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Acquired resistance to benomyl and some other systemic fungicides in a strain of Botrytis cinerea in cyclamen

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Abstract

A benomyl-resistant strain (R) of *Botrytis cinerea* was isolated from cyclamen that had been sprayed with relatively high doses of Benlate two weeks before. In vitro mycelial growth of this strain was less inhibited on PDA containing 1000 μg/ml benomyl (Benlate, 50% W.P.) than that of another, wild isolate of *B. cinerea* from cyclamen on PDA with 0.5 μg/ml of the fungicide.

The R-strain was also resistant to methyl-thiophanate, furidazol and to a lesser extent to thiabendazole. Mycelial growth of 5 other isolates was much more inhibited by benomyl than by thiabendazole.

Resistance was retained for at least 20 weeks after repeated subculturing on fungicide-free agar.

Introduction

Botrytis cinerea is one of the most serious pathogens in nurseries of cyclamen, causing soft rot at the base of the petioles. In order to prevent spread of *B. cinerea* in the hearts of the plants, the diseased parts must be carefully removed, especially when the plants are used for seed production. This removing can be omitted since the time benomyl has been introduced, for this fungicide proved to be very useful in controlling the disease, when applied as a spray of 0.15% Benlate (50% W.P.). Also in other flower crops this fungicide was found to be very effective against *B. cinerea,* for instance in geranium (Manning and Glickman, 1969). In vitro too, mycelial growth of this fungus was inhibited by benomyl (Bollen and Fuchs, 1970).

In a nursery in the Netherlands, where benomyl had been used only three times viz in October 1969 and subsequently, in the next crop, in April and June 1970, infections of *B. cinerea* were observed on cyclamen in September 1970. Two sprays, with a fortnight's interval, of 0.15% and 0.25% aqueous suspensions respectively, failed to control the soft rot. On the contrary, losses reached unprecedented proportions. After parts of diseased petioles had been plated out, all pieces revealed colonies of *B. cinerea.* Hence, the question arose whether this strain of *B. cinerea* was resistant or whether benomyl had not been taken up by the plant. Therefore benomyl and some other systemic fungicides were tested in vitro as to their effectiveness against this pathogen.

Materials and methods

The isolates. The isolates of *B. cinerea* used were obtained from petioles of cycla-

men, flowers of dahlia and sunflower, leaves of lettuce and from greenhouse soil. The pathogens were isolated by placing diseased parts after surface sterilization with sodium hypochloride (1%) onto potato dextrose agar (PDA), to which Vendarcin (50 μg/ml; active ingredient oxytetracyclin) was added to suppress bacterial growth; in the case of the benomyl-resistant strain Benlate was also added (5 μg/ml). The isolate from soil was obtained using the soil dilution plate technique.

In all experiments hyphal-tip cultures of these isolates were used.

The fungicides. For in vitro tests the following systemic fungicides were used. Benlate 50% W.P. (benomyl; 1-(butylcarbamoyl)-2-benzimidazole carbamic acid, methyl ester), NF 44 50% W.P. (methyl thiophanate; 1,2-bis-(3′-methoxycarbonyl - 2′-thioureido)-benzene), Bay 33173 50% W.P. (furidazol; 2-(2′-furyl)-benzimidazole) and Tecto 90% W.P. (thiabendazole; 2-(4-thiazolyl)-benzimidazole).

The in vitro activity against fungi was tested at 25 °C on PDA (Oxoid CM 139; pH 5.6) to which aqueous suspensions of the fungicides (all concentrations based on 50% active ingredient) were added to give a series of concentrations required. To obtain consistent results it was necessary to add the fungicides to the molten agar after cooling to 45 °C. The PDA-plates were inoculated by placing 5 mm discs of agar with young mycelium upside down in the centre. Growth of mycelium was measured in each of three replicates.

Effects of benomyl and thiabendazole on mycelial growth of six isolates of *B. cinerea* were also determined by inoculating agar discs with mycelium of the isolates at equal distances from the centre of large petri dishes (diam. = 15 cm; Fig. 2).

Assay of benomyl. In order to detect benomyl or its conversion product BCM (2-benzimidazole carbamic acid, methyl ester) in the petioles of cyclamen, these parts were squeezed out after having been washed thoroughly to remove the fungicidal remains possibly still present on the surface after the last spray. The fungistatic substances were estimated in the sap using the thin-layer chromatographic bioassay described by Homans and Fuchs (1970), with *Penicillium frequentans* as test organism.

Results

After having been plated out onto PDA with benomyl (5 μg/ml), all 20 pieces of tissue taken from petioles of cyclamen, sprayed with the fungicide two weeks before, yielded colonies of *Botrytis cinerea.* They resembled each other so closely that we surmized that they belonged to the same strain, further to be indicated R. The rate of mycelial growth was considerably lower than that of an isolate (S) of *B. cinerea* obtained from petioles of an unsprayed cyclamen on benomyl-free PDA.

Chromatography of the sap from petioles of the benomyl-treated plants revealed that this contained 0.05 to 0.1 μM BCM (Rf 0.45 in ethyl-acetate). Benomyl itself could not be detected. This is in accordance with literature data, e.g. Sims et al. (1969). The sap from unsprayed cyclamen did not yield any compound toxic to the test-fungus *P. frequentans.* Thus, although the concentration of BCM was rather low, it was shown that benomyl or BCM had been taken up by the diseased cyclamen plants.

Fig. 1. Inhibition of mycelial growth of R-strain and S-isolate of *Botrytis cinerea* by benomyl.

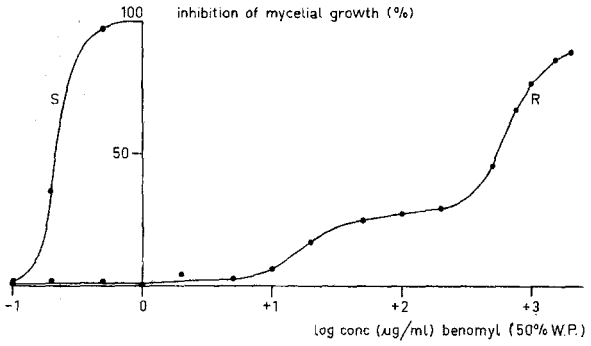

Fig. 1. Remming van de myceliumgroei van de R-stam en het S-isolaat van Botrytis cinerea door benomyl

The effect of benomyl on growth of strain R and isolate S is shown in Fig. 1 and Table 1.

It is obvious that the R-strain was very resistant to the fungicide. Even with 1000 µg/ml Benlate mycelial growth of this strain on PDA was less inhibited than that of the S-isolate on PDA with 0.5 µg/ml of the fungicide.

A comparison of the sensitivity to benomyl of six *B. cinerea* isolates revealed that only the R-strain was resistant. Growth of the other isolates was completely inhibited at a concentration of 1 µg/ml Benlate (Fig. 2).

In order to establish whether the R-strain was also resistant to other systemic

Table 1. Effect of benomyl on mycelial growth of a benomyl-resistant strain (R) and another isolate (S) of *Botrytis cinerea* on PDA. The cultures were incubated at 25 °C.

Conc. of Benlate 50% W.P.[1] (µg/ml)	Diameter of colony (mm)		Conc. of Benlate 50% W.P. (µg/ml)	Diameter of colony (mm)	
	R (5 days)	S (4 days)[2]		R (5 days)	S (4 days)
0	72.3 ± 1.6[3]	84.7 ± 1.3	50	54.7 ± 1.8	0
0.1	71.2 ± 1.8	83.3 ± 0.9	100	52.7 ± 1.8	0
0.2	70.7 ± 0.9	54.3 ± 9.2	200	51.2 ± 0.8	0
0.5	71.0 ± 1.3	4.0 ± 0	500	39.7 ± 0.4	0
1.0	72.2 ± 0.3	0	750	23.7 ± 1.1	–
2.0	69.3 ± 0.9	0	1000	14.7 ± 0.4	–
5.0	70.3 ± 0.4	0	1500	10.0 ± 0.3	–
10	67.0 ± 3.3	0	2000	8.2 ± 0.6	–
20	60.0 ± 2.7	0			

[1]The concentration of the active ingredient, benomyl, can be obtained by dividing the figures by two.
[2]It was not possible to measure the diameter after 5 days of incubation because of the size of the petri dishes (diam. = 90 mm).
[3]Median deviation.

Tabel 1. Invloed van benomyl op de myceliumgroei van een stam R, resistent tegen benomyl, en een willekeurig isolaat S van Botrytis cinerea op aardappel-glucose-agar. De cultures werden geïncubeerd bij 25 °C.

Fig. 2. Growth of six isolates of *Botrytis cinerea* in presence of benomyl and thiabendazole. C-PDA without fungicide; B1 and B100 – PDA with 1 and 100 μg/ml benomyl (50% W.P.); T1 and T100 – PDA with 1 and 100 μg/ml thiabendazole (50% W.P.). For explanation of number of isolate see Table 3. N.B. In this experiment isolate 5 shows a small colony of the 'sporulating type' (see text).

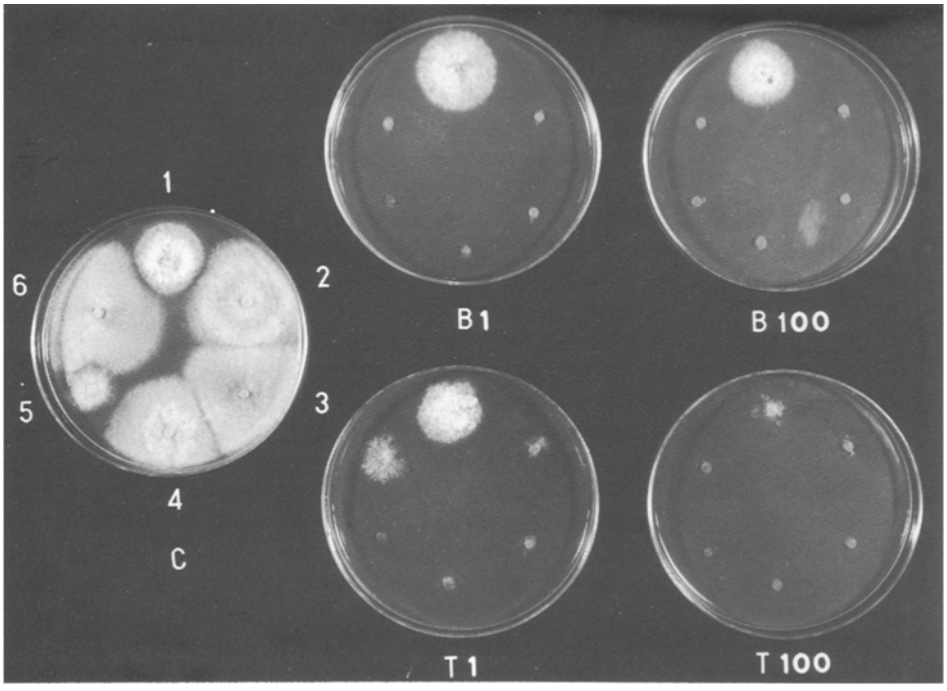

Fig. 2. De groei van zes isolaten van Botrytis cinerea in aanwezigheid van benomyl en thiabendazol. C–PDA zonder fungicide; B1 en B100 – PDA met 1 en 100 μg/ml benomyl (50% W.P.); T1 en T100 – PDA met 1 en 100 μg/ml thiabendazol (50% W.P.). Zie Tabel 3 voor de verklaring van het nummer van het isolaat. N.B. In deze proef toont isolaat 5 een kleine kolonie van het 'sporulerende type' (zie de tekst).

fungicides, this strain and the S-isolate were tested against two other derivatives of benzimidazole and one thiophanate. For comparison an isolate of the very sensitive species *Trichoderma viride* was included in this experiment. From the results, shown in Table 2, it appears that the R-strain was even more resistant to methyl-thiophanate, to a lesser extent to furidazol and only slightly resistant to thiabendazole, although it was not as sensitive to this fungicide as the S-isolate or the isolate of *Trichoderma*.

As can be seen in Fig. 2 the isolates differed in their sensitivity to thiabendazole. Therefore, the latter was further tested in a second experiment (Table 3). Here, the isolate from sunflower was the most sensitive one. The isolates 2 through 6, unlike strain R, were more sensitive to benomyl than to thiabendazole.

The exact quantitative determination of inhibition of mycelial growth by the fungicides was sometimes difficult in the case of the resistant strain and the isolate from sunflower, because of the appearance of sectors with very slowly growing mycelium associated with abundant sporulation. This occurred as well on PDA supplied with fungicides as on this medium without these substances. The ap-

Table 2. Effect of four systemic fungicides on mycelial growth of *Botrytis cinerea* (R and S see text) and *Trichoderma viride* (T).

Concentration (μg/ml) wettable powder, based on 50% active ingredient	Inhibition of mycelial growth (%)					
	benomyl			*methyl-thiophanate*		
	R	S	T	R	S	T
1	0	100	90.8 ± 0.5	0	17.4 ± 3.4	13.7 ± 0.5
10	6.3 ± 1.0	100	100	9.4 ± 1.0	100	79.2 ± 1.0
100	23.6 ± 3.6	100	100	16.3 ± 1.3	100	100
500	46.9 ± 1.9	100	100	31.3 ± 0.9	100	100
1000	87.8 ± 0.4	–	–	39.9 ± 1.7	–	–
	furidazol			*thiabendazole*		
	R	S	T	R	S	T
1	0	13.5 ± 3.4	10.6 ± 3.0	13.5 ± 0.7	92.9 ± 2.1	66.6 ± 0.5
10	16.3 ± 2.5	95.7 ± 0.8	76.7 ± 0.8	25.8 ± 2.8	100	100
100	57.1 ± 4.2	100	100	90.7 ± 3.0	100	100
500	88.3 ± 1.6	100	100	100	100	100
1000	100	–	–	100	–	–

Tabel 2. Invloed van vier systemische fungiciden op de myceliumgroei van Botrytis cinerea (R en S, zie de tekst) en Trichoderma viride (T).

pearance of sectors with slowly growing mycelium does not seem to be related with resistance to the fungicides.

The R-strain retained its resistance to benomyl for at least 20 weeks when sub-cultured weekly.

Table 3. Effect of benomyl and thiabendazole on mycelial growth of six isolates of *Botrytis cinerea* on PDA.

B. cinerea isolated from	Diameter of colony (mm) after 3 days at 25 °C		
	no fungicide	benomyl 0.5 μg/ml[1]	thiabendazole 0.5 μg/ml[1]
1 cyclamen (R)	39.0 ± 1.3	42.5 ± 1.3	37.5 ± 1.0
2 cyclamen (S)	58.7 ± 0.4	3.0 ± 0	52.2 ± 1.6
3 dahlia	52.0 ± 2.0	12.0 ± 3.3	44.7 ± 1.6
4 lettuce	50.0 ± 1.3	22.0 ± 0.7	44.7 ± 0.9
5 sunflower	50.7 ± 1.2	3.3 ± 0.9	38.7 ± 5.8
6 soil	53.0 ± 0.7	20.0 ± 0	46.0 ± 0.8

[1] The concentrations given are based on 50% wettable powder.

Tabel 3. Invloed van benomyl en thiabendazol op de myceliumgroei van zes isolaten van Botrytis cinerea op aardappel-glucose-agar.

Discussion

Although most fungal pathogens are known to be sensitive to benomyl, there are some groups of fungi, which are resistant to this fungicide (Bollen and Fuchs, 1970;

Edgington and Khew, 1970; Edgington et al., 1971). Until now, in practice only very few examples of acquired resistance against this fungicide are known. Schroeder and Provvidenti (1969) described resistance of cucurbit powdery mildew, presumably incited by *Sphaerotheca fuliginea,* in the United States. In Israel Netzer et al. (1970) reported that benomyl-treated pepper plants became heavily infected with powdery mildew, *Oidiopsis taurica.*

Working under laboratory conditions, Bartels and MacNeill (1970) obtained some induced resistance to benomyl, thiabendazole and furidazol among UV-induced mutants of *Fusarium oxysporum* f. sp. *melonis.* The fungus could tolerate 10, 30 and 80 μM, respectively, growing on potato sucrose agar. However, our resistant strain of *B. cinerea,* growing on PDA, tolerated at least 2000 μg/ml Benlate 50% W.P., which corresponds to 3448 μM benomyl. In this case the level of acquired resistance is similar to that of the group-specific resistance as for instance within Oomycetes, Mucorales etc. (Bollen and Fuchs, 1970; Edgington et al., 1971).

Resistance of *B. cinerea* to fungicides is not uncommon. Parry and Wood (1959 a, b) reported adaptation to captan and ferbam. Webster et al. (1970) obtained variants of *B. cinerea* resistant to DCNA (Botran; 2,6 dichloro-4-nitroaniline). Some of their isolates also showed resistance to other fungicides, but not to benomyl. It would be of particular interest to carry out cross-resistance tests with the R-strain in order to determine whether this strain is also resistant to the same fungicides towards which the *B. cinerea* strains of Webster and co-workers are resistant. In experiments on chemical control of the benomyl-resistant *Botrytis* in cyclamen, dichlofluanide (Eupareen) and even more the recently developed fungicide dichlozoline (Sclex) turned out to be very effective (Scholten and Bollen, 1971). Thus there does not exist cross-resistance with these fungicides.

Because of the sudden appearance of the resistant strain in practice [1] and the large difference in sensitivity to benomyl as compared to other *B. cinerea* isolates the strain might have arisen by mutation (sensu lato).

The order of effectiveness of the fungicides used, starting with the most effective one, was as follows (see Table 2):

B. cinerea, R: thiabendazole - furidazol - *benomyl* - methyl-thiophanate;

B. cinerea, S and *Trichoderma viride: benomyl* - thiabendazole - methyl-thiophanate - furidazol.

Although the R-strain is resistant to all four systemic fungicides, its resistance to benomyl is more striking than to the other ones. Especially its relative sensitivity to thiabendazole should be mentioned. Hence, the R-strain offers an interesting tool in a comparative study on the mode of action of benomyl and related fungicides.

Edgington (personal communication) and Edgington et al. (1971) compared 16 fungal species out of various groups with regard to their sensitivity to thiabendazole, benomyl and furidazol. They found a similar pattern of selective fungitoxicity for the three fungicides. However, in some cases benomyl was more effective than thiabendazole and furidazol e.g. to *Polyporus giganteus;* on the other hand, benomyl was less effective than these fungicides to *Alternaria solani.* In this respect the R-

[1] During the course of this investigation the phenomenon has been reported from several nurseries in different parts of the Netherlands.

strain can be considered to behave like *Alternaria,* the *Botrytis* isolate S and *Trichoderma* like *Polyporus.*

The very disastrous effect of the R-strain in cyclamen, when sprayed with benomyl, is not necessarily due to its high degree of virulence, but might also be caused by the inhibition of benomyl-sensitive antagonistic fungi at the base of the petioles.

It is questionable, whether the R-strain can maintain itself if plants are not sprayed with the fungicides mentioned. As compared to the other *Botrytis* isolates tested, mycelial growth is relatively slow indeed. However, survival of the R-strain in competition with other strains of *B. cinerea* on cyclamen depends also on other characteristics e.g. virulence and sporulation.

Samenvatting

Verworven resistentie van Botrytis cinerea in cyclamen tegen benomyl en enkele andere systemische fungiciden

In een kwekerij, waar bespuiting met benomyl (Benlate, 50% W.P.) drie maal was toegepast ter bestrijding van *Botrytis*rot in cyclamen, bleek de laatste bespuiting niet meer effectief. Integendeel, de ziekte breidde zich sneller uit dan onder normale omstandigheden het geval is. Uit bloemstelen van de aangetaste planten werd een *B. cinerea*-stam (R) geïsoleerd, die zeer resistent bleek tegen benomyl. In vitro werd de groei van deze stam op aardappel-glucose-agar met 1000 μg/ml benomyl (Benlate 50% W.P.) minder geremd dan die van een willekeurig *B. cinerea*-isolaat van cyclamen op het medium met 0.5 μg/ml van het fungicide (Tabel 1, Fig. 1).

De R-stam bleek eveneens resistent tegen methyl-thiophanaat, furidazol en in mindere mate tegen thiabendazol (Tabel 2).

De myceliumgroei van vijf isolaten van *B. cinerea,* verkregen van verschillende waardplanten, bleek in tegenstelling tot die van de R-stam juist sterker geremd te worden door benomyl dan door thiabendazol (Tabel 3).

De R-stam bleef gedurende tenminste 20 weken resistent na regelmatig overenten op voedingsbodems zonder het fungicide.

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