Acquired resistance to systemic fungicides of Septoria nodorum
and Cercosporella herpotrichoides in cereals.

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ACQUIRED RESISTANCE TO SYSTEMIC
FUNGICIDES OF SEPTORIA NODORUM AND
CERCOSPORELLA HERPOTRICHOIDES IN CEREALS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas, hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op woensdag 14 februari 1979 des namiddags te vier uur
in de aula van de Landbouwhogeschool te Wageningen.

Aan Yvon

STELLINGEN

1.

Onder de huidige praktijkomstandigheden is het optreden van resistentie van Cercosporella herpotrichoides tegen benzimidazool fungiciden, in die zin dat daardoor de effectiviteit van deze verbindingen verminderd wordt, niet waarschijnlijk.

Dit proefschrift.

2.

De door Rashid en Schlösser gebruikte methode naar de aanwezigheid van benomyl resistente stammen van Cercosporella herpotrichoides in het veld, wettigt niet de door hen getrokken kwantitatieve konklusies.

T. Rashid und E. Schlösser, 1975.
Z. Pflanzenkrankh. 11, 765-766.
T. Rashid und E. Schlösser, 1977.
Med. Fac. Landbouww. Gent, 1057-1065.

3.

Het feit, dat Chidambaram en Bruehl er niet in slaagden, benomyl resistente stammen te isoleren uit nakomelingschappen van veldisolaten van Cercosporella herpotrichoides, berust op een te gering aantal geteste sporen.

P. Chidambaram and G.W. Bruehl,1973
Plant Dis. Reptr. 57, 935-936.

4.

In het pathogeniteitsonderzoek van schimmelstammen, die resistent zijn tegen fungiciden, dient bij voorkeur ook hun gedrag in mengpopulaties met gevoelige stammen onderzocht te worden.

5.

De bewering van Gareth Jones en Jenkins, dat infektie van tarweplanten door Cercosporella herpotrichoides deze gevoeliger maakt voor Septoria nodorum, wordt onvoldoende door hun resultaten ondersteund.

D. Gareth Jones and P.D. Jenkins,
1978, Ann.appl.Biol., 90, 45-49.

6.

Ter vermijding van ongewenste sluikimporten van gewasbeschermingsmiddelen verdient het aanbeveling, de B.T.W. van deze produkten tussen de verschil-lende lidstaten van de E.E.G. op hetzelfde nivo te brengen.

7.

Het toevoegen van benzimidazool fungiciden aan voedingsoplossingen van hydro-
cultures dient met het oog op een mogelijke resistentie ontwikkeling te
worden afgeraden.

8.

Het gebruik van herbiciden, die vóór opkomst van het onkruid moeten worden
toegepast, dient waar mogelijk te worden beperkt.

9.

Het gebruik van elektroshocks met een hoog voltage, als een middel om
onkruiden te doden, is zeer beperkt in zijn praktische toepasbaarheid.

10.

De hypothese van van der Plank, dat vatbaarheid in pathogeen-waard systemen
berust op een copolymerisatie van een specifiek eiwit in het pathogeen
en een specifiek eiwit in de waardplant, is niet waarschijnlijk.

J.E. van der Plank, 1978.
Genetic and molecular basis of plant
pathogenesis. Springer Verlag,Berlin.

11.

De verspreiding van non-persistente virussen in het veld wordt na een
bespuiting met minerale olie tegengegaan, doordat de vermeerdering van het
virus in de bespoten plant wordt belemmerd.

12.

Het stichten van nationale landschapsparken is diskutabel, mede doordat
dit ten koste kan gaan van de landschapsbouw in andere delen van Nederland.

J.A.H.M. Horsten,
14 februari 1979.

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CONTENTS

1. INTRODUCTION

1.1. General

Since the end of the 19th century, fungicides have played an important role
in the control of fungal plant diseases. The fungicides first developed were cha-
racterized by the fact that they protected the plant or the seed by superficial
action and did not penetrate into the plant tissue. They are called "conventional
fungicides". Their action is based on the prevention of a fungal infection.
Examples of this type of fungicides are: copper and sulphur compounds, dithio-
carbamates, organic tin compounds, dodine and other organic and inorganic
compounds.

Approximately fifteen years ago a new type of fungicide was introduced, which
is characterized by the fact that these compounds are taken up in the plant tissue
and are transported in the plant. They are not only able to prevent infection but
may also exert a curative action by eliminating pathogens which have penetrated
into the plant tissue or seeds. They are called "systemic fungicides".

After the introduction of the systemic fungicides in practice, it has been
frequently reported that the effect of a new compound, which originally provided
excellent disease control, decreased considerably after a certain period of use.
In most of these cases, the pathogen proved to have acquired resistance to the
fungicide. The development of resistance to systemic fungicides has been recently
reviewed in a number of articles: DEKKER (1976[b], 1977); FEHRMANN (1976); OGAWA et al.
(1977). It should be noted that the development of resistance to conventional fungi-
cides was mostly of less importance (ASHIDA, 1965; GEORGOPOULOS and ZARACOVITIS, 1967).

Development of resistance to a fungicide may be due to a change at the site of
action, which decreases the affinity between the chemical and this site (DEKKER, 1976[a])
or to a change in the fungal cell, which inhibits the fungicide to reach the site
of action, e.g. a change in permeability of the fungal cell membrane. It has been
argued that the chance of a mutation to resistance is related to the mechanism of
action of the fungicide involved. Most conventional fungicides are known to act at
many sites in the fungal cell; for this reason they are called "multi-site inhibitors".
It is, therefore, very unlikely that the fungal cell can mutate to resistance at all
these sites. Investigations on the mechanism of action of systemic fungicides have
revealed that these compounds, as a rule act primarily at one or only a few sites in
the fungal cell metabolism (GEORGOPOULOS, 1976; DEKKER, 1977); for this reason they
are called "specific-site inhibitors".

It has been shown in several cases that resistance to a "specific-site inhibitor"

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may be brought about by a single gene mutation (HASTIE and GEORGOPOULOS, 1971; VAN TUYL, 1977). The rate at which resistant mutants appear may vary with the type of fungicide, the fungal species and the environmental conditions which includes the presence or absence of the fungicide concerned (DEKKER, 1976[b]). The fungicide will exert a selective action on the fungal population, favouring the increase of spontaneous resistant mutants, which may constitute a, usually, small part of the normal fungal population. The emergence of resistant forms does not necessarily imply that a fungicide resistance problem will arise in practice. This will largely depend on the properties of the resistant strain, the type of disease and the selection pressure by the fungicide (DEKKER, 1976[b]).

1.2. Review of the literature

Resistance to fungicides may emerge as a consequence of genetic or non-genetic changes in the fungal cell. As the latter type of resistance, which is called adaptation or transient resistance, seldom reaches a high level and is usually lost again after transfer to a fungicide-free medium, it would appear to be of limited practical importance. Aspects of this phenomenon have been relatively little studied. Of more importance is stable fungicide resistance, caused by mutations. The frequency with which fungicide mutants emerge depends on the number of mutations required for resistance and the mutability of the genes at the loci concerned. It may be influenced by the type of fungicide, the nature of the pathogen and by environmental conditions, e.g. the presence of U.V. or other radiation.

In natural spore populations of sensitive fungi, the frequency of specifically resistant spores is very small: BRASIER and GIBBS (1975) determined that only $1:1.3 \times 10^8$ spores of Ceratocystis ulmi were resistant to carbendazim (methyl-2-benzimidazole-carbamate). In Botryotinia fuckeliana this relation was $1:5 \times 10^7$ (POLACH and MOLIN, 1975) In Colletotrichum lindemuthianum the relative proportion of carbendazim resistant spores varied from $1:10^6$ to $1:8 \times 10^7$, depending on the strain examined (MEYER, 1976). These figures are valid only for the extremely small initial frequency of resistant spores in fungal populations which have not yet been exposed to the selection pressure of the respective fungicide.

The emergence of resistant strains, however, does not necessarily imply that problems with fungicide resistance will arise in practice. This may depend, amongst other things, on the type of disease, the characteristics of the resistant mutants and control management.

Leaf pathogens can be more easily eliminated by fungicides than pathogens which

infect the roots or the haulm base. In the latter case, the sensitive portion of the
population can be eliminated to a smaller extent only, so that more sensitive spores
are able to survive. Furthermore, spread of spores will be favoured by a short latent
period when coupled with a sufficient spore production and aerial spore dispersal.
This is the case with powdery mildews and Botrytis spp., but not with Ceratocystis
ulmi or Cercosporella herpotrichoides, which have a rather long latent period and
limited aerial spore dispersal. Whether the crop is grown in closed biotopes, such
as greenhouses, or as a field crop, is also of importance. In greenhouses, it is
rather improbable that sensitive spores will be supported from outside populations;
under field conditions, natural populations of a plant pathogen may be supplemented
from other locations. Of further great importance are the characteristics of the re-
sistant strains, especially pathogenicity and fitness, which determine the compe-
titive ability of resistant strains in natural fungal populations. According to
FUCHS and VIETS-VERWEY (1975), triforine resistant strains of Cladosporium cucumerinum
exhibited a reduced ability to produce viable spores. Triforine, together with other
fungicides such as fenarimol, triarimol, nuarimol and triadimefon, belongs to a
group of chemically different toxicants which inhibit ergosterol biosynthesis
(BUCHENAUER, 1977). FUCHS and DRANDAREVSKI (1976) concluded from their results that
"development of resistance to fungicides which inhibit ergosterol synthesis under
practical conditions is rather unlikely". During the 8 years that triforine has been
used in practice, no reports on the development of fungicide resistance in the field
have appeared. Fenarimol resistant mutants of Aspergillus nidulans obtained by
DE WAARD (1977) also showed a reduced fitness. As strongly reduced pathogenicity
was found by DEKKER and GIELINK (1979) in pimaricin resistant strains of Cladosporium
cucumerinum. On the basis of the mechanism of action of this fungicide, it was possi-
ble to suggest that reduced pathogenicity was linked to increased resistance.
The build-up of a resistant population of the pathogen in the field would, in this
case, be unlikely.

Finally, the management of disease control may influence the development of
fungicide resistance in the field as it determines the selection pressure exerted
on a fungal population. Since most systemic fungicides are readily transported up to
the transpiring parts of the plant (PETERSON and EDGINGTON, 1971), those fungi which
infect the haulm-base of a plant will be exposed for only a short time to the selection
pressure of the fungicide, if this were sprayed onto the top of the plant. After seed-
treatment or soil drench, there will be a supply of a fungicide for a longer period.
Not only the way a fungicide is applied but also other characters, such as persistance
in the plant, mode of accumulation and the effectiveness of the fungicide, play an
important role in this respect. Finally, the concentration used is important. Application
of high doses of a fungicide at frequent intervals will favour a rapid build-up

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of any resistant strains, even those with a lower pathogenicity or fitness
(DEKKER, 1976[a]). The selection pressure of a particular fungicide may be lowered
by alternate or combined use of two different fungicides.

Many examples of acquired resistance have been reported for fungicides whose
active principle is carbendazim (cf. FEHRMANN, 1976 ; DEKKER, 1977; OWAGA et al.,
1977). The mode of action of these fungicides is based on the inhibition of mitosis
as carbendazim binds to the protein subunits of the spindle microtubules (DAVIDSE,
1976). The mechanism of resistance is based on a decreased affinity of these subunits
to carbendazim. Genetic studies by HASTIE and GEORGOPOULOS (1971) and VAN TUYL
(1977) have shown that resistance in Aspergillus nidulans to carbendazim could be
attributed to different single gene mutations. By using UV radiation, VAN TUYL (1977)
was able to demonstrate, that a number of fungi has the potential to develop resis-
tance to carbendazim.

The development of resistance to fungicides is not confined to the carbendazim
group. Resistance to other systemic fungicides such as the oxathiins, the pyrimidine
derivatives ethirimol and dimethirimol, and the inhibitors of sterol biosynthesis
have also been reported. In most of these cases, however, the resistant strains had
been obtained after UV treatment or other laboratory experiments. Except dimethirimol
with these chemicals a widespread occurrence of resistant strains in field populations
of fungal pathogens has not been reported until now (cf. DEKKER, 1976[a], 1977; FEHRMANN,
1976 ; OGAWA et al., 1977).

1.3. Aim and outline of the present study.

In Europe, the control of some cereal pathogenic fungi with systemic fungicides
has become more and more common over the past ten years. In contrast to other crops,
however, cereals are not frequently sprayed with fungicides, usually once or twice in
a growth period.

The aim of the present study was to examine the possibility of the development
of resistance in cereal pathogens to systemic fungicides in the field which could
hamper adequate disease control.

Two cereal pathogens, Septoria nodorum, the causal agent of glume blotch of wheat,
and Cercosporella herpotrichoides, the causal agent of eyespot, were used in this study
In practice, eyespot is controlled mainly with carbendazim fungicides, whereas carben-
dazim fungicides or the protective substance captafol may be employed against S. nodorum

As the type of disease and pathogen may influence the development of fungicide
resistance, it seemed of interest to compare these two pathogens, which have a rather
different biology. C. herpotrichoides only attacks the haulm base, whereas S. nodorum

successively attacks the haulm base, the leaves and finally the ears and the grains. Thus, C. herpotrichoides will be exposed to the fungicide for a shorter time than S. nodorum, as carbendazim fungicides are readily transported acropetally to the upper parts of the plant (PETERSON and EDGINGTON, 1971). Under favourable climatic conditions, S. nodorum has a much shorter latent period, about 10-14 days, than C. herpotrichoides, the latent period of which is between six and ten weeks. Spread of spores of C. herpotrichoides is very limited (FEHRMANN and SCHRÖDTER, 1971) compared with S. nodorum (FAULKNER and COLHOUN, 1976).

As the type of fungicide may also influence the development of resistance, fungicides with a different mode of action were used. With S. nodorum experiments were carried out using carbendazim and edifenphos. Carbendazim inhibits mitosis in fungi (DAVIDSE, 1976), edifenphos interferes with cell membrane permeability (DE WAARD, 1974). Edifenphos is a systemic fungicide, used in Japan for the control of rice blast, Pyricularia oryzae (UMEDA, 1973). Although edifenphos is active against S. nodorum, there is no official registration for use against glume blotch in Germany. With C. herpotrichoides carbendazim was mainly used. For sake of comparison, the pyrimidine derivative nuarimol was used in the experiments. In field experiments this fungicide exhibited, at a high dosage, some effect against the eyespot fungus in wheat (FEHRMANN, unpublished). Nuarimol is an ergosterol biosynthesis inhibitor and in this respect is comparable with triarimol, triadimefon and triforine (BUCHENAUER, 1977). Edifenphos was not effective against C. herpotrichoides.

It was not possible to perform genetic studies to determine whether the fungicide resistance, found in this study, had a genetic basis. In this work the term "resistance" is used for each form of decreased sensitivity of a pathogen to a fungicide, whether this phenomenon is controlled by chromosomal genes or not.

The effect of spraying with carbendazim and edifenphos on the development of symptoms and on the length of the latent period in S. nodorum is presented in chapter 3.

To follow the effect of time of continuous and alternate treatments with two fungicides with a different mode of action, or of a mixture of both, on the development of fungicide resistance, a climate chamber system is used. With S. nodorum, the development of resistance to carbendazim and edifenphos is examined in an in vivo and an in vitro model. The term "in vivo" will be used for experiments with living and inoculated host plants. In C. herpotrichoides, development of resistance to carbendazim and nuarimol is examined in vitro only (chapter 4).

Experiments are not confined to controlled conditions in climate chamber models, but are also extended in field experiments, to determine the effect of field sprayings on the frequency of resistant strains (chapter 5).

Resistant strains of both pathogens are examined in agar and plant tests, in

order to compare them with sensitive ones with respect to important characteris-·
tics such as pathogenity and spore production, in the presence and absence of the
fungicide concerned (chapter 6).

To test, to what extent resistant strains are able to survive in mixed popu-
lations with sensitive ones, the relative proportion of resistant spores in mixtures
of resistant and sensitive spores is examined for three successive plant passages.
The results are presented in chapter 7.

2. MATERIALS AND METHODS

2.1. Fungi

Strains of the following fungi were used in the experiments: Septoria nodorum
(Berk.) Berk., perfect state Leptosphaeria nodorum E. Müll., the causal agent of
glume blotch of wheat.

Cercosporella herpotrichoides Fron, recently newly named as Pseudocercosporella
herpotrichoides (Fron) Deighton (cf. BOEREMA and VERHOEVEN, 1977), the causal agent
of eyespot of wheat, barley, rye and other grasses.

All isolates were made in this laboratory.

2.2. Plants

In the experiments with Septoria nodorum, the winter wheat variety 'Jubilar' was
used. This cultivar was chosen because of the susceptible response of its leaves to
the fungus.

Plants were grown in peat-amended standard soil in 7 cm square plastic pots. To
prevent overlapping of the leaves during inoculation, 7 kernels were sown in a straight
line and at equal spacing in the middle of each pot. The pots were then filled to the
rim with sterilized sand. Immediately after sowing, 10 ml of a 0.5% chlormequat (Cyco-
cel, CCC) suspension in water was added to each pot in order to prevent a strong cell
elongation of the leaves. The plants were kept in an incubator at $16\pm1^{\circ}C$, and exposed
to a light intensity of 3350 lux provided by fluorescent tubes (Philips TL 65 W/33 RS)
for 16 hours per day. After 8 days, plants at the one leaf stage were inoculated with
a spore suspension of S. nodorum.

In the experiments with Cercosporella herpotrichoides, the spring wheat variety
'Kolibri' was used. Plants were grown in 11 cm square plastic pots, in a mixture of one
part peat-amended standard oil and two parts compost, with 16 plants per pot. After
sowing, the pots were filled to the rim with sterilized sand. The plants were grown in
a walk-in climate chamber at $12^{\circ}C$, and exposed to a light intensity of 7200 Lux provided
by HQL lamps (Osram 400 W/R) for 12 hours per day. The mean relative humidity was 85%.
After 21 days, plants at the three leaf stage were inoculated with a spore suspension
of C. herpotrichoides.

2.3. Culture media.

Wheat meal agar (2% wheat meal + 2% agar, SHEARER, 1967) was used for culturing

both S. nodorum and C. herpotrichoides. The medium was autoclaved twice for 40 min. before the plates were poured.

Water agar (2%) was used for the isolation of S. nodorum from wheat leaf segments showing typical symptoms caused by this fungus. The medium was autoclaved for 60 min. To suppress bacterial growth, the medium was acidified to pH 4 with lactic acid (25%) and streptomycin sulphate was added up to a concentration of 300 μg/ml.

Wheat meal decoct agar was used for the determination of the relative proportion of fungicide resistant spores in spore suspensions of field isolates. Fourty g of wheat meal were added to two liters of distilled water and boiled for 60 min with continuous stirring. The suspension was then filtered through two layers of cheese cloth and made up to two liters with distilled water; 40 g agar were then added and the medium autoclaved for 60 min.

An agar medium with the following composition was used to isolate C. herpotrichoides from haulm segments:

> 750 ml distilled water
> 250 ml salt solution according to Hansteen-Cranner (SCHROPP, 1951)
> 1 ml A-Z Hoagland-solution
> 20 g agar

The medium was autoclaved for 60 min. To suppress bacterial growth, it was acidified to pH 4 with lactic acid (25%) and streptomycin sulphate was added up to a concentration of 300 μg/ml.

2.4. Fungicides

The chemical name and formulation of the fungicides used are listed in table 1.

Tab. 1. Name and formulation of the fungicides used.

common name	trade name	formulation	chemical name
thiophanate methyl	Cercobin M	70% WP	1,2-di-(3-methoxycarbonyl-2-thioureido)benzene
carbendazim	Derosal	60% WP	methyl benzimidazol-2-ylcarbamate
edifenphos	Hinosan	50% EC	O-ethyl-S,S-diphenyl-phosphorodithioate
nuarimol	Trimidal	9% EC	α-(2-chlorophenyl)-α-(4 fluorophenyl)-5-pyrimidine methanol

WP = wettable powder (w/w)
EC = emulsifiable concentrate (w/v)

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Cercobin M and Derosal were purchased, Hinosan was kindly provided by Bayer AG, Germany and Trimidal by Eli Lilly Ltd, Germany.

Since thiophanate methyl is converted readily to carbendazim in water (SELLING et al., 1970), laboratory experiments were carried out with carbendazim only.

2.5. Culturing of the fungi

2.5.1. Septoria nodorum

Isolation of the fungus, maintainance of strains and all in vitro experiments were performed in a walk-in climate chamber at $20 \overset{+}{-} 1^{\circ}$C. The 9 cm diameter plastic Petri dishes were exposed to a light intensity of 5000 lux provided by fluorescent tubes (Osram 65 W/ 55 TL) for 16 hours per day.

2.5.2. Cercosporella herpotrichoides

Isolation of the fungus, maintainance of strains and all in vitro experiments were performed in a walk-in climate chamber at $10 \overset{+}{-} 1^{\circ}$C. The 9 cm diameter plastic Petri dishes were continuously illuminated with Osram-L-Fluora fluorescent tubes (Osram L 65 W/ 77). The light spectrum of these tubes did not allow the light intensity to be measured with the aid of a normal lux meter.

2.6. Inoculation and fungicide application in climate chamber experiments with living plants.

2.6.1. Septoria nodorum

Plates with 14-day-old cultures extruding orange-pink spores from pycnidia were flooded with 10 ml of distilled water and the pycnidia with the pycnidio-spores were removed by gently rubbing the agar surface with a scalpel. The spore suspension was filtered through one layer of monyl-gauze (NY 30 HD) with a square mesh size of 30 μm, and diluted to a concentration of 10^6 spores per ml with the aid of a haemocytometer. To facilitate adhesion of the spore suspensions on the leaves, a gelatine solution in distilled water was added up to concentration of 0.5%.

Plants of the susceptible winter wheat variety 'Jubilar' were used in all experiments (cf. 2.2). With the aid of a de Vilbiss no. 15 adjustable tip atomizer, attached to a pressure pump, the spore suspension was sprayed evenly onto the

leaves, at a pressure of 1 atm. Six pots of plants, equally spaced and in a
straight line, were inoculated with 25 ml of spore suspension sprayed over a
period of 60 seconds. The distance between the atomizer and the plants was kept
constant (50 cm) in order to get a uniform droplet size on the leaves. Only in this
way were all leaves inoculated uniformly. The spray volume was too small to cause
the droplets to run off the leaves.

After inoculation, the pots of plants were placed in an incubator which had
the following dimensions: length 132 cm; depth 74 cm; height 65 cm. It was made of
zinc tin. The top consisted of a glass plate above which fluorescent tubes
(Philips TL 65 W/ 33 RS) were placed; these gave a light intensity of 3350 lux at
plant height. The front of the incubator was closed with a sheet of plastic. On the
bottom of the incubator there was a one cm deep layer of peat, which was saturated
with water before inoculation began. To insure 100% relative air humidity and a
continuous wetness on the leaves, a cold water humidifier (type ISMET LB 300) was
placed in the incubator. The humidifier was attached to an interval time clock,
programmed to disperse fine water droplets for 5 minutes each hour. In this way it
was possible to maintain a permanent film of water on the leaf surface and 100%
RH during the dark as well as during the light periods – conditions necessary for
a good infection and development of symptoms of <u>S. nodorum</u>.

The incubator was placed in a walk-in climate chamber at $16 \pm 1^{\circ}C$. During the
first 48 hours after inoculation, plants were kept in the dark, thereafter they
were illuminated for 16 hours per day as described. The mean temperatures in the
incubator were as follows: during the dark period $16 \pm 1^{\circ}C$, during the light period
$20 \pm 1^{\circ}C$.

If the experimental design required spraying of fungicides, the incubator
was opened 48 hours after inoculation. After evaporation of the water droplets on
the leaves, pots were removed from the incubator and the plants were sprayed with
the fungicides in the same way as described for the inoculation. The plants were
allowed to dry for two hours and then replaced in the incubator, which was then
reclosed.

2.6.2. <u>Cercosporella herpotrichoides</u>

Spores of 5 week-old cultures of the fungus were removed from the agar surface
by gently scratching with a scalpel. The spores were transferred to a flask con-
taining 25 ml of distilled water. The suspension was filtered through one layer of
monyl-gauze, in order to separate mycelial fragments from the conidia, and then
diluted to a concentration of $3x10^{5}$ spores per ml with the aid of a haemocytometer.

A de Vilbiss no. 15 adjustable tip atomizer, attached to a pressure pump, was used to spray the spore suspension onto the haulms of 21 day-old wheat plants (cf. 2.2), at a constant pressure of 1 atm. Pots of plants were inoculated separately. During inoculation, each pot was placed on a turntable turning at 30 revolutions per minute. Each pot was sprayed with 12.5 ml of spore suspension dispersed over 30 seconds. The distance between plants and atomizer was kept constant at 50 cm. After inoculation, plants were incubated in the dark for 7 days at 12°C and at a relative air humidity of 100%. The plants were then exposed to a light intensity of 7200 lux at pot height provided by HQL lamps (Osram 400 W/R) for 12 hours per day. The mean daily temperature in the climate chamber was 12°C and the mean relative air humidity was 85%.

If spraying with fungicides was required, plants were removed from the climate chamber 7 days after inoculation. The plants were sprayed with the fungicides in the same way as described for inoculation. The plants were allowed to dry for 2 hours, they were then returned to the climate chamber.

2.7. Isolation of the fungi

2.7.1. Septoria nodorum

From wheat leaves. Nine days after inoculation, wheat leaves were harvested and shaken for 15 minutes in 30 ml of a 7.5% H_2O_2 solution in sterile distilled water to which a small droplet of Triton-X was added as a wetting agent. The leaves were then washed three times by shaking them for three minutes with an equal volume of sterile distilled water. The surface sterilized and washed leaves were allowed to dry superficially and then placed on Petri dishes of water agar. The plates were incubated in a climate chamber at 20°C. After 5 days, leaf segments with pycnidia of S. nodorum were transferred to wheat meal agar at pH 4 supplemented with streptomycin sulphate (300 μg/ml). After another 14 days of incubation in the climate chamber, abundant spores had been formed which were used for further experiments.

From kernels. Wheat kernels, originating from field plots with different fungicide treatments, were surface sterilized by shaking them for 15 minutes in 30 ml of a 9% solution of Chloramin-T in sterile distilled water. Again, a small droplet of Triton-X was added. The kernels were then washed by shaking them three times for three minutes in the equal volume of sterile distilled water. After the kernels had dried superficially, they were placed on wheat meal agar at pH 4 containing streptomycin sulphate (300 μg/ml). After 7 days incubation

in the climate chamber, small colonies of S. nodorum were visible with charac-
teristic brownish pycnidia, extruding orange-pink spores. In order to get a
heterogeneous population, as many pycnidia as possible from one kernel were
transferred to new plates of wheat meal agar. Each subculture of this kind
was considered to be a separate isolate. These were transferred to plates with
wheat meal agar after another five days, if no contaminants were present. Two
weeks later, abundant conidia were present for further experiments.

2.7.2. Cercosporella herpotrichoides

Haulm segments of wheat or barley with characteristic Cercosporella-eyespot
symptoms were thoroughly washed for two hours in running tap water at high
pressure in order to remove external contaminants. Each haulm segment was cut in
5 small pieces with a scalpel and these were transferred to one agar plate
(cf. 2.3.), which was then incubated at $10^{o}C$ for 21 days. After incubation, the
plates were examined microscopically for the presence of C. herpotrichoides.
Due to its typical spore aggregates, with the rod-like, longish conidia, the
fungus was easily identified. As previously mentioned, subcultures were made on
wheat meal agar at pH 4 supplemented with streptomycin sulphate (300 μg/ml).
These heterogeneous subcultures were placed in a climate chamber at $10^{o}C$, 5
weeks later abundant spores were present which could be used in further experi-
ments.

2.8. Tests on acquired fungicide resistance in field isolates

2.8.1. Septoria nodorum

2.8.1.1. Spore germination tests

Plates with sporulating cultures of the fungus were flooded with 5 ml of
sterile distilled water and the pycnidia and the pycnidiospores removed from
the agar surface by gently scratching with a scalpel. The spore suspension was
filtered through one layer of sterile monyl-gauze and diluted to a concentration
of $2x10^{5}$ spores per ml with the aid of a haemocytometer. One ml of the spore
suspension was pipetted into each of three test tubes, containing either 1 ml
of sterile distilled water, 1 ml of carbendazim suspension at 2 μg/ml a.i. or
1 ml of edifenphos suspension at 50 μg/ml a.i.. The tubes were gently shaken
for a short time and then 0.03 ml droplets of each suspension were pipetted
onto glass slides. These were incubated at $20^{o}C$ in the dark and at 100% RH to

prevent the droplets evaporating. After 16 hours incubation, the percentage of germinated conidia was determined by counting 100 spores in each of three replicates. The germ tube length was also measured. A spore was classified as having germinated if the length of the germ tube exceeded 1/3 of the length of the spore.

2.8.1.2. Selection of resistant conidia from spore suspensions

Three wheat meal agar plate cultures of each kernel isolate of S. nodorum (cf. 2.7.1.) were flooded with 4 ml of sterile distilled water and the pycnidia with the pycnidiospores were carefully scratched off the agar surface with a scalpel. The suspensions were then poured into sterile 100 ml flasks. After one hour, by which time all spores had been released from the pycnidia, the suspensions were filtered through one layer of monyl-gauze, previously autoclaved for 20 minutes. The filtered spore suspension was pipetted onto the surface of wheat meal decoct agar plates, 1 ml per plate, containing either carbendazim at a concentration of $5 \mu g/ml$ a.i., or edifenphos at a concentration of $200 \mu g/ml$ a.i. These concentrations are lethal to sensitive spores of S. nodorum. The fungicides were added to the molten agar at $45^{o}C$, prior to pouring the plates. Moreover, 0.1 ml of each spore suspension was pipetted into a test tube containing 4.9 ml of distilled water, to determine the total number of spores pipetted onto the plates. The plates were incubated at $20^{o}C$. After 10 days, the colonies were counted and if necessary, they were subcultured on wheat meal agar plates without fungicide for further experiments.

2.8.2. Cercosporella herpotrichoides

For the quantitative determination of resistant spores in spore suspensions from field isolates (cf. 2.7.2.) spores were scratched off the agar surface of cultures, with a scalpel and transferred to a sterilized flask containing 12 ml of sterile distilled water. After 1 hour, the suspension was filtered through one layer of monyl-gauze, previously autoclaved for 20 minutes. The spore suspension was then pipetted onto the surface of wheat meal decoct agar plates, 1 ml per plate, containing carbendazim at a concentration of $3 \mu g/ml$ a.i. This concentration is lethal to sensitive spores of C. herpotrichoides. The fungicide had been added to the molten agar at $45^{o}C$ prior to pouring the plates. Moreover, 0.1 ml of each spore suspension was pipetted into a test tube containing 4.9 ml of distilled water. With the aid of a haemocytometer, the total number of tested

spores for each isolate was calculated. Plates were incubated at 20°C in the dark. Four weeks later, colonies of C. herpotrichoides were counted and if necessary they were subcultured on wheat meal agar plates without fungicide, for further experiments.

2.9. In vitro tests with sensitive and resistant strains.

2.9.1. Septoria nodorum

Resistant strains of S. nodorum, obtained from field isolates using the methods described above (2.8.1.2.), were subcultured at least three times in the absence of the respective fungicide. The phenotypic stability and the degree of the fungicide resistance were examined by growing both resistant and sensitive strains on wheat meal agar plates containing either carbendazim at concentrations from 0 to 1000 μg/ml a.i., or edifenphos at concentrations from 0 to 200 μg/ml a.i. The fungicides were added to the molten agar prior to pouring the plates, after it had cooled to 45°C. A 5 mm diameter agar disc of actively growing mycelium was used to centrally inoculate 4 replicate plates of each strain. Plates were incubated at 20°C. After 12 days, mycelial growth and spore production were measured. Spores were counted as follows: 10 ml of distilled water were pipetted onto the surface of each plate and the pycnidia with the pycnidiospores were carefully scratched off the agar surface with a scalpel. Each spore suspension was poured into a test tube, which was then shaken regularly for 4 hours. The spore concentration of each suspension was determined with the aid of a haemocytometer.

2.9.2. Cercosporella herpotrichoides

Essentially the same method was employed to test the stability and the degree of carbendazim resistance of C. herpotrichoides isolates. Plates were incubated at 10°C for three weeks (cf. 2.5.2.), then growth-rate was determined and spores were counted.

2.10. Tests with sensitive and resistant strains on living plants

2.10.1. Septoria nodorum

Wheat plants (cv. 'Jubilar') were inoculated with sensitive or resistant strains of S. nodorum and subsequently sprayed with fungicides as described under 2.6.1. Percentage of leaf necrosis was estimated 16 days after inoculation

and related to the percentage of leaf necrosis of control plants without fungici-
dal treatment. Five leaves from each pot were harvested and placed in a test tube
containing 5 ml of distilled water. The tubes then were placed in a thermostat at
4°C for 24 hours. The tubes were then shaken for 30 seconds and the total number
of spores counted by means of a haemocytometer.

2.10.2. Cercosporella herpotrichoides

The methods for inoculation and fungicidal treatment have been described
earlier (cf. 2.6.2.). After two months, plants were removed from the pots and
the haulms and roots carefully washed under running tap water. The plants were
classified into the following groups, according to the severity of the eyespot
symptoms on the haulm base.

0: no symptoms visible
1: light eyespot symptoms: haulms girdled by symptoms by less than 50%
2: severe eyespot symptoms: haulms girdled by symptoms by more than 50%

The disease index was calculated as follows:

$$\text{Disease index} = \frac{50 \times \left\{ \begin{array}{l} \text{(number of severely} \\ \text{diseased haulms)} \end{array} \times 2 + \begin{array}{l} \text{(number of lightly} \\ \text{diseased haulms)} \end{array} \times 1 \right\}}{\text{total number of haulms}}$$

The disease index ranges from 0 to 100.

3. EFFECT OF FUNGICIDE TREATMENTS ON THE DEVELOPMENT OF SYMPTOMS AND ON THE LATENT PERIOD OF SEPTORIA NODORUM

3.1. Introduction

Septoria nodorum (Berk.) Berk. is the imperfect form of Leptosphaeria nodorum E. Müll.

For successful infection and development of symptoms, the fungus requires a relative air humidity of 100%, coupled with longer periods of leaf wetness (EYAL et al., 1977). Hence, the pathogen becomes epidemic mainly in maritime regions and/or rainy seasons; it may cause substantial yield losses (BECKER, 1963; BRÖNNIMANN, 1968; BAKER, 1971; COOKE and JONES, 1971; BRÖNNIMANN et al., 1972; NELSON et al., 1976). Infection of winter wheat may already occur in autumn and winter, when pycnidia on wheat debris serve as a primary inoculum source (BECKER, 1963; SCHAREN, 1966; BRÖNNIMANN, 1968; HOLMES and COLHOUN, 1975); these are capable of producing viable pycnidiospores over a period of at least 18 months (WEBER, 1922).

Although S. nodorum is able to overwinter in young wheat plants (HOLMES and COLHOUN, 1975), spread of the disease will be rather limited under these conditions due to the fact that for reasonable development of symptoms, temperatures between about 15 and 25°C are required; only under such conditions is the latent period rather short (SHEARER and ZADOKS, 1972). Further spread of the pathogen to new leaves and to neighbouring plants is said to occur via splashing rain drops. Since rain droplets are transported by air movements, such as wind and other turbulences, a long-distance spread in the crop is possible (FAULKNER and COLHOUN, 1976).

After colonization of the green leaf parts, S. nodorum finally attacks the ears. Only minor yield losses are caused by leaf lesions which diminish the green leaf surface. Substantial losses are always caused by the infection of the glumes and by partial infection of the grain, leading to shriveled kernels with a low one-thousand-grain-weight.

S. nodorum can be controlled with carbendazim-generating fungicides or with different protective fungicides such as maneb, mancozeb or captafol. Fungicide combination may be useful. Control of glume blotch with carbendazim-fungicides in Germany often proved to be less successful than with captafol. Carbendazim treatment lead to an increase in yield however, which could be partially due to a side-effect against powdery mildew on the ears. With captafol however, timing of the

application has to be very accurate.

It was necessary to develop an accurate and reproducible inoculation method for the experiments. This method had to satisfy the specific humidity and temperature requirements of the pathogen, in order to be able to test the effect of different spray programmes on the development of fungicide resistance and also to compare resistant and sensitive strains with respect to their pathogenicty.

3.2. Experiments

Wheat seedlings, cv. 'Jubilar' (cf. 2.2.), were inoculated with a spore suspension of S. nodorum as described under 2.6.1. and 48 hours later, plants were sprayed with carbendazim at concentrations of 1000 μg/ml a.i. and 3000 μg/ml a.i., or with edifenphos at concentrations of 100 μg/ml a.i. and 250 μg/ml a.i.; control plants were not sprayed. Development of symptoms was assessed at regular intervals during a period of 26 days after inoculation. Each treatment consisted of 10 pots each with 7 plants. The average values are presented in figure 1.

The results, presented in figure 1, demonstrate that the described inoculation method enables a good and uniform development of S. nodorum symptoms on wheat seedlings. The standard errors, not presented here, never exceeded the 10% level of the average values. It may be concluded, therefore, that with this method good reproducible results can be obtained.

Both systemic fungicides delayed the development of symptoms, the extent of which depending on the concentration and on the fungicide used. However, it should be pointed out, that the necessary dosage of carbendazim is unusually high. This supports the observation that carbendazim-fungicides in the field exert only a slight influence on visible symptoms of glume blotch.

The effect of the described method on the length of the latent period was also examined. According to its definition, the latent period is the time needed for one generation. Hence in case of S. nodorum this means the time from inoculation until the formation of the first mature pycnidium. The criterion for pycnidial maturity is the extrusion of spores in a cirrhus after mounting a leaf in water between two glass slides (SHEARER and ZADOKS, 1972). Leaves were examined daily for the presence of mature pycnidia from the 4th day after inoculation. On each day, three leaves from different pots were examined. The results are listed in table 2. Within the limits of the described system, the values coincided quite well with the results which SHEARER and ZADOKS (1972) obtained for unsprayed plants under continuously wet conditions. Depending on the con-

centration employed, both fungicides extended the length of the latent period by 5 to 9 days.
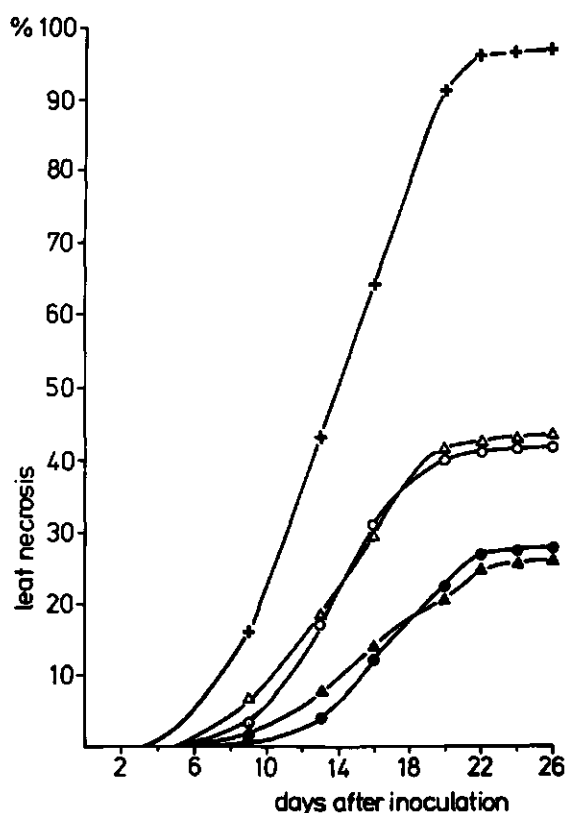
Fig. 1.
S. nodorum. Effect of sprayings with either carbendazim or edifenphos on the development of symptoms on wheat seedlings. For experimental details see text.
(+———+) unsprayed control; (△———△) carbendazim 1000 μg/ml;
(▲———▲) carbendazim 3000 μg/ml; (O———O) edifenphos 100 μg/ml;
(●———●) edifenphos 250 μg/ml.

Tab. 2. S. nodorum. Effect of spraying with either carbendazim or edifenphos on the length of the latent period. For experimental details see text.

Treatment	Latent period (days)
unsprayed control	8
carbendazim 1000 µg/ml	13
carbendazim 3000 µg/ml	17
edifenphos 100 µg/ml	14
edifenphos 250 µg/ml	17

3.3. Discussion

The reported findings confirm that the described method enables a good development of S. nodorum symptoms on wheat seedlings; the reproducibility of the results is good. Spraying with carbendazim or edifenphos exerts a twofold effect: both the symptom development as well as the length of the latent period are influenced, the effect being dependent on the concentration of fungicide used. It was observed that pathogenesis was initially more effectively delayed after application of edifenphos than of carbendazim (cf. fig. 1). After foliar spraying, carbendazim accumulates in the margins of the leaves (PETERSON and EDGINGTON, 1971). On the other hand, edifenphos remains largely at the site of application after being taken up by the leaf tissue and is hardly distributed to the other parts of the leaves (ISHIZUKA et al., 1973). Thus, the initial symptom development will be retarded to a greater extent by edifenphos than by carbendazim, the latter permitting a more rapid colonization of the non-marginal leaf area. These differences disappear with time due to the decreasing action of both fungicides.
As stated by EYAL and SCHAREN (1977) and as became clear from the results described above, the following parameters are important in order to achieve a good and reproducible development of S. nodorum symptoms on wheat seedlings. Firstly, it is advisable to raise the plants in such a way that the leaves do not overlap during inoculation and fungicide spraying. To this end, kernels were sown in a straight line and at equal spacing. Furthermore it is important that the spore suspension, diluted to a standardized concentration, is uniformly sprayed onto the plants with constant pressure and for a fixed time, keeping

the distance between plants and atomizer constant, in order to obtain a uniform droplet size on the leaves. The total volume sprayed should not be so large that the droplets run off the leaves. For a good development of symptoms the surface of the leaves should be wet for sufficiently long periods; this can be achieved by placing a cold water humidifier in the incubator.

4. EFFECT OF DIFFERENT SPRAY PROGRAMMES ON THE DEVELOPMENT OF FUNGICIDE RESISTANCE EXAMINED IN A CLIMATE CHAMBER SYSTEM.

4.1. Introduction

Development of fungicide resistance in plant pathogens may result in insufficient disease control. In some cases, the fungicide concerned had to be replaced by another one (cf. GEORGOPOULOS and DOVAS, 1973; WICKS, 1974; EICHHORN, 1975; KIEBACHER and HOFFMANN, 1976). The question arises, therefore, whether disease control might be managed in such a way, that the probability of the development of a resistant fungal population is minimized, without having to abandon the use of the fungicide concerned. It has been pointed out by several authors that the development of fungicide resistance may be reduced by the combined use of two or more fungicides, which must have a different mode of action to avoid failure of the control due to cross-resistance (WOLFE, 1971; DEKKER, 1973, 1976[a], 1976[b], 1977; FEHRMANN, 1976). Although not impossible, it still seems less likely, that a fungal strain will develop which is simultaneously resistant to two specific site inhibitors which have a different mode of action. If, under the selection pressure of one such substance, specifically resistant strains accumulate in a fungal population, then their further reproduction might be prevented by the simultaneous use of another systemic chemical. The combination of a systemic fungicide with a conventional, multisite inhibiting fungicide is another possibility. The latter might reduce sporulation on the plant surface and decrease the spread of the pathogen. However, when a conventional fungicide alone provides satisfactory disease control, it has to be taken into consideration whether it is desirable to add a systemic fungicide. STAUNTON and KAVANAGH (1975) suggested to use a tankmix of benomyl and dichlofluanid for the control of <u>Botrytis cinerea</u> on strawberries, although the effectiveness of dichlofluanid was comparable with that of benomyl alone and with the tankmix.

Development of fungicide resistance may also be counteracted by alternate application of two or more fungicides with a different mode of action. In this case it is also important to use various fungicides, in order to reduce the yearly selection pressure of a particular fungicide on the pathogen population. In such an alternate spraying programme, both systemic and non-systemic fungicides can be used.

It is rather questionable in how far the above mentioned methods can be used, when a great part of the fungal population consists of spores, resistant to one of the used fungicides. In case of combination of two or more compounds,

the disease will be controlled, but the percentage of specific resistant individuals will increase. JORDAN and RICHMOND (1975) observed, that a better control of benomyl insensitive Botrytis, affecting strawberries, was achieved by using a tankmix of benomyl and dichlofluanid than by using these compounds alternately. However, with an alternate spraying programme, the situation is somewhat different. It is rather dangerous to continue the use of a particular fungicide in an alternate spraying programme, when a great part of the fungal population consists of spores, resistant to this fungicide. This will result in insufficient disease control at that times on which the fungicide concerned is applied. This was also observed in experiments of EBBEN and SPENCER (1973). Dimethirimol and benomyl either alternately or together were applied to cucumber plants inoculated with powdery mildew strains, resistant to each of the fungicides. During the course of the experiments, a sharp increase in dimethirimol resistant strains after dimethirimol spraying was noticed, which could only be controlled by a benomyl spraying. On average, best results were obtained by a simultaneous application of the two fungicides. So, when a part of the population consists of resistant individuals it will be better to switch to a spraying programme with other fungicides. It will depend on the fitness of the resistant strains, whether the abandoned fungicide can be reused or not after a few years (cf. DOVAS et al., 1976). Considering all this information it would appear that it is important to employ such spray programmes which can avoid or delay the development of fungicide resistant populations. In order to examine whether such spray programmes would not finally lead to the development of resistance to the fungicides used, model experiments on this problem seemed desirable in which the resistance level of a fungal population was examined for a longer period of time. Investigations with S. nodorum were performed in models on living plants and agar plates and with C. herpotrichoides on agar plates only.

4.2. Septoria nodorum

4.2.1. Experiments on plants

The effect of continuous and alternate applications of carbendazim and edifenphos and of a mixture of both fungicides on the development of S. nodorum symptoms on wheat leaves was examined as follows.

Wheat seedlings were inoculated with S. nodorum for nine successive passages. To guarantee as much genetic heterogeneity as possible, the initial spore suspension used to inoculate the test plants was a mixture of 8 field isolates.

Fourty eight hours after inoculation, the plants were sprayed with the systemic
fungicides carbendazim and/or edifenphos at sublethal concentrations. After 7
days, a sample of leaves was harvested, disinfected and plated out in order to
isolate the pathogen, needed for inoculation of a second batch of plants. This
procedure was repeated for 9 passages on plants. In each passage the fungicides
were applied separately or as a mixture, or they were applied alternately from
passage to passage. In each successive passage the plants were inoculated with
a spore suspension obtained from the pycnidia of the previous batch of plants
in the same variant. The succession of experimental procedures was as follows:

Day 1: sowing of kernels (cf. 2.2.)

    8: inoculation (cf. 2.6.1.)

   10: fungicide application (cf. 2.6.1.)

   17: disinfection of a sample of the leaves (cf. 2.7.1.)

   24: assessment of symptoms

   28: sowing of kernels for the next batch of plants

   36: inoculation

   etcetera

The spraying programmes were as follows:

1. Plants in each passage sprayed with carbendazim (3000 μg/ml a.i.)
2. Plants in each passage sprayed with edifenphos (250 μg/ml a.i.)
3. Plants alternately sprayed with carbendazim (3000 μg/ml a.i.) and edifenphos
   (250 μg/ml a.i.), beginning with carbendazim in the first passage.
4. Plants in each passage sprayed with a mixture of carbendazim (1500 μg/ml a.i.)
   and edifenphos (125 μg/ml a.i.).
5. Unsprayed plants were used as controls.

    Changes in the percentage of leaf necrosis from passage to passage were
chosen as criterion for fungicidal resistance. The results are presented in
figure 2. In order to determine whether the observed difference were statisti-
cally significant, Wilcoxon's rank tests were performed on the results; the
corresponding significancies are listed in table 3.

    Within the limits of this model, it may be concluded that control of S.
nodorum with carbendazim and edifenphos became less effective, when this is
expressed by an increase in the percentage of leaf necrosis in subsequent plant
passages (fig. 2). The increase was faster and the final disease level was higher
after continuous carbendazim treatment than in the edifenphos variant. Continuous
carbendazim and edifenphos stress led to a statistically significant increase in
the percentage of leaf necrosis by the second and third passages, respectively.

Alternately spraying with carbendazim and edifenphos to a large extent delayed
the development of symptoms. In the variant with the alternate treatment the
relative percentage of necrotic leaf area increased from 19% in the first pas-
sage to 21% in the seventh, 29% in the eighth and finally 38% in the ninth pas-
sage. Thus, a significant increase was noticed in only the last two passages.
A signigicant difference between the data of the alternate treatment and those
of the continuous treatments, compared within the respective passages, was first
apparent in the fourth passage (tab. 3).
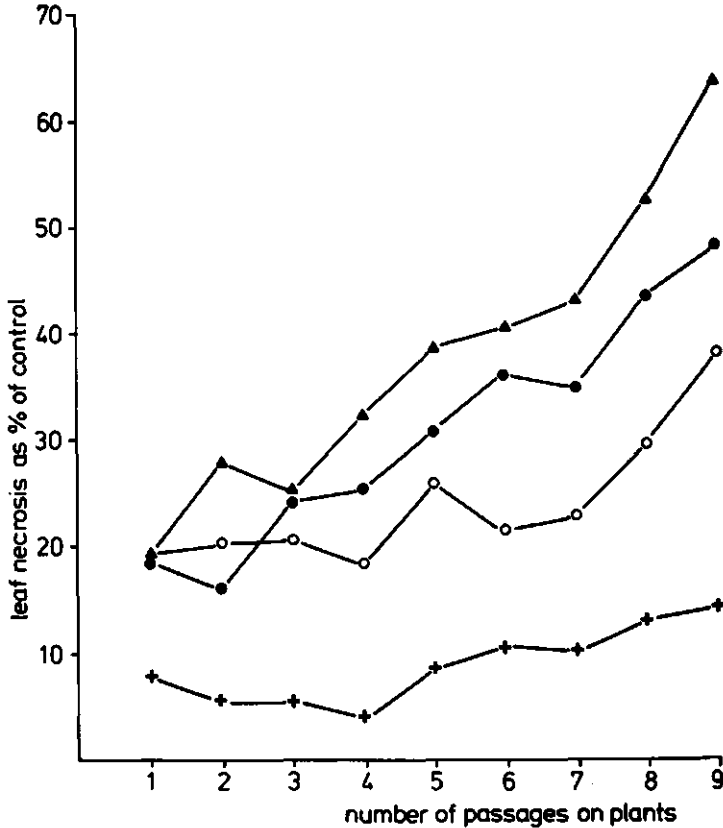
Fig. 2. <u>S. nodorum</u>. The effect of continuous and alternate spraying with carbenda-
zim and edifenphos and with a mixture of both on the percentage leaf necro-
sis of wheat plants, examined for nine successive passages. The values,
taken 16 days after inoculation, are expressed as a percentage of the con-
trol and are an average from 20 pots each containing 7 plants.
(▲────▲) carbendazim 3000 μg/ml; (●────●) edifenphos 250 μg/ml;
(O────O) alternately carbendazim 3000 μg/ml and edifenphos 250 μg/ml;
(+────+) mixture of carbendazim 1500 μg/ml and edifenphos 125 μg/ml.

Tab. 3. <u>S. nodorum</u>. Statistical significancies, calculated from the data in figure 2 by means of the Wilcoxon's rank test, for differences in relative percentages of leaf necrosis at different stages of the experiment. The data of each passage is compared with that of the first passage of each treatment.

Passages

treatment	2	3	4	5	6	7	8	9
carbendazim	xxx	xx	xxx	xxx	xxx	xxx	xxx	xxx
edifenphos	-	x	x	xxx	xxx	xxx	xxx	xxx
mixture	-	-	-	-	-	-	x	x
alternate	-	-	-	xx	-	-	xxx	xxx
carbendazim to alternate (y)		-		xx		xxx		xxx
edifenphos to alternate (y)	-		x		xxx		xxx	

- : not significantly different

x; xx; xxx : significant at P≤0.05; P≤0.01; P≤0.001 respectively.

(y) : within the corresponding passage.

Most promising results were obtained when a mixture of both fungicides was applied at each time. Although the concentration of each fungicide in the mixture was only half that of the other treatments, a better control of the disease was obtained (fig. 2). The development of symptoms was considerably delayed, compared with the other treatments. Only in passages 8 and 9 was there a slight but significant increase in the percentage of leaf necrosis (tab. 3).

During the course of the experiment, no substantial differences in symptom development were observed in the control variants with plants without fungicide application.

It is assumed, that reduced disease control in successive passages on plants was due to a decrease in fungicide sensitivity of the pathogen populations. In order to examine, whether this development of resistance was a consistent one, the ninth transfer populations were inoculated onto plants for three further passages in the absence of any fungicide. The different fungal populations were then again exposed to the respective fungicides. The results are presented in table 4. They show that after an interruption of the fungicide selection pressure for three successive passages on plants, the fungicides again provided

better disease control compared with the values of passage 9.

Tab. 4. <u>S. nodorum</u>. Effect of an interruption in the selection pressure of fungi-
cides on the resistance level of the fungal populations. Leaf necrosis
as a percentage of the unsprayed control; average of 20 pots each con-
taining 7 plants.

Treatment	Passage			Data of passage 13 significantly different from passage	
	1	9^y	13^z	1	9
carbendazim	19.3	64.8	48.4	xxx	xxx
edifenphos	19.6	47.6	38.1	xxx	xx
alternate	19.3 (c)	37.9 (c)	26.2 (c)	xx	xx
mixture	8.1	14.6	10.9	-	xx

y : after 9 passages with fungicide selection pressure

z : after 3 passages without fungicide selection pressure

(c): plants sprayed with carbendazim in these passages

- : not significant; xx and xxx: significant at $P \leqslant 0.01$ and $P \leqslant 0.001$
respectively.

4.2.2. Experiments on agar media.

The effect of continuous and alternate treatments with two systemic fungi-
cides with a different mode of action, and a mixture of both, on the development
of fungicide resistance in <u>S. nodorum</u> was also examined <u>in vitro</u>. For this, a
sensitive strain of <u>S. nodorum</u> was cultured for nine successive passages on wheat
meal agar plates containing either carbendazim or edifenphos, or a mixture of both.
The concentration of the fungicides was kept constant for all passages.

The different variants were as follows:

1. <u>S. nodorum</u> in each passage cultured on plates with carbendazim (1.5 μg/ml a.i.)
2. <u>S. nodorum</u> in each passage cultured on plates with edifenphos (25 μg/ml a.i.)
3. <u>S. nodorum</u> from passage to passage alternately cultured on plates with carben-
dazim (1.5 μg/ml a.i.) or edifenphos (25 μg/ml a.i.), beginning with carbendazim.
4. <u>S. nodorum</u> in each passage cultured on plates with a mixture of carbendazim
(0.75 μg/ml a.i.) and edifenhos (12.5 μg/ml a.i.)
5. Plates without fungicide served as controls.

A 5 mm diameter agar disc of freshly grown mycelium, taken from the culture
of the previous passage of the same variant was placed in the middle of each
plate. In each passage, radial mycelial growth was measured after 12 days. The
increase in mycelial growth from passage to passage was employed as the crite-
rion for the development of fungicide resistance. The results are presented in
figure 3.
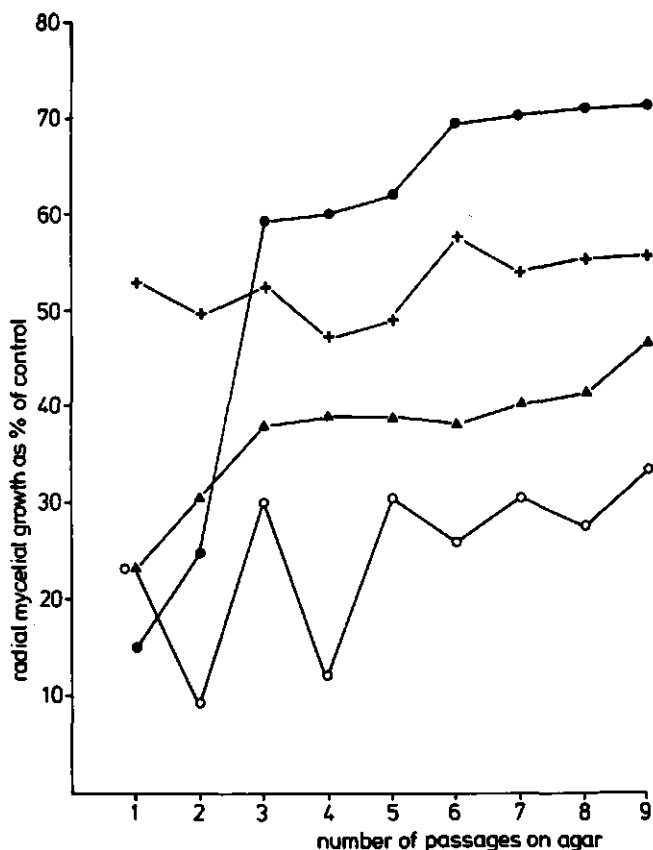
Fig. 3. S. nodorum. The effect of continuous and alternate exposure to carben-
        dazim and edifenphos and to a mixture of both on the mycelial growth
        on agar, determined during 9 successive passages. The data represent
        the radial mycelial growth after 12 days as a percentage of the control
        without fungicide (averages from 8 replicates).
        (▲———▲) carbendazim 1.5 μg/ml; (●———●) edifenphos 25 μg/ml;
        (O———O) alternately carbendazim 1.5 μg/ml and edifenphos 25 μg/ml;
        (+———+) mixture of carbendazim 0.75 μg/ml and edifenphos 12.5 μg/ml.

In order to determine whether there were differences between the values obtained,
the results were analysed statistically by means of the Wilcoxon's rank test.

The results are listed in table 5.

Tab. 5. S. nodorum. Statistical significancies, calculated from the data in figure 3 by means of the Wilcoxon's rank test, for differences in radial growth rate at different stages of the experiment. The data of each passage is compared with that of the first passage of each treatment.

	Passages							
Treatment	2	3	4	5	6	7	8	9
carbendazim	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
edifenphos	x	xxx	xxx	xxx	xxx	xxx	xxx	xxx
mixture	-	-	x^a	-	-	-	-	-
alternate	xxx^a	xxx	xxx^a	xxx	-	xx	-	xxx
carbendazim to alternate (b)		x		x		xx		xxx
edifenphos to alternate (b)	xx		xxx		xxx		xxx	

a : significant decrease

(b): within the same passage

- : not significantly different

x; xx; xxx : significant at $P \leq 0.05$; $P \leq 0.01$; $P \leq 0.001$ respectively

Within the limits of this system it may be concluded that S. nodorum was able to develop resistance to carbendazim and edifenphos in vitro when the pathogen was continuously exposed to the fungicides. This development of fungicide resistance was reflected by the increase in mycelial growth rate during successive passages (fig. 3). In contrast to the results from the experiments with living plants (cf. fig. 2), the resistance to edifenphos finally reached a higher level than to carbendazim. In the third passage of the treatment with continuous edifenphos exposure, a considerable increase in mycelial growth could be detected. This increase could be ascribed to the occurrence of many growth-sectors, which showed a better mycelial growth. It could be assumed that the resistance level of the S. nodorum population in these sectors was greater than that of the initial population. Agar discs from these sectors were used for the further passages. In the other treatments, sectors with a better mycelial growth did not occur.

Continuous treatment with either carbendazim or edifenphos resulted in significantly increased mycelial growth in the subsequent passages (tab. 5).

Alternate exposure to carbendazim and edifenphos considerably delayed the development of fungicide resistance. Only in those passages where carbendazim was added to the medium was a slight but significant increase in mycelial growth detected compared with the data of the first passage. In the passages of this variant where edifenphos was added, the mycelial growth rate was significantly smaller than after the same number passages within the continuous edifenphos variant. This was especially true for the second and fourth passages. Compared with the second passage there was a steady increase in growth until the eighth. It should be noted that the concentration of edifenphos in this medium was exactly the same as in the variant with continuous exposure to edifenphos. In the latter variant however, the growth rate was much higher than in the former, already from the first passage on. At first sight this would appear to incicate that pretreatment of S. nodorum with carbendazim increases its sensitivity to edifenphos.

After growing S. nodorum continuously on plates with a mixture of the two fungicides, a slight increase in mycelial growth rate could be detected in the last passages. However, when compared with the first passage, these differences were not statistically significant (tab. 5).

There were no significant differences between the populations with respect to their mycelial growth rate in the absence of the fungicides.

To examine to what extent the development of fungicide resistance was consistent, the populations of the ninth passage were cultured for three successive passages on agar plates without added fungicide and they were then again exposed to the respective fungicides. The results are listed in table 6. The results showed that an interruption in the selection pressure for three successive passages hardly influenced the resistance level of the different populations, subsequently reexposed to the fungicides. The selection pressure was, however, only relaxed for three passages after nine successive passages with fungicide treatment.

Tab. 6. **S. nodorum**. Effect of an interruption in the selection pressure by fungicides on the resistance level of the fungal populations. Mycelial growth rate after 12 days as a percentage of the untreated control; average of 8 replicates.

Treatment	Passage			Data of passage 13 significantly different from passage	
	1	9^y	13^z	1	9
carbendazim	23.8	50.9	46.2	xxx	-
edifenphos	15.6	74.8	69.4	xxx	-
alternate	23.8 (c)	35.6 (c)	30.8 (c)	xx	x
mixture	53.1	57.6	54.2	-	-

y : after 9 passages with fungicide selection pressure
z : after 3 passages without fungicide selection pressure
(c): cultured on plates with carbendazim
- : not significant
x; xx; xxx : significant at $P \leqslant 0.05$; $P \leqslant 0.01$ and $P \leqslant 0.001$ respectively.

4.3. Cercosporella herpotrichoides

Similar in vitro experiments were performed with Cercosporella herpotrichoides. The test fungicides were carbendazim and nuarimol. Investigations with living plants were not advisable for two reasons: the incubation time is unusually long, and assessment of the eyespot symptoms on greenhouse plants is rather inaccurate. The variants were as follows:

1. C. herpotrichoides in each passage cultured on plates with carbendazim (0.1 μg/ml a.i.)

2. C. herpotrichoides in each passage cultured on plates with nuarimol (2 μg/ml a.i.)

3. C. herpotrichoides from passage to passage alternately cultured on plates with carbendazim (0.1 μg/ml a.i.) or nuarimol (2 μg/ml a.i.) beginning with carbendazim.

4. C. herpotrichoides from passage to passage alternately cultured on plates with carbendazim (0.1 μg/ml a.i.) or nuarimol (2 μg/ml a.i.) beginning with nuarimol.

5. C. herpotrichoides in each passage cultured on plates with a mixture of carbendazim (0.05 μg/ml a.i.) and nuarimol (1 μg/ml a.i.)

6. Plates without fungicide served as controls.

A 5 mm diameter agar disc of freshly grown mycelium, taken from the culture of the previous passage was placed in the middle of each plate. As the criterion for the development of fungicide resistance, the increase of mycelial growth in

the presence of the fungicide from passage to passage was used. The results are presented in figure 4.
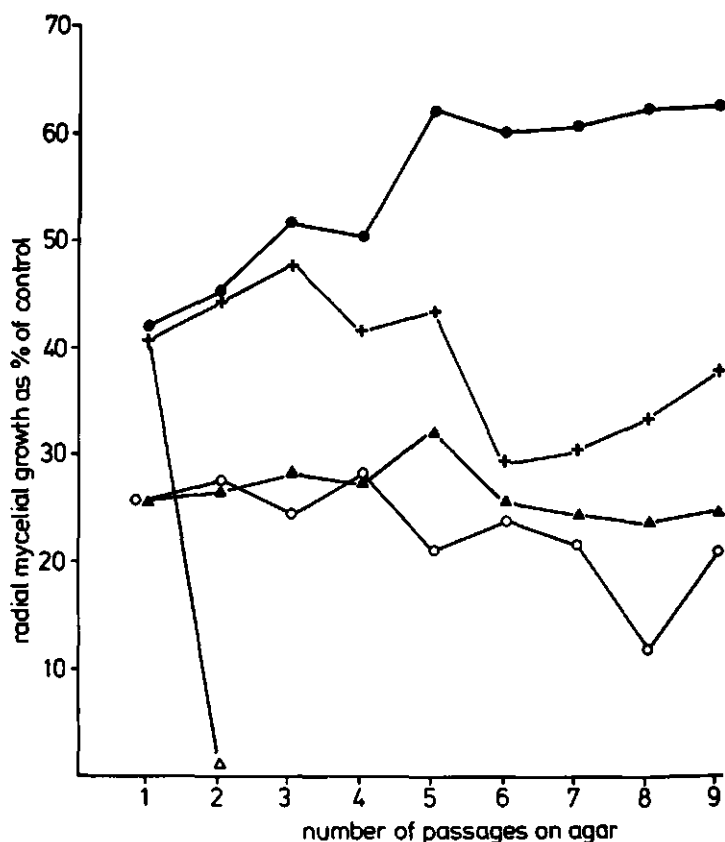
Fig. 4.   C. herpotrichoides. The effect of continuous and alternate exposure to carbendazim and nuarimol and to a mixture of both on the mycelial growth on agar, determined during 9 successive passages. The data represent the radial mycelial growth after 21 days as a percentage of the control without fungicide (averages from 16 replicates). (▲————▲) carbendazim 0.1 μg/ml; (●————●) nuarimol 2 μg/ml; (△————△) alternately carbendazim 0.1 μg/ml or nuarimol 2 μg/ml, beginning with nuarimol; (O————O) alternately carbendazim 0.1 μg/ml or nuarimol 2 μg/ml, beginning with carbendazim; (+————+) mixture of carbendazim 0.05 μg/ml and nuarimol 1 μg/ml.

In order to determine whether the values obtained were statistically different, the results were subjected to the Wilcoxon's rank test. The data are listed in table 7.

Tab. 7. C. herpotrichoides. Statistical significancies, calculated from the data in figure 4 by means of the Wilcoxon's rank test, for differences in radial growth rate at different stages of the experiment. The data of each passage is compared with that of the first passage of each treatment.

	Passages							
Treatment	2	3	4	5	6	7	8	9
carbendazim	-	x	-	xxx	-	-	-	-
nuarimol	xx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
mixture	x	xxx	-	-	xxx^a	xxx^a	xxx^a	-
alternate (b)	-	-	-	xxx^a	-	xxx^a	xxx^a	xxx^a
carbendazim to alternate (c)		-		xx		-		x
nuarimol to alternate (c)	xxx		xxx		xxx		xxx	

a : significant decrease

(b) : alternate treatment beginning with carbendazim

(c) : within the same passage

- : not significantly different

x; xx; xxx: significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively.

The finding that C. herpotrichoides was not able to develop resistance to carbendazim in these experiments, does not imply that the fungus has not the potential to do so. As will be shown in chapter 5, carbendazim resistant strains were present in field populations of the fungus.

Within the limits of the described system it may be concluded that C. herpotrichoides was able to develop resistance to nuarimol, when the pathogen was continuously exposed to the fungicide. This development of fungicide resistance was reflected by the increase in the mycelial growth rate during successive passages (fig. 4). This increase in mycelial growth was consistently significantly different from the values of the first passage (tab. 7).

In the variants with alternate exposure, several effects were apparent (fig. 4 and tab. 7). When C. herpotrichoides was first cultured on plates with nuarimol and subsequently on plates with carbendazim, hardly any mycelial growth could be detected. This phenomenon was also observed when other sensitive strains of the fungus were exposed to this alternate treatment with nuarimol and carben-

dazim. Alternating exposure to the two fungicides, beginning with carbendazim, led to a statistically significant decrease in the mycelial growth rate in the last passages; differences within this variant were, however, rather small. Here, a very interesting phenomenon was noticed. The fungicide concentrations in this treatment were exactly the same as in the treatments with continuous exposure to either one of the chemicals. Growth rates with continuous or alternating exposure were comparable for carbendazim but not so for nuarimol. Under continuous nuarimol stress relative growth rates were considerably higher than in case of nuarimol treatment in each second passage of this alternate treatment only.

When C. herpotrichoides was cultured on plates with a mixture of carbendazim and nuarimol, a significant increase in mycelial growth occurred in the first two passages. Later a decrease could be detected. After 9 passages, however, the growth rate did not differ significantly from that of the first passage (tab. 7).

Tab. 8. C. herpotrichoides. Effect of an interruption in the selection pressure of fungicides on the resistance level of the fungal populations. Mycelial growth rate after 21 days as a percentage of the untreated control; average of 16 replicates.

Treatment	Passage			Data of passage 13 significantly different from passage	
	1	9[y]	13[z]	1	9
carbendazim	25.9	24.9	25.8	−	−
nuarimol	41.7	62.4	61.4	xxx	−
alternate	25.9 (c)	21.2 (c)	24.1 (c)	−	x
mixture	40.9	38.3	37.8	−	−

y : after 9 passages with fungicide selection pressure

z : after 3 passages without fungicide selection pressure

(c): cultured on plates with carbendazim

− : not significant

x; xx; xxx : significant at $P \leqslant 0.05$ and $P \leqslant 0.001$ respectively.

To examine to what extent the described development of fungicide resistance was consistent, the different populations of the ninth passage were cultured for three further successive passages on agar plates without any fungicide added

and then were reexposed to the respective fungicides. The results are listed in table 8. The results demonstrate the stability of the acquired resistance to nuarimol. It should be pointed out, however, that the selection pressure had been relaxed only for three passages after nine successive passages with fungicide treatment.

4.4. Discussion

Experiments were carried out _in vitro_ and on the plant to determine whether development of fungicide resistance could be prevented or delayed by alternate or combined use of two systemic fungicides with a different mechanism of action.

Within the limits of the models used, it can be concluded, that <u>S. nodorum</u> was able to develop resistance to carbendazim and edifenphos after continuously being exposed to either fungicide. This tendency was counteracted by alternate or combined use of these two compounds.

In the _in vivo_ model with <u>S. nodorum</u> it was observed, that the level of resistance, acquired during 9 passages with different exposure to the fungicides carbendazim and edifenphos, significantly decreased after relaxation of the fungicide selection pressure for three passages. After these passages, however, the major part of the resistance was still present. The decrease of the resistance level after relaxation of the selection pressure might be explained in two ways. (i) The acquired resistance of the pathogen is not stable, but due to a reversible adaptation of the fungal population to either fungicide. This type of resistance, however, is not considered to be of importance for practice. (ii) There might be a decrease in the resistant population of the pathogen, due to the fact that the resistant individuals, on average, may be less competitive than the sensitive ones in absence of the fungicide. In that case, fungicide resistance might be a stable character, which could cause problems at a continuous selection pressure by one fungicide.

A decrease of the resistance level after relaxation of the fungicide selection pressure was hardly observed in the experiments on agar media.

The results obtained show, that the build-up of fungicide resistance in a pathogen population, on plants or on agar media, can be counteracted by alternate use of the two fungicides or by a mixture of them. As suggested earlier, the chance that a fungus will mutate for resistance to two different specific-site inhibitors, is far less than to one such inhibitor.

With <u>C. herpotrichoides</u>, the effect of the alternate exposure was very pronounced. Isolates of the fungus, first cultured on plates with nuarimol were

then hardly able to grow on plates with carbendazim. This reduction in growth was so complete, that no subcultures could be made to examine this effect over further passages. This effect was not observed when C. herpotrichoides was first exposed to carbendazim and subsequently to nuarimol and thereafter alternately to carbendazim and nuarimol. It is possible that an initial adaptation by the fungus to carbendazim inhibits the nuarimol-effect detected in the other treatment.

Another and permanent effect of carbendazim with respect to nuarimol does, however, exist. In those passages of the variant with alternate exposure- starting with carbendazim- where C. herpotrichoides was grown on plates contai- ning nuarimol, mycelial growth never reached the level of the first passage in the treatment with continuous nuarimol exposure. This could be explained by suggesting that carbendazim influences the fungal population in such a way that it becomes more sensitive to nuarimol. A synergism between the two fungicides might thus be present. Further experiments are required to determine whether the observed synergism between carbendazim and nuarimol in vitro is also operative under field conditions. Only carbendazim fungicides are available for the chemo- therapeutic control of the eyespot fungus in practice. The effect of nuarimol against C. herpotrichoides under field conditions is insufficient to control the disease (FEHRMANN, unpublished). This would appear to rule out the possibility of utilizing nuarimol with carbendazim, either as an alternate spray or as part of a fungicide mixture, to reduce the possible development of a fungicide resis- tant population of C. herpotrichoides. At present, resistance with the eyespot fungus in practice appears to be of little importance; this matter will be dis- cussed in detail later.

5. EFFECT OF FUNGICIDE APPLICATION ON THE INCIDENCE OF RESISTANT SPORES IN ISOLATES FROM THE FIELD.

5.1. Introduction

In field populations of plant pathogens, mutation for resistance to a particular fungicide may occur spontaneously. Without any selection pressure of the chemical, the frequency of resistant mutants will be extremely small- in the order of the natural mutation rate. Application of the fungicide concerned will selectively favour the growth and multiplication of these strains, due to the elimination or reduction of the sensitive individuals. Although the presence of resistant spores in a fungal population will not necessarily lead to an insufficient control of the disease, it is of interest to examine the resistance situation in the field: information concerning the presence and the relative proportion of fungicide resistant conidia in a fungal population may help to elucidate the consequences of fungicide application. This knowledge might be applied in the development of managing methods which can prevent or delay a rapid build-up of a resistant fungal population.

Monitoring for resistance is carried out by isolation of the fungus from diseased plant material. Assessment for the occurrence of resistance may be done in the following ways.

A. Subculturing of mycelium on fungicide-amended agar.

In order to test whether isolates are resistant to a particular fungicide, mycelium is transferred to agar plates, containing various concentrations of this compound. Growth of the isolate is compared with that of a standard sensitive strain. This technique has been used by most authors.

An advantage of this method is that the resistant strains can be kept in culture for further studies, e.g. on the stability of the acquired resistance, sporulation and pathogenicity. The procedure, however, does not allow any conclusions on the resistance situation in a fungal population in a quantitative way, but only in a qualitative one. Furthermore the method is laborious, such that the number of isolates tested will usually be limited and consequently the detection of a low incidence of resistance will be minimized. This may explain why CHIDAMBARAM and BRUEHL (1973) did not find any benomyl resistant strains of Cercosporella herpotrichoides within a field collection of 13 isolates. TATE et al. (1974) were unable to detect any fungicide resistant strains amongst 37 field isolates of Monilinia fructicola and 26 of M. laxa, and SMITH and SEARCY (1975)

were unsuccessful in finding resistant strains in 57 isolates of Cercospora arachidicola. WOLFE (1973), however, who examined more than one thousand single spore isolates of Erysiphe graminis on barley, was able to find a correlation between the intensity of ethirimol application in the field and the quantity of resistant strains.

B. Spore germination tests.

In spore germination tests, spore suspensions are mixed with the respective fungicide at different concentrations and subsequently incubated on glass slides or on agar plates. The criteria for fungicide resistance are the percentage of germinated spores and/or the length of the germ tubes, compared with those of sensitive standard strains. This method can only be applied if spores are available and if the fungicide concerned influences spore germination. It is, therefore, less frequently used than the former method. Examples may be found in the following publications: BENT et al., 1971, GILPATRICK and BLOWERS, 1974; WICKS, 1974, 1976; HOLLOMON, 1975; COOK and PEREIRA, 1976; KIEBACHER and HOFF-MANN, 1976; JONES and WALKER, 1976; TATE and SAMUELS, 1976; CHO, 1977; SZOLNIK and GILPATRICK, 1977.

On account of the fact that the percentage of germinated spores and the germ tube length are most frequently measured after 16 to 24 hours incubation, this method provides rapid information about the occurrence of resistant spores in a population. The accuracy of the test also enables small differences between resistant and sensitive spores to be detected. A disadvantage of the test is that only a small proportion of the total population is examined, since only a few hundred spores are counted. The probability of finding resistant conidia is therefore rather small, unless more than about 1% of the spores are resistant. Compared to the method first described, assessment of spore germination will provide most success in those cases where control of the disease is no longer effective. Another disadvantage of this method is that resistant spores present in the population can not be subcultured, if the test is done on glass slides. No further information can thus be obtained about the stability and the level of the acquired resistance.

C. Selection of resistant conidia from spore suspensions.

For conclusions on a quantitative basis, conidia from field isolates are mass produced in vitro, if the fungus is suitable for such a procedure. Highly

concentrated spore suspensions are spread over agar plates containing the fungicide at a concentration lethal for sensitive strains. This method is usually applied to determine the number of resistant spores in a fungal population after UV treatment (BEN-YEPHET et al., 1974; VAN TUYL, 1977). It has only been applied in a few cases to determine the relative proportion of resistant spores in a fungal population of a field isolate (ESURUOSO and WOOD, 1971; MACNEILL and SCHOOLEY, 1973; BRASIER and GIBBS, 1975; POLACH and MOLIN, 1975; MEYER, 1976; SHABI and BEN-YEPHET, 1976). An advantage of the method is that resistant spores can be detected even when they are present at a very low frequency in the fungal population. It is, therefore, very suited in those cases where a decreasing efficiency of the fungicide used is not yet visible. Furthermore, the resulting resistant colonies can be subcultured in order to compare them with sensitive strains, for characters such as pathogenicity and production of viable spores. If the number of resistant spores is expected to occur in the order of the natural mutation rate, i.e. smaller than $1:10^6$, then a huge quantity of spores has to be produced. These spores should be produced by a representative number of genetically heterogeneous isolates which are subcultured in vitro. This requirement makes the method quite laborious and not applicable to each fungus.

As pointed out earlier, for the control of Septoria nodorum and Cercosporella herpotrichoides, fungicides are mostly applied once or twice in a growth period. The purpose of the experiments to be described was to determine whether the percentage of resistant spores of both pathogens would increase under the influence of different levels of fungicide pressure on field grown crops and whether a correlation exists between the number of fungicide applications and the occurrence of resistant strains.

Spore suspensions of both pathogens, made from isolates originating from field plots which had received different fungicide sprays, were examined for the presence of resistant spores using the methods described previously under points B and C.

5.2. Septoria nodorum.

5.2.1. Spore germination tests.

To examine whether there was a correlation between different fungicide sprayings and the frequency of resistant strains, field plots of the winter wheat variety "Topfit" were sprayed with the systemic fungicides thiophanate methyl (350 g a.i./ha) and edifenphos (750 ml a.i./ha) in commercial prepara-

tions. The plots were approximately 20 m^2 in size. No artificial inoculation
with S. nodorum was carried out. Each fungicide was applied two or four times
at the following growth stages of the crop (LARGE, 1954).

    1. thiophanate methyl (2x):   9; 10.5.1.
    2. thiophanate methyl (4x):   9; 10.5; 10.5.1; 10.5.4.
    3. edifenphos (2x):   9; 10.5.1.
    4. edifenphos (4x):   9; 10.5; 10.5.1.; 10.5.4.
    5. unsprayed control

After ripening, ears were collected at random from the plots. S. nodorum
was reisolated from the kernels as described under 2.7.1. With these isolates,
spore germination tests were performed as described under 2.8.1.1. Eight isolates
were tested from each field plot. For each isolate, the percentage of germinated
spores and the length of the germ tubes were measured in three replicates of 100
spores, in the presence of carbendazim (1 μg/ml a.i.) and edifenphos (25 μg/ml
a.i.). The results are presented in figures 5 and 6.
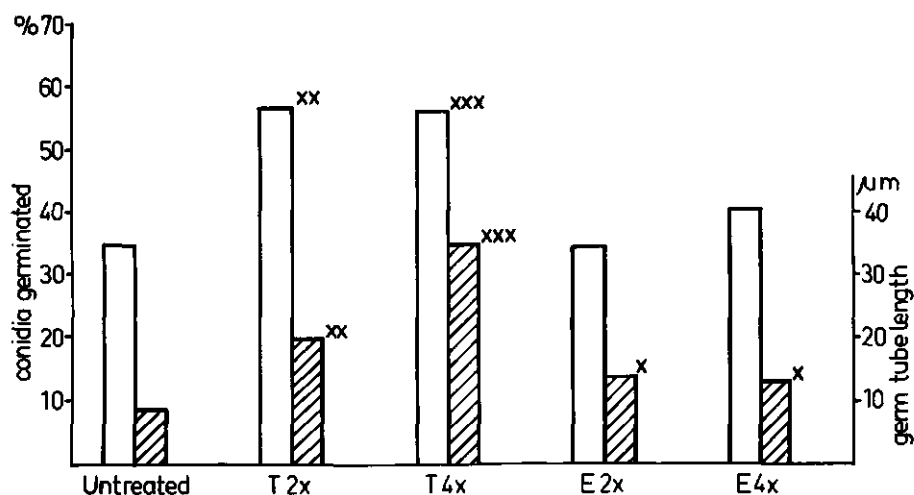
Fig. 5. S. nodorum. Percentage of germinated spores (☐) and germ tube
        length (▨) of isolates from field plots after two and four appli-
        cations of thiophanate methyl (T) or edifenphos (E), tested in the pre-
        sence of carbendazim (1 μg/ml a.i.). Winter wheat variety "Topfit", lo-
        cation Bursfelde, growth period 1973/1974. Average of three replicates
        for each of 8 isolates.
        x; xx; xxx: significant at $P \leqslant 0.05$; $P \leqslant 0.01$; $P \leqslant 0.001$ respectively.
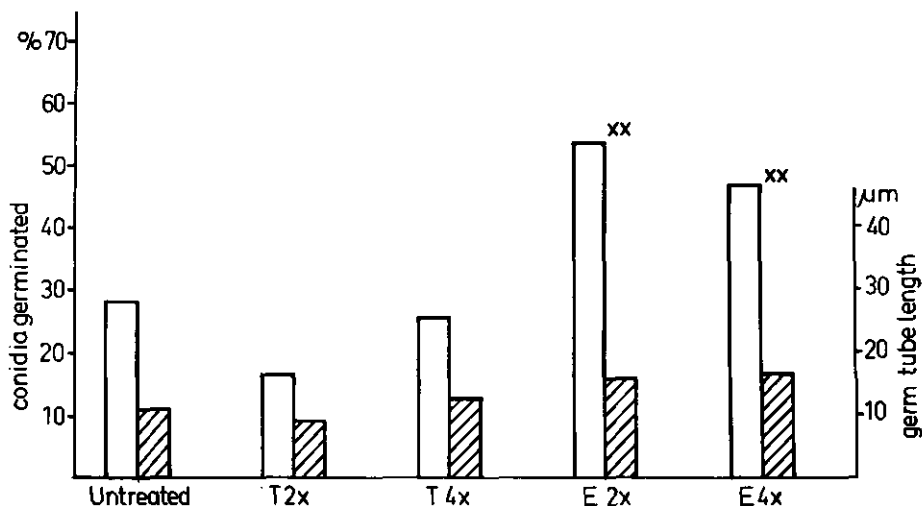
Fig. 6. S. nodorum. Percentage of germinated spores (☐) and germ tube
length (▨) of isolates from field plots after two and four
applications of thiophanate methyl (T) or edifenphos (E), tested in
the presence of edifenphos (25 μg/ml a.i.). Winter wheat variety "Topfit",
location Bursfelde, growth period 1973/1974. Average of three replicates
for each of 8 isolates.
xx: significant at $P \leqslant 0.01$.

In the presence of carbendazim, the percentage of germinated spores, and
their germ tube length, of isolates from field plots sprayed two or four times
with thiophanate methyl was significantly increased compared with isolates from
unsprayed plots (fig. 5). Spraying the plots two or four times did not influence
the percentage of spores germinated at 1 μg/ml carbendazim. There were no diffe-
rences between isolates from edifenphos sprayed plots and those from unsprayed
plots, with respect to their spore germination in the presence of carbendazim.
On average, germ tubes of isolates from edifenphos treated plots were slightly
longer than those from untreated plots.

If field plots had been sprayed two or four times with edifenphos, isolates
from these plots exhibited a significant increase in the percentage of germinated
spores in the presence of edifenphos, when compared with those from untreated
plots, but not in case of germ tube elongation. Differences between isolates
from thiophanate methyl sprayed plots and those from unsprayed plots were not
noticed when spores germinated in the presence of edifenphos (figure 6).

Summarizing it might be concluded from these results that the resistance level of the different S. nodorum populations, expressed as the ability of their spores to germinate at certain fungicide concentrations, was enhanced by field application of either thiophanate methyl or edifenphos. There was no indication that cross-resistance exists between carbendazim and edifenphos. This was not likely to be the case since both fungicides have a different mode of action.

The stability of the acquired resistance was not examined. It could, therefore, be due to reversible adaptation, or a genetic mutation to resistance to either fungicide. Further tests were carried out to obtain more information about the influence of fungicide application in cereals on the frequency of resistant strains.

5.2.2. Selection of resistant conidia from spore suspensions

Isolates of S. nodorum which originated from field plots with different fungicide treatments, were examined for the presence of resistant strains by spreading highly concentrated spore suspensions over agar plates which contained a lethal concentration of carbendazim or edifenphos.

Field plots, approximately 20 m^2 in size, were sprayed with thiophanate methyl (350 g a.i./ha) or edifenphos (750 ml a.i./ha) at the following growth stages (LARGE, 1954).

Location Bursfelde, winter wheat variety "Topfit"

1973	1. unsprayed control
	2. thiophanate methyl (4x): 9;10.1;10.5;10.5.4
1974, 1975, 1976 and 1977	1. unsprayed control
	2. thiophanate methyl (2x): 9;10.5.1.
	3. thiophanate methyl (4x): 9;10.5;10.5.1;10.5.4.
	4. edifenphos (2x) : 9;10.5.1.
	5. edifenphos (4x) : 9;10.5;10.5.1;10.5.4
1977, moreover	6. thiophanate methyl : 9
	+ edifenphos : 10.5

Location Hilwartshausen, winter wheat variety "Lapis".

1974 and 1975	1. unsprayed control
	2. thiophanate methyl (2x): 9;10.5.1
	3. thiophanate methyl (4x): 9;10.5;10.5.1;10.5.4.

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4. edifenphos (2x)       : 9;10.5.1.
5. edifenphos (4x)       : 9;10.5;10.5.1;10.5.4
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No artificial inoculation with S. nodorum was carried out. After ripening
ears were collected at random from the plots. The fungus was isolated from the
kernels as described under 2.7.1. After subculturing the isolates on agar, to
produce a sufficient number of spores, the pycnidiospore suspensions were
spread on the surface of agar plates as described under 2.8.1.2. On wheat
meal decoct agar containing carbendazim at a concentration of $5 \mu g$/ml a.i.,
two types of carbendazim resistant strains were observed: weakly carbendazim
resistant strains, showing only some mycelial growth, and highly carbendazim
resistant strains, showing a much more pronounced growth of mycelium with
sporulating pycnidia (see figure 7).
Characteristics of these strains are presented in chapter 6.

The frequencies of weakly carbendazim resistant spores in spore suspensions
of field isolates after field treatment with thiophanate methyl, are listed in
table 9. Within each year, spraying with thiophanate methyl raised the frequency
of weakly carbendazim resistant spores. The differences between isolates from
unsprayed and sprayed plots were, in all years except 1976, significant. The
extremely dry conditions in summer 1976 resulted in a low S. nodorum infection.
Only a few strains could be isolated from sprayed plots, so that no reliable
statistical analysis was possible. The results in figure 8 show that the increase
in the relative proportion of resistant spores after fungicide treatment obviously
was due to a gradual shift in the total population towards a higher frequency of
resistant spores.
The tendency for an increase in the frequency of weakly carbendazim resistant
conidia in the populations after two or four applications of thiophanate methyl
is clearly demonstrated. It should be noted, however, that even four thiophanate
methyl sprayingsdid not lead to any population with more than 10^{-4} resistant
spores.

With respect to the relative proportion of resistant conidia, whether the
crop was sprayed two or four times with thiophanate methyl had no influence on
theresult (tab. 9). Alternate sprayings with thiophanate methyl and edifenphos,
which were applied in 1977, did not significantly change the frequency of resis-
tant spores, compared with the results from unsprayed plots, but they did cause
significant differences compared with plots sprayed only with thiophanate methyl.
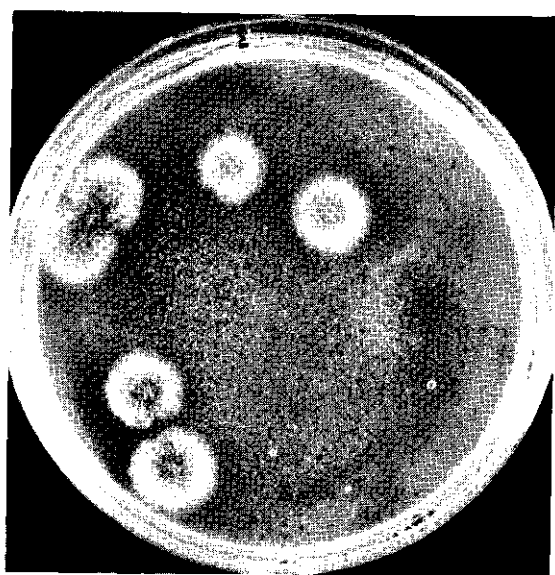
Fig. 7.   S. nodorum. Growth habit of weakly carbendazim (top) and highly carben-
dazim (bottom) resistant colonies after spreading spore suspensions on
agar plates containing carbendazim (5μg/ml a.i.). Growth after 10
days at 20°C.

Tab. 9. S. nodorum
Frequency of weakly carbendazim resistant spores in spore suspensions of
field isolates and the percentage of isolates yielding resistant spores
after two and four field applications of thiophanate methyl. Winter wheat
variety "Topfit", location Bursfelde, growth periods 1973, 1974, 1975,
1976 and 1977.

Fungicide treatment	Number of isolates	Total number of spores x 10^6	Number of resistant spores	Frequency	percentage of isolates with resistant spores
1973					
unsprayed	30	3944	504	$1{:}78{\times}10^5$	53
thiophanate methyl 4x	29	3440	1806	$1{:}19{\times}10^{5\,xxxx}$	89
1974					
unsprayed	10	1143	248	$1{:}46{\times}10^5$	60
thiophanate methyl 2x	9	2052	1206	$1{:}17{\times}10^{5\,xx}$	100
thiophanate methyl 4x	10	1736	996	$1{:}17{\times}10^{5\,x}$	100
1975					
unsprayed	33	1992	783	$1{:}25{\times}10^5$	82
thiophanate methyl 2x	28	2662	6413	$1{:}\,4{\times}10^{5\,xxx}$	94
thiophanate methyl 4x	22	1594	5635	$1{:}\,3{\times}10^{5\,xxx}$	91
1976					
unsprayed	17	2278	842	$1{:}27{\times}10^5$	88
thiophanate methyl 2x	4	464	338	$1{:}14{\times}10^5$ −	100
thiophanate methyl 4x	5	738	739	$1{:}10{\times}10^5$ −	100
1977					
unsprayed	16	875	59	$1{:}150{\times}10^5$	43
thiophanate methyl 2x	15	3039	787	$1{:}\,38{\times}10^5$ x	73
thiophanate methyl 4x	13	1256	526	$1{:}\,24{\times}10^5$ xx	77
alternate	12	961	90	$1{:}107{\times}10^5$ −	42

Wilcoxon's rank test for differences between sprayed and unsprayed plots within
each year: − not significantly different; x: significant at $0.025 \leqq P \leqq 0.10$;
xx: significant at $0.010 \leqq P \leqq 0.025$; xxx: significant at $0.005 \leqq P \leqq 0.010$;
xxxx: significant at $P \leqq 0.005$.
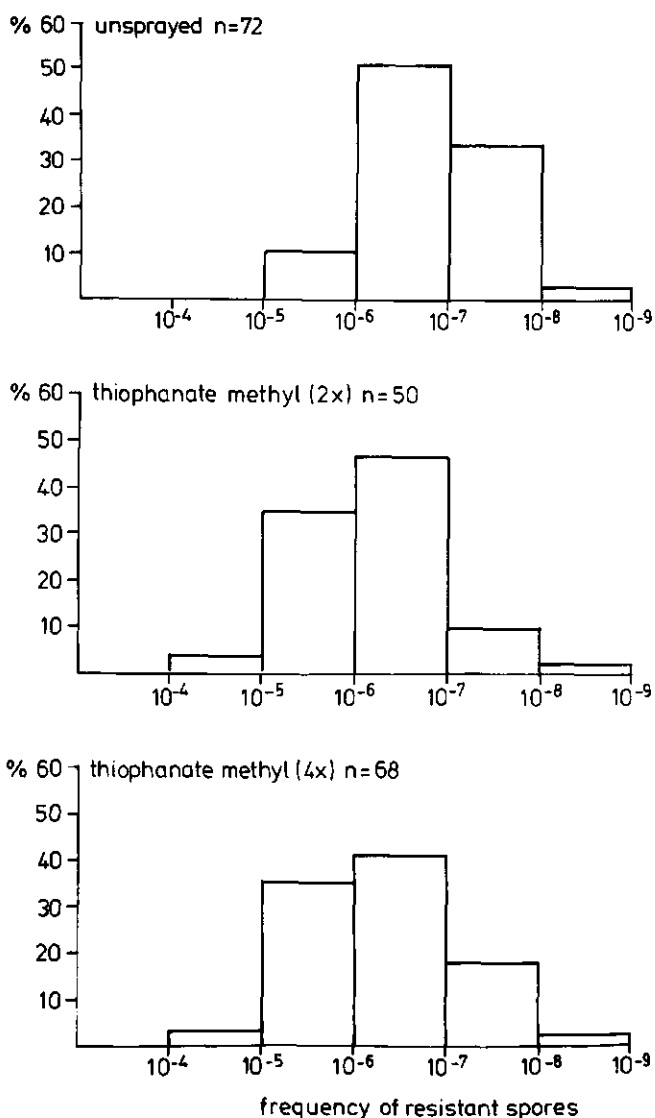
Fig. 8. S. nodorum. Frequency distribution of weakly carbendazim resistant spores in isolates from untreated field plots (top) or from plots sprayed two (middle) or four times (bottom) with thiophanate methyl. Winter wheat variety "Topfit", location Bursfelde. Summarized data of the growth periods 1973, 1974, 1975, 1976 and 1977. n = number of isolates.

It should be noted, that the frequency of resistant spores in unsprayed plots increased from 1973 until 1976. The differences between the values of 1973 and those of 1974, 1975 and 1976 were significant at $0.005 \leqslant P \leqslant 0.010$. The same tendency was also observed in the results from sprayed plots. This effect might be ascribed to an overall use of carbendazim fungicides in this region which conferred an extra selection pressure on the total S. nodorum population.

The 1977 values were in all cases lower than those of the preceding years. This might be due to the fact that, in this year, the field plots were approximately 5 km distant from those of the previous years. It is possible that another resistance level in the total S. nodorum population was present at this location.

Field application of thiophanate methyl influenced not only the frequency of resistant spores, but also the percentage of isolates yielding resistant spores (see table 9). The increase in the frequency of resistant spores in isolates from unsprayed plots between 1973 and 1976 was associated with a gradual increase in the percentage of isolates which yielded resistant spores. An application of thiophanate methyl followed by one of edifenphos had no influence on the percentage of such isolates.

The results obtained with the isolates from the winter wheat variety "Lapis" at the Hilwartshausen location were in good agreement with those from Bursfelde (table 10). In contrast to the Bursfelde results with the isolates from unsprayed plots, there was no significant yearly increase in neither the frequency of resistant spores nor the percentage of isolates which yielded re-sistant spores.

In addition to weakly carbendazim resistant spores, highly carbendazim resistant conidia were also present in subcultured field isolates. Their frequency, however, was too small for any reliable information about the influ-ence of field application of thiophanate methyl on the yearly incidence of these strains. Therefore, the results of the different years were summarized and pre-sented in table 11 (Bursfelde) and table 12 (Hilwartshausen).

Strains highly resistant to carbendazim were able to grow and produce spores even at a concentration of $1000 \mu g/ml$ a.i. carbendazim. The characteris-tics of these strains are given in chapter 6.

Tab. 10. S. nodorum. Frequency of weakly carbendazim resistant spores in spore suspensions of field isolates and the percentage of isolates yielding resistant spores after two and four field applications of thiophanate methyl. Winter wheat variety "Lapis", location Hilwartshausen, growth periods 1974 and 1975.

Fungicide treatment	Number of isolates	Total number of spores $\times 10^6$	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
1974					
unsprayed	20	2751	240	$1:114 \times 10^5$	60
thiophanate methyl 2x	27	6650	1868	$1: 35 \times 10^5$ x	82
thiophanate methyl 4x	25	5554	1562	$1: 35 \times 10^5$ -	72
1975					
unsprayed	35	4574	528	$1: 86 \times 10^5$	54
thiophanate methyl 2x	17	2070	2966	$1: 7 \times 10^5$ xxxx	88
thiophanate methyl 4x	28	5251	5779	$1: 9 \times 10^5$ xxxx	89

Wilcoxon's rank test for differences between unsprayed and sprayed plots within each year: - not significantly different; x: significant at $0.025 \leqslant P \leqslant 0.10$; xxxx: significant at $P \leqslant 0.005$.

Tab. 11. S. nodorum. Frequency of highly carbendazim resistant spores in spore suspension of field isolates and the percentage of isolates yielding resistant spores after two and four field applications of thiophanate methyl. Winter wheat variety "Topfit", location Bursfelde. Summarized data of the growth periods 1973, 1974, 1975, 1976 and 1977.

Fungicide treatment	Number of isolates	Total number of spores $\times 10^6$	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
unsprayed	106	10232	7	$1:14.6 \times 10^8$	6
thiophanate methyl 2x	56	8217	94	$1: 0.9 \times 10^8$	20
thiophanate methyl 4x	79	8764	100	$1: 0.9 \times 10^8$	15

Tab. 12. S. nodorum. Frequency of highly carbendazim resistant spores in spore
suspensions of field isolates and the percentage of isolates yielding
resistant spores after two and four field applications of
thiophanate methyl. Winter wheat variety "Lapis", location Hilwarts-
hausen. Summarized data of the growth periods 1974 and 1975.

Fungicide treatment	Number of isolates	Total number of spores x 10^6	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
Unsprayed	55	7325	6	1:12.2x10^8	9
thiophanate methyl 2x	45	8720	34	1: 2.6x10^8	16
thiophanate methyl 4x	53	10805	32	1: 3.4x10^8	15

When the data of the different years were summarized, it appeared that
spraying a wheat crop with thiophanate methyl influenced the incidence of highly
carbendazim resistant strains in two different ways. Firstly, thiophanate methyl
spraying enhanced the frequency of highly carbendazim resistant strains, compared
with the data from unsprayed plots. Secondly, the percentage of isolates yielding
resistant spores was increased.

S. nodorum isolates from unsprayed and edifenphos sprayed plots (cf. 2.7.1.)
were examined for the presence of edifenphos resistant spores. Highly concentrated
spore suspensions were spread over the surface of wheat meal decoct agar, con-
taining edifenphos at a concentration of $200 \mu g$/ml a.i., according to the methods
described under 2.8.1.2. Resistant colonies developed within 10 days and showed
a growth habit rather similar to that of weakly carbendazim resistant strains
(figure 9).
    Detailed characterization of the edifenphos resistant strains is presented
in chapter 6.
    The frequencies of edifenphos resistant conidia in spore suspensions of
field isolates after field applications of edifenphos, are listed in table
13 (Bursfelde) and table 14 (Hilwartshausen).
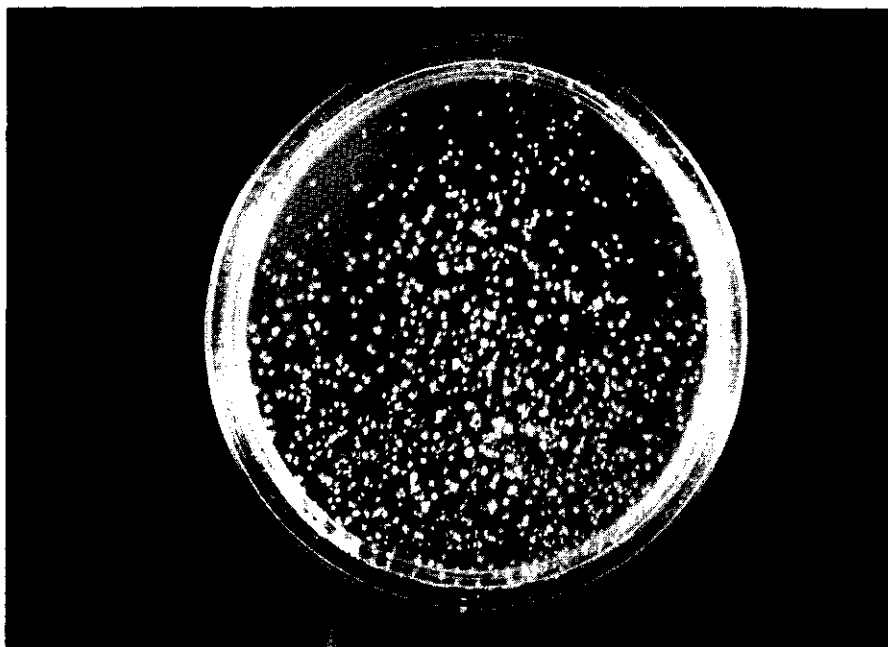
Fig. 9. <u>S. nodorum</u>. Growth habit of edifenphos resistant strains after spreading spore suspensions on agar plates which contained edifenphos (200μg/ml a.i.). Growth after 10 days at 20°C.

The results in table 13 show that, within each year, the frequency of edifenphos resistant conidia increased after field applications of edifenphos. In most cases, this increase was highly significant and could be attributed to a gradual shift in the total population towards a higher relative proportion of resistant spores (cf. figure 10). There were no differences between the results of isolates from plots sprayed two and four times with edifenphos. Alternate spraying with thiophanate methyl and edifenphos, carried out in 1977, did not significantly change the relative proportion of edifenphos resistant spores, neither compared with unsprayed nor with other sprayed plots, although the frequency appeared to be lower than that from other plots.

Field applications of edifenphos influenced both the frequency of edifenphos resistant spores and also the percentage of isolates which produced resistant spores. Table 13 shows that nearly all isolates from sprayed plots yielded resistant spores, whereas about 75% of the isolates from unsprayed plots did so. With the isolates from unsprayed plots, there was no yearly increase in the percentage of isolates which yielded resistant spores.

Tab. 13. S. nodorum. Frequency of edifenphos resistant spores in spore suspen-
sions of field isolates and the percentage of isolates yielding resis-
tant spores after two and four field applications of edifenphos. Winter
wheat variety "Topfit", location Bursfelde. Growth periods 1974, 1975,
1976 and 1977.

Fungicide treatment	Number of isolates	Total number of spores x 10^6	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
1974					
unsprayed	10	1143	96	1:119x10^5	70
edifenphos 2x	10	1781	766	1: 23x10^5 xxxx	100
edifenphos 4x	10	2502	1025	1: 24x10^5 xxxx	100
1975					
unsprayed	32	2358	192	1:123x10^5	75
edifenphos 2x	28	2955	1949	1: 15x10^5 xxxx	93
edifenphos 4x	30	3023	1540	1: 19x10^5 xxx	93
1976					
unsprayed	14	506	62	1: 82x10^5	78
1977					
unsprayed	16	791	119	1: 66x10^5	75
edifenphos 2x	17	1111	592	1: 18x10^5 -	93
edifenphos 4x	7	686	346	1: 20x10^5 xx	100
alternate	12	944	99	1: 95x10^5 -	83

Wilcoxon'x rank test for differences between sprayed and unsprayed plots
within each year: - not significantly different; xx: significant at $0.10 \leq P \leq 0.025$;
xxx: significant at $0.005 \leq P \leq 0.010$; xxxx: significant at $P \leq 0.005$.

Tab. 14.   <u>S. nodorum</u>. Frequency of edifenphos resistant spores in spore
           suspensions of field isolates and the percentage of isolates
           yielding resistant spores after two and four field applications
           of edifenphos. Winter wheat variety "Lapis", location Hilwartshausen.
           Growth periods 1974 and 1975.

Fungicide treatment	Number of isolates	Total number of spores $\times 10^6$	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
1974					
unsprayed	20	3228	189	$1:171 \times 10^5$	55
edifenphos 2x	13	2397	2055	$1: 12 \times 10^5$ xx	85
edifenphos 4x	12	1968	1552	$1: 13 \times 10^5$ x	83
1975					
unsprayed	33	3948	237	$1:166 \times 10^5$	58
edifenphos 2x	27	4115	3091	$1: 13 \times 10^5$ xxxx	85
edifenphos 4x	31	4385	2939	$1: 15 \times 10^5$ xxxx	87

Wilcoxon's rank test for differences between unsprayed and sprayed plots within
each year: x: significant at $0.025 \le P \le 0.05$; xx: significant at $0.010 \le P \le 0.025$;
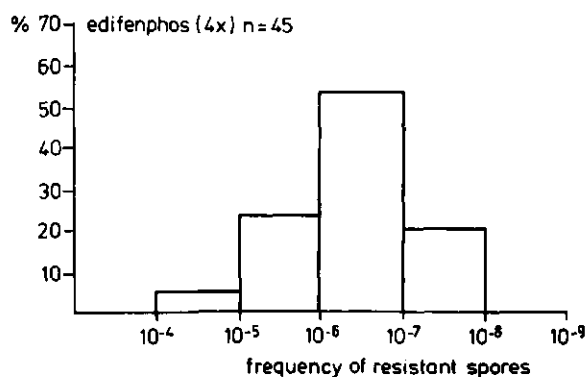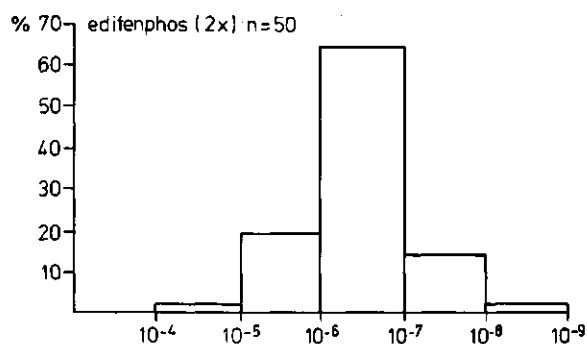xxxx: significant at $P \le 0.005$.
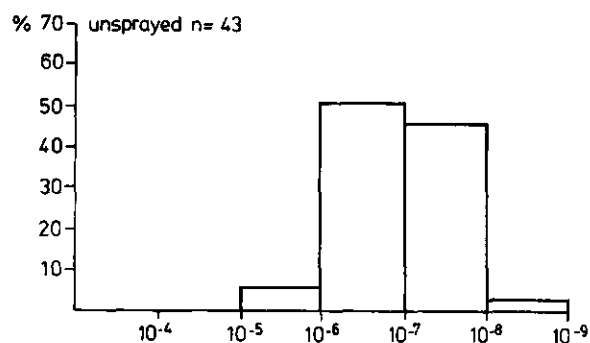
Fig. 10. S. nodorum. Frequency distribution of edifenphos resistant spores in isolates from untreated field plots (top) or from plots sprayed two (middle) or four times (bottom) with edifenphos. Winter wheat variety "Topfit", location Bursfelde. Summarized data of the growth periods 1974, 1975, 1976 and 1977.
n = number of isolates.

There was a good agreement between the results from Hilwartshausen, where the variety "Lapis" was grown, and those from Bursfelde, where the variety "Topfit" was grown.

The tests used have the disadvantage in that they only give information about the presence of resistant conidia in spore suspensions from in vitro subcultured isolates. This subculturing was necessary in order to obtain a sufficient number of spores. It can not be concluded, therefore, that resistant strains will occur in the different field populations at exactly the same frequency as in the in vitro subcultured populations. For this reason tests were carried out to examine the effect of subculturing the isolates. Mono-spore isolates of S. nodorum were subcultured in the same way as described for the normal field isolates. Subsequently the spore suspensions were pipetted onto the surface of agar plates which contained carbendazim or edifenphos as previous-ly described. Weakly carbendazim resistant spores were present in 25% of the isolates at a frequency of $1:370 \times 10^5$ spores; highly carbendazim resistant spores were not found. Edifenphos resistant spores were present in 27% of the isolates at a relative proportion of $1:340 \times 10^5$ spores. The incidence of resistant spores in these tests might be ascribed to spontaneous mutations occurring during the in vitro cultures. As the frequencies found were rather low, it could be concluded that subculturing has only a minor influence on the frequency of resistant spores. The influence on the percentage of isolates yielding resistant spores might be greater, since subculturing mono-spore cultures resulted in the presence of re-sistant spores in approximately 25% of the isolates tested. In spite of this, the differences between the respective populations may stay the same, but at a some-what lower level.

By using only those spores which are formed on diseased plant material the problem of subculturing in vitro would be avoided. With S. nodorum, wheat plants were sprayed two times with thiophanate methyl at growth stages 8 and 9. Two weeks after the last spraying, leaves were harvested, surface disinfected and the spores, which later formed on the leaves were tested for carbendazim resis-tance in the same way as described before. Although with this method information might be obtained about the incidence of resistant spores in the field, it proved to be less suitable due to the fact that the number of spores formed on the leaves was too small to detect differences between populations from unsprayed and sprayed plots.

Tab. 15. S. nodorum. Effect of field applications of thiophanate methyl and
edifenphos on the incidence of S. nodorum in kernels from the
treated plots. Winter wheat variety "Topfit", location Bursfelde.
Growth periods 1973, 1974, 1975, 1976 and 1977.
n = number of successful isolations per 1000 kernels.

Fungicide treatment	1973		1974		1975		1976		1977	
	n	%	n	%	n	%	n	%	n	%
unsprayed	178	100	213	100	120	100	12	100	128	100
thiophanate methyl 2x	–	–	159	75	109	91	6	50	59	46
thiophanate methyl 4x	72	40	109	51	122	102	6	50	54	42
edifenphos 2x	–	–	130	61	108	90	0	0	46	36
edifenphos 4x	–	–	107	50	89	74	0	0	35	27
alternate	–	–	–	–	–	–	–	–	24	19

Tab. 16. S. nodorum. Effect of field application of thiophanate methyl and
edifenphos on the incidence of S. nodorum in kernels from the
treated plots. Winter wheat variety "Lapis", location Hilwartshausen.
Growth periods 1974 and 1975.
n = number of successful isolations per 1000 kernels.

Fungicide treatment	1974		1975	
	n	%	n	%
unsprayed	195	100	127	100
thiophanate methyl 2x	103	53	95	75
thiophanate methyl 4x	109	56	88	70
edifenphos 2x	125	64	98	77
edifenphos 4x	73	37	70	55

54

As shown earlier, field applications of thiophanate methyl or edifenphos effected a significant increase in the percentage of specifically resistant spores. The absolute figures, however, remained rather small, since no isolates were found with more than $1:10^4$ resistant spores. It seemed of interest to examine whether the limited increase in the relative proportion of both carbendazim and edifenphos resistant spores, after field sprayings with thiophanate methyl and edifenphos respectively, influenced the effectiveness of either fungicide. For this purpose, 1000 randomly selected kernels from each field plot were placed on wheat meal agar plates, according to the method described under 2.7.1. After 10 days at 20^oC, the resulting isolates of S. nodorum were counted. The results are listed in tables 15 and 16.

If the successful isolation of S. nodorum from kernels is taken as a criterion for the effectiveness of thiophanate methyl or edifenphos applications in the field, it might be concluded from the results in table 15 that there was a pronounced tendency for a decrease in fungicide effectiveness in 1975 only (table 15). Best results were obtained if thiophanate methyl and edifenphos were applied alternately. Data of this treatment are available only from 1977. In this case, the incidence of the fungus was approximately 80% lower than in untreated plots (table 15).

The results from Hilwartshausen (table 16), where the field plots were treated for only two seasons, showed the same tendency.

It should be pointed out, that the figures represent the percentage of infected kernels. No conclusion can be drawn on the extent of presence of S. nodorum in the individual kernel.

5.3. Cercosporella herpotrichoides.

To examine the effect of field applications of carbendazim on the incidence of resistant strains of C. herpotrichoides, field plots, approximately 100 m^2 in size, of the winter barley variety "Vogelsanger Gold" were sprayed with carbendazim (180 g a.i./ha) at growth stage 8. At the milky-rips stage (10.11.1), plants were randomly sampled from the plots. The pathogen was isolated from haulm segments with characteristic eyespot symptoms, according to the method described under 2.7.2. After mass production of conidia of the different isolates on wheat meal agar, spore suspensions were spread on the surface of agar plates which contained carbendazim (3μg/ml a.i.), as described under 2.8.2. After four weeks incubation at 20^oC in the dark, carbendazim resistant colonies were visible as a profuse growth of greyish mycelium. Detailed characterization of these strains is given in chapter 6.

The frequencies of carbendazim resistant conidia in spore suspensions of field isolates after application of carbendazim are presented in table 17.

Tab. 17. C. herpotrichoides. Frequency of carbendazim resistant spores in spore suspensions of field isolates and the percentage of isolates yielding resistant spores after one field application of carbendazim. Winter barley variety "Vogelsanger Gold", location Bursfelde. Growth periods 1975, 1976 and 1977.

Fungicide treatment	Number of isolates	Total number of spores x 10^6	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
1975					
unsprayed	40	8647	11	$1:78x10^7$	12
carbendazim	40	7941	10	$1:79x10^7$ -	17
1976					
unsprayed	34	16171	23	$1:70x10^7$	12
carbendazim	46	23493	40	$1:58x10^7$ -	46
1977					
unsprayed	19	5087	20	$1:25x10^7$	21
carbendazim	18	4712	39	$1:12x10^7$ xxx	67

Wilcoxon's rank test for differences between unsprayed and sprayed plots within each year: -: not significantly different; xxx: significant at $0.005 \leq P \leq 0.010$.

The results show that field application of carbendazim had only a slight influence on the frequency of carbendazim resistant spores in field isolates of C. herpotrichoides. Only in 1977 was there a significant increase in their frequency after fungicide application, but it remained rather low. A more pronounced effect was apparent in the percentage of isolates yielding resistant spores, which was largely enhanced after carbendazim treatment in 1976 and 1977.

In a separate field trial at Münster, half of a rye field (total area approximately 1 ha) received a yearly application of thiophanate methyl to control eyespot, over four successive seasons; the reminder of the field was not

sprayed. At the end of the fourth season, rye stubble was collected and examined for the presence of carbendazim resistant strains of C. herpotrichoides. The methods used were the same as those already described. The results are listed in table 18.

Tab. 18.   C. herpotrichoides. Frequency of carbendazim resistant spores in spore suspensions of field isolates and the percentage of isolates yielding resistant spores after one field application of thiophanate methyl. Rye variety "Kustro", location Münster. Rye was grown on the same field for four successive years, in which half of that field received a yearly application of thiophanate methyl; the remainder of the field was not sprayed. Samples were drawn in 1977.

Fungicide treatment	Number of isolates	Total number of spores x 10^6	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
unsprayed	30	4353	5	$1:87 \times 10^7$	13
thiophanate methyl	29	3987	25	$1:16 \times 10^7$ x	35

Wilcoxon's rank test for differences between the unsprayed and the sprayed plot: x: significant at $0.025 \leqslant P \leqslant 0.05$.

After four yearly applications of thiophanate methyl, the frequency of carbendazim resistant spores was approximately 5 to 6 times greater than that found in the untreated control. The absolute figures were, however, so small that no economic significance could be attached to this finding.

To supplement the results found in the trial at Münster, a second field trial south of Hannover was begun in spring 1976. Here wheat will be grown continuously for five years and during this period one half of the plot (approximately 0.5 ha in size and square in format) will be sprayed twice in each season with carbendazim, in 1976, however, the plot received only one application of the fungicide. The remainder of the plot will be maintained as a non-sprayed control. Isolates were tested as previously described. The results are listed in table 19. The data from the first two seasons essentially confirm the conclusions drawn from table 18. The comparatively small selection pressure of carbendazim will favour some increase in the resistant strains, but only on an insignificant scale.

The follow-up of these experiments will show, what will happen when carbendazim is applied year after year during a longer period.

Tab. 19. C. herpotrichoides. Frequency of carbendazim resistant spores in spore suspensions of field isolates and the percentage of isolates yielding resistant spores after one field application of carbendazim in early May 1976 and of two field applications of carbendazim in early May and late June 1977. Winter wheat variety "Saturn", location Hannover. Wheat was grown on the same field. One half of the 1 ha field was sprayed, the other half remained unsprayed. Stubble samples were drawn in August 1976 and 1977.

Fungicide treatment	Number of isolates	Total number of spores x 10^6	Number of resistant spores	Frequency	Percentage of isolates with resistant spores
1976					
unsprayed	30	16623	7	1:237x10^7	10
carbendazim	29	9670	32	1: 30x10^7 xxx	55
1977					
unsprayed	30	7613	10	1: 76x10^7	20
carbendazim	30	7385	32	1: 23x10^7 x	47

Wilcoxon's rank test for differences between the sprayed and the unsprayed plot within each year: x: significant at $0.025 \leq P \leq 0.05$; xxx: significant at $0.005 \leq P \leq 0.010$.

5.4. Discussion

The effect of applications of thiophanate methyl and edifenphos on the development of fungicide resistance in field populations of S. nodorum was investigated. Similar experiments were carried out with carbendazim and C. herpotrichoides.

Spore germination tests with S. nodorum showed that two or four field applications of thiophanate methyl or edifenphos caused a significant increase in the percentage of spores, able to germinate at the tested fungicide concentration, compared with spores of isolates from unsprayed plots. The level of this increase was of a magnitude similar to that which has often been found after fungicide application (cf. JONES and WALKER, 1976; KIEBACHER and HOFFMANN, 1976; TATE and SAMUELS, 1976).

The experiments showed that in S. nodorum two types of carbendazim resistant strains were present. Firstly, weakly carbendazim resistant strains, occurring at a higher frequency, which were inhibited at concentrations of $5 \mu g/ml$ carbendazim, and secondly, highly carbendazim resistant strains, occurring at a much lower frequency, which were hardly inhibited at concentrations up to $1000 \mu g/ml$ carbendazim. Although there was some variation in carbendazim resistance within each group, there were no intermediates between both types (cf. chapter 6).

The presence of two types of resistance to a fungicide has also been reported for other fungi. GEORGOPOULOS and DOVAS (1973) found two levels of benomyl resistance in Cercospora beticola, some isolates being inhibited at a concentration of $5 \mu g/ml$, others being able to grow at $100 \mu g/ml$ benomyl. In Ceratocystis ulmi, BRASIER and GIBBS (1975) isolated spores capable of growth at $5 \mu g/ml$ carbendazim, whereas spores from other isolates were resistant at $1000 \mu g/ml$ carbendazim-HCL (SCHREIBER and TOWNSEND, 1976). FLETCHER and YARHAM (1976) found benomyl resistant spores in Verticillium fungicola which had an ED_{50} between 1 and $5 \mu g/ml$ and others with an ED_{50} between 100 and $500 \mu g/ml$.

It is possible that these differences in resistance level are related to different genes conferring resistance. The recognition of different resistance loci is possible only if mutant strains are crossed among themselves and the progeny analyzed for resistance to the toxicant in question. Experimentally, the existence of more than one locus for resistance to the same fungicide has been proved. HASTIE and GEORGOPOULOS (1971) demonstrated the presence of two genes for benomyl resistance in Aspergillus nidulans, one conferring a low and one conferring a high level of resistance. VAN TUYL (1977) has established a third gene in A. nidulans which confers a low level of benomyl resistance.

The results from field experiments in Bursfelde and Hilwartshausen indicated that two or four applications of thiophanate methyl within a season resulted in a distinct increase in the frequency of weakly carbendazim resistant spores in spore suspensions of in vitro subcultured field isolates. It was remarkable, that this frequency was not influenced by the number of applications of the fungicide. This can only be explained by the fact that the - almost weekly - sprayintervals were rather short, compared with the length of the latent period of the fungus. The fact that the selection pressure of a single fungicide application was rather moderate may also play an important role in this respect. The absence of a strong selection pressure was also reflected by the rather limited increase in the frequency of resistant spores. In no case, an isolate with more than $1:10^4$ resistant spores was found.

The percentage of isolates yielding resistant spores also increased after fungicide spraying. This has also been reported with other fungi. SCHUEPP and LAUBER (1977) found that the percentage of such isolates of Botrytis cinerea on grape increased from 31% to 73% after carbendazim sprayings. WOLFE (1973) observed that, after ethirimol treatment, the percentage of Erysiphe graminis isolates on barley which yielded resistant spores, increased from 4% in untreated plots to about 20% in treated plots. Comparable results with other fungi have been obtained by POLACH (1973), LITTRELL (1974), MILLER and FLETCHER (1974), JONES and EHRET (1976), FLETCHER et al. (1977), NOVACKA et al. (1977).

Although resistance to edifenphos in Pyricularia oryzae was reported to occur under laboratory conditions (UESUGI et al., 1973), this might be the first report of resistance to edifenphos in a field population of a pathogen. Treatment with edifenphos increased the frequency of strains resistant to this fungicide. There are, however, differences between the effects of field applications of edifenphos and thiophanate methyl. In each year there was an increase in the frequency of edifenphos resistant strains after edifenphos spraying and also in the percentage of isolates yielding resistant spores. From year to year, however, there was no significant increase in the frequency of edifenphos resistant spores in isolates from unsprayed plots, nor in the percentage of isolates yielding resistant spores (tables 13 and 14). This could be attributed to the fact that edifenphos is not used on a commercial scale to control S. nodorum and was only sprayed on the field plots. The absence of an overall selection pressure on the total population thus caused a shift back to its previous state.

Carbendazim resistant conidia were present in field populations of Cercosporella herpotrichoides, but the relative proportion was rather low. Application

of carbendazim increased this frequency. However, the absolute figures indicated that the danger of an economically important carbendazim resistance in C. herpotrichoides is minimal at these concentrations of the fungicide used.

A more pronounced effect of carbendazim was apparent in the percentage of isolates yielding resistant spores. This percentage increased after field applications of carbendazim. This was in contrast to the findings of RASHID and SCHLÖSSER (1977) who were unable to detect a correlation between the number of treatments on one hand and the percentage of isolates with carbendazim resistant spores on the other. The same authors (RASHID and SCHLÖSSER, 1975) at first reported the presence of carbendazim resistant strains of C. herpotrichoides. On the other hand, CHIDAMBARAM and BRUEHL (1973) were unable to find benomyl resistant spores of C. herpotrichoides, although they used the same method as described in the experiments above. This result was probably due to the fact that the total amount of tested spores, approximately 7×10^{7}, was too small to detect any resistance, since carbendazim conidia of the pathogen are normally present at frequencies about 10 times that low.

No practical problems of carbendazim resistance in C. herpotrichoides should be expected, even in the future, since development and spread of any fungicide resistance in a fungal population depends on the fitness of the resistant strains, the type of the disease and on the selection pressure exerted on the pathogen population. Even when resistant strains appear which have a fitness greater than the sensitive strains, their development and spread will be limited: the infection threshold is high, which minimizes the relative importance of single carbendazim resistant conidia; spread of spores in the field is rather   slow; the latent period is extremely long. Under practical circumstances the selection pressure, exerted by one or two carbendazim applications each season, is too small for a continuous selection of resistant strains. Furthermore, as this haulm attacking fungus can not be completely eliminated by only one or two fungicide sprayings, most of the sensitive population will survive, due to an insufficient covering by the fungicide.

6. CHARACTERIZATION OF RESISTANT STRAINS

6.1. Introduction

In chapter 5 it was reported that resistant strains are present in field populations of both S. nodorum and C. herpotrichoides. This does not necessarily imply that these resistant strains might cause practical problems. As pointed out in chapter 1, pathogenicity and fitness of resistant strains are important for their ability to survive in populations or to build up a resistant population. The question, whether a gene mutation to resistance at the same time influences fitness and virulence is, therefore, important.

Several authors have reported a decrease in pathogenicity and sporulation, correlated with a strain mutation to fungicide resistance. Only some information on such a phenomenon is available and mostly the employed methods could not exclude the possibility of several different mutations in one cell, e.g. by using U.V.-irradiation to produce resistant mutants.

By a single-gene change in the genetic code leading to specific fungicide resistance, the structure of the fungicide receptor within the cell might be altered in such a way, that it influences the fitness of the cell. This may result in a less pronounced pathogenicity and/or in a decreased sporulation capacity. An example of this phenomenon is given by DEKKER and GIELINK (1979), who worked with pimaricin resistant mutants of Cladosporium cucumerinum. They obtained indications that increased resistance was linked to decreased pathogenicity or sporulation capacity. Pimaricin is known to bind to ergosterol in the membrane and cause leakage of the cell. It was suggested that in pimaricin resistant strains ergosterol was replaced by another sterol, which showed less affinity to pimaricin and which was, at the same time, also responsible for lowered fitness. Triforine resistant mutants of the same pathogen showed a decreased ability to produce viable spores (FUCHS and VIETS-VERWEY, 1975).

Normally pathogenicity and fungicide resistance should be coded for by different genes and in that case the probability of simultaneous mutations in all of these is small.

From what has already been described it would appear that several important factors have to be considered in order to obtain reliable information about the fitness of both resistant and sensitive strains of a fungus. First of all, it is important that, if possible, only strains are tested, selected from naturally occurring field populations. If possible, genetic analysis should

be done to elucidate the nature of pathogenicity. Furthermore, a great number of strains should be tested, on account of the great variability in pathogenicity that may exist between isolates.

Sensitive and resistant strains of S. nodorum and C. herpotrichoides were examined with respect to their mycelial growth, development of symptoms and spore production. In most cases, the same strains were tested both on agar medium and on plants.

6.2. Septoria nodorum

6.2.1. Agar tests with carbendazim resistant strains.

Strains of S. nodorum weakly resistant to carbendazim which had been selected from field isolates from plots with different fungicide treatments as described under 2.8.1.2, were subcultured for three passages on wheat meal agar plates without added fungicide. The stability and the level of the acquired resistance were examined by culturing both resistant and sensitive strains on wheat meal agar plates containing carbendazim in concentrations form 0 to 5 μg/ml a.i. Plates were incubated at 20°C. After 8 days, mycelial growth was measured. The results are listed in table 20.

At all concentrations, the differences between the growth rates of weakly carbendazim resistant and sensitive strains are significant at P $\leqslant$ 0.001.

Examination of all data obtained revealed that nine percent of the tested resistant strains had lost their resistance after subculturing for three passages in the absence of the fungicide; it could be assumed that their acquired resistance was due to a reversible adaptation to carbendazim.

Weakly carbendazim resistant strains from plots sprayed four times with thiophanate methyl grew slightly better at the carbendazim concentrations tested than those from the other plots.

To obtain information about the mycelial growth rate and the spore production of both sensitive and resistant strains, 16 strains of each group were cultured on agar plates with and without added carbendazim. A 5 mm diameter agar disc of freshly grown mycelium was placed in the centre of each plate. Plates were incubated at 20°C for 12 days. The extent of mycelial growth and spore production were then measured. Each strain was tested in 4 replicates. The resul ts are presented in figure 11.

Tab. 20. <u>S. nodorum</u>. Degree of resistance of a number of weakly carbendazim resistant strains isolated from field plots with different fungicide treatments. Strains were subcultured for three passages in the absence of the fungicide, then they were exposed to different concentrations of carbendazim. Mycelial growth rate after 8 days as a percentage of the control, compared with the growth of carbendazim sensitive strains.

Treatment in field plots	isolates tested	carbendazim concentration (μg/ml)				
		1.0	1.5	2.0	3.0	5.0
unsprayed	39	70	38	17	3	0
thiophanate methyl 2x	79	77	38	19	7	1
thiophanate methyl 4x	127	72	43	25	9	3
sensitive strains	47	40	12	5	0	0
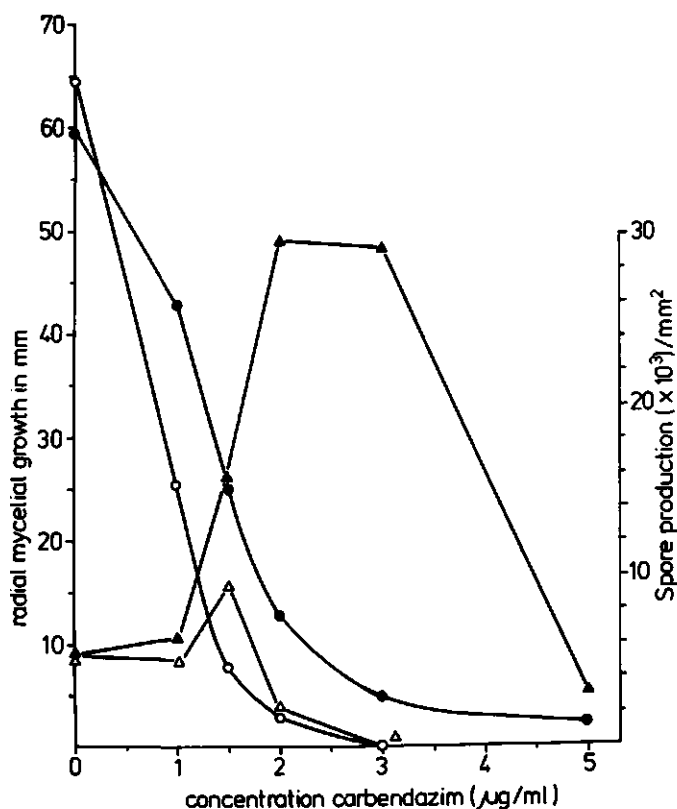
Fig. 11. <u>S. nodorum</u>. Radial mycelial growth (mm) and spore production per mm^2 mycelium of carbendazim sensitive and of weakly resistant strains at different carbendazim concentrations. Averages of 16 strains each replicated four times. Mycelial growth (●———●) and spore production (▲———▲) of resistant strains. Mycelial growth (O———O) and spore production (Δ———Δ) of sensitive strains.

As a group, the sensitive strains grew a little faster in the absence of the fungicide, than did the weakly resistant ones. The difference is statistically significant at P≤0.01. There was no difference between resistant and sensitive strains, with respect to their spore production in the absence of carbendazim. With resistant strains, a significant increase in spore production was noticed at concentrations between 1.5 and 3.0 µg/ml of the fungicide, compared with the spore production in its absence. This increase in the spores produced per mm^2 mycelium was counteracted by a simultaneous inhibition of the mycelial growth.

As demonstrated in chapter 5, strains with a high resistance level to carbendazim were also present in fieldpopulations of S. nodorum. Their characterization is presented below. Highly carbendazim resistant strains, obtained from field populations from plots with different fungicide treatments as described under 2.8.1.2, were subcultured for three passages on wheat meal agar plates without added fungicide. The stability and the level of the acquired resistance were examined by culturing the strains on wheat meal agar plates containing carbendazim at concentrations from 0 to 1000 μg/ml a.i. A 5 mm diameter agar disc of freshly grown mycelial culture was placed in the middle of each plate. Plates were incubated at 20°C. After 12 days, mycelial growth rate and spore production per mm^2 mycelium were measured. The results are presented in figure 12. They demonstrate that highly resistant strains of S. nodorum were able to grow and produce spores even at relatively high concentrations of up to 1000 μg/ml carbendazim.

To determine whether sensitive and highly resistant strains differ with respect to their mycelial growth rate and spore production in the absence of carbendazim, strains of both groups were cultured for 12 days on wheat meal agar plates at 20°C. Then mycelial growth and spore production per mm^2 mycelium were measured. The results are presented in table 21. The data demonstrate a moderate variability in the growth rate, but a high variability in the sporulation capacity of both groups. The most interesting result might be that there were resistant strains with a mycelial growth and spore production equal to that of sensitive ones. Fitness of these strains did not appear to have been influenced by the mutation to fungicide resistance. On average, the growth of sensitive strains was faster than that of resistant strains, the difference being statistically significant, whereas there was no significant difference between the 2 groups with respect to their spore production.

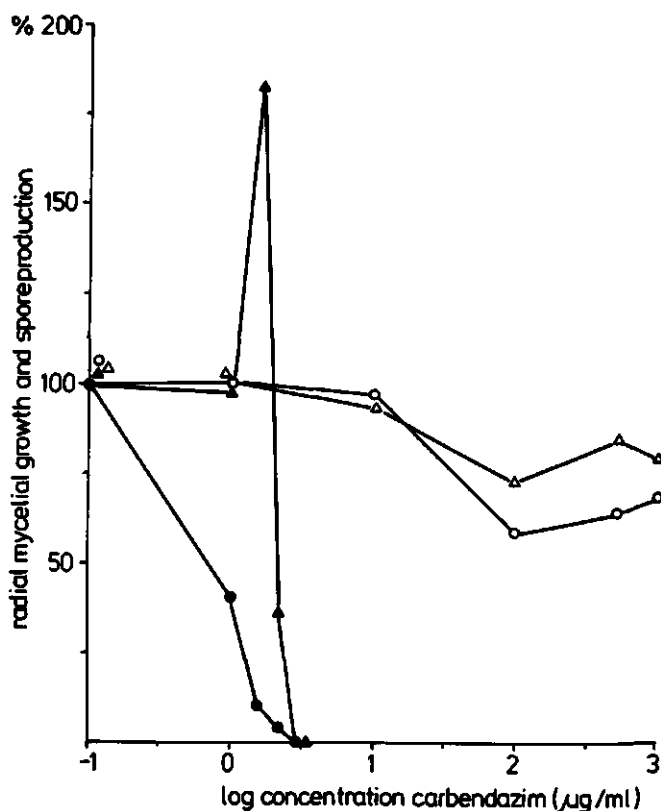Fig. 12. S. nodorum. Radial mycelial growth and spore production per mm^2
mycelium, relative to the control without fungicide, of highly
carbendazim resistant and sensitive strains.
Averages of 19 resistant and 16 sensitive strains, each tested in
four replicates. Mycelial growth (O———O) and spore production
(Δ———Δ) of resistant strains.
Mycelial growth (●———●) and spore production (▲———▲) of
sensitive strains.

Tab. 21. <u>S. nodorum</u>. Mycelial growth (mm) and spore production per mm^2 mycelium of carbendazim sensitive and highly resistant strains, tested <u>in vitro</u> in the absence of the fungicide. Each strains was tested in 4 replicates. Plates were incubated for 12 days at $20^{\circ}C$.

Strain	sensitive		resistant	
	mm	spores/mm^2	mm	spores/mm^2
1	63	2728	51	7349
2	62	2652	65	3016
3	64	8709	53	4036
4	65	6031	64	7465
5	66	8189	64	11197
6	59	5490	43	4135
7	66	4211	43	6891
8	65	6031	65	10856
9	65	7237	66	11699
10	65	10856	43	8270
11	68	6611	45	15104
12	65	10856	46	8428
13	65	6755	37	13953
14	65	6031	37	11162
15	60	3538	45	10069
16	63	15409	43	11026
17	65	14475	53	45
18	65	9650	54	328
19	66	8189	43	6202
20	65	12062	62	7954
21	65	7238	62	11932
22	61	6573	65	9650
23	67	4540	45	8559
24	63	3852	40	6369
25	65	6031	53	4535
26	65	7238	45	7552
27	63	11557	49	2654
28	64	1089	65	12062
29	65	1508	46	9632
30	63	3210	45	7552
$\bar{x}$	64.3	6951	51.2	7989
$s\bar{x}$	0.35	656	1.75	683

6.2.2. Tests with strains highly resistant to carbendazim on living plants.

Wheat plants were inoculated with carbendazim sensitive and highly resistant
strains of S. nodorum, according to the methods described under 2.6.1., in order
to examine their pathogenicity. Two days after inoculation one half of the plants
was sprayed with carbendazim (3000 μg/ml a.i.). After further 14 days, percentage
leaf necrosis was assessed and subsequently the spore production on the leaves
measured (cf. 2.10.1.). The results are listed in table 22. They show that
highly resistant strains were present with a fitness at least equal to sensitive
ones in the absence of carbendazim, if as the criterion for fitness the percentage
leaf necrosis and/or the number of spores produced is taken. It would appear that
the mutation to resistance was not correlated with a decreased fitness.

On unsprayed plants, sensitive strains as a group proved to be somewhat
more pathogenic than ones highly resistant to carbendazim. Sensitivity to carben-
dazim tended to be accompanied by a somewhat greater sporulation capacity.
Postinfectional application of carbendazim largely suppressed the development
of symptoms by sensitive strains, but not with highly resistant ones. There was
very little sporulation from necrotic leaf areas which resulted from an infection
by sensitive strains. Formation of pycnospores by the resistant strains was not
affected by the application of the fungicide.

Tab. 23. S. nodorum. Mycelial growth rate and spore production per mm^2 mycelium
on agar medium and percentage of leaf necrosis and spore production
per mm^2 necrosis on plants of a sample of highly carbendazim resistant
strains, tested in the absence of carbendazim.

Strain	agar tests		plant tests	
	mycelial growth (mm)	spores per mm^2	leaf necrosis (%)	spores per mm^2
a	71	2290	53	553
b	57	28225	53	60
c	57	14885	53	95
d	54	4426	65	718
e	67	12255	67	139
f	65	25901	61	779
g	65	24312	60	101

Tab. 22. S. nodorum. Pathogenicity tests on living plants with carbendazim sensitive (S) and highly resistant (R) strains, with or without fungicide treatment. Percentage of leaf necrosis and number of spores per mm^2 leaf necrosis. Each strain was tested in 10 replicates with 7 plants each. For experimental details see text.

Strain number	leaf necrosis (%)				spores per mm^2			
	unsprayed		carbendazim 3000 μg/ml		unsprayed		carbendazim 3000 μg/ml	
	S	R	S	R	S	R	S	R
1	70.6	52.8	23.5	55.5	408	553	0	438
2	69.9	46.8	22.9	39.4	570	440	0	629
3	56.1	52.8	13.4	47.3	775	60	0	110
4	76.4	52.7	28.7	49.9	349	95	0	172
5	67.4	59.0	19.5	54.7	332	623	0	684
6	72.0	54.5	27.1	48.6	338	444	9	493
7	71.2	53.6	22.1	50.1	2747	373	3	697
8	54.9	64.7	15.1	65.0	186	718	0	517
9	57.4	56.3	13.8	53.7	963	427	0	384
10	65.1	51.8	12.1	47.3	76	602	10	308
11	65.9	67.4	13.1	62.3	1378	139	0	225
12	71.3	64.2	19.4	59.9	42	271	0	130
13	59.1	63.5	10.2	65.2	471	236	0	254
14		58.1		54.4		427		411
15		63.1		56.3		187		277
16		52.1		46.2		288		229
17		60.8		48.9		779		520
18		69.2		62.8		234		251
19		59.5		54.0		101		118
$\bar{x}$	65.9	58.0	18.5	53.8	664	368	1.7	360
$s\bar{x}$	1.9	1.4	1.7	1.6	201	49	1.0	43

The same resistant strains were examined with respect to their mycelial growth and sporulation in vitro. To give an impression of the difference that may occur within these strains with respect to their relevant characteristics on agar and on plants, the corresponding values of a sample of these strains are listed in table 23. There was no correlation between neither mycelial growth rate on agar and the percentage leaf necrosis, nor the sporulation on agar and on leaves, respectively.

6.2.3. Agar tests with edifenphos resistant strains.

Edifenphos resistant strains of S. nodorum, obtained from field plots after different fungicide applications as described under 2.8.1.2, were subcultured for three passages on wheat meal agar plates without added fungicide. The stability and the level of the acquired resistance were examined by culturing both resistant and sensitive strains on wheat meal agar plates containing edifenphos in concentrations from 0 to 200 μg/ml a.i. A 5 mm diameter agar disc of freshly grown mycelial culture was placed in the centre of each plate. Plates were incubated at 20°C. After 8 days, mycelial growth was measured. The results are listed in table 24.

Tab. 24. S. nodorum. Degree of resistance of a number of edifenphos resistant strains isolated from field plots with different fungicide treatments. Strains were subcultured for three passages in the absence of the fungicide, then they were exposed to different concentrations of edifenphos. Mycelial growth rate after 8 days as a percentage of the control, compared with the growth of edifenphos sensitive strains.

Treatment in field plots	Isolates tested	edifenphos concentration (μg/ml)				
		10.0	25.0	50.0	100.0	200.0
unsprayed	49	72	36	18	6	3
edifenphos 2x	72	81	45	29	14	8
edifenphos 4x	232	80	47	29	17	11
sensitive strains	71	27	6	1	0	0

At all concentrations, the differences, between the growth rates of edifenphos resistant and sensitive strains were significant at P $\leq$ 0.001.

Examination of all data obtained revealed that seven percent of the resis-

tant strains tested had lost their resistance after subculturing for three
passages in the absence of the fungicide. It is probable, that their resistance
was due to a reversible adaptation to edifenphos. Edifenphos resistant strains
from plots sprayed four times grew slightly better at the concentrations tested,
than those from other plots.

To obtain information about the mycelial growth and spore production of
both sensitive and resistant strains, 18 strains of each group were cultured
on agar plates with and without edifenphos. A 5 mm diameter agar disc of freshly
grown mycelium was placed in the centre of each plate. The plates were incubated
at 20°C for 12 days. Mycelial growth and spore production were then measured. Each
strain was tested in 4 replicates. The results are presented in table 25.

Tab. 25. <u>S. nodorum</u>. Mycelial growth (mm) and spore production per mm^2 mycelium
of edifenphos sensitive and resistant strains at different edifenphos
concentrations. Averages of 18 strains in four replicates each.
Plates were incubated at 20°C for 12 days.

Strains	edifenphos concentration (μg/ml)					
	0.0	10.0	25.0	50.0	100.0	200.0
<u>sensitive</u>						
mycelium (mm)	$65^+_-0.4$	$30^+_-1.8$	$12^+_-0.8$	$5^+_-0.6$	$1^+_-0.3$	0
spores per mm^2	5085^+_-628	2640^+_-471	2585^+_-706	1776^+_-637	0	0
<u>resistant</u>						
mycelium (mm)	$66^+_-0.5$	$61^+_-0.6$	$51^+_-1.1$	$35^+_-1.0$	$16^+_-0.9$	$10^+_-0.5$
spores per mm^2	5760^+_-689	3770^+_-599	2065^+_-394	1417^+_-189	1691^+_-329	2286^+_-837

There were no significant differences between the two groups of strains with
respect to their mycelial growth and spore production in the absence of edifenphos.
Although mycelial growth of sensitive strains was significantly suppressed by
edifenphos, compared with the resistant strains, there were no significant
differences between the two groups of strains with respect to their spore pro-
duction per mm^2 mycelium, at concentrations of up to 50 μg/ml edifenphos.

The results in table 26 show the presence of edifenphos resistant strains
with a comparable   fitness   as   some edifenphos sensitive strains, when

mycelial growth or spore production is taken as the criterion for fitness.

Tab. 26. <u>S. nodorum</u>. Mycelial growth (mm) and spore production per mm^2 mycelium of edifenphos sensitive and resistant strains, tested <u>in vitro</u> in the absence of the fungicide. Each strain was tested in 4 replicates. Plates were incubated at 20°C for 12 days.

Strain number	sensitive		resistant	
	mm	spores per mm^2	mm	spores per mm^2
1	65	3558	67	4285
2	65	6030	67	2837
3	67	2838	67	3973
4	63	1284	67	4541
5	64	4976	67	6811
6	68	4408	65	4221
7	65	1809	66	4152
8	63	8056	66	4679
9	67	2837	65	9648
10	63	7703	66	4679
11	66	8188	69	5351
12	65	3015	68	9918
13	64	7464	61	51
14	69	7492	65	4100
15	66	3275	62	10604
16	65	8442	65	5547
17			68	11019
18			70	7279
$\bar{x}$	65.3	5085	66.2	5760

The mycelial growth of a sensitive and a resistant strain at different edifenphos concentrations, are presented in figure 13.
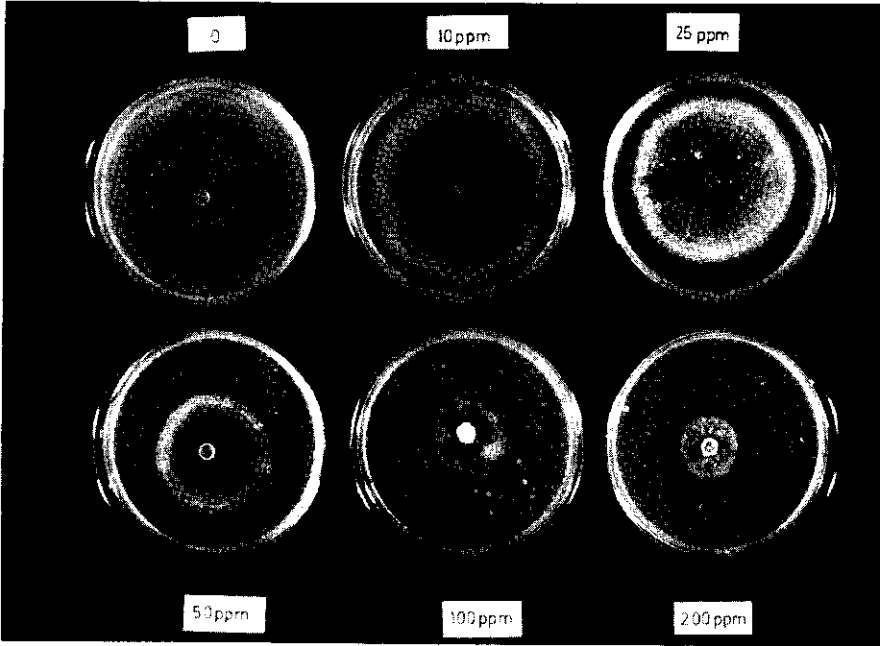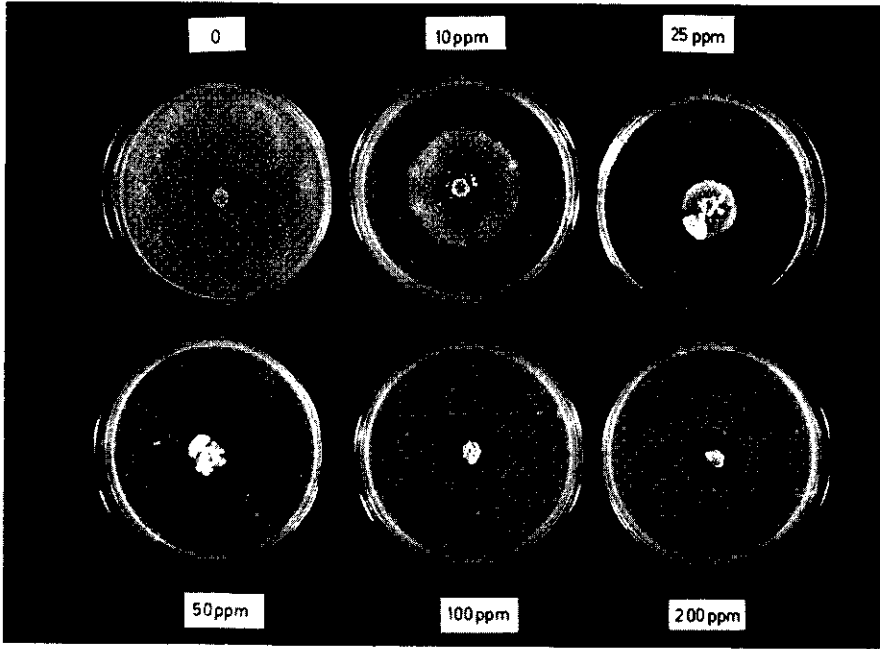
Fig. 13. <u>S. nodorum</u>. Mycelial growth of an edifenphos sensitive (top) and an edifenphos resistant (bottom) strain at different edifenphos concentrations.

6.2.4. Tests with edifenphos resistant strains on living plants.

Wheat plants were inoculated with edifenphos sensitive and resistant strains of S. nodorum, according to the methods described under 2.6.1, in order to examine their pathogenicity. Two days after inoculation, one half of the plants was sprayed with edifenphos 250 μg/ml a.i. After a further 14 days, percentage of leaf necrosis was assessed and subsequently the spore production on the leaves measured (cf. 2.10.1). The results are listed in table 27.

Tab. 27. <u>S. nodorum</u>. Pathogenicity tests on living plants with edifenphos sensitive (S) and resistant (R) strains, with or without fungicide treatment. Percentage of leaf necrosis and number of spores per mm^2 necrotic leaf area. Each strain tested in 10 replicates with 7 plants each.

Strain number	leaf necrosis (%)				spores per mm^2			
	unsprayed		edifenphos 250 μg/ml		unsprayed		edifenphos 250 μg/ml	
	S	R	S	R	S	R	S	R
1	70.6	54.8	13.6	26.7	408	394	225	457
2	69.9	69.7	7.7	32.1	570	1164	0	714
3	56.1	56.7	13.6	26.8	775	1263	367	870
4	76.4	53.5	17.8	13.7	349	441	224	203
5	67.4	51.3	8.4	12.5	332	92	1011	50
6	72.0	62.2	13.7	32.7	338	1173	471	655
7	71.2	62.9	13.9	46.6	2747	1606	1321	513
8	54.9	62.3	10.6	27.9	186	1389	0	705
9	57.4		4.2		963		178	
10	65.1		11.1		76		22	
11	65.9		6.2		1378		0	
12	71.3		12.0		42		0	
13	59.1		8.1		471		281	
$\bar{x}$	65.9	59.2	10.8	27.4	664	940	315	521
$s\bar{x}$	1.9	2.2	1.1	3.8	201	194	114	98

They show that edifenphos resistant strains were present with a comparable fitness as sensitive ones, in the absence of edifenphos, if as criterion the percentage of leaf necrosis and/or the spore production is used. The mutation to

edifenphos resistance did not appear to affect fitness. In the absence of the fungicide, sensitive strains as a group produced more symptoms on wheat seedlings than edifenphos resistant strains, this difference being significant at $P \leq 0.05$. On the average, resistant strains produced more spores than did the sensitive ones, although this difference is not statistically significant. Spraying with edifenphos significantly suppressed the development of symptoms, both with sensitive and resistant strains. The difference in leaf necrosis between the two strain types is, however, significant at $P \leq 0.001$. In case of edifenphos application, the mean values did show a greater spore production by the resistant strains. Edifenphos did not significantly suppres sporulation by sensitive strains. This is in contrast to a postinfectional treatment of plants with carbendazim where the formation of conidiospores by carbendazim sensitive strains was almost completely inhibited.

6.3. Cercosporella herpotrichoides

6.3.1. Agar tests with carbendazim resistant strains.

Carbendazim resistant strains of C. herpotrichoides, selected from field plots after different fungicide treatments as described under 2.8.2., were subcultured for three passages on wheat meal agar plates without added fungicide. The stability and the level of the acquired resistance were examined by culturing both resistant and sensitive strains on wheat meal agar plates containing carbendazim at concentrations between 0 and 100 μg/ml a.i. A 5 mm diameter disc of freshly grown mycelial culture was placed in the centre of each plate. Plates were incubated at 10°C. After 21 days, mycelial growth was measured. The results are presented in figure 14. Whereas sensitive strains were completely inhibited at a concentration of 1 μg/ml carbendazim, resistant strains were still able to grow at 100 μg/ml of the fungicide.

Mycelial growth and spore production per mm^2 mycelium of resistant strains were measured after growing them on wheat meal agar plates containing carbendazim at concentrations between 0 to 1000 μg/ml. The results are listed in table 28. Even at the very high carbendazim concentration of 1000 μg/ml the strains were able to grow. Here growth reduction was only about 40%, compared with the control without fungicide. More interesting is the finding that in the presence of the fungicide considerably more spores per mm^2 mycelium were produced than in its absence. This phenomenon was observed in all strains tested. Despite quite large standard errors, values from the 10 μg/ml, 100 μg/ml and 1000 μg/ml variants

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were significantly (P ≤ 0,001) different from the corresponding values of the control without carbendazim.

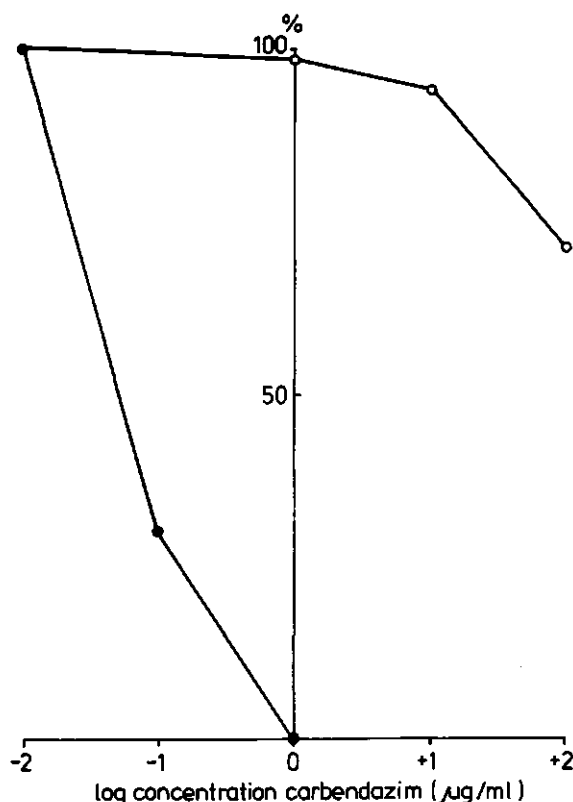Fig. 14. C. herpotrichoides. Radial mycelial growth as a percentage of the
control without fungicide   of carbendazim sensitive and resistant
strains, tested at different concentrations of carbendazim. Average
values from 10 strains, each tested in four replicates.
(O———O) resistant strains, (●———●) sensitive strains.

To determine whether resistant and sensitive strains differ with respect
to their mycelial growth and spore production, in the absence of the fungicide,
strains of both groups were cultured for 21 days on wheat meal agar plates at
10°C. Mycelial growth and spore production were then measured. To guarantee as
much genetic homogeneity as possible, the resistant strains were selected from
the progeny of the sensitive ones. The results are listed in table 29.

There is a tendency for sensitive strains to grow slightly faster and to
produce some more spores than resistant ones; however, in the latter case, this
difference was not statistically significant. Fifteen resistant strains grew
faster than their sensitive mother strains, whereas 21 grew slower.
Fifteen resistant strains produced more spores than their sensitive mother
strains, whereas 24 produced less. Summarizing it can be concluded that car-
bendazim sensitive and resistant strains differ only slightly, if at all, in
growth rate and sporulation capacity. This is valid for in vitro conditions
without fungicide present.

Tab. 28.   C. herpotrichoides. Mycelial growth rate (mm) and spore production
           per mm^2 mycelium of carbendazim resistant strains, tested at dif-
           ferent carbendazim concentrations. Average values from 34 strains
           each tested in 4 replicates. Plates were incubated at 10^{0}C for 21
           days.

| | carbendazim concentration (µg/ml) | | | | | | | | | |
| | 0.0 | | 1.0 | | 10.0 | | 100.0 | | 1000.0 | |
	mm	spores per mm^2	mm	spores per mm^2	mm	spores per mm^2	mm	spores per mm^2	mm	spores per mm^2
resistant strains	36	7250	34	8787	31	13444	26	27045	21	41677
s$\bar{x}$	0.7	1030	1.0	946	0.8	1519	1.0	2713	1.0	5109

Tab. 29. <u>C. herpotrichoides</u>. Mycelial growth rate (mm) and spore production per mm^2 mycelium of sensitive mother strains and carbendazim resistant daughter strains, tested <u>in vitro</u> in the absence of the fungicide. Each strain was tested in 4 replicates. Plates were incubated for 21 days at $10^{\circ}C$.

Strain number	Mycelial growth (mm)		Spore production/mm^2	
	sensitive	resistant	sensitive	resistant
1	28	26	18048	14444
		26		22830
2	30.5	22.5	16575	21410
		25.5		25490
3	29	29	14545	181
4	29	29.5	11515	11420
5	30.5	30.5	40136	3835
		32		8955
6	30	30.5	14164	26438
7	30.5	27	10136	6293
		30		5524
		28		10675
8	29.5	30	26793	14305
9	30	29	12181	17575
10	31	28.5	11538	18838
11	30	26.5	8640	22686
12	29.5	32	12005	932
		28.5		18995
		29.5		22547
		31.5		10398
13	29	30.5	5303	3689
		24.5		37791
		29.5		4099
		28.5		3296
		30		4815
		27		9615
		29		4696
		30		5099
		25		3061
14	28.5	29	20879	9545
15	33	28.5	585	5805
16	34.5	29	835	20909
17	33	34	14168	1984
18	32.5	32.5	20386	8082
19	32	34.5	5223	2676
20	32	33.5	14925	2497
21	34	32.5	17420	16646
22	33.5	33.5	5788	5334
23	33.5	32.5	5107	10735
24	31.5	33	9114	9133
25	31.5	30.5	19512	18219
26	33	31	10070	20159
$\bar{x}$	31.1*	29.5	13292	11706
$s\bar{x}$	0.36	0.42	1637	1338

*: significantly different from the values of the resistant strains at $P \leq 0.05$

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6.3.2. Tests with carbendazim resistant strains on living plants.

Wheat plants were inoculated with carbendazim resistant and sensitive
strains of C. herpotrichoides, according to the methods described under 2.6.2.,
in order to examine their pathogenicity. Seven days after inoculation, one half
of the plants was sprayed with carbendazim 50 μg/ml a.i. Eight weeks after inocu-
lation, the disease index was assessed (cf. 2.10.2). The results are listed in
table 30.

Tab. 30. C. herpotrichoides. Disease indices of wheat plants after inoculation
with carbendazim sensitive and resistant strains, respectively, after
an incubation time of 8 weeks. Resistant strains were selected from the
progeny of the sensitive strains. Each strain was tested in 4 replicates
with 16 plants each. For experimental details see text.

Strain number	sensitive		resistant	
	unsprayed	carbendazim 50 μg/ml	unsprayed	carbendazim 50 μg/ml
1	72.9	1.6	27.4	19.3
2	68.2	8.1	88.2	93.7
			93.6	95.3
3	91.9	30.6	98.4	88.3
4	46.7	3.1	93.7	98.4
5	73.3	10.9	76.8	26.6
6	70.3	15.6	96.8	96.7
7	81.2	8.6	88.9	98.4
9	75.8	17.7	96.8	82.3
11	96.7	4.8	90.3	95.3
12	93.5	6.2	67.7	100.0
			92.2	93.5
13	80.0	12.5	51.6	82.1
23	82.8	6.7	93.3	88.3
$\bar{x}$	77.8	10.5	82.6	82.7

Examining the values of the strains separately reveals, that three sensitive
strains (1, 12, 13) were more pathogenic than their resistant daughter strains;

four sensitive strains (2, 4, 6, 9) were clearly less pathogenic. On average, however, there were no significant differences between the two groups of strains with respect to the development of symptoms in the absence of carbendazim. Spraying with carbendazim greatly suppressed development of symptoms by sensitive strains, whereas pathogenesis of resistant strains was not inhibited at all.

6.4. Discussion

Fungicide sensitive and resistant strains of S. nodorum and C. herpotricho-ides were compared with respect to their mycelial growth rate, their sporulation capacity and their pathogenicity on agar medium and/or living wheat plants.

Tests with S. nodorum showed that two types of carbendazim resistant strains exist, one with a rather low level of resistance and another with a high resistance level. There was no indication of intermediates between these two types.

The resistance level of the weakly carbendazim resistant strains was rather low, since the mycelial growth was nearly completely inhibited at 5 μg/ml carbendazim. This growth reduction was accompanied by a sharp increase in the number of spores, produced per mm^2 mycelium at lower concentrations of the fungicide. Translated to the field situation, this would mean that weakly resistant strains would have more possibilities to survive in mixed populations with sensitive ones in the presence of the fungicide than in its absence.

In vitro experiments revealed that sensitive strains of S. nodorum grew slightly faster on agar medium without fungicide, than strains either weakly or highly resistant to carbendazim, but that there were no differences in spore production. This was confirmed by tests with living plants without application of carbendazim. There was a greater area of leaf necrosis following inoculation with sensitive strains than with highly resistant strains.

The great variation between different resistant strains with respect to their mycelial growth (37 mm versus 65 mm) and spore production (45 versus 15104 spores per mm^2) as presented in table 21 demonstrates that it is important to test a sufficient number of strains in order to give reliable information about these properties.

Although the average values of a number of strains provide some information about differences in mycelial growth, pathogenicity and spore production between groups of strains, it seems of more importance to examine whether resistant strains are present with a fitness at least equal to sensitive strains. The results showed, that strains highly resistant to carbendazim were present which had an equal mycelial growth and/or spore production in vitro and an equal

development of symptoms and/or spore production in vivo as sensitive strains, when tested in the absence of the fungicide. It is possible that they would be able to survive in field populations of S. nodorum, even in the absence of any fungicide selection pressure. There were no indications at all that degree of resistance to carbendazim was correlated with fitness.

Experiments were also carried out with edifenphos resistant strains of S. nodorum. Although the resistance level was not very high, the differences in mycelial growth and the percentage leaf necrosis after plantinoculation were statistically significant, when edifenphos sensitive and resistant strains were compared in the presence of the fungicide. However, there were no distinct differences in spore production. Edifenphos resistant strains were present with at least a comparable fitness as sensitive ones, when grown in the absence of edifenphos.

Since the tests with C. herpotrichoides were performed with sensitive mother strains and corresponding carbendazim resistant daughter strains, they might provide the most reliable information about the intrinsic characteristics of these strains. The properties of carbendazim resistant strains in vitro and in vivo did not differ very much from those of sensitive strains, in the absence of the fungicide.

That carbendazim resistant strains of C. herpotrichoides produced more spores in the presence of the fungicide than in the absence was an interesting result. Extrapolating these laboratory results to conditions in the field should be made with caution. A positive stimulation in sporulation by carbendazim was achieved at a concentration of $10 \mu g/ml$ in the medium, but not at $1 \mu g/ml$. Under natural conditons a carbendazim concentration within the tissue of the haulm base of $10 \mu g/ml$ would only rarely be expected to occur and, if so, for only a limited time: the fungicide is transported acropetally and accumulated in marginal tissues of the host. This stimulation of spore production was also reported by POLACH and MOLIN (1975), who found that a resistant strain of Botryotinia fuckeliana produced more spores in the presence of the fungicide than in the absence.

7. Survival ability of resistant strains in mixed populations.

7.1. Introduction

As shown in chapter 6, differences in the development of symptoms may exist
between resistant and sensitive strains, in the absence of the fungicides. Although
many of the factors which determine fitness may be investigated in laboratory
experiments, they do not provide all the information necessary in order to predict
the behaviour of resistant strains in mixed populations under field conditions.
WOLFE (1971) suggested that "although particular individuals such as fungicide
resistant ones may be selected, the whole population does not easily shift in this
direction, since there are many characteristics in a population, which are held in
a complex balance in the existing environmental situation. Thus, if the new selec-
tion pressure is relaxed, the population may tend to shift back to its previous
state of balanced adaptation. It is only when the resistant strains have a large
and continuous selective advantage that the population gradually becomes fully
adapted".

The survival ability of resistant strains in the population of a fungus
can be studied by inoculating host plants with a mixture of sensitive and resis-
tant strains. This is repeated for several passages without any fungicide stress
and in each passage the ratio of resistant to sensitive strains is determined.
RUPPEL (1975) found that a benomyl resistant strain of Cercospora beticola had
a lower competitive ability than a sensitive one. To inoculate sugar beet plants,
a spore suspension was applied which contained conidia from a sensitive and a
resistant strain at equal concentrations. However the ratio resistant/sensitive
was 9:51 after reisolation. This was in contrast with the results obtained by
DOVAS et al. (1976) with a resistant strain of the same pathogen. They found that
the relative proportion of a benomyl resistant strain in a mixed population in-
creased in the absence of benomyl, as well as in its presence. These contradictory
results may not be surprising, since in both experiments only a mixture of one
resistant and one sensitive strain was used. Independent of fungicide sensitivity
the strains may also vary in their pathogenicity and/or sporulation capacity.
Although MEYER (1976) also worked with mixtures of one benomyl resistant and one
sensitive strain of Colletotrichum lindemuthianum the results obtained might give
more reliable information, since the mixture consisted of a sensitive mother
strain and the corresponding resistant daughter strain. In this way, the probabi-
lity of a high variation in pathogenicity and/or sporulation capacity was reduced.

Four experiments were carried out with an initial bean plant inoculum consisting of a mixture of both strains at equal concentrations. In one experiment the resistant strain almost completely disappeared from the mixture after 4 successive plant passages. On average, however, the differences in the composition of the respective mixtures after 4 successive passages were not very pronounced.

To test the survival ability of resistant strains of S. nodorum and C. herpotrichoides in mixed populations, plants were inoculated with mixtures of resistant and sensitive strains in different ratios. The composition of each mixture was examined for three successive passages in the presence and absence of the respective fungicide.

7.2. Septoria nodorum

7.2.1. Experiments with carbendazim resistant strains.

To test the survival ability of strains of S. nodorum highly resistant to carbendazim, spore suspensions were made from 10 resistant strains and from 10 sensitive strains. The suspensions from sensitive and resistant strains were mixed as follows:

	sensitive		resistant
1	90	:	10
2	75	:	25
3	50	:	50

Wheat seedlings were subsequently inoculated, according to the methods described under 2.6.1. After 48 hours, one half of the plants in each variant was sprayed with carbendazim (1000 μg/ml), the other half remained unsprayed. Nine days after inoculation, leaves were harvested and disinfected as described under 2.7.1. and placed on water agar. After two weeks, sufficient spores had been formed on the leaf segments, such that further subculturing on wheat meal agar was not necessary. Spore suspensions from each variant were used to inoculate a second batch of wheat seedlings. Prior to inoculation, a 5 ml sample was taken from each spore suspension and diluted to the desired concentration of 100 spores per ml. One ml of each suspension was then pipetted onto the surface of ten water agar plates, one ml per plate. These were incubated for 5 days at 20°C, when small colonies of S. nodorum became visible. At least 100 colonies from each variant were transferred to plates of wheat meal agar containing carbendazim at 10 μg/ml a.i.,

a concentration lethal to sensitive spores of S. nodorum. The plates were again incubated at 20°C and after a further 7 days, the surviving colonies were counted. The composition of each mixture was examined in this way for three successive passages on plants, in the presence and in the absence of the fungicide. The results are presented in figure 15.
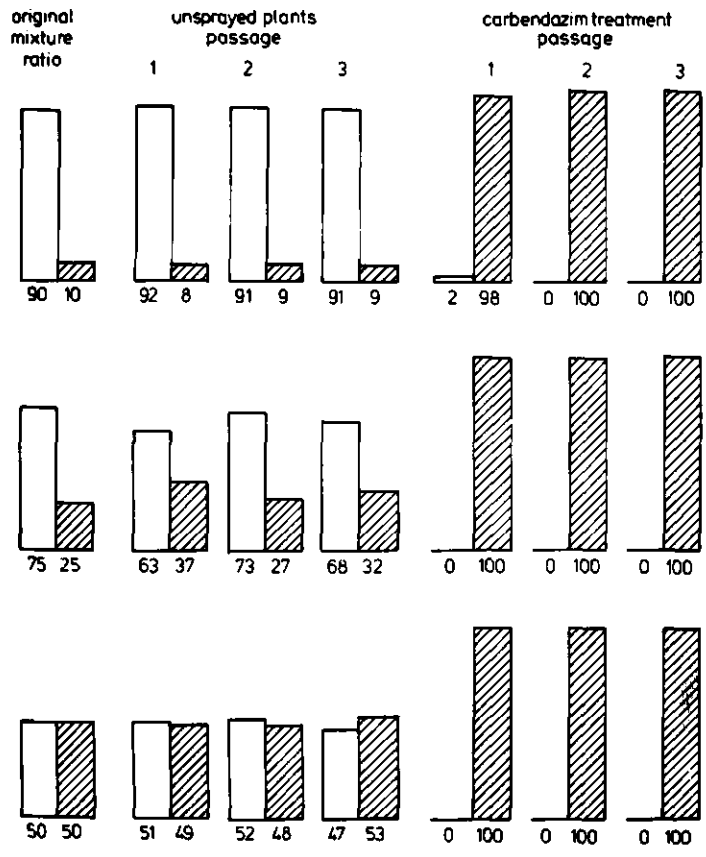

Fig. 15. S. nodorum. Survival of carbendazim resistant strains in mixed populations during three successive passages on plants. Plants were either sprayed with carbendazim (1000 μg/ml) or remained unsprayed. For experimental details see text.
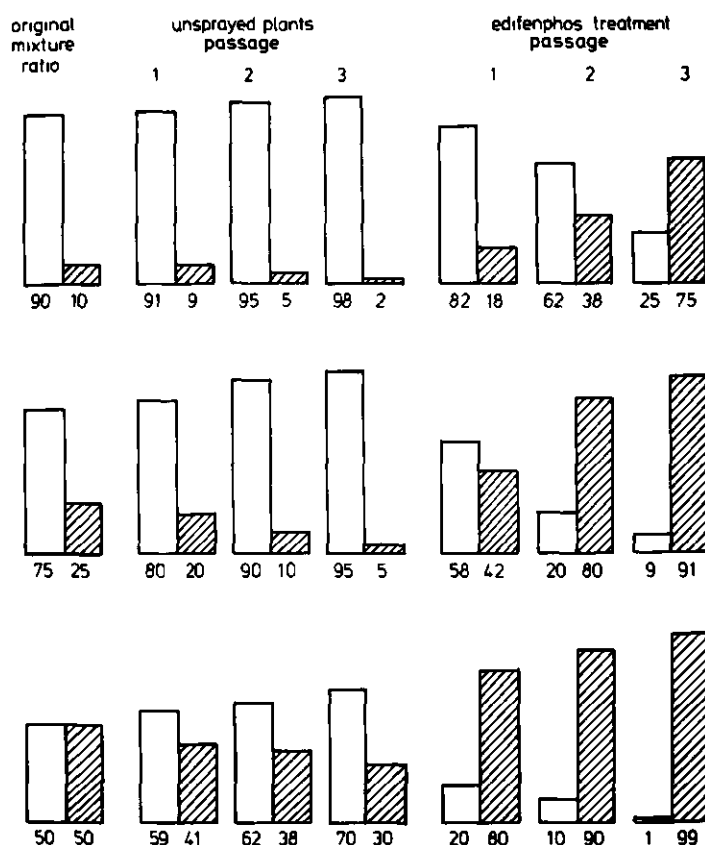
⬜ sensitive strains

▨ resistant strains

Fig. 16. S. nodorum. Survival of edifenphos resistant strains in mixed
populations during three successive passages on plants.
Plants were either sprayed with edifenphos (100 μg/ml) or remained
unsprayed. For experimental details see text.

☐ sensitive strains

▨ resistant strains

In the **variant** without carbendazim application, there were little differences in the composition of each mixture. This would suggest that the fitness of the resistant and sensitive strains as a group were comparable. Although the selection pressure exerted by spraying with carbendazim at 1000 μg/ml was rather low (cf. chapter 3), sensitive spores had already completely disappeared from the respective mixed populations after the first passage. Thus, spraying with carbendazim at this concentration imposed such a selection pressure on the population that only resistant spores were able to survive.

7.2.2. Experiments with edifenphos resistant strains.

The survival ability of edifenphos resistant strains of S. nodorum was examined in the same way as described for the carbendazim resistant strains under 7.2.1. Here the plants were sprayed postinfectionally with edifenphos at 100 μg/ml. Finally the colonies of each variant were transferred to wheat meal agar plates containing edifenphos at 200 μg/ml a.i., a concentration which is lethal to sensitive spores of S. nodorum. The results are presented in figure 16. On average, edifenphos resistant strains had a lower competitive ability than sensitive ones, since resistant strains gradually disappeared from the mixed populations in the absence of any selection pressure. This does not necessarily imply that all the resistant strains had a lower fitness, since a mixture of 10 strains was tested. This phenomenon was counteracted by treating the populations with edifenphos. In this case resistant strains maintained themselves and their relative proportion increased gradually from passage to passage. This is in contrast to the results obtained in the former experiment. Here spraying the plants with carbendazim resulted in only carbendazim resistant spores being reisolated after just one passage.

To determine the difference in fitness between the edifenphos sensitive and resistant strains, the models, given by LEONARD (1969) could be used.

$$\frac{P_n}{P_o} = (1-s)^n \frac{q_n}{q_o} \qquad (1)$$

P_o = initial relative proportion of the weaker genotype

q_o = initial relative proportion of the stronger genotype

n = number of passages

(1-s) = difference in fitness between the weaker and the stronger genotype

defined as the relative reproduction ratio between the two
genotypes.

The number of passages n, needed to decrease the proportion P_o to the proportion
P_n is given by:

$$n = \frac{\ln \dfrac{P_n \, (1-P_o)}{P_o \, (1-P_n)}}{\ln (1-s)} \qquad (2)$$

The sigmoid curve obtained in this way can be transformed to a straight line
by the following equation:

$$\ln \frac{P_n}{1-P_n} = \ln \frac{q_n}{1-q_n} + n \ln (1-s) \qquad (3)$$

In this way, the difference in fitness (1-s) can be estimated if $\ln \dfrac{P}{1-p}$
is plotted against the number of passages. Using the last equation
(3), the results presented in figure 16 were transformed. The results are
presented in figure 17. The resulting values for differences in fitness are
listed in table 31.

Tab. 31. <u>S. nodorum</u>. Differences in fitness, defined by the relative reproduction
ratio (1-s), between edifenphos resistant and sensitive strains, calcu-
lated from the results in figure 17.

Original ratio			Relative reproduction ratio	
sensitive	:	resistant	unsprayed	sprayed
90	:	10	0.55	2.88
75	:	25	0.54	3.22
50	:	50	0.77	4.22

In populations from unsprayed plants, the reproduction ratio was always
smaller than 1, indicating that resistant strains had a smaller reproduction
than the sensitive ones. Therefore, resistant strains would slowly disappear
from the mixed population. If the plants were sprayed with edifenphos, reproduction

of the resistant strains was greater than that of the sensitive strains. In
this case, resistant strains will finally completely dominate the population.
The gradual increase in the proportion of the resistant spores after spraying
with fungicide was due to the fact that not only resistant but also sensitive
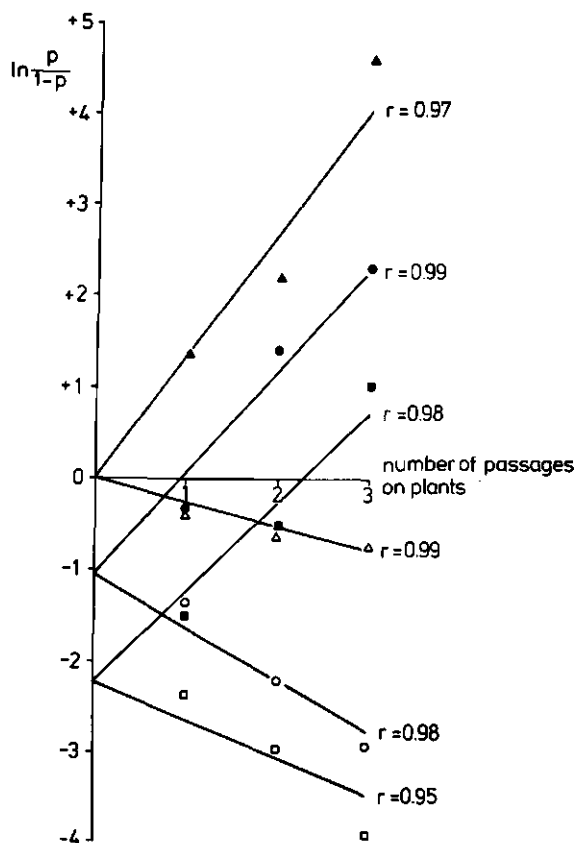strains were able to produce spores at the edifenphos concentrations used.

Fig. 17.   S. nodorum. Transformation of the results of figure 16 by means of
           the equation (3) given in the text.

sensitive/resistant			unsprayed	sprayed
90	:	10	□———□	■---■
75	:	25	O———O	●———●
50	:	50	Δ———Δ	▲———▲

7.3. Cercosporella herpotrichoides

Monospore isolates of C. herpotrichoides were subcultured on wheat meal
agar and resistant strains selected according to the method described under
2.8.2. In this way, sensitive mother strains and corresponding carbendazim
resistant daughter strains could be examined for differences in their competi-
tive ability. Again, spore suspensions from 10 strains of both groups were
mixed in the following ratios:

	sensitive		resistant
1	90	:	10
2	75	:	25
3	50	:	50

Wheat plants were inoculated as described under 2.6.2. Seven days later, one
half of the plants of each variant was sprayed with carbendazim ($25\,\mu$g/ml a.i.),
the other half remained unsprayed. After another seven weeks, the fungus was
reisolated from the haulms as described under 2.7.2. After subculturing the
isolates from each treatment on wheat meal agar for 5 more weeks, sufficient
spores were available for the inoculation of the next batch of plants. Prior
to inoculation, a 5 ml sample was taken from each spore suspension, which was
diluted to the desired concentration of 100 spores per ml. One ml of each sus-
pension was then pipetted onto the surface of ten water agar plates. The plates
were incubated at 20°C in the dark. One week later, colonies of C. herpotrichoides
became visible. At least 100 colonies from each treatment were transferred to
plates of wheat meal agar containing carbendazim at $3\,\mu$g/ml a.i., a concentration
lethal to sensitive spores of C. herpotrichoides. Plates were incubated at 10°C
and after a further 2 weeks, the surviving colonies were counted. In this way,
the relative proportion of carbendazim resistant and sensitive spores in the
conidial suspensions of isolates was determined for three successive passages on
plants. The isolates were obtained from plants with or without postinfectional
carbendazim treatment. The results are presented in figure 18.

There were hardly any differences in the relative composition of the spore
suspensions in the successive passages in which the host plants had not been post-
infectionally treated with carbendazim. From this result it can be concluded that,
on average, the fitness of carbendazim resistant and sensitive strains of C. herpo-
trichoides was comparable. Spraying with carbendazim at $25\,\mu$g/ml had a pronounced
effect on the composition of each conidial suspension. In this case, only resistant

strains were able to survive, due to the selection pressure exerted by the fungicide on the total population.
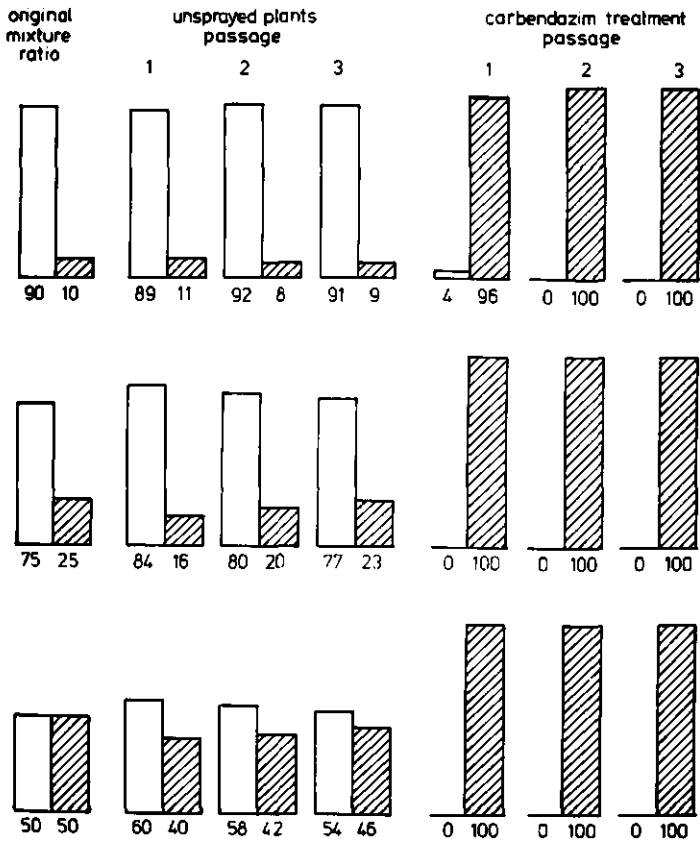

Fig. 18. C. herpotrichoides. Survival of carbendazim resistant strains in mixed populations during three successive passages on plants. Plants were either sprayed with carbendazim (25 μg/ml) or remained unsprayed.
For experimental details see text.

☐   sensitive strains

▨   resistant strains

7.4. Discussion.

With S. nodorum, the competitive ability of carbendazim and edifenphos resistant strains was studied by repeated inoculation of plants with mixtures of resistant and sensitive spores.

Under the selection pressure of carbendazim, the sensitive spores had disappeared from the mixed populations already after one passage. Without carbendazim application, however, the ratio between resistant and sensitive spores did not change significantly during three passages. This suggests that the fitness of the resistant strains, as a group, is comparable with that of the sensitive strains.

In contrast to carbendazim resistant strains, those resistant to edifen-phos cannot maintain themselves in mixed populations on living plants without fungicidal treatment. This was proven to be due to a smaller relative repro-duction ratio of the edifenphos resistant strains. The occurrence of edifen-phos resistant strains in field populations of S. nodorum would probably not lead to serious problems, if the fungicide were not frequently applied.

With C. herpotrichoides, no significant changes in the ratio between carbendazim resistant and sensitive strains were detected during three passages in the absence of the fungicide. After carbendazim treatment, however, nearly only carbendazim resistant spores could be reisolated already after the first passage.

GENERAL DISCUSSION

Since the introduction of the systemic fungicides in the sixties, a series of examples became kwown, in which fungicide application in the field showed a decreasing effectiveness. In most cases, this was proven to be caused by the development of specific fungicide resistance by the fungus (cf. FEHRMANN, 1976 , DEKKER, 1977, OGAWA et al., 1977). It should be mentioned, that the large scale use of non-systemic fungicides only rarely led to any important development of fungicide resistance.

Resistance to systemic fungicides is a complex phenomenon, in which many factors are involved. The rate at which resistant mutants are selected may vary with the type of fungicide, the fungal species and the environmental conditions, which includes the presence or absence of the fungicide concerned. The spread and the enrichment of resistant strains in a fungal population will largely depend on the properties of the resistant strains, the type of disease and the selection pressure by the chemical (DEKKER, 1976[b]).

In view of this it seemed of interest to study the development of fungicide resistance in two different cereal pathogens e.g. Septoria nodorum, the causal agent of glume blotch of wheat, and Cercosporella herpotrichoides, the causal agent of eyespot in wheat, barley and rye. As the rate at which resistant strains are selected may vary with the type of fungicide, different fungicides were used. In S. nodorum, development of resistance to thiophanate methyl and edifenphos, in C. herpotrichoides to carbendazim and nuarimol was studied.

The aim of the present study was to examine, whether S. nodorum and C. herpotrichoides were able to develop resistance to these fungicides, at the conditions of a field grown crop and at a rather moderate selection pressure, exerted on the pathogen populations by only a few fungicide sprayings, and if so, to perform model experiments on managing methods which might counteract this.

From a theoretical point of view one should expect a development of fungicide resistance in the field more likely to occur in S. nodorum than in C. herpotrichoides. Since most systemic fungicides are readily transported acropetally to the upper parts of the plant, S. nodorum, attacking leaves and glumes, will be exposed for a longer time to the action of a fungicide than C. herpotrichoides which only attacks the haulm base. Furthermore one must keep in mind, that in practice of pathogen control in wheat, fungicides generally are applied only once in one growth period. As a rule, carbendazim-generating fungicides are applied against both pathogens. At growth stage 6, at which C. herpotrichoides in wheat might

most effectively be controlled (FEHRMANN, unpublished), S. nodorum already appears in the haulms and younger leaves (FEHRMANN et al., 1977). In this way, a selection in the S. nodorum population takes place in favour of more resistant forms at the beginning of the local development of an epidemic, which then is characterized by an explosive increase in the number of spores released from the pycnidia shortly before blossom (FEHRMANN, 1974).

As C. herpotrichoides, which attacks the haulm base, cannot be eliminated completely by one fungicide spraying, part of both sensitive and resistant individuals will be able to survive. In contrast, the population of S. nodorum, which is a leaf pathogen, can be more effectively reduced, since leaves are better covered by a fungicide than haulms, when applied as a foliar spray.

As far as the spread of resistant strains is concerned, there are differences between both pathogens. Spread of resistant strains in a field is favoured by a short latent period, coupled with a sufficient production of viable spores and an aerial spread of the spores. S. nodorum has a rather short latent period of 10-14 days, dependent on climatic conditions (SHEARER and ZADOKS, 1972) whereas the latent period of C. herpotrichoides is rather long. Conidia of S. nodorum can be dispersed over long distances in the crop, when they become air-borne (FAULKNER and COLHOUN, 1976) whereas spore dispersal of C. herpotrichoides is rather limited (FEHRMANN and SCHRÖDTER, 1971).

From the foregoing it might be clear that resistant spores in a S. nodorum population find more favourable conditions for their development and spread than those in a C. herpotrichoides population.

To a certain extent, these theoretical statements were confirmed by the results obtained in the present study, in which the effect of fungicide sprayings in the field on the incidence of resistant spores on both pathogens was examined (chapter 5).

It was demonstrated that carbendazim resistant strains were present in field isolates of C. herpotrichoides. Both the percentage of isolates yielding resistant spores and the frequency of resistant spores in the respective isolates tended to be greater in isolates from carbendazim sprayed plots than in those from unsprayed plots. The absolute figures, however, were so extremely small, that at least at this selection pressure, no problems with carbendazim resistance in C. herpotrichoides seem immanent for the future. It cannot be excluded that, at a higher selection pressure, a pronounced increase in the frequency of carbendazim resistant spores will occur, especially in view of the finding that the fitness of carbendazim resistant and sensitive strains appears comparable (chapter 6). Enrichment of resistant individuals under high selection pressure was indeed demonstrated in

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chapter 7. Due to the limited spread of the C. herpotrichoides spores, these problems will then have a local significance only. In agreement herewith, no reports of failure of eyespot control by carbendazim-generating fungicides have yet appeared, although carbendazim resistant strains of C. herpotrichoides were found in the field (RASHID und SCHLÖSSER, 1975).

With S. nodorum, the situation was somewhat more complicated since two types of carbendazim resistant spores were found: those with a low-level resistance, inhibited at $5 \mu g/ml$ carbendazim in vitro, occurring at a frequency of about 7×10^{-6} in populations from unsprayed plots, and those with a high-level resistance, occurring at a frequency of about 100 times that low and hardly inhibited at $1000 \mu g/ml$ carbendazim in vitro. The frequencies of both types increased significantly after field application of thiophanate methyl, but this increase did not seem to influence the effectiveness of the fungicide in the field significantly (chapter 5). The selection pressure, exerted on the populations by the fungicides, was rather moderate. This was also reflected by the observation that in no case an isolate with more than $1 : 10^4$ resistant spores was found. Although an increase of the selection pressure by carbendazim may initially give a better disease control, the selection of highly carbendazim resistant strains may be favoured and finally lead to severe problems.

Experiments on agar media as well as pathogenicity tests on plants showed, that carbendazim resistant strains do emerge in populations of S. nodorum which, within the limits of these experiments, did not differ from sensitive wild type strains in mycelial growth, development of symptoms and spore production, in the absence of the fungicide (chapter 6). Therefore one might expect, that they are able to survive in mixed populations with sensitive strains even in the absence of any fungicide pressure. The results of chapter 7 confirm this. The relative proportion of highly carbendazim resistant strains in mixed populations with sensitive ones is not changing during several passages on living plants in the absence of the fungicide. When, however, a moderate dosis of carbendazim is applied postinfectionally, only resistant spores could be reisolated, even after already the first passage. This finding might explain, why after relatively short effective use of some fungicides the whole fungal population at a given location proved to have become resistant rather suddenly, such as was the case with Cercospora beticola (GEORGOPOULOS, 1973), Venturia inaequalis (KIEBACHER und HOFFMANN, 1976) and Botrytis cinerea in grape (EICHHORN, 1975). When there are no differences between resistant and sensitive strains in competitive ability, relaxation of the selection pressure will not lead to a renewed succesful use of the fungicide.

The frequency of spores, resistant to edifenphos was significantly greater in isolates from edifenphos treated field plots than in those from untreated plots (chapter 5). The absolute figures, however, remained rather small. Although several edifenphos resistant strains proved to have an apparent equal fitness as sensitive strains, when mycelial growth, development of symptoms or spore production are taken as a criterion for fitness (cf. chapter 6), on average the tested edifenphos resistant strains were not able to survive in mixed populations with sensitive ones in the absence of edifenphos (chapter 7). This was proven to be due to a smaller relative reproduction ratio of the tested resistant strains compared with the sensitive ones. Only when edifenphos was applied postinfectionally, resistant strains were able to dominate numerically the mixed population by the time. This might explain, why the frequency of edifenphos resistant spores in spore suspensions of isolates from unsprayed plots did not increase from one year to another, as was the case with weakly carbendazim resistant strains of S. nodorum (chapter 5). A second factor may contribute to this phenomenon: in the area where the field experiments were performed, use of carbendazim-generating fungicides is quite common, but no edifenphos is applied by the farmers.

Although the observed increase in the frequency of fungicide resistant spores did not result in a decrease in the effectiveness of the respective compounds, a resistance problem can not be ruled out completely. Therefore it is important to apply such spray programmes which can avoid or delay the development of fungicide resistance. In accordance with the theories of WOLFE (1971), DEKKER (1972) and FEHRMANN (1976), a spraying programme in which two fungicides with a different mode of action are applied alternately or as a mixture, proved to retard the development of fungicide resistance, compared with continuous application of either fungicide alone (chapter 4). These results, obtained under controlled conditions, were supplemented by those from field experiments (cf. chapter 5). In 1977, alternate sprayings with thiophanate methyl and edifenphos proved to control glume blotch even more effectively than four sprayings with each fungicide alone. Furthermore, the frequency of resistant spores in isolates from these plots ranged at the level of those from unsprayed plots. It seems clear, that in this way an important tool is given to retard a development of fungicide resistance. More attention has to be paid to the introduction of more than one fungicide for the control of a given pathogen.

Another possibility to prevent the development of fungicide resistance might be given by crop rotation. At the location Bursfelde where winter wheat was grown after winter wheat, the frequency of carbendazim resistant spores with a low-level

resistance in isolates of <u>S. nodorum</u> from unsprayed or thiophanate methyl sprayed plots increased from one year to another, so did also the percentage of isolates yielding resistant spores. In Hilwartshausen, where a crop rotation was performed, in which winter wheat was grown after sugar beets, this tendency seemed not to be present. The effect of a crop rotation might thus be considered as a relaxation of the fungicide selection pressure, leading to a disappearance of resistant strains with a smaller competitive ability than sensitive ones.

In such cases, where only one fungicide is available for the control of a pathogen or where crop rotation is not possible, the mentioned methods cannot be applied. In these cases it is advisable to apply the fungicide only if it is very urgent. Anyway, crop losses can not be avoided, even when fungicides are applied. On longer sight it might be more profitable to accept a somewhat higher crop loss by lowering the dosis of a fungicide or by less frequent application. This will help at least part of the sensitive population to survive also, which then can compete with the resistant part of the population. On contrast, application of high doses of a fungicide at frequent intervals will favour a rapid build-up of highly resistant forms.

Finally, it should be pointed out, that also the use of less susceptible cultivars may be of value, which will allow to apply a lower selection pressure of the fungicide. In this way, plant breeders and phytopathologists might cooperate to tackle the problem of resistance to systemic fungicides.

SUMMARY

Since the introduction of the systemic fungicides in the sixties, the phenomenon of resistance to these compounds has become a serious problem in the control of fungal plant diseases. The aim of this study was to contribute to the knowledge about the consequences of fungicide applications in cereals with respect to the emergence of fungicide resistant strains of Septoria nodorum, the causal agent of glume blotch of wheat, and of Cercosporella herpotrichoides, the causal agent of eyespot of wheat, barley and rye.

CHAPTER 3.

An accurate and reproducible inoculation method was used to inoculate wheat seedlings with spore suspensions of S. nodorum. The effect of post-infectional applications of the systemic fungicides carbendazim and edifenphos with respect to the development of symptoms and the length of the latent period was studied. Both fungicides suppressed the development of symptoms and elongated the duration of the latent period, the extent to which depending on the concentration of each fungicide used.

CHAPTER 4.

It is suggested that alternate or combined use of two systemic fungicides with a different mode of action can reduce the enrichment of specific resistant forms in a fungal population. To verify these statements, model experiments were carried out under controlled conditions.

During 9 passages on living wheat plants, inoculated with spore suspensions of S. nodorum, the fungicides carbendazim and edifenphos were applied either alone or as a mixture in each passage, or applied alternately from passage to passage. After 9 passages, disease control by either fungicide alone was reduced. This tendency was not so pronounced in that variant where the fungicides were applied alternately and nearly absent in that variant where a mixture was applied.

Comparable results were obtained in experiments on agar media. A stable edifenphos resistant strain was selected in that variant, where S. nodorum in each passage was cultured on plates with edifenphos.

With C. herpotrichoides, experiments were carried out on agar media only, using carbendazim and nuarimol. Here, a strong effect of the alternate exposure

of the fungus to these two compounds was present, since the fungus was hardly
able to grow on plates with a moderate dosis of carbendazim, after first being
cultured on plates with nuarimol.

CHAPTER 5.

The effect of field applications of thiophanate methyl and edifenphos on
the frequency of resistant spores in field isolates of S. nodorum was examined,
during five vegetation periods between 1973 and 1977. Two types of carbendazim
resistant strains were present in S. nodorum. Weakly carbendazim resistant
strains were inhibited at 5 μg/ml carbendazim in vitro and occured at a frequency
of about 1: $7x10^6$ in populations from unsprayed plots. Highly carbendazim resis-
tant strains occurred at a frequency of about 100 times that low and were hardly
inhibited at 1000 μg/ml carbendazim in vitro. In each year, there was a signifi-
cant increase in the frequency of weakly carbendazim resistant spores after
thiophanate methyl applications in the field, but the absolute figures remained
rather low. In no case, an isolate with more than 1:10^4 weakly resistant spores
was found.

There was no clear yearly increase in the frequency of highly carbendazim
resistant spores after thiophanate methyl application.

Edifenphos resistant spores were present in field isolates of S. nodorum.
Their frequency- about 1:10^7 in isolates from unsprayed plots-, increased signi-
ficantly after edifenphos application in the field, but the absolute figures
remained rather low.

During three years, field trials were carried out with C. herpotrichoides
to examine the effect of carbendazim application in the field on the frequency
of carbendazim resistant spores in field isolates. Their frequency in isolates
from unsprayed plots was about 1: $7x10^8$. This frequency increased after carbenda-
zim applications, but mostly on an insignificant scale only.

CHAPTER 6.

Fungicide sensitive and resistant strains of S. nodorum were compared with
respect to their mycelial growth rate, their sporulation capacity and their
pathogenicity on agar medium and/or living wheat plants.

Weakly carbendazim resistant strains showed a significantly faster mycelial
growth on agar media with carbendazim, than sensitive ones, but were almost
completely inhibited at 5 μg/ml carbendazim. This growth reduction was accompanied

by an increase in the number of spores, produced per mm^2 mycelium.

Highly carbendazim resistant strains were able to grow and produce spores at 1000 μg/ml carbendazim in vitro. Resistant strians were present with a mycelial growth and spore production equal to that of sensitive ones. Pathogenicity tests with sensitive and highly carbendazim resistant strains on living wheat plants showed the same tendency, as far as the development of symptoms and sporeproduction is concerned.

Edifenphos resistant strains of S. nodorum were compared with sensitive ones in tests on agar media and on wheat plants. Resistant strains were strongly inhibited at 200 μg/ml edifenphos in vitro. Edifenphos resistant strains were present with an equal fitness as sensitive ones, when grown in the absence of edifenphos, on agar media and on living wheat plants.

The tests with C. herpotrichoides were performed with sensitive mother strains and the corresponding carbendazim resistant daughter strains. These resistant strains were able to grow and produce spores even at 1000 μg/ml carbendazim in vitro. They produced more spores per mm^2 mycelium when cultured in the presence of carbendazim than in its absence. The properties of carbendazim resistant strains in vitro and in vivo did not differ very much from those of sensitive strains, in the absence of the fungicide.

CHAPTER 7.

The competitive ability of resistant strains was studied by inoculating wheat plants with different mixtures of resistant and sensitive spores. Carbendazim resistant strains of S. nodorum and C. herpotrichoides could maintain themselves in the mixed population for three passages on wheat plants, even in the absence of any fungicide selection pressure.

In case of a postinfectional fungicide application, only carbendazim resistant strains could be reisolated.

Edifenphos resistant strains disappeared from the respective populations in the absence of the fungicide, due to a smaller relative reproduction ratio.

SAMENVATTING

Sedert de introduktie van de systemische fungiciden in de jaren zestig is het optreden van resistentie tegen deze middelen een ernstig probleem geworden in de bestrijding van schimmelziekten. Het doel van dit onderzoek was een breder inzicht te verkrijgen in de gevolgen van bespuitingen met systemische fungiciden in granen, met betrekking tot het optreden van resistente stammen van _Septoria_ _nodorum_, de veroorzaker van het kafjesbruin bij tarwe, en van _Cercosporella_ _herpotrichoides_, de veroorzaker van de oogvlekkenziekte bij tarwe, gerst en rogge.

HOOFDSTUK 3.

Een nauwkeurige en reproduceerbare inoculatiemethode werd toegepast om tarwezaailingen te inoculeren met sporesuspensies van _S. nodorum_. Het effect van bespuitingen met de systemische fungiciden carbendazim en edifenphos op de symptoomontwikkeling en op de duur van de latente periode werd onderzocht. Beide fungiciden onderdrukten de symptoomontwikkeling en verlengden de duur van de latente periode; de mate waarin dit gebeurde was afhankelijk van de gebruikte concentratie.

HOOFDSTUK 4.

Een afwisselende of gekombineerde bespuiting met 2 fungiciden met een verschillend werkingsmechanisme kan de vermeerdering van resistente sporen in een schimmelpopulatie tegengaan. Dit werd nagegaan in experimenten onder klimaatkameromstandigheden.

Gedurende 9 passages op tarweplanten, geinoculeerd met sporesuspensies van _S. nodorum_, werden de fungiciden carbendazim en edifenphos in iedere passage afzonderlijk of gemengd gespoten, dan wel van passage tot passage afwisselend. Na 9 passages bleek de ziektebestrijding geringer te zijn in die variant waar de planten in iedere passage met een der beide fungiciden afzonderlijk gespoten waren. Deze tendens was minder duidelijk in die variant waar de fungiciden afwisselend gespoten waren en bijna afwezig daar waar in iedere passage een mengsel gespoten was.

Vergelijkbare resultaten werden behaald in proeven op agarmedia.
Een stabiele edifenphos resistente stam werd geselecteerd in die variant, waar _S. nodorum_ in iedere passage op platen met edifenphos groeide.

Proeven met _C. herpotrichoides_ werden alleen op agar media gedaan, met de fungiciden carbendazim en nuarimol. Een duidelijk effect van de afwisselende

behandeling was aanwezig, omdat de schimmel nauwelijks in staat was te groeien op platen met een relatief geringe dosis carbendazim, na eerst gegroeid te hebben op platen met nuarimol.

HOOFDSTUK 5.

Het effect van veldbespuitingen met thiofanaat methyl en edifenphos op de frequentie van resistente sporen in veldisolaten van S. nodorum, werd gedurende 5 vegetatieperiodes tussen 1973 en 1977 onderzocht. Bij S. nodorum bleken 2 types stammen met carbendazim resistentie voor te komen. Zwak carbendazim resistente stammen werden bijna volledig geremd in vitro bij $5\,\mu$g/ml carbendazim en waren aanwezig in een frequentie van ongeveer 1: $7x10^6$ in populaties van onbespoten percelen. Sterk carbendazim resistente stammen waren aanwezig in een ongeveer 100 keer zo lage frequentie en werden nauwelijks geremd in vitro bij 1000 μg/ml carbendazim. In ieder jaar was er een signifikante toename te konstateren van zwak resistente sporen, na thiofanaat methyl bespuiting in het veld, maar de absolute aantalen bleven tamelijk laag. In geen geval werd een isolaat met meer dan 1: 10^4 resistente sporen gevonden.

Er was geen duidelijke jaarlijkse toename in de frequentie van sterk carbendazim resistente sporen na bespuiting met thiofanaat methyl.

Edifenphos resistente sporen werden eveneens gevonden in veldisolaten van S. nodorum. Hun frequentie - ongeveer 1: 10^7 in isolaten van onbespoten percelen - nam significant toe na edifenphos bespuiting, maar de absolute aantallen bleven tamelijk gering.

Gedurende een periode van 3 jaar werden proeven uitgevoerd met C. herpotrichoides, om het effect te onderzoeken van veldbespuitingen met carbendazim op de frequentie van carbendazim resistente sporen in veldisolaten. Deze frequentie in isolaten van onbespoten percelen was ongeveer 1: $7x10^8$. Deze frequentie nam toe na carbendazim bespuiting in het veld, maar meestal was deze toename niet significant.

HOOFDSTUK 6.

Fungiciden resistente en gevoelige stammen van S. nodorum werden vergeleken in mycelium groei, sporulerend vermogen en pathogeniteit in proeven op agarmedia en op tarweplanten.

Zwak carbendazim resistente stammen vertoonden een significant snellere groei dan gevoelige stammen op agarmedia met carbendazim, maar werden bijna geheel

geremd bij 5 μg/ml carbendazim. Deze groeiremming ging gepaard met een toename van het aantal sporen, geproduceerd per mm^2 mycelium.

Sterk carbendazim resistente stammen waren in staat te groeien en te sporuleren bij 1000 μg/ml carbendazim _in vitro_. Er bleken resistente stammen aanwezig te zijn met een groeisnelheid en sporenproduktie _in vitro_, die vergelijkbaar waren met die van gevoelige stammen. Dit werd ook gekonstateerd in proeven op tarweplanten, waar het de snelheid van symptoomontwikkeling en de sporenproduktie betreft.

Edifenphos resistente en gevoelige stammen van S. nodorum werden onderling vergeleken in proeven op agarmedia en op tarweplanten. Resistente stammen werden sterk geremd bij 200 μg/ml edifenphos _in vitro_. Edifenphos resistente stammen bleken aanwezig te zijn, die een gelijke fitness als sensitieve stammen hadden, indien getest in de afwezigheid van edifenphos op agarmedia of op tarweplanten.

De proeven met C. herpotrichoides werden uitgevoerd met gevoelige moederstammen en de bijbehorende carbendazim resistente dochterstammen. Deze resistente stammen waren in staat te groeien en te sporuleren bij concentraties van 1000 μg/ml carbendazim _in vitro_. Zij produceerden meer sporen per mm^2 mycelium in de aanwezigheid van carbendazim dan in de afwezigheid.

De relevante eigenschappen van carbendazim resistente stammen _in vitro_ en _in vivo_ verschilden niet veel van die der gevoelige stammen, indien getest in afwezigheid van het fungicide.

HOOFDSTUK 7.

Het competitieve vermogen van resistente stammen werd onderzocht door tarweplanten te inoculeren met verschillende mengsels van resistente en gevoelige sporen.

Carbendazim resistente stammen van S. nodorum en C. herpotrichoides konden zich handhaven in de mengpopulaties gedurende tenminste drie passages op tarweplanten, zelfs in afwezigheid van het fungicide. Indien na de infectie een gematigde dosis carbendazim op de planten werd gespoten, konden alleen nog resistente sporen geïsoleerd worden.

Edifenphos resistente stammen konden zich niet handhaven in de mengpopulaties in afwezigheid van het fungicide, omdat zij een geringer reproducerend vermogen bleken te hebben.

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CURRICULUM VITAE

Jacobus Aloysius Henricus Maria Horsten werd op 13 juli 1946 te Tilburg geboren.
In 1965 behaalde hij het Gymnasium-B diploma aan het Sint Odulphus Lyceum te
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