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# Resistance to benomyl and some chemically related compounds in strains of Penicillium species

G. J. BOLLEN

Laboratory of Phytopathology, Agricultural University, Wageningen

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**Abstract**

Two *Penicillium* species, viz *P. brevicompactum* and *P. corymbiferum*, were isolated from senescent petioles of cyclamen and from bulbs of lilies, respectively, both samples treated previously with benomyl. The isolates turned out to be very resistant to this fungicide when grown on malt agar, supplied with the fungicide; at a concentration of 2000 μg/ml they were less inhibited than randomly chosen isolates of the same species on agar with 1 μg/ml.

The strains retained their resistance at the same level for at least 3 months after repeated subculturing on fungicide-free agar.

Resistance to benomyl coincided with resistance to methyl-thiophanate and, to a lesser extent, also to thiabendazole and furidazol.

## Introduction

The genus *Penicillium* belongs to the group Phialosporae within the Moniliales, which is characterized by the formation of conidia on phialides. In previous studies on the specificity of the *in vitro* antifungal spectrum of benomyl about 40 species of Phialosporae were tested for their sensitivity to this fungicide (Bollen and Fuchs, 1970; Edgington *et al.*, 1971), but no resistant species were found. With regard to the effectiveness of benomyl *in vivo* especially successful application against *Penicillium* storage disease of fruits, should be mentioned (Harding, 1968; Spalding *et al.*, 1969 and others). Excellent results were also obtained against *Penicillium corymbiferum*, causing rot in bulbs of iris (De Rooy, 1969).

In December 1970 we received senescent leaves from cyclamen, which had been sprayed with Benlate (aqueous suspension 0.2%) three months before and affected by a resistant strain of *Botrytis*. These plants had previously been used as inoculum sources by Dr G. Scholten, Research Station for Floriculture at Aalsmeer, to test the effectiveness of several fungicides against a resistant strain of *Botrytis cinerea* (Scholten and Bollen, 1971). After having been plated out on PDA most parts of petioles of the leaves revealed colonies of *Penicillium*. The level of resistance of these *Penicillia* to benomyl has now been studied.

In January 1971 in two samples of bulbs of lilies of different origin, treated previously with Benlate (0.2% aqueous suspension) a dry rot on the bulb scales was observed. A *Penicillium* species was isolated from the diseased parts of both samples. They were also tested for their resistance to benomyl together with the isolates from cyclamen.

In addition, the effect of benomyl *in vitro* was compared with that of three other systemic fungicides.

## Materials and methods

The *in vitro* activity against the isolates was tested on malt agar (Oxoid CM 59, pH 5.4) to which aqueous suspensions of the fungicides were added to give a series of concentrations required. All concentrations were based on 50% active ingredient of the formulated product. The fungicides were added to the molten agar after cooling to circa 45 °C. The plates were inoculated with a drop of a spore suspension. Diameter of the colonies was measured in each of three replicates after 4 and 7 days incubation at 25 °C.

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

## Results

In the isolates from cyclamen two types or strains could be distinguished. Both were determined as belonging to the *Penicillium brevicompactum* series. One strain was a typical *Penicillium brevicompactum* Dierckx. Although the growth of the other one was less restricted on malt agar and Czapek's agar, we yet considered this strain to belong to the same species. However, if one wants to differentiate between *P. brevi-*

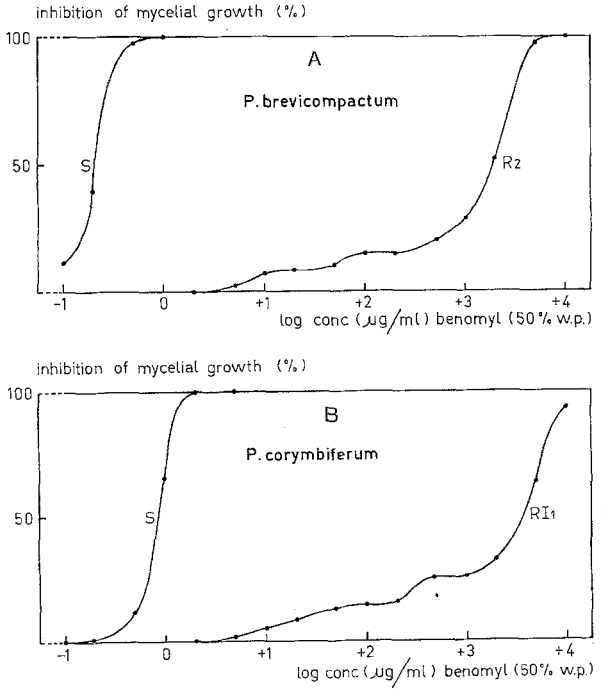


Fig. 1. Effect of benomyl on growth of isolates of benomyl-resistant strains (R2 and RI1) and of randomly chosen isolates (S) of *Penicillium brevicompactum* (A) and *P. corymbiferum* (B) on malt agar. The plates were incubated at 25 °C. Growth was measured after 7 days incubation. Each point represents the average of three replicates. (In all cases median deviation in diameter of colonies was < 5%).

*Fig. 1. Invloed van benomyl op de groei van resistente stammen (R2, resp. RI1) en willekeurige isolaten (S) van Penicillium brevicompactum (A) en P. corymbiferum (B) op moutagar. De cultures werden geïncubeerd bij 25 °C. De groei werd 7 dagen na het enten gemeten. Elk punt geeft het gemiddelde weer van drie herhalingen. (In alle gevallen was de gemiddelde afwijking in de diameter van de kolonies < 5%).*

Fig. 2. Inhibition of growth of *Penicillia* by three benzimidazole fungicides.
B-benomyl; F-furidazol; T-thiabendazole; C-control (without fungicide).
1, 2 and 3 – *P. brevicompactum:* S, R1 and R2, respectively;
4, 5 and 6 – *P. corymbiferum:* S, R*I1* and RI2, respectively.
The concentrations of the fungicides (1, 10, 100 and 1000) are given in μg/ml of the wettable powder, 50%.

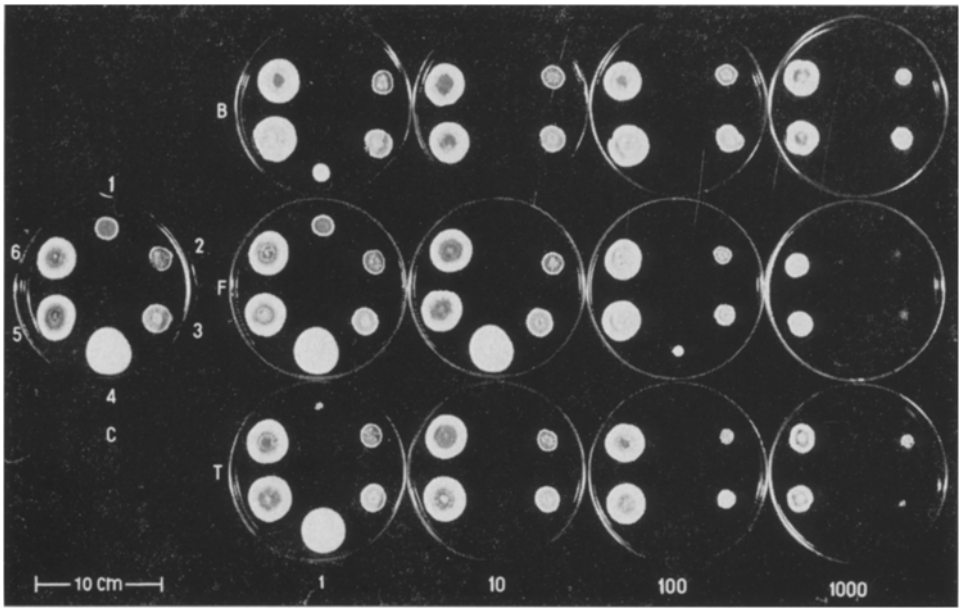


*Fig. 2. Remming van de groei van Penicillia door drie fungiciden uit de benzimidazol-groep.*
*B-benomyl; F-furidazol; T-thiabendazol; C-controle (zonder fungicide).*
*1, 2 en 3 – P. brevicompactum, resp. S, R1 en R2.*
*4, 5 en 6 – P. corymbiferum, resp. S, RI1, en RI2.*
*De concentraties van de fungiciden (1, 10, 100 en 1000) zijn gegeven in μg/ml.*

*compactum* and *P. stoloniferum*, this strain could be considered to be the *P. stoloniferum*.

In comparison with an isolate of *P. brevicompactum* from soil (S) both strains ($R_1$ and $R_2$) proved to be very resistant to benomyl (Table 1 and Fig. 1A). Growth of S was completely suppressed at 1 μg/ml, whereas growth of $R_1$ and $R_2$ was not even half inhibited at 1000 μg/ml of the fungicide.

Both isolates from lilies were identified as belonging to *P. corymbiferum* Westling by the 'Centraal bureau voor Schimmelcultures', although they did not show the yellow discoloration of the agar at the reverse of the colony as described by Raper and Thom (1949). Because of the great similarity in morphology and cultural characteristics we surmized that the isolates from both samples belonged to the same strain. Whether this strain of *P. corymbiferum* is the only causal organism of the rot observed in the bulbs of lilies, will be further studied elsewhere during the next season. The fungus is known to be pathogenic to bulbous iris (Saaltink, 1968).

As with *P. brevicompactum*, sensitivities to benomyl of both isolates ($RI_1$ and $RI_2$) from lilies were compared with that of an isolate (S) of *P. corymbiferum*, obtained from

Table 1. Effect of four systemic fungicides on mycelial growth of isolates of *Penicillium brevicompactum* and *P. corymbiferum* on malt agar. The plates were incubated at 25 °C. Growth was measured 7 days after incubation in each of three replicates. +– Growth very restricted. *Median deviation.

| Concentration of fungicide, 50% W.P. (μg/ml) | Diameter of colony (mm) | | | | | |
|---|---|---|---|---|---|---|
| | *P. brevicompactum* | | | *P. corymbiferum* | | |
| | S | R1 | R2 | S | RI1 | RI2 |
| No fungicide | 20.0±0.5* | 19.0±0.3 | 23.3±0.5 | 39.0±0.7 | 36.7±1.1 | 36.8±1.2 |
| Benomyl: | | | | | | |
| 1 | 0 | 17.7±0.2 | 22.7±1.1 | 15.5±0.7 | 35.0±0.7 | 35.8±0.8 |
| 10 | 0 | 17.7±0.3 | 21.7±0.6 | 0 | 34.7±1.1 | 35.3±1.6 |
| 100 | 0 | 16.2±0.6 | 19.5±0.7 | 0 | 31.7±0.9 | 31.3±0.4 |
| 1000 | 0 | 13.0±0.7 | 16.8±0.2 | 0 | 27.3±1.1 | 27.7±1.1 |
| Methyl-thiophanate: | | | | | | |
| 1 | 16.3±0.4 | 18.0±0.7 | 22.3±0.4 | 37.7±0.4 | 35.0±0.7 | 33.7±0.2 |
| 10 | 0 | 18.7±1.6 | 22.3±1.2 | 13.3±0.4 | 34.0±0.7 | 33.5±1.0 |
| 100 | 0 | 17.2±0.6 | 21.5±0.3 | + | 35.3±1.1 | 33.7±0.3 |
| 1000 | 0 | 16.0±0.7 | 21.5±0.0 | 0 | 33.0±2.7 | 30.2±1.2 |
| Furidazol: | | | | | | |
| 1 | 18.5±0.7 | 18.0±0 | 21.3±0.4 | 37.0±1.3 | 35.2±0.8 | 35.3±0.4 |
| 10 | 0 | 17.2±0.9 | 21.7±1.1 | 36.3±1.2 | 34.3±0.4 | 36.5±1.0 |
| 100 | 0 | 13.2±0.2 | 17.0±0.0 | 9.3±1.3 | 32.5±1.0 | 32.0±0.0 |
| 1000 | 0 | 0 | 0 | 0 | 19.2±0.4 | 19.3±0.4 |
| Thiabendazole: | | | | | | |
| 1 | 5.3±1.4 | 18.7±0.4 | 22.0±0.0 | 36.7±0.6 | 34.7±1.1 | 36.3±0.4 |
| 10 | 0 | 17.3±0.2 | 21.0±0.0 | 0 | 36.0±0.7 | 34.7±0.4 |
| 100 | 0 | 11.3±0.4 | 14.3±0.9 | 0 | 32.8±0.2 | 32.0±0.3 |
| 1000 | 0 | 7.3±1.4 | + | 0 | 21.3±0.4 | 21.0±0.0 |

*Tabel 1. Invloed van vier systemische fungiciden op de groei van isolaten van Penicillium brevicompactum en P. corymbiferum op moutagar. De cultures werden geïncubeerd bij 25 °C. De groei werd 7 dagen na het enten gemeten. + – Zeer beperkte groei, moeilijk te meten. * Gemiddelde afwijking.*

the 'Centraal bureau voor Schimmelcultures' (CBS 135.41). This isolate appeared slightly less sensitive to benomyl than the S-isolate of *P. brevicompactum* (Table 1, Fig. 1). As in the case of *P. brevicompactum*, the two resistant isolates were even less inhibited when growing on malt agar with 2000 μg/ml Benlate than the S-isolate on agar with1 μg/ml.

To compare the mechanism of resistance in the *Penicillium* strains with that in a benomyl-resistant strain of *Botrytis cinerea*, the order of effectiveness of the same four systemic fungicides as earlier tested for this fungus (Bollen and Scholten, 1971) was established. From the results (Table 1, Fig. 2) it appears that the benomyl-resistant strains were also resistant to methyl-thiophanate and, although to a lesser extent, to furidazol and thiabendazole.

The order of effectiveness of the fungicides against the non-resistant isolates of both *Penicillium* species was:

benomyl (B) > thiabendazole (T) > methyl-thiophanate (M) > furidazol (F).

For the resistant strains of both *Penicillia* this order was T and F > B > M.

At a concentration of 1000 μg/ml, furidazol was slightly more effective than thiabendazole (Fig. 2).

The resistance of furidazol and thiabendazole attained a higher level in the strain of *P. corymbiferum* than in those of *P. brevicompactum*. However, the S-isolate of either *P. corymbiferum* was also less sensitive.

The strains retained their resistance to the four fungicides at the same level for at least 3 months after repeated subculturing on fungicide-free agar.

## Discussion

Some cases of acquired and induced resistance to benomyl have been reported earlier (cf. Bollen and Scholten, 1971). This resistance was also found in *Fusarium roseum* (Hoitink and Schmitthenner, 1970) and *Neurospora crassa* (Sisler, 1971). In addition some laboratory resistance has been obtained for *Fusarium oxysporum* f. sp. *lycopersici* (Thanassoulopoulos *et al.*, 1971).

As in the case of the resistant strain of *B. cinerea*, resistance to benomyl in *Penicillia* coincides with resistance to the other benzimidazol derivatives furidazol and thiabendazole and also to methyl-thiophanate. Cross-resistance to benomyl and methyl-thiophanate may be explained by the fact that methyl-thiophanate in tap water gives rise to 2-benzimidazole carbamic acid, methyl ester (BCM) (Selling *et al.*, 1970). The same compound is formed rapidly in aqueous solutions of benomyl (Clemons and Sisler, 1969).

The order of effectiveness of the fungicides towards the S-isolates of the *Penicillium* species was the same as towards the wild strain of *B. cinerea*, viz B > T > M > F. For the resistant strains in both *Penicillium* species this order has been altered nearly in the same way as for the resistant strain of *B. cinerea*. In all these cases the R-strains were much more resistant to B and M than to T and F (Fig. 2). Hence, it can be inferred that the same mechanism of resistance is operating, whatever this mechanism may be. Additional evidence is shown by the similarity in the shapes of the inhibition curves of the resistant strains of the *Penicillium* species and *B. cinerea*.

In our experiments the agar plates with fungicide were inoculated with a drop of a spore suspension. In resistant strains both processes, spore germination and mycelial growth, must be insensitive to the fungicide. On malt agar with 100 and 1000 μg/ml of thiabendazole and furidazol very small colonies of *P. brevicompactum* were observed (Fig. 2). Thus mycelial growth was strongly inhibited, while germination still occurred. Spore germination appears to be less sensitive to the fungicides than mycelial growth. This is in accordance with the findings of Gottlieb and Kumar (1970), that growth of germ tubes of some *Penicillia* was much more inhibited by thiabendazole than spore germination.

Concerning the frequency at which resistance in *Penicillia* appears, it is worth mentioning that from a soil rich in *Penicillia*, no resistant strains could be isolated up to 8 months after applying benomyl to it.

Earlier, we have reported the disastrous effect of a resistant strain of *B. cinerea* in cyclamen, sprayed previously with benomyl (Bollen and Scholten, 1971). It is currently studied whether the resistant strains of *P. brevicompactum*, also isolated from cyclamen, may contribute towards a 'biological' control of *B. cinerea* on plants sprayed with the fungicide.

In view of the antifungal spectrum of benomyl, the application of this fungicide obviously results in a temporary disturbance of the ecological equilibrium (cf. Van den Berg and Bollen, 1971). Because we can always assume a natural control of pathogenic fungi by their antagonists, the appearance of resistance in saprophytic fungi is of equal phytopathological importance as that in pathogenic ones.


## Samenvatting

*Resistentie tegen benomyl en enkele chemisch verwante verbindingen in stammen van Penicillium-soorten*

Uit afstervende bladstelen van cyclamen en uit schubben van leliebollen, welke eerder met benomyl waren behandeld, konden respectievelijk *Penicillium brevicompactum* en *Penicillium corymbiferum* worden geïsoleerd. De isolaten bleken *in vitro* zeer resistent tegen het fungicide. De myceliumgroei van deze isolaten werd op moutagar met 2000 µg/ml benomyl minder geremd dan die van willekeurige isolaten van dezelfde soorten op agar met 1 µg/ml (Fig. 1).

De isolaten bleven gedurende tenminste 3 maanden resistent na regelmatig overenten op voedingsbodems zonder het fungicide.

De resistente stammen van de beide *Penicillium*-soorten bleken eveneens resistent tegen methyl-thiophanaat en in mindere mate ook tegen thiabendazol en furidazol (Tabel 1). De volgorde van de groeiremmende werking van deze fungiciden was voor de willekeurig gekozen (gevoelige) isolaten: benomyl > thiabendazol > methyl-thiophanaat > furidazol. Voor de resistente stammen was deze: thiabendazol en furidazol > benomyl > methyl-thiophanaat. In het feit dat een dergelijke verandering in volgorde van remmend effect ook voor *Botrytis cinerea* geldt, ligt een aanwijzing, dat de wijze waarop de resistentie werkt, voor deze schimmels gelijk is.



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## Address

Laboratorium voor Fytopathologie, Binnenhaven 9, Postbus 85, Wageningen, the Netherlands