MECHANISMS OF ACTION
OF THE ORGANOPHOSPHORUS
FUNGICIDE PYRAZOPHOS

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LIST OF ABBREVIATIONS

A	absorbance
ATP	adenosine 5′-triphosphate
Ci	Curie
^{14}C-phe	L-phenylalanine-^{14}C (U)
^{14}C-ur	uridine-2-^{14}C
^{14}C-thy	thymidine-2-^{14}C
DBLS	diazoblue laurylsulphate
DNA	deoxyribonucleic acid
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylene diaminotetraacetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)
Gly-agar	glucose yeast extract agar
k_i	bimolecular rate constant
K_m	Michaelis constant
NA	α-naphthylacetate
NAD^+	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
PCA	perchloric acid
PDA	potato dextrose agar
PO-pyrazophos	phosphate analogue of pyrazophos
PP	2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)-pyrimidine
RNA	ribonucleic acid
SDH	succinate dehydrogenase (EC 1.3.99.1)
TCA	trichloroacetic acid
TLC-bioassay	thin-layer chromatographic bioassay
v	reaction velocity at a finite substrate concentration

LIST OF STRUCTURAL FORMULA'S
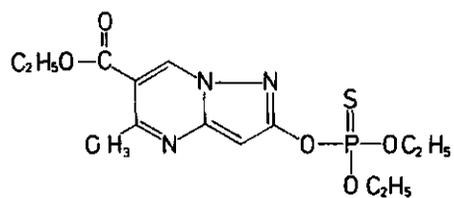
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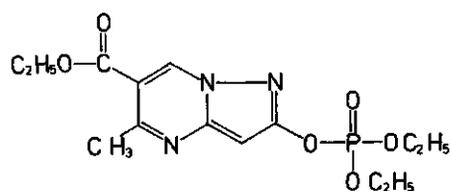
PO-pyrazophos

PP
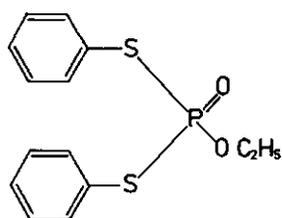
Hinosan

Kitazin

CONTENTS

1. INTRODUCTION

1.1. GENERAL

Plant pathogenic fungi cause serious damage to many crops of world importance. Worldwide losses of crops as a result of fungal diseases have been estimated at 25,000 million dollars (BENT, 1969). Chemical control of these diseases has found widespread use in the 19th and the beginning of the 20th century, mainly by the application of inorganic sulphur, copper and mercury compounds. These chemicals are still important in the control of various plant diseases. Since the discovery of dithiocarbamates in 1934 organic sulphur compounds also find a wide application in disease control (OWENS, 1969).

The compounds mentioned above are primarily protectant fungicides; treatments of plants are intended to prevent or protect against infections. Such fungicides are not taken up through the leaf surface and can only kill a fungus before it enters the plant. During the last decade fungicides have been developed, which are taken up by the plant via leaves or roots, and can be transported within the plant body to tissues remote from the site of application. They are called systemic fungicides. Systemic fungicides have the advantage in that they may eradicate established infections, may protect newly developing parts of the plant and are sometimes less subject to loss by weathering. The control of vascular and wilt diseases and the protection of plants against diseases caused by soil-borne fungi would become possible through the use of systemic fungicides (BENT, 1969). The first review and classification of this type of compounds was published by KIRBY in 1972. In his classification, benzimidazoles, anilides, pyrimidines, morpholines, piperazines, organophosphorus compounds and several miscellaneous chemicals are distinguished. Furthermore, a number of antibiotics may be considered as a special group of systemic fungicides (DEKKER, 1971). Many of the compounds listed in the latter reviews have found a frequent use in practice against a broad range of fungal pathogens.

1.2. MODE OF ACTION OF SYSTEMIC FUNGICIDES

In recent years increasing attention is paid to the mechanism of action of systemic fungicides. It has been suggested that their mode of action involves mainly an interaction with biosynthetic processes, although this has not yet been demonstrated for each of the compounds separately (KAARS SIJPESTEIJN, 1970). An inhibitory effect on biosynthetic processes has indeed been described for several of the first systemic fungicides like 6-azauracil, chloramphenicol, cyclo-heximide, griseofulvin, streptomycin and sulphonamides. After the introduction of these compounds a number of other systemic fungicides have been developed. Most of them are already in agricultural use against various plant pathogens.

Among these fungicides are benzimidazoles, anilides (oxathiins), pyrimidines, organophosphorus compounds, antibiotics and several other, miscellaneous compounds. Data regarding their mechanism of action are listed below.

The antibiotics blasticidin-S, kasugamycin and the polyoxins which are used against fungal diseases of rice, were shown to interfere with protein or chitin synthesis (HUANG et al., 1964; TANAKA et al., 1965; OHTA, 1970).

The toxicity of chloroneb, 1,4-dichloro-2,5-dimethoxybenzene, to *Rhizoctonia solani* appears to involve a direct or indirect inhibition of DNA synthesis (HOCK and SISLER, 1969).

Carboxin, 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide, a compound which is selectively toxic to a number of *Basidiomycetes*, causes an inhibition of DNA, RNA and protein synthesis, and also affects energy production and precursor synthesis in the tricarboxylic acid cycle in *Ustilago maydis* and *R. solani* (RAGSDALE and SISLER, 1970). More or less similar results were obtained by MATHRE (1970) who found that beside inhibition of respiration by a blockage of pyruvate and acetate oxidation, also inhibition of nucleic acid synthesis is a major toxic effect of carboxin and its sulphone analogue, oxycarboxin. Experiments in vitro with isolated mitochondria of *U. maydis* demonstrated that carboxin inhibits mitochondrial respiration at or close to the site of succinate oxidation (MATHRE, 1971).

Kitazin-P, O,O-diisopropyl S-benzyl phosphorothioate, acting mainly against *Pyricularia oryzae* on rice, causes an accumulation of intermediates of chitin synthesis in this fungus, suggesting an interference with the latter process (MAEDA et al., 1970).

The benzimidazole fungicides benomyl, 1-(butylcarbamoyl)-2-benzimidazole carbamic acid methyl ester, thiabendazole, 2-(4'-thiazolyl)benzimidazole and fuberidazole, 2-(2-furyl)benzimidazole probably share a common mechanism of action, as can be deduced from their similar in vitro fungitoxic spectrum and the fact that mutants resistant to one of these fungicides, are usually also resistant to the others (BOLLEN and FUCHS, 1970; EDGINGTON et al., 1971; BARTELS-SCHOOLEY and MACNEILL, 1971; HASTIE and GEORGOPOULOS, 1971). CLEMONS and SISLER (1969) showed that benomyl breaks down rapidly in aqueous solutions to form methyl benzimidazol-2-yl carbamate (MBC), and suggested that MBC would be the toxic principle of benomyl; this breakdown product is at least as fungitoxic as the parent compound to a number of fungi like *Neurospora crassa* and *U. maydis*. It has once been assumed that the toxicity is brought about by an interference with DNA synthesis (CLEMONS and SISLER, 1971). Afterwards, it has been reported that in synchronous cultures of *Saccharomyces cerevisiae* MBC did not inhibit DNA synthesis during the first cell cycle after addition of the chemical, but prevented the completion of cell division resulting in the formation of typical doublets of mother and daughter 'cells' (HAMMER-SCHLAG and SISLER, 1973). This inhibition of cytokinesis, which has also been observed upon incubation of *U. maydis* sporidia with MBC, is probably caused by an interference with mitosis (HAMMERSCHLAG and SISLER, 1973). Antimitotic activity of MBC has also been found by DAVIDSE (1973) with *Aspergillus nidulans*.

MBC treatment of mycelial samples resulted in an almost doubling of the DNA content, while no increase in the number of nuclei was noted. This gave rise to an increase of the average DNA content per nucleus till nearly twice the initial content.

The mechanism of fungitoxicity of thiophanate, 1,2-bis(3-ethoxycarbonyl-2-thioureido)-benzene and thiophanate methyl, 1,2-bis(3-methoxycarbonyl-2-thioureido)-benzene is probably similar to that described above for MBC, since the latter fungicide is readily converted into this compound (SELLING et al., 1970). Although the benzimidazole fungicides benomyl, thiabendazole and fuberidazole probably have a common mechanism of action, it may not be excluded that some of these chemicals exert additional toxic effects. For instance, in the case of benomyl the formation of butyl isocyanate, a volatile compound toxic to *U. maydis* and *S. cerevisiae* has been demonstrated. The inhibitory effect of butyl isocyanate on the respiration of both fungi resembled that of benomyl (HAMMERSCHLAG and SISLER, 1972). An additional mode of action has also been suggested for benomyl and thiabendazole against *Fusarium oxysporum* f. sp. *melonis*, since spontaneous and induced mutants of this fungus, resistant to benomyl and thiabendazole, did exhibit cross-tolerance with these chemicals and with fuberidazole; a fuberidazole mutant, however, was shown to be as sensitive as the wild-type *Fusarium* to benomyl and thiabendazole (BARTELS-SCHOOLEY and MACNEILL, 1971). Additional effects of thiabendazole can also be ascribed to interference with respiration as ALLEN and GOTTLIEB (1970) have shown with mitochondria of *Penicillium atrovenetum*; in this instance effects on biosynthetic processes in the fungus seemed to be secondary, because they only appeared at relatively high concentrations of the compound.

The pyrimidine derivative ethirimol, 5-butyl-2-ethylamino-4-hydroxy-6-methylpyrimidine and its 2-dimethylamino analogue dimethirimol, both selectively toxic towards powdery mildew fungi, possibly act by blocking pyridoxal-catalyzed transfer of C-1 units to tetrahydrofolic acid (BENT, 1970; CALDER-BANK, 1970).

1.3. AIM OF THE INVESTIGATIONS

As indicated by MARSH (1972) in his preface to 'Systemic Fungicides' investigations on the mode of action of systemic fungicides are now in progress in several research centres in the world. It appears that attention has especially been focused on compounds already used on a rather broad scale like the benzimidazoles, oxathiins and pyrimidines.

With respect to organophosphorus fungicides only reports on the mode of action of Hinosan, Kitazin and saligenin cyclic phosphates, all mainly used in Japan against fungal diseases on rice, have been published (OHKAWA and ETO, 1969; MAEDA et al., 1970; DE WAARD, 1972). Other representatives of this group of compounds, used in the Netherlands and some other Western countries against powdery mildews on a number of ornamental plants and vegetables,

have not been investigated extensively. One of these is triamiphos, which may be regarded as the first chemical developed as a systemic fungicide (KOOPMANS, 1960). Other familiar compounds of this group are pyrazophos and Dowco 199 (CLARE et al., 1968; MARIOUW SMIT, 1969). Fungicidal characteristics of these chemicals will be described in chapter 3. Their economic importance is restricted because of the fact that they display a rather selective toxic action to powdery mildew fungi. Although this selectivity may be a disadvantage with regard to their economic production, it certainly is an advantage from an ecological point of view. Highly selective fungicides will probably disturb the microbial balance in the plant environment to a lesser extent than broad spectrum fungicides. Another important aspect of this type of fungicides is their structural similarity with insecticidal organophosphorus compounds. It can not be denied that the knowledge on the mechanism of insecticidal activity might provide clues for the elucidation of their fungicidal action.

Considerations as mentioned above have led to the present study on the mechanism of action of organophosphorus compounds. It was supposed that the results could afford information on toxicological aspects and possible side-effects of these compounds and might be of use for the synthesis of new fungicides. Furthermore, knowledge on the mechanism of action is also necessary to elucidate the mechanism of resistance in fungi towards systemic fungicides, a phenomenon, which is often reported since the introduction of this type of compounds.

1.4. OUTLINE OF THE PRESENT STUDY

The present study on the mechanism of action of organophosphorus fungicides has been started by investigating the fungitoxic properties of a number of fungicidal and insecticidal compounds and some of their derivatives (chapter 3). Because of its relatively high fungitoxicity both against powdery mildew fungi like *Sphaerotheca fuliginea* and against *P. oryzae*, pyrazophos has been selected to study its mechanism of action in more detail.

Many organophosphorus compounds have the capacity to inhibit, to a variable extent, the activity of esterases (O'BRIEN, 1960; 1967). In the case of Kitazin also an effect on chitin synthesis has been described (MAEDA et al., 1970). In the latter instance, the authors suggested that the interference with chitin synthesis might be related with an effect on cell membrane permeability. These data prompted us to investigate effects of pyrazophos and some related compounds on non-specific esterases of *S. fuliginea* (chapter 4) and on chitin synthesis and cell membrane permeability of *P. oryzae* (chapter 5). In further experiments, effects of pyrazophos on various important cellular processes like respiration, DNA, RNA, and protein synthesis have been studied (chapter 6).

In view of the possibility that fungal metabolites might be responsible for the fungitoxic action of pyrazophos, its metabolic conversion in *P. oryzae* and in some insensitive fungi has been investigated (chapter 7). One of the meta-

bolites found, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP), seemed to be of major importance and, therefore, its in vitro fungitoxicity and effects on respiration, and DNA, RNA, and protein synthesis of the fungus have been studied (chapters 8 and 9). Finally, in chapter 10 a general discussion of the results presented has been given.

2. MATERIALS AND METHODS

2.1. PLANTS

Cucumber plants, cv. 'Lange Gele Tros' and barley seedlings, cv. 'Balder', were used throughout all experiments. Cucumber plants, two in each pot, were grown in steamed soil in climate rooms, at 20 °C and under white fluorescent light (Philips TL MF 40 W/33 RS). Plants were irradiated from 6 a.m. till 6 p.m.

Barley seedlings, seven per pot, were grown in sand in a greenhouse at a temperature from 16 to 18 °C.

2.2. FUNGI

Cucumber and barley powdery mildew were maintained under greenhouse conditions at 16–22 °C. Each week, 4–6 weeks old cucumber and 2–4 weeks old barley plants, grown as described under 2.1., were inoculated. During winter, additional fluorescent (Philips TLF 65 W/33) and mercury (Philips HPLR 400 W) light was given from 4 p.m. till midnight.

Pyricularia oryzae, strain CBS 433.70, which proved to be non-pathogenic to rice, was maintained on an agar medium containing 2% glucose and 0.5% yeast extract. *P. oryzae* strains Japan and BASF, generously supplied by Farbwerke Hoechst A.G., Frankfurt, Germany, were cultured on medium containing 1.5% oatmeal, 0.5% water-soluble starch, 0.1% yeast extract, 0.2% streptomycin sulphate and 2.5% agar, pH 6.1–6.4. Spores of both strains were obtained from cultures on medium containing 7.5% malt extract, 0.2% streptomycin sulphate, and 2.5% agar, pH 6.1–6.4. Mixtures of spore suspensions of both strains, pathogenic to rice and barley, were employed in in vivo pathogenicity tests.

Saccharomyces cerevisiae was maintained on an agar medium containing 3% malt extract and 0.5% peptone and grown in a liquid medium of the same composition, without agar.

Pythium debaryanum was cultured on PDA or grown in a liquid medium containing 1.7% glucose and the aqueous extract of 200 g peeled potatoes per liter medium.

All other fungi used were cultured on PDA.

2.3. CULTURE MEDIA AND CHEMICALS

Malt agar, potato dextrose agar (PDA), peptone, yeast extract and agar were obtained from Oxoid Ltd., London, England. Chemicals were obtained from several companies, the names of which have been abbreviated in the list as given below: B: Brocades-Stheeman & Pharmacia, Amsterdam, The Netherlands;

6

BDH: British Drug Houses, Poole, England; C: Calbiochem A.G., Lucerne, Switzerland; C-P: Chemisch-Pharmaceutische Fabriek en Groothandel N.V., 's-Hertogenbosch, The Netherlands; F: Fluka A.G., Buchs, Switzerland; G: G.T. Gurr Ltd., London, England; H. Hoffmann-La Roche, Basle, Switzerland; M: Mycofarm, Delft, The Netherlands; N: Nutritional Biochemical Corporation, Cleveland, U.S.A.; S: Sigma Chemical Co., St. Louis, U.S.A.

Acetaldehyde (BDH); acetylcholinesterase (EC 3.1.1.7) (S); acetylthiocholine iodide (S); adenine (S); adenosine (S); 3'-adenylic acid (H); 5'-adenylic acid (S); ATP grade I (S); catechol (BDH); L-cysteine (S); cytochrome c from horse heart type II (S); cytosine (S); diazoblue (di-ortho anisidine, tetrazotized) (G); diphenylamine (BDH); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (S); DNA from calf thymus sodium salt type II (S); EDTA (F); folic acid (N); fumaric acid (BDH); glutathione (reduced) (N); glycine (H); guanine (S); guanosine (S); guanylic acid (N); hydrogen peroxide (BDH); hypoxanthine (S); inosine (N); sodium laurylsulphate (S); D-methionine (C); L-methionine (S); α-naphthol (S); α-naphthylacetate (S); NAD$^+$ grade II (S); NADH grade III (S); sodium oleate (BDH); orcinol (BDH); orotic acid (N); yeast 3-phosphoglycerate kinase (EC 2.7.2.3) (B); D(-)3-phosphoglyceric acid grade II (S); pyrogallol (C-P); RNA (F); streptomycin sulphate (M); succinic acid (B); terramycin (Vendarcin) (M); thymine (S); uracil (S); xanthine (N); xanthosine (N).

The radiochemicals D-glucosamine-1-^{14}C hydrochloride, L-phenylalanine-^{14}C (U), uridine-2-^{14}C and thymidine-2-^{14}C were obtained from The Radiochemical Centre, Amersham, England and ^{32}P-orthophosphate from the Institute for Atomic Sciences in Agriculture (ITAL) at Wageningen, The Netherlands.

Pyrazophos, crystalline and in a 30% (w/v) formulation, pyrazophos-2-^{14}C, the phosphate analogue of pyrazophos, and 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine were generously supplied by Farbwerke Hoechst A.G., Frankfurt, Germany. Hinosan 51.4% (w/v), demeton-S, demeton-S-methyl, demeton-O and demeton-O-methyl were kindly provided by Bayer-Agrochemie N.V., Arnhem, The Netherlands. Wepsyn 25% WP was obtained from Philips-Duphar N.V., 's-Graveland, The Netherlands. Dowco 199 50% WP and Kitazin 48% (w/v), manufactured by Dow Chemical, U.S.A. and Kumiai Chemical Industry Co., Japan, were obtained through the courtesy of Dr. L. Scholten and Dr. A. Tempel, respectively (Institute of Phytopathological Research, Wageningen, The Netherlands). Azinphos-ethyl, coumaphos, dimethoate, malathion, paraoxon, O,O-diethyl O-phenylphosphorothioate and the phosphate analogues of these compounds were generously supplied by the Laboratory for Research on Insecticides, Wageningen, The Netherlands.

2.4. FUNGITOXICITY TESTS

2.4.1. *In vivo tests against powdery mildew fungi*
The effects of chemicals against cucumber and barley powdery mildew were

tested in foliar spray, root application and leaf disc tests (DEKKER, 1961; NIE-MANN, 1964; NIEMANN and DEKKER, 1966). Unless otherwise stated, cucumber and barley plants of 4 and 2 weeks old, respectively, and grown as described under 2.1., were used in all experiments. The chemicals examined were dissolved or suspended in acetone or water; the final concentration of acetone in treatments and controls never exceeded 1 %.

In foliar spray tests plants were sprayed two times per day during two days. Inoculation was carried out by dusting dry conidia on the leaf surface one day or more before application of the compounds (curative spray) or one day after the treatment (protective spray). For each concentration tested two pots were employed.

In root application tests cucumber and barley plants were immersed with their roots in flasks containing the fungicide solution or suspension and subsequently inoculated as described above. After four days plants were transferred to a Hoagland nutrient solution.

Leaf disc tests were only performed with cucumber powdery mildew. Leaf discs cut from the first or second leaf of cucumber plants were transferred in Petri dishes onto solutions or suspensions of compounds to be tested and subsequently inoculated. After four days the fungicide solution or suspension was replaced by water. For each concentration tested two Petri dishes with seven discs each were used.

The assessment of powdery mildew in the tests mentioned above took place when surfaces of leaves or leaf discs in control treatments were covered for 70–100 % with mycelium, about 5–7 days after inoculation in the case of barley powdery mildew and 7–14 days in the case of cucumber powdery mildew, depending on the season. The infection of leaves or discs was recorded using a scale ranging from 0 to 5. The symbols used in this scale have the following meaning: 0: no visible fungal growth, 0–1: less than 10 %, 1–2: 10–30 %, 2–3: 30–50 %, 3–4: 50–70 % and 4–5: 70–100 % of the leaf covered with mycelium, respectively.

Assessment of phytotoxicity took place at the same day. The symbols used are tt: very toxic; plants or discs dead, or almost dead; t: necrotic spots, chlorosis, growth reduction, browning of margins; s: only weak symptoms of toxicity, like slight yellow discoloration, slight browning of leaf margins.

2.4.2. In vitro tests against powdery mildew fungi

Spore germination tests of cucumber and barley powdery mildew were carried out according to methods of DE WAARD (1971a). Young, viable conidia were dusted on 25 μ thick cellulose membranes laid on modified Czapek Dox agar, containing 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g KCl, 1.05 g $MgSO_4.7H_2O$, 0.02 g $FeSO_4.7H_2O$, 30 g sucrose, 1 g yeast extract, 0.025 g terramycin and 10 g agar per liter distilled water. The compounds to be tested were mixed through the molten agar at a temperature of c. 45 °C, just before pouring the agar into Petri dishes (DE WAARD, 1971b). Germination of cucumber and barley powdery mildew conidia was assessed after 44 and 20 hours of incubation in the dark at

20 °C, respectively. Two hundred and fifty conidia were counted to determine germination percentage and length of 20 germ tubes (appressoria), selected at random, was measured. All experiments were carried out in duplicate.

Effects of chemicals on elongation of germination hyphae of *Sphaerotheca fuliginea* were investigated according to VAN 'T LAND and DEKKER (1972).

2.4.3. *In vitro tests against non-obligate parasitic fungi*

The effect of chemicals on spore germination of different non-obligate parasitic fungi was established by suspending spores in 8 % orange juice and mixing equal volumes of these suspensions with fungicide solutions or suspensions in water. Small drops of these mixtures were transferred onto glass-slides in Petri dishes and incubated at 100 % RH and 20 °C. After varying times, germination counts were made and length of 25 germ tubes, selected at random, was measured. All tests were carried out in duplicate.

The effect of compounds on radial mycelial growth on agar was determined in the following way. Chemicals were added as a suspension or solution in acetone to molten agar medium at 45 °C; the final acetone concentration never exceeded 1 % in treated and control samples. To suppress bacterial growth the media were supplied with terramycin to a final concentration of 25 mg/l, except when fungi belonging to the *Oomycetes* were tested. The agar was poured in Petri dishes and inoculated by placing an inverted 5 mm-agar disc of young mycelium on the agar surface. Incubation was at 23 °C, with the exception of *Pyricularia oryzae*, which was incubated at 25 °C. Radial growth was measured in triplicate and in the case of *P. oryzae* always after seven days (BOLLEN and FUCHS, 1970).

Toxicity of chemicals to fungi in shake cultures was established using 24 ml nutrient medium in 100 ml flasks with 1 ml spore suspension. Chemicals were added as solutions or suspensions in acetone, the final concentration of acetone being 1 %. In the control samples 0.25 ml acetone was added. The flasks were incubated in a New Brunswick or Gallenkamp orbital shaker at 25 °C and about 200 rev/min. for four days. Dry weight of mycelium was determined after washing with distilled water on a Büchner funnel and drying overnight at 60 °C.

Fungitoxicity of chemicals was also determined by spotting solutions of the chemicals in ethanol on Merck TLC aluminium sheets, pre-coated with silicagel F 245. The sheets were sprayed with spore suspensions of different fungi in a tap water solution containing 1.00 % KH_2PO_4, 0.43 % $Na_2HPO_4.2H_2O$, 0.57 % KNO_3, 0.14 % $MgSO_4.7H_2O$, 0.14 % NaCl and 4.3 % glucose, and incubated for two days at 100 % RH and 23 °C (HOMANS and FUCHS, 1970).

2.4.4. *In vivo test against* Pyricularia oryzae

Effects of chemicals on *P. oryzae* in vivo were investigated, using barley in a foliar spray test. The plants were sprayed as described under 2.4.1. and thereafter inoculated with spore suspensions in 0.05 % sodium oleate and 0.25 % gelatin solution. The suspension contained about equal quantitites of spores of *P. oryzae* strain Japan and of strain BASF. The concentration varied from 10^5

to 10^6 spores/ml. After inoculation, plants were incubated for two days in plastic boxes at 100% RH and 25°C. Assessment was made after four days by counting the number of lesions of *P. oryzae* on the first leaf. All experiments were carried out in triplicate.

2.5. IN VITRO PREPARATIONS OF SPHAEROTHECA FULIGINEA

2.5.1. *Conidial suspensions*

One to two days old viable conidia were collected by washing them from cucumber leaves infected with *Sphaerotheca fuliginea* with tap water onto 100 and 20 μ mesh sieves. Contaminants remained on the 100 μ mesh sieve, while conidia were collected from the 20 μ mesh sieve. They were suspended carefully in 0.04 M phosphate buffer pH 7.0, containing 0.0025% Tween 20, in 250 ml centrifuge tubes. On standing, heavy particles settled to the bottom, while aggregates of spores floated on the surface. Homogeneous suspensions were obtained by sucking away the liquid beneath the surface. Spore concentrations were estimated by means of a haemocytometer.

2.5.2. *Haustorial suspensions*

Haustoria were isolated according to methods modified after DEKHUIJZEN (1966). Mildew hyphae and conidia were removed from infected leaves by cutting them into pieces of about 1–2 cm^2 and stirring these pieces in tap water with 0.0025% Tween 20 at 500 rpm for 1 hour. The pieces were collected on a 400 μ mesh sieve and washed thoroughly under running tap water. Haustoria were released from the leaves by homogenizing the latter twice for 30 sec at 5300, 9500, 12000 and 16000 rpm in an ice-cold buffer containing 0.3 M sucrose, 0.03 M $Na_2HPO_4.2H_2O$, 0.07 M KH_2PO_4 and 0.003 M $MgSO_4.7H_2O$, pH 6.5. The homogenate was filtered twice through 50, 40 and 30 μ mesh sieves. The resulting filtrates were centrifuged at 3500 × g for 10 min. The pellets were suspended in buffer and, within about 60 sec, filtrated through a 5 μ micro-mesh sieve using air pressure and ultrasonic vibration. The haustoria remaining on the sieve were collected and stored in buffer at 0°C. To this crude haustorial suspension Tween 20 was added to a final concentration of 0.0025%. Subsequently, the suspension was filtered through a 10 μ micro-mesh sieve, again using air pressure and ultrasonic vibration. The haustoria, passing through the sieve, were collected in ice-cold buffer without Tween 20 and spun down at 3500 × g for 10 min, after which they were again suspended in a small volume of buffer.

The suspension obtained in a representative experiment, contained 93.4% haustoria, 1.2% hyphal fragments, 5.0% empty cucumber leaf cells and other cell fragments, and 0.3% intact cucumber cells. From 100 g of cucumber leaves about 20–50 × 10^6 haustoria were recovered.

2.5.3. *Cell-free extracts*

Suspensions of 1–2 days old viable conidia were centrifuged at 3000 × g for

10

15 min and the pellets suspended in a small volume of the appropriate homogenization medium. Cell-free extracts were made by ultrasonic disintegration for 3 min at 0°C, using a MSE 'Ultrasonic Power Unit' or by means of a hand-driven Potter-Elvehjem homogenizer at 4°C. The homogenates were centrifuged at 3000 × g for 10 min. and then at 20000 × g for 60 min.

In some experiments the protein content of cell-free extracts was determined according to methods of LOWRY et al. (1951).

2.6. PREPARATION OF MYCELIAL SUSPENSIONS OF *PYRICULARIA ORYZAE*

Standard mycelial suspensions of *Pyricularia oryzae* strain CBS 433.70 were prepared from 3 days old vigorously growing cultures of the fungus in a liquid 2% glucose, 0.5% yeast extract medium. The mycelium was isolated by filtration on 100 μ sieves and washed with an excess of water. After suspending in 2% glucose, the mycelium was partly homogenized in a Virtis-homogenizer at about 10000 rpm. for 20 sec. Subsequently, the suspension was centrifuged at 3000 × g for 5 min and the pellet taken up in a 2% glucose, 0.5% yeast extract medium. The approximate mycelial concentration was determined by measuring the wet volume of 10 ml of the suspension after 5 min of centrifugation in a Homef centrifuge at 3000 rpm. Unless otherwise stated, mycelial suspensions were used with a mycelial wet volume of 0.7–0.8 ml/10 ml mycelial suspension, which corresponds with a dry weight of about 1.5 mg/ml.

Mycelial suspensions of 2 days old liquid cultures of *Pythium debaryanum* were prepared in the same way, using a 1.7% glucose, potato extract culture medium instead of a glucose, yeast extract medium.

2.7. DETERMINATION OF ENZYME ACTIVITIES

Activity of various enzymes in cell-free extracts of *Sphaerotheca fuliginea* were determined in standardized assay mixtures using a Beckman DB-G spectrophotometer in combination with a Sargent SRL recorder. In all tests, the reaction was started by the addition of cell-free extracts to the assay mixtures. With the exception of the determination of esterase activity, activities of enzymes were calculated from the initial reaction velocity, which was measured by recording the increase or decrease in absorbance of one of the substrates or reaction products. Maximal absorbance changes at highest substrate concentrations were 0.3–0.4 per min. Usually, reaction velocities were reasonably linear under these conditions for at least the first 20 sec. All experiments were carried out in duplicate at 25°C, unless otherwise stated.

2.7.1. *Glyceraldehydephosphate dehydrogenase* (EC 1.2.1.12)
Cell-free extracts of *S. fuliginea*, prepared in 0.05 M triethanolamine buffer

pH 7.6 containing 5 mM EDTA, were used. Enzyme activities were determined according to a modified method of BEIZENHERZ et al. (1953) in a medium containing 0.05 M triethanolamine buffer pH 7.6, 5 mM EDTA, 3.3 mM MgSO$_4$.7H$_2$O, 0.15 mM NADH, 2.5 mM reduced glutathione, 1.5 mM ATP, 7.0 mM phosphoglycerate and 4.5 units/ml 3-phosphoglycerate kinase (EC 2.7.2.3). Initial velocities were measured by spectrophotometric recording of the oxidation of NADH at 340 nm.

2.7.2. *Succinate dehydrogenase* (EC 1.3.99.1)

Cell-free extracts of *S. fuliginea* were prepared in 0.1 M phosphate buffer pH 7.4, containing 0.32 M sucrose and 5 mM EDTA. After centrifugation at 3000 × g for 10 min. and at 25000 × g for 60 min the pellet was suspended in the original volume of buffer. Succinate dehydrogenase activity was measured in a medium containing 0.1 M phosphate buffer pH 7.4, 5 mM EDTA, 10 mM KCN, 0.1 % cytochrome c and 20 mM succinate (DE KORT, 1969). Initial velocities were measured by spectrophotometric recording of the reduction of cytochrome c at 550 nm.

2.7.3. *Catechol oxidase* (EC 1.10.3.1)

Catechol oxidase activity was determined in cell-free extracts of *S. fuliginea* in 0.1 M McIlvaine buffer pH 6.0 using catechol as a substrate. Upon addition of 1.0 ml of the extract to 2.0 ml of 1 % catechol, enzyme activity was determined by measuring the increase in absorbance at 400 nm for at least 5 minutes (FUCHS, 1965).

2.7.4. *Peroxidase* (EC 1.11.1.7)

Peroxidase activity was measured according to KIERMEYER and KAYSER (1960). Cell-free extracts of *S. fuliginea*, in 0.04 M phosphate buffer pH 6.0 were incubated in duplicate with 2.5 ml of a reaction mixture, which was made immediately before use and consisted of 10 ml 0.5 M pyrogallol solution in water and 12.5 ml 0.066 M phosphate buffer pH 6.0 in 100 ml of water. After mixing 2.25 ml of the reaction medium with 0.50 ml of cell-free extract, 0.25 ml of a 1 % H$_2$O$_2$ solution was added and the increase in absorbance at 420 nm measured immediately.

2.7.5. *Carboxyl- and arylesterases* (EC 3.1.1.1. and 3.1.1.2)

Unless otherwise stated, esterase activity was measured according to VAN ASPEREN (1962) by incubation of 1.0 ml of a cell-free extract of *S. fuliginea* conidia in 0.04 M phosphate buffer pH 7.0 with 5.0 ml of 3.0×10^{-4} M α-naphthylacetate (NA), dissolved in the same buffer. If smaller amounts of cell-free extracts were used, the volume was made up to 1 ml with buffer. The reaction was always stopped after 20 min of incubation at 22 °C by the addition of 1 ml diazoblue laurylsulphate (DBLS) solution, which was composed of a 1 % diazoblue and a 5 % sodium laurylsulphate solution, both in water, in a proportion of 2 to 5. The absorption of the reaction product, present as a α-naphthol-

diazoblue complex was measured at 600 nm. The reaction velocity v of NA hydrolysis in the incubation mixture was arbitrarily expressed as $\triangle A_{600\,nm}/20$ min. If necessary, the concentration of α-naphthol in the samples could be calculated by reference to a calibration curve, obtained with 6.0 ml α-naphthol solutions in 0.034 M phosphate buffer pH 7.0 and 1.0 ml DBLS solution. A quantity of 0.1 μmole of α-naphthol in the reaction mixture causes an absorption at 600 nm of 0.56.

Inhibition of esterase activity was determined by measuring residual esterase activity after pre-incubation of 0.1 ml of the cell-free extract and 0.1 ml inhibitor solution in 0.04 M phosphate buffer pH 7.0 during various times (inhibition time). Pre-incubation was stopped by the addition of 5.0 ml 3.12×10^{-4} M NA, resulting in a final concentration of 3.0×10^{-4} M. Residual esterase activity was determined in the same way as described above by incubation for 20 min with the substrate solution and subsequent addition of the DBLS solution.

2.7.6. *Acetylcholinesterase* (EC 3.1.1.7)

Acetylcholinesterase activity was determined according to ELLMAN et al. (1961). The reaction mixture contained 20 μl 0.075 M acetylthiocholine iodide, 100 μl 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid), and varying amounts of cell-free extracts of *S. fuliginea* conidia in 0.1 M phosphate buffer pH 8.0; the volume was made up to 3.0 ml with the same buffer. After addition of the cell-free extract, enzyme activity was determined by recording the increase in absorbance at 412 nm.

2.8. HISTOCHEMICAL DEMONSTRATION OF ESTERASE ACTIVITY

Esterases of cucumber powdery mildew were demonstrated histochemically according to PEARSE (1961). Leaf discs infected with the fungus were fixed for 2 hours in acetone at 4°C and evacuated afterwards. After fixation they were washed in water and incubated for 30 min in a solution consisting of a 0.04 M phosphate buffer which contained 1.0 ml 4% α-naphthylacetate and 0.5 g diazoblue per 100 ml. After 15 min of incubation the solution was refreshed. Incubation was stopped by washing the discs with an excess of water. They were embedded in a glycerol-gelatin mixture and assayed for esterase activity microscopically. Enzyme activity in samples from leaves treated with organophosphorus compounds was arbitrarily indicated according to a scale varying from $-$: no activity, $\pm$: slight activity, up to $+++++$: activity of control treatment.

2.9. MEASUREMENTS OF OXYGEN UPTAKE

Measurements of oxygen uptake by *Sphaerotheca fuliginea* conidia and by mycelium of *Pyricularia oryzae* were performed according to UMBREIT et al.

(1951). Per flask, 2.0 or 3.0 ml of conidial or mycelial suspensions were used. In the center well, 0.2 ml of 20% KOH and in the side-arm 0.1 ml of a fungicide solution were pipetted. The experiments were performed in fourfold at 25°C. In later experiments, oxygen uptake of mycelial suspensions of *P. oryzae* was measured polarographically using a Clark recording oxygen cathode in a 3 ml chamber. If necessary, respiratory rates were calculated on the basis of 240 μM O_2 in the aerated medium (IKUMA and BONNER, 1967). The measurements were carried out in duplicate at 25°C.

2.10 MEASUREMENTS OF RADIOACTIVITY

Radioactivity in liquid samples was counted in a Nuclear Chicago, Model Mark I liquid scintillation counter. A scintillation solution according to BRUNO and CHRISTIAN (1961) was used. Radioactivity of the samples was counted three times for 10 min. and corrected for background radioactivity. All data were expressed as disintegrations per min.

Radioactivity in thin-layer chromatograms was recorded by means of a Nuclear Chicago Actigraph III. From the scans the proportional distribution of radioactivity in the chromatograms could be computed. If necessary, the absolute amount of radioactivity on the chromatogram was determined by counting a sample of the solution, spotted on the chromatogram, in the liquid scintillation counter.

2.11. DETERMINATION OF LEAKAGE OF ^{32}P-ORTHOPHOSPHATE FROM MYCELIAL CELLS OF *PYRICULARIA ORYZAE*

Standard mycelial suspensions of *Pyricularia oryzae* were incubated in portions of 90 ml with 10 ml ^{32}P-orthophosphate solution in 300 ml flasks on an orbital shaker at 180 rpm. at 25°C. The final radioactivity in the mycelial suspension amounted to about 5 nCi/ml. After about 4 hours of incubation when 60–70% of the ^{32}P-orthophosphate had been taken up by the mycelial cells, the suspensions were centrifuged and washed three times with equal volumes of a 2% glucose solution in distilled water. Then, the suspensions were resuspended in 100 ml of a glucose, yeast extract medium and again incubated on the orbital shaker. After about 30–60 min of incubation, fungitoxic chemicals were added. After various incubation times, samples of 5 ml were taken from these suspensions, and immediately centrifuged. Radioactivity was measured in samples of the supernatants and was expressed as a percentage of radioactivity present in the mycelial suspension at zero time. The natural half-life of ^{32}P-orthophosphate during an experiment was taken into account by measuring the radioactivity in the mycelial suspension just before measuring all other samples.

2.12. Incorporation of ^{14}C-pyrazophos in *Pyricularia oryzae*

2.12.1. *Fractionation of mycelial cells*

Standard mycelial suspensions of *Pyricularia oryzae* incubated with ^{14}C-pyrazophos in portions of 20 ml in 100 ml flasks on an orbital shaker at 180 rpm and 25°C were centrifuged and washed five times with 25 ml of distilled water. The mycelial pellets were fractionated according to modified methods of Munro and Fleck (1966). The pellets were cooled in ice and extracted five times for 3 min with 5 ml of cold acetone. Subsequently, the pellets were extracted three times for 15 min with 2.5 ml ice-cold 5% perchloric acid (PCA). The PCA extracts were combined, while the residues were extracted with 5 ml of ethanol, ethanol/ether 3:1, and ether, respectively; also these extracts were combined. The residues were suspended in 4 ml of 0.5 N KOH and extracted for one night at 37°C. The next day, the suspensions were cooled in ice and acidified with 25% PCA. After 30 min the suspensions were centrifuged. The supernatants were combined with those of a following washing of the residues with 4 ml cold 5% PCA. The remaining pellets were suspended again in 4 ml 5% PCA and heated at 70°C for 20 min. After cooling, the suspensions were centrifuged. The supernatants obtained were combined with those of a following washing with the same PCA solution. The remaining mycelial residues were suspended in 4 ml of water. All centrifugations were carried out in a Homef centrifuge at 3000 rpm. The fractions obtained were always made up to a known volume, samples of which were taken for measuring radioactivity in a liquid scintillation counter.

2.12.2. *Detection and identification of metabolites*

Standard mycelial suspensions incubated with ^{14}C-pyrazophos in portions of 10 ml in 50 ml flasks on an orbital shaker at 180 rpm and 25°C were centrifuged. The culture media were decanted while the pellets were rinsed five times with 10 ml of distilled water. Subsequently, the pellets were suspended in 5 ml of acetone and extracted overnight. Then, the suspensions were centrifuged and the pellets washed again two times with 5 ml of acetone. The acetone extracts were combined, while the remaining residues were suspended in 5.0 ml of water. All centrifugations were carried out with a Homef centrifuge at 3000 rpm. From all fractions obtained samples were taken for measuring radioactivity in a liquid scintillation counter.

Identification of ^{14}C-components in the culture media took place as follows: after acidifying with 1.0 ml of 5% PCA, the culture media were extracted three times with equal volumes of toluene. Preliminary experiments showed the extraction of chemically pure pyrazophos, the phosphate analogue of pyrazophos (PO-pyrazophos) and of their hydrolysis product 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP) to be quantitative under these conditions. Radioactivity in the toluene extracts and the remaining water phase (residual toluene-extracted culture medium) was also determined. Samples of the toluene extracts were evaporated to a small volume and spotted

on silicagel F 254 thin-layer plates (Merck). The combined wash waters of the beforementioned mycelial pellets were freeze-dried and their residues extracted twice with 5 ml of toluene. The extracts were combined, evaporated to a small volume and aliquots spotted on thin-layer plates. The combined acetone extracts of the mycelial residues were also concentrated and spotted on thin-layer plates. The plates were developed using cyclohexane/acetone 7:3 and ether as solvents. Rf-values of pyrazophos, PO-pyrazophos, and PP with cyclohexane/acetone as the solvent were about 0.57, 0.25, and 0.16, respectively and with ether 0.77, 0.33, and 0.60, respectively.

Radioactivity on the chromatograms was scanned using an Actigraph III. From these data the distribution of radioactivity among the different components in a particular fraction could be calculated. This also enabled calculation of the absolute radioactivity of each component.

In preliminary experiments it was shown that pyrazophos and some metabolites to be expected like PO-pyrazophos and PP, were chemically stable during the extraction procedures.

2.13. INCORPORATION OF ^{14}C-LABELLED SUBSTRATES IN *PYRICULARIA ORYZAE*

2.13.1. ^{14}C-glucosamine

Standard mycelial suspensions of *Pyricularia oryzae* were incubated in triplicate on an orbital shaker in portions of 9 ml in 50 ml flasks. To the flasks 1 ml of a D-glucosamine-1-^{14}C hydrochloride solution with a specific activity of 9.26 mCi/mmole was added. The final activity of the chemical in the mycelial suspensions was 2.14 nCi/ml. After incorporation of the radiochemical, the samples were centrifuged at 3000 × g for 2 min and washed three times with distilled water. The incubation media, which contained residual ^{14}C-glucosamine, were stored until use while the rinse waters were discarded. The mycelial pellets were extracted overnight with 5 ml of 5% trichloroacetic acid (TCA) and centrifuged again; this provided TCA-extracts and mycelial residues, which were processed further according to slightly modified methods of MAEDA et al. (1970). The TCA-extracts which contained acid-soluble intermediates of chitin synthesis, were washed three times with equal volumes of diethylether. The mycelial pellets were extracted for one night with 10 ml of 1 N NaOH and the next day for 30 min with 10 ml of 50, 75, and 96% ethanol and with methanol/chloroform 3:1, respectively. Then, the mycelial residues were suspended in 5.0 ml of distilled water using a Potter-Elvehjem homogenizer. Radioactivity was assayed in incubation media, ether-washed TCA-extracts and in suspensions of the mycelial residues.

2.13.2. ^{14}C-uridine and ^{14}C-thymidine

Standard mycelial suspensions of *P. oryzae* were incubated in triplicate with uridine-2-^{14}C and thymidine-2-^{14}C as described for ^{14}C-glucosamine incorporation. The radiochemicals had a specific activity of 56 and 62 mCi/mmole,

16   *Meded. Landbouwhogeschool Wageningen 74-14 (1974)*

respectively. The final activity of both chemicals in the mycelial suspensions was about 0.57 nCi/ml. After incorporation of the radiochemicals the mycelium was precipitated by centrifuging at 3000 × g for 2 min and washed three times with cold distilled water. For the extraction of DNA and RNA the mycelial precipitates were processed further according to methods of MUNRO and FLECK (1966). To this end, the pellets were extracted three times for 10 min with 5.0 ml of ice-cold 0.2 N perchloric acid (PCA) at 0–4 °C.

In the case of the ^{14}C-uridine experiments the PCA-extracted mycelial pellets were suspended in 2.0 ml 0.3 N KOH and incubated overnight at 37 °C. The next day, the suspensions were acidified with 2.5 N PCA to pH 4.0 and kept in ice for 30 min. Then, the suspensions were centrifuged at 3000 × g for 5 min at 4 °C and washed with 2.0 ml 0.2 N PCA. The supernatants of these extracts were combined and made to 5.0 ml with 0.2 N PCA. This fraction is referred to as the RNA-extract.

In the case of the ^{14}C-thymidine experiments, the PCA-extracted mycelial pellets were suspended in 2.0 ml of 1.0 N PCA and heated for 20 min at 70 °C. After centrifugation at 3000 × g for 3 min, the pellet was resuspended again in 2.0 ml 1.0 N PCA and heated for another 20 min at 70 °C. After centrifugation, the hot PCA-extracts were combined and made to 5.0 ml with 1.0 N PCA. This fraction is referred to as the DNA-extract. Radioactivity was assayed in incubation media, PCA-extracts and RNA- and DNA-extracts.

2.13.3. ^{14}C-phenylalanine

Standard mycelial suspensions of *P. oryzae* were incubated in triplicate with L-3-phenylalanine-^{14}C as described for ^{14}C-glucosamine incorporation. The ^{14}C-phenylalanine had a specific activity of 513 mCi/mmole; the final activity of the radiochemical in the mycelial suspensions was about 0.66 nCi/ml. After incorporation of the radiochemical, the mycelium was precipitated by centrifugation at 3000 × g for 2 min and washed three times with cold distilled water. Subsequently, the mycelial pellets were extracted according to methods of CLEMONS and SISLER (1971) with 5 ml of 96 % ethanol and two times with 5 ml of 50 % ethanol, respectively. The ethanol extracts were combined, while the mycelial residues were suspended in 5.0 ml of distilled water. Radioactivity was assayed in incubation media, the combined ethanol extracts and mycelial residues.

2.14. DETERMINATION OF NUCLEIC ACIDS AND PROTEINS

Nucleic acids in mycelium of *Pyricularia oryzae* were determined in fourfold by extraction of mycelial suspensions as described under 2.13.2. The final RNA-extract was made to 10 ml instead of 5 ml and assayed for RNA by means of the orcinol reaction according to the description of MUNRO and FLECK (1966). Samples of 0.5 ml were made to 3.0 ml with 0.2 N PCA and combined with 3.0 ml of an orcinol reagent, which consisted of 0.6 % orcinol

and 0.02% $FeCl_3.6H_2O$ in approximately 12 N HCl. The mixtures were heated for 30 min at $100\,°C$ and their absorption at 660 nm was read against a blank consisting of 3.0 ml 0.2 N PCA and 3.0 ml orcinol reagent. The concentrations of RNA in the samples were calculated, using a calibration curve, made with 0.01% yeast RNA solution in 0.2 N PCA.

In the case of DNA determinations the final DNA-extracts of 5 ml were prepared from 40 ml of the mycelial suspension instead of 10 ml. DNA was determined with diphenylamine according to BURTON (1956). To samples of 2.0 ml of the DNA-extract, 2.0 ml of a 4% solution of diphenylamine in glacial acetic acid and 0.1 ml 0.16% (w/v) aqueous acetaldehyde were added. After mixing the components by swirling, they were incubated overnight at $30\,°C$. Then, their absorption at 595 nm was read against a blank which consisted of 2.0 ml 1.0 N PCA, 2.0 ml of diphenylamine reagent and 0.1 ml acetaldehyde solution. The concentrations of DNA in the samples were calculated using a calibration curve, made with a 0.01% calf thymus DNA suspension in 1.0 N PCA.

Protein determinations of mycelium of *P. oryzae* were performed in duplicate by washing and extracting 10 ml of mycelial suspensions as described under 2.13.3. The extracted mycelial residues were suspended in 1 ml water and assayed for proteins according to GORNAL et al. (1949) by adding 4.0 ml of a biuret reagent. This reagent was prepared by combining a solution of 1.5 g cupric sulphate and 6.0 g of sodium potassium tartrate in 500 ml of water with 300 ml of a 10% NaOH solution, after which the solution was made to 1 l with distilled water. The protein samples were mixed with the reagent by swirling and allowed to stand overnight. The next day the samples were centrifuged and the absorption of the supernatant at 540 nm was measured against a blank consisting of 1.0 ml water and 4.0 ml of biuret reagent. Protein concentrations in the samples were read from a calibration curve, established with a clear solution of BSA, the protein concentration of which was assayed by means of its ultraviolet light absorption at 280 nm (LAYNE, 1957).

3. FUNGITOXICITY OF ORGANOPHOSPHORUS COMPOUNDS

3.1. INTRODUCTION

In crop protection organophosphorus compounds are mainly used as insecticides. The development of these compounds was initiated by SCHRADER in Germany in 1934 and since then a large number of insecticidal, acaricidal and nematicidal organophosphorus compounds have found acceptance in practical use. Generally, these organophosphorus pesticides have a low fungal toxicity; fungicidal activity has been found in only a small number of organophosphorus insecticides. Reviews on fungicidal effects of organophosphorus compounds have been published by SCHEINPFLUG and JUNG (1968) and by MEL'NIKOV (1969). One of the first publications on this subject described the fungicidal activity of the systemic insecticide phorate (Thimet) when used against *Rhizoctonia solani* in cotton (ERWIN and REYNOLDS, 1958). In vitro efficacy of phorate against *R. solani* and some other fungi has also been reported (HACSKAYLO and STEWART, 1962). Other organophosphorus insecticides with fungicidal activity in vivo are: demeton (Systox) and disulfoton (Disyston), against *Verticillium* wilt in potato (HOYMAN and DINGMAN, 1964, 1965 and 1967); demeton, demeton-S-methyl, thiometon, phosphamidon and metaisosystox, against different powdery mildew fungi, although the addition of lead arsenate appeared essential for good activity (KIRBY, 1957; BESEMER and TACONIS, 1961); parathion, against rust fungi, especially *Puccinia coronata* on oats, and against bean powdery mildew (YARWOOD, 1954; GONDRAN, 1966; FLEISCHMANN et al., 1968; SIMONS and BROWNING, 1970) and diazinon and phorate, against *Helminthosporium oryzae* on rice (ANIMA PAL, 1969).

Some investigations have been carried out to study effects of organophosphorus compounds on bacteria (SWART-FÜCHTBAUER, 1955; EL-HELALY et al., 1963; NAUMANN, 1970). In general, the in vitro toxicity of the compounds tested was low; only chlorthion showed a relatively high toxicity against some phytopathogenic bacteria like *Pseudomonas* spp., *Erwinia carotovora* and *Agrobacterium tumefaciens*.

Probably the first organophosphorus compound[1] developed as a fungicide is triamiphos (1-[bis(dimethylamido)phosphoryl]-3-phenyl-5-aminotriazole-1, 2, 4; Wepsyn). Triamiphos is systemic; it is selectively active against powdery mildew fungi on a number of ornamental plants. Under practical conditions it has a useful side-effect on aphids and some effects on spider mites (KOOPMANS, 1960; ELINGS, 1961). Microscopic observations revealed that development of *Erysiphe graminis* f. sp. *tritici* on triamiphos treated barley plants was

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[1] The chemical names of the organophosphorus compounds are according to American usage, as given by FEST and SCHMIDT (1973). The compounds are sometimes also referred to as phosphates $(RO)_2P(O)OR$, phosphorothionates $(RO)_2P(S)OR$, phosphorothiolates $(RO)_2P(O)SR$ and phosphorothiolothionates $(RO)_2P(S)SR$.

usually stopped because of malformation of haustoria (MAGENDANS and DEKKER, 1966). The bis-(dialkylamido)phosphoryl group of the fungicide appeared to be essential for the anti-mildew activity. The fungitoxicity also depends on the substituents in the triazole ring. Maximum activity was found when the substituent at C(3) is H, alkyl or phenyl and at C(5) amino (VAN DEN BOS et al., 1960).

A related group of compounds, 1- and 2-[bis(dimethylamido)phosphoryl]-3-alkyl-5-anilino-1,2,4-triazoles with fungicidal and insecticidal activity have been described by the same research group (TEMPEL et al., 1968).

Since the introduction of triamiphos a number of other organophosphorus fungicides have been developed and used in practice. Among these are fungicides which are specially used for the control of blast and other diseases on rice, e.g. Kitazin-P (O,O-diisopropyl S-benzyl phosphorothioate), Hinosan (O-ethyl S,S-diphenyl phosphorodithioate) and Cerezin (O-methyl O-cyclohexyl S-(4-chlorophenyl) phosphorothioate. These fungicides display both a curative and protective effect. Kitazin-P is systemic; to control rice blast it can be applied in a granular formulation in paddy water or as a spray. The compound is also effective against *H. oryzae*, *H. sigmoideum* and *Pellicularia sasakii* on rice (KADO and YOSHINAGA, 1969; YOSHINAGA, 1969). The phosphate analogue of Kitazin-P shows fungicidal activity only in vitro. Probably the poor in vivo activity is attributable to rapid decomposition of this compound under practical conditions. O,O-diisopropyl O-benzyl phosphorothioate and O,O-diisopropyl S-benzyl phosphorodithioate, both analogues of Kitazin-P, are fungicidal neither in vivo nor in vitro; the O,O-diethyl analogue (Kitazin), however, displays again a strong fungitoxicity both in vivo and in vitro (MAEDA et al., 1970). According to UESUGI (1970) Hinosan and Cerezin are also fungitoxic to *Pyricularia oryzae*. Because the latter compound is toxic to fish, it can not be used in ordinary paddy fields. The former compound has also an effect against *H. sigmoideum*, *P. sasakii* and *Cochliobolus miyabeanus* and some insecticidal activity (UESUGI, 1970; UMEDA, 1973). Other compounds recently found to be active against diseases on rice are Conen (O-n-butyl S-ethyl S-benzyl phosphorodithioate), Inezin (O-methyl S-benzyl phenylphosphonothioate and Phosber (O-methyl O-(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate, a compound also active against insects on rice (UESUGI, 1970; KAJIWARA, 1971; YAMAGUCHI, 1971; UESUGI and TOMIZAWA, 1972).

Other organophosphorus fungicides active against *P. oryzae* belong to the group of saligenin cyclic phosphorus esters. Saligenin cyclic S-methyl and S-ethyl phosphorothioate show fungicidal activity, while on the other hand, cyclic O-methyl phosphate and its thiophosphate analogue (salithion) display a low fungitoxicity but a high insecticidal action (ETO et al., 1968).

In addition to the compounds mentioned above, which are mainly active against the rice blast fungus, other compounds were found which primarily show activity against powdery mildew fungi. One of these is O,O-diethyl phthalimidophosphorothioate (Dowco 199). This compound effectively controlled powdery mildew on rose, red cherry, cucumber, apple and turf and was also

active against apple scab and some leaf spot diseases (CLARE et al., 1968; SEMENIUK and PALMER, 1970; THEUNE, 1970). The fungicidal activity is associated with the N-phosphorylated dicarboximide moiety. Substitution of the thiono group by oxygen, and the introduction of methylene groups or of an oxygen atom between the nitrogen and phosphorus atom enhance mammalian toxicity and lead to a marked decrease in fungicidal activity (TOLKMITH and MUSSELL, 1967; TOLKMITH and SENKBEIL, 1967). TOLKMITH and MUSSELL (1967) also described the fungicidal activity of imidazolyl phenylphosphonoamidothioates against *Erysiphe cichoracearum* and *Phytophthora infestans* on tomato plants; N,N-diethyl N'-(2-methylimidazol-1-yl) and N,N-dimethyl N'-imidazol-1-yl phenylphosphonoamidohtioate appeared to be the most interesting of these compounds. Later also the fungitoxicity of N,N-dialkyl N'-imidazol-1-yl phosphoroamidochloridothioates was described (BUDDE and TOLKMITH, 1969).

Another chemical mainly active against powdery mildews is pyrazophos (Curamil, Afugan, Hoe 2873, O,O-diethyl O-(5-methyl-6-ethoxycarbonyl-pyrazolo[1,5-a]pyrimidin-2-yl) phosphorothioate). Protection of more than 30 crops against different powdery mildew fungi has been described. The compound is systemic and exerts a protective and strong curative action (MARIOUW SMIT, 1969). The control of apple powdery mildew by pyrazophos was reported by other research workers (COVEY, 1968; HAY, 1971). Replacement of ethyl in the ethoxycarbonylester group by alkyl chains of varying length up to $C_{12}H_{25}$ does not influence the activity against powdery mildew on wheat. Substitution of methyl by ethyl in the diethyl phosphorothioate group causes a slight reduction in fungitoxicity and replacement of sulphur by oxygen at the phosphorus atom (PO-pyrazophos) results in a nearly complete loss of fungicidal activity (MARIOUW SMIT, 1969). The related compound 2-hydroxy-5-methyl-6-ethoxycarbonyl(1,5-a)pyrimidine (PP) is not fungitoxic at all against powdery mildew fungi (BELAL, 1971).

Trichlorex, an O,O,O-tributyl phosphate derivative of 2,4,5-trichlorophenol was tested in vitro against some fungi among which *R. solani* showed a relatively high sensitivity (BOUILLENNE-WALRAND et al., 1968).

Other, simple organophosphates with fungitoxic properties are a number of phosphorotetrathioates, trimethyl phosphorothioate and O,O-diethyl S-methyl phosphorodithioate. The latter compound was successfully tested against seedling diseases in cotton caused by *R. solani* (JACOB, 1967). Trimethyl phosphorothioate is a selective fungicide used to control *Pythium* spp. (FEST and SCHMIDT, 1973). In vitro, some phosphorotetrathioates displayed an aselective toxicity to a number of fungi tested. The most active compound proved to be S,S,S-trimethyl phosphorotetrathioate. The activity decreased with increasing length of the alkyl chains (SCOTT et al., 1960).

The aim of the experiments, described in this chapter is to extend the investigations on the fungitoxicity of organophosphorus compounds, in order to obtain fungus-fungicide combinations which are most suitable to study the mode of action of these compounds. Because of their relatively high toxicity,

attention was especially focused on the antifungal activity of pyrazophos, Hinosan and Kitazin. Since the phosphate analogues of phosphorothionate insecticides play an important role in the mode of action of these compounds, also the fungitoxicity of the phosphate analogue of pyrazophos (PO-pyrazophos) and of some of the other compounds used, were investigated.

3.2. RESULTS

3.2.1. *In vivo toxicity to powdery mildew fungi*

The fungitoxicity of a number of organophosphorus fungicides, insecticides and structurally related compounds have been studied in the leaf disc test; some of these were investigated further in a foliar spray and root application test. The tests have been described under 'Materials and methods' 2.4.1.

In the leaf disc test *Sphaerotheca fuliginea* appeared to be most sensitive to pyrazophos (table 3.1.). Even a concentration of 10^{-6} M completely inhibited fungal growth on the leaf discs. PO-pyrazophos was fungitoxic only at a concentration of 10^{-4} M but at the same time very phytotoxic. Of the other fungicides tested, Kitazin and triamiphos were not active; Dowco 199 and Hinosan were fungitoxic at a concentration of 10^{-4} M, but also slightly phytotoxic. Of the insecticides tested only parathion inhibited fungal growth (table 3.1.). Azinphos-ethyl, coumaphos, demeton-S, demeton-S-methyl, dimethoate, malathion, O,O-diethyl O-phenyl phosphorothioate and their phosphate analogues and diethoxyphosphorothioate, which are not included in table 3.1., did not show activity against the fungus.

The activity of pyrazophos (30% w/v formulation) against *S. fuliginea* in a foliar spray test can be seen in table 3.2. Applied as a protective spray, a concentration of 10^{-4} M completely prevented fungal growth, while in the case of a curative spray applied about 5–6 days after inoculation a concentration of 4×10^{-4} M was necessary for disease control. Similar results were

TABLE 3.1. Effect of some organophosphorus fungicides, insecticides and related compounds against cucumber powdery mildew in the leaf disc test. For assessment see 2.4.1.; (t) t = (very) phytotoxic.

compound	concentrations (M)				
	control	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Dowco 199	5	0^t	5	5	–
Hinosan	5	3^t	5	5	–
Kitazin	5	5	5	5	–
parathion	5	0^t	4	5	–
paraoxon	5	5	5	5	–
pyrazophos	5	0	0	0	5
PO-pyrazophos	5	0^{tt}	5	5	5
triamiphos	5	5	5	5	–

obtained with chemically pure pyrazophos, dissolved in water with 0.0025%
Tween 20. In the same tests, PO-pyrazophos, Hinosan and Kitazin were not
active even at the highest concentration tested (4×10^{-4} M). As a protective
spray against barley powdery mildew, pyrazophos again proved to be the most
active. In contrast to *S. fuliginea*, *E. graminis* f. sp. *hordei* was also sensitive to
Hinosan and, although to a lesser extent, to Kitazin and PO-pyrazophos
(table 3.3.).

TABLE 3.2. Effect of pyrazophos (30% w/v formulation) against cucumber powdery mildew
in foliar spray tests. For assessment see 2.4.1.

protective spray		curative spray	
concentration (M)	disease index	concentration (M)	disease index
1.0×10^{-4}	0	4.0×10^{-4}	0
0.5×10^{-4}	2	2.0×10^{-4}	1
0.1×10^{-4}	3	1.0×10^{-4}	2–3
control	5	0.5×10^{-4}	4–5
		control	5

TABLE 3.3. Effect of pyrazophos, PO-pyrazophos, Hinosan and Kitazin against barley pow-
dery mildew in foliar spray tests (protective) and root application tests. Figures represent
percentages of the leaves covered with mycelium. For assessment see 2.4.1.; (t)t = (very)
phytotoxic.

concen-tration (M)	foliar spray				root application			
	pyra-zophos	PO-pyra-zophos	Hino-san	Kita-zin	pyra-zophos	PO-pyra-zophos	Hino-san	Kita-zin
10^{-4}	1	44	18	42	27^t	45^{tt}	36^t	21^t
10^{-5}	6	68	68	67	51	51	49	42
10^{-6}	33	77	82	78	58	57	45	39
control	77	77	77	77	52	52	52	52

The systemic activity of pyrazophos against cucumber powdery mildew was
also studied using a root application test, at concentrations of 10^{-4}, 10^{-5} and
10^{-6} M. The average disease indexes observed 8 days after inoculation were
3–4, 4–5 and 5, respectively. At all concentrations tested, the non-infected
parts of the leaves were restricted to the veins. This mildew-free zone was
relatively broad at a concentration of 10^{-4} M and almost absent at 10^{-6} M
(fig. 3.1.). PO-pyrazophos, Hinosan and Kitazin did not show systemic fungici-
dal activity, even at the highest concentration tested (10^{-4} M).

The systemic activity of the same compounds against barley powdery
mildew is given in table 3.3. With the exception of PO-pyrazophos, all com-
pounds showed a moderate anti-mildew activity at the highest concentration
tested. At this concentration, however, phytotoxic symptoms like chlorosis and
necrotic spots, were observed in the upper half of the leaves.
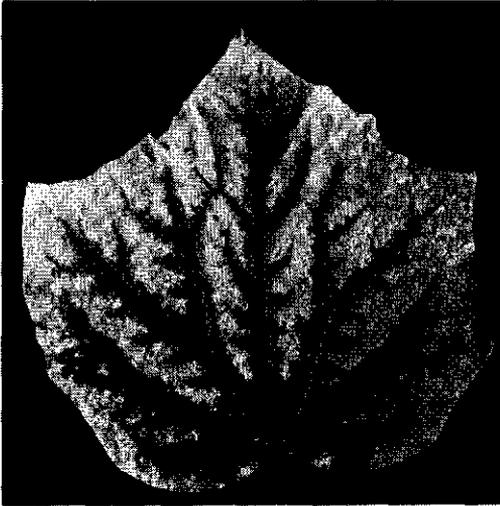
FIG. 3.1. Cucumber leaf infected with *Sphaerotheca fuliginea*. Leaf taken from a plant, placed in a 10^{-4} M suspension of pyrazophos for 2 days.

3.2.2. *In vitro toxicity to powdery mildew fungi*

The in vitro fungicidal activity of a number of organophosphorus compounds was tested in spore germination tests on cellophane membranes at a concentration of 10^{-4} M. It appeared that beside the organophosphorus fungicides tested, some insecticidal thio- and dithiophosphates show a significant effect on germination of both *S. fuliginea* and *E. graminis* f. sp. *hordei* conidia (table 3.4.). Most pronounced effects were observed with azinphos-ethyl and parathion. Substitution in the insecticides of the thiono group by oxygen generally reduced their fungitoxic activity.

Effects of pyrazophos, PO-pyrazophos, Hinosan and Kitazin on germination of *S. fuliginea* conidia are presented in fig. 3.2. The shape of the dosage-response curves shows that inhibition of germination by pyrazophos and PO-pyrazophos is much less concentration-dependent than that by Hinosan and Kitazin. The ED_{50}-values of pyrazophos, PO-pyrazophos, Hinosan and Kita-
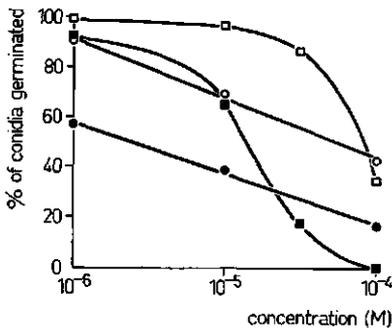
FIG. 3.2. Effect of pyrazophos (●—●), PO-pyrazophos (○—○), Hinosan (■—■) and Kitazin (□—□) on germination of *Sphaerotheca fuliginea* conidia in vitro on cellulose membranes.

TABLE 3.4. Effect of organophosphorus fungicides, insecticides and related compounds on germination percentages and length of germ tubes of *Sphaerotheca fuliginea* and *Erysiphe graminis* f. sp. *hordei* in spore germination tests and on radial growth of *Pyricularia oryzae* on Gly agar, at a concentration of 10^{-4} M. Percentages of germination, length of germ tubes and radial growth based on control as 100%.

	S. fuliginea	E. graminis f. sp. hordei		P. oryzae
	germination	germination	length of germ tubes	radial growth
fungicides and related compounds				
Dowco 199	0	0	0	95
Hinosan	0	0	0	3
Kitazin	35	0	0	0
pyrazophos	17	7	29	14
PO-pyrazophos	43	22	31	27
triamiphos	39	14	21	100
insecticides and related compounds				
azinphos-ethyl	14	4	59	45
PO-analogue of azinphos-ethyl	107	77	93	81
coumaphos	58	16	90	75
PO-analogue of coumaphos	78	49	81	73
demeton-S	86	39	51	94
demeton-O	84	10	43	94
demeton-S-methyl	23	53	67	99
demeton-O-methyl	96	56	61	98
dimethoate	96	87	93	87
PO-analogue of dimethoate	103	93	90	85
malathion	92	30	31	84
malaoxon	99	89	81	79
parathion	28	8	37	65
paraoxon	55	20	84	81
O,O-diethyl O-phenyl phosphorothioate	56	14	55	87
O,O-diethyl O-phenyl phosphate	103	86	80	94
diethoxyphosphorothioate	95	57	72	98

zin, derived from the curves, were 2×10^{-6}, 6×10^{-5}, 2×10^{-5} and 8×10^{-5} M, respectively.

The in vitro toxicity of pyrazophos and PO-pyrazophos to *S. fuliginea* was investigated further by determining their effect on elongation of germination hyphae (VAN 'T LAND and DEKKER, 1972). From the results, presented in fig. 3.3., the ED_{50}-values of the compounds were found to be 5×10^{-8} and 10^{-5} M, respectively. Thus, the ED_{50}-values of pyrazophos and PO-pyrazophos for spore germination are about 40 and $6 \times$ as high as those for growth of germination hyphae, respectively.

3.2.3. *In vitro antifungal spectrum of pyrazophos*

In the literature, pyrazophos is described only as an anti-mildew fungicide. Due to the obligate parasitic character of powdery mildew fungi, in vitro

TABLE 3.5. Effect of pyrazophos and PO-pyrazophos on radial growth of some fungi on PDA. *Ascomycetes are classified according to their conidial state. The fungi are arbitrarily distinguished in three categories according to their sensitivity: a. $ED_{50} < 10^{-4}$ M, b. 10^{-3} M $< ED_{50} < 10^{-4}$ M and c. $ED_{50} > 10^{-3}$ M.*

| | days after inoculation | diameter of colony (mm) | | | | | category of sensitivity to | |
| | | control | conc. of pyrazophos | | conc. of PO-pyrazophos | | pyrazophos | PO-pyrazophos |
			10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M		
OOMYCETES								
Phytophthora cryptogea	4	40.3	32.7	32.8	33.8	15.0	c	b
Pythium debaryanum	1	32.0	29.5	28.6	11.6	7.7	c	a
ZYGOMYCETES								
Mortierella elongata	4	68.0	44.8	29.8	46.2	7.5	b	b
Mortierella isabellina	6	47.5	27.7	12.0	36.2	2.5	b	b
Mortierella vinacea	6	32.0	17.0	3.7	15.7	0.0	b	a
Rhizopus nigricans	2	59.5	39.5	27.1	36.3	10.7	b	b
DEUTEROMYCETES								
Sphaeropsidales								
Ascochyta pisi	4	23.5	18.0	17.4	23.5	14.3	c	c
Melanconiales								
Colletotrichum acutatum	4	29.3	23.7	23.2	27.7	11.5	c	b
Colletotrichum lindemuthianum	4	18.7	5.0	4.6	17.5	0.0	a	b
Moniliales								
Blastosporae								
Botrytis cinerea	4	53.3	32.3	283.	47.5	16.8	c	b
Cladosporium cucumerinum	4	13.0	13.2	12.4	14.5	6.7	c	c
Monilia fructicola	4	32.5	25.3	24.3	23.6	0.1	c	b
Porosporae								
Helminthosporium oryzae	4	32.7	21.4	21.8	24.3	18.6	c	c
Phialosporae								
Aspergillus niger	4	33.0	27.7	25.8	25.6	20.5	c	c
Fusarium culmorum	5	73.2	51.0	39.0	37.0	15.2	c	b
Penicillium expansum	5	27.7	24.3	24.2	22.2	15.3	c	c
Pyricularia oryzae	5	24.6	2.0	0.0	5.2	0.0	a	a
Verticillium albo-atrum	5	18.2	13.5	13.6	13.0	7.1	c	b
MYCELIA STERILIA								
Rhizoctonia solani	4	72.1	39.5	39.3	40.0	16.5	c	b
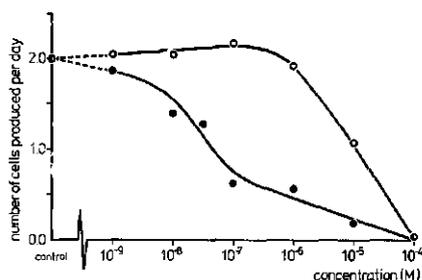
FIG. 3.3. Effect of pyrazophos (●—●) and PO-pyrazophos (○—○) on growth of germination hyphae of *Sphaerotheca fuliginea* in vitro.

investigations on the mode of action of pyrazophos against these organisms will be difficult. In order to find other fungi, sensitive to this compound, studies on the in vitro antifungal spectrum of pyrazophos were performed. In addition, also the antifungal spectrum of PO-pyrazophos was investigated. In the literature, some of the fungi used in these experiments, have been reported to be sensitive to other organophosphorus compounds (ch. 3.1.). From table 3.5. it is evident that all fungi tested were relatively insensitive to pyrazophos, with the exception of *Colletotrichum lindemuthianum* and *P. oryzae*. The ED_{50} of the compound to these fungi was lower than 10^{-4} M. With the exception of *P. oryzae*, the toxicity of PO-pyrazophos to the fungi tested was generally slightly higher than that of pyrazophos. This is most evident when the toxicities of the compounds are compared at a concentration of 10^{-3} M.

Some additional data on the antifungal spectrum of pyrazophos and its phosphate analogue were obtained by means of TLC-bioassays ('Materials and methods' 2.4.3.). Using this bioassay, minimal inhibitory amounts to mycelial growth of some fungi on thin-layer plates could be established (table 3.6.). The method proved not to be applicable to *P. oryzae*. Although the results of these experiments are difficult to compare with those mentioned above, it is evident that under these conditions some of the fungi tested do show a relatively high sensitivity, e.g. *C. acutatum* for pyrazophos and *Fusarium culmorum* for PO-pyrazophos, both with a minimal inhibitory amount of about 3 nmole.

TABLE 3.6. Minimal amount in nmoles of pyrazophos and PO-pyrazophos which inhibited mycelial growth of some fungi on thin-layer plates.

	pyrazophos	PO-pyrazophos
Ascochyta pisi	100	10
Aspergillus niger	30	10
Botrytis cinerea	>100	30
Cladosporium cucumerinum	>100	100
Colletotrichum acutatum	3	10
Colletotrichum lindemuthianum	100	10
Fusarium culmorum	100	3
Penicillium expansum	>100	30

The minimal amount of pyrazophos which suppressed growth of *C. lindemuthianum*, appeared to be relatively high, whereas radial growth of the fungus on PDA was shown to be relatively sensitive (table 3.5.). In the case of *C. acutatum* the opposite was found.

3.2.4. *In vitro toxicity to* Pyricularia oryzae

Investigations on the in vitro antifungal spectrum of pyrazophos showed that *P. oryzae* was one of the most sensitive non-obligate parasitic fungi tested. The fungus is also sensitive to other organophosphorus compounds, like Hinosan and Kitazin (KADO and YOSHINAGA, 1969; YOSHINAGA, 1969 and UESUGI, 1970). The in vitro toxicity of organophosphorus compounds to *P. oryzae* were, therefore, studied more extensively.

Results of experiments on the effect of pyrazophos and PO-pyrazophos on radial growth of *P. oryzae* on Gly agar are presented in fig. 3.4. From the dosage-response curves ED_{50}-values of pyrazophos and PO-pyrazophos were found to be 10^{-5} and 5×10^{-5} M, respectively. The dosage-response curves of both compounds for mycelial growth in shake cultures with liquid Gly media were also determined (fig. 3.4.). Under these experimental conditions the ED_{50}-values of pyrazophos and PO-pyrazophos were found to be 10^{-6} and 3×10^{-5} M, respectively.

Beside mycelial growth, germination of *P. oryzae* spores was also tested for sensitivity to pyrazophos and PO-pyrazophos. Only pyrazophos possessed a low inhibitory capacity for spore germination and germ tube growth (table 3.7.). Hence, mycelial growth is much more sensitive to pyrazophos and PO-pyrazophos than spore germination.

Results of experiments on the toxicity of other organophosphorus fungicides, insecticides and related compounds to *P. oryzae* are included in table 3.4. With
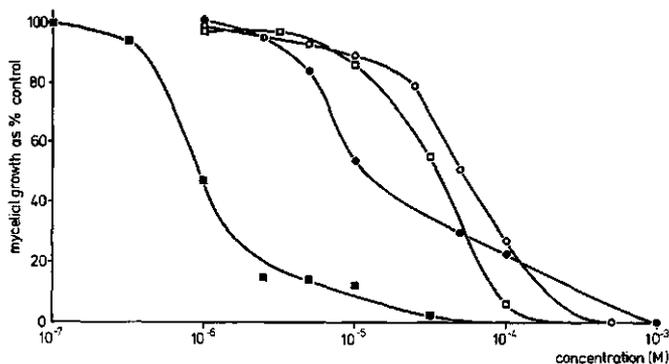
FIG. 3.4. Effect of pyrazophos and PO-pyrazophos on mycelial growth of *Pyricularia oryzae* in vitro on Gly agar and in shake cultures of the same composition without agar.
Agar medium with pyrazophos: (●—●) and PO-pyrazophos: (○—○); shake culture with pyrazophos: (■—■) and PO-pyrazophos: (□—□).

TABLE 3.7. Effect of pyrazophos, PO-pyrazophos, Hinosan and Kitazin on germination percentage and length of germ tubes of *Pyricularia oryzae* conidia in spore germination tests. Percentages of germination and length of germ tubes based on control as 100 %.

concen-tration (M)	pyrazophos		PO-pyrazophos		Hinosan		Kitazin	
	% of germina-tion	length of germ tubes	% of germina-tion	length of germ tubes	% of germina-tion	length of germ tubes	% of germina-tion	length of germ tubes
10^{-6}	101	94	103	97	96	92	101	100
10^{-5}	100	76	102	96	0	0	101	95
3.3×10^{-5}	99	93	99	93	0	0	93	39
10^{-4}	92	69	101	101	0	0	0	0

the exception of azinphos-ethyl and parathion, all insecticides and related compounds tested had only a weak antifungal activity. Beside pyrazophos, of the fungicides tested, only Hinosan and Kitazin proved to be fungitoxic. Both compounds had an ED_{50}-value of 3×10^{-5} M for radial growth (fig. 3.5.).

Hinosan and Kitazin also showed strong inhibitory effects towards spore germination and germ tube growth of *P. oryzae*. The results of these experiments indicate the occurrence of a rather critical concentration above which germination did not take place (table 3.7.). This phenomenon is in strong contrast with the effect of pyrazophos and PO-pyrazophos on these processes.

In order to find out whether the toxicity of the compounds described above has a fungistatic or fungicidal character, agar discs with mycelium of *P. oryzae* were placed on Gly agar containing 10^{-3} M of pyrazophos, PO-pyrazophos, Hinosan and Kitazin. For each chemical 5 Petri-dishes each with 3 agar discs were used. After 10 days mycelial growth on agar containing pyrazophos or PO-pyrazophos was 2 and 0.5 mm, respectively. After transferring the discs to agar without these chemicals the colonies grew out vigorously. Mycelial growth was neither observed on agar containing Hinosan and Kitazin,
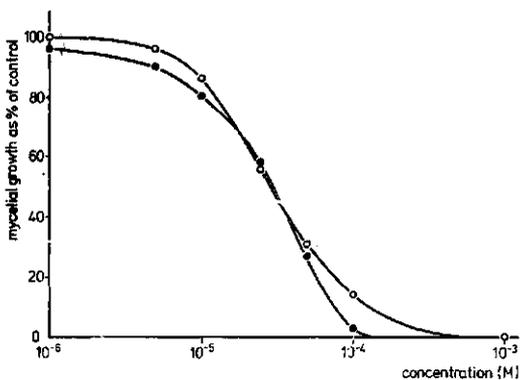
FIG. 3.5. Effect of Hinosan (●—●) and Kitazin (○—○) on mycelial growth of *Pyricularia oryzae* in vitro on Gly agar.

nor on fungicide free media after transfer of the discs. The results suggest that pyrazophos and PO-pyrazophos are fungistatic, while Hinosan and Kitazin are rather fungicidal.

3.2.5. *In vivo toxicity to* Pyricularia oryzae

In vivo fungicidal effects of pyrazophos, PO-pyrazophos, Hinosan and Kitazin against *P. oryzae* on barley were investigated in foliar spray tests. The results, presented in table 3.8. show that Hinosan and Kitazin, which are both described in the literature as compounds effective against rice blast (KADO and YOSHINAGA 1969; YOSHINAGA, 1969 and UESUGI, 1970), reduced infection significantly. Pyrazophos, which is described in the literature as an anti-mildew fungicide (MARIOUW SMIT, 1969), also accomplished disease control.

TABLE 3.8. Activity of some organophosphorus fungicides against *Pyricularia oryzae* on barley in a protective foliar spray test. Figures represent numbers of leaf spots based on control as 100%.

concentration (M)	pyrazophos	PO-pyrazophos	Hinosan	Kitazin
10^{-4}	12	34	6	28
10^{-5}	70	80	48	54
10^{-6}	99	84	52	64

3.3. DISCUSSION

The experiments described in this chapter show a strong protective and curative action of pyrazophos against cucumber powdery mildew after foliar sprays with 10^{-4} and 4×10^{-4} M solutions, respectively (table 3.2.). In a leaf disc test the compound proved to be fungitoxic even at a concentration of 10^{-6} M (table 3.1.). The systemic activity against cucumber powdery mildew is unsatisfactory because of the fact that the fungitoxicity is confined to the vein system. Possibly, the lipophylic character of pyrazophos restricts the distribution of the compound from the veins into the mesophyll of the leaves. This is in contrast with strongly hydrophylic compounds like p-methoxyphenyl methylsulphone, which are transported to the leaf margins (ROMBOUTS, 1971).

Protective foliar sprays of pyrazophos could also eradicate barley powdery mildew. After root application only an incomplete disease control was obtained, while at the same time symptoms of phytotoxicity in the leaves were observed (table 3.3.).

As a foliar spray both Hinosan and Kitazin showed a protective activity against *P. oryzae* on barley (table 3.8.).

The results are in agreement with data from the literature that phosphorothionate fungicides such as pyrazophos are mainly active against powdery

30

mildew fungi, whereas phosphorothiolate or phosphorodithiolate compounds like Kitazin, Cerezin and Hinosan are mainly toxic to *P. oryzae* and other fungi on rice (SCHEINPFLUG and JUNG, 1968). The experiments, however, showed that pyrazophos also exerts a fungitoxic action against *P. oryzae* on barley (table 3.8.). In their turn, Hinosan and Kitazin were found to be toxic to *S. fuliginea* and *E. graminis* f. sp. *hordei*; this could be observed in spore germination tests (table 3.4.). In vivo, only barley powdery mildew proved to be sensitive (compare tables 3.1. and 3.3.). Thus, the difference between the anti-fungal spectra of phosphorothionate, phosphorothiolate and phosphorodi-thiolate compounds only seems to be a gradual one.

The in vitro experiments show a rather selective toxicity of pyrazophos to *P. oryzae*. The toxicity to the fungus is strongly dependent on the test used. ED_{50}-values of pyrazophos for spore germination, radial growth on agar and mycelial growth in shake culture were found to be $> 10^{-4}$ (table 3.7.), 10^{-5} and 10^{-6} M (fig. 3.4.), respectively. Similar results were obtained for the in vitro toxicity of the compound to *S. fuliginea*. ED_{50}-values for spore germina-tion and growth of germination hyphae were 2×10^{-6} and 5×10^{-8} M, respec-tively (fig. 3.2. and 3.3.). It is not evident, which factors are responsible for this phenomenon.

Compounds possessing a phosphorothionate group proved to be generally more toxic to powdery mildew fungi than their oxygen analogues. This was evident for pyrazophos in all fungitoxicity tests performed and also for most of the organophosphorus insecticides tested against *S. fuliginea* and *E. graminis* f. sp. *hordei* (table 3.4.). This might be explained by the more lipophylic charac-ter of phosphorothionates and phosphorothiolothionates compared to the corresponding phosphates and phosphorothiolates. This hypothesis is suppor-ted by results of experiments of BELAL (1971), who found with pyrazophos, PO-pyrazophos and three other derivatives of pyrazophos a correlation between their partition coefficient in oleylalcohol and water and their fungicidal activity against barley and cucumber powdery mildew. The lipophylic character possibly facilitates the uptake of phosphorothionates through the gelatinous outer layer of the cell wall of powdery mildew fungi, which might explain the rather selective toxicity of these compounds (McKEEN et al., 1966 and 1967). It is not clear whether the same reasoning can be applied to explain the high toxicity of pyrazophos to *P. oryzae*.

The in vitro antifungal spectrum of PO-pyrazophos (table 3.5.) showed that *Pythium debaryanum*, *Mortierella vinacea* and *P. oryzae* are most sensitive to this compound, having an ED_{50}-value lower than 10^{-4} M. The toxicity of PO-pyrazophos to most of the fungi tested is of the same order of magnitude or even higher than that of pyrazophos. Again the difference in lipophylic or hydrophylic character of these compounds in relation to the cell wall composi-tion of these fungi might explain this phenomenon.

External factors which render it difficult to compare fungitoxicities of pyrazophos and PO-pyrazophos, as described above, are the water insolubility of pyrazophos at concentrations higher than about 5×10^{-5} M and the gener-

ally lower chemical stability of organophosphates in water as compared with the stability of thiophosphate analogues.

The antifungal spectrum of both compounds with regard to growth inhibition of different fungi in TLC-bioassays (table 3.6.) differs strongly from that in agar (table 3.5.). No explanation for these results can be given. It is possible that adsorption of the compounds on the silicagel layer affects their rate of uptake into the fungal mycelium.

The in vitro experiments indicate that with respect to *P. oryzae*, pyrazophos and PO-pyrazophos are rather fungistatic while Hinosan and Kitazin are distinctly fungicidal. In culture media, containing even 10^{-3} M pyrazophos or PO-pyrazophos the fungus is not killed, while above a certain, critical concentration Hinosan and Kitazin cause an irreversible inhibition of mycelial growth. The lethal effects of the latter compounds are also evident in spore germination tests with the same fungus.

These observations suggest that the mechanism of action of pyrazophos, Hinosan and Kitazin may be different. More detailed investigations on this subject will be described in following chapters.

3.4. SUMMARY

In vivo fungitoxicity tests showed a strong protective and curative action of pyrazophos against *Sphaerotheca fuliginea* on cucumber. However, the systemic activity of the compound proved to be unsatisfactory. When applied as a foliar spray pyrazophos also displayed a fungitoxic activity against *Erysiphe graminis* f. sp. *hordei* and *Pyricularia oryzae* on barley. The activity against *P. oryzae* equalled that of Hinosan and Kitazin, both well-known fungicides active against rice blast. On foliar application, Hinosan and Kitazin also displayed a toxicity to barley powdery mildew. All compounds displayed an in vitro toxicity to the fungi mentioned above. The in vitro effects suggested that pyrazophos is rather fungistatic, while Hinosan and Kitazin are fungicidal.

A study on the antifungal spectrum of pyrazophos indicated that the compound is rather selective in its fungitoxic action. Beside powdery mildews and *P. oryzae* only *Colletotrichum lindemuthianum* proved to be relatively sensitive.

In the case of pyrazophos and some phosphorothionate insecticides it was found that their phosphate analogues were generally less fungitoxic to *S. fuliginea*, *E. graminis* f. sp. *hordei* and *P. oryzae*, while usually the opposite results were found with the other fungi tested. These differences might be based on the lipophylic or hydrophylic character of these compounds in relation to the different composition of the fungal cell walls.

4. EFFECT OF PYRAZOPHOS ON ESTERASE ACTIVITY OF
SPHAEROTHECA FULIGINEA

4.1. INTRODUCTION

In a review on powdery mildew fungicides YARWOOD (1954) noticed a similarity between powdery mildews and spider mites as to their response to chemicals like sulphur and parathion. A strong correlation between anti-mildew and acaricidal activity has also been described for substituted nitrophenols like dinobuton (Acrex), dinocap (Karathane) and binapacryl (Acricid) and for quinoxalines like oxythioquinox (Morestan) and thioquinox (Eradex) (MARTIN, 1968). As cited in chapter 3 also some organophosphorus compounds possess both fungicidal and insecticidal or acaricidal properties. It is questionable whether the intrinsic mode of action of these compounds is the same in these different organisms.

With respect to organophosphorus insecticides it is known that their lethal effect against both vertebrates and invertebrates is due to inhibition of acetyl-cholinesterase, which gives rise to an interference with normal nervous activity through accumulation of acetylcholine at the nerve endings. The mode of action of organophosphorus fungicides must be different because of the absence of this enzyme in fungi. It is known, however, that still other sites of action of organophosphorus compounds exist. LORD and POTTER (1954) were the first to demonstrate the high sensitivity of other esterases for these chemicals. These esterases are described in the literature as aliesterases, B-esterases and finally as carboxylesterases (EC 3.1.1.1) (ALDRIDGE, 1953; GOMORI, 1953; DIXON and WEBB, 1967). They are insensitive to eserine and hydrolyse non-specifically a number of substrates like methyl butyrate, ethyl butyrate, phenyl acetate, phenyl propionate and α-naphthyl acetate (NA) (AUGUSTINSSON, 1958; VAN ASPEREN, 1964). In some cases also a lipase or proteolytic activity of esterases has been reported (AUGUSTINSSON, 1958; WIGGLESWORTH, 1958, 1966; COOK et al., 1969). NA is frequently used as a substrate because it is hydrolysed by different types of esterases and because it can also be applied for the histochemical demonstration of these enzymes (PEARSE, 1961; STEPHAN and CHELDELIN, 1970).

Studies on the presence, characteristics and sensitivity to organophosphorus compounds of esterases in insects and spider mites have frequently been made. The enzymes have also been demonstrated in a number of fungi and bacteria; however, with the exception of a study of SIERRA (1957), these publications merely describe their presence and characteristics in cell-free extracts or in situ by means of a histochemical demonstration (MANDELS, 1953; BYRDE and FIELDING, 1955; GREUALL and SIERRA, 1957; STOCK et al., 1961; SIERRA, 1963; ROBERTS and ROSENKRANZ, 1966; BAILLIE et al., 1967; WALKER et al., 1967; MORICHI et al., 1968; PEBERDY and TURNER, 1968; PITT, 1968; MEYER and

RENARD, 1969; PITT and COOMBES, 1969; BROWN, 1970; CHILD et al., 1971; EL-SHARKAWY and HUISINGH, 1971).

As implicated above an inhibition of esterase activity in fungi might be responsible for the mode of fungitoxic action of organophosphorus compounds. It was shown in chapter 3 that pyrazophos was one of the organophosphorus compounds which was most toxic to *Sphaerotheca fuliginea*. Therefore, the in vitro inhibitory potency of this phosphorothionate fungicide for substrate non-specific esterases in cell-free extracts of this fungus has been investigated. In insects, phosphorothionate insecticides are generally converted into their phosphate analogues; these are regarded as the actual insecticidal principles, because of their much higher potencies to inhibit acetylcholinesterase than that of the parent compounds. Therefore, also the inhibition of esterase activity by PO-pyrazophos and paraoxon has been investigated. Paraoxon was studied because of its well-known high esterase-inhibiting capacity. In addition, experiments were also carried out to determine the effect of these compounds on esterase activity in intact conidia, hyphae and haustoria, in order to determine whether they inhibit enzyme activity in vivo or not.

4.2. RESULTS

4.2.1. *Introductory experiments*

Cell-free extracts of *S. fuliginea* conidia were tested for the presence of acetylcholinesterase and substrate non-specific esterases according to methods described under 2.7.6. and 2.7.5. in 'Materials and methods', respectively. Under the test conditions used, no acetylcholinesterase activity could be detected. However, substrate non-specific esterases, carboxyl- and arylesterases, could be easily demonstrated. Using NA as a substrate, formation of α-naphthol during a 20 min incubation period appeared to be linear with the amount of cell-free extract added, up to an amount of about 1.6 μmole α-naphthol per 6.0 ml incubation mixture. This amount of α-naphthol corresponds with an
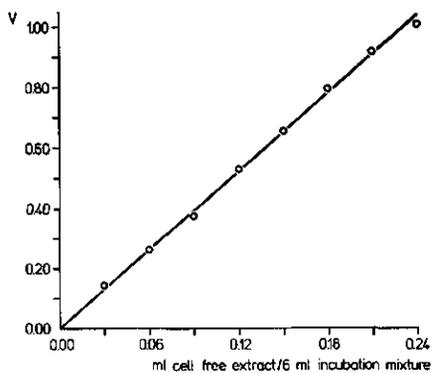
FIG. 4.1. Esterase activity (v) in relation to concentration of a cell-free extract of *Sphaerotheca fuliginea* conidia. Assay medium: 6 ml of 2.5 $\times$ 10^{-4} M NA in 0.04 M phosphate buffer pH 7, containing various amounts of cell-free extracts; v = α-naphthol-diazoblue complex formation in incubation mixture expressed as $\triangle A_{600nm}$ after 20 min of incubation.
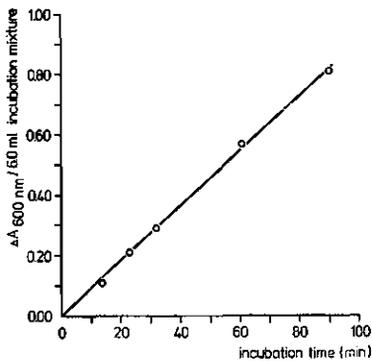
FIG. 4.2. NA hydrolysis by esterases in a cell-free extract of *Sphaerotheca fuliginea* conidia in relation to incubation time. Assay medium: 6 ml of 2.5×10^{-4} M NA in 0.04 M phosphate buffer pH 7, containing a fixed amount of cell-free extract; degree of hydrolysis measured as α-naphthol-diazoblue complex formation ($\triangle A_{600nm}$) after various incubation times.

absorbance of the α-naphthol-diazoblue complex of about 1.00 (fig. 4.1.). Using a fixed amount of cell-free extract a linear relationship was also found between product formation and incubation time (fig. 4.2.). The relation between esterase activity and substrate concentration has been presented in fig. 4.3. The K_m value of NA was calculated to be 5.6×10^{-4} M. In all subsequent experiments an NA concentration of 2.5 or 3.0×10^{-4} M was used because of the water insolubility of NA at higher concentrations (VAN ASPEREN, 1962).

4.2.2. *In vitro inhibitory potency*

Inhibition of cholinesterases or substrate non-specific esterases by organophosphorus compounds can be determined according to ALDRIDGE (1950) and MAIN (1964). ALDRIDGE expressed the irreversible phosphorylation reaction between esterase (E) and organophosphate (PX) by the following equation

$$E + PX \xrightarrow{\quad k_i \quad} EP + X \qquad (I)$$
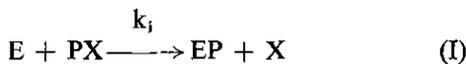
in which the bimolecular rate constant k_i is generally considered to be a
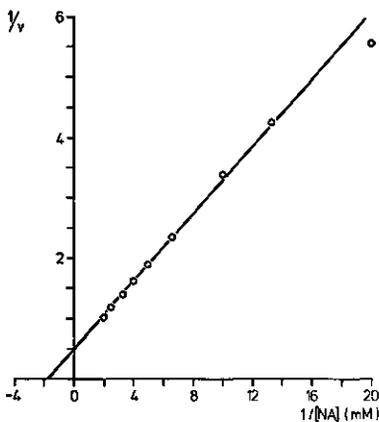
FIG. 4.3. Esterase activity (v) in a cell-free extract of *Sphaerotheca fuliginea* conidia in relation to NA concentration. Assay medium: 6 ml of $0.5–5.0 \times 10^{-4}$ M NA in 0.04 M phosphate buffer pH 7, containing a fixed amount of cell-free extract; v = α-naphthol-diazoblue complex formation in incubation mixture, expressed as $\triangle A_{600nm}$ after 20 min of incubation.

reliable criterion by which to measure the inhibitory power of an organo-phosphate for an esterase. The equation is based on the assumption that esterase and organophosphate react directly to form the irreversibly phosphorylated enzyme (EP); the formation of an enzyme-inhibitor complex is neglected. According to ALDRIDGE (1950) the time course of the straightforward, bimolecular phosphorylation reaction may simulate a first order reaction and thus seems to depend on the concentration of a single reactant. This is realized when in inhibition experiments the concentration of the inhibitor is essentially constant. As reviewed by SMISSAERT (1972) determinations of k_i can be based on the equation

$$\log P = 2 - \frac{k_i[I]}{2.3} t \qquad\qquad (II)$$

in which P indicates the percentage of non-inhibited esterase activity, [I] the inhibitor concentration and t a pre-incubation (inhibition) time of enzyme and organophosphate. The k_i should be determined over a range of inhibitor concentrations and should not vary significantly with change of [I].

As described above k_i values of pyrazophos, PO-pyrazophos and paraoxon for esterases in cell-free extracts of conidia of *S. fuliginea* were determined by measuring the non-inhibited esterase activity after various pre-incubation times of enzyme and inhibitor before addition of the substrate ('Materials and methods' 2.7.5.). A prerequisite is that at the moment of substrate addition the phosphorylation reaction has to be stopped. This was investigated by studying the relationship between α-naphthol formation and incubation time after a 3 min pre-incubation of 0.1 ml of cell-free extract and 0.1 ml of inhibitor solution at the highest concentrations used. After the pre-incubation time the mixture was diluted with 5.0 ml 3.12×10^{-4} M NA and assayed for α-naphthol after various times (fig. 4.4.). Product formation appeared to be almost linear
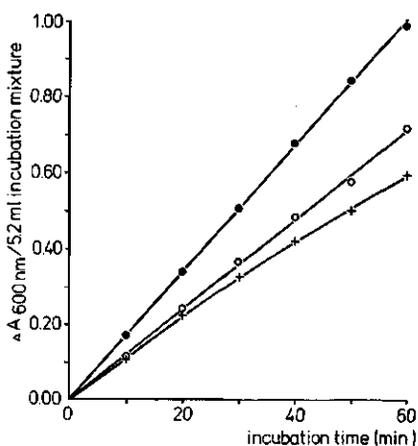
FIG. 4.4. NA hydrolysis by esterases in a cell-free extract of *Sphaerotheca fuliginea* conidia in relation to incubation time, after 3 min pre-incubation of 0.1 ml of extract with 0.1 ml of inhibitor solution, resulting in final concentrations of 5×10^{-5} M pyrazophos (O—O), 10^{-6} M PO-pyrazophos (●—●), and 5×10^{-5} M paraoxon (+—+). After pre-incubation, the mixture was diluted with 5 ml 3.12×10^{-4} M NA in 0.04 M phosphate buffer pH 7; degree of hydrolysis measured as α-naphthol-diazoblue complex formation ($\triangle A_{600nm}$) after various incubation times.
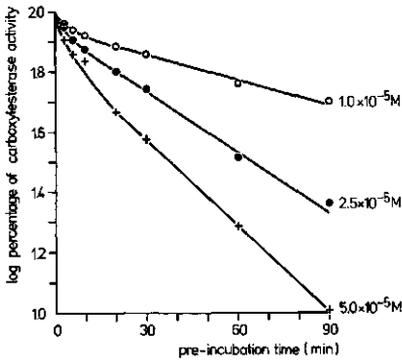
FIG. 4.5. Carboxylesterase activity in a cell-free extract of *Sphaerotheca fuliginea* conidia in relation to time of pre-incubation of a mixture of extract and pyrazophos, at concentrations of pyrazophos as indicated in figure. After pre-incubation, 0.2 ml of the mixture was diluted with 5 ml 3.12×10^{-4} M NA in 0.04 M phosphate buffer pH 7. Residual esterase activity was determined by measuring the α-naphthol-diazoblue complex formation in the incubation mixture after 20 min of incubation.

with incubation time, indicating that enzyme activity remained constant during the incubation period.

In further experiments, the relation between length of pre-incubation time and percentage of inhibition of esterase activity in the cell-free extracts at fixed inhibitor concentrations was studied. Plots of these relations each produced an asymptotic curve, the maximum value of which approaches 74% of inhibition. This indicates that esterase activity was due to at least two different components of the enzyme. The component which was inhibited by the organophosphates, can be regarded as carboxylesterase, while the other component most probably represents arylesterase. Therefore, inhibition percentages of total esterase activity were converted into inhibition percentages of carboxylesterase activity (MAIN, 1969). Next, carboxylesterase activity expressed as the logarithm of the percentages of residual activity has been plotted against pre-incubation time (fig. 4.5., 4.6. and 4.7.). The plots obtained proved to be not linear, but consisted of a straight and a curved part; this could suggest that at least two types of carboxylesterases were present in the cell-free extract, which were inhibited at significantly different rates (MAIN, 1964). According to equation II k_i values were calculated from the slope of the
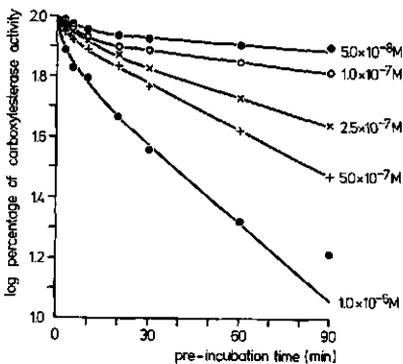
Fig. 4.6. Carboxylesterase activity in a cell-free extract of *Sphaerotheca fuliginea* conidia in relation to time of pre-incubation of a mixture of extract and PO-pyrazophos, at concentrations of PO-pyrazophos as indicated in figure. After pre-incubation, 0.2 ml of the mixture was diluted with 5 ml 3.12×10^{-4} M NA in 0.04 M phosphate buffer pH 7. Residual esterase activity was determined by measuring the α-naphthol-diazoblue complex formation in the incubation mixture after 20 min of incubation.
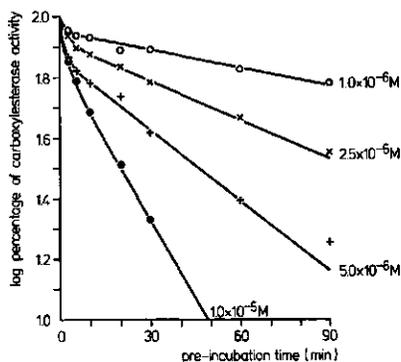
FIG. 4.7. Carboxylesterase activity in a cell-free extract of *Sphaerotheca fuliginea* conidia in relation to time of pre-incubation of a mixture of extract and paraoxon, at concentrations of paraoxon as indicated in figure. After pre-incubation, 0.2 ml of the mixture was diluted with 5 ml 3.12×10^{-4} M NA in 0.04 M phosphate buffer pH 7. Residual esterase activity was determined by measuring the α-naphthol-diazoblue complex formation in the incubation mixture after 20 min of incubation.

straight part of these plots; the values, calculated for the different inhibitor concentrations used, are presented in table 4.1. The average k_i values of pyrazophos, PO-pyrazophos and paraoxon were found to be 5.6×10^2, 2.9×10^4 and 4.1×10^3 M^{-1} min^{-1}, respectively.

4.2.3. *Inhibition of esterase activity in conidia and haustoria*

A suspension of freshly harvested conidia of *S. fuliginea* in 0.04 M phosphate buffer pH 7.0 proved to hydrolyse NA. The esterase activity amounted to about 95 % of the activity present in the same suspension after ultrasonic disintegration of the conidia. Product formation during a 20 min incubation time (v) proved to be linear up to concentrations of about 10^6 conidia per incubation mixture, which concentration gave rise to hydrolysis of about 1.6 µ mole NA. Similar results were obtained for the relation between product formation and incubation time; product formation of α-naphthol was linear with incubation time up to 80 min; then, an amount of 1.6 µmole of NA per incubation mixture, containing 3.4×10^5 conidia, has been hydrolysed.

Inhibition of esterase activity in intact conidia was investigated as described under 'Materials and methods' 2.7.5. by measuring residual esterase activity after pre-incubation of 0.2 ml conidial suspension with 0.2 ml inhibitor solution.

TABLE 4.1. Bimolecular rate constants of pyrazophos, PO-pyrazophos and paraoxon and carboxylesterases in cell-free extracts of conidia of *Sphaerotheca fuliginea*.

pyrazophos		PO-pyrazophos		paraoxon	
conc. (M)	k_i (M^{-1}min^{-1})	conc. (M)	k_i (M^{-1}min^{-1})	conc. (M)	k_i (M^{-1}min^{-1})
5.0×10^{-5}	4.3×10^2	1.0×10^{-6}	2.6×10^4	1.0×10^{-5}	4.0×10^3
2.5×10^{-5}	6.2×10^2	5.0×10^{-7}	2.3×10^4	5.0×10^{-6}	3.6×10^3
1.0×10^{-5}	6.4×10^2	2.5×10^{-7}	3.0×10^4	2.5×10^{-6}	4.2×10^3
		1.0×10^{-7}	2.9×10^4	1.0×10^{-6}	4.5×10^3
		5.0×10^{-8}	3.6×10^4		
average k_i:	5.6×10^2	average k_i:	2.9×10^4	average k_i:	4.1×10^3
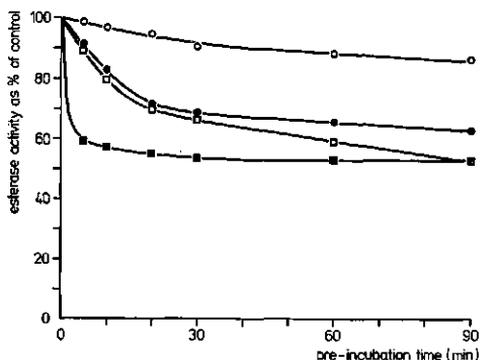
After the pre-incubation time 10 ml of 3.12×10^{-4} M NA were added. Results have been presented in fig. 4.8. As was found in the in vitro experiments, again PO-pyrazophos caused a significantly stronger inhibition of esterase activity than pyrazophos. Furthermore, it was evident that total esterase activity could be ascribed to carboxyl- and probably arylesterase activity, as no higher inhibition percentages could be obtained than 48%.

Upon incubation with NA, haustorial suspensions, prepared according to methods modified after DEKHUIJZEN (1966) as described under 'Materials and methods' 2.5.2., also displayed esterase activity. Product formation of α-naphthol was linear with incubation time up to 90 min; then, an amount of 0.8 μmole of NA per reaction mixture, containing 2×10^6 haustoria, was hydrolysed.

Inhibition of esterase activity in haustoria was studied by spraying plants, infected with *S. fuliginea*, one day before the isolation of the haustoria with water, 10^{-4} M pyrazophos or PO-pyrazophos, respectively. Per treatment 18 plants were used. The esterase activity of the three haustorial suspensions obtained, was determined as described for activity in cell free extracts and arbitrarily calculated as $\triangle A_{600\,nm}$ per 10^6 haustoria per 90 min of incubation of a reaction mixture containing 3×10^{-4} M NA. The esterase activities of haustorial suspensions from treated plants were expressed as a percentage of the control and were found to be 67 and 46% for pyrazophos and PO-pyrazophos, respectively. In another experiment, in which the plants were sprayed with 4×10^{-4} M fungicide solutions, these percentages proved to be 66 and 40, respectively. Thus, in both experiments inhibition by PO-pyrazophos was stronger than that by pyrazophos. Maximal inhibition of esterase activity was not obtained, because after pre-incubation of haustoria, obtained from control plants for 90 min with 5×10^{-5} M PO-pyrazophos, the residual esterase activity was found to be 18–21%.

4.2.4. *Histochemical demonstration of inhibition of esterase activity*

According to methods described under 'Materials and methods' 2.8., esterase

activity could be histochemically demonstrated in acetone fixed discs of cucumber leaves, whether or not infected with *S. fuliginea*. Macroscopically an intense black staining, due to an α-naphthol-diazoblue precipitate, was observed along a 1–2 mm margin of the discs. Microscopically, especially the vein system in this margin appeared to be densely stained. In infected discs, after 10 min of incubation a clearly visible, diffuse staining was also observed in the conidia and to a somewhat lesser extent in the hyphae. After prolonged incubation up to 30 min the conidia and hyphae were coloured black. The penetration hyphae were visible as black spots, while the haustoria did not show a significant staining.

Inhibition of esterase activity in the fungus by pyrazophos and PO-pyrazophos was demonstrated histochemically using the foliar spray, leaf disc and root application test with cucumber as the test plant.

In the foliar spray test, cucumber plants were inoculated with conidia. Two days after inoculation, when most of the conidia were germinated and had formed appressoria and hyphae, plants were sprayed with 10^{-4} M pyrazophos and PO-pyrazophos in water containing 0.0025% Tween 20. Treatment with 0.0025% Tween 20 in water was used as a control. For each treatment 6 cucumber plants were used. Before spraying and 1, 3, 5, 7 and 10 days after spraying discs were punched out of the leaves and kept at −22°C until use. Ten days after the fungicide application esterase activity was demonstrated histochemically in all discs and at the same time development of the fungus on the discs was assessed microscopically. Results are presented in table 4.2. Esterase activity in conidia and hyphae on plants treated with PO-pyrazophos was completely inhibited one day after application. Inhibition by PO-pyrazophos gradually decreased with time, while esterase inhibition in discs treated with pyrazophos increased; the latter effect was, however, probably caused indirectly by the lethal action of this chemical.

Fungal growth on the leaf discs was normal in the control and PO-pyrazophos treatments. In both cases, the first conidia were microscopically observed three days after the treatments and the macroscopical symptoms

TABLE 4.2. Histochemical demonstration of esterase activity in conidia and hyphae of *Sphaerotheca fuliginea* on cucumber leaves. Plants were sprayed with 10^{-4} M pyrazophos and PO-pyrazophos in water containing 0.0025% Tween 20, 2 days after inoculation. Esterase activity is given on an arbitrary scale from −: no activity, to $+++++$: activity as in control treatment.

days after application	control	pyrazophos	PO-pyrazophos
0	$+++++$		
1	$+++++$	$+++$	$+$
2	$+++++$	$+++$	$+$
5	$+++++$	$++$	$++$
7	$+++++$	$+$	$++$
10	$+++++$	$\pm$	$+++$

40

TABLE 4.3. Histochemical demonstration of the effect of pyrazophos and PO-pyrazophos on esterase activity in conidia and hyphae of *Sphaerotheca fuliginea* on cotyledons of cucumber. The compounds were applied to the roots at a concentration of 4×10^{-4} M at the time of inoculation of the seedlings; after 4 days esterase activity and fungitoxicity were assessed (I). In a second experiment the compounds were applied at a concentration of 2×10^{-4} M to diseased seedlings; after 2 days assessments for esterase activity were made (II). Esterase activity is given on an arbitrary scale from $-$: no activity, to $+ + + + +$: activity as in control treatment. For assessment of fungitoxicity see 2.4.1.

	esterase activity	disease index
experiment I		
control	$+ + + + +$	5
pyrazophos	$+ + + + +$	3[1]
PO-pyrazophos	$+ + +$	5
experiment II		
control	$+ + + + +$	
pyrazophos	$+ + +$	
PO-pyrazophos	$+$	

[1]) Infection restricted to the margins and the apical part of the cotyledons.

appeared after 10 days. Fungal growth on pyrazophos treated leaves proved to be completely stopped immediately after application.

In the leaf disc test, discs from infected leaves, which had been floated during 1 day on 10^{-5} M pyrazophos (fungitoxic) and PO-pyrazophos (non-fungitoxic) were stained histochemically for esterase activity. Esterase activity in conidia and hyphae on pyrazophos and PO-pyrazophos treated discs were arbitrarily assessed as $+ + +$ and $+$, respectively, while activity in the control treatment was $+ + + + +$ (Explanation of assessment under 2.8.). Thus, esterase activity is much less inhibited by pyrazophos than by PO-pyrazophos.

In the root application test cucumber seedlings were placed on solutions containing 4×10^{-4} M pyrazophos and PO-pyrazophos and inoculated with conidia of *S. fuliginea*. The experiment was performed in duplo with 3 seedlings per treatment. Four days after inoculation discs were punched out of cotyledons and stained histochemically. Results are presented in table 4.3. Pyrazophos, which only prevented fungal growth along the vein system of the leaves, hardly inhibited esterase activity, while PO-pyrazophos which was not fungitoxic at all, inhibited esterase activity significantly. In a similar experiment seedlings already infected with the fungus, were placed on 2×10^{-4} M pyrazophos and PO-pyrazophos. After 2 days esterase activity in the fungus was demonstrated (table 4.3.). Again, PO-pyrazophos proved to be a stronger inhibitor of esterase activity than pyrazophos.

4.3. Discussion

Substrate non-specific esterases, already demonstrated in a large number of different organisms, were also found to be present in *S. fuliginea*. Their activity could only partly be inhibited by organophosphates, indicating that beside carboxylesterases, which can be inhibited by these chemicals, probably also arylesterases were present.

Bimolecular rate constants of pyrazophos, PO-pyrazophos and paraoxon for carboxylesterases in cell-free extracts of conidia were calculated from the slopes of the log P against t plots. As can be derived from fig. 4.5., 4.6. and 4.7., the inhibition of esterase activity becomes progressively smaller and is linear with incubation from c. 10 min onwards. When the calculation of the k_i values was based on the slope of the linear parts of the curves, they did not vary significantly with change of [I] (table 4.1.), indicating that first order kinetics were present and the method used is applicable (MAIN, 1969). It is unlikely that the curved shape of the plots reflects a regeneration of the phosphorylated enzyme, since product formation after short pre-incubation times with the inhibitors at the highest concentrations used proved to be almost linear with time (fig. 4.4.). The most probable cause may be the presence of different carboxylesterases, each capable of hydrolysing the substrate but differing in their reactivity to organophosphates. In this case the plots can be resolved into several linear components as has been described for serum cholinesterases (MAIN, 1969). Esterases differing in their reactivity to organophosphorus compounds were also found in spider mites (SMISSAERT, 1965). Each linear component of the plots then represents a first order plot for the inhibition of a single enzyme. If this explanation is applicable for the inhibition of carboxylesterases of *S. fuliginea*, it should mean that the k_i values calculated, are based on the component of the enzymes, which possessed the lowest reactivity to the inhibitors used. The k_i values of the other component(s) are approximately 5–10 × higher but were not calculated because of the inaccuracy of measurements of the slope in the curved part of the plots.

The k_i values as given in table 4.1. show a relatively high inhibitory capacity of PO-pyrazophos compared with pyrazophos and paraoxon. The k_i value of pyrazophos was found to be 50 × as high as that of PO-pyrazophos; this is in agreement with the general finding that the inhibitory capacity of phosphorothionates for esterases is lower than that of their oxygen analogues (e.g. ALDRIDGE, 1950). These inhibitory capacities of pyrazophos and PO-pyrazophos are inversely correlated with their fungicidal action (ch. 3). This is also frequently found for phosphorothionate insecticides, which have a high in vivo insecticidal activity, but a low capacity to inhibit acetylcholinesterase in vitro. However, also in vivo PO-pyrazophos appeared to display a relatively strong inhibitory activity towards esterases in conidia, hyphae and haustoria, as compared with pyrazophos. This was demonstrated histochemically after their treatment with these compounds. Interference with penetration of PO-pyrazophos to the site of esterase activity in the fungus is, therefore, probably

not the cause of its lack of fungitoxicity. The apparent absence of a correlation between in vivo esterase inhibition and in vivo fungitoxicity of pyrazophos and PO-pyrazophos strongly indicates that the mode of fungitoxic action of pyrazophos is not based on inhibition of carboxylesterase activity.

Obviously esterase activity in *S. fuliginea* can be inhibited without a noticeable fungitoxic effect on the fungus. A comparable result has been found with ali-esterases of *Pseudomonas aeruginosa*, which could be completely inhibited by paraoxon without affecting growth (SIERRA, 1957).

Also in insects esterases can be specifically inhibited by triorthocresyl phosphate, without lethal effects; in this instance only symptoms of gastric malfunctions have been observed (STEGWEE, 1960). Therefore, the physiological role of substrate non-specific esterases in organisms is not yet well understood. Their localization in insects and mammalians suggests that they fulfil multiple functions of a secretory, digestive or resorptive character (f.i. THOMSON, 1966; FREYVOGEL et al., 1968; COOK et al., 1969; BONGERS and NOGGE, 1970). The presence of carboxylesterases in food vacuoles of protozoa also suggests a function in intracellular digestion of food in these organisms (GUTHWIN and KOPAC 1959; MÜLLER et al., 1960, 1967). Among the esterases found in plants, those which are present in sphaerosomes or lysosome-like particles may aleo be of importance for intracellular digestion (f.i. BALZ, 1966; MATILE, 1969; McLEAN and GAHAN, 1970).

In extensive histochemical studies on the localization of non-specific esterases in bacteria and fungi, mostly no suggestions were made about their function in these organisms (BAILLIE et al., 1967; WALKER et al., 1967; PITT, 1968; BROWN, 1970). Only in an investigation of LLOYD et al. (1971) on esterases in *Candida lipolytica*, *Aspergillus niger* and a yeast-like fungus their function has been described. Esterase activities were determined in intact cells and cell-free extracts, using NA as a substrate. The esterases of these organisms also hydrolysed tributyrin and hence can also arbitrarily be classified as lipases. In the case of *C. lipolytica* the natural substrate of these enzymes may be indeed lipid, since esterase activity was induced when the organism was grown on a solid tributyrin medium, while no activity could be detected when glucose replaced this substrate. Also the finding that intracellular enzyme activity in a liquid shaken glucose-mineral salts medium increased during growth, suggested that esterases of *C. lipolytica* may be involved in the metabolism of intracellular and extracellularly added lipids. In the yeast-like fungus three new esterases occurred at the onset of arthrospore formation. These enzymes are probably responsible for the increase in intracellular esterase activity and intracellular lipid utilization and thus, for the decrease in respiration quotient. In *A. niger* esterases were only found at late stages of conidiation when intracellular lipid decreased. In this fungus the level of lipolytic esterase activity and the intracellular lipid consumption during differentiation appeared to be interrelated. This again could mean, that in *A. niger* lipid functions as a source of carbon and energy during conidiation.

McKEEN (1970) estimated that osmiophylic bodies, consisting of lipids, in

hyphal cells of *Erysiphe graminis* f. sp. *hordei* occupied up to 25% of the cell volumes. They appeared about the fourth day after haustoria were formed and at the same time that conidiophores started to produce conidia and mycelial growth had ceased. In *Sphaerotheca macularis* conidia it was shown that the lipid bodies disappear upon germination (MITCHELL and McKEEN, 1970). The situation in the closely related cucumber powdery mildew fungus may be similar and, therefore, the possibility exists that the esterases described in this chapter also have a lipolytic function. However, because of the fact that after their inhibition by PO-pyrazophos no fungitoxic effects were observed, it does not seem probable that they play a vital role in the fungus. Hence, the even weaker esterase inhibition by pyrazophos has to be regarded as a side effect of the compound which does not seem to be responsible for its fungicidal action and the importance of which is not, as yet, understood.

4.4. SUMMARY

Esterase activity assayed by measuring the hydrolysis of NA, could be demonstrated in conidia, hyphae and haustoria of *Sphaerotheca fuliginea*. Activity in cell-free extracts of conidia could be partly inhibited by pyrazophos, PO-pyrazophos and paraoxon, indicating that both carboxyl- and probably arylesterases were present. Some evidence was obtained that carboxylesterase activity was probably caused by different enzyme components, differing in their reactivities to organophosphorus compounds. The bimolecular rate constants of pyrazophos, PO-pyrazophos and paraoxon for one of these components were calculated as 5.6×10^2, 2.9×10^4, and $4.1. \times 10^3$ M^{-1} min^{-1}, respectively, indicating a relatively high in vitro inhibitory capacity of PO-pyrazophos.

Also in in vivo experiments PO-pyrazophos displayed a stronger inhibition of esterase activity than pyrazophos, although PO-pyrazophos hardly possessed fungicidal activity. The absence of a correlation between in vivo esterase inhibition and fungitoxicity of pyrazophos and PO-pyrazophos suggests that the mode of action of pyrazophos is not based on an inhibition of carboxylesterases in the fungus. The results also indicate that these enzymes are probably of minor importance in the physiology of the fungus, because their activity could be inhibited without noticeable interference with fungal growth.

5. EFFECTS OF PYRAZOPHOS, HINOSAN AND KITAZIN ON CELL MEMBRANE PERMEABILITY

5.1. INTRODUCTION

In the previous chapter it was demonstrated that the mechanism of action of pyrazophos against *Sphaerotheca fuliginea* could not be explained by its esterase inhibitory capacity. Indications for other possible mechanisms of action were obtained from the literature in which the effect of Kitazin on the incorporation of ^{14}C-glucosamine in *Pyricularia oryzae* has been described (MAEDA et al., 1970). This fungicide induced an accumulation of UDP-N-acetylglucosamine, an intermediate of chitin synthesis, and caused an inhibition of the incorporation of ^{14}C-glucosamine into a mycelial cell wall fraction. The compound neither affected mycelial respiration nor nucleic acid and protein synthesis of the fungus (KAKIKI et al., 1969). It has been suggested, therefore, that Kitazin inhibits chitin synthetase activity or the passage of a lipophylic intermediate of chitin synthesis through the cytoplasmic membrane. An accumulation of intermediates of chitin synthesis was not found at relatively high concentrations of Kitazin; this might also indicate an effect on cytoplasmic membrane permeability (MAEDA et al., 1970).

Effects of Hinosan on the incorporation of ^{14}C-glucosamine in *P. oryzae* were found to be similar to those of Kitazin as described above (DE WAARD, 1972).

In a comparable way, effects of pyrazophos on chitin synthesis were investigated and compared with those of Hinosan and Kitazin. Because of their possible influence on cell membrane permeability also experiments on leakage of ^{32}P-orthophosphate from mycelial cells upon incubation with these compounds have been carried out.

5.2. RESULTS

5.2.1. *Effects on incorporation of* ^{14}C-glucosamine

Standard mycelial suspensions of *P. oryzae* in Gly agar were prepared as described under 'Materials and methods' 2.6. On addition of a D-glucosamine-1-^{14}C hydrochloride solution to the mycelial suspension (final radioactivity 2.14 nCi/ml), radioactivity was incorporated into the mycelial residue of the fungus linear with time, up to 150 min of incubation, when about 50% of the amount of radioactivity added, was found to be present in this fraction. Effects of pyrazophos, Hinosan and Kitazin were studied by adding these fungicides at various concentrations simultaneously with ^{14}C-glucosamine to mycelial suspensions. After a 2 hour incorporation period radioactivity was determined in incubation media, TCA-extracts of mycelial cells and mycelial residues as described under 2.13.1.
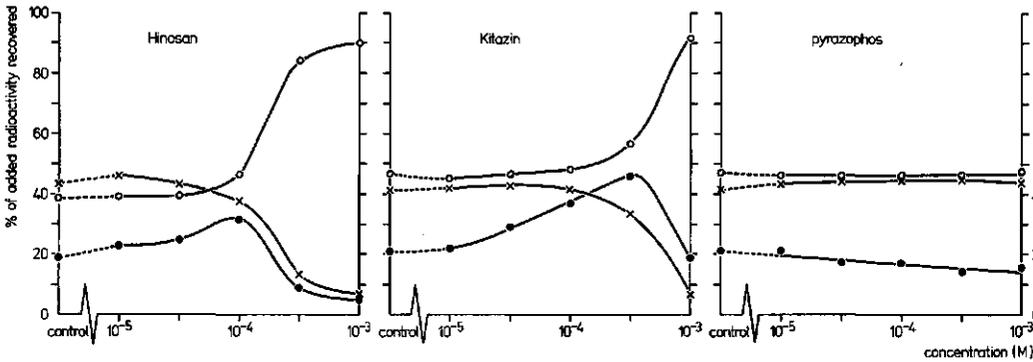
FIG. 5.1. Effects of Hinosan, Kitazin and pyrazophos on incorporation of ^{14}C-glucosamine by mycelium of *Pyricularia oryzae*. Percentages of added radioactivity recovered in incubation media (O—O), TCA-extracts of mycelium (●—●) and mycelial residues ($\times—\times$). Percentages of radioactivity recovered in TCA-extracts have been expressed on a 10 × enlarged scale.

Results of the experiments are shown in fig. 5.1. Both Hinosan and Kitazin gave rise to an accumulation of acid-soluble intermediates of chitin synthesis in the TCA-extract, which was maximal at a concentration of 10^{-4} M and 3.2×10^{-4} M, respectively. At higher concentrations, in both instances a decrease in percentage of radioactivity in this fraction was noticed, concomitantly with a decrease in the amount of ^{14}C-glucosamine incorporated into the mycelial residue and an increase in the amount of residual radioactivity in the incubation medium.

Data on the fungitoxicity of Hinosan and Kitazin under the test conditions used above are presented in fig. 5.2. On comparison of this figure with fig. 5.1. it appears that at a concentration of 10^{-4} M, which is more or less the ED_{50}-value of both compounds, the incorporation of ^{14}C-glucosamine into the mycelial residues was hardly reduced, indicating that chitin synthesis was much less inhibited than mycelial growth.

From fig. 5.1. it is also evident that pyrazophos did not affect chitin synthesis, even at a concentration of 10^{-3} M; at this concentration, mycelial growth was inhibited for only c. 50%, which is remarkable low, since the ED_{50}-value of the compound for mycelial growth during one week in shake cultures with media of the same composition, but inoculated with spores, was shown to be 10^{-6} M (ch. 3). The significance of this observation will be discussed in chapter 8.

5.2.2. *Effects on cell membrane permeability*

Standard mycelial suspensions incubated with ^{32}P-orthophosphate and washed thoroughly with a 2% glucose solution, were suspended in a Gly medium as described under 2.11. Then, the suspensions were incubated with various concentrations of pyrazophos, Hinosan and Kitazin. Untreated suspensions and suspensions with 1% chloroform were used as controls.
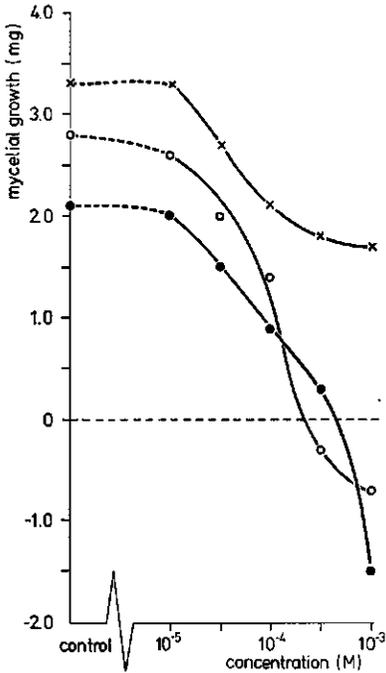
Fig. 5.2. Effect of Hinosan ($\bigcirc$—$\bigcirc$), Kitazin ($\bullet$—$\bullet$) and pyrazophos ($\times$—$\times$) on mycelial growth of *Pyricularia oryzae* during a 2 hour incubation period. Initial dry weight 19.2, 9.6 and 11.4 mg per 10 ml mycelial suspensions, respectively.

Results of the experiments are presented in fig. 5.3. The untreated mycelial cells hardly showed any leakage of ^{32}P-orthophosphate, while in the case of chloroform already after one hour of incubation more than 50% of the radioactivity initially present in the mycelium, was found in the incubation medium.

Mycelium treated with 3.2×10^{-4} and 10^{-3} M Hinosan and 10^{-3} M Kitazin also displayed a significant leakage of ^{32}P-orthophosphate. Fig. 5.2. shows that under the same conditions these treatments gave rise to a decrease of mycelial dry weight. After prolonged incubation also a low but significant leakage of the radio-chemical was observed at the ED_{50}-values of both compounds for mycelial growth, being c. 10^{-4} M. It was shown that these ED_{50}-concentrations did not vary significantly with duration of incubation.

Incubation of the mycelial suspension with 10^{-3} M pyrazophos (fig. 5.3.) did not induce any leakage of ^{32}P-orthophosphate, even after incubation for 18 hours, indicating that this fungicide does not affect cell membrane permeability.

5.2.3. *Toxicity to* Pythium debaryanum

Chitin is a well-known constituent in the cell wall of most fungi (ARON-SON, 1965). Probably, no chitin occurs in the cell wall of *Oomycetes* and in that case also no chitin synthetase will be present in these fungi. A lack of sensitivity of these fungi to Hinosan and Kitazin would, therefore, be in agreement with the hypothesis that the compounds affect chitin synthesis by
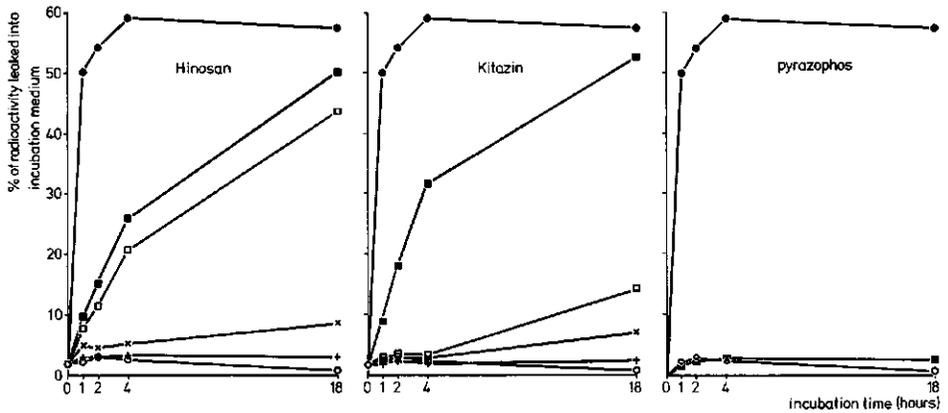
FIG. 5.3. Effect of Hinosan, Kitazin, pyrazophos and chloroform on leakage of ^{32}P-ortho-phosphate from mycelial cells of *Pyricularia oryzae* into incubation medium. Control ($\bigcirc$—$\bigcirc$), Hinosan, Kitazin or pyrazophos concentrations 3.2×10^{-5} M ($+$—$+$), 10^{-4} M ($\times$—$\times$), 3.2×10^{-4} M ($\square$—$\square$), and 10^{-3} M ($\blacksquare$—$\blacksquare$), and chloroform (1%) ($\bullet$—$\bullet$).

inhibition of the activity of this enzyme. However, experiments with *Pythium debaryanum*, used as a representative of the *Oomycetes*, showed that both Hinosan and Kitazin inhibit radial growth of this fungus; their ED_{50}-values were 10^{-4} and 2×10^{-4} M, respectively (fig. 5.4.). Obviously, other sites of attack in the fungus than chitin synthesis have to account for the fungitoxicity.

5.3. DISCUSSION

Incubation of mycelial suspensions of *P. oryzae* with ^{14}C-glucosamine in the presence of Hinosan and Kitazin gave rise to an accumulation of intermediates
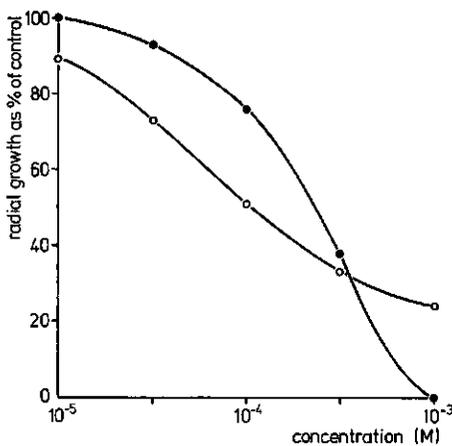
FIG. 5.4. Effect of Hinosan ($\bigcirc$—$\bigcirc$) and Kitazin ($\bullet$—$\bullet$) on radial growth of *Pythium debaryanum* on PDA.

of chitin synthesis. In the case of Kitazin the accumulated products appeared to consist primarily of UDP-N-acetylglucosamine (MAEDA et al., 1970). A similar effect on chitin synthesis of the fungus *Cochliobolus miyabeanus* has been found for polyoxin D. This antibiotic is an in vitro inhibitor of chitin synthetase and specifically inhibited the in vivo incorporation of ^{14}C-glucosamine into the fungal cell wall chitin; however, it did not influence the incorporation of ^{14}C-glucose, ^{14}C-amino acids and ^{14}C-sodium acetate. These data strongly suggest that the fungitoxicity of polyoxin D is due to an inhibition of the biosynthesis of fungal cell wall chitin (OHTA, 1970; KELLER and CABIB, 1971).

Although Hinosan and Kitazin also caused an accumulation of intermediates of chitin synthesis in *P. oryzae* (fig. 5.1.), it is doubtful whether this is a specific, direct effect of these fungicides. As has been demonstrated by MAEDA et al. (1970), Kitazin also inhibited the incorporation of ^{14}C-glucose into the cell wall. Furthermore, it has been shown in experiments, described in this chapter, that ED_{50}-concentrations of Hinosan and Kitazin for fungal growth hardly inhibited the incorporation of ^{14}C-glucosamine into the mycelial residue. At fungitoxic concentrations the incorporation was strongly inhibited but no accumulation of intermediates occurred. The latter finding might indicate that also other factors like interference with membrane permeability are involved in the inhibition of mycelial growth (MAEDA et al., 1970).

This hypothesis has been confirmed in the experiments described. Fungitoxic concentrations of Hinosan and Kitazin, which caused a decrease of the initial dry weight, at the same time gave rise to leakage of ^{32}P-orthophosphate from mycelium of *P. oryzae* (fig. 5.3.). Similar results have been described for *Neurospora crassa* treated with polyene antibiotics; also in this case leakage of fungal cell components was so strong that dry weight decreased (KINSKY, 1961). At the ED_{50}-values of Hinosan and Kitazin for mycelial growth also a low but significant leakage of ^{32}P-orthophosphate could be observed after 18 hours of incubation.

The question remains to be answered whether the effect of Hinosan and Kitazin on cell membrane permeability is primarily responsible for their toxicity. With regard to this it is suggested that even the slight effects on cell membrane permeability, observed at the ED_{50}-values of Hinosan and Kitazin might cause a considerable effect on mycelial growth, so that a direct correlation between effect on leakage of ^{32}P-orthophosphate and inhibition of growth may be difficult to demonstrate.

The observation that growth of *P. debaryanum*, a fungus which probably does not synthesize chitin, is inhibited by Hinosan and Kitazin to about the same extent as that of *P. oryzae* again suggests that other sites of action of the compounds than chitin synthesis must exist.

In conclusion, it seems more probable that interference of Hinosan and Kitazin with cell membrane permeability is responsible for their fungitoxicity than direct inhibition of chitin synthesis. The accumulation of intermediates of chitin synthesis then should be regarded as an indirect effect.

With respect to pyrazophos, it is evident that this compound at its ED_{50}-concentration for mycelial growth neither affected cell membrane permeability nor chitin synthesis. This finding demonstrates that the effects of Hinosan and Kitazin are rather specific and that, therefore, the mechanism of fungicidal action of organophosphorus compounds is not necessarily the same for all of them.

5.4. SUMMARY

Upon incubation of mycelial suspensions of *Pyricularia oryzae* with ^{14}C-glucosamine and 10^{-4} M Hinosan or 3.2×10^{-4} M Kitazin an accumulation of intermediates of chitin synthesis was observed. At these concentrations, mycelial growth was strongly inhibited; however, the incorporation of radio-activity into a mycelial residue fraction, which contains chitin, was hardly influenced. At higher concentrations a decrease in the amount of intermediates took place, concomitantly with a decrease in the amount of radioactivity incorporated into the mycelial residue and in the initial dry weight.

These results suggested that apart from chitin synthesis, also cell membrane permeability could be influenced by these compounds. This was confirmed by experiments in which the effect of both compounds on leakage of ^{32}P-orthophosphate from the mycelium into the culture medium was investigated. Treatments of Hinosan an Kitazin which gave rise to a decrease in mycelial dry weight also caused a leakage of the radiochemical. To a much lesser extent leakage was also observed at the ED_{50}-concentrations of both compounds for mycelial growth.

It is argued that the effect of Hinosan and Kitazin on cell membrane permeability is probably primarily responsible for their fungitoxicity and that the interference with chitin synthesis has to be regarded as a secondary effect. This hypothesis is strengthened by the observation that also *Pythium debaryanum* although probably not able to synthesize chitin, is sensitive to both compounds.

In contrast to Hinosan and Kitazin, pyrazophos neither affected cell membrane permeability nor chitin synthesis, which indicates that the mechanism of fungicidal action of organophosphorus compounds is not necessarily the same for all of them.

6. EFFECTS OF PYRAZOPHOS ON RESPIRATORY PROCESSES, AND NUCLEIC ACID AND PROTEIN SYNTHESIS

6.1. INTRODUCTION

In chapter 3 which deals with the fungitoxicity of organophosphorus compounds, it has been shown that those chemicals which possess a thionate group like pyrazophos, appeared to have a generally higher fungitoxicity to powdery mildew fungi than their oxygen analogues. It is known that a thiono group can be detached by various organisms by means of a microsomal desulfuration reaction. In the case of pyrazophos such a metabolic conversion possibly occurs in *Pyricularia oryzae* (ch. 7). Since sulphur is known to interfere with respiration of fungi (TWEEDY, 1969), this might mean that the sulphur detached from pyrazophos might also affect this process. Effects of pyrazophos on oxygen uptake by *Sphaerotheca fuliginea* and *P. oryzae* were, therefore, investigated.

Inhibition of oxygen uptake might be due to interference with several catabolic processes, as has been described for some organophosphorus compounds. Various chemicals of this type inhibited catalase activity in vivo in garden-cress seedlings (BEYE, 1960). The inhibitory activity of parathion and derivatives towards peroxidase increased in the order of parathion, paraoxon and aminoparathion (KIERMEYER et al., 1970). Dehydrogenases in different bacteria have been reported to be inhibited in vivo by chlorthion (EL-HELALY et al., 1963). Yeast alcohol dehydrogenase was inhibited in vitro by cyclic saligenin S-methyl and S-ethyl phosphorothioate (ETO et al., 1968). The latter authors indicated that the inhibitory effect is due to an alkylation of sulphhydryl groups of the enzyme. *Aspergillus niger*, growing on a medium containing 10^{-3} M pyrazophos, produced brown instead of black conidia (unpublished results). This might indicate that pyrazophos inhibits polyphenol oxidase (PPO), which is involved in melanin formation (MULDER, 1938).

In view of these data the inhibitory effect of pyrazophos on activities of peroxidase, PPO, glyceraldehydephosphate (GAPDH) and succinate dehydrogenase (SDH) were investigated.

As far as is known to us, effects of organophosphorus compounds on nucleic acid and protein synthesis have not been reported. The possibility, however, that the pyrazolo(1,5-a)pyrimidine moiety of pyrazophos interacts in some way with one of these biosynthetic processes may not be excluded; 4-amino- and 4-hydroxypyrazolo(3,4-d)pyrimidine, which are structural analogues of adenine and guanine, respectively, are well-known inhibitors of xanthine oxidase and probably also affect purine biosynthesis. These compounds have therapeutic anti-tumour, bactericidal and fungicidal activities (f.i. FEIGELSON et al., 1957; ALEXANDER et al., 1966; PICKERING and WOODS, 1972). Nucleosides of the related pyrazolo(4,3-d)pyrimidines are known as antibiotics. The

antibiotics like formycin (7-amino-3-(β-D-ribofuranosyl)-pyrazolo(4,3-d)py-rimidine are structural analogues of adenosine, inosine and xanthosine (SUHA-DOLNIK, 1970). In view of these reports effects of pyrazophos on nucleic acid and protein synthesis were also investigated.

6.2. RESULTS

6.2.1. *Effects on respiratory processes*

The effect of pyrazophos on oxygen uptake by *S. fuliginea* conidia was studied according to 'Materials and methods' 2.9. The results, presented in fig. 6.1., show that untreated conidia display a distinct endogenous respiration, which probably decreases gradually because of the damaging effect of water on the conidia. Upon incubation of the conidia with 10^{-4} M pyrazophos, oxygen uptake expressed as a percentage of control, slowly decreased with time. The experiment was also performed with *P. oryzae*, since in the case of *S. fuliginea* inhibitory effects on oxygen uptake cannot be correlated with those on conidial germination or mycelial growth. Oxygen uptake by mycelial suspensions of *P. oryzae* could also be inhibited by pyrazophos (fig. 6.2.). Contrary to that of *S. fuliginea*, oxygen uptake in the presence of 10^{-3} M pyrazophos, when expressed as a percentage of the control, gradually increased in the course of time, concomitantly with a decrease in inhibition of mycelial growth (fig. 6.3.).

In cell-free extracts of *S. fuliginea* conidia a significant activity of peroxidase, GAPDH and SDH could be observed. PPO activity could not be detected. Upon pre-incubation of the cell-free extracts with 10^{-4} M pyrazophos no pronounced effects on these enzyme activities could be observed (table 6.1.).

6.2.2. *Effects on nucleic acid and protein synthesis*

The influence of pyrazophos on nucleic acid synthesis was studied by adding
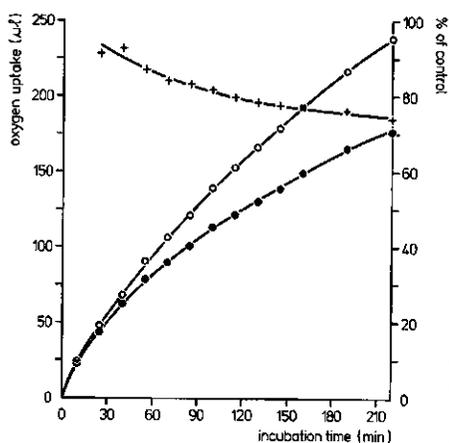
FIG. 6.1. Effect of 10^{-4} M pyrazophos on oxygen uptake by *Sphaerotheca fuliginea* conidia. Concentration of conidia: 19.6×10^6/ml. Control (○—○); pyrazophos (●—●); oxygen uptake in the presence of pyrazophos as % of control (+—+).
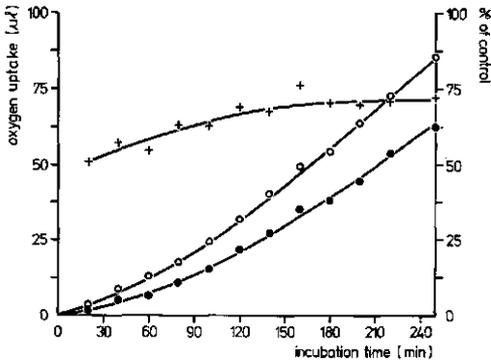
FIG. 6.2. Effect of 10^{-3} M pyrazophos on oxygen uptake by mycelium of *Pyricularia oryzae* in Gly medium. Initial dry weight of mycelial suspension 0.4 mg/ml. Control ($\bigcirc—\bigcirc$) pyrazophos ($\bullet—\bullet$); oxygen uptake in the presence of pyrazophos as % of control ($+—+$).

TABLE 6.1. Effects of 10^{-4} M pyrazophos on activities of peroxidase, glyceraldehydephosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDH) in enzyme preparations of conidia of *Sphaerotheca fuliginea* after pre-incubation of cell-free extracts with fungicide for 60 min.

	concentration of conidia ($\times 10^6$/ml) in disintegration medium	enzyme preparation (ml) added to incubation mixture	Δ A/min		activity as % of control
			control	pyrazophos	
peroxidase	4	0.50	0.185	0.178	96
GAPDH	6	0.02	0.358	0.335	94
SDH	6	0.05	0.105	0.100	95
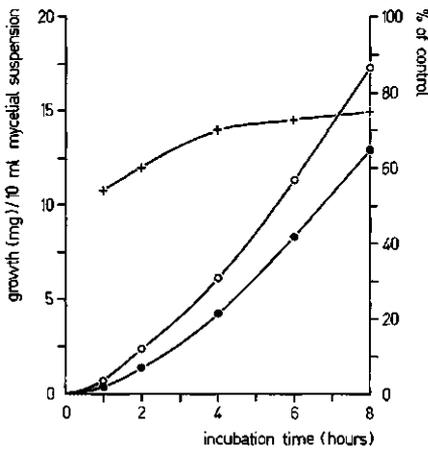
FIG. 6.3. Effect of 10^{-3} M pyrazophos on mycelial growth of *Pyricularia oryzae* in liquid Gly medium. Initial dry weight of mycelial suspension 1.5 mg/ml. Control ($\bigcirc—\bigcirc$); pyrazophos ($\bullet—\bullet$); mycelial growth in the presence of pyrazophos as % of control ($+—+$).
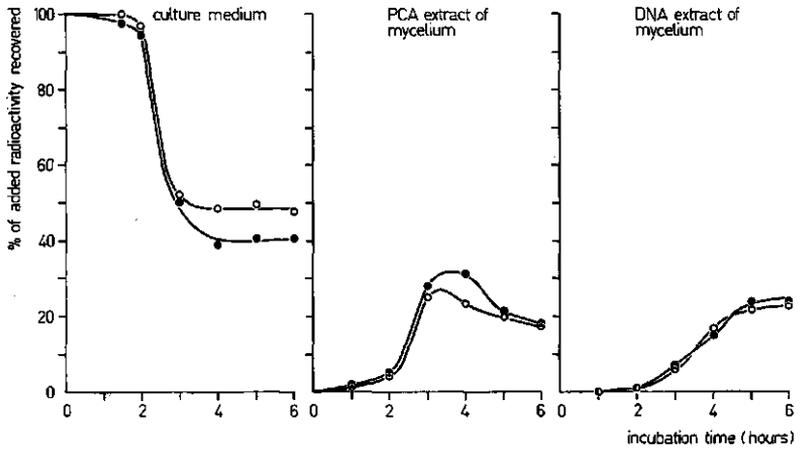
FIG. 6.4. Effect of 10^{-3} M pyrazophos on incorporation of ^{14}C-thy by mycelium of *Pyricularia oryzae*. Pyrazophos and ^{14}C-thy were added simultaneously to the mycelial suspension. Control (○—○); pyrazophos (●--●).

the fungicide and ^{14}C-thymidine (^{14}C-thy) or ^{14}C-uridine (^{14}C-ur) simultaneously to mycelial suspensions of *P. oryzae* as described under 'Materials and methods' 2.13.2. The final concentration of pyrazophos in the medium was 10^{-3} M; its effect on mycelial growth is shown in fig. 6.3. The incorporation of the radiochemicals was studied by assaying radioactivity after various inhibition times a. in culture media, which contained residual ^{14}C-thy or ^{14}C-ur, b. in
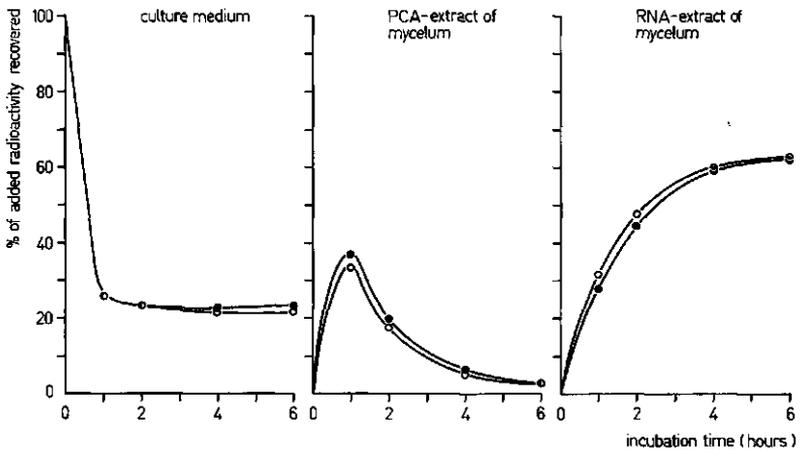
FIG. 6.5. Effect of 10^{-3} M pyrazophos on incorporation of ^{14}C-ur by mycelium of *Pyricularia oryzae*. Pyrazophos and ^{14}C-ur were added simultaneously to the mycelial suspension. Control (○—○); pyrazophos (●—●).

PCA extracts of the mycelium, which contained acid-soluble precursors of DNA or RNA and c. in DNA- and RNA-extracts of the mycelium, respectively.

The results show that incorporation of both radiochemicals was only slightly affected by pyrazophos (fig. 6.4. and 6.5.).

In order to investigate the effect of pyrazophos on protein synthesis its influence on incorporation of ^{14}C-phenylalanine (^{14}C-phe) by *P. oryzae* was studied (ch. 2.13.3.). Percentages of radioactivity recovered were determined in culture media, in ethanol extracts of mycelium and in mycelial residues; the fractions contained residual ^{14}C-phe, ethanol extractable precursors of proteins, and proteins, respectively. Incorporation of ^{14}C-phe was allowed to take place for 120 min of incubation immediately after addition of pyrazophos to the mycelial suspensions. Incorporation of radioactivity by the fungus was also measured for 60 min after preceding incubation during 6 hours with the fungicide; after this pre-incubation period the mycelium was precipitated by centrifugation and resuspended in the original volume of Gly medium containing again 10^{-3} M pyrazophos. Results, presented in table 6.2., show hardly any effect of pyrazophos on the incorporation of ^{14}C-phe.

6.2.3. *Reversal of fungitoxicity*

The fungitoxicity of a compound can sometimes be reversed by addition of an antagonist; this may provide information on the mechanism of action of these fungicides. In this connection the reversal of the toxicity of 6-azauracil, L-methionine, dimethirimol and ethirimol by some compounds can be mentioned (DEKKER and OORT, 1964; DEKKER, 1969a; BENT, 1970). Tests were made, therefore, to determine whether a number of chemicals would reverse the effect of 10^{-6} M pyrazophos on cucumber powdery mildew in the leaf disc test (ch. 2.4.1.). The chemicals tested at a concentration of 10^{-4} M were adenine, cytosine, guanine, hypoxanthine, thymine, uracil, xanthine, adenosine, guano-

TABLE 6.2. Effect of 10^{-3} M pyrazophos on incorporation of ^{14}C-phe by mycelium of *Pyricularia oryzae*. Incorporation of ^{14}C-phe was followed for 120 min of incubation, immediately after addition of pyrazophos (I) and for 60 min after a 6 hour pre-incubation of mycelium with pyrazophos (II).

| | % of added radioactivity recovered | | | | | |
| | culture filtrate | | ethanol extract of mycelium | | mycelial residue | |
	control	pyra-zophos	control	pyra-zophos	control	pyra-zophos
I experiment 1	72.2	73.1	3.60	3.61	5.73	6.03
I experiment 2	79.1	82.6	0.93	0.73	2.48	2.37
II experiment 1	70.7	74.6	0.76	0.75	2.81	2.72
II experiment 2	74.6	83.1	0.56	0.69	3.31	3.36

sine, inosine, xanthosine, 3'-adenylic acid, 5'-adenylic acid, guanylic acid, folic acid, lipoic acid, orotic acid, pyridoxal, D-ethionine, glycine, L-methionine, serine and glutathione. None of these chemicals reversed the fungitoxicity of pyrazophos. L-methionine and D-ethionine showed each a weak fungicidal activity in itself.

6.3. DISCUSSION

The fungitoxicity of pyrazophos to *P. oryzae* in mycelial suspensions proved to be surprisingly low, compared with its toxicity under other conditions (ch. 3). The significance of this observation will be discussed in chapter 8. In view of the fact that its relatively low toxicity even decreased in the course of time, primarily initial effects of pyrazophos on oxygen uptake and on the incorporation of ^{14}C-thy, ^{14}C-ur and ^{14}C-phe were investigated. Measurement of initial effects is also necessary to exclude as much as possible secondary effects due to interference with possible other sites of attack of the fungicide.

With regard to oxygen uptake it was found that in both conidia of *S. fuliginea* (fig. 6.1.) and mycelium of *P. oryzae* (fig. 6.2.) this process was significantly inhibited by pyrazophos at concentrations of 10^{-4} and 10^{-3} M, respectively. In the case of *P. oryzae* this inhibition was of the same magnitude as that of mycelial growth. The effect of pyrazophos on oxygen uptake can probably not be explained by inhibition of peroxidase, and of glyceraldehydephosphate or succinate dehydrogenase activities since these were not strongly affected upon incubation of cell-free extracts of *S. fuliginea* conidia with pyrazophos (table 6.1.). This also indicates that there is a discrepancy between pyrazophos and other organophosphorus compounds like parathion, chlorthion and cyclic saligenins regarding their effect on these enzymes (EL-HELALY et al., 1963; ETO et al., 1968; KIERMEYER et al., 1970).

Results from experiments with ^{14}C-thy and ^{14}C-ur revealed that their incorporation into DNA and RNA by *P. orzyae* was hardly affected by pyrazophos, indicating no appreciable inhibition of nucleic acid synthesis. Incorporation of ^{14}C-phe was not inhibited during 2 hours immediately after addition of the fungicide and during 1 hour after 6 hours pre-incubation with the fungicide. This indicates that protein synthesis was also not affected. The inability of pyrazophos to inhibit nucleic acid and protein synthesis is not in contradiction with the failure of a number of chemicals tested to reverse the fungitoxicity of pyrazophos.

In conclusion it can be stated that of the processes investigated oxygen uptake was most affected. This might indicate that interference of pyrazophos with respiratory processes is responsible for the fungitoxicity of the compound. This hypothesis was tested in experiments described in the next chapters.

6.4. SUMMARY

Upon incubation of mycelial suspensions of *Pyricularia oryzae* with 10^{-3} M pyrazophos hardly any effect on incorporation of ^{14}C-thymidine, ^{14}C-uridine and ^{14}C-phenylalanine by the fungus was found, indicating no pronounced inhibition of nucleic acid and protein synthesis. Under the same conditions oxygen uptake proved to be inhibited by the fungicide to about the same extent as mycelial growth. Oxygen uptake of conidia of *Sphaerotheca fuliginea* could also be inhibited by 10^{-4} M pyrazophos. Hence, of the metabolic processes tested respiration seems to be the only one affected.

7. METABOLIC CONVERSION OF PYRAZOPHOS IN FUNGI

7.1. INTRODUCTION

Metabolic conversion of organophosphorus compounds in insects has been studied extensively but relatively little is known about their breakdown in fungi. Recently, some studies have been published on the conversion of Hinosan, Kitazin and Inezin in *Pyricularia oryzae*.

Hinosan, O-ethyl S,S-diphenyl phosphorodithioate, is degraded by cleavage of one of the P–S bonds, followed by hydrolysis of the other thiophenyl- and ethylester bonds. To a smaller extent, Hinosan is also hydroxylated at the para-position of one of the phenyl groups. In addition to the known breakdown products which were detected in the culture medium, other non-identified water-soluble metabolites were found in the mycelium (UESUGI and TOMIZAWA, 1971).

Upon incubation of Kitazin–P, O,O-diisopropyl S-benzyl phosphorothioate, with mycelium of *P. oryzae* the relatively stable metabolite O,O-diisopropyl hydrogen phosphorothioate was formed; in addition O-isopropyl S-benzyl hydrogen phosphorothioate, diisopropyl hydrogen phosphate and isopropyl dihydrogen phosphate were detected to a much lesser extent. Hydroxylation of Kitazin-P occurred at the meta-position of the S-benzyl moiety. In mycelial cells unidentified metabolites were found, which were presumed to be neutral conjugates of metabolites with fungal components (TOMIZAWA and UESUGI, 1972).

Inezin, O-ethyl S-benzyl phenylphosphonothioate, is converted for the greater part into O-ethyl hydrogen phenylphosphonate and into O-ethyl hydrogen phenylphosphonothioate. Among a number of metabolites, produced in low amounts, are phenylphosphonic acid and O-ethyl S-m-hydroxybenzyl phenylphosphonothioate (UESUGI and TOMIZAWA, 1972). In none of these studies is any information presented on the fungitoxicity of the metabolites of Hinosan, Kitazin-P and Inezin.

Pyrazophos differs from the compounds mentioned above in being a phosphorothioate with a thiono group. These types of organophosphorus esters generally have insecticidal properties and are usually converted by a microsomal oxidation reaction into their phosphate analogues, which are characterized by their high anti-cholinesterase activities. This type of in vivo activation has been found in a number of organisms like insects and mammals (f.i. O'BRIEN, 1967; FUKUTO and METCALF, 1969; O'BRIEN and YAMAMOTO, 1970). Microsomal oxidation of thionate insecticides to their oxygen analogues has not been reported to occur in bacteria and fungi (FEST and SCHMIDT, 1973). Compounds like parathion, methyl-parathion and fenitrothion are metabolized by soil micro-organisms such as *Bacillus subtilis, Rhizobium japonicum* and *R. meliloti* primarily by reduction to the amino analogues and hydrolysis to dimethyl- or diethylphosphorothioic acid (LICHTENSTEIN and SCHULZ, 1964; MIYAMOTO

et al., 1966; MICK and DAHM, 1970).

Metabolic degradation of the phosphate esters, formed by microsomal oxidation from their thiono analogues, occurs in insects primarily by hydrolytic breakdown and generally results in non-toxic products. Major types of hydrolysis are cleavage of P-O-C or P-S-C bonds by phosphatases and of P-O-C bonds perhaps by microsomal mixed function oxidases (O'BRIEN, 1967; NAKATSUGAWA et al., 1969). In organophosphorus esters containing an additional carboxyester group, hydrolysis can also be performed by carboxyesterases. An example of this is the hydrolysis of the carboethoxy ester bond in malathion. This type of breakdown of malathion has also been demonstrated in *Trichoderma viride* and *Pseudomonas species* (MATSUMARA and BOUSH, 1966).

In this chapter, experiments on the metabolism of pyrazophos in *P. oryzae* are described. It was expected that these studies might give additional information on the mode of action of the compound, particular in view of the possibility that an activation reaction as described above for organothionate esters might also occur with pyrazophos in *P. oryzae*. In addition, studies on the fate of pyrazophos in other, non-sensitive fungi are reported.

7.2. RESULTS

7.2.1. *Distribution of* ^{14}C-*pyrazophos and its metabolites in mycelium of* Pyricularia oryzae

Standard mycelial suspensions of *P. oryzae* (ch. 2.6.) with a dry weight of 1.5 mg/ml were incubated in quadruplicate with ^{14}C-pyrazophos (spec. act. c. 1.09 mCi/mmole) in Gly medium. Pyrazophos was present in the suspensions at a non-toxic concentration of 10^{-5} M, which represented a radioactivity of 24145 dpm/ml. The distribution of ^{14}C-pyrazophos and possible ^{14}C-metabolites in the culture medium and in water rinses and fractions of the mycelial cells was determined as described under 'Materials and methods' 2.12.1.

The results shown in table 7.1. show that after 1 day of incubation 45% of the label added, was still present in the culture medium and 36% was in the water rinses. Even in the fifth water rinse a significant amount of radioactivity was found, indicating a strong adsorption of pyrazophos or its metabolites on the mycelium. The relatively high radioactivity in the acetone extracts could either be ascribed to extracellular pyrazophos not yet washed off by the water rinses or to intracellular pyrazophos extracted from the mycelial cells. In the other mycelial extracts, only a low amount of radioactivity was found, indicating no appreciable incorporation into fungal components such as lipids and nucleic acids. After extraction the mycelial residue still contained only 1% of the added radioactivity, which might mean a low incorporation of pyrazophos or its metabolites into proteins or other cell components.

7.2.2. *Metabolic conversion of* ^{14}C-*pyrazophos*

Mycelial suspensions of *P. oryzae* with a dry weight of 2.1 mg/ml were incu-

TABLE 7.1. Distribution of ^{14}C-pyrazophos and its metabolites in culture medium, water rinses and fractions of mycelium of *Pyricularia oryzae*, incubated with 10^{-5} M ^{14}C-pyrazophos (spec. act. c. 1.09 mCi/mmole) for 1 day in liquid Gly medium.

		% of added radio-activity recovered
culture medium		44.70
1st water rinse	24.45	
2nd water rinse	6.62	
3rd water rinse	2.70	
4th water rinse	1.50	
5th water rinse	1.07	
		36.34
1st acetone extract	8.77	
2nd acetone extract	2.07	
3rd acetone extract	0.62	
4th acetone extract	0.25	
5th acetone extract	0.14	
		11.85
combined cold 5% PCA extracts		0.24
combined cold ethanol, ethanol-ether and ether extracts		0.23
0.5 N KOH extract		0.44
hot 5% PCA extract		0.10
mycelial residue		1.02
recovery		94.92

bated as described above in Gly medium, containing 10^{-5} M ^{14}C-pyrazophos (spec. act. c. 0.36 mCi/mmole. The radioactivity amounted to 8037 dpm/ml suspension. After 1, 4, 11, and 22 hours of incubation, mycelial suspensions were fractionated as described under 'Materials and methods' 2.12.2.

In table 7.2., the percentages of radioactivity in various fractions of the culture medium and of the mycelium in relation to time of incubation are given. The most pronounced change was the decrease in radioactivity in the toluene extract of the culture medium (B) and the concomitant increase in the residual toluene-extracted water phase (C); the total label in the (non-extracted) culture medium (A), however, did not change to a large extent. Radioactivity in the combined water rinses of the mycelium (D) slowly decreased with time, while that in acetone extracts of mycelium (E) and in mycelial residues (F) gradually increased.

Some of the fractions indicated above were concentrated, and spotted on thin-layer silicagel plates and developed using ether and cyclohexane/acetone 7:3 as solvents. Chemically pure pyrazophos, PO-pyrazophos and a hydrolysis product of these compounds, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo-(1,5-a)pyrimidine (PP), were used as reference compounds. Upon scanning of the radioactivity in the chromatograms it appeared that in the toluene extracts of the culture medium (B) a sharply increasing portion of the radioactivity was

60

TABLE 7.2. Recovery of added radioactivity in ^{14}C-pyrazophos, ^{14}C-PO-pyrazophos, ^{14}C-PP and non-identified ^{14}C-labelled metabolites in culture media, combined rinse waters, and fractions of mycelium of *Pyricularia oryzae*, incubated with 10^{-5} M ^{14}C-pyrazophos (spec. act. c. 0.36 mCi/mmole) for 1, 4, 11 and 22 hours in liquid Gly medium. A: culture medium, B: toluene extract of culture medium, C: residual toluene-extracted culture medium, D: combined rinse waters of mycelium, E: combined acetone extracts of mycelium, and F: mycelial residue. Recovery of radioactivity obtained by summation of radioactivity in fractions B, C, D, E, and F.

incubation time (hours)	fraction	% of added radioactivity recovered	% of added radioactivity recovered in			
			pyrazophos	PP	PO-pyra-zophos	non-identi-fied meta-bolites
	A	46.9				
	B	42.8	41.0	0.0	0.8	1.0
	C	2.1	0.0	0.0	0.0	2.1
1	D	38.5	38.5	0.0	0.0	0.0
	E	10.1	10.1	0.0	0.0	0.0
	F	0.4				
	recovery	93.9	89.6	0.0	0.8	3.1
	A	45.0				
	B	38.7	28.7	0.0	7.8	2.2
	C	5.7	0.0	0.0	0.0	5.7
4	D	40.3	37.7	0.0	2.6	0.0
	E	12.9	12.9	0.0	0.0	0.0
	F	0.3				
	recovery	97.9	79.3	0.0	10.4	7.9
	A	45.5				
	B	32.8	12.8	2.0	14.4	3.6
	C	12.3	0.0	0.0	0.0	12.3
11	D	35.7	30.1	0.0	5.6	0.0
	E	16.0	16.0	0.0	0.0	0.0
	F	0.7				
	recovery	97.5	58.9	2.0	20.0	15.9
	A	47.8				
	B	27.3	5.4	0.0	17.7	4.2
	C	16.4	0.0	0.0	0.0	16.4
22	D	33.2	18.7	1.5	9.5	3.5
	E	19.4	19.4	0.0	0.0	0.0
	F	3.8				
	recovery	100.1	43.5	1.5	27.2	24.1

present as ^{14}C-PO-pyrazophos (fig. 7.1.; table 7.2.). ^{14}C-PP was found in a low amount after 11 hours of incubation. In later experiments, the identity of both metabolites in the toluene extracts was further substantiated by co-chromatography in benzene/acetone 3:1 and in ether/hexane 8:2. After chromatography of the toluene-extracted culture medium (C) all radioactivity present in this fraction proved to remain at the origin. So far, attempts to identify the metabolites involved have not been made.

FIG. 7.1. TLC-radioscans of toluene extracts of culture media of *Pyricularia oryzae*, incubated with 10^{-5} M pyrazophos (spec. act. c. 0.36 mCi/mmole) for 1 (A), 4 (B), 11 (C) and 22 (D) hours in Gly medium. Solvent: ether. I: ^{14}C-pyrazophos; II: ^{14}C-PO-pyrazophos; III: ^{14}C-PP; IV: non-identified ^{14}C-labelled metabolites.
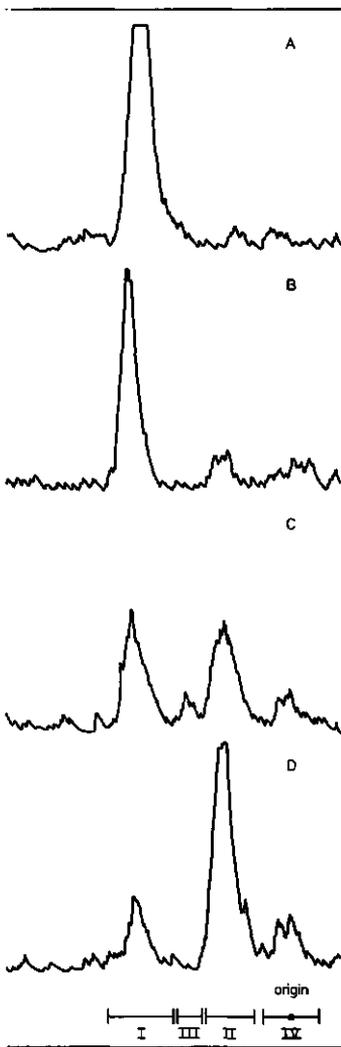
FIG. 7.1.   FIG. 7.3.

FIG. 7.3. TLC-radioscans of toluene extracts of culture media of *Pyricularia oryzae*, incubated with 10^{-5} M pyrazophos (spec. act. c. 0.51 mCi/mmole) for 1 (A), 4 (B), 11 (C) and 22 (D) hours in Gly medium and subsequently acidified with PCA to a final concentration of 0.5%. Solvent: benzene/acetone 3:1. I: ^{14}C-pyrazophos; II: ^{14}C-PO-pyrazophos; III: ^{14}C-PP; IV: non-identified ^{14}C-labelled metabolites.

Using the procedures described above for the toluene extract, it was found that the combined water rinses of the mycelium (D) contained predominantly pyrazophos; relatively large amounts of ^{14}C-PO-pyrazophos and minor amounts of PP and non-identified metabolites were found after prolonged incubation. From an experiment in which mycelium was incubated for 22 hours with ^{14}C-pyrazophos, the successive water rinses of the mycelium were assayed separately. The relative amount of radioactivity present as ^{14}C-pyrazophos increased in the successive rinses; the fifth water rinse contained almost exclusively this radiochemical.

Upon chromatography of the acetone extracts of the mycelium and scanning of the chromatograms, only ^{14}C-pyrazophos proved to be present.

From the distribution of the radioactivity in the chromatograms, percentages of added radioactivity recovered in the different fractions in ^{14}C-pyrazophos, ^{14}C-PO-pyrazophos, ^{14}C-PP and non-identified ^{14}C-labelled metabolites were calculated (table 7.2.). An overall picture of the recovery of these radiochemicals in relation to time is presented in fig. 7.2. In this way the results show even more clearly a strong conversion of pyrazophos into PO-pyrazophos and non-identified metabolites, and to a much lesser extent into PP.

Incubation of ^{14}C-pyrazophos in Gly medium without mycelium under the same conditions as described above for mycelial suspensions did not result in a breakdown of the fungicide. Neither any breakdown was observed upon incubation of 10^{-5} M ^{14}C-pyrazophos for 1 day in a sterile culture filtrate of the fungus.

7.2.3. 2-Hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP)

Further investigations on the formation of the breakdown products PO-pyrazophos and PP were carried out by incubating mycelial suspensions of *P. oryzae* for 1, 4, 11 and 22 hours with 10^{-5} M ^{14}C-pyrazophos (spec. act. c. 0.51 mCi/mmole) as described under 7.2.2. and thereafter acidified with PCA to a final concentration of 0.5%. After incubation of the suspension for 30 min and upon centrifugation for 5 min at 3000 × g, the supernatants were
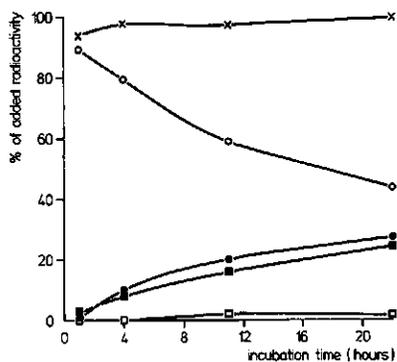
FIG. 7.2. Distribution of added radioactivity in ^{14}C-pyrazophos (○—○), ^{14}C-PO-pyrazophos (●—●), ^{14}C-PP (□—□) and non-identified ^{14}C-labelled metabolites (■—■) in liquid cultures of *Pyricularia oryzae* in Gly medium in relation to time. Recovery of added radioactivity (×—×); cultures incubated with 10^{-5} M pyrazophos (spec. act. c. 0.36 mCi/mmole).

TABLE 7.3. Recovery of added radioactivity in ^{14}C-pyrazophos, ^{14}C-PO-pyrazophos, ^{14}C-PP and non-identified ^{14}C-labelled metabolites in toluene extracts of culture media of *Pyricularia oryzae*, incubated with 10^{-5} M pyrazophos (spec. act. c. 0.51 mCi/mmole) for 1, 4, 11 and 22 hours in Gly medium. The culture media were analysed after acidification of the mycelial suspensions with PCA to a final concentration of 0.5%.

incubation time (hours)	percentages of radioactivity added, recovered in			
	pyrazophos	PO-pyrazophos	PP	non-identified metabolites
1	23.0	1.0	0.3	1.0
4	20.2	4.7	1.5	1.8
11	13.9	14.3	7.2	4.0
22	3.8	20.5	2.3	9.1

extracted 3 times with toluene; the toluene extracts were combined, concentrated, spotted and chromatographed as described in chapter 2.12.2. Upon scanning of the radioactivity in the chromatograms, ^{14}C-PP was detected in relatively large amounts in addition to ^{14}C-pyrazophos and ^{14}C-PO-pyrazophos. It appeared that maximal amounts of the metabolite were present after 4 and 11 hours of incubation of mycelium with pyrazophos (fig. 7.3.). From this figure the percentages of added radioactivity recovered in ^{14}C-pyrazophos, ^{14}C-PO-pyrazophos and ^{14}C-PP and non-identified ^{14}C-labelled metabolites were calculated (table 7.3.). The results again indicate large changes in concentration of pyrazophos and its conversion products during incubation.

Under UV irradiation spots of pyrazophos, PO-pyrazophos and PP could be seen on chromatograms of toluene extracts of culture media in which mycelium had been incubated with 10^{-3} M pyrazophos. In the TLC-bioassay, described under 'Materials and methods' 2.4.3., using *Ascochyta pisi*, *Cladosporium cucumerinum* and *Fusarium culmorum* as test fungi, these amounts of PO-pyrazophos and PP proved to be fungitoxic (fig. 7.4.). In these chromatograms, incidentally a third fungitoxic spot was present, which could neither be seen under UV irradiation nor detected as a ^{14}C-labelled metabolite of ^{14}C-pyrazophos in the metabolic conversion experiments (ch. 7.2.2.). In extracts of mycelium not incubated with pyrazophos, the same spot was also found occasionally; this indicates that this toxicant is not a metabolite of pyrazophos. It might be picolinic acid or piricularin; both compounds are toxins of *P. oryzae* which can be produced in shake cultures of the fungus. They have been investigated extensively by TAMARI (1966).

When the residual toluene-extracted culture medium was assayed with the same TLC-bioassay no fungitoxic spot was found, indicating that the non-identified metabolites, present in this fraction were probably not inhibitory to fungal growth.

7.2.4. *Fate of* ^{14}*C-pyrazophos in* Pythium debaryanum *and* Saccharomyces cerevisiae

In order to investigate whether ^{14}C-pyrazophos is metabolized in a similar

64
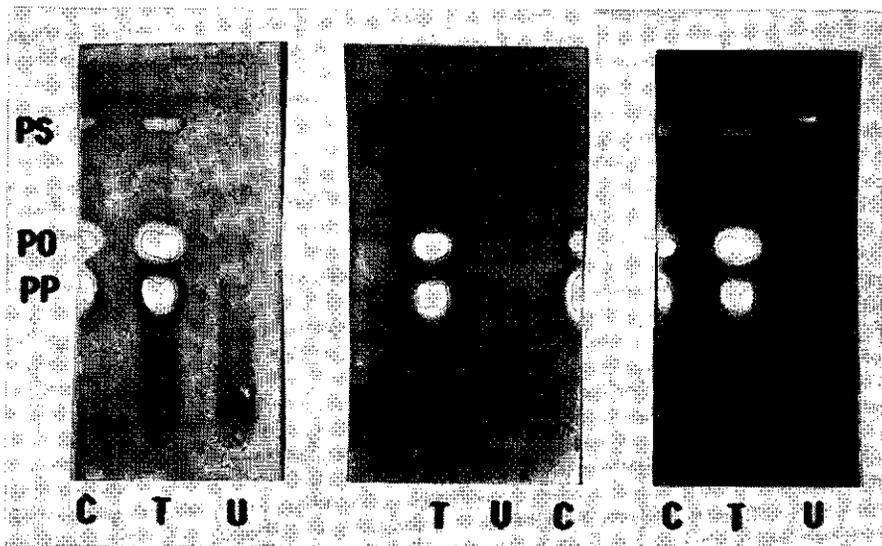
FIG. 7.4. TLC-bioassays of toluene extracts of culture media of *Pyricularia oryzae*, incubated for 22 hours with 10^{-3} M pyrazophos and subsequently acidified with PCA to a final concentration of 0.5%. Solvent: benzene/acetone 3:1. Test fungi: *Fusarium culmorum* (left), *Ascochyta pisi* (middle), *Cladosporium cucumerinum* (right). C: references of pyrazophos (PS), PO-pyrazophos (PO) and PP; T and U: extracts of mycelium with and without 10^{-3} M pyrazophos, respectively.

way in non-sensitive fungi, the fate of ^{14}C-pyrazophos was studied in *Pythium debaryanum* and *Saccharomyces cerevisiae*.

Radial growth of *P. debaryanum* on PDA with 10^{-3} M pyrazophos, PO-pyrazophos and PP was 89, 24 and 0% of the control, respectively, while growth of *S. cerevisiae* in a liquid malt extract-peptone medium with the same concentration of the compounds was 100, 84 and 73% of the control. Thus, both fungi are virtually insensitive to pyrazophos, but in contrast to *S. cerevisiae*, *P. debaryanum* is sensitive to PP. The fate of pyrazophos in both fungi was studied by incubation of mycelial or cell suspensions with 10^{-5} M ^{14}C-pyrazophos (spec. act. c. 0.36 mCi/mmole), respectively. The initial dry weight of the mycelial suspension of *P. debaryanum* was 1.03 mg/ml and that of the cell suspension of *S. cerevisiae* 0.47 mg/ml.

After 1, 4, 11 and 22 hours of incubation, radioactivity recovered in fractions of the culture medium and of fungal cells were determined as described under 'Materials and methods' 2.12.2. Results, presented in table 7.4., indicate a high radioactivity in the toluene extracts of the culture medium (B) and a low radioactivity in the residual toluene-extracted water phases (C), indicating that hardly any water-soluble metabolites were formed. The amount of label recovered in the combined water rinses (D), however, was relatively high; this might mean that pyrazophos and/or its metabolites were strongly adsorbed on

TABLE 7.4. Recovery of added radioactivity in culture media, combined rinse waters and fractions of mycelium or cells of *Pythium debaryanum* and *Saccharomyces cerevisiae*, incubated with 10^{-5} M pyrazophos (spec. act. c. 0.36 mCi/mmole) for 1, 4, 11 and 22 hours, respectively. Recovery of radioactivity obtained by summation of radioactivity in fractions B, C, D, E and F.

	% of added radioactivity recovered							
	P. debaryanum				*S. cerevisiae*			
	incubation time (hours)				incubation time (hours)			
	1	4	11	22	1	4	11	22
culture medium (A)	29.1	27.7	27.7	23.6	49.1	31.5	26.8	36.1
toluene extract of culture medium (B)	21.8	21.4	24.1	16.3	38.3	27.8	20.9	29.8
residual toluene-extracted culture medium (C)	4.0	4.4	1.9	2.6	0.9	0.9	0.9	1.1
combined water rinses of mycelium (D)	35.1	30.6	34.0	32.6	35.9	57.7	55.3	50.3
acetone extracts of mycelium (E)	21.8	22.1	23.6	26.1	1.9	3.2	2.5	3.5
mycelial residue (F)	0.2	0.2	0.2	0.6	0.5	3.1	2.7	1.9
recovery	82.9	78.7	83.8	78.2	77.5	92.7	82.3	86.6

the fungal cells. In the acetone extracts of the mycelial cells (E) a relatively large amount of radioactivity was found in only those of *P. debaryanum*.

Upon chromatography of the toluene extracts of the culture media, of the combined water rinses, and the mycelial acetone extracts only ^{14}C-pyrazophos appeared to be present. Apparently the compound was not taken up or converted by these fungi.

7.3. DISCUSSION

Time course studies on the metabolic conversion of ^{14}C-pyrazophos in *P. oryzae* demonstrated a rapid breakdown of the fungicide into PO-pyrazophos, PP and other non-identified metabolites under the experimental conditions used. The formation of PO-pyrazophos indicates that *P. oryzae*, like other organisms such as insects, is able to convert an organophosphorothionate ester into its oxygen analogue. Since incubation of pyrazophos in fungal culture filtrates did not result in any conversion into PO-pyrazophos, the process probably takes place intracellularly. It is suggested that the formation of PO-pyrazophos is due to a microsomal oxidation reaction, which is, to our knowledge, not previously reported to operate in fungi (FEST and SCHMIDT, 1973). Microsomal mixed function oxidases might also be responsible for a direct breakdown of pyrazophos into PP (NAKATSUGAWA et al., 1969). It seems, however, more probable that enzymatic hydrolysis of PO-pyrazophos is responsible for the formation of PP. Formation of non-identified metabolites from PP probably explains the decrease in amount of PP, found in toluene extracts of culture media after 22 hours of incubation (table 7.3.).

With respect to pyrazophos the high recovery in the water rinses is worth mentioning and points to a strong adsorption of the fungicide on the mycelium. Upon washing the mycelium, pyrazophos was gradually released into the water rinses. This phenomenon may also partly account for the relatively high radioactivity in the acetone extracts of the mycelium, in which only pyrazophos could be detected.

PO-pyrazophos was found in the culture medium and the water rinses, but not in the acetone extracts of the mycelium. It is supposed that this metabolite after its formation is rapidly released into the incubation medium, so that only low, non-detectable amounts of these metabolites are present in the mycelium.

PP was found incidentally and in low amounts in the culture medium and water rinses (table 7.2.). After acidification of the culture medium, containing the mycelium, large amounts of the metabolite could be detected in the toluene extract of the culture medium. This might be explained by a more efficient extraction of PP from the mycelium because of disruption of the cell membranes. It might also be caused by an immediate inhibition of those enzyme activities in the culture medium or mycelial cells, which are responsible for breakdown of PP.

The site of formation of PP might be both extra- or intracellular. No experiments were performed to elucidate this question. The same is true for the breakdown of PP into non-identified metabolites.

Upon fractionation of washed and acetone extracted mycelium of *P. oryzae* incubated with ^{14}C-pyrazophos, only low amounts of radioactivity were present in the cold acid-soluble extract, the ethanol and ether extracts and the DNA and RNA fractions of the mycelium, and in the mycelial residue. These results indicate no pronounced incorporation of pyrazophos or its metabolites into fungal components (table 7.1.).

P. debaryanum and *S. cerevisiae*, both insensitive to pyrazophos, were not able to metabolize the fungicide. It is not certain whether this should be ascribed to a lack of penetration to a possible site of conversion or to a lack of ability of these fungi to convert pyrazophos to other fungitoxic metabolites. In the case of *S. cerevisiae*, a low uptake of pyrazophos probably explains the lack of toxicity of the fungicide since only low amounts were found in the acetone extracts of the yeast cells. With regard to *P. debaryanum* it can be stated that the high amounts of pyrazophos found in similar acetone extracts, do not necessarily mean that the fungicide was taken up by the fungus. As was found with *P. oryzae*, this phenomenon can also be explained by a strong adsorption on the fungal cell walls. If uptake of pyrazophos by the mycelium of *P. debaryanum* takes place, it is, on the other hand, also possible that the insensitivity of the fungus is related to the absence of a metabolic conversion in the fungus, inasmuch as growth can be inhibited by PO-pyrazophos and PP.

In view of the rapid formation of PO-pyrazophos and PP from pyrazophos by *P. oryzae* but the lack of it by fungi insensitive to the fungicide, and because of the fungitoxicity of both metabolites in TLC-bioassays (fig. 7.4.), it was

hypothesized that they might play a role in the mechanism of action of pyrazophos. In the next chapter, experiments concerning the fungitoxicity of PP will, therefore, be described.

7.4. SUMMARY

Upon incubation of mycelial suspensions of *Pyricularia oryzae* with 10^{-5} M pyrazophos rapid metabolic conversion into PO-pyrazophos, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP) and water-soluble, non-identified metabolites took place. It is suggested that the breakdown might be due to activities of microsomal mixed function oxidases and hydrolytic enzymes.

Pyrazophos appeared to be strongly adsorbed to the mycelium, since the compound was gradually released in successive water rinses and acetone extracts. PO-pyrazophos, PP and non-identified metabolites were found in the culture medium and in the first water rinses of the mycelium, indicating that they were rapidly released from the mycelium into the culture medium. PP could be detected in relatively large amounts in the culture medium after acidification of the mycelial suspensions. Contrary to pyrazophos, both PO-pyrazophos and PP proved to be fungitoxic to a number of fungi in TLC-bioassays. Pyrazophos or its metabolites were not incorporated to an appreciable amount into fungal components.

Pythium debaryanum and *Saccharomyces cerevisiae*, which are relatively insensitive to pyrazophos, did not break down the fungicide. In the case of *S. cerevisiae* the insensitivity might be due to a low uptake of the chemical into the fungus. The same phenomenon might explain the low toxicity of pyrazophos to *P. debaryanum*. In view of the relatively high amounts of the compound found in acetone extracts of the mycelium, it is, however, also possible that pyrazophos is taken up, but that insensitivity of the fungus is related to absence of metabolic conversion.

It is suggested that PO-pyrazophos and PP might play a role in the mechanism of fungitoxic action of pyrazophos.

8. FUNGITOXICITY OF 2-HYDROXY-5-METHYL-6-ETHOXYCARBONYLPYRAZOLO(1,5-a)-PYRIMIDINE (PP)

8.1. INTRODUCTION

In chapter 7 the formation of 2-hydroxy-5-methyl-6-ethoxycarbonyl-pyrazolo(1,5-a)pyrimidine (PP) from pyrazophos by *Pyricularia oryzae* was described. In TLC-bioassays this metabolic conversion product appeared to be fungitoxic to different fungi. Data of BELAL (1971), on the other hand, show PP not to be fungitoxic to powdery mildew fungi in vivo.

In view of the unexpected fungitoxicity of PP in TLC-bioassays, effects of the chemical on mycelial growth of a number of fungi have been investigated further and described in this chapter.

8.2. RESULTS

8.2.1. *Fungitoxicity in TLC-bioassays*

Quantities of PP ranging from 1 to 100 nmole in acetone were spotted on silicagel thin-layer plates. The plates were bio-assayed as described in chapter 2.4.3. with spore suspensions of different fungi. After two days of incubation the minimal amount of PP which inhibited mycelial growth was assessed (table 8.1.). The results are comparable with those obtained with pyrazophos and PO-pyrazophos in similar tests (table 3.6.). Of the fungi tested, *Ascochyta pisi* and *Colletotrichum acutatum* were most sensitive; the minimal inhibitory amount for these organisms was about 10 nmole (2.2 μg). The fungitoxicity of PP equalled that of PO-pyrazophos (table 3.6.). In the case of *A. pisi* the minimal inhibitory concentration of PP was about $10 \times$ lower than that of pyrazophos. Also with regard to most of the other fungi tested, the toxicity of PP was higher than or at least equal to that of pyrazophos.

8.2.2. *Effect of pH on fungitoxicity*

The effect of PP on radial growth of *P. oryzae* was investigated at different pH's of Gly media, because it could be surmised that the relatively acid character of the C-2 hydroxyl group of PP could influence its toxicity. The pK-value of this hydroxyl group was determined to be 5.7 (OVEREEM, pers. comm.). The results presented in fig. 8.1. indicate that the inhibition of mycelial growth by PP was indeed pH-dependent. At pH 4.0–5.0, 10^{-3} M of the compound proved to be completely fungitoxic, while at higher pH levels a significant decrease in toxicity was observed.

The ED_{50}-value of PP for radial growth on Gly agar pH 4.0 was found to be 8×10^{-5} M (fig. 8.2.).

In a similar way as described above, the toxicity of PP was tested as to radial

TABLE 8.1. Minimal amount in nmoles of PP which inhibited mycelial growth of some fungi on thin-layer plates.

Ascochyta pisi	10
Aspergillus niger	30
Botrytis cinerea	100
Cladosporium cucumerinum	30
Colletotrichum acutatum	10
Colletotrichum lindemuthianum	30
Fusarium culmorum	30
Penicillium expansum	30

growth of other fungi on PDA at pH 4.0 and 6.3 (table 8.2.). The in vitro anti-fungal spectra revealed that fungi belonging to the *Zygomycetes* were not only relatively sensitive to this chemical, but also at both pH values to about the same degree. In general, most of the other fungi tested were somewhat stronger inhibited by PP at pH 4.0 than at pH 6.3. This was most evident in the case of *P. oryzae*.

8.2.3. *Fungitoxicity to* Pyricularia oryzae *in liquid cultures*

In chapter 5 and 6 it was observed that pyrazophos displayed a low toxicity as to growth of *P. oryzae* in standard mycelial suspensions during 2 hours of incubation with the fungicide. The ED_{50}-value of the compound for mycelial growth in these short-term experiments proved to be $100-1000 \times$ higher than for mycelial growth during about 1 week in long term experiments (fig. 3.4.).
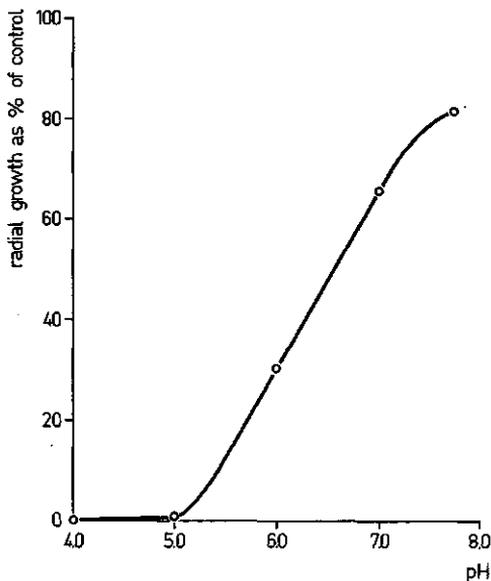
FIG. 8.1. Effect of pH on inhibition of radial growth of *Pyricularia oryzae* by 10^{-3} M PP. The fungus was grown on Gly-agar, prepared in 0.05 M McIlvaine buffer (pH 4.0, 5.0 and 6.0) or 0.05 M phosphate buffer (pH 7.0 and 8.0).

TABLE 8.2. Effect of PP on radial growth of some fungi on PDA made in 0.05 M McIlvaine buffer (pH 4.0) and on non-buffered PDA (pH 6.3). Ascomycetes are classified according to their conidial state. The fungi are arbitrarily distinguished in three categories according to their sensitivity: a. $ED_{50} < 10^{-4}$ M, b. 10^{-3} M $< ED_{50} < 10^{-4}$ M, and c. $ED_{50} > 10^{-3}$ M.

		diameter of colony (mm)								category of sensitivity	
		PDA buffered at pH 4.0				non-buffered PDA pH 6.3					
	days after inocu-lation	control	conc. of PP		days after inocu-lation	control	conc. of PP			pH 4.0	pH 6.3
			10^{-4} M	10^{-3} M			10^{-4} M	10^{-3} M			
OOMYCETES											
Phytophthora cryptogea	5	20.7	8.3	0.0	4	40.3	26.0	7.2		a	b
Pythium debaryanum	7	0.0	0.0	0.0	1	53.7	25.3	0.0		a	a
ZYGOMYCETES											
Mortierella elongata	5	32.0	17.0	0.0	4	48.3	20.3	0.0		b	a
Mortierella isabellina	5	36.2	30.3	7.5	6	47.5	32.0	17.5		b	b
Mortierella vinacea	5	26.0	13.8	3.5	6	32.0	12.0	5.2		b	a
Rhizopus nigricans	2	68.0	63.8	22.8	2	81.3	69.0	29.5		b	b
DEUTEROMYCETES											
Sphaeropsidales											
Ascochyta pisi	4	16.0	15.8	6.8	4	23.5	24.2	12.0		b	c
Melanconiales											
Colletotrichum acutatum	4	18.8	12.8	2.2	4	29.3	28.0	19.3		b	c
Colletotrichum lindemuthianum	7	7.7	1.7	0.0	4	18.7	18.3	8.2		a	b
Moniliales											
Blastosporae											
Botrytis cinerea	4	61.3	55.2	21.3	4	53.3	34.5	15.2		b	b
Cladosporium cucumerinum	7	18.0	17.8	2.3	4	21.7	20.7	17.5		b	c
Monilia fructicola	4	34.0	19.0	0.0	4	57.0	39.2	3.7		b	b
Porosporae											
Helminthosporium oryzae	5	42.5	22.7	8.7	4	27.8	25.0	21.0		b	c
Phialosporae											
Aspergillus niger	3	51.8	50.2	18.0	4	33.0	31.2	18.0		b	c
Fusarium culmorum	4	53.3	21.8	7.2	5	73.2	42.0	27.5		a	b
Penicillium expansum	3	19.2	19.3	9.8	5	27.7	28.2	18.5		c	c
Pyricularia oryzae	7	31.9	13.5	0.0	7	38.0	36.3	26.2		a	c
Verticillium albo-atrum	7	11.5	4.8	0.0	4	25.0	20.0	2.5		a	b
MYCELIA STERILIA											
Rhizoctonia solani	4	54.7	46.7	18.6	4	79.3	69.0	26.0		b	b
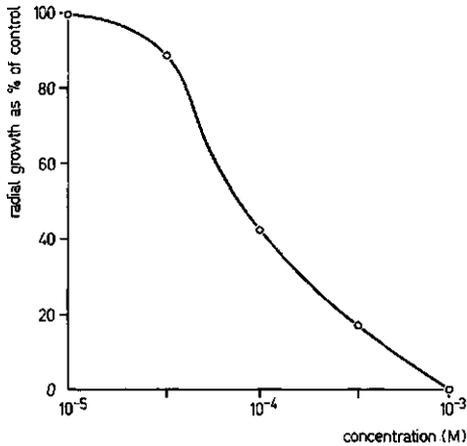
FIG. 8.2. Radial growth of *Pyricularia oryzae* on Gly-agar in 0.05 M McIlvaine buffer pH 4.0 in relation to concentration of PP.

With regard to the results obtained in chapter 7, it is suggested that this phenomenon can be due to a metabolic conversion of pyrazophos into fungitoxic breakdown products, one of which is PP. In the short-term experiments, the 2 hour growth period might, therefore, have been too short for the formation of inhibitory amounts of PP.

In view of these results, the fungitoxicity of PP in short-term experiments was investigated by determining the ED_{50}-values of PP for growth of mycelial suspensions in liquid Gly media at pH 4.0 and 7.0 during a 2 hour growth period. Treatments with pyrazophos and PO-pyrazophos were used as controls. The results again showed a higher fungitoxicity of PP at pH 4.0 than at pH 7.0 (table 8.3.). At pH 4.0 growth was inhibited about 50% at a concentration of 10^{-4} M PP; 3×10^{-4} M completely stopped growth (fig. 8.3.). It was of interest to note that at pH 4.0, PP proved to be considerably more fungitoxic than pyrazophos. This indicates a direct toxicity of PP under the experimental conditions used.

TABLE 8.3. ED_{50}-values of pyrazophos, PO-pyrazophos and PP for mycelial growth of *Pyricularia oryzae* in liquid Gly medium, made in 0.05 M McIlvaine buffer pH 4.0 and in 0.05 M phosphate buffer pH 7.0, respectively. Growth period: 2 hours. Initial dry weight: $\pm$ 20 mg/ 10 ml mycelial suspension.

	ED_{50}-value (M)	
	pH 4.0	pH 7.0
pyrazophos	$>10^{-3}$	$>10^{-3}$
PO-pyrazophos	5×10^{-4}	4×10^{-4}
PP	10^{-4}	$>10^{-3}$
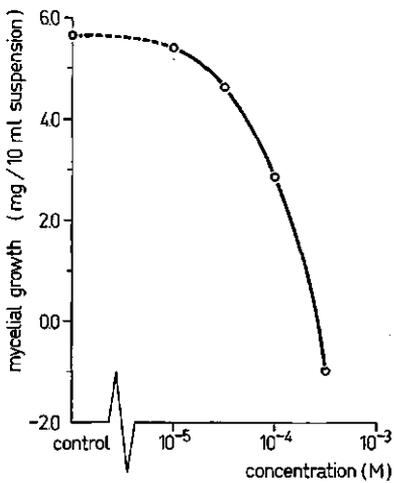
Fig. 8.3. Mycelial growth of *Pyricularia oryzae* in liquid Gly medium in 0.05 M McIlvaine buffer pH 4.0 in relation to concentration of PP. Incubation time: 2 hours. Initial dry weight 34.8 mg/10 ml suspension.

8.3. Discussion

For a number of fungi tested the toxicity of PP appeared to depend strongly on the pH of the nutrient medium. This was most evident in the case of *P. oryzae*. The ED_{50}-value of PP for radial growth of this fungus at pH 4.0 and 7.0 proved to be 8×10^{-5} and $> 10^{-3}$ M, respectively (fig. 8.1. and 8.2.). Comparable results were obtained for growth of mycelium in suspensions in short-term experiments (table 8.3., fig. 8.3.).

These differences in toxicity can probably be ascribed to the fact that usually, when solutions of PP in water or nutrient media are employed with a pH > 6.0, the compound will mainly be present in a dissociated form because the pK value of the C-2 hydroxyl group is 5.7 (OVEREEM, pers. comm.). Chemicals are usually taken up by microorganisms more slowly when dissociated than when in a non-dissociated form. This has been described for a number of weak acids and bases and toxicants such as 2,4-dinitrophenol and kasugamycin (SIMONS and BEEVERS, 1951; KOTYK, 1962; HAMADA et al., 1965). It is, therefore, tempting to suggest that a similar phenomenon may account for the fungitoxicity of PP to *P. oryzae* at pH 4.0. The same explanation may be applicable with regard to the relatively high toxicity of PP at pH 4.0 to radial growth of most of the other fungi tested (table 8.2.). In the case of fungi belonging to the *Zygomycetes*, PP inhibited radial growth at pH 4.0 and 6.3 to about the same degree; this different response in different fungi tested is not understood.

A comparison of the antifungal spectrum of PP in PDA at pH 4.0 (table 8.2.) with that of pyrazophos and PO-pyrazophos at pH 6.3. (table 3.5.), is not readily feasable. However, the minimal inhibitory amounts of the chemicals in TLC-bioassays can be compared to a limited extent (compare table 3.6.

with table 8.1.). From the tables it is evident that under these conditions *A. pisi*, *Cladosporium cucumerinum* and *Penicillium expansum* in particular are more sensitive to PP than to pyrazophos. With the exception of *Aspergillus niger* and *C. acutatum* this statement holds to a lesser degree for most of the other fungi tested.

The ED_{50}-value of PP for growth of *P. oryzae* in mycelial suspensions during 2 hours incubation appeared to be of the same order of magnitude as that for radial growth (c. 10^{-4} M). This similarity is in strong contrast with the difference in toxicity of pyrazophos in these tests. The ED_{50}-values of pyrazophos for growth in mycelial suspensions during 2 hours and for radial growth during 1 week on agar appeared to be c. 10^{-3} (table 8.3.) and 10^{-5} M (fig. 3.4.), respectively. For mycelial growth during 1 week in liquid media, inoculated with conidia an ED_{50}-value as low as 10^{-6} M was found (fig. 3.4.). These discrepancies might be due to a variable rate of breakdown of pyrazophos into toxic and non-toxic conversion products and to the different ratios between the amounts of fungal material and of pyrazophos present in these tests. The former reason is based on the assumption that pyrazophos has to be converted into a metabolite being the actual fungitoxic principle; the latter on the supposition that in the case of a high ratio between fungal material and pyrazophos, relatively high amounts of the chemical can be aspecifically adsorbed on the mycelial cells; this can result in a lower effective concentration of the compound. A similar phenomenon has been described for the toxicity of chloroneb to *Rhizoctonia solani* (HOCK and SISLER, 1969). The effective concentration of pyrazophos can also be influenced by its water-insolubility at concentrations higher than 5×10^{-5} M.

The relatively high direct inhibitory activity of PP towards growth of mycelium of *P. oryzae* in suspensions at pH 4.0 as compared to pyrazophos (table 8.3.) might indicate that this breakdown product is the actual fungitoxic principle. The fact that a number of the other fungi tested are also relatively sensitive to PP may be a confirmation of this hypothesis. Although PO-pyrazophos also possesses a considerably higher fungitoxicity to most of the fungi tested than pyrazophos, the significance of this metabolite for overall fungitoxicity was thought to be less than that of PP, since it is supposed that PO-pyrazophos can also be broken down into PP.

The cause of the relatively high sensitivity of *P. oryzae*, *Colletotrichum lindemuthianum* and some powdery mildew fungi to pyrazophos (ch. 3) compared to PP is not known, but it may be due to a relatively rapid uptake of compounds with a lipophylic character by these fungi. This suggestion is supported by experiments of BELAL (1971) who found a correlation between toxicity of pyrazophos and some of its derivatives to powdery mildew fungi and their partition coefficient in mixtures of oleylalcohol and water. Assuming that PP is the actual fungitoxic principle, then fungi sensitive to pyrazophos should convert this compound first into the toxic metabolite. This is in agreement with the results described in chapter 7 according to which *P. oryzae* degraded pyrazophos to a number of metabolites among which PP, while fungi insensi-

74

tive to pyrazophos did not decompose the chemical.

Conversion of a non-toxic compound to a toxic product by fungi has also been demonstrated for 6-azauracil. In *Colletotrichum lagenarium* this compound is converted to its nucleotide which interferes with the pyrimidine biosynthesis of the fungus (DEKKER and OORT, 1964).

Assuming that PP is the actual fungitoxic principle, this chemical might affect catabolic and anabolic processes in short-term experiments more strongly than pyrazophos. To substantiate this hypothesis experiments have been carried out on the effects of PP on oxygen uptake, nucleic acid and protein synthesis; they have been described in the next chapter.

8.4. SUMMARY

The fungitoxicity of 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)-pyrimidine (PP), a metabolic conversion product of pyrazophos in cultures of *Pyricularia oryzae*, to growth of a number of fungi in TLC-bioassays appeared to be considerably higher than that of pyrazophos. Its toxicity to mycelial growth of *P. oryzae* in liquid cultures or on agar was only evident when the media were buffered at pH 4.0–5.0. The latter results can probably be ascribed to the weakly acid character of the chemical.

The direct toxicity of PP in short-term experiments, using growth periods of 2 hours and media buffered at pH 4.0 proved to be also significantly higher than that of pyrazophos and PO-pyrazophos. This indicates a possible involvement of the breakdown product in the mechanism of toxic action of pyrazophos.

9. EFFECTS OF PP ON OXYGEN UPTAKE, AND NUCLEIC ACID AND PROTEIN SYNTHESIS

9.1. INTRODUCTION

In the previous chapters it was demonstrated that upon incubation of a mycelial suspension of *Pyricularia oryzae* with pyrazophos several metabolites were formed, of which PO-pyrazophos and PP were identified. In short-term experiments, using growth periods of 2 hours and a glucose, yeast extract culture medium pH 4.0 the toxicity of PP proved to be significantly higher than that of pyrazophos. This phenomenon was the main evidence that PP could be the actual fungitoxic principle of pyrazophos. The aim of the experiments described in this chapter was to determine the effects of PP on some cellular processes like oxygen uptake, and nucleic acid and protein synthesis and to attempt to localize the site of toxic action.

9.2. RESULTS

9.2.1. *Effect on oxygen uptake*

Oxygen uptake by mycelial suspensions of *P. oryzae* was measured polarographically with an oxygen electrode in a 3 ml chamber. Mycelial suspensions were made in 0.04 M phthalate buffer pH 4.0 and pH 7.0 containing 2 % glucose. Effects of pyrazophos, PO-pyrazophos and PP were studied by injecting 30 μl of 5×10^{-2} M solutions of the chemicals, dissolved in acetone, into the chambers. Injection of 30 μl acetone was used as a control treatment. At pH 4.0, addition of PP immediately decreased oxygen uptake to about 56 % of the control (fig. 9.1.); after 3 hours of incubation with PP oxygen uptake had decreased to about 36 % of the control (table 9.1.). At pH 7.0 hardly any effect of the chemical was observed. At pH 4.0 inhibition of oxygen uptake by pyrazophos and PO-pyrazophos was much lower than that by PP at both incubation times tested, while at pH 7.0, on the other hand, their effects were distinctly larger.

9.2.2. *Effects on nucleic acid and protein synthesis*

DNA, RNA and protein synthesis was measured as increase of DNA, RNA and protein contents of cultures of *P. oryzae*. To this end, mycelial suspensions of the fungus were made in a 2 % glucose, 0.5 % yeast extract medium in 0.05 M McIlvaine buffer pH 4.0 and pre-incubated for 2 hours. Subsequently, the suspensions were incubated with various concentrations of PP for 5–6 hours. Next, they were assayed for DNA, RNA and proteins, as described in chapter 2.14.

The results of these experiments, presented in tables 9.2., 9.3. and 9.4., indicate that the toxicant displays inhibitory effects on fungal DNA, RNA
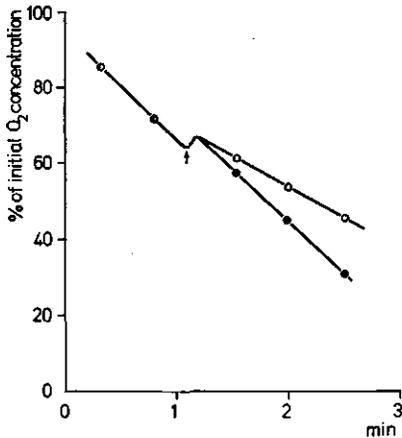
FIG. 9.1. Effect of 5×10^{-4} M PP on the rate of oxygen uptake of *Pyricularia oryzae*. The arrow indicates the time when a control amount of acetone (●—●) or PP, dissolved in acetone, (○—○) were added.

TABLE 9.1. Effect of 5×10^{-4} M pyrazophos, PO-pyrazophos, and PP on oxygen uptake of mycelial suspensions of *Pyricularia oryzae* in a 0.04 M phthalate buffer pH 4.0 and pH 7.0, containing 2% glucose. The compounds were added to the suspensions as solutions in acetone. Equal amounts of acetone were used in controls. * Median deviation.

	oxygen uptake as % of control		
	immediately after addition of the compounds at pH 4.0	3 hours after addition of the compounds at pH 4.0	immediately after addition of the compounds at pH 7.0
control	91.6 ± 8.4*	99.4 ± 2.1	93.4 ± 0.4
pyrazophos	75.6 ± 1.6	93.1 ± 5.5	66.6 ± 0.8
PO-pyrazophos	87.3 ± 2.1	100.7 ± 4.8	80.4 ± 0.2
PP	56.4 ± 1.5	35.6 ± 2.3	94.0 ± 3.0

TABLE 9.2. Effect of PP on increase of DNA in *Pyricularia oryzae* during a 6 hour incubation period.

	dry weight per 10 ml mycelial suspension (mg)	mycelial growth as % of control	µg DNA per 10 ml mycelial suspension	increase in DNA as % of control	DNA as % of mycelial dry weight
control (t=0 h)	24.5	–	46.1	–	0.19
control (t=6 h)	43.0	–	77.3	–	0.18
3×10^{-5} M PP (t=6 h)	40.2	84.9	70.5	78.2	0.18
1×10^{-4} M PP (t=6 h)	36.2	63.2	59.7	43.5	0.16
3×10^{-4} M PP (t=6 h)	25.5	5.4	46.1	0.0	0.18

TABLE 9.3. Effect of PP on increase of RNA in *Pyricularia oryzae* during a 6 hour incubation period.

		dry weight per 10 ml mycelial suspension (mg)	mycelial growth as % of control	mg RNA per 10 ml mycelial suspension	increase in RNA as % of control	RNA as % of mycelial dry weight
control	(t=0 h)	24.5	–	1.54	–	6.28
control	(t=6 h)	44.7	–	2.72	–	6.08
3×10^{-5} M PP (t=6 h)		40.7	80.2	2.52	83.0	6.19
1×10^{-4} M PP (t=6 h)		36.8	60.9	2.24	59.3	6.09
3×10^{-4} M PP (t=6 h)		28.8	21.3	1.70	13.5	5.90

TABLE 9.4. Effect of PP on increase of protein in *Pyricularia oryzae* during a 5 hour incubation period.

		dry weight per 10 ml mycelial suspension (mg)	mycelial growth as % of control	mg protein per 10 ml mycelial suspension	increase in protein as % of control	protein as % of mycelial dry weight
control	(t=0 h)	12.8	–	2.22	–	17.3
control	(t=5 h)	20.8	–	3.43	–	16.5
1×10^{-4} M PP (t=5 h)		16.9	51.3	2.96	61.2	17.5
3×10^{-4} M PP (t=5 h)		12.1	–8.8	2.36	11.6	19.5

and protein synthesis. Increases in these components were proportional to increases in dry weight.

9.3. DISCUSSION

At the fungitoxic concentrations used, PP exerted distinct effects on oxygen uptake, and nucleic acid and protein synthesis by *P. oryzae*. The effects on increases in DNA, RNA and protein were proportional to increases in dry weight. Furthermore, the contents of these fungal components, expressed as a percentage of mycelial dry weight do not vary significantly between the control and PP-treated samples (tables 9.2., 9.3. and 9.4.). These results indicate that inhibition of each of these biosynthetic processes is non-specific, contrary to what has been described for methyl benzimidazol-2-yl carbamate (MBC) which compound inhibits DNA synthesis in *Neurospora crassa* and *Aspergillus nidulans* (CLEMONS and SISLER, 1971; DAVIDSE, 1973). A specific site of inhibition has also been described for quinacrine, which inhibits specifically RNA

synthesis in *Bacillus cereus* and for triarimol, which specifically affects ergosterol synthesis in *Ustilago maydis* (SELIGMAN and MANDEL, 1971; RAGSDALE and SISLER, 1973). Therefore, the general inhibition of several biosynthetic processes observed in *P. oryzae* by PP might be caused by an aspecific reaction or by an indirect effect due to interference with another cellular process. This might be cell respiration because upon addition of 5×10^{-4} M PP to a mycelial suspension of the fungus, oxygen uptake was at once inhibited for 44% and after 3 hours of incubation for 64% (table 9.1.). In similar experiments inhibition of oxygen uptake by pyrazophos and PO-pyrazophos was lower than reported above for PP and decreased in the course of time. At pH 7.0, hardly any effect of PP on oxygen uptake was observed, which correlates well with the results presented in chapter 7 that the chemical is not toxic under these conditions.

On the basis of this reasoning the following hypothesis on the mechanism of action of PP against *P. oryzae* can be suggested. First, the action of PP is aspecific and inhibits directly both oxygen uptake and synthetic processes to a certain degree, which results in a complete inhibition of mycelial growth. In this case toxicity may be due to an aspecific reaction of PP with fungal cell components. Aspecific mechanisms of action have been suggested for many of the conventional, non-systemic fungicides like disulfides, dialkyldithiocarbamates, organic mercuries, quinones, captan and other related $R-SCCl_3$ compounds (TORGESON, 1969). These fungicides often have the capacity to react with sulphhydryl compounds and can be antagonized with cysteine and glutathione. After a chemical hydrolysis at the phenolic ester linkage, saligenin cyclic S-methyl and S-ethyl phosphorothioate can also react with cysteine (ETO et al., 1968; OHKAWA and ETO, 1969). However, upon incubation of 5×10^{-4} M PP in 0.1 M citrate-phosphate buffer pH 5.7 or phosphate buffer pH 8.0 no reaction with 10^{-4} M cysteine was observed (unpublished results). As was shown in chapter 6, the fungitoxicity of pyrazophos against *Sphaerotheca fuliginea* can also not be antagonized by cysteine or glutathione. A second hypothesis on the mechanism of action of PP might be that the chemical interferes specifically with oxygen uptake and hence indirectly inhibits biosynthetic processes. According to this view a partial inhibition of oxygen uptake could also result in a complete inhibition of fungal growth. Such a mechanism has also been established for the systemic fungicide carboxin. Carboxin inhibits mitochondrial respiration of *U. maydis* at or close to the site of succinate oxidation; inhibition of nucleic acid and protein synthesis was suggested to be a secondary effect due to the lack of the necessary high energy compounds produced during operation of the tricarboxylic acid cycle (RAGSDALE and SISLER, 1970; MATHRE, 1971; GEORGOPOULOS et al., 1972). In order to elucidate whether a similar mechanism of action would be applicable for PP against *P. oryzae* in further investigations attention should be focussed primarily on effects of PP on mitochondrial respiration of this or other sensitive fungi from which mitochondria can be readily isolated.

In preliminary experiments with isolated mitochondria of *P. oryzae* a significant inhibitory effect of 10^{-4} M PP on mitochondrial oxygen uptake,

using 0.01 M malate and pyruvate as a substrate, has already been noticed. Inhibitory effects of PP were much less evident when other substrates like 0.001 M NADH or 0.01 M succinate were used. It is hoped that research along these lines will provide a more exact indication for the location of the site of toxic action of PP.

9.4. SUMMARY

At concentrations fully inhibitory to mycelial growth of *Pyricularia oryzae*, PP inhibited oxygen uptake at pH 4.0 progressively with time. Inhibition of DNA, RNA and protein synthesis, measured as increases in DNA, RNA and protein contents with time, appeared to be proportional to inhibition of increase in dry weight. It is assumed that PP either inhibits specifically oxygen uptake and, hence, indirectly various biosynthetic processes, or affects both oxygen uptake and several biosynthetic processes by an aspecific reaction of this chemical with cellular components.

10. GENERAL DISCUSSION

Organophosphorus pesticides are well-known because of their generally strong insecticidal activities. The main basis for these toxic effects is their capacity to inhibit acetylcholinesterase. Since 1960 a number of fungicidal organophosphorus compounds have also been introduced. Their fungicidal activities towards *Sphaerotheca fuliginea*, *Erysiphe graminis* f. sp. *hordei* and *Pyricularia oryzae* have been described in chapter 3. Pyrazophos proved to be one of the most toxic compounds towards these fungi. Since some of the organophosphorus fungicides also display insecticidal or acaricidal side-activity, investigations on the mechanism of action of pyrazophos were initiated with a study of its effect on the activity of carboxylesterases. These were shown to occur in *S. fuliginea* and are related with acetylcholinesterase (ch. 4). Activity of these carboxylesterases could, in fact, be inhibited by pyrazophos and to an even larger extent by the phosphate analogue of pyrazophos. However, it was shown that these effects, due to phosphorylation of these enzymes, could not account for the fungitoxic action since no correlation could be established between fungitoxicity and inhibition of enzyme activity.

Investigations on the subject were, therefore, continued by studying effects of pyrazophos on cell membrane permeability using *P. oryzae* as the test fungus (ch. 5). It was shown that pyrazophos, in contrast to the organophosphorus fungicides Hinosan and Kitazin, did not influence cell membrane permeability of the fungus.

In short-term experiments, pyrazophos hardly affected nucleic acid and protein synthesis and only slightly inhibited oxygen uptake of *P. oryzae* (ch. 6). In the same experiments, toxicity of pyrazophos proved to be surprisingly low. Apparently, in long-term experiments pyrazophos is transformed to more toxic breakdown products in relatively large quantities. These products were shown to be PO-pyrazophos and PP. In contrast to pyrazophos, PP did exert a direct toxic action in short-term experiments and it was, therefore, supposed that this metabolite could be the actual fungitoxic principle (ch. 7 and 8). PP also displayed inhibitory effects towards mycelial oxygen uptake, and nucleic acid and protein synthesis (ch. 9). Hence, the toxic character of pyrazophos seems to be mainly due to its heterocyclic nucleus, while the phosphorylating capacity of the chemical probably is of lesser significance. This is in contrast to the insecticidal action of similar compounds, such as parathion. In these instances, the intrinsic insecticidal action is due to the O,O-diethylthionophosphate moiety which phosphorylates acetylcholinesterase after a metabolic conversion to the phosphate analogue.

A more or less comparable situation has been described for saligenin cyclic phosphate esters. The phosphorothionate analogues such as salathion have high insecticidal activity, while phosphorothiolate analogues such as saligenin cyclic S-methyl phosphorothioate display a strong fungitoxic activity. It was

also suggested that fungitoxicity of the latter compound to *P. oryzae* was not based on its ability to inhibit esterases, but on the property of a breakdown product, *viz.* an o-hydroxybenzyl ion, to react with sulphhydryl groups (OHKAWA and ETO, 1969).

As mentioned before, the fungicides Hinosan and Kitazin influence cell membrane permeability of *P. oryzae*, which probably indirectly influences chitin synthesis (MAEDA et al., 1970; ch. 5). Obviously, the mechanisms of fungitoxic action of organophosphorus fungicides are not identical with each other. Therefore, it is difficult to predict whether the mechanism of action of other organophosphorus fungicides like Dowco 199 and triamiphos, which were not investigated in these studies, is similar to one of those outlined above. In fact, in the case of Dowco 199 TOLKMITH and MUSSELL (1967) assumed that the toxophoric group of this fungicide is not the phosphorus containing moiety but the phthalimide group, or more specifically its unsaturated dicarboximide ring. These authors suggested that the compound might inactivate certain enzymes by formation of acylated derivatives or by its reactivity with sulphhydryl enzymes.

In the literature no suggestions on the mechanism of action of triamiphos are reported. The chemical only slightly reduced germination of *E. graminis* f. sp. *hordei* conidia on glass slides (KOOPMANS, 1960). Because of the relatively high toxicity to the same fungus in vivo, it was suggested that the compound might act indirectly via the host plant (KOOPMANS, 1960; MAGENDANS and DEKKER, 1966). However, germination of conidia dusted on cellulose membranes could be inhibited by triamiphos, but compared to other fungicides this occurred at relatively high concentrations (DE WAARD, 1971b).

In view of these data and in analogy with the mechanism of action of pyrazophos it is possible that in the case of both Dowco 199 and triamiphos, the hydrolysis products of these compounds, phenylaminotriazole and phthalimide, respectively, might be their actual fungitoxic principles. In cultures of *Saccharomyces pastorianus*, phthalimide was also shown to be a breakdown product of folpet [N-(trichloromethylthio)phthalimide]. However, in this case the fungitoxicity of phthalimide was found to be insignificant in comparison with that of the intact folpet molecule. Binding of sulphur-containing fragments to ethanol-insoluble entities of the cells is assumed to be primarily responsible for fungicidal effects of folpet (LUKENS and HORSFALL, 1967; SIEGEL and SISLER, 1968a and 1968b).

The mechanism of breakdown of fungitoxic organophosphorus compounds is an interesting phenomenon. In the case of saligenin cyclic S-methyl phosphorothioate, breakdown to the o-hydroxybenzyl ion appeared to be a nonenzymatic reaction (OHKAWA and ETO, 1969). The conversion of pyrazophos into PO-pyrazophos and PP by *P. oryzae* is a metabolic one. It is tempting to investigate whether a microsomal oxidation reaction and/or an enzymatic hydrolysis of pyrazophos or PO-pyrazophos are responsible for these conversions. Microsomal desulfuration reactions of organothionate compounds have been demonstrated in vitro in microsomal fractions of various organisms like

mammals, insects, spider mites and plants. So far, no such reactions have been found in microsomal fractions of fungi (FEST and SCHMIDT, 1973), although their existence is very probable on the basis of the results observed. Investigations in this field might contribute not only to the knowledge on the metabolic conversion of organophosphorus compounds, but could also provide information on the mechanism of breakdown of other fungicides to non-toxic products.

Resistance in fungi to systemic fungicides is frequently encountered. A review on acquired resistance of fungi to systemic fungicides has recently been published (DEKKER, 1973). The question may arise whether fungi like *P. oryzae* and powdery mildew fungi can develop resistance to organophosphorus fungicides. In this respect, the mechanism of action of fungicides may provide some information since generally no resistance in fungi has been found to develop to multisite inhibitors because of their rather aspecific inhibitory effect on fungal enzymes. This is in contrast to fungicides which affect the fungus at one specific site (DEKKER, 1969b, 1973). Hinosan and Kitazin are probably such specifically interfering compounds, which have been found to influence cell membrane permeability of *P. oryzae* (ch. 5). Resistance to these compounds, obtained by selection on fungicide containing PDA media has indeed been reported (UESUGI and TOMIZAWA, 1971; TOMIZAWA and UESUGI, 1972). Also after irradiation of the same fungus with ultraviolet light, strains less sensitive to pyrazophos were obtained. The ED_{50}-value of pyrazophos for these strains was about ten times higher than for the parent strain. This property, however, disappeared after a number of transfers on fungicide free media and has to be considered as tolerance rather than resistance (DE WAARD, unpublished). After 3–4 years of application in practice against a number of powdery mildews in the Netherlands no resistance against pyrazophos has been noted. These observations might be plausible in the light of a less specific mechanism of action of PP in the fungus.

With respect to the mechanism of action of PP, which involves an inhibition of oxygen uptake and, in addition, possibly an inhibition of biosynthetic processes like nucleic acid and protein synthesis (ch. 9), the question arises why pyrazophos has such a narrow spectrum of antifungal activity and generally such a low phytotoxicity. As it was found that the pyrazophos insensitive fungi *Pythium debaryanum* and *Saccharomyces cerevisiae* do not break down pyrazophos into PP, it was supposed that a selective uptake of pyrazophos and/ or a specific metabolic conversion of the fungicide into PP in *P. oryzae*, and perhaps other sensitive fungi forms the explanation for the selective fungitoxicity. This suggestion is strengthened by the fact that fungi insensitive to pyrazophos often can be inhibited by PO-pyrazophos and PP, indicating an 'intrinsic sensitivity' of these organisms. The same reasoning might account for the low phytotoxicity of pyrazophos. As has been demonstrated in chapter 3, the systemic activity of the compound against cucumber powdery mildew, when applied via the roots, is only apparent in narrow zones around the vein system, indicating a limited transport to the leaf margins. Apart from this

factor the usually lower metabolic level in plants may also render plants less sensitive to systemic fungicides (KAARS SIJPESTEIJN, 1970). The 'intrinsic sensitivity' of plants is also indicated by the considerably higher phytotoxicity of PO-pyrazophos to cucumber and barley plants, than of pyrazophos (ch. 3).

On the basis of these considerations, it might be assumed that the diethyl-thionophosphate group plays an important role in the toxic action of pyrazophos towards plants and fungi. It seems likely that for sensitive fungi this substituent acts as an inbuilt formulation factor, which facilitates the uptake of pyrazophos. Intracellularly, the chemical has to be broken down into PP, in order to exert its toxic action. By changing the diethylthionophosphate group or by introducing other substituents in the pyrazolo-pyrimidine nucleus of PP it might be possible to obtain derivatives with a broader spectrum of antifungal activity than pyrazophos, since PP is also less selective in its toxicity towards fungi. Because fungi might less readily acquire resistance against this type of compounds, such derivatives will be valuable for practical use, provided that they do not display phytotoxic effects. It should be emphasized that in toxicological studies of these compounds the toxicity and possible side-effects of breakdown products should receive equal attention as the parent compound.

SUMMARY

In plant protection organophosphorus compounds are well-known for their insecticidal and acaricidal action. Since 1960, a number of organophosphorus fungicides have also been introduced. In an in vivo screening of these and some related insecticidal compounds against *Sphaerotheca fuliginea* on cucumber, pyrazophos (O,O-diethyl O-(5-methyl-6-ethoxycarbonylpyrazolo[1,5-a]pyrimidin-2-yl) phosphorothioate) proved to be most active. The chemical displayed both a protective and curative action against the disease; in addition, considerable toxicity to *Pyricularia oryzae* on barley was also found. Pyrazophos proved to be rather selective because hardly any other fungi sensitive to the chemical were found in a study on the in vitro spectrum of antifungal activity.

The organophosphorus fungicides Hinosan (O-ethyl S,S-diphenyl phosphorodithioate) and Kitazin (O,O-diethyl S-benzyl phosphorothioate) were about as toxic as pyrazophos to *P. oryzae*, but were much less toxic than pyrazophos to *S. fuliginea*. In view of these results, investigations on mechanisms of fungitoxic action of organophosphorus fungicides were mainly focused on pyrazophos.

Both pyrazophos and its phosphate analogue (PO-pyrazophos) were found to inhibit the activity of carboxylesterases of *S. fuliginea*. However, because no correlation could be established between in vivo inhibition of the activity of these enzymes by pyrazophos and PO-pyrazophos, and their fungitoxicity, this effect probably does not account for the mechanism of action of pyrazophos.

Neither could the toxicity of pyrazophos, in all other experiments studied with *P. oryzae*, be attributed to an effect on cell membrane permeability, as was shown to be present upon incubation of mycelium of the fungus with Hinosan and Kitazin. In addition, pyrazophos hardly affected nucleic acid and protein synthesis, and only slightly inhibited oxygen uptake.

In short-term experiments, using an incubation time of 2 hours, pyrazophos was $100-1000 \times$ less toxic to fungal growth in mycelial suspensions than to radial growth on agar and growth in liquid media inoculated with conidia. In the latter tests, growth was assayed after 1 week of incubation. These results can be partly ascribed to the fact that pyrazophos is metabolically converted in the fungus into two fungitoxic breakdown products, PO-pyrazophos and 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP). In short-term experiments the toxicity of PP for mycelial growth in suspensions buffered at pH 4.0 even proved to be considerably higher than that of pyrazophos and PO-pyrazophos. PP might, therefore, be regarded as the actual fungitoxic principle of pyrazophos. This hypothesis is supported by the finding that PP, in contrast to pyrazophos, also displayed an inhibitory activity towards nucleic acid and protein synthesis and towards oxygen uptake of the fungus. The weak effects of pyrazophos on these processes and on mycelial growth in short-term experiments can probably be ascribed to an insufficient conversion

of pyrazophos into PP under these conditions.

Regarding the site of fungitoxic action of PP in *P. oryzae*, two hypotheses could be suggested. First, PP could inhibit specifically oxygen uptake and hence indirectly cellular synthetic processes like nucleic acid and protein synthesis. Secondly, PP might react aspecifically with cellular components and hence, directly affect both oxygen uptake and biosynthetic processes.

Pythium debaryanum and *Saccharomyces cerevisiae* are practically insensitive to pyrazophos. Upon incubation of these fungi with the fungicide no breakdown products could be detected. Therefore, sensitivity of fungi for pyrazophos seems to be the result of a selective uptake of pyrazophos and/or of its conversion into PO pyrazophos and PP as has been demonstrated for *P. oryzae*.

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SAMENVATTING

Bij de bestrijding van planteziekten worden organische fosforverbindingen vooral gebruikt als insecticiden en acariciden. Vanaf 1960 zijn ook een aantal fungicide organische fosforverbindingen geïntroduceerd. In een onderzoek naar de werking van deze fungiciden en sommige verwante insecticide verbindingen tegen *Sphaerotheca fuliginea* op komkommer, bleek pyrazofos (O,O-diethyl O-(5-methyl-6-ethoxycarbonylpyrazolo[1,5-a]pyrimidin-2-yl) fosforothioaat) het meest actief te zijn. De verbinding werkte zowel preventief als curatief tegen deze ziekte en was bovendien fungitoxisch voor *Pyricularia oryzae* op gerst. Aangezien in een in vitro onderzoek naar het werkingsspectrum vrijwel geen andere gevoelige schimmels werden gevonden, is de fungicide werking klaarblijkelijk vrij selectief.

Andere organische fosforverbindingen die tegen *P. oryzae* ongeveer even toxisch bleken als pyrazofos, maar in een veel mindere mate tegen *S. fuliginea*, zijn Hinosan (O-ethyl S,S,-difenyl fosforodithioaat) en Kitazin (O,O-diethyl S-benzyl fosforothioaat). Op grond van deze resultaten werd besloten het onderzoek naar werkingsmechanismen van fungicide organische fosforverbindingen te richten op pyrazofos.

Zowel pyrazofos als zijn fosfaat-analoog (PO-pyrazofos) remmen de activiteit van carboxylesterasen van *S. fuliginea*. Dit effect kan echter waarschijnlijk het werkingsmechanisme niet verklaren, aangezien er geen verband kon worden vastgesteld tussen de in vivo activiteitsremming van deze enzymen en hun fungitoxiciteit. De toxiciteit van pyrazofos kon evenmin worden toegeschreven aan een effect op de permeabiliteit van de celmembraan, althans bij *P. oryzae* die in alle volgende experimenten als toetsschimmel werd gebruikt. Een effect op de permeabiliteit werd daarentegen wel gevonden bij incubatie van schimmelmycelium met Hinosan en Kitazin. Pyrazofos vertoonde bovendien nauwelijks een effect op de nucleinezuur- en eiwitsynthese en slechts een zwak effect op de zuurstofopname van de schimmel.

In kortdurende proeven, met een incubatietijd van 2 uur, bleek de toxiciteit van pyrazofos voor groei van mycelium in suspensies $100-1000 \times$ lager vergeleken met de remming van de radiale groei op agar en de groei in vloeibare media, geënt met conidiën. In de laatstgenoemde toetsen werd de groei bepaald na 1 week incubatietijd. Deze resultaten kunnen waarschijnlijk gedeeltelijk worden toegeschreven aan het feit dat pyrazofos door de schimmel kan worden omgezet in twee fungitoxische afbraakproducten, PO-pyrazofos en 2-hydroxy-5-methyl-6-ethoxycarbonyl-pyrazolo(1,5-a)pyrimidine (PP). In kortdurende proeven bleek de toxiciteit van PP voor myceliumgroei in suspensies, gebufferd op pH 4.0, aanzienlijk hoger te zijn dan van pyrazofos en PO-pyrazofos. Op grond hiervan werd de mogelijkheid aanwezig geacht dat PP het werkelijke fungitoxische principe van pyrazofos is. Deze hypothese wordt gesteund door het resultaat dat PP, in tegenstelling tot pyrazofos, ook een remmende werking

bezat op de nucleinezuur- en proteinesynthese en op de zuurstofopname van de schimmel. De zwakke effecten van pyrazofos op deze processen en op de myceliumgroei in kortdurende proeven kunnen waarschijnlijk worden toegeschreven aan een onvoldoende omzetting van pyrazofos in PP onder deze omstandigheden.

Op basis van de verkregen resultaten werden voor de plaats van werking van PP tegen *P. oryzae* twee hypothesen opgesteld. In de eerste plaats wordt verondersteld dat PP specifiek de zuurstofopname van de schimmel remt en daardoor indirect uiteenlopende biosynthetische processen, zoals nucleinezuur- en eiwitsynthese. In de tweede plaats bestaat de mogelijkheid dat PP aspecifiek met celbestanddelen reageert en daardoor aspecifiek zowel de zuurstofopname als bovengenoemde biosynthetische processen beïnvloedt.

Pythium debaryanum and *Saccharomyces cerevisiae* zijn vrijwel ongevoelig voor pyrazofos. Bij incubatie van deze schimmel met het fungicide werden geen afbraakproducten gevonden. Gevoeligheid van schimmels voor pyrazofos lijkt dan ook een gevolg te zijn van een selectieve opname van pyrazofos en/of de omzetting ervan in PO-pyrazofos en PP, zoals is aangetoond bij *P. oryzae*.

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