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A comparison of the in vitro antifungal spectra of thiophanates and benomyl

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Abstract

The antifungal spectra of thiophanate, thiophanate-methyl and its derivative 2-(3-methoxycarbonyl-2-thioureido) aniline (NF 48), were similar to that of benomyl. The order of effectiveness *in vitro* was: benomyl > NF 48 > thiophanate-methyl > thiophanate, benomyl being by far the most and thiophanate by far the least active compound.

The effect on mycelial growth of two fungi with an irregular inhibition pattern are presented in detail, viz. of *Colletotrichum acutatum* and *Gliocladium roseum*. In the first case mycelial growth was inhibited to over 50% at low concentrations; the inhibition, however, was not further enhanced with increasing concentrations. Mycelial growth of *Gliocladium roseum* was maximally suppressed at low concentrations of the fungicides, whereas in this case an increase of the concentrations resulted in a decrease of inhibitory activity until a constant level had been reached.

Conidiobolus eurymites, unlike the other Zygomycetes tested, proved to be sensitive to the four fungicides.

Introduction

In a study on the selectivity of fungicides with regard to soil mycoflora some recently introduced systemic fungicides were included. One of these was benomyl. The antifungal spectrum of this fungicide appeared to be very characteristic. Especially Oomycetes, Mucorales and the porosporic Deuteromycetes proved to be resistant; blastosporic and phialosporic Deuteromycetes were very sensitive to this fungicide (Bollen and Fuchs, 1970; Edgington et al., 1971).

According to literature data thiophanate and thiophanate-methyl are active against many plant-pathogenic fungi, e.g. *Fusarium oxysporum* (Aelbers, 1971); *Botrytis cinerea, Phialophora cinerescens, Venturia* spp. and *Verticillium albo-atrum* (Formigoni et al., 1970); *Cercospora beticola, Gloeosporium* spp. and *Penicillium* spp. (Ishii, 1970). Diseases caused by *Pythium* spp., *Phytophthora infestans* and *Alternaria* spp. could not be controlled by either compound (Aelbers, 1971). Interestingly, the latter species are also resistant to benomyl. Moreover, acquired resistance to benomyl coincided with resistance to thiophanate-methyl in *Botrytis cinerea* (Scholten and Bollen, 1971) and in two *Penicillium* species (Bollen, 1971).

To assess the degree of their similarity in antifungal activity, the fungitoxic spectra of the four fungicides were studied in more detail. For this purpose 36 fungal isolates were chosen, representing a series in which the characteristic spectrum of benomyl is expressed. Homans and Fuchs (1970) tested a strain of *Colletotrichum acutatum* – in their paper indicated as *Glomerella cingulata* – which proved to be non-sensitive to

benomyl in a thin-layer chromatographic bio-assay. Also this strain and some other isolates of that species have been included.

Materials and methods

The isolates. The fungi were obtained from greenhouse soil (17 isolates, viz Table 1, No 3, 4, 5, 6, 10, 11, 12, 15, 20, 21, 23, 24, 26, 29, 31, 32, 33), from diseased roots (9 isolates, viz Table 1, No 1, 2, 7, 8, 27, 28, 30, 34, 35), from seeds (3 isolates, viz Table 1, No 9, 18, 19) or from other plant material (6 isolates, viz Table 1, No 14, 16, 17, 22, 25, 36), whereas the origin of *Colletotrichum acutatum* (Table 1, No 13) is unknown. Only a single isolate of every species has been tested, except in the case of species with benomyl-resistant strains.

The fungicides. The following systemic fungicides were used: benomyl (Benlate; 1-(butylcarbamoyl)-2-benzimidazole carbamic acid, methyl ester), thiophanate (Orga Topsin, NF 35; 1,2-bis (3-ethoxycarbonyl-2-thioureido) benzene), thiophanate-methyl (Orga Topsin-M, NF 44; 1,2-bis-(3-methoxycarbonyl-2-thioureido) benzene) and its derivative, yet without common name, 2-(3-methoxycarbonyl-2-thioureido) aniline (NF 48).

All species were tested on potato-dextrose-agar (PDA; Oxoid CM 139, pH 5.3) to which aqueous suspensions of the fungicides, available as 50% wettable powders, were added, giving a series of 0, 1, 10, 100 and 200 μM, respectively. In order to get consistent results, it was necessary to add the fungicides to the molten agar after cooling to c. 45 °C.

The plates were inoculated by placing a 5 mm-agar disc with young mycelium upside down on the agar surface and incubated at c. 23 °C, unless otherwise indicated. To suppress bacterial growth the medium was supplied with Vendarcin (50 µg/ml; active ingredient oxytetracyclin) except in the case of *Phytophthora cryptogea.* Radial growth was measured in each of three replicates.

Results

The results shown in Table 1 reveal a striking similarity in the antifungal spectra of the four fungicides tested. Resistance to benomyl coincided with resistance to thiophanates, see Oomycetes (No 1 and 2), Mucorales (No 3–5), *Phoma betae* (nr 9), porosporic Moniliales (No 17–19), *Geotrichum* sp. (No 20), *Doratomyces microsporus* (No 33) and *Polyporus betulinus* (No 36). The benomyl-resistant strains of *Botrytis cinerea* (No 16) and *Penicillium brevicompactum* (No 25) were also resistant to all three thiophanates, whereas the randomly chosen isolates of both species were very sensitive to all four fungicides.

The highest sensitivity was recorded for the S-isolate of *Penicillium brevicompactum* (No 24), *Phialophora cinerescens* (No 27) and *Trichophyton terrestre* (No 32), the ED_{50}-values of all four compounds being below 1 μM.

The order of effectiveness of the fungicides was: benomyl > NF 48 > thiophanate-methyl > thiophanate (Table 1, Fig. 1–4). Benomyl was by far the most and thiophanate by far the least active compound. NF 48 was only slightly more active than thiophanate-methyl. For some fungi the differences in antifungal activity of the four

Table 1. In vitro fungitoxic spectrum of benomyl and three thiophanates.

Fungal species tested (names between brackets refer to perfect state)	ED_{50} (in μM) of the fungicide			
	benomyl	thiophanate	thiophanate-methyl	NF 48
OOMYCETES				
1. *Phytophthora cryptogea*	> 200	> 200	> 200	> 200
2. *Pythium debaryanum*	> 200	> 200	> 200	> 200
ZYGOMYCETES				
Mucorales				
3. *Mortierella elongata*	10–100	> 200	> 200	100–200
4. *Mucor racemosus*	> 200	> 200	> 200	> 200
5. *Zygorhynchus moelleri*	> 200	> 200	> 200	> 200
Entomophthorales				
6. *Conidiobolus* cf. *eurymites*	1–10	1–10	1–10	1–10
ASCOMYCETES				
7. *Gaeumannomyces graminis* (syn. *Ophiobolus graminis*) Other Ascomycetes are classified according to their conidial state.	c. 1	1–10	1–10	1–10
DEUTEROMYCETES				
Sphaeropsidales				
8. *Ascochyta lycopersici* (*Didymella lycopersici*)	1–10	c. 200	10–100	10–100
9. *Phoma betae* (*Pleospora bjoerlingii*)	> 200	> 200	> 200	> 200
10. *Phoma doliolum* (*Leptosphaeria doliolum*)	1–10	> 200	10–100	10–100
11. *Phoma eupyrena*	1–10	> 200	c. 100	10–100
12. *Phoma herbarum*	1–10	> 200	10–100	10–100
Melanconiales				
13. *Colletotrichum acutatum*	1–10*	c. 100*	c. 10*	c. 10*
14. *Colletotrichum gloeosporioides*	< 1	1–10	1–10	c. 1
Moniliales				
Blastosporae				
15. *Botrytis cinerea* S (*Sclerotinia fuckeliana*)	< 1	1–10	< 1	< 1
16. *B. cinerea* R	> 200	> 200	> 200	> 200
Porosporae				
17. *Curvularia trifolii*	c. 200	> 200	> 200	> 200
18. *Drechslera avenae* (Pyrenophora avenae)	100–200	> 200	> 200	> 200
19. *Helminthosporium sativum*, syn. *Drechslera sorokiana* (*Cochliobolus sativus*)	100–200	> 200	> 200	> 200

* see, however, Fig. 1.

Fungal species tested (names between brackets refer to perfect state)	ED_{50} (in μM) of the fungicide			
	benomyl	thiophanate	thiophanate-methyl	NF 48
Arthrosporae				
20. *Geotrichum* sp.	> 200	> 200	> 200	> 200
Phialosporae				
21. *Aspergillus flavus*	1–10	100–200	10–100	1–10
22. *Fusarium redolens*	1–10	10–100	10–100	10–100
23. *Gliocladium roseum*	1–10	10–100	c. 10	1–10
24. *Penicillium brevicompactum* S	< 1	c. 1	< 1	< 1
25. *P. brevicompactum* R	> 200	> 200	> 200	> 200
26. *Penicillium vermiculatum* (*Talaromyces vermiculatus*)	1–10	10–100	c. 10	1–10
27. *Phialophora cinerescens*	< 1	< 1	< 1	< 1
28. *Thielaviopsis basicola*	1–10	c. 100	10–100	1–10
29. *Trichoderma viride*	> 1	1–10	1–10	1–10
30. *Verticillium dahliae*	c. 1	c. 10	1–10	1–10
Aleuriosporae				
31. *Gilmaniella humicola*	< 1	1–10	1–10	1–10
32. *Trichophyton terrestre*	< 1	< 1	<1	< 1
Annellosporae				
33. *Doratomyces microsporus*	> 200	> 200	> 200	> 200
MYCELIA STERILIA				
34. *Rhizoctonia solani*	1–10	c. 100	10–100	10–100
BASIDIOMYCETES				
Aphyllophorales				
35. *Fomes annosus*	10–100	> 200	10–100	10–100
36. *Polyporus betulinus*	> 200	> 200	> 200	> 200

Tabel 1. Het fungitoxisch spectrum van benomyl en drie thiofanaten op grond van proeven in vitro.

compounds were much less than for other species (compare the No 6 and 23 on the one hand with No 8, 10 and 12 on the other hand). Hence it was not possible to express their relative effectiveness in consistent ratios, applicable to any given fungal species.

The inhibition of mycelial growth and spore germination by fungicidal agents is often plotted as logarithm of dosis versus probit of percentages of inhibition. In this way as a rule straight lines are obtained. However, the inhibition of growth of many species by the fungicides used in this study did not result in such straight lines. The most striking examples were:

1. *Colletotrichum acutatum.*

At low concentrations of the fungicides mycelial growth was strongly inhibited, but raising the concentrations in the medium above the ED_{50}-doses had hardly any effect (Fig. 1, Table 2). For comparison the inhibition curves of *C. gloeosporioides* are given. Five other isolates of *C. acutatum* responded more or less in the same way. (Table 3). Thus, this remarkable behaviour seems to be characteristic for the entire species.

Fig. 1. Effect of benomyl and thiophanate-methyl on mycelial growth of *Colletotrichum acutatum* and *C. gloeosporioides*. Radial growth was measured after 9 days incubation at 23 °C in each of 3 replicates. In all cases median deviation was < 5% of the average.

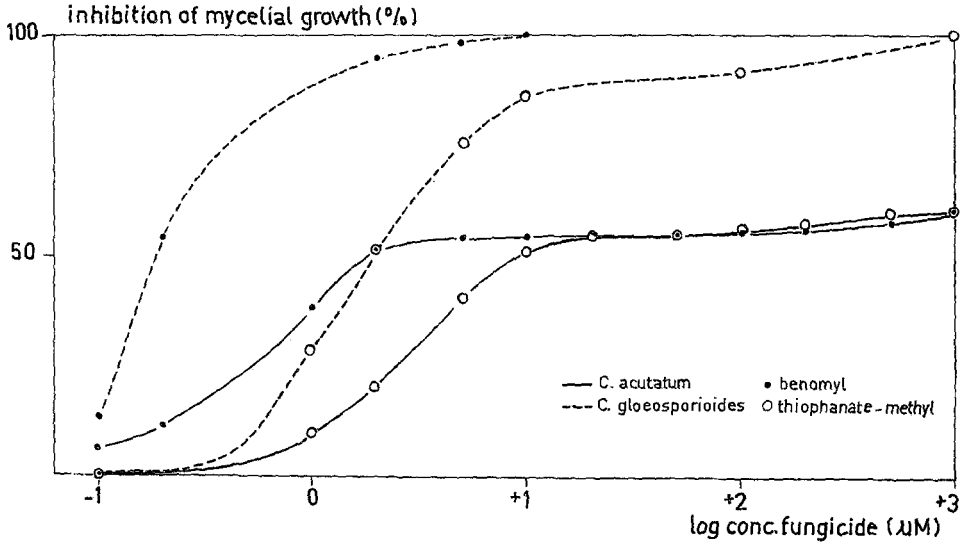

Fig. 1. Invloed van benomyl en thiofanaat-methyl op de myceliumgroei van Colletotrichum acutatum en C. gloeosporioides. De radiale groei werd gemeten in 3 herhalingen na 9 dagen incubatie bij 23 °C. In alle gevallen was de gemiddelde afwijking < 5% van het gemiddelde.

Tabel 2. Effect of benomyl and thiophanates on mycelial growth of *Colletotrichum acutatum*. Radial growth was measured after 9 days incubation at 23 °C in each of 3 replicates. Median deviation was in all cases < 5% of the average.

Concentration of fungicide (µM)	Inhibition of mycelial growth (%)			
	benomyl	thiophanate	thiophanate-methyl	NF 48
1	37.8	9.4	11.6	14.8
10	53.0	46.3	50.8	50.0
100	53.7	49.7	56.7	58.1
200	55.9	51.5	57.2	55.8

Tabel 2. Invloed van benomyl en thiofanaten op de myceliumgroei van Colletotrichum acutatum. De radiale groei werd gemeten in 3 herhalingen na 9 dagen incubatie bij 23 °C. In alle gevallen was de gemiddelde afwijking < 5% van het gemiddelde.

2. *Gliocladium roseum.*

As in the case of *C. acutatum* the fungicides were very effective at low concentrations (Fig. 2). Mycelial growth was maximally suppressed at c. 4, 125 and 30 µ*M* of benomyl, thiophanate-methyl and NF 48, respectively. Increasing the concentrations of benomyl and NF 48 resulted in a decrease of inhibitory activity until at about 50 and 200 µ*M*, respectively, a constant level of c. 75% inhibition had been reached. The curve for thiophanate-methyl also indicates an inversion. Although less pronounced than for *G. roseum*, this inversion was also found in the inhibition of *Ascochyta lycopersici* by the thiophanates (Fig. 4).

The shapes of the dosis-response curve of benomyl and NF 48 show a significant resemblance. This is also evident for *Mortierella elongata* (Fig. 3). At lower concentrations of the fungicides the curves for thiophanate and thiophanate-methyl are more or less parallel, but at higher concentrations they diverge, the activity of thiophanate-methyl increasing more rapidly (Fig. 2 and 4).

Table 3. Effect of benomyl and thiophanate-methyl on mycelial growth of 5 isolates of *Colletotrichum acutatum*. Growth was measured after 7 days incubation at 23 °C in each of three replicates. * Median deviation.

Isolate	Source	Diameter of colony (mm)						
		no fungicide	benomyl (μM)			thiophanate-methyl (μM)		
			1	10	100	1	10	100
1	*Capsicum annuum*	50.7±0.9*	33.5±0.3	15.0±0.3	14.2±0.6	41.3±0.9	32.5±3.0	27.0±0.0
2	*Persea americana*	52.3±1.3	40.0±0.7	34.5±3.0	35.0±3.7	43.0±2.0	40.0±0.0	39.0±0.7
3	*Carica papaya*	16.0±0.7	13.7±0.9	10.3±0.4	9.5±0.3	17.3±1.1	15.3±2.4	12.5±0.3
4	*Fragaria sp.*	55.0±0.0	23.8±1.5	16.3±1.1	15.7±1.1	46.3±1.6	25.3±2.1	18.3±1.1
5	*Coffea sp.*	52.0±0.0	34.0±2.7	16.0±1.0	15.0±0.0	46.3±1.8	33.7±1.8	24.2±1.2

*Tabel 3. Invloed van benomyl en thiofanaat-methyl op de myceliumgroei van 5 isolaten van Colletotrichum acutatum. De groei werd gemeten in 3 herhalingen na 7 dagen incubatie bij 23 °C. *Gemiddelde afwijking.*

Fig. 2. Effect of benomyl and thiophanates on mycelial growth of *Gliocladium roseum*. Radial growth was measured after 7 days incubation at 24 °C in each of 3 replicates. In all cases median deviation was < 5% of the average.

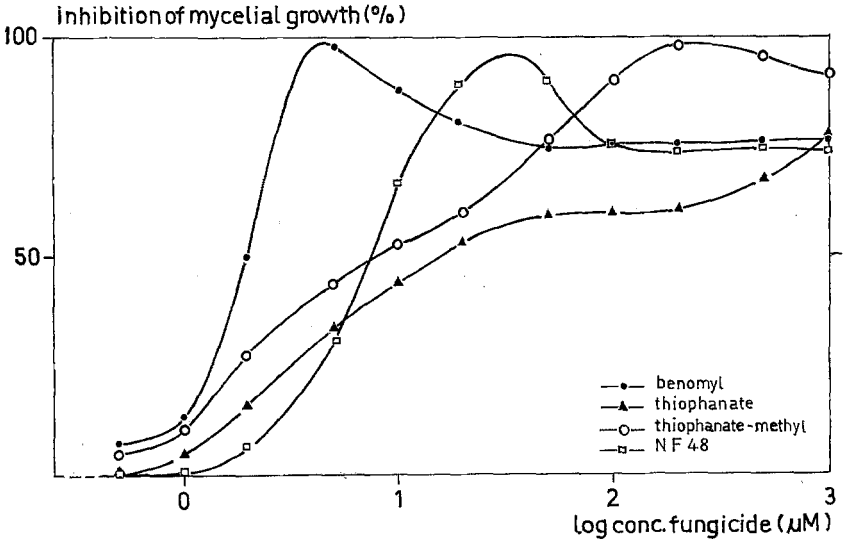

Fig. 2. Invloed van benomyl en thiofanaten op de myceliumgroei van Gliocladium roseum. De radiale groei werd gemeten in 3 herhalingen na 7 dagen incubatie bij 24 °C. In alle gevallen was de gemiddelde afwijking < 5% van het gemiddelde.

Fig. 3. Effect of benomyl and thiophanates on mycelial growth of *Mortierella elongata*. Radial growth was measured after 4 days incubation at 23 °C in each of 3 replicates. In all cases median deviation was < 5% of the average.

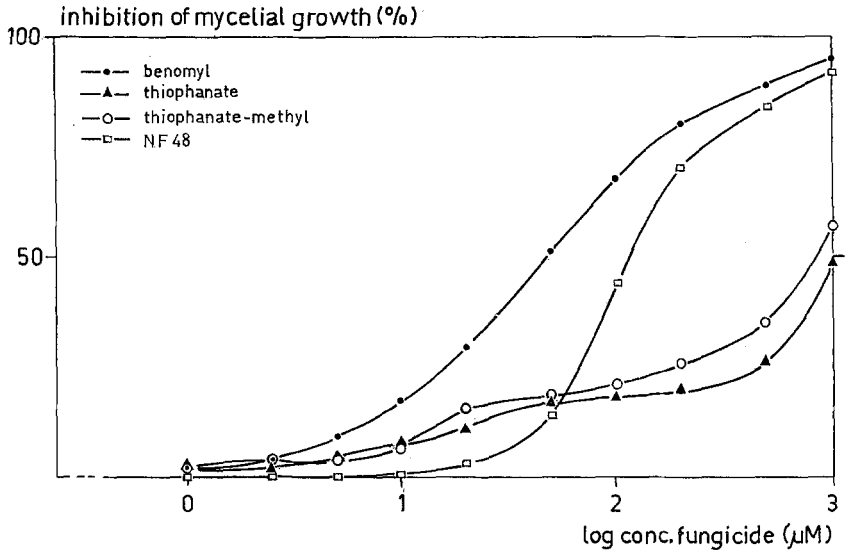

Fig. 3. Invloed van benomyl en thiofanaten op de myceliumgroei van Mortierella elongata. De radiale groei werd gemeten in 3 herhalingen na 4 dagen incubatie bij 23 °C. In alle gevallen was de gemiddelde afwijking < 5% van het gemiddelde.

Fig. 4. Effect of benomyl and thiophanates on mycelial growth of *Ascochyta lycopersici*. Radial growth was measured after 6 days incubation at 20 °C in each of 3 replicates. In all cases median deviation was < 10% of the average.

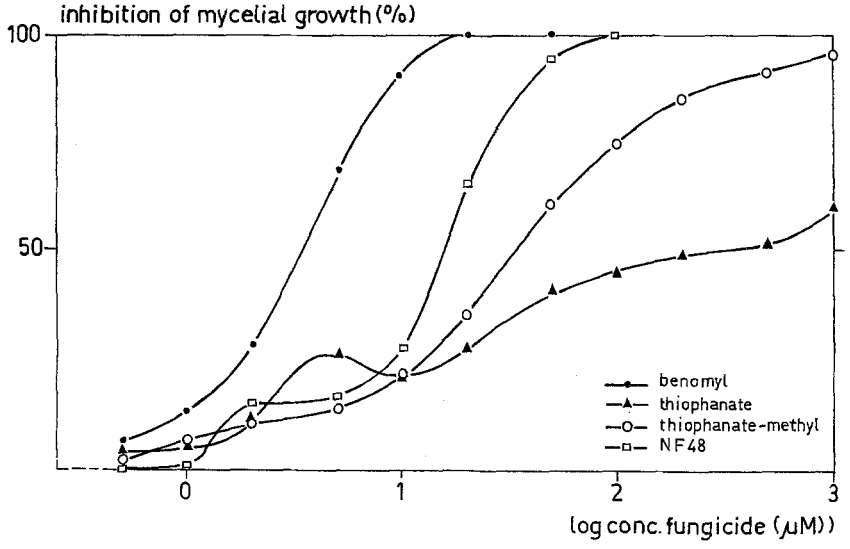

Fig 4. Invloed van benomyl en thiofanaten op de myceliumgroei van Ascochyta lycopersici. De radiale groei werd gemeten in 3 herhalingen na 6 dagen incubatie bij 20 °C. In alle gevallen was de gemiddelde afwijking < 10% van het gemiddelde.

Discussion

While this study was in progress, it was demonstrated by several authors that thiophanate-methyl and NF 48 in aqueous solutions, especially when heated, give rise to 2-benzimidazole carbamic acid, methyl ester, abbreviated as BCM (Selling et al., 1970; Noguchi et al., 1971; Fuchs et al., 1972). This highly fungitoxic compound is also readily formed in aqueous solutions of benomyl (Clemons and Sisler, 1969). In a similar way the somewhat less fungitoxic ethyl ester (BCE) is formed from thiophanate (Vonk and Kaars Sijpesteijn, 1971). In view of these findings the similarity of the antifungal spectra of the four fungicides can easily be understood.

For some fungi, such as *Phoma eupyrena* (Table 1, No 11), the difference in inhibitory activity between thiophanates and benomyl was greater than for other species, for instance *Conidiobolus eurymites* (Table 1, No 6). Working with *Aspergillus niger* and *Cladosporium cucumerinum*, Vonk and Kaars Sijpesteijn (1971) found an increased fungitoxicity and rate of conversion of the thiophanates to BCE and BCM at higher pH. So it is possible that in our tests, in which commercial PDA with relatively low buffering capacity has been used, the fungus by excreting metabolites in the medium and altering its pH, influenced the rate of conversion of the fungicides. Since the inhibitory effect of the thiophanates is only due to BCE or BCM formed, much lower activity of benomyl and thiophanates can be expected for those fungi, which excrete acid products.

In our tests *in vitro* the order of effectiveness was: benomyl > NF 48 > thiophanatemethyl > thiophanate. This order completely reflects the rate of conversion of benomyl and thiophanates at room temperature and neutral pH (Fuchs et al., 1972). However, working with powdery mildew infected pea, barley and cucumber plants the same authors reported a significantly better control with thiophanate-methyl than with NF 48. The order of effectiveness *in vivo* became: benomyl > thiophanate-methyl > NF 48 > thiophanate. They demonstrated that this difference in *in vitro* and *in vivo* activity was due to the final distribution pattern of the fungitoxicant within the plant. Although the latter results are obtained for obligate parasites, it is quite possible that the distinct correlation between the activity *in vitro* and the effectiveness *in vivo* as described for benomyl (Bollen and Fuchs, 1970) is not as clear-cut for the thiophanates, particularly for NF 48.

An inversion of the inhibitory activity against *Gliocladium roseum* at higher concentrations of the fungicides, as shown in Fig. 2 for benomyl, thiophanate-methyl and NF 48 is not unique. The classical example is presented by the type of inhibition of a group of fungi, called 'moulds with inversion growth', by dialkyldithiocarbamates. Kaars Sijpesteijn and Jansen (1959) convincingly demonstrated that in the case of *Aspergillus niger* this conversion was due to formation of two Cu-dimethyldithiocarbamate complexes, differing in toxicity and solubility. To our knowledge, no data about a similar phenomenon for benomyl, the thiophanates or their conversion products are available.

The remarkable dosis-response curve found for *Colletotrichum acutatum* (Fig. 1), showing that inhibition of mycelial growth is not increased at increasing concentrations, can neither be explained with our present knowledge of the fungicides and from their mode of action.

With respect to earlier conclusions on resistance to benomyl of Phycomycetes

(Edgington et al., 1971) or Zygomycetes (Bollen and Fuchs, 1970), the sensitivity of *Conidiobolus eurymites* (Tabele 1, No 6) is noteworthy. No other data on activity of benomyl on Entomophthorales are available, most species belonging to this order being parasites on small soil animals, insects or predaceous fungi and therefore difficult to cultivate on common media. In this respect the saprophytic *C. eurymites* is an exception. Because of the sensitivity of this species to the fungicides tested, the statement that the Zygomycetes are resistant or tolerant to benomyl must be restricted to the Mucorales at most.

Samenvatting

Een vergelijking van de fungitoxische spectra van thiofanaten en benomyl

Het fungitoxisch spectrum van de systemische fungiciden thiofanaat, thiofanaat-methyl en een derivaat hiervan, NF 48, bleek gelijk te zijn aan dat van benomyl. *In vitro* was de volgorde in remmende werking op de myceliumgroei: benomyl > NF 48 > thiofanaat-methyl > thiofanaat. Van deze verbindingen was benomyl verreweg het meest en thiofanaat duidelijk het minst fungitoxisch. Thiofanaat-methyl en NF 48 gaven in dit opzicht slechts weinig verschil te zien.

De invloed van de fungiciden op de myceliumgroei van twee schimmels met een onregelmatig remmingspatroon is weergegeven in de Fig. 1 en 2. Bij geringe concentraties van de fungiciden in het medium werd de myceliumgroei van *Colletotrichum acutatum* reeds tot ruim 50% geremd. Verdere toename van de fungiciden, zelfs tot zeer hoge concentraties (1000 μM), had echter geen sterkere remming ten gevolge. Bij *Gliocladium roseum* bleek de myceliumgroei het sterkst geremd bij lage concentraties (bv. voor benomyl bij ca 4 μM). Verhoging van de concentraties had een afname in remmende werking ten gevolge tot een niveau van 75% remming was bereikt.

In tegenstelling tot de andere Zygomyceten, die tot nu toe op gevoeligheid ten aanzien van benomyl onderzocht werden, bleek *Conidiobolus eurymites*, een saprofyt uit de bodem, gevoelig te zijn voor alle vier fungiciden.

Acknowledgement

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