapter 2

Natural products in plant protection

Ad B.K. Jespers
Maarten A. de Waard

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ABSTRACT

Natural products can be used to control pests and diseases in crops. These products include anorganic compounds, but also a variety of plant extracts. In the beginning of this century active microbial extracts were discovered as well. Synthetic crop protection chemicals were developed from about 1940 onwards and sustained progress in modern agriculture. The first generation chemicals have aspecific modes of toxic action and are in many instances deleterious to the environment. The second generation chemicals have specific modes of action and meet modern environmental requirements. A disadvantage of these chemicals is the potency of target organisms to acquire resistance. This condition urged agrochemical industry to develop chemicals with new modes of action. Such chemicals can be developed by using natural bioactive products as leads in synthesis programmes.

This paper describes the progress that has been made in the development of natural bioactive compounds in pest and disease control.

INTRODUCTION

After World War II, the agrochemical industry provided agriculture with a vast array of crop protection chemicals such as fungicides, insecticides, herbicides, and nematicides. Random synthesis, biological screening and empirical optimization yielded many effective compounds (Cremlyn, 1991). Among the first crop protection chemicals were multi-site inhibitors such as the dithiocarbamate fungicides (Dekker, 1987). Selective action of these compounds between target organism and plants is mainly based on differences in uptake. The more recently developed protection chemicals are more potent in terms of doses required to control the pest or disease, and selectivity between target and non-target organisms. They usually have a specific mode of action. Therefore, such compounds can often be used in integrated plant protection strategies (Oudejans, 1991). Since selective compounds are specific site inhibitors in the metabolism of the target organism, the risk of development of resistance in the target organisms is high. This occurred in a number of pest insects (Georgiou and Lagunes, 1988) and fungal plant pathogens (Delp, 1988). Resistance problems and the lack of promising new lead compounds, presses the plant protection industry to find new solutions to control pests and diseases (Fromtling, 1987). Furthermore, there is growing concern about crop protection chemicals because of their undesirable side effects on humans, other non-

target organisms and their behaviour and fate in the environment (Anonymous, 1991; Anonymous, 1988; Norman, 1988, Metcalf, 1987).

These conditions caused renewed interest in the use of 'natural' compounds in plant protection because they are inherently regarded degradable in the natural environment (Isono, 1990; Keyserlingk *et al.*, 1985). The compounds originate from defence systems of virtually all living organisms, but are primarily of bacterial, fungal and plant origin (Hostettmann and Lea, 1987; Cutler, 1988). Living organisms producing antibiotic compounds, or extracts of the organism can be used in spray applications. Compounds can also be used as leads in chemical synthesis of novel crop protection agents with new modes of action (Marco *et al.*, 1991). The potential value of natural compounds in crop protection will be reviewed in this paper.

HISTORY

Anorganic compounds.

Crop losses due to pests and diseases are already described in The Old Testament (Dekker, 1987). In those days compounds found in the natural environment were already used to control pests and diseases. Homer (1000 B.C.) mentioned the use of elemental sulphur. The Greek physician Pliny the Elder (A.D. 79) and Chinese in the 16th century recommended arsenic as an insecticide. All well known poisons have been used to control pests at one time or another (Cremlyn, 1991). For instance, Hamberg mentioned mercuric chloride as a wood preservative in 1705 and Schulthess described the inhibition of smut spores by copper sulphate in 1761 (McCallan, 1969).

Copper is very toxic to all living organisms and therefore not suitable to control leave-borne diseases. In 1882, Millardet noticed that leaves treated with a mixture of lime and copper sulphate (the Bordeaux mixture) effectively controlled *Plasmopara viticola* in vines (Millardet, 1885). Phytotoxicity of copper in the mixture was reduced because of its presence as insoluble copper oxide. Fungal acidification of the leaf surface results in solubilisation of copper oxide and fungitoxicity. The Bordeaux mixture can be regarded as the first selective fungicide (Martin, 1969). This success stimulated the search for other chemical pesticides

based on copper, mercury, tin or sulphur. Despite their obvious burden on the environment, they are still being used (Cremlyn, 1991).

Organic compounds from random screening.

In 1931, the 'rubber accelerator' tetramethylthiuram (thiram) was found to inhibit growth of fungi and microbes (McCallan, 1969). This led to the discovery of dialkyldithiocarbamates, monoalkyldithiocarbamates, esters of dinitrophenols and phtalimides (Kittleson, 1952). These chemicals are not transported in plants and act as protectants. Attempts were also made to develop systemic compounds which are translocated throughout the plant, thereby eradicating established pathogens and providing protection from further attack. The break-through came in the late 1960's with the development of the first 1,4-oxathiins (*e.g.* carboxin). These fungicides bind to mitochondrial complex II and inhibit succinate dehydrogenase activity. Despite the fact that this enzyme is present in all living organisms, 1,4-oxathiins are selectively toxic to *Basidiomycete* fungi (White and Georgopoulos, 1992). In the following decades, many new classes of selective fungicides were discovered. For example, hydroxy-pyrimidines (*e.g.* ethirimol) selectively inhibit adenosine deaminase in powdery mildews (Hollomon, 1992), benzimidazoles (*e.g.* benomyl) inhibit microtubules assembly by binding to ß-tubulin of a broad range of fungi but not the *Oomycetes* (Davidse, 1986), phenylamides (*e.g.* metalaxyl) inhibit r-RNA synthesis in *Oomycetes* by specific interference with a chromatin bound RNA-polymerase complex (Davidse *et al.*, 1983a) and the DMI's, a group of chemically unrelated heterocyclic compounds such as azoles, imidazoles and pyridines inhibit sterol 14α-demethylation of a wide range of fungi (Köller, 1992a).

The first synthetic organic insecticide is probably hexachlorocyclohexane (HCH), synthesised by Michael Faraday in 1825 (Brooks, 1990). However, its insecticidal properties were only appreciated in 1942, after the discovery of DDT in 1939. These organochlorine insecticides clearly are nerve poisons because they prolongate action potentials, but their primary site of action remains unclear (Metcalf, 1976). Organophosphorous insecticides originated from research on nerve gasses in World War II (Fest and Schmidt, 1973), concomitantly with the development of insecticidal methylcarbamate esters. These compounds inhibit acetylcholine esterase activity in nerval synapses. Specificity can be manipulated by variation of the substituents on the organophosphorous or methylcarbamate moiety. Their

development continued for many decades (Cremlyn, 1991). Another important group of insecticides that originated from random screening were the benzoylphenylureas (*e.g.* diflubenzuron). These compounds prevent moulting in insects by inhibition of chitin biosynthesis (Mauchamp and Perrineau, 1987). An overview of fungicidal and insecticidal compounds is shown in Table 1.

In concert with improved fertilisation, cultural practises and disease resistant cultivars, crop protection products highly contributed to present-day high-yield agriculture (Cremlyn, 1991).

Resistance.

In order to reduce their impact on the environment, modern agrochemicals are developed towards ecological selectivity (*i.e.* discrimination between target and non-target organisms), physiological selectivity (specific enzyme inhibition) and good degradability in the environment (Köller, 1992b). The introduction and extensive use of these compounds in plant pest and disease control led to development of resistance (Delp, 1988; Georgiou and Lagunes, 1988). Though DDT does not have a very specific mode of action, resistance developed in many insect species after only a few years of widespread use. The mechanism of resistance is based on increased detoxification of the compound by oxidation, dechlorination or by knock-down resistance (kdr), possibly based on a change of its target site. Kdr also results in cross-resistance to pyrethroids. Resistance to methylcarbamate and organophosphorous insecticides is often caused by reduced concentrations of the active compound in the nerval synapse as a result of oxidation, decarboxylation or conjugation. It can also be caused by modification of the target enzyme choline esterase (Brattsten, 1990).

Although a few decades later, the speed of resistance development to fungicides was similar to that of insecticides (*e.g.* benzimidazoles [Davidse, 1986], hydroxypyrimidines [Hollomon, 1981] and phenylamides [Davidse *et al.*, 1989]). Resistance to benzimidazoles is usually caused by a single amino acid change in the target protein ß-tubulin (Fujimura *et al.*, 1992). The same is true for carboxin resistance (Broomfield, 1992). As yet, the only case of field-resistance has been found in *Puccinia hordei*. Other fungicide-resistance mechanisms are: decreased inhibitor concentration at the target site as a result of a reduced uptake (polyoxins [Misato *et al.*, 1977]), increased efflux (sterol demethylation inhibitors [DMI; De Waard and

Table 1 Chemical groups of registered insecticides and fungicides (adapted from Kidd and James, 1991).

Class	Chemical group	Number of compounds	Year of first introduction	Example of group
Insecticide	organophosphate	76	1944	parathion
	pyrethroid	31	1949	allethrin
	methylcarbamate	29	1953	carbaryl
	inorganics	11	< 1900	HCN
	organochlorine	9	1939	DDT
	acylurea	7	1972	diflubenzuron
	biological	6	< 1900	nicotine
	isoprenoid	2	1975	isoprene, methoprene
	nitrophenol	2	1932	DNOC
	thiadiazine	2	1952	dazomet
	antibiotic	1	1979	abamectin
	formamidine	1	1971	amitraz
	nitroguanidine	1	1990	imidacloprid
	nereistoxin	1	1983	bensultap
	other	10		
Fungicide	triazole	21	1973	triadimefon
	aromatic	18	1936	pentachlorophenol
	inorganic	15	1882	copper mixtures
	dithiocarbamate	12	1931	thiram
	organophosphate	6	1969	pyrazophos
	antibiotic	5	1968	pimaricin
	benzimidazole	5	1968	benomyl
	phenylamide	4	1977	metalaxyl
	imidazole	4	1973	imazalil
	morpholine	4	1967	dodemorph
	phtalimide	4	1952	captan
	pyrimidine	4	1970	triarimol
	carbamate	3	1978	propamocarb
	dicarboximide	3	1975	vinclozolin
	guanidine	2	1968	guazatine
	hydroxypyrimidine	2	1968	ethirimol
	oxathiin	2	1966	carboxin
	phenylpyrrole	2	1988	fenpiclonil
	piperidine	2	1986	fenpropidin
	triazine	2	1955	anilazine
	acetamide	1	1976	cymoxanil
	benzothiazole	1	1975	tricyclazole
	piperazine	1	1967	triforine
	pyridine	1	1975	buthiobate
	pyrrole	1	1990	fluoromide
	thiadiazole	1	1969	etridiazole
	other	26		

Van Nistelrooy, 1987]) and reduced activation (organophosphorous fungicides [De Waard and Van Nistelrooy, 1980]). Fungicidal action can be compensated (captan [Barak and Edgington, 1984] or circumvented (polyene macrolides [Grindle and Farrow, 1978].

In many cases, resistant organisms have a normal comparative fitness (*c.f.* DDT [Oppenoorth, 1976], benzimidazoles [Davidse, 1986] and hydroxypyrimidines [Hollomon, 1992]). Fortunately, the opposite has also been reported. For instance, fungi readily acquire resistance to DMIs in the laboratory, but notwithstanding their extensive use, loss of field control has only been reported in a limited number of cases (Hollomon *et al.*, 1990). Phenylamide-resistant *Phytophthora infestans* without any apparent reduction in fitness, dominated the population in the Netherlands after only one year of spraying with metalaxyl (Davidse *et al.*, 1983b). However, after withdrawal of the fungicide from the market for 4 years, the majority of the population was metalaxyl-sensitive and the fungicide displayed again good control (Davidse *et al.*, 1989). Similar results were obtained in the control of *Botrytis cinerea* with other fungicides such as the dicarboximides (Beever, 1982; Davis and Dennis, 1981). Apparently, resistant populations are less fit under field conditions as compared with the wild-type population. This phenomenon has important implications for anti-resistance strategies.

Resistance management.

Nowadays, the occurrence of resistance does not trigger immediate panic reactions or alarming publications. Resistance management has become an integrative part of modern agriculture. Since the major part of newly introduced agrochemicals has a high resistance risk, resistance management does start even before the introduction of a compound on the market (Staub, 1991; Highwood, 1990).

The basis for prevention of resistance development is integrated pest and disease control. The use of chemicals is only justified, if control cannot be achieved by other means. The imposed selection pressure of chemicals should be as short and diverse as possible. This implicates the use of mixtures and rotation of compounds of different resistance groups. They often include conventional non-specific compounds (Staub, 1991; Phillips *et al.*, 1989; Dekker, 1987). Many of these 'old'non-specific compounds have inferior environmental and toxicological properties (Anonymous, 1988). Therefore, considerable pressure is put on

withdrawal of these 'old' compounds, but one should realize that these measures at the same time hamper anti-resistance strategies.

Currently, pest and disease control is dependent of compounds belonging to a limited number of resistance classes. In order to make anti-resistance strategies based on variation in use of compounds more successful, a range of compounds from different resistance groups should be available. Therefore, agrochemical industry is urged to develop compounds for which target organisms do not posses cross-resistance. At the same time, biological activity and environmental properties should be at least similar to those of existing compounds. However, many new compounds introduced in the last decade are derivatives of groups discovered before the 1970s, and belong to resistance groups already known. Hence, screening of more than a million compounds in the last decade resulted in the introduction of only a very limited number of compounds with novel modes of action and resistance (Table 1). This explains the renewed interest of the agrochemical industry in the area of natural compounds with a variety of unique characteristics, waiting to be exploited (Pillmoor *et al.*, 1993; Keyserlingk, 1985).

Natural compounds as pesticides

Plant extracts.

Medicinal plants are known for many centuries and extracts were used in folklore and modern medicine (Marston and Hostettmann, 1987). Therefore, it is not surprising that plant extracts were tested for their ability to control pests and diseases as well. In Greek folklore, it was recommended to steep seed grain in wine or a mixture of bruised cypress leaves to control 'mildew' which presumably was smut (Mason, 1928). In medieval times, dregs of olive oil called amurca were used. Tobacco leaf extracts containing nicotine were employed in the seventeenth century to control 'plum curculio' and the lace bug (McCallan, 1969). The first two 'real' insecticides from plant origin, were already introduced around 1850: rotenone from roots of Derris and pyrethrum from flower heads of *Chrysanthemum* spp. At present, they are still being used (Cremlyn, 1991).

Interest in plant extracts as sources for compounds to control pests and diseases is

increasing (Cutler, 1988). Neem trees (*Azadirachta indica*) were used for medicinal and insecticidal purposes in India for a long time. They are commercially grown from 1985 onwards. Their extracts contain several anti-feeding compounds and growth regulators, including azadirachtin. The extract is used to control army worms (*Spodoptera* spp.) (Bell *et al.*, 1990). In a programme to screen extracts from medicinal plants for fungicidal activity, BASF found that aqueous extracts of *Reynoutria sachalinensis* (*Polygonaceae*) showed good protectant control of powdery mildews. The dried extract is marketed under the trade name Milsana® (Herger *et al.*, 1988). The active principle has not been identified. The involvement of a glycolipid with tenside properties acting as an inducer of resistance in the host plant was postulated (Ammermann and Scherer, 1993).

Phytoalexins may play a role in natural resistance of plants (Ebel, 1986). They are only weakly active when applied as conventional fungicides, since localized accumulation near the site of infection appears to be an important part of the resistance mechanism (Rathmell, 1984). In general, the complex structure of most phytoalexins make them inaccessible for chemical synthesis. Recently, it was shown that genetic transformation of tobacco with the gene encoding stilbene synthase from grapevine enhanced resistance to *B. cinerea* by synthesis of increased levels of the phytoalexin resveratrol (Hain *et al.*, 1993). The concentration of resveratrol attained at the site of infection also inhibited fungal growth *in vitro*.

Microbial extracts.

Most bacteria and fungi produce a wide variety of secondary metabolites with antibiotic activity. These antibiotics are used to control other organisms which compete for the same nutrients in their natural environment (Bennet and Bentley, 1989). The first report of an antibiotic was published in 1908. It was found that culture filtrates of *Pseudomonas destructans*, the cause of turnip rot, could kill the causal organism (Potter, 1908). The first identified antibiotic was gliotoxin, isolated from *Gliocladium fimbriatum* (Johnson *et al.*, 1943).

A wealth of literature exists on the discovery and potential use of antibiotics in agriculture (*e.g.* Gutterson, 1990; Berdy, 1980). The discovery of antibiotic activity by penicillin in extracts of *Penicillium notatum* by Fleming in 1929, stimulated the search for

new antibiotics such as streptomycin, tetracyclines, chloramphenicol. Hundreds of new antibiotics are discovered each year (Saksena *et al.*, 1989). The use of these antibiotics had a tremendous impact on successful treatment of human and animal diseases (Lowe and Elander, 1983). They also made internal (systemic) therapy of plants possible (Dekker, 1963). However, their use in agriculture remained limited, because widespread application in crops might simultaneously select for resistance to these antibiotics in human pathogens (Isono, 1990). Streptomycin is an example of an antibiotic used for control of bacterial diseases in both mammals and plants. The insecticide/acaricide abamectin and the fungicides blasticidin S, kasugamycin, pimaricin and validamycin A are used in agriculture (Kidd and James, 1991). Currently, most antibiotics are applied in rice disease control (Isono, 1990).

Reasons for the fact that only a few antibiotics have been developed as commercial agricultural compounds are quite diverse. Efficacy, stability, (eco)toxicity and production costs of the antibiotic are important factors. Production by fermentation can be difficult to scale up, or be expensive due to special nutritional requirements of the producing organism (Lievens *et al.*, 1989). In general, antibiotics are regarded as easy degradable because they are of natural origin. However, this is not necessarily true as practical application rates are significantly higher than the maximal attainable concentration in nature (Gutterson, 1990). Instability can be disadvantageous when it reduces efficacy too much (Simpson *et al.*, 1991). Toxic side effects may be present as well. For instance, blasticidin S causes considerable eye conjunctivitis (Isono, 1990). Therefore, registration procedures of antibiotics do not differ from those of synthetic pesticides (Marco *et al.*, 1991).

Microbial biocontrol agents.

Biocontrol of pests and diseases has advantages over chemical control because of its presumed non-hazardous nature. Cultural practices are traditionally used to stimulate the natural suppressive population of microorganisms in soils (Edwards and Stinner, 1990). Disease suppressiveness can also be increased upon artificial introduction of new biocontrol organisms (Sivan and Chet, 1992). The mechanism of biocontrol can be based on hyperparasitism of the host, competition for limiting amounts of nutrients or killing of the target organism with toxins.

The best known biocontrol organism is *Bacillus thuringiensis* (BT; Entwistle *et al.*,

1993). It was responsible for a bacterial disease in the Japanese silkworm culture around 1900. Bacterial cells contain crystals of proteins toxic to specific *Lepidoptera* species. In the last decade, BT is used for control of various pests, especially in natural vegetations. In terms of formulation and application, it is regarded as a common insecticide. The major disadvantages of BT are its relatively high price, slow speed of kill, rapid inactivation in the field and short shelf life. The narrow pest spectrum of insecticidal activity often requires BT to be mixed with conventional insecticides to control all pests present (Engel *et al.*, 1990). At present, BT represents more than 90% of the worldwide sales in biocontrol agents. Bacterial strains can contain different types of crystal protein selectively toxic to various insect species. Specificity of the toxin is governed by distinct regions in the protein which can be manipulated at the gene level, either by mutation, or chimaerization of toxin genes with divergent selective toxicities (Lisansky and Coombs, 1992). The gene encoding the protein has been expressed in crop plants, with variable results with regard to insect resistance. Transgenic cultivars are in the process of being registered for commercial use (Ely, 1993). Since BT acts like a conventional insecticide, the continuous selection pressure imposed by transgenic plants is undesirable from a resistance management point of view, since they impose a continuous selection for resistant individuals. Resistance to BT already occurs in practice (Marrone and Macintosh, 1993).

The insect hyperparasite *Verticillium lecanii* is commercialized in Europe as an insect biocontrol agent. Registration has been hampered because its conidia can be allergenic to man (Ravensberg *et al.*, 1990). Other fungi have been evaluated as well, but their development is slow due to problems of low pathogenicity, low viability of inoculum, differences in virulence within a pest species, production problems and constraints imposed by temperature and humidity requirements (Gillespie and Claydon, 1989). The potential of *V. lecanii* to control fungal diseases is under evaluation as well (Grabski and Mendgen, 1986). The most important factor for fungicidal activity is probably based on cell wall degrading activity of glucanases and chitinases produced by this hyperparasite.

The level of hydrolytic enzymes such as glucanase and chitinase in plants increases considerably upon infection of a pathogen. Therefore, they are thought to play a role as a defense mechanism of plants (Boller, 1987). Tobacco plants with elevated chitinase activity, were less susceptible to *Rhizoctonia solani* (Broglie *et al.*, 1991). *Escherichia coli* transformed

with a bean chitinase was able to reduce fungal root infection by soil application (Shapiro *et al.*, 1989). However, in many plant pathogen interactions this mechanism does not seem to be important (*c.f.* Wubben *et al.*, 1993).

Bacteria of fluorescent *Pseudomonas* species are often associated with a decline in severity of the take-all disease caused by *Gäumannomyces graminis* in continuous cropping of wheat. The disease suppression is primarily caused by the production of iron-chelating siderophores and an array of antibiotics such as phenazines, phenylpyrroles and 2,4-diacetylphloroglucinol (Défago and Haas, 1990). Additional disease suppression may be obtained after seed or soil bacterization with strains selected for high biocontrol activity (Thomashaw and Weller, 1991). Antifungal activity of the bacterial strains was improved after transformation with additional genes which resulted in the production of additional antibiotics (Défago and Haas, 1990).

Recently, the biofungicide Mycostop® was introduced. It contains lyophilized fermentation cultures of *Streptomyces griseoviridis* and controls a range of seed- and soil-borne fungal pathogens by releasing a range of unidentified antibiotics (White *et al.*, 1990).

Despite the fact that the number of biofungicides is increasing, biocontrol of plant pathogens is more difficult and less advanced than biocontrol of insects (McDonald, 1991).

BIOLOGICAL COMPOUNDS AS LEADS FOR CHEMICAL SYNTHESIS

Chemotherapeutic agents related to endogenous compounds.

In the discovery of new chemotherapeutic agents, it is quite common to start synthesis of compounds based on endogenous compounds. For instance, the discovery of the first ß-blocker propanolol started from adrenaline (Roberts, 1989). Juvenile hormones (JH) regulate metamorphosis in insects. The titre of JH in insects must decline before metamorphosis of the last larval stage to adult can take place. Chemical modification of JH resulted in compounds such as methoprene and prodrone, which mimic JH activity. If applied at the last larval or nymphal stage, they have insecticidal properties by preventing metamorphosis. Fenoxycarb also possesses JH activity (Sparks, 1990). Such a specific mode of action makes these compounds suitable for use in Integrated Pest Management systems. The efficacy of JH

analogues is limited if larval stages of the pest insect impose most crop damage (Edwards and Stinner, 1990). Biogenic amines (serotonin, octopamin, γ-aminobutryc acid [GABA], *etc.*) are important neuromodulators in insects. Formamidin insecticides (*e.g.* chlordimeform and amitraz) are analogues of octopamin and agonize its receptors in the neuromuscular synapses (Evans, 1985).

Various nucleotide analogues such as 8-azaguanine (Bull and Faulkner, 1964) and 6-azauracil (Dekker and Oort, 1964) have been evaluated for their selective action against fungi in agriculture. They showed control of cucumber powdery mildew under laboratory conditions, but were not effective enough under field conditions. Fluoro analogues of endogenous compounds often inhibit methylation reactions (Abeles and Alston, 1990). In sensitive fungi, 5-fluorocytosine is metabolised to 5-fluorodeoxyuridinemonophosphate which inhibits thymidylatekinase, a key enzyme in DNA biosynthesis (Polak, 1988). This compound is successfully used to treat patients with *Candida* infections, but does not have applications in agriculture (Kerridge, 1986).

Pyrethrum.

The extract of dried flower heads of *Chrysanthemum cinerariaefolium* has been used as an insecticide since 1850. It possesses a knock-down effect on flying insects (Elliot, 1989). The active ingredient is a mixture of the related compounds pyrethrin, cinerin, and jasmolin. They consist of esters of cyclopropanecarboxylic acid and cyclopentenolone (Fig. 1). Pyrethrum is often regarded as an ideal pest-control agent because of its powerful insecticidal activity and low toxicity to mammals. However, pyrethrum is too unstable to economically control pests of agricultural crops (Davies, 1985). It is often used as an household insecticide.

Initial efforts aimed to elucidate the structure of the active ingredients resulted in the synthesis of pyrethroids such as allethrin (Schechter *et al.*, 1949). Instability of these pyrethroids is mainly caused by their liability to oxidation and sensitivity to light. The most labile sites are the vinylcyclopropane moiety in the acid and the cyclopentenolone moiety in the alcohol (Elliot, 1989). The stability of the vinyl group could be increased by halogenation and the cyclopentenolone group by substitution with a diphenylether. These structural modifications resulted in permethrin. Introduction of an α-cyano substituent enhanced activity and resulted in compounds such as deltamethrin and cypermethrin (Elliot *et al.*, 1973).

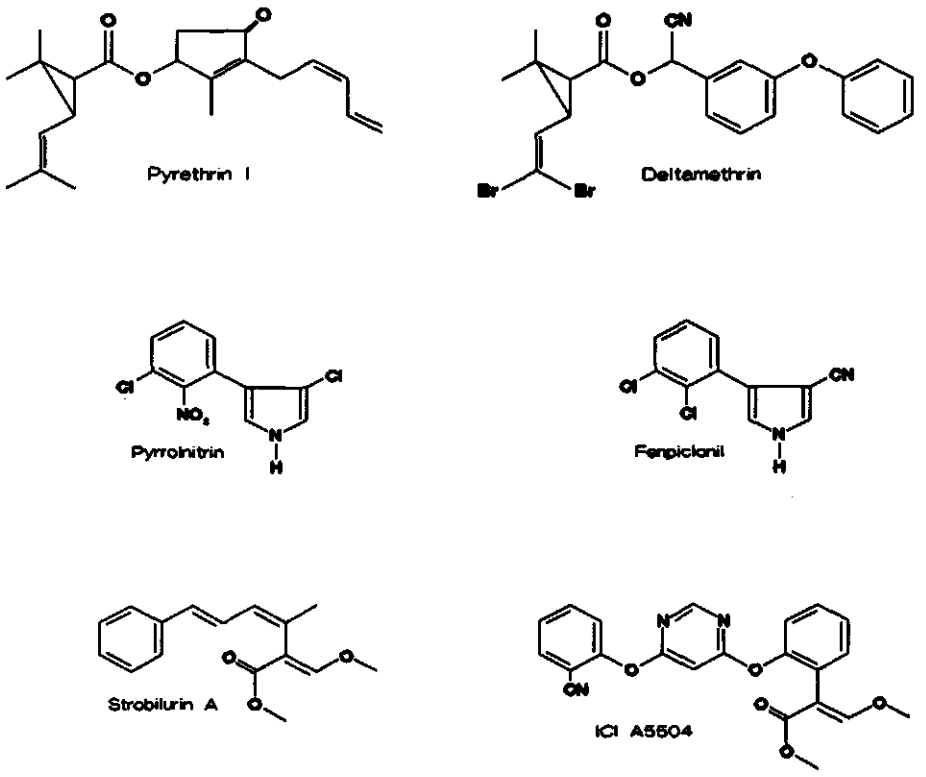

Fig. 1 Structural formulae of the natural products pyrethrin I, pyrrolnitrin, strobilurin A and their synthetic analogues deltamethrin, fenpiclonil and ICI A5504.

Additional modifications of the basic backbone changed the selectivity and patentability of the pyrethroids (Ford *et al.*, 1989). This resulted in the synthesis and widespread use of synthetic pyrethroids in insect control (Davies, 1985).

Pyrrolnitrin.

The phenylpyrrole antibiotic pyrrolnitrin (Fig. 1) is a potent antifungal compound first isolated from *Pseudomonas pyrrocinia* (Arima *et al.*, 1965). It is produced by a number of *Pseudomonas* spp. (Lively *et al.*, 1969). A number of related phenylpyrrole antibiotics are

produced as well (Roitman *et al.*, 1990). The use of some of these bacteria in biocontrol is under investigation (Thomashow and Weller, 1990; Défago and Haas, 1990). Pyrrolnitrin is regarded to play a major role in biocontrol activity by *Pseudomonas* spp. (Thomashow and Weller, 1991; Lam *et al.*, 1987).

The oldest example of an antifungal phenylpyrrole produced by these type of bacteria is pyoluteorin, isolated from *P. aeruginosa* in 1958 (Takeda, 1958). It has bactericidal and fungicidal activity, especially against *Oomycetes* (Howell and Stipanovic, 1980). Therefore, seed bacterization with species producing this antibiotic is evaluated for its potency to control *Pythium* spp. (Whipps and Lumsden, 1991). Two fairly new phenylpyrrole antibiotics, dioxapyrrolomycin (Nakamura *et al.*, 1987) and neopyrrolomycin (Nogami *et al.*, 1990), are produced by *Streptomyces*. They have bactericidal properties, but are also active against a limited number of fungi such as *Magnaporthe grisea*.

Phenylpyrroles are among the few natural antibiotics containing chlorine atoms. Well known other examples are griseofulvin produced by *Penicillium griseofulvum* (Brian *et al.*, 1946), chloramphenicol (Ehrlich *et al.*, 1948), 7-chlorotetracycline (Duggar, 1948), and vancomycin (McCormick *et al.*, 1956) produced by *Streptomyces* species. Chlorination is mediated by chloroperoxidases (Pee and Lingens, 1988).

Pyrrolnitrin is effective in control of several post-harvest diseases (Hammer and Evensen, 1993; Janisiewicz, 1991) and has been used to treat infections by opportunistic fungi in humans (Tawara *et al.*, 1989). It cannot be used in agriculture since the pyrrole ring is unstable in light which results in biologically inactive products (Roitman *et al.*, 1990). Furthermore, it appeared to be very difficult to artificially synthesize the pyrrole ring (Nyfeler and Ackermann, 1991). Both these problems could be solved by substitution of the chlorine with a more potent electron withdrawing group such as cyanide (Leusen *et al.*, 1972). It was established that 3-cyanopyrroles were at least as active fungicides as pyrrolnitrin and much more stable in light (Nyfeler *et al.*, 1990). Further optimization led to the introduction of the highly active fungicides fenpiclonil (Nevill *et al.*, 1988) and fludioxonil (Gehman *et al.*, 1990)

Strobilurin.

The development of ß-methoxyacrylate related fungicides is another elegant example of the optimization of a natural compound (Beautement *et al.*, 1991). These analogues of strobilurins

and oudemansins are produced by several genera of small agarics such as *Strobilurus* and *Oudemansiella* (Anke *et al.*, 1977). These antibiotics have the ß-methoxyacrylate moiety in common and proved to be unique inhibitors of mitochondrial respiration by binding to cytochrome B (Mansfield and Wiggins, 1990). Practical application of the antibiotics is not possible, because they can not be produced on a large scale, are relatively volatile and very instable (Godwin *et al.*, 1992). A chemical optimization programme at ICI and BASF started from the simplest molecule strobilurin A (Fig. 1).

After establishment of fungicidal activity of strobilurin A, the structure and geometric configuration had to be determined. The triene (E,Z,E)-geometry was not initially recognized which explained the initial failures to synthesize the antibiotic. Based on comparison of structures of natural analogues, the ß-methoxyacrylate moiety was thought to be the toxophore (Becker *et al.*, 1981).

Light sensitivity of the natural compound is primarily caused by the conjugated diene structure in the lipophilic α-substituent of the ß-methoxyacrylate toxophore (Anke & Steglich, 1989). It was stabilized without loosing fungicidal activity upon introduction of the double bond in the stilbene moiety. Further optimization of the α-substituent was also possible and resulted in the systemic fungicide ICIA5504 (Godwin *et al.*, 1992). A similar programme at BASF resulted in the methoximino fungicide BAS 490 F (Ammermann *et al.*, 1992).

OUTLOOK

Interest in plant protection agents from biological origin is increasing because in public perception they are regarded as environmentally safe and less toxic than synthetic chemicals (Ames *et al.*, 1987). However, the first rule in toxicology states: not the compound, but its dose makes it toxic. Ames and coworkers (1990a,b) gave a number of arguments for the statement that essential differences between synthetic and natural pesticides are absent. For instance, the feared bioaccumulation of DDT is not unique to synthetic pesticides. Natural compounds from potato such as solanine and chaconine inhibit cholinesterase and are persistent and teratogenic. They accumulate in a similar way in fat as DTT. For these reasons, they would never be registered as a pesticide. In consequence, a pest resistant potato cultivar

with high levels of these toxins had to be withdrawn from the market. A particular insect-resistant celery cultivar caused skin irritation upon exposure to daylight. This cultivar contains an 8-fold higher concentration of carcinogenic psoralens. Toxicity data for natural bioactive compounds are often lacking, but penicillin is well known for its allergenic nature. Fungal spores, as experienced with the registration of *V. lecanii*, may cause allergic reactions as well. Eye conjunctivitis caused by blasticidin S has already been mentioned. These examples are not intented to obscure the toxicity of synthetic pesticides, but illustrate that the registration procedure of so-called natural agents should be similar to their synthetic counterparts.

Natural products can contribute to pest and disease control because of their enormous diversity. Therefore, most companies involved in the development of agrochemicals are active in this field. Many new antibiotics, some of them displaying new modes of action, are being discovered each year. Though many of these antibiotics have a complex structure and are probably difficult to synthesize, they may guide chemists to compounds with novel selective target sites.

Biocontrol agents themselves are still primarily used in niche markets and their performance is in general still inferior to chemical control. In future, these problems may gradually be overcome by improvement of strains and application methods.

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Chapter 3

Biochemical effects of the phenylpyrrole fungicide fenpiclonil in *Fusarium sulphureum* (Schlecht)

Ad B.K. Jespers
Leen C. Davidse
Maarten A. de Waard

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ABSTRACT

Fenpiclonil (CGA 142705) is the first commercially developed phenylpyrrole fungicide, based on chemical modifications of the antibiotic pyrrolnitrin. The fungicide is toxic to representatives of Ascomycetes, Basidiomycetes and Deuteromycetes. In this report, we describe the effect of fenpiclonil on several physiological processes using the target fungus *Fusarium sulphureum* as a sensitive test organism. EC_{50} of fenpiclonil for radial growth on PDA and mycelial growth in liquid medium was 0.5 and 10 μM, respectively. At low inhibitory concentration, fenpiclonil did not immediately affect oxygen consumption, nuclear division and DNA-, RNA-, protein-, chitin-, ergosterol- and (phospho)lipid biosynthesis. However, the accumulation of amino acids and sugars was instantaneously affected at concentrations ranging from 4.2 -42 μM. The reduction in accumulation was accompanied by an increased accumulation of the membrane potential probe tetraphenylphosphonium bromide and a marginal change of the proton gradient probe propionic acid. These results suggest that the biochemical mechanism of action of fenpiclonil may be related to membrane dependent transport processes. To our knowledge, such a mechanism has not been described before for any fungicide.

INTRODUCTION

Fenpiclonil (CGA 142705), 4-(2,3-dichlorophenyl)pyrrole-3-carbonitrile (Fig. 1) is the first phenylpyrrole fungicide commercially developed for seed treatment in cereals by CIBA-GEIGY A.G., Basel. It is commercially available under the trade names 'Beret®' and 'Galbas®' (1). Seed treatment for control of pathogens in other crops is also promising. Fungi among the Ascomycetes, Basidiomycetes and Deuteromycetes are sensitive to the fungicide. The more potent analogue CGA 173506, 4-(2,3-difluoro-1,3-benzodioxol-4-yl)pyrrole-3-carbonitrile has been developed as a foliar fungicide as well and will be marketed under the trade name 'Saphire®' (2). Benzimidazole- and dicarboximide resistant isolates of various fungal species are sensitive to phenylpyrroles (3).

Fenpiclonil is an analogue of the antifungal antibiotic pyrrolnitrin, which is produced by several *Pseudomonas* species (4,5). Fungicidal activity of these bacteria is caused by the antibiotics produced, pyrrolnitrin being a predominant and very effective one (6,7,8). The use of pyrrolnitrin-producing bacteria in biological control programs is currently being investigated (9,10). Direct application of pyrrolnitrin to crops is hampered by a low chemical stability of the compound (11). Chemical modifications of pyrrolnitrin resulted in the more

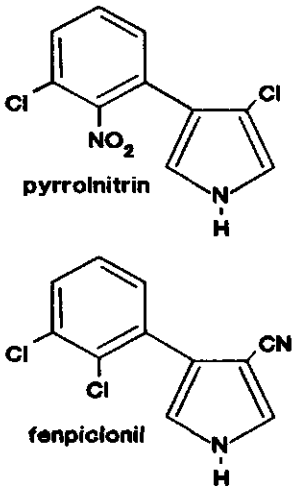

Fig. 1 Structural formulae of pyrrolnitrin and fenpiclonil (CGA 142705).

stable and highly active compounds fenpiclonil and CGA 173506.

In the present study the biochemical effects of fenpiclonil have been investigated in the fungus *Fusarium sulphureum* (Schlecht) (syn. *Fusarium sambucinum* [Fuckel]; teleomorph *Gibberella cyanogena* [Desm] Sacc.), one of the causal organisms of damping-off in cereals, of which control by fenpiclonil is recommended (1). This fungus was selected because it is easy to handle in physiological studies and highly sensitive to fenpiclonil.

The goal of this study was to investigate the biochemical mechanism of action of fenpiclonil by testing various physiological processes for their sensitivity to the compound. In this way, several potential target sites were identified.

MATERIALS AND METHODS

Organism and culture methods.

The isolates used, were obtained from the fungal collections of the Research Institute for Plant Protection (IPO) in Wageningen, CIBA-GEIGY in Basel and our laboratory. Isolates were weekly subcultured on potato dextrose agar (PDA) at 20°C. Czapek Dox Liquid cultures were inoculated with macro conidia, which were obtained from two-week-old cultures on PDA, to a final concentration of 2×10^5 conidia ml^{-1}. Cultures were incubated in a rotary shaker at 120 rpm and 20°C for 2 days. Mycelium was harvested and washed with demineralised water by vacuum filtration over a filter paper in a Büchner funnel. Subsequently, washed mycelium was resuspended in an appropriate incubation medium at an average rate of 3.3 mg wet weight ml^{-1} (0.9 mg dry weight ml^{-1}) and was allowed to settle for at least 1 hour, unless

stated otherwise.

Chemicals.

Czapek Dox liquid medium was purchased from Oxoid Ltd. (London, UK). PDA and TLC plates (Silicagel 60 F_{254}) were obtained from Merck (Darmstadt, FRG). Radiochemicals [2-^{14}C]acetic acid (sp. act. 2.0 GBq mmol^{-1}), N-acetyl-[1-^{14}C]glucosamine (sp. act. 2.0 GBq mmol^{-1}), L-[U-^{14}C]aspartic acid (sp. act. 8.1 GBq mmol^{-1}), 2-deoxy-D-[1-^{14}C]glucose (sp. act. 2.0 GBq mmol^{-1}), [1-^{14}C]glucose (sp. act. 2.1 GBq mmol^{-1}), L-[U-^{14}C]glycine (sp. act. 2.0 GBq mmol^{-1}), L-[U-^{14}C]phenylalanine (sp. act. 19 GBq mmol^{-1}), [*methyl*-^{3}H]thymine (sp. act. 1.74 GBq mmol^{-1}), and [5,6-^{3}H]uracil (sp. act. 1.78 GBq mmol^{-1}) were obtained from Amersham International Ltd. (Amersham, UK), [1-^{14}C]propionic acid (sp. act. 2.1 GBq mmol^{-1}), and [*phenyl*-^{14}C]tetraphenylphosphonium bromide (sp. act. 0.71 GBq mmol^{-1}) from Dupont Research Products (Den Bosch, The Netherlands). Hydroluma and LumaSolve were purchased from Lumac BV (Schaesberg, The Netherlands). Unlabelled tetraphenylphosphonium bromide was bought from ICN Pharmaceuticals (Plainville N.Y., US). Technically pure fenpiclonil (98.49%, CGA 142705) was a gift of CIBA-GEIGY AG (Basel, Switzerland). All other chemicals were of technical grade.

Toxicity studies.

Fungicides were added to the medium from 400-fold concentrated stock solutions in methanol. Control treatments obtained equivalent quantities of methanol. Radial growth analysis was carried out in triplicate on PDA at 20°C. Mycelial growth experiments were carried out in triplicate in Czapek Dox liquid medium. After various incubation times, samples (10 ml) were collected on preweighed Whatmann GF/A filters using a Millipore sampling manifold, washed twice with water (10 ml), dried overnight at 65°C and weighed. Toxicity of fungicides was represented as residual mycelial dry weight increase.

Oxygen consumption and ATP content.

Oxygen consumption of mycelial suspensions in fresh Czapek Dox liquid medium at a density of 1 mg wet weight ml^{-1} (0.3 mg dry weight ml^{-1}), was measured using a Yellow Springs Instrument recording oxygen cathode (Model 53) at 22°C. Mycelial suspensions (10 ml) were

allowed to equilibrate in the cuvettes for 30 min. Fungicides were added and in course of time the cathode was placed in the cuvet and the oxygen consumption was monitored (12). Oxygen consumption was expressed as μmol O_2 min^{-1} mg^{-1} mycelium. ATP content of mycelium was determined in perchloric acid (1 N) extracts according to Carver (13).

Lipid and sterol synthesis.

Mycelial suspensions in Czapek Dox liquid medium without saccharose at a density of 3 mg wet weight ml^{-1} (0.8 mg dry weight ml^{-1}) were preincubated in the presence or absence of fenpiclonil (4.2 μM) for 15 min and incubated with D-glucose (100 μM) and [2-^{14}C]acetate (74 kBq) in a rotary shaker at 120 rpm and 22°C for another 3 hours. Mycelium was collected on Whatmann GF/A filters and washed twice with water (10 ml). Total lipids were extracted directly from collected mycelium with chloroform/methanol (2/1) according to Weete *et al.* (14). One part of the extracted lipids was used to separate phospholipids, sterols, acyl-sterols, free fatty acids and glycerolipids by thin layer chromatography (TLC) with hexane/diethyl ether/acetic acid (79/20/1) as solvent system (15). Silica gel with phospholipids at the origin of the TLC plates was scraped off and extracted with chloroform/methanol/acetic acid (5 ml; 60/30/1) and separated by two-dimensional TLC as described by Weete *et al.* (14). Another part of the extracted total lipids was saponified by alkaline hydrolysis (14). Sterols were extracted with diethylether and separated in the same system as described for total lipids. Fatty acids were extracted from the acidified water phase, methylated with methanolic HCl (16) and separated by argentation TLC with benzene/ethylacetate (9/1) as solvent system (17). Radioactive spots on TLC-plates were located by autoradiography using Kodak X-AR films. Silicagel containing radioactive compounds was scraped from the plates and counted for radioactivity in a mixture of water (1 ml) and Hydroluma (10 ml) using a Beckmann LS 2800 scintillation counter.

Ergosterol content.

Standard mycelial suspensions (10 ml) were incubated in Czapek Dox liquid medium in the presence of various fenpiclonil concentrations for 24 hours. Sterols were extracted from the mycelium as previously described. They were separated by Si-HPLC using a Ultraspheres-Si column (4.6 mm i.d. × 25 cm; particles of 5 μm) at 25 °C using heptane/isopropanol (98/2) as solvent at a flow rate of 1 ml min^{-1} and UV-detection at 210 and 280 nm. The sterols were

identified by their relative retention time (RRT) with cholesterol as reference. The RRTs of the fraction corresponding to the C4-desmethyl, C4-methyl and C4,4-dimethyl sterols were 1.07, 0.77 and 0.68, respectively. The three fractions were collected and analyzed by GC using a HP-5890 A, HP-52 column (30 m × 0.53 mm × 0.88 μm film thickness; 5% phenylsilicone), a split-splitless mode (1/5) of injection, a flame ionisation detector and helium as carrier gas. The column temperature was initially 35 °C and subsequently increased to 230 °C and 280 °C at a rate of 30 °C min^{-1} and 2 °C min^{-1}, respectively. The temperature was decreased after 20 min to 35 °C at a rate of 70 °C min^{-1}. GC standards were cholesterol, ergosterol, obtusifoliol, lanosterol, and eburicol with RRT of 1.00, 1.08, 1.18, 1.21, and 1.28, respectively.

DNA, RNA, protein and chitin synthesis.

Accumulation and incorporation of DNA, RNA and protein precursors was conducted according to Davidse *et al.* (18). DNA and RNA precursors used, were the pyrimidine bases [*methyl*-^{3}H]thymine and [5,6-^{3}H]uracil, respectively, since accumulation of corresponding nucleosides was too low (19). L-[U-^{14}C]phenylalanine was used as protein precursor. Fenpiclonil was added from 200-fold concentrated stock solutions in methanol, 15 min before a 15-min incubation period with precursors. Control cultures obtained equivalent amounts of methanol. Chitin synthesis was estimated from the incorporation of radiolabeled N-acetyl-glucosamine in 10% TCA and ethanol/ether insoluble material (20). Accumulation never reached substrate limiting conditions.

Accumulation studies.

Accumulation of amino acids was assessed with standard mycelial suspensions (10 ml) in Czapek Dox liquid medium and accumulation of sugars in 5 mM Mes/Tris buffer pH 5.5 in the presence of appropriate amounts of D-glucose. Final substrate concentrations were for L-[U-^{14}C]glycine, L-[U-^{14}C]aspartic acid and L-[U-^{14}C]phenylalanine 0.5 mM and for 2-deoxy-D-[1-^{14}C]glucose 0.1 mM. In all cases, substrates were added from 200-fold stock solutions in water, fenpiclonil from 200-fold stocks in methanol. Accumulation was stopped by filtrating the mycelium over Whatmann GF/A glass fibre filters using a Millipore sampling manifold. The mycelium was washed twice within 15 s with ice-cold water (5 ml) and submersed in Hydroluma (4 ml). The radioactivity of the samples was determined by

scintillation counting after incubation at room temperature for 15 h. Accumulation was calculated in nmol amino acid or sugar mg^{-1} mycelial dry weight in time (Fig. 3). In comparative experiments between fungal isolates, phenylalanine was added at zero time. Accumulation was assessed at t = 15 min (time addition of fenpiclonil), and at t = 30 min. Increase in accumulation of phenylalanine between t = 15 and 30 min was plotted against concentration of fenpiclonil (Fig. 4).

Distribution of tetraphenylphosphonium (TPP^+) and propionic acid (PPA) over mycelium and extracellular medium was assessed according to Budd (21). Standard mycelial suspensions (150 ml) in Mes/Tris buffer (5 mM; pH 5.5) containing D-glucose (50 mM) were incubated with the probes (5 μM), added from 100-fold concentrated stock solutions in water. After equilibration (15 min), fenpiclonil was added. At intervals, samples (10 ml) were withdrawn and mycelium was collected on Whatman GF/A filters. TPP^+ treated mycelium was washed twice with 1 mM $MgSO_4$ (5 ml) within 10 sec; PPA treated mycelium was not washed (Kashket, 1985). The accumulated amount of probe was radioassayed as described above. Routinely, accumulation of both probes was assessed 15 min after addition of fenpiclonil in non-equilibrium conditions. Accumulation levels of the probes were corrected for aspecific binding in mycelium, measured after incubation in the same buffer with toluene (1%) at 37 °C for 1 h (22,23).

Miscellaneous.

Effects of fenpiclonil on nuclear division were studied in germinating macro conidia fixed in ethanol/acetic acid (3/1). Nuclei were visualized with Giemsa stain according to Ziogas *et al.* (24). Membrane permeability was assessed by measuring fenpiclonil-induced leakage of electrolytes with a Radiometer conductivity meter (25). Lipid peroxidation was assessed with the thiobarbituric acid reaction according to Keppler and Novacky (26).

Table 1 Toxicity of fenpiclonil, vinclozolin and sodium chloride to isolates of various fungi in radial growth tests with PDA.

Fungal species	Number of isolates	EC_{50} Fenpiclonil (μM)		Vinclozolin(μM)		Sodium chloride (M)	
Fusarium sulphureum							
Field isolates	6	0.75[a]	(0.41 - 1.23)[b]	56	(45 - 78)	0.89	(0.78 - 0.99)
Sector isolates	7	41	(21.4 - 49.2)	> 175	(> 175)	0.18	(< 0.08 - 0.21)
Fusarium solani f.sp. *coeruleum*							
Isolate 1649	1	1.7		43		0.92	
Isolate 1643	1	> 40		16		0.86	
Sector isolates	3	> 40	(> 40)	> 175		< 0.18	
Botrytis cinerea							
Dicarboximide S[c]	8	0.28	(0.13 - 0.60)	0.77	(0.46 - 1.6)	0.45	(0.17 - 0.64)
Dicarboximide R[c]	5	0.40	(0.21 - 0.68)	22	(10 - 32)	0.34	(0.17 - 0.56)
Sector isolates	2	20	(19 - 21)	> 175	(> 175)	< 0.08	(< 0.08)
Rhizoctonia solani							
Field isolates	3	0.28	(0.16 - 0.41)	n.d.		n.d.	
Alternaria porri							
Field isolate	1	0.82		n.d.		n.d.	
Fusarium oxysporum f.sps.	3	> 40		n.d.		n.d.	

[a] Mean EC_{50}.

[b] Between brackets: Toxicity range, most extreme values shown.

[c] S and R: dicarboximide sensitive and field resistant, respectively.

RESULTS

Effects on growth and development.

Several fungal species were tested for sensitivity to fenpiclonil (Table 1). *Fusarium oxysporum* fsps. were the least sensitive and *Botrytis cinerea* and *Rhizoctonia solani* the most. EC_{50} values of all fungi tested ranged from 0.13 to values above 400 µM. A number of test fungi readily showed fast growing sectors upon growth on plates amended with fenpiclonil. Isolates from these sectors were cross-resistant to the dicarboximide fungicide vinclozolin and showed osmotic sensitivity. Similarly, sector isolates of *B. cinerea* with vinclozolin-resistance, showed cross-resistance to fenpiclonil. However, dicarboximide resistant field isolates of *B. cinerea* were not fenpiclonil resistant. Furthermore, with respect to fenpiclonil and vinclozolin, sensitivities of *F. solani* f.sp. *coeruleum* isolates 1643 and 1649 were negatively correlated. *Fusarium sulphureum* (isolate 1743) was chosen as the test organism, because it is amongst other *Fusaria* being controlled in cereals with fenpiclonil, it is sensitive to the fungicide (EC_{50} 0.5 µM, Fig. 2) and easy to handle in biochemical studies. The less sensitive isolate 1743.1 (EC_{50} 40 µM) was isolated from a sector in isolate 1743 growing on PDA amended with fenpiclonil (4 µM).

Germination of macro conidia in Czapek Dox was highly inhibited by fenpiclonil. Minimal inhibitory concentrations were similar as for radial growth. Germ tube growth rate in the presence of fenpiclonil (0.42 µM) was reduced about 50%; these germ tubes did not show major deformations or loss of cytoplasm. Control germ tubes (6 h incubation) and germ tubes in the presence of fenpiclonil (0.42 µM, after

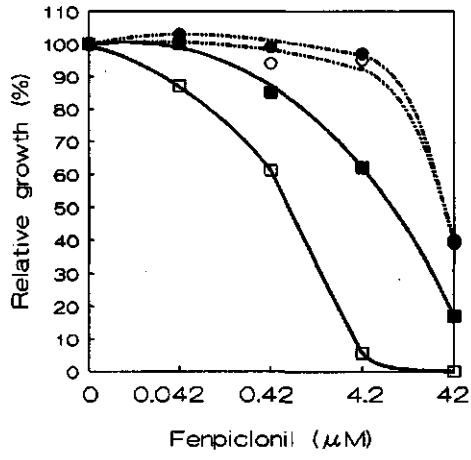

Fig. 2 Effect of fenpiclonil on radial growth on PDA (□,○) and mycelium dry weight increase in Czapek Dox liquid medium (■,•) of *Fusarium sulphureum* isolates 1743 (□,■) and 1743.1 (○,•), expressed as percentage of control.

12 h incubation) contained 2-3 nuclei, which were not deformed in the fenpiclonil-treated germlings. Only high rates of fenpiclonil (42 μM) induced a slight leakage of electrolytes from standard mycelial suspensions in water of both *F. sulphureum* isolates 1743 and 1743.1. However, compared with dodine (50 μM), which fungicidal action is based on increased leakage of electrolytes from mycelium (25), the leakage caused by fenpiclonil was at least a 1000-fold lower.

EC_{50} values of fenpiclonil to mycelial growth of *F. sulphureum* isolates 1743 and 1743.1 in liquid cultures were 10 and 40 μM, respectively (Fig. 2). EC_{50}'s increased when higher initial inoculum densities of the fungus were employed.

Effect on oxygen consumption and ATP content

Measurement of oxygen consumption started at about 80% oxygen saturation of the suspension and remained linear in time to 10% saturation. Fenpiclonil did not instantaneously affect oxygen consumption of mycelium. Oxygen consumption was considerably reduced when mycelium was exposed to the fungicide for 30 min or longer (Table 2). ATP content of the mycelium varied between experiments, but was not reduced by fenpiclonil. Typical values obtained for both control and fenpiclonil-treated mycelium were 5.5 μmol ATP g^{-1} dry

Table 2 Effect of pre-incubation time with fenpiclonil on oxygen consumption of *Fusarium sulphureum* isolate 1743 in Czapek Dox.

Fenpiclonil (μM)	Pre-incubation time (min)							
	0		30		180		540	
	units[a]	%[b]	units	%	units	%	units	%
0	8.5	100	9.9	100	11.6	100	10.8	100
0.42	8.4	99	8.7	88	8.3	72	7.7	71
4.2	8.1	95	8.8	89	9.2	79	8.2	77
42	7.5	88	5.3	53	6.2	53	5.2	49

[a] Unit: μmol O_2 min^{-1} mg^{-1} dry weight of mycelium

[b] Percentage of control treatment with methanol

Table 3 Effect of fenpiclonil on the incorporation of [2-^{14}C]acetate into lipids of *Fusarium sulphureum* isolates 1743 and 1743.1[a]

Lipid	Isolate 1743				Isolate 1743.1				Standard error
	Control		Fenpiclonil[b]		Control		Fenpiclonil[b]		
	Total lipids	Specific lipids	Total lipids	Specific lipids	Total lipids	Specific lipids	Total lipids	Specific lipids	
Total lipids	31.5[c]		18.5		26.5		26.6		2.1
Phospholipids	11.3[d]		10.3		26.9		28.0		0.9
phosphatidyl-inositol		0.7[e]		0.4		1.7		1.3	0.1
phosphatidyl-choline		40.7		32.8		44.4		44.2	5.6
phosphatidyl-glycerol		1.4		0.8		1.2		1.2	0.2
phosphatidyl-ethanolamine		34.9		42.7		36.3		38.8	5.6
phosphatidyl-serine		19.6		20.2		15.4		13.4	5.2
Glycerolipids	79.8		78.7		62.6		61.2		3.3
Free fatty acids	0.4		0.9		0.6		0.5		0.1
Free sterols	6.9		7.0		8.2		8.4		0.1
Acyl sterols	0.3		0.5		0.3		0.2		0.2
Sterols									
C4-desmethyl sterols		92.8		79.1		93.8		93.4	6.3
C4-methyl sterols		2.3		4.0		1.3		1.5	0.3
C4,4-dimethyl sterols		4.9		16.8		4.9		5.1	1.1
Fatty acids									
no double bond		29.3		29.7		31.2		31.0	2.7
1 double bond		35.3		34.9		32.4		31.6	3.0
2 double bonds		26.2		28.4		27.6		29.5	2.5
3 double bonds		9.1		7.1		8.8		7.9	0.7

[a] Data are mean results of two experiments carried out in duplicate. Incorporation time 3 h.
[b] Fenpiclonil (4.2 μM) added 15 min prior to addition of [2-^{14}C]acetate
[c] Percentage incorporation in total lipids of [2-^{14}C]acetate added (0.27 GBq)
[d] Percentage of total lipids
[e] Percentage of specific lipids

Table 4 Sterol composition of *Fusarium sulphureum* isolates 1743 and 1743.1 after incubation with fenpiclonil for 24 hours.

	Isolate 1743 Fenpiclonil (μM)				Isolate 1743.1 Fenpiclonil (μM)			
Sterols	0	0.42	4.2	42	0	0.42	4.2	42
ergosterol	97[a]	97	88	88	93	94	99	96
lanosterol	< 1	< 1	2	3	< 1	< 1	0	0
eburicol	1	1	4	3	1	2	< 1	< 1
unidentified	2	2	5	7	5	4	< 1	< 1

[a] Percentage of total sterols

mycelium and 28% ATP relative to total adenylate nucleotides. Similar values were reported for *Trichoderma reesei* (27).

Effects on lipid biosynthesis and composition

Effects of fenpiclonil (4.2 μM) on lipid biosynthesis in mycelial suspension were studied by measuring the effect on incorporation of [2-^{14}C]acetate for 3 hours. Under these conditions, fenpiclonil reduced mycelial growth of *F. sulphureum* isolates 1743 and 1743.1, for 48 and 11%, respectively. Fenpiclonil did not notably affect the incorporation of acetate into phospholipids and glycerolipids. Phospholipid composition and saturation of fatty acid residues were also not significantly changed (Table 3). However, the relative proportion of C4,4-dimethyl sterols to total saponified sterols and the proportion of free fatty acids relative to total lipids increased significantly in isolate 1743, but not in isolate 1743.1. No major changes in sterol composition were recorded after incubation with fenpiclonil for 24 h (Table 4).

Effects on DNA, RNA, protein and chitin synthesis

Accumulation of thymine into mycelium was very low, and increased at high fenpiclonil concentrations (42 μM). Incorporation in DNA was not greatly affected (Table 5). Most of

Table 5 Effect of fenpiclonil on accumulation and incorporation of precursors of DNA, RNA, and protein synthesis by mycelium of *Fusarium sulphureum* isolate 1743 in Czapek Dox liquid medium.

Fenpiclonil[a]	[*methyl*-^{3}H]Thymine[b]		[5,6-^{3}H]Uracil[b]		[U-^{14}C]Phenylalanine[b]		N-acetyl[1-^{14}C]glucosamine[b]	
(μM)	Accumulation[c]	Incorporation[c]	Accumulation	Incorporation	Accumulation	Incorporation	Accumulation	Incorporation
0	0.93 (100)	0.014 (100)	86 (100)	10.3 (100)	87 (100)	80 (100)	41 (100)	29 (100)
0.42	0.91 (98)	0.014 (99)	81 (94)	9.3 (90)	88 (101)	80 (100)	31 (75)	26 (89)
4.2	0.74 (79)	0.014 (102)	65 (75)	6.0 (58)	87 (100)	78 (98)	27 (65)	23 (80)
42	1.80 (193)	0.012 (84)	52 (60)	3.5 (34)	74 (85)	70 (87)	13 (32)	7 (27)

[a] Fenpiclonil added 45 min prior to a 15-min incubation period with the precursors.

[b] Radioactivity added and final substrate concentration per flask with mycelial suspension (10 ml): [*methyl*-^{3}H]thymine, 1.1 x 10^4 Bq, 0.6 nM; [5,6-^{3}H]uracil, 1.1 x 10^4 Bq, 0.6 nM; [U-^{14}C]phenylalanine, 9.5 x 10^2 Bq, 5.0 nM; N-acetyl[1-^{14}C]glucosamine, 1.0 x 10^3 Bq, 51 nM.

[c] Percentage of radioactivity added. In brackets: percentage of control.

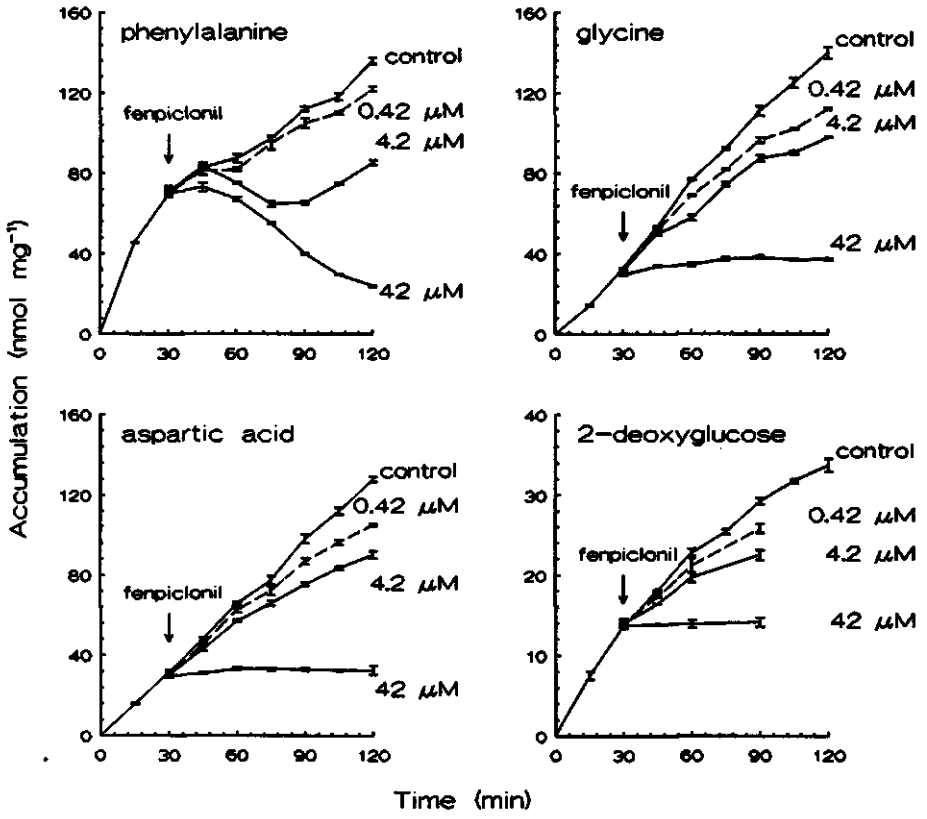

Fig. 3 Effect of fenpiclonil on accumulation of phenylalanine (0.5 mM), glycine (0.5 mM), aspartic acid (0.5 mM), and 2-deoxy-glucose (0.1 mM) in *Fusarium sulphureum* isolate 1743. Fenpiclonil added at the time and rate indicated.

the uracil added accumulated in the mycelium; fenpiclonil significantly reduced accumulation and concurrent incorporation in RNA (Table 5).

Accumulation and incorporation of phenylalanine was not greatly affected (Table 5). Accumulation and incorporation of N-acetylglucosamine into chitin were reduced to a similar extend upon fenpiclonil-treatment (Table 5).

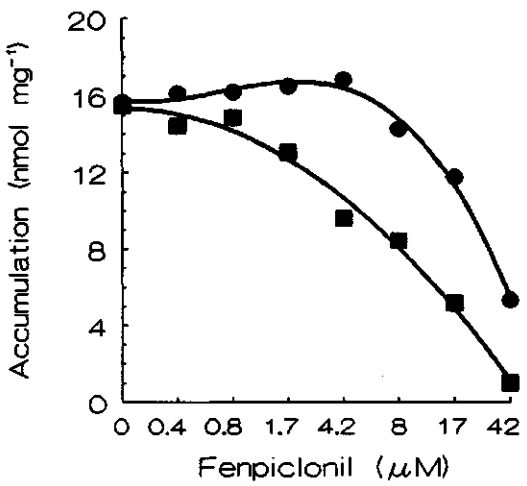

Fig. 4 Effect of fenpiclonil on accumulation of phenylalanine (0.25 mM) in *Fusarium sulphureum* isolates 1743 (■) and 1743.1 (•).

Effects on amino acid and sugar accumulation

Fenpiclonil significantly reduced the accumulation of neutral (glycine and phenylalanine), basic (lysine) and acidic amino acids (aspartic acid) and of 2-deoxy-D-glucose by mycelium of *F. sulphureum* isolate 1743 (Fig. 3). The effect was almost instantaneous. At relatively high concentrations, the fungicide fully inhibited accumulation in time and even caused a reduction in accumulation of the lipophilic amino acid phenylalanine. The less sensitive isolate 1743.1 showed a similar, but less significant reaction (Fig. 4).

Effects on accumulation of TPP^+ and PPA

TPP^+ accumulated readily in mycelium of *F. sulphureum*. Equilibrium conditions were established within 15 min. The accumulated amount of TPP^+ increased within one minute after addition of fenpiclonil (Fig. 5). A steady-state in accumulation of the probe was only reached after 1-2 hours. PPA accumulated also readily in mycelium, but attained a continuously slow accumulation in time, which lasted for several hours (Fig. 5). PPA was not metabolised, because all accumulated PPA could be extracted from mycelium with 50%

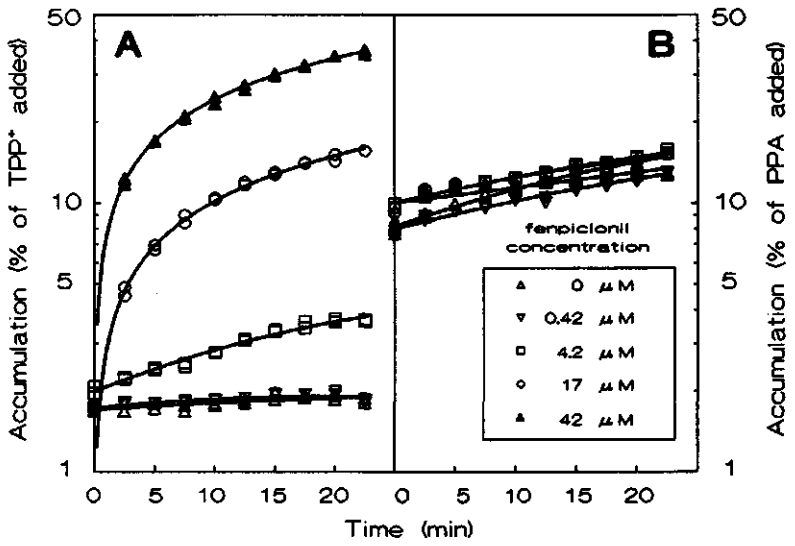

Fig. 5 Effect of fenpiclonil on accumulation of tetraphenylphosphonium bromide (TPP^+; A) and propionic acid (PPA; B) by mycelial suspensions of *Fusarium sulphureum* isolate 1743. TPP^+ and PPA were added 15 min prior to addition of fenpiclonil (t = 0).

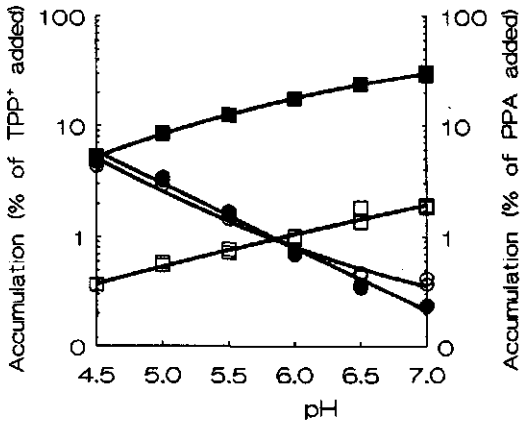

Fig. 6 Relation between pH of MES/Tris medium on accumulation of TPP^+ (□,■) and PPA (○,•) by mycelial suspensions of *Fusarium sulphureum* isolate 1743 in the presence (■,•) and absence of fenpiclonil (17 µM; □,○).

methanol. Accumulation of PPA was hardly affected by fenpiclonil (Fig. 5). TPP^+- and PPA accumulation responded to changes in pH of the medium. Increase in pH value correlated with a higher accumulation of TPP^+ and a lower accumulation of PPA (Fig. 6). At all pH values tested, fenpiclonil induced an increased accumulation of TPP^+ but hardly affected the accumulation of PPA (Fig. 6). Incubation of all fenpiclonil-treated mycelialsuspensions with 1% toluene for 1 h, reduced accumulation of both TPP^+ and PPA to the same background levels as control suspensions. Fluorescent membrane potential probes (28,29) could not be used in this study, because fenpiclonil quenched the fluorescence of carbocyanine dyes.

DISCUSSION

Toxicity tests revealed fenpiclonil as an effective inhibitor of mycelial growth in many imperfect fungi at concentrations below 4 μM. *F. sulphureum* isolates 1743 (fenpiclonil-sensitive) and 1743.1 (fenpiclonil-resistant) were selected as test organisms.

Fenpiclonil caused a significant reduction of accumulation of amino acids and sugars (Fig. 3 and 4). The reduction occurred immediately after addition of fenpiclonil and the magnitude agreed with growth reduction of mycelium in liquid medium (*cf.* Fig. 2 and 4). These results suggest that accumulation related processes are the primary target of fenpiclonil. Competitive or irreversible inhibition of transmembrane carriers by fenpiclonil is not likely, because accumulation of different substrates (amino acids and sugars) was inhibited.

The reduction in accumulation of the substrates might be due to instantaneous cell death. However, as exemplified by the distribution of TPP^+ and PPA over the intra- and extracellular space, membrane potentials and proton gradients in mycelium were not dissipated (Fig. 5). This should be the case if cells would die upon fenpiclonil-treatment (28,30).

In fungi, the distribution of the probes TPP^+ and PPA are sometimes considered to be indicative for the plasma membrane potential and proton gradient only (21,31,32,33,34). However, eukaryotic organisms contain various compartments surrounded by membranes. In equilibrium conditions, the probes are distributed across all separate organelle membranes according to their specific potentials (23,34). Therefore, accumulation of these probes in mycelium is only indicative for the overall change in membrane potential and proton gradient in mycelium and not for the plasma membrane only. For this reason TPP^+ and PPA

accumulation were not represented as the plasma membrane potential ($\Delta\Psi$) and proton gradient (ΔpH), the two constituents of the proton motive force (Δp; 29). As a result of an increased medium pH, $\Delta\Psi$ and ΔpH are usually hyperpolarized and dissipated, respectively (29). In our experiments a positive correlation between medium pH and TPP^+ and a negative correlation between medium pH and PPA accumulation was found (Fig. 6). Therefore, accumulation of TPP^+ and PPA might still be indicative for the $\Delta\Psi$ and ΔpH. However, the hyperpolarization of mycelium upon fenpiclonil treatment did not correlate with a decrease in ΔpH at all pH values tested.

Fenpiclonil instantaneously inhibited accumulation of substrates such as amino acids and 2-deoxyglucose at relatively high concentrations (Fig. 3). The relation between the plasma membrane potential and accumulation of these substrates is not fully understood. For instance, while the Δp energizes amino acid (35) and pyrimidine base accumulation (19), the accumulation of sugars can be energized by both Δp (36,37,38) and ATP (39,40). Hence, a decrease in substrate accumulation would usually correlate with a decrease in Δp instead of the supposed increase as suggested by the present experiments. Therefore, the mechanism involved is not understood. It might relate to direct action of the fungicide on transmembrane carriers or to indirect action via energization of those carriers. Such fenpiclonil-induced defects on components of the plasma membrane bilayer will be a topic for future research.

Accumulation studies with precursors at low substrate concentrations were carried out to study effects of fenpiclonil on incorporation in DNA, RNA protein and chitin (Table 5). Effects of fenpiclonil on accumulation and incorporation of the precursors were similar in most treatments. This indicates that none of the biosynthetic processes involved were inhibited stronger than the accumulation of the corresponding substrates. In consequence, none of these processes seems to be involved as the primary target site of fenpiclonil. The increased accumulation of thymine at high fenpiclonil dosage (42 µM) may be related to the very low accumulation of the base, combined with possible membrane damage at this high fungicide concentration. This may be the case, since under similar conditions electrolyte leakage was observed. Uracil incorporation into RNA was slightly more reduced by fenpiclonil than uracil accumulation. However, as compared with other RNA synthesis inhibitors, this reduction was low (18).

Inhibition of oxygen consumption by fenpiclonil was only evident after several hours incubation and not complete at the highest fenpiclonil concentration tested. It also did not

affect ATP levels in mycelium, as well as the proportional content of ATP in relation to total adenylate nucleotides. The inhibitory effects of fenpiclonil on oxygen consumption were regarded as secondary. It was reported that pyrrolnitrin, the natural antibiotic analogue of fenpiclonil, inhibited electron transport in the oxidative respiratory chain of fungi (41,42,43). However, inhibition of endogenous respiration was only evident at antibiotic concentrations exceeding the EC_{50} more than 1000-fold (44). Therefore, in these instances respiration should also not be regarded as a plausible primary target site.

Ergosterol biosynthesis was inhibited to some extent by fenpiclonil. Eburicol accumulated, which is typical for filamentous fungi treated with ergosterol biosynthesis inhibitors (45). The less fenpiclonil-sensitive strain 1743.1, did not show this effect. However, the effect of known ergosterol biosynthesis inhibitors on the sterol composition was much greater than the effects observed with fenpiclonil (*cf.* 46,47,48). Therefore, ergosterol biosynthesis did not seem to be the primary target of fenpiclonil. Changes in composition of other lipid fractions and membrane integrity were even smaller. Still, the increased free fatty acid concentration might contribute to the fungicidal action (49). Increased lipid peroxidation as observed after dicarboximide treatment (49) was not observed.

Leroux (50) hypothesised that the modes of action of fenpiclonil and dicarboximide fungicides are possibly related. This was based on the observation that wild type and laboratory-induced osmotic-sensitive isolates of *B. cinerea* showed cross-resistance to both types of fungicides. This was confirmed in our experiments (Table 1). In contrast, we found that the sensitivity of two *F. solani* f.sp. *coeruleum* isolates to fenpiclonil and the dicarboximide vinclozolin was negatively correlated. This suggests that both fungicides may have a different action. In such a case fenpiclonil and its more potent analogue CGA 173506 can be used in anti-resistance strategies for control of dicarboximide-resistant pathogens. Only biochemical studies with phenylpyrroles and dicarboximides can really identify the mode of action of these fungicides and confirm the validity of these hypotheses.

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Chapter 4

Interference of the phenylpyrrole fungicide fenpiclonil with membranes and membrane function

Ad B.K. Jespers
Leen C. Davidse
Maarten A. de Waard

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ABSTRACT

The phenylpyrrole fungicide fenpiclonil accumulates to high levels in mycelium of *Fusarium sulphureum* and artificial liposomes. The accumulation is a result of a physico-chemical distribution of the fungicide over lipid like material of the mycelium and the medium. Accumulation is reversible as the fungicide can easily be removed from mycelium by washing with water. Fenpiclonil is not metabolized by the fungus upon incubation for 24 hr. The fungicide does neither affect membrane fluidity in artificial liposomes nor amino acid accumulation in bacterial vesicles. Thus, accumulation of the fungicide does not seem to affect the functioning of membranes.

Fenpiclonil induces the accumulation of the membrane potential probe tetraphenylphosphonium bromide by *Fusarium sulphureum*. Since accumulation of this probe probably reflects the cumulative potential over all cell membranes, the increased accumulation is more likely a result of changes of potentials over membranes of intracellular organelles rather than plasma membrane hyperpolarization. Hence, the previously described effects of fenpiclonil on amino acid and saccharide uptake cannot be explained by plasma membrane hyperpolarization.

INTRODUCTION

Fenpiclonil (CGA 142705; 4-(2,3-dichlorophenyl) pyrrole-3-carbonitrile) is the first phenylpyrrole fungicide developed by CIBA-GEIGY A.G., Basel[1]. Control of cereal pathogens such as *Gerlachia nivalis*, *Tilletia caries*, and *Septoria nodorum* by seed treatment is at least equivalent to standard products[2]. Treatment of seed potatoes gave excellent control of *Rhizoctonia solani*, *Helminthosporium solani*, *Fusarium solani f.sp. coeruleum*, and *Fusarium sulphureum*[3]. Commercial formulations of fenpiclonil such as 'Beret®', 'Galbas®', and 'Gambit®' are or will be marketed worldwide.

Fenpiclonil instantaneously inhibits the accumulation of amino acids and sugars in the test organism *Fusarium sulphureum* (Schlecht)[4]. Simultaneously, the fungicide enhances the accumulation of tetraphenylphosphonium bromide (TPP^+), suggesting that it increases the plasma membrane potential. These data are indicative for a direct interference of the fungicide with the plasma membrane. This action has also been suggested for the analogous antibiotic pyrrolnitrin[5]

The present study describes the accumulation and fate of fenpiclonil in fungal mycelium and the effect of fenpiclonil on membranes and membrane function such as accumulation of

amino acids and fluidity of artificial liposomes. In addition, the fenpiclonil-induced accumulation of TPP^+, which suggested hyperpolarization of fungal plasma membranes[4] is described in more detail.

MATERIALS AND METHODS

Organism, culture methods and sample preparation.

F. sulphureum isolate 1743 was obtained from the fungal collection of the Research Institute for Plant Protection (IPO) in Wageningen. The isolates were weekly subcultured on potato dextrose agar (PDA) at 20°C. Czapek Dox liquid cultures were inoculated with macroconidia obtained from two-week-old cultures on PDA, to a final concentration of 2×10^5 conidia ml^{-1}. Cultures were incubated in a rotary shaker at 120 rpm and 20°C for 2 days. Mycelium was harvested and washed with demineralised water by vacuum filtration over a filter paper in a Büchner funnel. Subsequently, washed mycelium was resuspended in the appropriate incubation medium and allowed to settle for at least 1 hr. All experiments were performed at least three times in duplicate in a rotary shaker at 120 rpm and 20 °C.

Bacillus subtilis isolate B25 was obtained from the culture collection of the Department of Microbiology, Wageningen Agricultural University, Wageningen. It was maintained by bi-weekly subculturing on PDA slants. To obtain a starter culture, LB medium[6] (100 ml) was inoculated with *B. subtilis* and incubated overnight in a rotary shaker at 200 rpm at 37°C. The starter culture (10 ml) was added to LB medium (1 l) and incubated under continuous flux of air (1 bubble s^{-1}) at 37°C for 3 h. When cultures had an OD_{660} between 0.5 - 1.5, the cells were harvested by centrifugation (4000g, 5 min) and washed twice with the appropriate medium[7].

Chemicals.

Czapek Dox liquid medium was purchased from Oxoid Ltd. (London, UK). PDA and TLC plates (Silicagel 60 F_{254}) were obtained from Merck (Darmstadt, FRG). Radiolabelled [*phenyl*-^{14}C]tetraphenylphosphonium bromide (sp act 0.71 GBq $mmol^{-1}$), [carboxyl-^{14}C]dextran (sp act 21 MBq g^{-1}), and [U-^{3}H]H_2O (sp act 3.3 TBq mol^{-1}) were obtained from Du Pont Research

Products (Den Bosch, The Netherlands). Hydroluma and LumaSolve were purchased from Lumac BV (Schaesberg, The Netherlands), and trifluoperazine (TFP), carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) and cetylpyridinium bromide (CPB) from Sigma (St. Louis, MO, USA). Tetraphenylphosphonium bromide (TPP^+) was purchased from ICN Biomedicals (Plainville, NY), and sodium orthovanadate from Janssen Pharmaceuticals (Beerse, Belgium). Technically pure fenpiclonil (98.49%, CGA 142705) and [4-*pyrrole*-^{14}C]fenpiclonil (sp act 0.41 GBq $mmol^{-1}$) were gifts of CIBA-GEIGY AG (Basel, Switzerland). All other chemicals were of technical grade.

Accumulation of fenpiclonil

Mycelial suspensions in Czapek Dox medium (100 ml) were equilibrated for 1 hr. Accumulation of fenpiclonil in mycelium was measured at intervals after addition of the fungicide (400 Bq ml^{-1}, from a 200-fold concentrated stock solution in methanol). Mycelial samples (5 ml) were collected on Whatmann GF/A filters using a Millipore sampling manifold. Mycelium was washed twice with water (2 ml) and mixed with Hydroluma (10 ml). Radioactivity was determined by scintillation counting after 15 hr. The effect of test chemicals on accumulation of fenpiclonil was studied by incubation of mycelium with these chemicals for 30 min before adding fenpiclonil. Test chemicals were added from 200-fold concentrated stock solutions.

Fate of fenpiclonil

Mycelial suspensions (30 ml) were allowed to accumulate fenpiclonil for 24 h. Mycelium was washed, collected as described above, subsequently extracted with 70% methanol (3 times) and ice-cold 10% TCA (w/v)[8]. The mycelial residue was suspended in 5% TCA and incubated at 90°C for 15 min. The residue obtained after filtration over a GF/A filter was suspended in 0.5 N NaOH and incubated at 36°C for 16 h. The final mycelial residue was obtained after filtration. Radioactivity was determined in all extracts. The culture medium and the combined methanol extracts were extracted twice with chloroform. The chloroform extracts were analyzed by TLC using dichloromethane or water saturated *n*-butanol as solvent systems. Radioactive spots on TLC plates were located by autoradiography using Kodak X-AR films.

Potassium efflux

Mycelial suspensions in 10 mM MES/Tris buffer pH 5.5 (50 ml) were amended with fenpiclonil or TFP from 200-fold concentrated stock solutions in methanol. Control treatments obtained equivalent quantities of methanol. Mycelium was harvested after 15 min of incubation, washed with ice-cold demineralised water, and boiled in 15 mM CsCl for 10 min. After centrifugation (10,000*g*, 10 min), the potassium concentration in the supernatants was determined with an Eppendorf ELEX 6361 atomic emission spectrometer using KCl as standard[9]. The potassium concentration in culture filtrates mixed with CsCl (final concentration 15 mM) was determined as well.

Determination of cell volume

The effect of fenpiclonil and TFP on the intracellular volume of mycelium was determined from the distribution of [U-^{3}H]water and membrane impermeable [^{14}C]carboxyl-dextran over mycelium and medium[10]. Mycelial suspensions in Czapek Dox (10 ml) were amended with the test compounds and the two probes. After incubation (15 min), mycelium was collected over Whatman GF/A filters, and dissolved in LumaSolve (1 ml) at 30°C for 30 min. Radioactivity was determined with a Beckman LS6000 TA scintillation counter in the dual channel mode (windows 0-350 and 350-670, respectively). The system was calibrated with quenched [^{3}H] and [^{14}C] standards. The intracellular volume was expressed relative to mycelial dry weight.

Preparation of membrane vesicles

Plasma membrane vesicles of *B. subtilis* were essentially prepared according to the procedure of Kaback[11]. Bacterial cells (10 g wet cells l^{-1}) were suspended in 25 mM K_2HPO_4/H_3PO_4 buffer pH 8.0 at 37°C. Membrane vesicles were formed after incubation in Lysozyme (100 mg l^{-1}), RNase (30 mg l^{-1}), DNase (30 mg l^{-1}), and $MgSO_4$ (10 mM) under continuous stirring at 37°C for 30 min. Incubation was continued with EDTA (10 mM) and $MgSO_4$ (30 mM) for 2 and 15 min, respectively. All components were added from 200-fold concentrated stock solutions in the same buffer. After cooling to 0°C, the cell debris was removed by centrifugation (2,000*g*, 5 min). Vesicles remaining in the supernatant were pelleted in a second centrifugation step (20,000*g*, 45 min) and washed in 50 mM K_2HPO_4/H_3PO_4 buffer

pH 6.6. Finally, the vesicles were resuspended in the same buffer (±20 mg protein ml^{-1}).

Aspartic acid transport assay with membrane vesicles

Concentrative transport of aspartic acid into *B. subtilis* membrane vesicles was energized with PMS reduced by ascorbate[12]. The system generates an electrical potential (proton motive force) across the membrane by reducing the respiratory chain of the vesicles. The proton motive force drives the active transport of amino acids[13]. The assay mixture consisted of 25 mM K_2HPO_4/H_3PO_4 buffer pH 6.6, 10 mM $MgSO_4$, inhibitor (specified concentration), vesicles (1.09 mg membrane protein ml^{-1}), 20 mM potassium-ascorbate, pH 6.6, 100 µM PMS, and 100 µM aspartic acid (100 Bq). The components were sequentially added to the buffer resulting in a final volume of 550 µl. Timing was started with the addition of aspartic acid. At intervals, samples (100 µl) were filtered over Millipore AA nitrocellulose filters (0.8 µm pore size) and washed twice with 0.1 M LiCl (2 ml). Radioactivity was determined as described above.

Anisotropy of membrane vesicles

Reversed phase asolectin liposomes were prepared in 10 mM K_2HPO_4/H_3PO_4 pH 7.0 according to Szoka and Papahadjopoulos[14]. Dioleoylphosphatidylcholine (DOC) liposomes loaded with 1,6-diphenyl-1,3,5-hexatriene (DPH) were prepared in 10 mM K_2HPO_4/H_3PO_4 pH 7.0 by sonication in a bath type sonicator for 5 min[15,16]. Final concentrations of DOC and DPH were 40 and 0.08 µM, respectively. The fluorophore DPH has an all-*trans*-polyene structure with absorption and fluorescence transitions moments along the major axis of the molecule. The degree of anisotropy of fluorescence polarization relates to the viscosity which oppose the rotations of this long axis. Therefore, membrane fluidity of the liposomes is proportional to the fluorescence anisotropy of DPH. Polarized fluorescence intensities were measured parallel and perpendicular to the direction of polarization of the excitation beam in a SLM 8000 spectrofluorometer at 20°C[17]. The excitation and emission wavelength were 340 and 431 nm, respectively. The steady-state anisotropy was calculated according to Pazoutova *et al.*[18]. Fenpiclonil was added to the liposomes 15 min prior to the measurement. Equivalent amounts of methanol were added to control treatments.

Membrane potential

The overall membrane potential (E_{TPP}) was estimated by the distribution of TPP^+ over mycelium and extracellular medium as described before[19], and tentatively expressed in mV[20]. TPP^+ and test compounds were added to the mycelium, 15 min prior to addition of fenpiclonil. The distribution of TPP^+ over mycelium, washed twice with 1 mM $MgSO_4$ (5 ml) and external medium, was assessed after incubation with fenpiclonil for 15 min. Values were corrected for aspecific binding of the probe to mycelium deenergized with 0.8 mM CPB[21].

RESULTS

Accumulation and fate of fenpiclonil

Mycelial suspensions of *F. sulphureum* isolate 1743 (0.9 mg dry wt ml^{-1}) rapidly accumulated fenpiclonil (0.42 µM) from the external medium. The maximum accumulation level (179 nmol g^{-1} mycelium dry wt) was reached within 1 min after addition of the fungicide (Fig. 1). The internal cell volume was assessed to be 1.26 µl mg^{-1} mycelial dry weight. Hence, the maximal accumulation level mentioned above corresponds with an internal fungicide concentration of 142 µM indicating a 525-fold accumulation as compared with the concentration in the external medium. Similar results were obtained with the less sensitive isolate 1743.1 (EC_{50} 40 µM). Compounds known to affect respiration and the proton motive force did not reduce accumulation (Table 1). Accumulation of fenpiclonil into mycelium and artificial asolectin-liposomes with the same lipid content was similar (Fig. 2). The lipid content of mycelium was assessed to be 3.4%. Hence, under equilibrium conditions the ratio between the amount of fenpiclonil accumulated in lipids of mycelium (6 µmol g^{-1} lipid) and the amount of fenpiclonil in the external medium (0.27 nmol g^{-1} medium) in equilibrium conditions was calculated to be 21,500.

The major part of fenpiclonil accumulated in mycelium could be extracted with methanol (Table 2). More than 99% of the label in the methanol fraction could be extracted with chloroform. TLC analyses with two different solvent systems, dichloromethane and water saturated *n*-butanol identified the labelled material present as fenpiclonil. A minor quantity of fenpiclonil accumulated (2.7%) could be extracted from the mycelium by NaOH treatment.

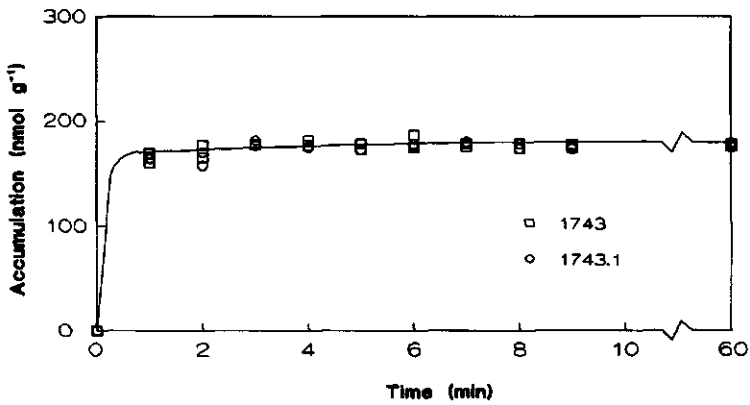

Fig. 1 Accumulation of fenpiclonil (0.42 μM) by mycelium (0.9 mg dry wt ml^{-1}) of *Fusarium sulphureum* isolates 1743 and 1743.1 in Czapek Dox liquid medium in time.

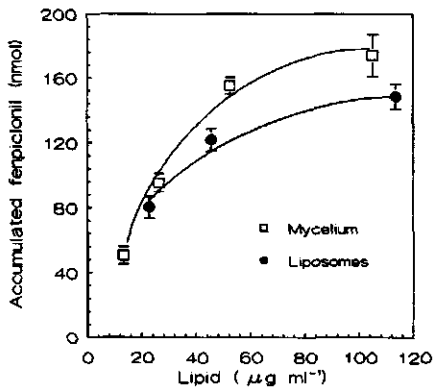

Fig. 2 Relation between lipid content of mycelial suspensions of *Fusarium sulphureum* isolate 1743 (3.4% lipid) or asolectin liposomes and accumulation of fenpiclonil from the external medium (0.42 μM) assessed after incubation with the fungicide for 15 min.

Table 1 Effect of compounds on accumulation of fenpiclonil by mycelium of *Fusarium sulphureum* isolate 1743[a].

Compound	Concentration (μM)	Accumulation[b] (nmol g^{-1})
Control		201 ± 1.3
TFP	42	211 ± 3.8
CCCP	100	200 ± 1.3
KCN[c]	500	205 ± 0.9

[a] Mycelial concentration 0.8 mg mycelium dry wt ml^{-1}.

[b] Initial fenpiclonil concentration 0.42 μM (525 nmol g^{-1} mycelial dry wt).

[c] Water as solvent.

Table 2 Recovery of fenpiclonil from the culture filtrate and various fractions of mycelium of *Fusarium sulphureum* isolate 1743[a] incubated with the fungicide (0.42 μM) for 24 hr.

Fraction	Volume (ml)	Recovery		
		Bq	%	%[b]
Culture filtrate	34.5	2980	46.3	
Methanol extract	15.0	3193	49.6	93.2
TCA (0°C) extract	4.5	7	0.1	0.2
TCA (90°C) extract	8.5	33	0.5	1.0
NaOH extract	6.3	175	2.7	5.1
Mycelial residue		15	0.2	0.4
Total		6403	99.4	

[a] Mycelium dry wt. concentration, 3 mg ml^{-1}

[b] % of fenpiclonil accumulated.

Table 3 Effect of fenpiclonil and CCCP on accumulation of aspartic acid by membrane vesicles of *Bacillus subtilis* isolate B25 energized with ascorbate-PMS[a].

Time Min	Energized				Not energized
	Control	Fenpiclonil (μM)		CCCP (μM)	
		4.2	42	100	
1	2.88 ± 1.50[b]	2.38 ± 1.08	2.24 ± 1.19	0.14 ± 0.25	0.06 ± 0.20
3	5.72 ± 2.31	4.76 ± 2.09	4.73 ± 1.28	0.07 ± 0.18	0.08 ± 0.11
5	7.27 ± 2.04	6.64 ± 2.39	7.02 ± 2.65	0.10 ± 0.17	0.12 ± 0.12
7	9.44 ± 3.17	9.18 ± 2.31	9.32 ± 2.85	0.08 ± 0.12	0.14 ± 0.12

[a] Membrane protein concentration, 1.09 mg ml^{-1}; aspartic acid concentration, 100 μM (91.7 nmol mg^{-1} membrane protein);

[b] Accumulation expressed as nmol mg^{-1} membrane protein

Table 4 Effect of several compounds on fenpiclonil-induced hyperpolarization of the plasma membrane potential (E_{TPP}) in *Fusarium sulphureum* isolate 1743[a].

Compound	Concentration	Control	Fenpiclonil (17 µM)	
		E_{TPP}	E_{TPP}	ΔE_{TPP}
Control		-70 ± 2	-131 ± 1	-61
Valinomycin	50 µM	-104 ± 2	-128 ± 7	-24
Nigericin	50 µM	-84 ± 1	-118 ± 1	-34
Gramicidin S	50 µM	-25 ± 7	-35 ± 8	-10
TFP	42 µM	-116 ± 3	-165 ± 3	-49
CCCP	42 µM	-44 ± 4	-80 ± 2	-36
Na_3VO_4	42 µM	-64 ± 3	-129 ± 2	-65
KCN	500 µM	-67 ± 2	-130 ± 1	-63
KCl	10 mM	-38 ± 3	-97 ± 3	-59
KCl	250 mM	-11 ± 2	-72 ± 2	-61
NaCl	10 mM	-48 ± 2	-103 ± 4	-55
NaCl	250 mM	-23 ± 3	-91 ± 3	-68
$MgCl_2$	10 mM	-2 ± 1	-63 ± 4	-61
CPB	0.8 mM	0 ± 2	0 ± 3	0

[a] Figures indicate the putative plasma membrane potential (mV) as calculated from the distribution of TPP^+ and are mean values of three experiments.

Table 5 Effect of fenpiclonil and trifluoperazine on intra- and extracellular potassium concentration and mycelial volume of *Fusarium sulphureum* isolate 1743[a].

Treatment	Concentration (µM)	Potassium concentration		Mycelial volume[b]
		Medium (µM)	Mycelium (mM)	
Control	0	3	188	1.26
Fenpiclonil	4.2	3	191	n.d.[c]
	17	4	187	n.d.[c]
	42	7	184	1.25
TFP	42	166	42	1.02

[a] Mean values of two experiments

[b] µl mg^{-1} mycelial dry weight

[c] Not determined

The majority of the label in the culture filtrate (97%) could be extracted with chloroform and was also identified as fenpiclonil.

Most of the fenpiclonil accumulated (> 80%) could be released from mycelium by repeated washing with water. After this procedure the fungus continued to grow normally.

Effect on membrane fluidity

The effect of fenpiclonil on the fluidity of membranes was quantified by measuring changes in fluorescence anisotropy of DPH in artificial phospholipid liposomes. Control anisotropy values were 0.109 ± 0.007. These were not affected by treatment with fenpiclonil at concentrations ranging from 0.4 to 40 µM.

Effect on aspartic acid accumulation by bacterial vesicles

Functional plasma membrane vesicles of *B. subtilis* were readily formed after lysis of cells in the presence of lysozyme. Accumulation of aspartic acid into these vesicles increased upon ascorbate-PMS energization (Table 3). Fenpiclonil did not significantly reduce the accumulation. CCCP, known to dissipate the proton motive force, was used as a reference. This compound, in contrast to fenpiclonil, inhibited accumulation of aspartic acid effectively.

Membrane potential

Previous studies showed that fenpiclonil enhanced the accumulation of the membrane potential probe TPP^+ [4]. Since this may be caused by an hyperpolarization of the plasma membrane potential, the effect was studied in more detail using various compounds with known effects on cell membrane processes. The putative membrane potential (E_{TPP}) of control mycelium was -70 mV (Table 4). The ionophores valinomycin and nigericin, and the antipsychotic drug TFP hyperpolarized the plasma membrane potential, whereas gramicidin S and CCCP dissipated the membrane potential considerably. The membrane potential was also reduced by high concentrations of electrogenic cations in the medium. Addition of the respiration inhibitor KCN to mycelium did not dissipate the membrane potential, but did reduce oxygen consumption (results not shown).

Fenpiclonil (17 µM) hyperpolarized the membrane potential by about 60 mV. Similar values were found in the presence of most of the test compounds. In contrast,

hyperpolarization by fenpiclonil in the presence of valinomycin, nigericin, gramicidin S and CCCP was relatively low (Table 4).

Fenpiclonil had no effect on efflux of potassium and cell volume. TFP was used as a control. This antipsychotic drug caused a significant efflux of potassium and reduced the cell volume (Table 5).

DISCUSSION

Fenpiclonil instantaneously accumulated into mycelium of *F. sulphureum* to a maximum level. The accumulation was not influenced by compounds that increase (TFP) or decrease (CCCP) the membrane potential and by the respiration inhibitor KCN. Therefore, active influx via an ATP driven transport protein, as is the case with polyoxins[22,23], or energy-dependent efflux of passive accumulation as described for ergosterol biosynthesis inhibiting fungicides[24], is not likely. Accumulation of fenpiclonil driven by either the membrane potential (*c.f.* cationic fungicides such as *sec*-butylamine[25] and aminoglycosides[26,27]) or the proton gradient (*c.f.* benzoic and sorbic acid[28]) is also not likely, since the nitrogen in the pyrrole ring of fenpiclonil has neither a basic nor acid character[1].

The accumulation level of fenpiclonil was positively correlated with the amount of mycelial lipid in the culture medium as is the case with most fungicides (*c.f.* benzimidazoles[8,29], chlorinated nitrobenzenes[30] and dodine[30-32]). The ratio between the fenpiclonil concentration in mycelial lipids or liposomes and medium was about 20,000. This value is comparable with the *n*-octanol - water partition coefficient of the fungicide (log P = 4.3)[1]. Binding to intracellular components as is the case with captan[33,34] did not occur since the fungicide could almost quantitatively be extracted from mycelium with methanol or water.

Accumulation of xenobiotics may cause malfunctioning of fungal membranes. This has been described for polyene macrolide antibiotics, which rearrange membrane sterols[35], and suggested for pyrrolnitrin which would interact with membrane phospholipids[5]. The action of fenpiclonil was tested in a well-defined model system of *B. subtilis* vesicles. In these experiments, the fungicide neither affected membrane fluidity of artificial liposomes, nor transport of aspartic acid, despite of its high accumulation level (Tables 2 and 3). Therefore,

the fungicidal action of fenpiclonil is not likely based on an aspecific interaction of the fungicide with the phospholipid bilayer. Such an aspecific interaction would also imply a lack of selective fungitoxicity. Since this is not the case, a putative action of fenpiclonil on fungal membranes should be more specific.

The concentrative accumulation of essential compounds such as sugars and minerals is an important function of the fungal plasma membrane[36,37]. In many cases, this accumulation is energized by the membrane potential and the proton gradient, the two constituents of the proton motive force[19,38,39]. Fenpiclonil induced an increased accumulation of TPP^+ and reduced the accumulation of sugars and amino acids[4]. TPP^+ is often regarded as a probe to quantify the plasma membrane potential (E_{TPP}) in both pro- and eukaryotic cells[20,40,41,42]. Therefore, fenpiclonil may effect the plasma membrane potential of *F. sulphureum*.

The apparent plasma membrane potential of mycelium is about -70 mV, which is comparable to values reported for other fungi[20,40,43], but considerably less negative than the plasma membrane potential of *Neurospora crassa*[44] and yeast[41,45]. The ionophores valinomycin and nigericin hyperpolarized the plasma membrane potential which may be related to direct effects on potassium fluxes[46,47]. The antipsychotic drug TFP also caused an hyperpolarization, which may be related to potassium fluxes caused by inhibition of proton ATPase in the plasma membrane[48]. These types of hyperpolarization are usually counteracted by dissipation of the proton gradient[19]. Gramicidin S and CCCP dissipated the membrane potential considerably. This is probably due to a dissipation of all monovalent cation gradients[19,46,49]. Addition of the electrogenic cations K^+, Na^+, or Mg^{2+} to the medium reduced the membrane potential as expected.

Fenpiclonil hyperpolarized the apparent membrane potential by 60 mV in the control as well as in most of the treatments (Table 4). The strongly dissipating agents gramicidin S and CCCP reduced the fenpiclonil-induced hyperpolarization. The same effects were observed with the potassium ionophores valinomycin and nigericin. Hyperpolarization of the plasma membrane potential is usually caused by fluxes of potassium over the plasma membrane, in exchange for protons[48,50]. Fenpiclonil did not induce such effects, because neither a change in proton gradient[4] nor an efflux of potassium was observed (Table 5). Potassium efflux was significantly enhanced by the reference compound TFP. The fungicide did not decrease the cell volume as is the case with TFP, even not at high concentrations. (Table 5). Therefore,

the apparent hyperpolarization might be caused by an intracellular flux of potassium from the cytoplasm to mitochondria[51]. This conclusion would corroborate with the observation that the fenpiclonil analogue pyrrolnitrin induces swelling of isolated mitochondria by the presumed accumulation of potassium[52]. However, it is improbable that this phenomenon would be the primary action of fenpiclonil, since the fungicide only affacts oxygen consumption[4] and oxidative phosphorylation[53] at high concentrations.

The observed changes in accumulation of TPP^+ caused by fenpiclonil are compatible with the idea that TPP^+ accumulation reflects changes in potentials over intracellular membranes, the mitochondrial membrane in particular, rather than that of the plasma membrane. It would imply that the effect of fenpiclonil on accumulation of monosaccharides and amino acids as described in a previous study[4], is not caused by plasma membrane hyperpolarization, but by a mechanism not yet known.

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Chapter 5

Effect of fenpiclonil on macromolecule biosynthesis in *Fusarium sulphureum* (Schlecht)

Ad B.K. Jespers
Maarten A. de Waard

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ABSTRACT

Fenpiclonil is a phenylpyrrole fungicide used for control of *Fusarium sulphureum*. At a low inhibitory concentration, the fungicide specifically inhibited incorporation of glucose and mannose into fungal macromolecules. This effect was not observed with a fenpiclonil less sensitive laboratory isolate of the *F. sulphureum*. Toxicity of phenylpyrrole analogues ranked with their inhibitory effect on mannose incorporation.

The highest inhibitory effect was found for incorporation of glucose into hyphal wall glycans. Biosynthesis of these glycans is mediated by glycan synthetases in the plasma membrane. However, these enzymes are probably not the target site of fenpiclonil, since fenpiclonil neither inhibited glycan synthetase activity in cell-free extracts nor caused accumulation of uridinediphosphoglucose in mycelium.

Fenpiclonil also inhibited incorporation of [^{14}C] label in glucose into other fungal macromolecules such as proteins. These results suggest that fenpiclonil does not interfere with various polymerization steps in biosynthesis of macromolecules, but with an initial metabolization step of glucose such as glucose phosphorylation. This hypothesis would also explain the inhibitory effect of fenpiclonil on incorporation of [^{14}C]uracil into uridine and UDP-activated sugars.

INTRODUCTION

Fenpiclonil is the first phenylpyrrole fungicide developed by CIBA-GEIGY AG, Basel (Fig. 1; 1). The fungicide, at concentrations which reduced growth by about 50% (4 μM), instantaneously inhibits accumulation of substrates such as amino acids and monosaccharides in *Fusarium sulphureum* (2). This observation suggests that fungitoxicity may be a consequence of a yet unknown primary effect of the fungicide on membrane function (2). In the present study, accumulation and incorporation of various substrates into macromolecules of *F. sulphureum* were studied at a concentration of fenpiclonil (0.4 μM) which inhibited growth by only 15%. Similar experiments were carried out with a

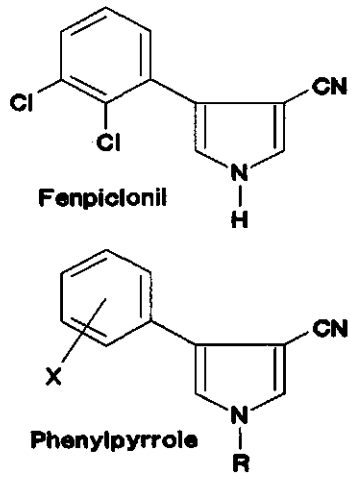

Fig. 1 Structure of fenpiclonil and general structure of phenylpyrrole fungicides.

fenpiclonil less sensitive isolate. Various phenylpyrrole analogues were tested as well. The site of inhibition in the various biosynthetic processes was determined by isolation and characterization of mycelial fractions containing different types of macromolecules, especially those containing various types of polysaccharides.

MATERIALS AND METHODS

Organism, culture methods and sample preparation.

The *F. sulphureum* isolate 1743 was obtained from the fungal collection of the Research Institute for Plant Protection (IPO) in Wageningen. The fenpiclonil less sensitive isolate 1743.1 originated from a fast growing sector in a culture of isolate 1743 on PDA amended with fenpiclonil (4 μM). Reduced sensitivity to fenpiclonil correlates with high sensitivity to osmotic stress and loss of pathogenicity (2). The isolates were weekly subcultured on potato dextrose agar (PDA) at 20°C. Czapek Dox liquid cultures were inoculated with macroconidia, which were obtained from two-week-old cultures on PDA, to a final concentration of 2×10^5 conidia ml^{-1}. Cultures were incubated in a rotary shaker at 120 rpm and 20°C for 2 days. Mycelium was harvested and washed with demineralised water by vacuum filtration over a filter paper in a Büchner funnel. Standard mycelial suspensions were obtained by resuspending the washed mycelium in Czapek Dox liquid medium without glucose. Before starting experiments, suspensions were allowed to settle for 1 hr. In all experiments, mycelial suspensions were treated by preincubation with fenpiclonil from 200-fold stock solutions in methanol for 15 min. Methanol (0.5%) served as control. All experiments were performed at least two times in duplicate in a rotary shaker at 120 rpm and 20°C.

Chemicals

Czapek Dox liquid medium was purchased from Oxoid Ltd (London, UK). PDA and TLC plates (Silicagel 60 F_{254} and PEI-cellulose F_{254}) were obtained from Merck (Darmstadt, FRG). Radiolabelled N-acetyl-[1-^{14}C]glucosamine (sp. act. 2.0 GBq $mmol^{-1}$), L-[U-^{14}C]aspartic acid (sp. act. 7.88 GBq $mmol^{-1}$), [1-^{14}C]glucose (sp. act. 2.1 GBq $mmol^{-1}$), [6-^{14}C]glucose (sp. act. 2.0 GBq $mmol^{-1}$), D-[U-^{14}C]glucose (sp. act. 10.8 GBq $mmol^{-1}$), D-[U-^{14}C]mannose (sp. act.

9.3 GBq mmol^{-1}), D-[2-^{3}H]mannose (sp. act. 570 GBq mmol^{-1}), [*methyl*-^{3}H]thymine (sp. act. 1.74 GBq mmol^{-1}), [2-^{14}C]uracil (sp. act. 1.92 GBq mmol^{-1}) and uridine diphospho-D-[U-^{14}C]glucose ammonium salt (sp. act. 12.0 GBq mmol^{-1}) were obtained from Amersham International Ltd (Amersham, United Kingdom). Aqualuma was purchased from Lumac BV (Schaesberg, The Netherlands). ß-1,3-glucanase from *Helix pomatia* was obtained from Fluka (Buchs, Switzerland). Technically pure fenpiclonil (98.49%, CGA 142705) and the phenylpyrrole analogues CGA 106877, CGA 106884, CGA 117106, CGA 129973, CGA 152908, CGA 153322, CGA 173506, and CGA 266258 were gifts of Ciba-Geigy AG (Basel, Switzerland). All other chemicals were of technical grade.

Accumulation and incorporation studies

Substrates were added to standard mycelial suspensions (10 ml) from 200-fold concentrated stock solutions in water. Final concentrations were 0.0017, 10, 25, 10, 200, and 200 µM for [*methyl*-^{3}H]thymine, [2-^{14}C]uracil, [U-^{14}C]aspartic acid, [2-^{14}C]mannose, [1-^{14}C]glucose, and [6-^{14}C]glucose, respectively. Incubation was stopped after 15 min. Accumulation into mycelium and subsequent incorporation of substrates into macromolecules were assessed as described previously (2,3).

The ratio of accumulated and incorporated mannose and aspartic acid in mycelium of *F. sulphureum* isolates 1743 or 1743.1 was determined in single Erlenmeyer flasks. The substrate mixture consisted of glucose, [2-^{3}H]mannose and [U-^{14}C]aspartic acid at final concentrations of 100, 25, and 25 µM, respectively. The experiment was performed as described previously (2). Differentially treated mycelium was hydrolyzed in 0.6 N perchloric acid (1 ml) at 90°C for 30 min and suspended in 10 ml Aqualuma Plus (3). Radioactivity of both isotopes was determined with a Beckman LS6000 TA scintillation counter in the dual channel mode (windows 0-350 and 350-670) after 15 hrs. The system was calibrated with quenched [^{3}H] and [^{14}C] standards (4).

Fractionation of mycelium

Incorporation of mannose into (glyco)proteins was assessed in standard mycelial suspensions (10 ml) incubated with 100 µM D-glucose and [U-^{14}C]mannose (5,000 Bq ml^{-1}) for 15 min. After incubation, mycelium was collected on Whatman GF/A filters, washed with ice-cold

demineralised water and resuspended in ice-cold water (0.5 ml). Subsequently, ice-cold $CHCl_3/CH_3OH$ (1/1, 3 ml) was added within 30 sec (5,6). This procedure yielded homogeneously dispersed mycelium and further mechanical disruption was not necessary. Mycelial residues were washed twice by centrifugation with $CHCl_3/CH_3OH/H_2O$ (1/1/0.3, 3 ml). All extracts were combined and water soluble metabolites of low molecular weight (low Mr) were obtained from the upper layer after phase separation with water (3 ml).

Water soluble and cell wall proteins were extracted three times from mycelial residues with phenol/acetic acid/water (2/1/1; 2 ml) in a shaking water bath at 70°C for 30 min (PAW extraction, 7,8). The combined extraction fluids were mixed with 300 µl ammonium formate (10%) and 100 µl ovalbumin (50 mg ml^{-1}). (Glyco)proteins were precipitated by addition of acetone (35 ml) at -20°C for 1 hr and hydrolyzed in 1 N NaOH and 1 N $NaBH_4$ at 100°C for 1 hr (9). Oligosaccharide side chains were separated from proteins by passing the mixture over anion exchange columns (0.5 × 2 cm, AG 1-X8, 100 - 200 mesh). The columns were equilibrated and saccharides eluted with the hydrolysation medium.

Incorporation of glucose into fungal macromolecules was assessed in standard mycelial suspensions incubated in 100 µM D-[U-^{14}C]glucose (10 ml, 7,500 Bq ml^{-1}) for 15 min. The incubation was stopped and metabolites (low Mr) were extracted according to the procedure described above. Water soluble macromolecules (glycoprotein, protein, DNA, RNA, *etc.*) were extracted with 3 ml sodium lauryl sulphate (SLS; 1%) in 50 mM Tris·HCl pH 8.0 at 0°C for 1 hr (10). Mycelial residues (hyphal walls) were subsequently fractionated in two steps with 1 N NaOH (3 ml) at 60°C for 20 min (fraction I), with 0.5 N HCl (3 ml) at 100°C for 1 hr (fraction II), and with 1 N NaOH (3 ml) at 60°C for 20 min (fraction III, 11). Between changes of extraction fluid, mycelial residues were washed with demineralized water (3 ml). Wash water was discarded. The extracts were neutralized and (glyco)proteins were extracted with equal volumes of phenol saturated with 10 mM acetic acid. The water phase contained primarily glycans.

The size of the extracted glycoprotein attached oligosaccharides and cell wall glycan fragments was estimated by Biogel-P4 gel filtration chromatography (range from 1 to 12 sugar residues). The column (1.0 × 100 cm) was eluted with 50 mM Tris·HCl pH 7.5, 50 mM NaCl, and 0.02% NaN_3 at a flow rate of 8 ml hr^{-1} (12). Extracted glycans were hydrolyzed to monomers by complete acid hydrolysis in 6 N H_2SO_4 at 120°C for 3 hrs (13). The nature of

the glycan monomers was determined by paper chromatography using ethanol/ pyridine/ water (8/2/1) as solvent system (14). Digestion of ß-1,3-glucan was performed with purified ß-1,3-glucanase (0.1 mg ml^{-1}) from *Helix pomatia* in 25 mM Tris·Cl pH 7.0 at 25°C for 24 hrs (10).

Uracil metabolites

Standard mycelial suspensions were incubated as described for the glucose metabolism experiments, except for the presence of [2-^{14}C]uracil (200 Bq ml^{-1}) in the substrate mixture. Uracil metabolites were extracted from incubated mycelium with 50% ethanol (5 ml) at 0°C for 1 hr and dried in an Eleya rotary vacuum evaporator (15). Uracil metabolites were separated by thin layer ion-exchange chromatography on PEI-cellulose plates using 0.25 M LiCl as solvent system (16). Radioactive spots were located by autoradiography using Kodak X-AR films and identified by R_f value using authentic standards.

Cell free glycan biosynthesis

Washed mycelium was suspended in FAGE buffer (25 mM HEPES, pH 7.4, 0.1 M glycerol, 10 mM NaF, 1 mM α-toluenesulphonyl fluoride, 5 mM EGTA, and 1% bovine serum albumin fraction V) at a density of 0.1 g wet wt ml^{-1} and disrupted four times at 0°C for 30 sec with 30 sec intervals in a Bead-Beater (Biospec Products, Bartlesville, OK, 17). ß-1,3-Glucan synthetase activity in the supernatant fraction (500g, 5 min) was determined by the method of Quigley and Selitrennikoff (18,19). Assay mixtures in FAGE contained protein and UDPGlc at final concentrations of 2 mg ml^{-1} and 100 µM, respectively.

RESULTS

Accumulation and incorporation

Accumulation of substrates into mycelium and incorporation into macromolecules was studied at a fenpiclonil concentration of 0.4 µM. Under these condition, the fungicide inhibited mycelial growth in liquid medium by 15% (2). The substrate concentrations used, allowed linear accumulation during the experiment. Accumulation levels never exceeded 50%. Inhibition of

Table 1 Effect of fenpiclonil (0.4 μM) on accumulation of radiolabelled substrates by *Fusarium sulphureum isolate 1743* and incorporation of the radiolabel into macromolecules during 15 min of incubation.

		Accumulation[a]			Incorporation[a]		
	Concentration	Control	Fenpiclonil[b]		Control	Fenpiclonil[b]	
Precursor	μM	%[c]	%[c]		%[d]	%[d]	
[*methyl*-^{3}H]Thymine	-[e]	0.16	0.12	(78)	33.1	33.5	(101)
[2-^{14}C]Uracil	10	4.5	4.2	(93)	5.3	5.6	(106)
[U-^{14}C]Aspartic acid	25	26.7	24.3	(91)	7.9	8.4	(106)
N-Acetyl-[1-^{14}C]glucosamine	10	41.8	31.5	(75)	70.6	83.3	(118)
[2-^{14}C]mannose	200	2.7	2.2	(83)	26.8	14.2	(52)
[1-^{14}C]glucose	200	20.4	14.7	(72)	14.6	8.8	(60)
[6-^{14}C]glucose	200	20.0	14.4	(72)	28.4	18.2	(64)

[a] Mean results of two experiments. Variation in repetitions less than 5%.

[b] Fenpiclonil added 15 min prior to addition of substrates.

[c] Accumulation in mycelium as percentage of label added.

[d] Incorporation in macromolecules as percentage of label accumulated.

[e] [*methyl*-^{3}H]Thymine was added at the highest specific activity available (final concentration 1.7 nM).

accumulation of the various substrates ranged from 7 to 28% (Table 1). The fungicide did not inhibit incorporation of the accumulated substrates thymine, uracil, aspartic acid, and N-acetylglucosamine into DNA, RNA, protein, and chitin, respectively (Table 1). In contrast, incorporation of glucose and especially of mannose into fungal macromolecules was significantly inhibited (Table 1).

In a following experiment, effects of various concentrations of fenpiclonil on accumulation and incorporation of [2-^{3}H]mannose and [U-^{14}C]aspartic acid were tested (Fig. 2). The accumulation of mannose and aspartic acid in untreated mycelium of isolate 1743 was 1.4 and 37% and in untreated mycelium of isolate 1743.1 1.3 and 30% of the label added, respectively. The values for incorporation of mannose and aspartic acid into fungal macromolecules were 0.54 and 1.6% for isolate 1743 and 0.56 and 1.8% for isolate 1743.1, respectively. Fenpiclonil inhibited accumulation of mannose and aspartic acid in isolate 1743 to about the same degree

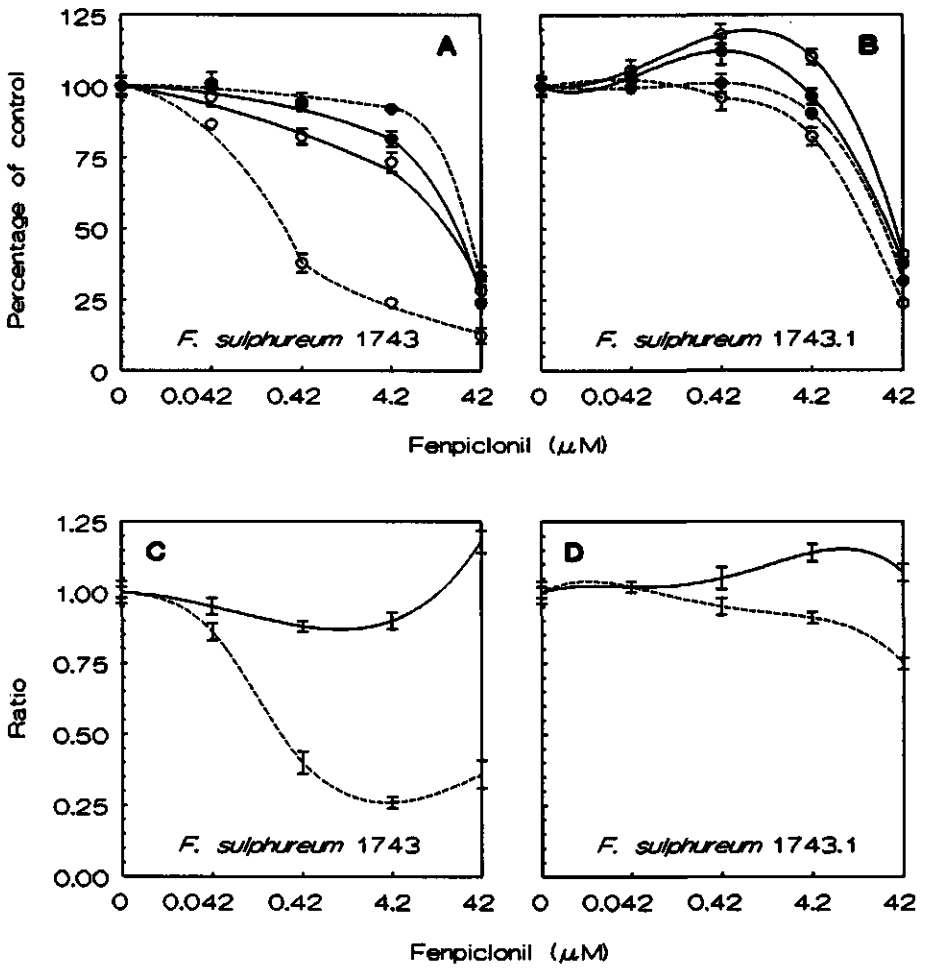

Fig. 2 Effect of fenpiclonil on accumulation (—) and incorporation (---) of [U-^{14}C]aspartic acid (25 μM, •) and [2-^{3}H]mannose (25 μM, ○) in *Fusarium sulphureum* isolates 1743 and 1743.1 in a medium containing cold glucose (100 μM). Figures expressed as percentage relative to the untreated control (Top panels). The 2 bottom panels represent the ratio between accumulated mannose and accumulated aspartic acid (—), and between incorporated mannose and incorporated aspartic acid (- - - -) for isolate 1743 and 1743.1, respectively. Data are mean values of 3 experiments carried out in duplicate.

Table 2 Effect of various fenpiclonil-analogues on radial growth (EC_{50}), accumulation of [2-^{3}H]mannose (25 µM) and [U-^{14}C]aspartic acid (25 µM) in mycelium and incorporation of the radiolabel into macromolecules of *Fusarium sulphureum* isolate 1743 in a medium containing cold glucose (100µM).

Compound		EC_{50}	Aspartic acid[a]		Mannose[a]		Ratio	
X	R	µM	Acc[b]	Inc[c]	Acc[b]	Inc[c]	Inc[d]	%[e]
Control		0	41	4.5	0.72	47	0.187	0
4-C_6H_5	H	> 40	41	4.2	0.83	39	0.189	-1
2,3-Cl_2	CH_3	> 40	42	4.1	0.90	35	0.185	1
4-$N(CH_3)_2$	H	> 40	42	4.3	0.77	42	0.180	5
3-$N(CH_3)_2$	H	> 40	42	4.2	0.84	35	0.168	13
3-OC_6H_5	H	27	41	4.2	0.77	37	0.166	15
H	H	27	41	4.4	0.82	35	0.162	17
2,4-Cl_2	H	14	40	4.8	0.71	40	0.149	26
2,3-Cl_2[f]	H	0.7	38	4.3	0.70	10	0.044	100
2-O(CF_2)O-3	H	0.2	37	4.2	0.69	8	0.038	104

[a] Mean results of four experiments. Variation in repetitions less than 5%.
[b] Accumulation in mycelium as percentage of label added.
[c] Incorporation into macromolecules as percentage of accumulation.
[d] Ratio between mannose and aspartic acid incorporated into macromolecules.
[e] As [c] relative to fenpiclonil.
[f] Fenpiclonil

Table 3 Effect of fenpiclonil (0.4 µM) on the fate of the radiolabel in [U-^{14}C]mannose in *Fusarium sulphureum* isolate 1743 during 15 min of incubation in a medium containing 100 µM glucose[a].

	Control		Fenpiclonil[b]			
Fraction	%[c]	%[c]	%[c]	%[c]	%[d]	%[d]
CO_2	65.2		62.0		95	
$CHCl_3$/MeOH extract	24.0		32.7		136	
PAW extract	2.8		1.8		64	
Protein		2.0		1.4		70
Oligosaccharide		0.8		0.4		50
Residue	8.0		3.5		44	
Accumulation	13.4[e]		10.5[e]		78	

[a] Mean results of three experiments. Variation in repetitions less than 5%.
[b] Fenpiclonil added 15 min prior to addition of [U-^{14}C]mannose.
[c] Incorporation as percentage of radioactivity accumulated.
[d] Effect of fenpiclonil as percentage of control.
[e] Percentage of total radioactivity added.

Table 4 Effect of fenpiclonil (0.4 μM) on the fate of the radiolabel in [U-^{14}C]glucose (100 μM) in *Fusarium sulphureum* isolate 1743 during 15 min of incubation[a].

Fraction	Control		Fenpiclonil[b]			
	%[c]	%[c]	%[c]	%[c]	%[d]	%[d]
CO_2	8.8		4.8		54	
$CHCl_3$/MeOH extract	60.9		77.4		127	
SLS extract	14.2		8.1		57	
Fraction I	4.7		2.6		56	
Protein		2.1		1.5		73
Glycan		2.6		1.1		42
Fraction II	4.9		2.5		52	
Protein		0.6		0.7		116
Glycan		4.3		1.8		43
Fraction III	1.1		1.2		109	
Protein		0.3		0.6		211
Glycan		0.8		0.5		68
Chitin	0.9		1.1		123	
Accumulation	84.0[e]		71.6[e]		85	
Recovery	98.7[e]		99.3[e]			

[a] Mean results of two experiments. Variation in repetitions less than 5%.
[b] Fenpiclonil added 15 min prior to addition of [U-^{14}C]glucose.
[c] Incorporation as percentage of radioactivity accumulated.
[d] Effect of fenpiclonil as percentage of control.
[e] Percentage of total radioactivity added.

Table 5 Effect of fenpiclonil (0.4 μM) on the fate of [2-^{14}C]uracil (10 μM) in *Fusarium sulphureum* isolate 1743 during 15 min of incubation[a].

Metabolite	Control		Fenpiclonil[b]		
	%[c]		%[c]		%[d]
Uracil	1.0	(7.8)	2.2	(19.7)	223
Uridine	0.44	(3.5)	0.18	(1.6)	40
Nucleotides	1.7	(13.1)	1.7	(15.5)	104
RNA	0.77	(6.0)	0.52	(4.6)	67
UDPGlc	2.5	(19.4)	2.4	(20.9)	95
UDPGlcNAc	6.5	(50.3)	4.3	(37.8)	66
Accumulation	12.8		11.3		88

[a] Mean results of two experiments. Variation in replicate values less than 5%.
[b] Fenpiclonil added 15 min prior to addition of uracil.
[c] Percentage of uracil added (percentage of uracil accumulated).
[d] Effect of fenpiclonil (percentage of control).

(Fig. 2A). In contrast, incorporation of mannose into fungal macromolecules was stronger inhibited than incorporation of aspartic acid. The concentration of fenpiclonil which reduced the incorporation ratio by 50% was 0.1 µM (Fig. 2C). Hardly any inhibitory effects on these parameters of the less-sensitive isolate 1743.1 were observed (Fig. 2B and 2D).

Various analogues differing in fungitoxicity from fenpiclonil were tested in the same system. The inhibitory effect on mannose incorporation ranked positively with their toxicity (Table 2).

Effect on composition fungal macromolecules

When mycelium was allowed to accumulate [2-^{14}C]mannose for 15 min, the major part of radioactivity taken up (65%) was recovered in CO_2 released from the culture medium (Table 3). Accumulation of the label in the $CHCl_3/CH_3OH$ fraction, containing the various biosynthetic intermediates, increased upon fenpiclonil treatment. A minor part (10.8%) of the accumulated label in mannose was incorporated into macromolecules. Since mannose in mycelium is mainly present in the sugar side chains of glycoproteins (20), the PAW fraction was isolated. Fenpiclonil (0.4 µM) appeared to inhibit incorporation of the label in mannose into this fraction by 50%. Incorporation of the label in mannose into other mycelial macromolecules (residue) was inhibited by 56%.

Strong acid hydrolysis of the total macromolecule fraction (PAW extract and residue) yielded sugar monomers. The major part (> 70%) was identified as glucose, and a minor part mannose (results not shown).

The effect of fenpiclonil (0.4 µM) on incorporation of the label in [U-^{14}C]glucose into macromolecules was also studied. In this experiment, the main components of the mycelial macromolecular fractions were analyzed by sequential extraction and digestion (11, Table 4). The SLS extract contained water soluble glycans, protein, RNA and DNA (14), fraction I (first NaOH digest) proteins and long chain ß-1,3-glucans (> 12 sugar residues, ß-1,3-glucanase digestion yielded exclusively sugar monomers, results not shown). The predominant part of fraction II (HCl digest) contained short chain glycans. Glycans in fraction III (second NaOH digest) consisted of long chain glycans (probably complex ß-1,3-1,6-glucans) and monosaccharides. The remaining fraction was chitin (11).

Hence, at low growth inhibitory concentrations (0.4 µM), fenpiclonil inhibited incorporation

of glucose into almost all macromolecular fractions (Table 4). The highest reduction was observed in the structural hyphal wall ß-1,3-glucans of fraction I and II.

Glucan synthetase activity

ß-1,3-glucan synthesis is mediated by ß-1,3-glucan synthetases in the plasma membrane (18,22). Inhibition of activity of these enzymes would result in accumulation of its substrate UDPGlc (23). The concentration of UDPGlc was estimated by incubating mycelial suspensions with [2-^{14}C]uracil. After extraction the metabolites were quantified (Table 5). Upon fenpiclonil treatment, the internal uracil concentration considerably increased. The major part of the accumulated uracil was metabolised to UDPGlc and UDPGlcNAc. Upon incubation with fenpiclonil, a reduction of incorporation into these metabolites was noticed.

Cell-free glucan synthetase activity of mycelial extracts was 4.3 nmole min^{-1} mg^{-1} protein. Activity could not be inhibited by fenpiclonil, even not at 40 µM.

DISCUSSION

Fenpiclonil, at a concentration which only caused a marginal growth inhibition (15%), inhibited the incorporation of glucose and mannose into various macromolecules. Incorporation of mannose was stronger inhibited than that of glucose (Table 1). Under similar conditions, accumulation of these sugars and of various other precursors tested was only slightly influenced. This is in contrast to results previously reported, in which high concentrations of fenpiclonil inhibited accumulation of a wide variety of precursors. Results of the present experiments confirm this, since at relatively high concentrations of fenpiclonil, accumulation and incorporation of all substrates became aspecifically inhibited (Fig. 2A and 2B). Therefore, specific effects on sugar metabolism were corrected for aspecific effects by plotting the ratio between accumulated mannose and aspartic acid and between incorporated mannose and aspartic acid. These figures confirm that the accumulation ratio is hardly affected, while the incorporation ratio is significantly reduced (Fig. 2C and 2D). This was not the case with the less sensitive isolate 1743.1. Inhibition of mannose incorporation by various fenpiclonil analogues ranked with their fungitoxicity (Table 2). Therefore, the mode of action of fenpiclonil

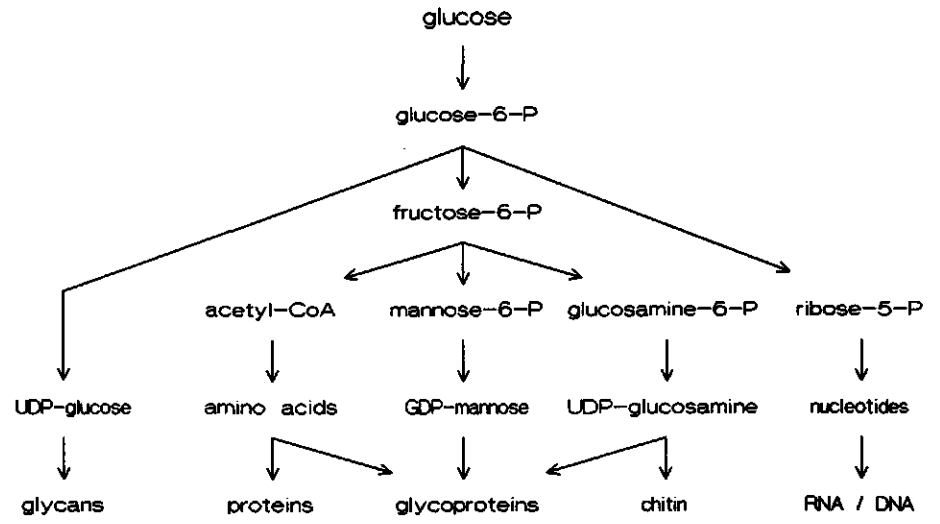

Fig. 3 The main biosynthetic pathways of macromolecules in fungi (4, 5, 10, 11, 20, 24, 33, 36, 39).

may rather be based on incorporation of sugars into macromolecules than on inhibition of accumulation of sugars and amino acids into mycelium as suggested previously (2).

The main biosynthetic pathways for various fungal macromolecules starting from glucose are shown in Fig. 3. One arrow does not necessarily indicate a single enzymatic step and the steps may be reversible. The major part of mannose in mycelium is present in saccharide side chains of glycoproteins (20) and can be marked by feeding mycelium with radiolabelled mannose (14). Incorporation of mannose into glycoprotein sugars was considerably inhibited by fenpiclonil (Table 3). However, the major part of accumulated mannose-6-phosphate apparently isomerized to fructose-6-phosphate and glucose-6-phosphate (24), since a high proportion of accumulated mannose was metabolized to CO_2, and a high proportion of macromolecules consisted of labelled glucose (Table 3). Therefore, experiments with radiolabelled glucose were expected to have similar results. Since glucose accumulated more readily into mycelium than mannose (*c.f.* Table 3 and 4), glucose was used in following experiments.

In these experiments, mycelial macromolecules were isolated and fractionated into water

soluble macromolecules (SLS extract) and cell wall macromolecules (fraction I - III and chitin) according to Sietsma *et al.* (11,25). Cell walls of filamentous fungi primarily consist of glycoproteins and glycans embedded in a crystalline chitin microfibril matrix (26-29). The glycans generally have a ß-1,3 and ß-1,3-1,6 structure (10,11). *F. sulphureum* does not contain ß-1,4-glucans (21). The nature of compounds in the various fractions was partly verified and appeared comparable to data published for *Schizophyllum commune* (11). Fenpiclonil inhibited incorporation of glucose into almost all fractions obtained, but inhibition of incorporation into structural hyphal wall glycans (fractions I and II) was most significant (Table 4). Polymerization of UDPGlc is mediated by glucan synthetases located in the fungal plasma membrane (18,22). However, activity of these enzymes is probably not directly affected by fenpiclonil, since cell-free polymerization could not be inhibited by the fungicide. Furthermore, *in vivo* inhibition of synthetase activity by fenpiclonil would result in accumulation of its precursor, UDPGlc (23). This was not the case (Table 5). In addition, depletion of structural glycans in the hyphal wall would result in morphological changes of the mycelium upon fenpiclonil treatment (30-32). This has not been observed (2).

Enzymes which mediate the polymerization of amino acids to proteins, nucleotides to DNA and RNA, and N-acetylglucosamine to chitin were not inhibited by fenpiclonil (2). Therefore, it is not likely that fenpiclonil interferes with the final polymerization steps of the various biosynthetic pathways, but more likely with early metabolisation steps of glucose. A putative target site may be the phosphorylation of glucose, the first step in its metabolization. Glucose-6-phosphate is the precursor of various biosynthetic routes (Fig. 3, 33-35). Inhibition of glucose phosphorylation would lead to accumulation of non-phosphorylated sugars, which may explain the accumulation of radiolabel in the $CHCl_3/CH_3OH$ extract of fenpiclonil-treated mycelium (Table 3 and 4). It would also explain the reduced levels of glycans (Table 4). A reduction in the glucose-6-phosphate content may also lower the ribose-5-phosphate content and explain the reduced glycosylation of uracil to uridine (Table 5). The normal biosynthetic route for pyridine nucleotides involves glycosylation of orotate to orotidylate before decarboxylation to uridine takes place (36,37). A decrease in ribose-5-phosphate content may inhibit this route as well and would also result in reduced levels of uridine and UDP-activated sugar donors. Hence, inhibitory effects of fenpiclonil on initial steps of glucose metabolism may effect biosynthesis of glycans in various ways (38,39).

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Chapter 6

Effect of fenpiclonil on phosphorylation of glucose in *Fusarium sulphureum* (Schlecht)

Ad B.K. Jespers
Maarten A. de Waard

Experimental Mycology: submitted

ABSTRACT

The phenylpyrrole fungicide fenpiclonil inhibited the metabolism of glucose in mycelium of *Fusarium sulphureum* (Schlecht) at a concentration which only slightly inhibited mycelial growth (EC_{15}). At the same concentration, fenpiclonil also inhibited accumulation and, to a higher extent, phosphorylation of 2-deoxy[U-^{14}C]glucose in starved mycelium loaded with cold 2-deoxyglucose. Fenpiclonil did not affect cell-free phosphorylation of 2-deoxyglucose and the ATP content of mycelium. Therefore, the primary mode of action of the fungicide may be based on inhibition of transport-associated phosphorylation of glucose. This may cause a cascade of metabolic events which eventually lead to fungal growth inhibition and death. One major event affected by inhibition of transport-associated phosphorylation is the accumulation of neutral polyols, such as glycerol and mannitol in mycelium. This was not observed in an osmotically sensitive, fenpiclonil-resistant laboratory isolate of the fungus.

INTRODUCTION

Fenpiclonil is the first phenylpyrrole fungicide developed by CIBA-GEIGY A.G., Basel (1). At a concentration (4 μM) which inhibit growth of *Fusarium sulphureum* by about 50%, the fungicide instantaneously inhibits accumulation of substrates such as amino acids and monosaccharides (2). At a concentration (0.4 μM) which causes only growth inhibition of about 15%, the incorporation of radiolabelled glucose and mannose into macromolecules of *F. sulphureum* was significantly inhibited. The highest inhibitory effect was found for incorporation of glucose into hyphal wall glycans (3). Fenpiclonil also inhibited incorporation of [^{14}C] in glucose into other fungal macromolecules such as proteins. Therefore, fenpiclonil does probably not interfere with synthesis of various macromolecules, but more likely in early events such as phosphorylation of glucose (3). In the present study, this hypothesis was investigated by assessing fenpiclonil-induced effects on glucose metabolism in *F. sulphureum*.

MATERIALS AND METHODS

Organism, culture methods and sample preparation

F. sulphureum isolate 1743 was obtained from the fungal collection of the Research Institute for Plant Protection (IPO) in Wageningen. The less fenpiclonil-sensitive isolate 1743.1 originated from a fast growing sector in a culture of isolate 1743 on PDA amended with fenpiclonil (4 μM). Reduced sensitivity to fenpiclonil correlates with high sensitivity to osmotic stress and loss of pathogenicity (2).

The isolates were weekly subcultured on potato dextrose agar (PDA) at 20°C. Czapek Dox liquid media were inoculated with macroconidia, obtained from two-week-old cultures on PDA, to a final concentration of 2×10^5 conidia ml^{-1}. Cultures were incubated in a rotary shaker at 120 rpm and 20°C for 2 days.

Mycelium of *F. sulphureum* isolate 1743 was harvested and washed with demineralised water by vacuum filtration over filter paper in a Büchner funnel. Standard mycelial suspensions were obtained by resuspending washed mycelium in Czapek Dox liquid medium without glucose and by starvation for 1 hr in a rotary shaker at 120 rpm and 20°C. Then, fenpiclonil was added from 200-fold concentrated stock solutions in methanol. Equivalent amounts of methanol were added in control treatments. All experiments were performed at least two times in duplicate in a rotary shaker at 120 rpm and 20°C.

Chemicals

Czapek Dox liquid medium was purchased from Oxoid Ltd (London, UK). PDA and TLC plates (Silicagel 60 F_{254} and PEI-cellulose F_{254}) were obtained from Merck (Darmstadt, FRG). Radiolabelled [1-^{14}C]acetate (sp. act. 1.97 GBq $mmol^{-1}$), 2-deoxy-D-[U-^{14}C]glucose (sp. act. 1.96 GBq $mmol^{-1}$), [1-^{14}C]glucose (sp. act. 2.1 GBq $mmol^{-1}$), [6-^{14}C]glucose (sp. act. 2.0 GBq $mmol^{-1}$), D-[U-^{14}C]glucose (sp. act. 10.8 GBq $mmol^{-1}$) were obtained from Amersham International Ltd (Amersham, United Kingdom). Aqualuma was purchased from Lumac BV (Schaesberg, The Netherlands). Technically pure fenpiclonil (98.49%, CGA 142705) was a gift of Ciba-Geigy AG (Basel, Switzerland). All other chemicals were of technical grade.

Incubation of mycelium with sugars

After pre-incubation of standard mycelial suspensions with fenpiclonil for 15 min, [U-^{14}C]glucose (100 µM; 7500 Bq ml^{-1}) or 2-deoxy[U-^{14}C]glucose (2-D[U-^{14}C]G; 10 µM; 7500 Bq ml^{-1}) mixed with glucose (100 µM) was added from 200-fold concentrated stock solutions. Metabolism of radiolabelled monosaccharides was assessed after 15 min of incubation in a rotary shaker at 120 rpm and 20 °C. Non-radioactive treatments were performed under the same conditions in a medium containing 5 mM glucose.

After incubation, mycelium was collected on Whatman GF/A filters, washed with ice-cold demineralised water and resuspended in ice-cold water (0.5 ml). Subsequently, the water soluble metabolites of low molecular weight (low Mr) were extracted by adding ice-cold $CHCl_3/CH_3OH$ (1/1, v/v; 3 ml) within 30 sec (3). This procedure yielded homogeneously dispersed mycelium as a result of which mechanical disruption was not necessary. Mycelial residues were washed twice with 3 ml $CHCl_3/CH_3OH/H_2O$ (1/1/0.3, v/v/v). The $CHCl_3$ containing extracts were combined and mixed with water (3 ml). The resulting upper CH_3OH/H_2O phase was collected by aspiration and CH_3OH was evaporated under a stream of nitrogen at 30°C. Resulting solutions in water were described as the low Mr extract and stored at -20°C. The $CHCl_3$ phase was discarded. The reduction of radiolabel in the external medium after incubation was used to calculate the amount of glucose accumulated by mycelium. Radiolabel in carbon dioxide was trapped by filter paper soaked in KOH (1 N) present in a central well of Erlenmeyer flasks (4).

Decarboxylation of glucose and acetate radiolabelled at different carbon positions, was studied in standard mycelial suspensions at final concentrations of 100 and 10 µM, respectively (5).

Steady state accumulation of 2-DG was studied in standard mycelial suspensions (10 ml) preincubated with cold 2-DG (10 µM) and acetate (1 mM) for 30 min. Immediately after addition of fenpiclonil, 2-D[U-^{14}C]G (< 1 nM, 500 Bq ml^{-1}) was added. At intervals, samples were collected and fractionated as described before (6, 7). Aliquots (0.4 ml) of all fractions were mixed with Aqualuma Plus (4 ml) and equilibrated for 15 hr. Radioactivity was determined with a Beckman LS6000 TA scintillation counter (windows 0-670). The system was calibrated with quenched [^{14}C] standards.

Fractionation of extracts

Cation exchange columns (AG 50W-X8, 100-200 mesh, 0.4 × 2 cm) were converted to the hydrogen form with 5 N HCl (10 ml). Similarly, anion exchange columns (AG 1-X8, 100-200 mesh) were converted to their hydroxide form with 5 N NaOH (10 ml). Columns were neutralized by elution with deionized water (20 ml).

Low Mr extracts were fractionated by sequential elution over cation and anion exchange columns. Neutral carbohydrates do not bind to either of the columns. Cationic and anionic carbohydrates were eluted with ammonia (10%, w/v) and TFA (5 N) from the cation and anion exchange columns, respectively (8). All fractions were lyophilized and redissolved in a small volume of water.

Separation of neutral carbohydrates

Neutral carbohydrates were separated by isocratic HPLC on an Aminex HPX-87P column (300 × 7.8 mm). After injection (100 µl), the column was eluted with deionized water at a flow rate of 0.6 ml min^{-1}. Eluted carbohydrates were quantified with a Waters 410 differential refractive index detector. Radioactive fractions were quantified with a Radiomatic Flo-One\Beta A200 radiodetector. Compounds were tentatively identified by comparison of their retention time with those of standard trehalose, glucose, glycerol and mannitol (9).

Separation of sugar phosphates

Anionic carbohydrates and sugar phosphates in the low Mr fraction were separated from other compounds by liquid DEAE-Sepharose anion exchange chromatography. The column (70 × 5 mm) was equilibrated with 0.01 M lithium formate buffer, pH 3.5 (10 ml), loaded with sample (1 ml), and eluted with a linear gradient from 0.01 to 0.15 M lithium formate buffer, pH 3.5.

In experiments on the metabolism of 2-DG, a rapid quantitative separation of 2-DG from 2-DG6P in the low Mr fraction (1 ml) was achieved with a smaller DEAE-Sepharose column (20 × 5 mm) and successive elutions with 0.01 M lithium formate buffer, pH 3.5 (4 ml) and 6 N HCOOH (3 ml).

Sugar phosphates in the various fractions were also separated with TLC using silica gel plates and ethyl acetate/2-propanol/water (6/3/1, v/v/v) as solvent system (10). Radioactive

spots were visualized by autoradiography. Compounds in spots were identified by comparison of their Rf value with those of authentic standards.

Cell-free 2-DG phosphorylation

Washed mycelium was suspended in disruption buffer (50 mM HEPES, pH 7.5, 25 mM $MgSO_4$, and 10 mM EGTA) at a density of 0.1 g wet wt ml^{-1} and disrupted four times at 0°C for 30 sec with 30 sec intervals in a Bead-Beater (Biospec Products, Bartlesville, OK (11)). Cell debris was removed by centrifugation at 5,000g for 5 min (twice). The phosphorylation assay mixture (100 µl) contained 2-D[U-^{14}C]G (100 µM, 500 Bq ml^{-1}), Na_2ATP (5 mM) and protein (1 mg ml^{-1}) in disruption buffer. The reaction was started with the addition of Na_2ATP. In treatments, fenpiclonil was added 15 min prior to the start of the experiment. Samples were incubated at 25°C for 15 min. Reactions were stopped with 1 ml $CHCl_3/CH_3OH$ (1/1, v/v). Reaction products were analyzed as described above.

RESULTS

Metabolism of glucose

Standard mycelial suspensions accumulated 65% of [U-^{14}C]glucose added within 15 min (Table 1). The accumulation rate was linear in time. Fenpiclonil (0.4 µM) reduced accumulation by 26%. Fractionation of mycelium demonstrated that fenpiclonil most strongly reduced the amount of radiolabel into the methanol insoluble macromolecular fraction. Fenpiclonil also inhibited the incorporation of radiolabel into carbon dioxide and in the anionic and cationic fractions. The incorporation of [^{14}C] in glucose into the neutral fraction increased upon fenpiclonil treatment.

Neutral carbohydrates

Standard mycelial suspensions of both isolates 1743 and 1743.1 were incubated with cold glucose for 2 hr. Neutral carbohydrates from mycelial extracts of both isolates were separated by HPLC with an Aminex HPX-87P column (Fig. 1). These carbohydrates primarily consisted of mannitol and trehalose (Fig. 1; Table 2). Mycelium of isolate 1743 also contained low

Table 1 Effect of fenpiclonil on incorporation of the radiolabel in [U-^{14}C]glucose into various fractions of *Fusarium sulphureum* isolate 1743 and on [^{14}C]carbondioxide production during 15 min of incubation[a].

Fraction	Control		Fenpiclonil (0.4 μM)[b]			
	%[c]		%[c]		%[d]	
Accumulated	64.6		48.1		74	
Carbon dioxide	6.3	(9.8)	3.6	(7.5)	57	(77)
Anions	3.1	(4.9)	1.6	(3.3)	51	(69)
Cations	20.5	(31.7)	11.2	(23.3)	55	(74)
Neutral	22.2	(34.3)	26.7	(55.4)	120	(162)
Macromolecules	12.5	(19.3)	5.0	(10.4)	40	(54)

[a] Experiments repeated three times. Incorporation of radiolabel into the various fractions varied between experiments (results of one representative experiment is shown), but the effect of fenpiclonil on the incorporation never varied more than 5%.

[b] Fenpiclonil added 15 min prior to addition of [U-^{14}C]glucose (100 μM; 8500 Bq sample^{-1}).

[c] Percentage of radiolabel added (percentage of radiolabel accumulated).

[d] Percentage of control.

amounts of glycerol and only trace amounts of glucose. Isolate 1743.1 contained a relative low amount of mannitol and hardly any glycerol and glucose. Upon fenpiclonil treatment (0.4 μM), the glycerol and mannitol content in isolate 1743 considerably increased, whereas these effects were not observed in isolate 1743.1.

The radiolabel in [U-^{14}C]glucose incorporated into the neutral carbohydrate fraction, appeared to be present in glucose and mannitol (Fig. 1; Table 3). Upon fenpiclonil treatment, the concentration of these compounds increased. An additional yet unidentified peak eluted 90 s before mannitol (Fig. 1). Incorporation of label into glycerol was not observed.

Accumulation and metabolism of glucose

[1-^{14}C]Glucose, [6-^{14}C]glucose, and [U-^{14}C]glucose accumulated into mycelium to about the same level (Table 4). [^{14}C]Carbon dioxide production with these substrates differed

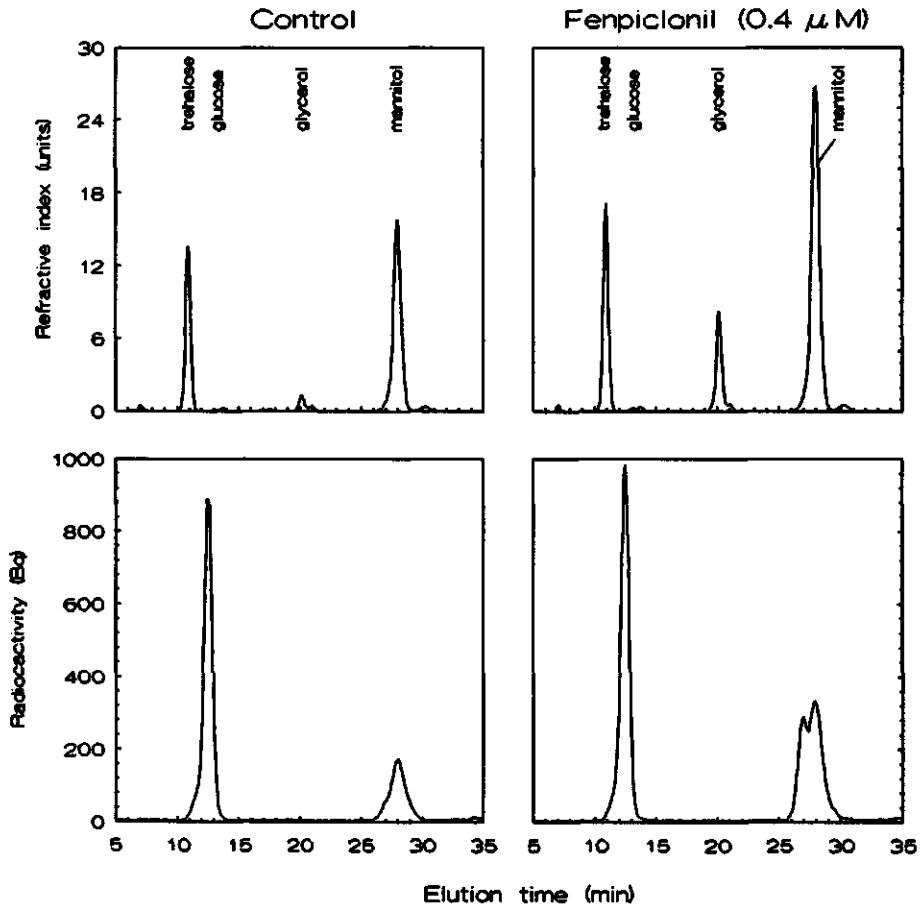

Fig. 1 Effect of fenpiclonil (0.4 μM) on elution profiles of neutral carbohydrates in mycelium of *Fusarium sulphureum* isolate 1743 after 2 hr incubation with cold glucose (top panels) and [U-^{14}C]glucose (bottom panels). Reference compounds eluted at the time indicated. Neutral carbohydrates separated on an Aminex HPX-87P HPLC column (300 × 7.8 mm, mobile phase water, flow rate 0.6 ml min^{-1}) and detected by measuring the refractive index (top panels) or radioactivity (bottom panels).

Table 2 Effect of fenpiclonil on the content of neutral carbohydrates (mM) in mycelium of *Fusarium sulphureum* isolates 1743 and 1743.1, 2 hr after incubation with cold glucose (100 μM).

	Isolate 1743 Fenpiclonil (μM)			Isolate 1743.1 Fenpiclonil (μM)		
	0	0.42	4.2	0	0.42	4.2
Trehalose	121	148	97	134	116	103
Glucose	6	11	7	1.7	2.1	1.3
Glycerol	50	311	216	1.6	2.1	2.3
Unidentified	14	16	8	4.8	2.9	7.2
Mannitol	463	766	526	269	241	237
Total	654	1251	854	411	365	351
Recovery[a]	98	98	98	94	93	95

[a] Data calculated from a representative experiment shown in Fig. 1 (top panels).

Table 3 Effect of fenpiclonil on incorporation of radiolabel in [U-^{14}C]glucose into glucose and mannitol of *Fusarium sulphureum* isolate 1743 after 15 min of incubation.

	Control		Fenpiclonil (0.4 μM)	
Carbohydrate	Bq	%[a]	Bq	%[a]
Glucose	3092 ± 102	75	4064 ± 115	58
Mannitol[b]	1007 ± 77	25	2904 ± 101	42

[a] Percentage of total carbohydrates. Data calculated from a representative experiment shown in Fig. 1 (bottom panels).

[b] Also includes an unidentified compound in the fenpiclonil treatment.

Table 4 Effect of fenpiclonil (0.4 μM) on accumulation of glucose (100 μM) or acetate (10 μM) in mycelium of *Fusarium sulphureum* isolate 1743 and on [^{14}C]carbon dioxide production after 15 min of incubation[a].

Substrate	Accumulation (%[b])			[^{14}C]Carbon dioxide (%[c])		
	Control	Fenpiclonil[d]		Control	Fenpiclonil[d]	
[1-^{14}C]glucose	62.9 ± 0.5	47.3 ± 0.9	(75)	10.3 ± 0.4	4.9 ± 0.2	(47)
[6-^{14}C]glucose	64.4 ± 0.1	45.1 ± 1.2	(70)	1.4 ± 0.1	0.6 ± 0.1	(43)
[U-^{14}C]glucose	63.8 ± 0.7	45.6 ± 0.5	(72)	6.1 ± 0.2	2.7 ± 0.2	(45)
[1-^{14}C]acetate	78.6 ± 0.6	68.1 ± 0.7	(87)	6.0 ± 0.5	6.1 ± 0.6	(103)

[a] Mean results of four experiments.
[b] Percentage ± S.E. of label added.
[c] Percentage of radiolabel accumulated.
[d] Fenpiclonil added 15 min prior to addition of radiolabelled substrates (percentage of control).

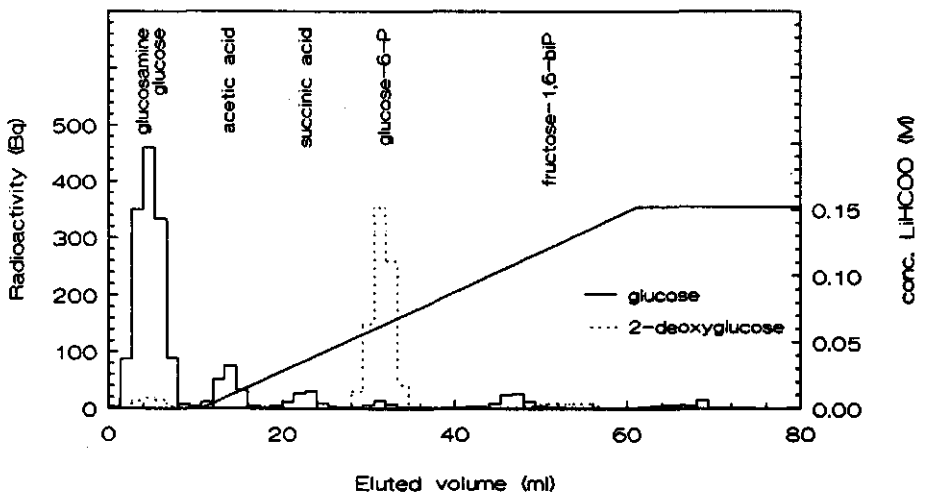

Fig. 2 Elution profiles of the low molecular weight fraction from mycelium of *Fusarium sulphureum* isolate 1743 incubated with [U-^{14}C]glucose (100 μM) or 2-deoxy[U-^{14}C]glucose (10 μM) for 15 min on DEAE-Sepharose column (70 × 5 mm; flow rate 15 ml hr^{-1}; eluent: lithium formate gradient, pH 3.5). Reference compounds eluted as indicated.

Table 5 Effect of fenpiclonil on accumulation of the radiolabel in [U-^{14}C]glucose (100 μM) and 2-D[U-^{14}C]G (10 μM) into mycelium of *Fusarium sulphureum* isolate 1743 after 15 min of incubation[a].

Sugar	Control	Fenpiclonil (μM)			
		0.42		4.2	
Glucose	64.3 ± 0.4	54.6 ± 0.5	(85)	42.4 ± 0.4	(66)
2-DG	22.0 ± 0.2	16.3 ± 0.2	(74)	13.6 ± 0.2	(62)

[a] Mean results of four experiments. Percentage of label added ± S.E. (percentage of control).

Table 6 Effect of fenpiclonil (0.4 μM) on accumulation and metabolism of 2-D[U-^{14}C]G in mycelium of *Fusarium sulphureum* isolate 1743 loaded with 2-DG (10 μM) for 30 min[a].

Metabolite	Incubation time (min)					
	1			5		
	Control	Fenpiclonil		Control	Fenpiclonil	
Accumulation	22.6 ± 0.6	20.0 ± 0.8	(88)	62.5 ± 1.6	50.8 ± 2.0	(81)
2-DG[b]	5.6 ± 0.2	7.8 ± 0.2	(138)	8.8 ± 0.5	11.5 ± 0.7	(131)
2-DG6P[b]	15.8 ± 0.4	11.8 ± 0.5	(75)	50.5 ± 1.4	37.4 ± 1.5	(74)
Macromolecules	1.2 ± 0.1	0.4 ± 0.1	(33)	3.3 ± 0.4	1.9 ± 0.4	(59)

[a] Figures indicate percentage of label added (5000 Bq sample^{-1}) ± S.E.; in brackets percentages relative to control.

[b] 2-DG and 2-DG6P separated on a DEAE-Sepharose column (20 × 5 mm).

significantly, but the inhibitory effect of fenpiclonil (0.4 μM) on [^{14}C]carbon dioxide production was similar for all substrates tested. Production of [^{14}C]carbon dioxide was stronger inhibited than accumulation of the radiolabel in mycelium. Accumulation of [1-^{14}C]acetate was also inhibited by fenpiclonil, but [^{14}C]carbon dioxide production was not affected.

The low Mr fraction obtained from standard mycelial suspensions of isolate 1743 incubated with [U-^{14}C]glucose (100 μM) for 15 min was separated by DEAE-Sepharose chromatography with a lithium formate gradient. The neutral and cationic carbohydrates eluted in the void volume and comprised the major part of the low Mr fraction (Fig. 2). Monovalent (acetic acid) and divalent organic acids (succinic acid) eluted shortly after the void volume, while sugar phosphates eluted at higher lithium formate concentrations. Upon fenpiclonil treatment, the amount of label in the void volume (mainly neutral carbohydrates) increased (Table 1). The other fractions such as the one containing glucose-6-P showed lower radioactivity upon fenpiclonil treatment (results not shown).

Metabolism of 2-DG

In order to study the effect of fenpiclonil on phosphorylation of glucose, standard mycelial suspensions were incubated with [U-^{14}C]glucose (100 μM) or 2-D[U-^{14}C]G (10 μM). The inhibitory effect of fenpiclonil on accumulation of the radiolabel was slightly stronger for 2-D[U-^{14}C]G than for [U-^{14}C]glucose. 2-D[U-^{14}C]G accumulated slower than [U-^{14}C]glucose (Table 5). Separation of 2-DG and 2-DG metabolites in low Mr fractions by DEAE-Sepharose (70 × 5 mm columns) revealed two radiolabel-containing fractions: one in the void volume and one eluting after 32 ml (Fig. 2). TLC analysis showed that the fraction in the void volume contained 2-D[U-^{14}C]G and the second one 2-D[U-^{14}C]G6P. Upon fenpiclonil treatment, accumulation of 2-D[U-^{14}C]G was reduced, but the

Table 7 Effect of fenpiclonil on phosphorylation of 2-D[U-^{14}C]G in mycelial extracts from *Fusarium sulphureum* isolate 1743 after 5 min of incubation[a].

Fenpiclonil μM	Phosphorylated %	
0	43.8 ± 0.9	(100)
4.2	43.8 ± 0.7	(100)
42	42.0 ± 1.0	(96)
420	41.3 ± 0.6	(94)

[a] Mean results of four experiments. Percentage of label added ± S.E. (percentage of control).

ratio between 2-D[U-^{14}C]G and 2-D[U-^{14}C]G6P did not change considerably (results not shown).

In order to study phosphorylation of 2-DG in more detail, accumulation of 2-D[U-^{14}C]G was studied in mycelium loaded with cold 2-DG. This was achieved by incubation of standard mycelial suspensions with 2-DG (10 μM) for 30 min. By that time 95% of the 2-DG added had accumulated and no net accumulation took place any more. Under these conditions, the addition of a negligible amount of 2-D[U-^{14}C]G (< 1 nM) resulted in a linear accumulation of the label in time for about 5 min. The predominant sugars 2-DG and 2-DG6P in the low Mr extracts were quantitatively separated on small DEAE-Sepharose columns (20 × 5 mm). Upon fenpiclonil treatment, the concentration of 2-DG increased, whereas the incorporation of the label into 2-DG6P decreased (Table 6).

Phosphorylation of 2-DG was also studied in a cell-free system. Fenpiclonil only slightly inhibited phosphorylation at high concentrations (Table 7).

DISCUSSION

The prototrophic fungus *Fusarium sulphureum* is able to grow on a minimal medium containing glucose and mineral salts. The main metabolic pathways of glucose are depicted in Fig. 3. Elimination of the carbons in glucose by decarboxylation is mediated by enzymes operating in various routes. The amount of carbon dioxide released is indicative for the relative importance of the various metabolic pathways (12, 13). Carbons 3 and 4 in glucose are decarboxylated upon oxidation of pyruvate by pyruvate dehydrogenase. The other carbons may be eliminated in the TCA-cycle. A relatively high [^{14}C]carbon dioxide production with [1-^{14}C]glucose as the substrate indicates an active pentose phosphate pathway. This was the case in the present experiments (Table 4). The pentose phosphate pathway is stimulated by nitrate and intensive aeration (14). These conditions were used in the present experiments. Upon fenpiclonil treatment, inhibition of [^{14}C]carbon dioxide production from [1-^{14}C]glucose and [6-^{14}C]glucose was similar (Table 4). Therefore, the fungicide does not interfere specifically with the pentose phosphate pathway. Pyruvate dehydrogenase was also not affected, as the release of [^{14}C]carbon dioxide from [U-^{14}C]glucose was not different from [6-^{14}C]glucose

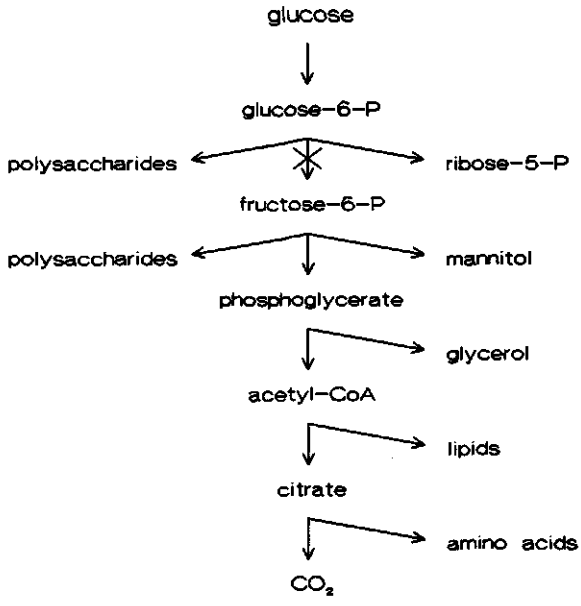

Fig. 3 Main metabolization pathways of glucose in fungal mycelium. Metabolization of 2-deoxyglucose does not proceed after the step indicated (×).

(Table 4). Fenpiclonil did not inhibit the generation of [^{14}C]carbon dioxide from externally added [1-^{14}C]acetate. Therefore, the fungicide does not inhibit a step in the TCA-cycle, as is the case with carboxin (15). Hence, results suggest that the fungicide inhibits initial steps of glucose metabolism. This hypothesis would explain the inhibitory effect of fenpiclonil on incorporation of radiolabelled glucose in macromolecules as well (3).

Metabolism of 2-DG was used to study early effects of fenpiclonil in glucose metabolism. Like glucose, 2-DG is rapidly phosphorylated into 2-DG6G. Isomerization to 2-deoxyfructose can not take place. Other metabolic conversions such as dehydrogenation and polymerization occur, but at low rates (16). The accumulation rate of 2-DG appeared to be lower than that of glucose, but the inhibitory effect of fenpiclonil (0.4 μM) was slightly higher (Table 5). These results confirm the suggestion that fenpiclonil inhibits metabolism of glucose during initial steps.

Starved mycelium accumulates and phosphorylates 2-D[^{14}C]G very rapidly (Fig. 2). Effects on these processes can be studied more effectively under equilibrium conditions (17). Therefore, mycelium was loaded with cold 2-DG until no net accumulation occurred. Acetate was used as the energy source, since its metabolism was not inhibited by fenpiclonil (Table 4). Upon addition of 2-D[^{14}C]G, the major part of accumulated 2-D[^{14}C]G was phosphorylated within 1 min. Upon fenpiclonil treatment, the proportion of intracellular 2-DG increased, while the proportion of 2-DG6P proved to be considerably reduced (Table 6). Therefore, fenpiclonil appears to inhibit the conversion of 2-DG to 2-DG6P. Since phosphorylation of glucose is mediated by the same enzyme, fenpiclonil is most probably also an inhibitor of glucose phosphorylation.

Sugar transport systems in filamentous fungi are well studied in *Neurospora crassa* (18). This fungus has a low-affinity facilitated diffusion system and a repressible high-affinity active transport system. Normal or starved hyphae maintain a high resting membrane potential (-160 to -250 mV), which energize the latter transport system (19). Like *Aspergillus nidulans*, *F. sulphureum* has a much lower membrane potential ranging from -80 to -50 mV (3, 18, 20). The main accumulation system in these fungi seems to be transport-associated phosphorylation of glucose (17). It is possible that fenpiclonil inhibits this process (Table 6). A direct effect on activity of hexokinase is not likely since inhibition of cell-free phosphorylation was not observed (Table 7). At the low fenpiclonil concentrations used, a decrease in ATP content was also not detected (3). Bisson *et al.* proposed that cytosolic hexokinase can be associated with transmembrane sugar carriers, which behaves like a transport-associated phosphorylation complex (Fig. 4, 22). Inhibition of the activity of this complex may be a consequence of the high accumulation rate of fenpiclonil into membranes (23) and can result in a gradual reduction of the glucose-6-phosphate content in mycelium. This effect leads to a gradual starvation of the fungus, and result in a steady reduction in oxygen consumption in time upon prolonged incubation with the fungicide as described previously (2). Reduction of the glucose-6-phosphate content of mycelium upon fenpiclonil treatment may also explain the inhibition of synthesis of macromolecules such as glycans. This implicates that inhibition of glucose phosphorylation starts a cascade of metabolic events culminating in the toxic action of the fungicide.

Another metabolic event following inhibition of glucose phosphorylation, is the increase in content of neutral sugars (Table 2). The osmotic consequences are significant and can

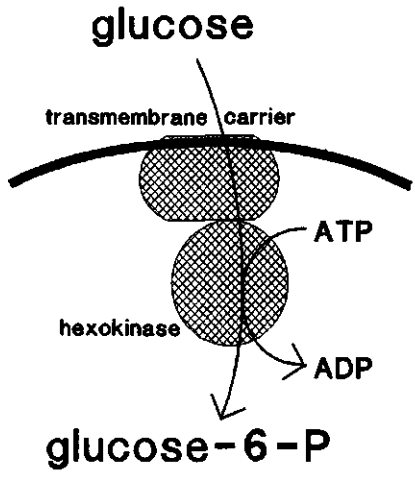

Fig. 4 Model of transport-associated phosphorylation of glucose in fungi (after Bisson; 22).

contribute to the toxic effect of fenpiclonil. In this context, it is striking to note that the resistant strain does not accumulate polyols upon fenpiclonil treatment and hence may escape this toxic effect. A different osmoregulation may be a common feature in the modes of resistance to phenylpyrroles and dicarboximide fungicides (24).

Under laboratory conditions both phenylpyrrole and dicarboximide fungicides readily select for resistant mutants such as isolate 1743.1. These mutants are sensitive to osmotic stress, cross-resistant to both types of fungicides, and not pathogenic (25). Based on these observations, it was hypothesised that both classes of fungicides have similar modes of action (26). In practice, *B. cinerea* acquired resistance to dicarboximides after a few years of commercial use. However, these resistant isolates are not osmotically sensitive, not cross-resistant to phenylpyrroles, and suffer only from a minor fitness penalty (27). This makes the two classes of fungicides biologically different. Resistance to these fungicides in *B. cinerea* is due to allelic mutations at the *daf*-1 locus (29, 29). In *Neurospora crassa*, osmotic sensitive mutants (*i.e.* *os*-1, *os*-2, *os*-4, *os*-5, *smco*-8, and *smco*-9) showed a reduced sensitivity to dicarboximides (30), but mutants selected on medium amended with vinclozolin, exclusively contained an allelic mutation at the *os*-1 locus (31). Hence, the *daf*-1 locus of *B. cinerea* and the *os*-1 locus

of *N. crassa* are presumably homologous. The *os*-1 mutant is osmotically sensitive, as it does not retain compounds such as mannitol and glycerol, which counteract the imposed osmotic pressure (24, 32). This corroborates with our observation that the fenpiclonil-resistant *F. sulphureum* isolate 1743.1 has a lower content of the same polyols (Table 2). This finding indicates a similar resistance mechanism in the fungal isolates, but does not necessarily indicate a similar mode of action of both classes of fungicides. This hypothesis could not be tested, since *F. sulphureum* is insensitive to dicarboximides.

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Chapter 7

GENERAL DISCUSSION

GENERAL DISCUSSION

In the last few decades plant disease control has become heavily dependent on fungicides (Chapter 1). This development was favoured by the introduction of several classes of fungicides with specific modes of action, such as benzimidazoles, phenylamides, dicarboximides and sterol biosynthesis inhibitors (1). A disadvantage of these chemicals is the potency of target organisms to acquire resistance. This condition urged the agrochemical industry to develop chemicals with new modes of action (Chapter 2). Examples of such chemicals are the highly active phenylpyrrole fungicides fenpiclonil (CGA 142705) and fludioxonil (CGA 173506) commercially developed by CIBA-GEIGY AG, Basel. They are analogues of the antifungal antibiotic pyrrolnitrin produced by several *Pseudomonas* species.

In general, understanding of the mode of action of new fungicides is not required in initial stages of their development. However, it becomes important when the fungicides reaches commercial use. At first, to ascertain the novelty of the site of action. The attractiveness of new compounds is considerably reduced if they would have a mode of action similar to that of existent commercial products. Secondly, to develop *in vitro* bioassays to measure the intrinsic activity of the compound. In this way, data can be generated for use in quantitative structure activity relationships (QSAR) and computer modelling studies. Such data are of importance in the rational design of new inhibitors based on the lead compound (1). Knowledge on the mode of action of fungicides is also useful to evaluate the risk for resistance development and to understand the selective action against target and non-target organisms. Therefore, the mode of action of fenpiclonil was studied in the project described in this thesis.

The mode of action of fenpiclonil was studied by a biochemical approach in which the sensitivity of putative sites of action was analysed in a systematic step-by-step procedure. Toxicity tests revealed fenpiclonil as an effective inhibitor of mycelial growth of many imperfect fungi at concentrations below 4 μM. *F. sulphureum* isolate 1743 was selected as a sensitive test organism. This fungus is easy to handle in biochemical studies. A seed treatment with fenpiclonil is recommended for control of this pathogen in cereals (2). Culturing *F. sulphureum* on PDA amended with fenpiclonil (4 μM) allowed the development of sectorial growth. Isolates from such sectors proved to be resistant to fenpiclonil. In some experiments,

the representative fenpiclonil-resistant isolate 1743.1 was used to compare the biochemical effects of fenpiclonil in sensitive and resistant isolates.

Laboratory isolates of fungi resistant to phenylpyrrole and dicarboximide fungicides are sensitive to osmotic stress, cross-resistant to both types of fungicides, and not pathogenic (3). Based on these observations, it was hypothesised that both classes of fungicides have similar modes of action (4). In practice, *Botrytis cinerea* acquired resistance to dicarboximides after a few years of commercial use. However, these resistant isolates are not osmotically sensitive, not cross-resistant to phenylpyrroles, and suffer only from a minor fitness penalty (5). This suggests that the two types of resistant isolates are biologically dissimilar. In all isolates, resistance is based on allelic mutations at the *daf*-1 locus (6, 7). In *Neurospora crassa*, osmotic sensitive mutants (*i.e. os*-1, *os*-2, *os*-4, *os*-5, *smco*-8, and *smco*-9) showed a reduced sensitivity to dicarboximides (8), but mutants selected on medium amended with lethal concentrations of vinclozolin, contained exclusively one allelic mutation at the *os*-1 locus (9). Hence, the *daf*-1 locus of *B. cinerea* and the *os*-1 locus of *N. crassa* are presumably homologous.

Fenpiclonil instantaneously accumulated into mycelium of *F. sulphureum* to the maximum level attainable. The accumulation could not be influenced by various compounds affecting metabolic activity of mycelium. The accumulation level was positively correlated with the amount of mycelial lipid in the culture medium. Therefore, binding of fenpiclonil to cell components is probably not significant. Accumulation appears to be a physico-chemical distribution of the fungicide between lipids in mycelium and extracelluar medium. This is the case with many other classes of fungicides, such as the benzimidazoles (10), chlorinated nitrobenzenes (11), and dodine (12).

For the fenpiclonil-related antibiotic pyrrolnitrin it has been suggested that it interacts with membrane phospholipids (13). Fenpiclonil neither affected membrane fluidity of artificial asolectin liposomes, nor transport of aspartic acid into isolated bacterial vesicles of *Bacillus subtilis* (Chapter 3). Therefore, the fungicidal action of fenpiclonil is not likely based on an aspecific interaction of the fungicide with the phospholipid bilayer. Such an aspecific interaction would also imply a lack of selective fungitoxicity. Since this is not the case, a putative action of fenpiclonil on fungal membranes should be more specific.

In the first experiments on the effect of fenpiclonil on metabolic processes of *F. sulphureum*, concentrations above the EC_{50} value (>4 μM) of the fungicide were used. At

early steps, oxygen consumption and ATP levels in mycelium were not affected. Ergosterol biosynthesis became slightly inhibited, but insignificantly as compared with specific ergosterol biosynthesis inhibitors (14). Effects of fenpiclonil on the composition of other lipids were even smaller. Increased lipid peroxidation as reported for dicarboximide treatment of fungal mycelium (15) was not observed. Other main biosynthetic processes such as DNA, RNA protein and chitin synthesis were also not significantly affected (Chapter 3). However, at these relatively high concentrations, fenpiclonil caused a significant reduction in accumulation of amino acids and sugars. The reduction occurred immediately after addition of the fungicide and the magnitude corresponded to the reduction of mycelial growth in liquid medium. It was hypothesised that the reduction could be due to instantaneous cell death, but the accumulation of TPP^+ and PPA in mycelium was not reduced. Accumulation of these probes is considered to be indicative for the magnitude of the plasma membrane potential ($\Delta\Psi$) and proton gradient (ΔpH), the two constituents of the proton motive force (Δp; 16). TPP^+ accumulation by mycelium even increased upon fenpiclonil treatment, which suggests an hyperpolarization of the $\Delta\Psi$. A reduction in accumulation of essential substrates such as sugars and amino acids would suggest a decrease in $\Delta\Psi$ (17, 18). Therefore, the hyperpolarization observed is not easily understood.

The apparent $\Delta\Psi$ of mycelium was calculated to be -70 mV, which is comparable to values reported for other fungi (19, 20), but considerably less negative than the $\Delta\Psi$ of *Neurospora crassa* (21). Fenpiclonil hyperpolarized the apparent $\Delta\Psi$ to -130 mV. This was also the case upon the treatment of mycelium with ionophores and electrogenic cations. Hyperpolarization of the $\Delta\Psi$ is usually caused by fluxes of potassium over the plasma membrane, in exchange for protons (22). Fenpiclonil did not induce such effects, because neither a change in ΔpH nor an efflux of potassium was observed. Therefore, the apparent hyperpolarization might be caused by an intracellular flux of potassium from the cytoplasm to mitochondria (23). This assumption would corroborate with the observation that the fenpiclonil analogue pyrrolnitrin induces swelling of isolated mitochondria by the presumed accumulation of potassium (24). The observed changes in accumulation of TPP^+ caused by fenpiclonil are also compatible with the concept that TPP^+ accumulation reflects changes in potentials over intracellular membranes, the mitochondrial membrane in particular, rather than a change in potential across the plasma membrane.

At its EC_{15} (0.4 μM), fenpiclonil selectively inhibited incorporation of various monosaccharides into macromolecules of the fungus. Inhibition of mannose incorporation by various fenpiclonil analogues rank-correlated with their fungitoxicity. Under similar conditions, accumulation of these sugars and of various other precursors tested was only slightly influenced. The major part of mannose in mycelium is present in saccharide side chains of glycoproteins (25) and can be marked by feeding mycelium with radiolabelled mannose (26). Using this technique it could indeed be shown that fenpiclonil inhibited incorporation of mannose into glycoprotein. However, the major part of accumulated mannose-6-phosphate apparently isomerized to fructose-6-phosphate and glucose-6-phosphate (27), since a high proportion of accumulated mannose was metabolized to carbon dioxide, and a high proportion of macromolecules contained labelled glucose. Cell walls of filamentous fungi primarily consist of glycoproteins and glucans embedded in a crystalline chitin microfibril matrix (28). The glucans generally have a ß-1,3 and ß-1,3-1,6 structure (29). *F. sulphureum* does not contain ß-1,4-glucans (30).

Fenpiclonil (0.4 μM) also inhibited incorporation of glucose into almost all fractions tested, but inhibition of incorporation into structural hyphal wall glycans was most significant. Effects were observed within 15 min of incubation of mycelium with the fungicide. Polymerization of uridinediphosphoglucose (UDPGlc) is mediated by glucan synthases located in the fungal plasma membrane (31). However, activity of these enzymes is probably not directly affected by fenpiclonil, since cell-free polymerization could not be inhibited by the fungicide. Furthermore, *in vivo* inhibition of synthase activity by fenpiclonil would result in accumulation of its precursor, UDPGlc (32). This was not the case. In addition, depletion of structural glucans in the hyphal wall would result in morphological changes of the mycelium upon fenpiclonil treatment (33). This has not been observed (Chapter 2). Therefore, it is likely that fenpiclonil interferes with initial steps in glycan synthesis.

Elimination of carbons in glucose as carbon dioxide is mediated by enzymes operating in various metabolic pathways. The amount of carbon dioxide released is indicative for the relative importance of the various pathways (34). Carbons 3 and 4 in glucose are decarboxylated upon oxidation of pyruvate by pyruvate dehydrogenase. The other carbons may be eliminated in the TCA-cycle. A relatively high [^{14}C]carbon dioxide production with [1-^{14}C]glucose as the substrate indicates an active pentose phosphate pathway. This was the

case in the present experiments. The pentose phosphate pathway is stimulated by nitrate and intensive aeration (35). These conditions were used in the present experiments. Upon fenpiclonil treatment, inhibition of [^{14}C]carbon dioxide production from [1-^{14}C]glucose and [6-^{14}C]glucose was similar (Chapter 6). Therefore, the fungicide does not interfere specifically with the pentose phosphate pathway. Pyruvate dehydrogenase was also not affected, as the release of [^{14}C]carbon dioxide from [U-^{14}C]glucose was not different from that of [6-^{14}C]glucose. Fenpiclonil did not inhibit the elimination of [^{14}C]carbon dioxide from externally added [1-^{14}C]acetate. Therefore, the fungicide does not inhibit a step in the TCA-cycle. It is more likely that the fungicide inhibits initial steps of glucose metabolism. This would corroborate with the inhibitory effect of fenpiclonil on incorporation of radiolabelled glucose in fungal macromolecules.

Metabolism of 2-deoxyglucose (2-DG) was used to study early effects of fenpiclonil in glucose metabolism. Like glucose, 2-DG is rapidly phosphorylated into 2-deoxyglucose-6-P (2-DG6G). However, isomerization to 2-deoxyfructose can not take place. Other metabolic conversions such as dehydrogenation and polymerization occur, but at low rates (17). The accumulation rate of 2-DG appeared to be lower than that of glucose, but the inhibitory effect of fenpiclonil (0.4 μM) was slightly higher. These results confirm the suggestion that fenpiclonil inhibits metabolism of glucose during initial steps.

Starved mycelium accumulates and phosphorylates 2-D[^{14}C]G very rapidly. Effects on these processes can better be studied under equilibrium conditions. Therefore, mycelium was loaded with cold 2-DG until no net accumulation occurred. Acetate was used as the energy source, since its metabolism was not inhibited by fenpiclonil. Upon addition of 2-D[^{14}C]G, the major part of accumulated 2-D[^{14}C]G was phosphorylated within 1 min (Chapter 6). Upon fenpiclonil treatment, the proportion of intracellular 2-DG increased, while the proportion of 2-DG6P proved to be considerably reduced. Therefore, fenpiclonil appears to inhibit the conversion of 2-DG to 2-DG6P. Since phosphorylation of glucose is mediated by the same enzyme, fenpiclonil is most probably also an inhibitor of glucose phosphorylation. This inhibitory effect would explain the various metabolic effects reported for fenpiclonil.

Sugar transport systems in filamentous fungi are well studied in *Neurospora crassa* (17). This fungus has a low-affinity facilitated diffusion system and a repressible high-affinity active transport system. Normal or starved hyphae maintain a high resting $\Delta\Psi$ (-250 to -160 mV), which energize the latter transport system (22). Like *Aspergillus nidulans*, *F. sulphureum* has

a much lower $\Delta\Psi$ ranging between -80 to -50 mV (17). The main accumulation system in these fungi seems to be transport-associated phosphorylation of glucose (36). It is possible that fenpiclonil inhibits this process (Chapter 6). A direct effect on activity of hexokinase or related enzymes is not likely since inhibition of cell-free phosphorylation was not observed. At the low fenpiclonil concentrations used, decrease in ATP content was also not detected. Bisson *et al.* proposed that cytosolic hexokinase can be associated with transmembrane sugar carriers, which behaves like a transport-associated phosphorylation complex (37). Inhibition of the activity of this complex may be a consequence of the high accumulation rate of fenpiclonil into membranes (Chapter 4) and can result in a gradual reduction of the glucose-6-phosphate content in mycelium. This effect leads to a gradual starvation of the fungus, and result in a steady reduction in oxygen consumption in time upon prolonged incubation with the fungicide as described previously (Chapter 3). Reduction of the glucose-6-phosphate content of mycelium upon fenpiclonil treatment may also explain the inhibition of synthesis of macromolecules such as glycans (Chapter 5). This implicates that inhibition of glucose phosphorylation starts a cascade of metabolic events culminating in the toxic action of the fungicide.

Another metabolic event following inhibition of glucose phosphorylation, is the increase in content of neutral sugars (Chapter 6). These effects were observed in short term pulse-chase experiments, in which mycelium of the wild type isolate of *F. sulphureum* was incubated with [U-^{14}C]glucose. The radiolabel could be recovered in the neutral carbohydrates glucose and mannitol. Upon fenpiclonil treatment, the content of both these compounds increased. In addition, a new compound with an elution time similar to that of mannitol was detected. Under the starvation conditions used in the present experiments, no radiolabel was incorporated in trehalose, a glucose dimer used for storage of glucose (38). Instead, glucose was mainly metabolised to carbon dioxide. The main non-labelled neutral carbohydrates in mycelium were mannitol, trehalose and glycerol. Upon fenpiclonil treatment, the content of the polyols, especially that of glycerol, increased considerably. The osmotic consequences may be significant and can contribute to the toxic effect of fenpiclonil. In this context, it is striking to note that the resistant strain does not accumulate polyols upon fenpiclonil treatment and hence may escape this toxic effect. Effects on osmoregulation may be a common feature in the mode of resistance to phenylpyrroles and dicarboximide fungicides (39). However, this does not necessarily imply that their modes of action are similar.

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ABSTRACT

In the last few decades, plant disease control has become heavily dependent on fungicides. Most modern fungicides were discovered by random synthesis and empirical optimization of lead structures. In general, these fungicides have specific modes of action and meet modern environmental criteria. A disadvantage of modern fungicides is the potency of plant pathogens to acquire fungicide resistance. This phenomenon urges the agrochemical industry to search for chemicals with new modes of action. Natural products often have unique modes of action and can be used as lead structures in chemical synthesis programmes. One natural lead structure is the antifungal antibiotic pyrrolnitrin, produced by several *Pseudomonas* species. Its use in an optimization programme of CIBA-GEIGY A.G., Basel, Switzerland, led to the development of the highly active phenylpyrrole fungicides fenpiclonil (CGA 142705) and fludioxonil (CGA 173506). The elucidation of the mode of action of fenpiclonil is the topic of this thesis.

Fenpiclonil is toxic to representatives of Ascomycetes, Basidiomycetes and Deuteromycetes. Its effect on several physiological processes was studied using the fungus *Fusarium sulphureum* as a sensitive target organism. The EC_{50} of fenpiclonil to radial growth on PDA and mycelial growth in Czapek Dox liquid medium is 0.5 and 4 μM, respectively.

Fenpiclonil accumulates to a high level in mycelium of *F. sulphureum* and in artificial liposomes. The accumulation appears to be the result of a physico-chemical partitioning of the fungicide over lipids in mycelium and the medium. Accumulation is reversible as the fungicide is readily released from mycelium by washing with water. The fungus does not metabolize fenpiclonil upon incubation for 24 hours.

At its EC_{50} (4 μM), fenpiclonil does not immediately affect oxygen consumption, nuclear division, and DNA-, RNA-, protein-, chitin-, ergosterol- and (phospho)lipid biosynthesis. However, accumulation of amino acids and sugars is instantaneously inhibited at concentrations ranging from 4.2-42 μM. The reduction in accumulation is accompanied by an increased accumulation of the membrane potential probe tetraphenylphosphonium bromide (TPP^+) and a marginal change of the proton gradient probe propionic acid. This suggests that the biochemical mechanism of action of fenpiclonil may be related to membrane dependent transport processes. The increased accumulation of TPP^+ is probably the result of changes of potentials over membranes of various cell compartments rather than from plasma membrane hyperpolarization. The fungicide neither influences membrane fluidity in artificial liposomes nor amino acid accumulation in bacterial vesicles. Thus, accumulation of the fungicide does not aspecifically effect functioning of various types of membranes.

At its EC_{15} (0.4 μM), fenpiclonil selectively inhibits accumulation and incorporation of various

monosaccharides into macromolecules of *F. sulphureum*. This effect was not observed with a fenpiclonil-resistant laboratory isolate of the fungus. Various less active structural analogues of fenpiclonil also inhibit accumulation and incorporation ot monosaccharides into macromolecules, but to a lesser extent. Strongest inhibition (58%) was observed for the incorporation of [U-^{14}C]glucose in hyphal wall glycan fractions. Biosynthesis of these glycans is catalysed by glycan synthases in the plasma membrane. However, fenpiclonil does not directly interfere with these enzymes, since their activity is not inhibited in a cell-free assay. Furthermore, the precursor of glycans, uridinediphosphoglucose, does not accumulate upon fenpiclonil treatment. All effects described were observed within 15 min of incubation with the fungicide, and indicate that the mechanism of action of fenpiclonil may be related to glucose metabolism.

Fenpiclonil (0.4 µM) inhibits the production of [^{14}C]carbon dioxide in mycelium incubated in a medium with glucose as the carbon source. However, no differential effect on [^{14}C]carbon dioxide production with glucose labelled at different carbon positions was observed. The fungicide does not inhibit [^{14}C]carbon dioxide production using acetate as the carbon source. These results indicate that metabolisation of glucose is neither affected by inhibition of pyruvate dehydrogenase activity nor of enzymes in the TCA-cycle. Therefore, the site of action of fenpiclonil is most likely located in early steps of glycolysis.

Fenpiclonil inhibits the accumulation of 2-deoxy[U-^{14}C]glucose in starved mycelium loaded with 2-deoxyglucose. Fungicide treatment results within one min of incubation in an increased content of 2-deoxy[U-^{14}C]glucose and a decreased content of 2-deoxy [U-^{14}C]glucose-6-phosphate. The fungicide does not effect cell-free phosphorylation of glucose and the mycelial ATP concentration. These results indicate that the mode of action of fenpiclonil is due to inhibition of transport-associated phosphorylation of glucose. Inhibition of glucose phosphorylation will cause a cascade of metabolic events leading to the toxic action of the fungicide. A major event may be the accumulation of polyols which was not observed in an osmotically-sensitive and fenpiclonil resistant laboratory isolate of the fungus.

SAMENVATTING

In de moderne landbouw is de bestrijding van planteziekten in hoge mate afhankelijk van fungiciden. Dergelijke verbindingen worden vrijwel altijd ontdekt op basis van *'trial and error'* in uitgebreide screeningsprogramma's. Door synthese van structuuranalogen worden *'lead'* stoffen vervolgens geoptimaliseerd tot commerciële fungiciden. In het algemeen bezitten deze verbindingen een specifiek werkingsmechanisme in de te bestrijden plantpathogene schimmels en voldoen aan moderne milieu-criteria. Een nadeel van moderne fungiciden is dat schimmels resistentie kunnen ontwikkelen tegen deze verbindingen. Deze resistentieproblematiek noopt de agrochemische industrie om steeds weer fungiciden met een nieuw werkingsmechanisme te ontwikkelen. Natuurstoffen met antibiotische werking bezitten vaak een nog onbekend werkingsmechanisme en kunnen daarom als start gebruikt worden in chemische synthese programma's. Een voorbeeld hiervan is de ontwikkeling van de fenylpyrrool fungiciden fenpiclonil (CGA 142705) and fludioxonil (CGA 173506) door CIBA-GEIGY A.G., Bazel, Zwitserland. Deze verbindingen zijn afgeleid van het antibioticum pyrrolnitrin dat wordt geproduceerd door *Pseudomonas* spp. Het doel van dit proefschrift is om het werkingsmechanisme van het fenylpyrrool fungicide fenpiclonil op te helderen.

Fenpiclonil is werkzaam tegen diverse schimmels die tot de klassen van de Ascomyceten, Basidiomyceten en Deuteromyceten behoren. Het fungicide wordt op de markt gebracht als zaaizaadontsmettingsmiddel in granen, onder andere ter bestrijding van *Fusarium sulphureum*. Deze schimmel werd in het uitgevoerde onderzoek als testorganisme gebruikt. De EC_{50} van fenpiclonil voor remming van de radiale groei op PDA en myceliumgroei in vloeibaar medium is respectievelijk 0,5 en 4 µM.

Fenpiclonil accumuleert in sterke mate in mycelium van *F. sulphureum* en in kunstmatige liposomen. Dit is waarschijnlijk een gevolg van een fysisch-chemische verdeling van het fungicide over lipiden in mycelium en het medium. De accumulatie is reversibel aangezien het fungicide vrij gemakkelijk uit het mycelium verwijderd kan worden door het te wassen met water. De schimmel is niet in staat om fenpiclonil af te breken gedurende een incubatietijd van 24 uur.

Bij de EC_{50} (4 µM) heeft fenpiclonil geen onmiddellijk effect op de zuurstofopname, kerndeling en synthese van DNA, RNA, eiwit, chitine, ergosterol en (phospho)lipiden. Bij concentraties van 4.2 tot 42 µM wordt de opname van aminozuren en suikers onmiddellijk geremd. Deze reductie gaat gepaard met een hogere accumulatie van tetrafenylfosfonium bromide (TPP^+), een stof die vaak gebruikt wordt om de membraanpotentiaal te meten. Bij dezelfde concentratie werden geen effecten gevonden op de opname van propionzuur dat een indicator is voor de protongradiënt. Dit suggereert

dat het biochemisch werkingsmechanisme van fenpiclonil gerelateerd zou kunnen zijn aan membraanafhankelijke transportprocessen. De hogere accumulatie van TPP^+ is waarschijnlijk het resultaat van veranderingen in potentiaal over verschillende celcompartimenten en niet van een verandering in de plasmamembraanpotentiaal. Fenpiclonil heeft geen invloed op de vloeibaarheid van kunstmatige liposomen en de opname van aminozuren in bacteriële vesicles. Accumulatie van het fungicide leidt dus niet tot een aspecifieke verstoring van het functioneren van verschillende typen membranen.

Fenpiclonil remt bij de EC_{15} (0.4 μM) specifiek de incorporatie van diverse monosacchariden in macromoleculen van *F. sulphureum*. Een laboratoriumisolaat van *F. sulphureum* met een verminderde gevoeligheid voor fenpiclonil vertoonde dit effect niet. Diverse minder actieve analogen van fenpiclonil vertonen hetzelfde effect, maar in mindere mate. De inbouw van [^{14}C]glucose in celwandglycanen wordt het sterkst geremd (58%). De biosynthese van deze glycanen wordt gekatalyseerd door glycaan synthasen in de plasmamembraan. Fenpiclonil interfereert echter niet direct met deze enzymen, omdat de activiteit ervan niet geremd wordt in een celvrij systeem. Bovendien leidt incubatie van mycelium met fenpiclonil niet tot accumulatie van uridinedifosfoglucose, een precursor van glycanen. Alle beschreven effecten werden binnen 15 min waargenomen en suggereren dat fenpiclonil het glucose metabolisme remt.

Fenpiclonil (0.4 μM) remt de productie van [^{14}C]koolstofdioxide door mycelium, geïncubeerd in een medium met glucose als koolstofbron. Bij incubatie met glucose, radioactief gemerkt op verschillende posities werd echter geen verschil gevonden in de mate van [^{14}C]koolstofdioxide productie. De productie van [^{14}C]koolstofdioxide wordt niet geremd wanneer acetaat als koolstofbron wordt gebruikt. Deze resultaten duiden erop dat het effect op het metabolisme van glucose niet wordt veroorzaakt door remming van de activiteit van pyruvaatdehydrogenase of van enzymen in de citroenzuurcyclus. Fenpiclonil interfereert daarom waarschijnlijk met initiële stappen van de glycolyse.

Fenpiclonil remt de opname en, in sterkere mate, de fosforylering van 2-deoxy [^{14}C]glucose in gehongerd mycelium dat is geladen met 2-deoxyglucose. Een korte behandeling met het fungicide (1 min) leidt tot een verhoging van het 2-deoxy[^{14}C]glucose- en verlaging van 2-deoxy[^{14}C]glucose-6-fosfaatgehalte. In een celvrij systeem heeft het fungicide geen effect op de fosforylering van 2-deoxyglucose. Fenpiclonil beïnvloedt evenmin het ATP gehalte van mycelium. Deze resultaten wijzen erop dat het fungicide aangrijpt op de transportafhankelijke fosforylering van glucose. Remming van de fosforylering van glucose kan een reeks van metabolische effecten veroorzaken die uiteindelijk leidt tot de fungicide werking van het middel. Eén van de belangrijkste metabolische effecten kan de ophoping van polyolen zijn, hetgeen niet werd waargenomen bij een osmotisch-gevoelig en fenpiclonil-resistent laboratoriumisolaat van de schimmel.

CURRICULUM VITAE

Ad Jespers werd geboren op 24 april 1964 te Breda. In 1982 behaalde hij het diploma Atheneum B aan het Sint Odulphus Lyceum te Tilburg. Aansluitend begon hij de studie Landbouwplantenteelt aan de toenmalige Landbouwhogeschool te Wageningen. Tijdens de doctoraalfase werkte hij gedurende 5 maanden bij het toenmalige Consulentschap in Algemene Dienst voor de Gewasbescherming te Wageningen en 3 maanden bij de Stichting Nederlands Graan Centrum te Wageningen. In augustus 1987 slaagde hij voor het ingenieursexamen met als afstudeervakken Landbouwplantenteelt, Fytopathologie en Plantenfysiologie. Van 15 mei tot 31 december 1987 werkte hij als toegevoegd onderzoeker bij de vakgroep Fytopathologie van de Landbouwuniversiteit in Wageningen aan de ontwikkeling van resistentie tegen metalaxyl in *Phytophthora infestans* en *Bremia lactucae*. Aansluitend werd de militaire dienstplicht vervuld. Op 1 maart 1989 werd hij wederom aangesteld als toegevoegd onderzoeker bij de vakgroep Fytopathologie met het onderzoek waarvan de resultaten in dit proefschrift staan beschreven. Sinds 28 april 1993 is hij werkzaam als *insecticides registration manager* bij *Household and Personal Care Research* B.V. te Den Haag, een werkmaatschappij van Sara Lee | D·E.

Correspondentie adres: Dovenetelweg 15, 2555 TA Den Haag.