Neth. J. Pl. Path. 81 (1975) 157–167

Resistance to benzimidazole fungicides in pathogenic strains of Verticillium fungicola

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Accepted 29 April 1975

Abstract

Effectiveness of benzimidazole fungicides in controlling *Verticillium* disease of the commercial mushroom, *Agaricus bisporus*, was closely correlated with the differential in vitro activity. Thiophanate-methyl was an exception as greater disease control was achieved than expected from its in vitro activity.

About one year after clearance for use of benzimidazole fungicides in mushroom growing, resistance in *V. fungicola* (Preuss) Hassebr. (syn. *V. malthousei* Ware) appeared on Dutch farms. In vitro, tests were made to determine the degree of resistance of two isolates, R1 and R2, obtained from diseased sporocarps from benomyl-sprayed crops. Mycelial growth of R2 was considerably less inhibited by MBC-fungicides than that of R1. In vivo, R1 was more pathogenic to mushrooms than a freshly made benomyl-sensitive isolate. Both benomyl-resistant isolates were cross-resistant to other MBC-fungicides and to thiabendazole and cypendazole. Likewise, two new experimental fungicides, imazalil and vinchlozolin, did not provide promising alternatives. When appearance of resistance in *V. fungicola* is suspected on mushroom farms, additional emphasis should be laid on farm hygiene to achieve disease control.

Introduction

Soon after the introduction of benomyl for disease control, Snel and Fletcher (1971) made the suggestion based on results of in vitro experiments, that fungal pathogens of the cultivated mushroom, *Agaricus bisporus* might be controlled by this fungicide. Its effectiveness in controlling two of the most important fungal diseases has since been confirmed. Dry bubble, caused by *Verticillium fungicola* (Preuss) Hassebr. (syn. *V. malthousei* Ware; for nomenclature see Gams, 1971) could successfully be controlled by a single fungicide application after casing (Holmes et al., 1971; Gandy, 1972). Recently, Fletcher et al. (1975) reported excellent control of wet bubble, caused by *Mycogone perniciosa*, after mixing benzimidazole fungicides with the casing soil before casing.

Wuest et al. (1972) even found an increase in yield for one out of four strains of *Agaricus bisporus* after benomyl application to a disease-free crop.

In the Netherlands, about one year after registration of benzimidazole compounds and thiophanate-methyl for application in mushroom growing, these fungicides failed to control dry bubble in some farms. This possibly could indicate tolerance of *Verticillium* to these chemicals. Similar observations in English mushroom farms were reported by Gandy and Spencer (1974).

Tolerance to benomyl of one isolate of *V. fungicola* in vitro had already been signal-

ized by Wuest et al. (1974). This concerned a morphologically atypical isolate, obtained from a white mushroom in 1958. Because the isolation date antecedes the introduction of benomyl, Wuest et al. (1974) concluded that tolerant strains occur in natural populations of *V. fungicola*. In their experiments the isolate was 'neither aggressive nor (a) virulent pathogen'.

The present study deals with the in vitro and 'in vivo' tolerance of isolates obtained from infected mushrooms out of benomyl-treated crops.

Materials and methods

Fungicides. The chemicals used in mushroom growing trials and in in vitro experiments are listed in Table 1. For convenience in comparing the activity of the different benzimidazole fungicides, molecular weights and the weight of each fungicide equivalent to 100 g benomyl have been included. Folcidin and the experimental fungicide BAS 35200F (proposed common name vinchlozolin) were kindly provided by Dr R. Wäckers from Bayer Agrochemie N.V. (Arnhem), and Ir W. J. Scholtens from BASF-Nederland (Arnhem), respectively. Imazalil was obtained from Janssen Pharmaceutica (Beerse, Belgium).

Mushrooms were grown by the single zone system. Standard mushroom trays (0.27 m^2) were filled with 21.6 kg (c. 80 kg/m^2) compost. The compost was peak-heated for 10 days. A maximum air temperature of 57 °C was recorded on the first day. Subse-

Table 1. Fungicides applied.

Trade mark or code	Common name of active ingredient	Molecular weight	Quantity of active ingredient equivalent to 100 g benomyl (g)	Active ingredient in wettable powder (%)
AAzinam	maneb	465	–	80
Systemic fungicides:				
Bavistin	carbendazim = MBC	191	66	50
Benlate	benomyl	290	100	50
Derosal	carbendazim	191	66	60
Folcidin	cypendazole	329	113	50
Lirotect-60	thiabendazole (TBZ)	201	69	60
Topsin-M	thiophanate-methyl (TM)	342	120	70
–	MBC, pure; methyl benzimidazol-2-yl-carbamate	191	66	–
Experimental fungicides:				
BAS 35200 F	vinchlozolin; 3-(3-dichlorphenyl)-5 methyl-5-vinyl-1, 3-oxazolidin-2, 4-dion	286	–	50
R 18 531	imazalil; 1(β-(allyloxy)-2,4-dichlorophenethyl) imidazole nitrate	360	–	100

Tabel 1. Toegepaste fungiciden.

quently, the compost was spawned with a commercial spawn (snow white strain, Sinden A1). The spawning rate was 5 liter spawn/1000 kg compost. Twelve days after spawning the compost was fully colonized by mushroom mycelium. At that time the compost was covered with a 4 cm layer of casing soil, consisting of about 60% black peat, 34% sphagnum peat, 3% sand, and 3% marl. Immediately after casing, fungicides were sprayed (1 liter/m^2) onto the surface of the casing soil.

Nine to twelve days after casing, plots were inoculated with *V. fungicola* by spraying a conidial suspension over it (c. 15×10^5 conidia/m^2). Conidia were obtained from cultures grown on malt agar (Oxoid CM 59, pH 5.9) at 24°C for 2 weeks. During inoculation, the trays used for uninoculated controls were covered with plastic sheets. To prevent adjacent growing rooms from becoming infected, precautions were observed according to Dieleman-van Zaayen (1972).

Mushrooms appeared 19 to 21 days after casing. During the next 5 weeks they were harvested every 2 to 3 days. Healthy mushrooms were weighed in each of 8 (trial 1) and 9 (trial 2) replicates in randomized blocks. Diseased mushrooms were counted. The yields and the number of diseased mushrooms were processed by analysis of variance.

In vitro studies. The four isolates of *V. fungicola* were obtained from diseased sporocarps of *A. bisporus*. S1 isolated by W. Gams in 1969 was kindly provided by the Centraal Bureau voor Schimmelcultures, Baarn, code CBS 992.69. Isolates S2, R1 and R2 were isolated in 1974, R1 and R2 from mushrooms taken from benomyl-sprayed crops in a farm where the fungicide had failed to control *Verticillium*. In vitro sensitivity of mycelial growth of the isolates was tested on malt agar to which aqueous suspensions (1/50 v/v) of the formulated fungicides were added in a series of concentrations. Pure MBC was dissolved in acetone because of its low solubility in water. The suspensions were added to the molten agar cooled to c. 45°C. This was especially done to prevent unequal conversion of thiophanate-methyl (TM) into MBC in the various series, as the conversion rate is highly dependent on temperature (Fuchs et al., 1972). In exp. 1 the plates contained 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 μg benomyl per ml agar. In the test on cross-resistance (exp. 2) the activity of the fungicides was compared for equivalent amounts on molecular base (1, 10 and 100 μM). In exp. 3 the plates contained 1, 10, 20 and 100 μg of the chemicals per ml agar. To suppress bacterial growth, the medium was supplied with 50 μg/ml of oxytetracyclin (commercially available as Vendarcin).

The plates were inoculated with an inverted disc (diam. 5 mm) of agar with mycelium. For *Verticillium* the discs were cut from plates with germinated conidia, seeded one day previously. For *Agaricus* the discs were cut from the periphery of 2 wk-old cultures. The plates were incubated at 22°C (exp. 1 and 2) and 25°C (exp. 3). Radial growth was measured in each of 3 replicates. The percentages of growth inhibition were plotted on a probability scale against the logarithm of the concentrations of the fungicides and the ED_{50} determined. Exp. 1, 2 and 3 were repeated 4, 2 and 2 times, respectively. The data of one representative series of each experiment are presented.

Results

Trials in mushroom growing houses. In the plots inoculated with conidial suspensions

Fig. 1. Symptoms of dry bubble disease in a mushroom crop 3 weeks after the casing soil had been inoculated with a spore suspension of a benomyl-resistant isolate of *V. fungicola*.

Fig. 1. Droge mollen in een champignonteelt 3 weken nadat de dekaarde besmet was met een sporesuspensie van een benomyl-resistent isolaat van V. fungicola.

Table 2. Effect of systemic fungicides on mushroom yield and incidence of *Verticillium* disease during 5 weeks of harvesting in a crop inoculated with a benomyl-sensitive isolate of *V. fungicola*.

Treatment	Quantity applied (g a.i./m²)	Yield (kg/m²)	Number of diseased sporocarps (average/tray)
uninoculated control	–	18.47	74
inoculated:			
no treatment	–	12.57	278
benomyl	0.75	17.06	97
carbendazim (Bavistin)	0.50	18.49	89
cypendazole	0.75	16.15	158
thiabendazole (TBZ)	0.90	12.60	231
thiophanate-methyl (TM)	0.70	14.92	165
thiophanate-methyl (TM)	1.05	16.47	114
thiophanate-methyl (TM)	1.40	17.84	62
L.S.D. P = 0.05		2.15	54
P = 0.01		2.96	75

Tabel 2. Invloed van systemische fungiciden op de opbrengst aan champignons en het optreden van Verticillium-ziekte gedurende 5 plukweken in een teelt die met een benomyl-gevoelig isolaat van V. fungicola was besmet.

brown spots on the mushroom pilei occurred in the first week of harvesting. Deformed sporocarps were present from the second week of harvesting onwards (Fig. 1).

Trial 1. In this trial the effectiveness of benzimidazole fungicides and TM were compared in controlling *Verticillium*. The trial was performed when tolerance to benomyl was not yet in the picture. The results are shown in Table 2. Applications of fungicides to the inoculated plots, with the exception of TBZ, resulted in a significant increase of yield. In some instances the same or nearly the same yield as in uninoculated plots was obtained.

The number of diseased mushrooms in the uninoculated control plots demonstrates that infection by *V. fungicola* could not be kept restricted to the inoculated plots only. Diseased sporocarps in the uninoculated plots were found particularly in the last week of the crop. The infection is probably due to conidial dispersion during watering. In the experimental design the plots were distributed at random. Hence, control trays often adjoined inoculated ones.

Trial 2. Isolate R1 was used for inoculation. The yields of various plots are shown in Table 3. Benomyl, whether or not combined with maneb, completely failed to control the disease. The fungicide even promoted the incidence of diseased mushrooms.

Experiments in vitro. Isolate R2 like isolate R1 was obtained from diseased sporocarps from a crop in a farm, where application of benomyl did not result in any control of dry bubble. The morphological characteristics of both isolates conformed with those of *V. fungicola* as described by Gams (1971).

Experiment 1. Effect of benomyl on mycelial growth of the four isolates of V. fungicola. The sensitivity to benomyl of isolates S1 and S2 was markedly different from that of R1 and R2 (Table 4). Isolates R1 and R2 also differed in degree of resistance. Likewise the sensitivity of the S-isolates differed, S2 being less sensitive than S1.

Table 3. Effect of benomyl on mushroom yield and incidence of *Verticillium* disease during 4 weeks of harvesting in a crop inoculated with a benomyl-resistant isolate (R1) of *V. fungicola.*

Treatment and quantity applied (g a.i./m²)	Yield (kg/m²)	Number of diseased sporocarps (average/tray)
uninoculated control	16.08	65
inoculated:		
no treatment	4.32	248
benomyl (0.75)	4.42	331
maneb spray (0.40)	5.37	284
benomyl (0.75) + maneb spray (0.40)	3.92	336
L.S.D. P = 0.05	1.81	49
P = 0.01	2.48	67

Tabel 3. Invloed van benomyl op de opbrengst aan champignons en het optreden van Verticillium-ziekte gedurende 4 plukweken in een teelt die met een resistent isolaat (R1) van V. fungicola was besmet.

Table 4. Differential sensitivity to benomyl of mycelial growth of S and R isolates of *Verticillium fungicola*. Radial growth was measured after 9 days incubation at c. 22 °C in each of 3 replicates.

Isolate	ED_{50} (μg a.i./ml)	Highest concentration at which mycelial growth was observed
S1	1.0	2
S2	1.2	5
R1	13	500
R2	200	5000

Tabel 4. Gevoeligheid voor benomyl van de myceliumgroei van de S- en R-isolaten van Verticillium fungicola. De radiale groei werd gemeten in 3 herhalingen na 9 dagen incubatie bij ca. 22 °C.

Table 5. Effect of benzimidazole fungicides and thiophanate-methyl on mycelial growth of S and R isolates of *Verticillium fungicola*. Radial growth was measured after 9 days incubation at 22 °C in each of 3 replicates. Standard deviations varied from 2 to 13 % of the mean values.
+) very restricted mycelial growth (< 1 mm) from the agar disc onto the fungicide-amended agar.

Fungicide	Conc. of fungicide (μM)	Mean diameter of colonies excl. inoculum discs (mm)			
		S1	S2	R1	R2
no fungicide					
water (1/50 v/v)		31.8	29.2	31.5	29.5
acetone (1/50 v/v)		33.0	28.3	33.0	29.4
benomyl	1	25.9	27.8	30.0	28.4
	10	+	4.0	26.2	29.2
	100	0	0	7.7	20.2
MBC (pure)	1	9.3	14.8	32.0	31.0
	10	0	0	23.2	29.2
	100	0	0	+	22.1
carbendazim = MBC	1	32.3	29.6	32.8	32.0
(Derosal formulation)	10	0	+	28.8	32.4
	100	0	0	16.7	29.2
carbendazim = MBC	1	26.4	24.0	31.0	28.1
(Bavistin formulation)	10	0	+	26.0	27.0
	100	0	0	14.3	29.2
cypendazole	1	24.8	24.0	31.6	33.2
	10	0	0	26.0	28.0
	100	0	0	8.8	24.4
TBZ	1	32.1	28.1	31.9	30.3
	10	9.0	20.3	33.5	32.1
	100	0	0	22.3	23.9
TM	1	29.2	28.6	30.2	23.8
	10	24.0	29.0	31.2	29.8
	100	3.2	23.2	28.8	26.8

Tabel 5. Invloed van benzimidazoolfungiciden en thiofanaat-methyl op de myceliumgroei van de S- en R-isolaten van Verticillium fungicola. De radiale groei werd gemeten in 3 herhalingen na 9 d incubatie bij 22 °C. De standaardafwijkingen varieerden van 2 tot 13 % van de gemiddelde waarden.
+) enige myceliumgroei (< 1 mm) vanuit het ponsstukje over de fungicidehoudende agar.

Fig. 2. Effect of benzimidazole configuration fungicides on S and R isolates of *V. fungicola*. Concentration of the fungicides (a.i.) in the medium 1, 10 and 100 μMol. A) benomyl (Benlate); B) MBC; C) carbendazim (Derosal); D) carbendazim (Bavistin); E) cypendazole (Folcidin); F) thiabendazole (Lirotect-60); G) thiophanate-methyl (Topsin-M). Control above.

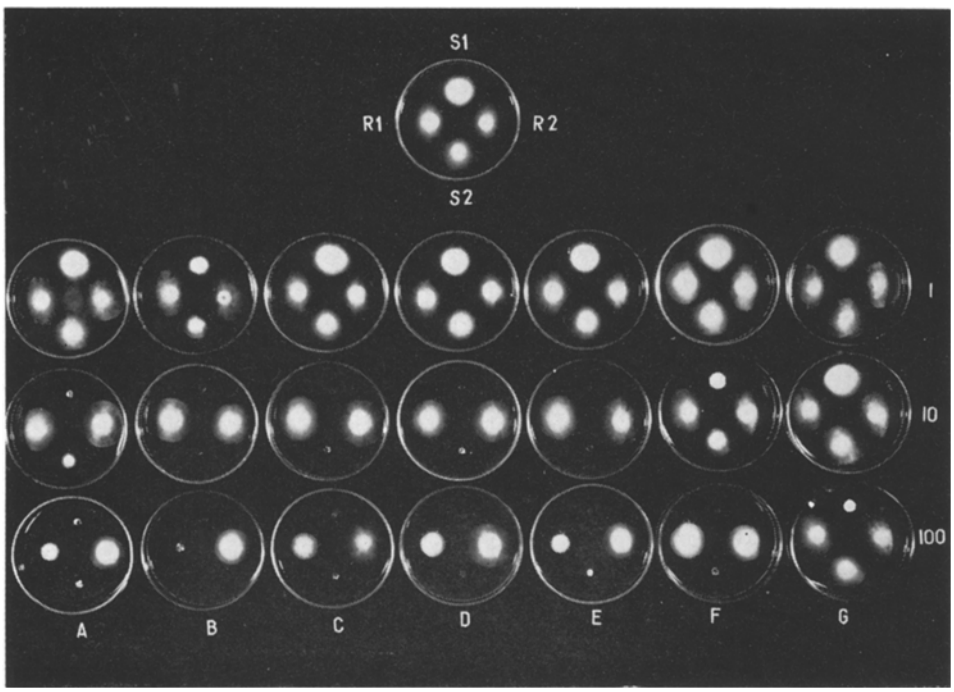

Fig. 2. Invloed van fungiciden met een benzimidazoolconfiguratie op S- en R-isolaten van V. fungicola. Fungicideconcentratie (actieve stof) in het medium 1, 10 en 100 μMol.

Experiment 2. Test on cross-resistance. The isolates used in the previous experiment were tested for their sensitivity to two other MBC-fungicides, carbendazim and TM, registered for application in mushroom growing in the Netherlands. The benzimidazole compounds cypendazole and TBZ, which are not used for *Verticillium* control in mushroom growing, were also included. TBZ was chosen because some of the laboratory induced benomyl-resistant isolates of *Aspergillus nidulans* and *A. niger* were still sensitive to TBZ (Van Tuyl, 1975). Table 5 and Fig. 2 show the intrinsic activity of the various compounds. Mycelial growth of both R-isolates was resistant to all of the 7 fungicides. The isolates showed the same response as to benomyl namely S1 was more sensitive than S2 and R2 more resistant than R1.

Experiment 3. Sensitivity of Agaricus spp. and V. fungicola to other chemicals. Differential sensitivity of *A. bisporus* and *A. bitorquis* as well as that of an R- and an S-isolate of *V. fungicola* were tested to two experimental fungicides, imazalil and vinchlozolin. The mycelial growth of both *Agaricus* species was more inhibited than that of the isolates of *V. fungicola*.

Discussion

The effectiveness of disease control by the fungicides was closely related with the differential in vitro activity to *V. fungicola* (Table 2 and 5, Fig. 2). An exception was found for TM which was more effective in controlling the disease than would be expected from its low in vitro activity. This is especially evident, when its activity to *V. fungicola* is compared with that of TBZ (Fig. 2, F and G). On agar, mycelial growth was more inhibited by TBZ than by TM, but the effectiveness in disease control turned out to be inverse (Table 2). If, like in the in vitro experiments on cross-resistance, equivalent amounts of active units of both fungicides (in μMol) are compared in their effectiveness, this is even more obvious. On the base of the molecular weight, the equivalent of 0.90 g TBZ/m^2, used in trial 1 (Table 2), is 1.53 g TM/m^2. Application of even the lowest dose of TM, i.e. 0.70 g/m^2, resulted in a significant increase of yield, whereas that of 0.90 g TBZ/m^2 did not.

The low in vitro activity of TM to *V. fungicola* may by due to a slow conversion rate of the chemical into MBC. Because this conversion rate is highly dependent on temperature (Fuchs et al., 1972), the chemicals were added to the molten agar cooled to at least 45 °C. Since mycelial growth on the MBC-plates was considerably more inhibited than on agar provided with equivalent amounts of TM (Fig. 1, G and B) it can be concluded that only minimal amounts of MBC had been formed from TM during preparation of the plates.

In soil, TM is readily converted to MBC. Five days after adding TM to two field soils (pH 5.7 and 5.9, respectively) in dosages near those recommended for disease control, Kosaka et al. (1972) found that nearly all methanol-extractable fungicide was present in the form of MBC. In casing soil, conversion of TM into MBC is probably even more rapid than in the field soils used by Kosaka et al. for two reasons. The pH of the casing soil (pH 7.1) was higher and, as has been shown by Kaars Sijpesteijn (1972), the rate of MBC formation from TM strongly increased with pH in the range of pH 5 to pH 7. Secondly, in casing soil microbial activity is probably higher than in normal field soils. Kosaka et al. (1972) found that MBC was very slowly formed from TM in autoclaved field soils. Therefore, they ascribed the rapid conversion of TM into MBC in natural soils mainly to microbial activity. Hence, in casing soil a high rate of conversion can be assumed.

Unlike in the benomyl-resistant isolate described by Wuest et al. (1974), in our isolates R1 and R2 resistance to benomyl is associated with pathogenicity to mushrooms. Both isolates were obtained from heavily infected benomyl-sprayed crops. Moreover, the pathogenicity of isolate R1 has been reconfirmed in trial 2.

Since their resistant non-pathogenic strain of *Verticillium* was isolated before the introduction of benomyl, Wuest et al. concluded that resistance can occur in the original population of the fungus. The amount of the benzimidazole fungicides used in mushroom growing will be crucial for selection of the resistant strains.

Fig. 3 depicts the effects of benomyl on incidence of the disease and on crop yield in the presence of a sensitive and a resistant isolate of *V. fungicola*, as these were found in trials 1 and 2. Because similar environmental conditions and equal rates of inoculum were applied, the results of trial 1 are comparable with those of trial 2. A comparison of yield reductions in the plots S2,0 and R1,0 indicates that isolate R1 was more pathogenic than S2.

Fig. 3. Differential effect of benomyl on mushroom yield; the casing soil was inoculated with a sensitive and a resistant isolate of *V. fungicola*, respectively. 0) no fungicide; B) benomyl (0.75 g a.i./m²); R1 and S2) inoculated with benomyl-resistant isolate R1 and benomyl-sensitive isolate S2. Trials 1 and 2 were done in 8 and 9 replicates, respectively. * and ** L.S.D. for P = 0.05 and P = 0.01.

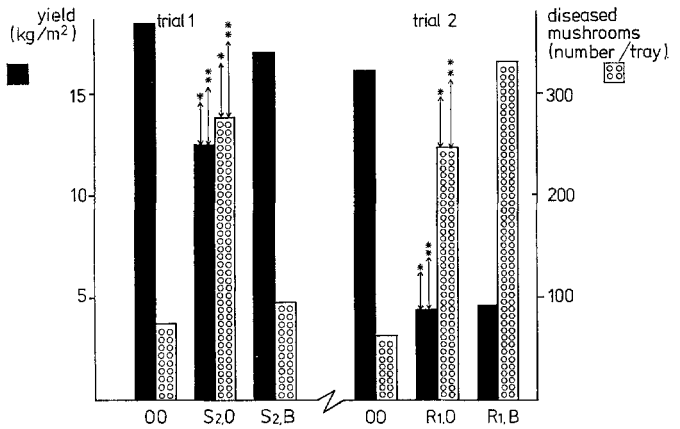

Fig. 3. Invloed van benomyl op de opbrengst aan champignons na besmetting van de dekaarde met een gevoelig resp. een resistent isolaat van V. fungicola.

The unequal degree of resistance in vitro found for the isolates R1 and R2 seems to be an exception rather than the rule for resistant isolates occurring under field conditions. Harding (1972) tested TBZ-resistant isolates of *Penicillium digitatum* and *P. italicum*. Four isolates of *P. italicum* were equally resistant to TBZ and, to a lesser extent, equally resistant to benomyl. Within eight isolates of *P. italicum* two levels of resistance to TBZ were found. These isolates were not cross-resistant to benomyl. In *P. brevicompactum* and *P. corymbiferum* both benomyl-resistant isolates of each species tested were resistant at the same level (Bollen, 1971). Jarvis and Hargreaves (1973) reported that all isolates of *Botrytis cinerea* obtained from raspberry and strawberry, where standard benomyl sprays did not control grey mold, grew immediately on 1000 μg/ml benomyl agar. In a study of the incidence of tolerant strains of *B. cinerea* on glasshouse crops, Miller and Fletcher (1974) found 7 tolerant in a total of 41 isolates. The resistance of one of these was intermediate between sensitive isolates and the very resistant ones.

After UV irradiation of sensitive strains of 10 fungi, Van Tuyl (1975) obtained resistant isolates in all species. Although he found that in *Aspergillus nidulans* and *A. niger* one gene was responsible for benomyl resistance, in all species the degree of resistance of different mutants varied from low to high levels. Under field conditions strains with different levels of resistance may be present like the mutants after UV irradiation in the laboratory. Whether or not only the most resistant strain of a saprophytic fungus will dominate is dependent on the amount of fungicide present in the substrate. The occurrence of a pathogenic fungus in a benomyl-sprayed crop depends on its pathogenicity along with its benomyl-resistance (cf. Wuest et al., 1974).

Because of cross-resistance with other benzimidazole configuration fungicides and the higher sensitivity to recently developed fungicides of mycelial growth of *Agaricus* spp. than that of *V. fungicola*, at present no alternatives for effective chemical control

of resistant *Verticillium* strains are known. Therefore, to control these strains, emphasis has to be placed on farm hygiene including partial sterilization of the casing soil by formaldehyde as carried out before the introduction of the selective fungicides.

Acknowledgments

We would like to thank Miss Martje Verf and Mr Theo Staaks for technical assistance.

Samenvatting

Resistentie tegen benzimidazoolfungiciden in pathogene stammen van Verticillium fungicola

De mate, waarin *V. fungicola* in de champignonteelt door verschillende benzimidazoolfungiciden wordt bestreden, is gecorreleerd met de remmende werking op de myceliumgroei in vitro. Thiofanaat-methyl vormt daarop een uitzondering. De ziekte werd door dit fungicide beter bestreden dan op grond van de remming van de myceliumgroei kon worden verwacht.

Ongeveer een jaar na de toelating van benzimidazoolfungiciden in de champignonteelt trad in de praktijk resistentie in *V. fungicola* op. Al in 1973 hadden Amerikaanse onderzoekers (Wuest et al., 1974) resistentie gevonden in een isolaat van deze schimmel. Dit isolaat bleek echter niet pathogeen voor de champignon te zijn.

De mate van resistentie van twee isolaten, afkomstig van zieke champignons van bedrijven waar toepassing van benomyl geen bestrijding van de ziekte tengevolge had, bleek zeer ongelijk (Fig. 2). In inoculatieproeven bleek het matig resistente isolaat zeer pathogeen. Ook in een fungicidevrije teelt was de aantasting na inoculatie met dit isolaat groter dan na inoculatie met een pas geïsoleerd benomyl-gevoelig isolaat (Fig. 3).

De beide benomyl-resistente isolaten waren eveneens resistent tegen andere MBC-fungiciden en tegen thiabendazool en cypendazool. Twee nieuwe experimentele fungiciden boden evenmin een veelbelovend alternatief. Daarom zal in de bestrijding van droge mollen, met name waar men resistentie vermoedt, het accent moeten blijven liggen op de hygiëne.

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