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A Comparison of Benomyl and Thiophanates with Respect to Some Chemical and Systemic Fungitoxic Characteristics

A. FUCHS, G. A. VAN DEN BERG, AND L. C. DAVIDSE

Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands

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Some chemical and systemic fungitoxic characteristics of benomyl, thiophanate (NF 35), thiophanate-methyl (NF 44) and the related compound 2-(3-methoxycarbonyl-thioureido)-aniline (no common name; NF 48) were compared. All four compounds were converted to the same fungitoxic compound benzimidazole carbamic acid, methyl ester (BCM) [or the closely related ethyl ester (BCE)], at a rate depending on pH and temperature. In 400 μM solutions at room temperature, the transformation rates (T) decreased in the order: $T_{\text{benomyl}} > T_{\text{NF 48}} > T_{\text{thiophanate-methyl}} > T_{\text{thiophanate}}$, with the latter two compounds only being transformed at high pH (9.1). Concentrations (C) of fungitoxicant (BCM, respectively, BCE) in aerial parts of plants, which had been immersed with their roots in such solutions for 2 days, however, did not reflect the above sequence: $C_{\text{NF 48}} > C_{\text{benomyl}} > C_{\text{thiophanate-methyl}} > C_{\text{thiophanate}}$. Neither did the effectiveness (E) of these fungicides in protecting plants against fungal attack: $E_{\text{benomyl}} > E_{\text{thiophanate-methyl}} > E_{\text{NF 48}} > E_{\text{thiophanate}}$. No appreciable protection resulted from application of the same solutions when heated under reflux for 1 h, although their fungitoxic activity *in vitro* increased rather than decreased. These phenomena could be ascribed to differences in distribution of BCM, resulting from application of the various fungicides via the roots. Benomyl, which is an N-1 substituted BCM, and thiophanate-methyl which, most likely, produces an intermediate, that is another N-1 substituted BCM, caused a more or less homogeneous distribution of fungitoxicant over the aerial plant parts. However, with NF 48 and the heated solutions of the fungicides, which contained almost exclusively BCM (respectively, BCE), the fungitoxicant accumulated almost quantitatively in the leaves that were present at the time of treatment so that the leaves that expanded later were virtually unprotected. An hypothesis comprising retention of BCM only if substituted at N-1 (benomyl, intermediate conversion product of thiophanate-methyl) in (or on) the roots and continuous gradual release of BCM, is put forward to explain the behaviour *in vivo* of these fungicides.

The antifungal spectrum of the systemic fungicides thiophanate and thiophanate-methyl (1, 2) is essentially similar to that of benomyl (3, 4). As has been shown (5, 6), this similarity must be ascribed to the fact, that the thiophanates, by degradative cyclization (7, 8), and benomyl, by hydrolysis (9–11), are both readily converted to 2-alkoxycarbonyl aminobenzimidazoles, viz., 2-ethoxy-, respectively, 2-methoxycarbonyl aminobenzimidazole. The latter compound is more usually named benzimidazole carbamic acid, methyl ester (BCM), whereas the ethoxy analog is also designated as benzimidazole carbamic acid, ethyl ester (BCE). BCM is also rapidly formed from 2-(3-methoxycarbonyl-thioureido)-aniline (6), a compound closely related to the thiophanates. Figure 1 depicts the four parent compounds as well as their conversion products.

BCM and BCE are now generally accepted as the actual fungitoxic principles

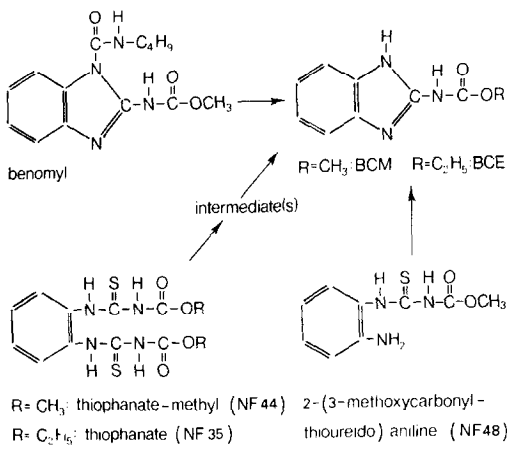

FIG. 1. *Structural formulae of benomyl and thiophanates, and of their conversion products BCM (benzimidazole carbamic acid, methyl ester) and BCE (benzimidazole carbamic acid, ethyl ester) (code names between brackets).*

of benomyl and the thiophanates, BCM being slightly more fungitoxic than BCE (6–8). Contrary to what was expected on account of their conversion to BCM, respectively, BCE, these fungicides rather differed in effectivity in protecting plants against fungal diseases. Therefore, both types of systemic fungicides were compared with respect to their behaviour within plants. Some experiments *in vitro* were carried out to provide the necessary background information for the studies *in vivo*.

MATERIALS AND METHODS

Plants, fungi. Experiments *in vivo* were carried out using barley (cv. Cambrinus), bean (cv. Dubbele Witte z. dr.), Chinese aster (cv. Prinses Madeleine), cucumber (cv. Lange Gele Tros), pea (cv. Mansholt) and tomato (cv. Bonner Beste) plants. In experiments *in vitro* strains of the following fungal species were used: a. strains sensitive to benomyl (3): *Botrytis cinerea, Cladosporium cucumerinum, Penicillium brevicompactum, P. corymbiferum, P. expansum, P. frequentans*; b. strains tolerant or resistant to benomyl (3): *Alternaria brassicae, Botrytis cinerea* [resistant strain, cf. (12)], *Cochliobolus sativus, Colletotrichum acutatum*,[1] *Doratomyces microsporus, Penicillium brevicompactum, P. corymbiferum* [both resistant strains, cf. (13)], *Phytophthora cryptogea.* The effectiveness of the various fungicides was tested in the following host–parasite combinations: barley-*Erysiphe graminis* f. sp. *hordei*, Chinese aster-*Phytophthora cryptogea*, cucumber-*Cladosporium cucumerinum* and cucumber-*Sphaerotheca fuliginea*, and pea-*Erysiphe pisi.*

Chemicals. 1-(Butylcarbamoyl)-2-benzimidazole carbamic acid, methyl ester [common name benomyl; Du Pont de Nemours & Co. (Inc.)] and 1,2-bis-(3-ethoxycarbonyl-2-thioureido)-benzene and 1,2-bis-(3-methoxycarbonyl - 2 - thioureido) - benzene (common names thiophanate and thiophanate-methyl; in Figs. 3–5, which were made before the common names were adopted, still designated with the original code names NF 35 and NF 44, respectively; Nippon Soda Co., Ltd., through Orgachemia N.V.) were available as technical grade chemicals and as 50 % wettable powders; 2-(3-methoxycarbonyl-thioureido)-aniline (no common name proposed; coded NF 48; Nippon Soda Co., Ltd., through Orgachemia N.V.) was only available as a 50% wettable powder.

Although in most experiments the formulated chemicals were employed, the common names benomyl, thiophanate, and thiophanate-methyl, and the code name NF 48 will be used throughout this article.

BCM was obtained by boiling aqueous solutions of technical grade benomyl for 1 h under reflux; after filtering, the filtrate was purified by recrystallization. A reference sample of BCM was kindly provided by Dr. C. W. Pluijgers, Institute of Organic Chemistry T.N.O., Utrecht, The Netherlands.

All concentrations of fungicides given in

[1] Formerly (14) erroneously indicated as *Glomerella cingulata.*

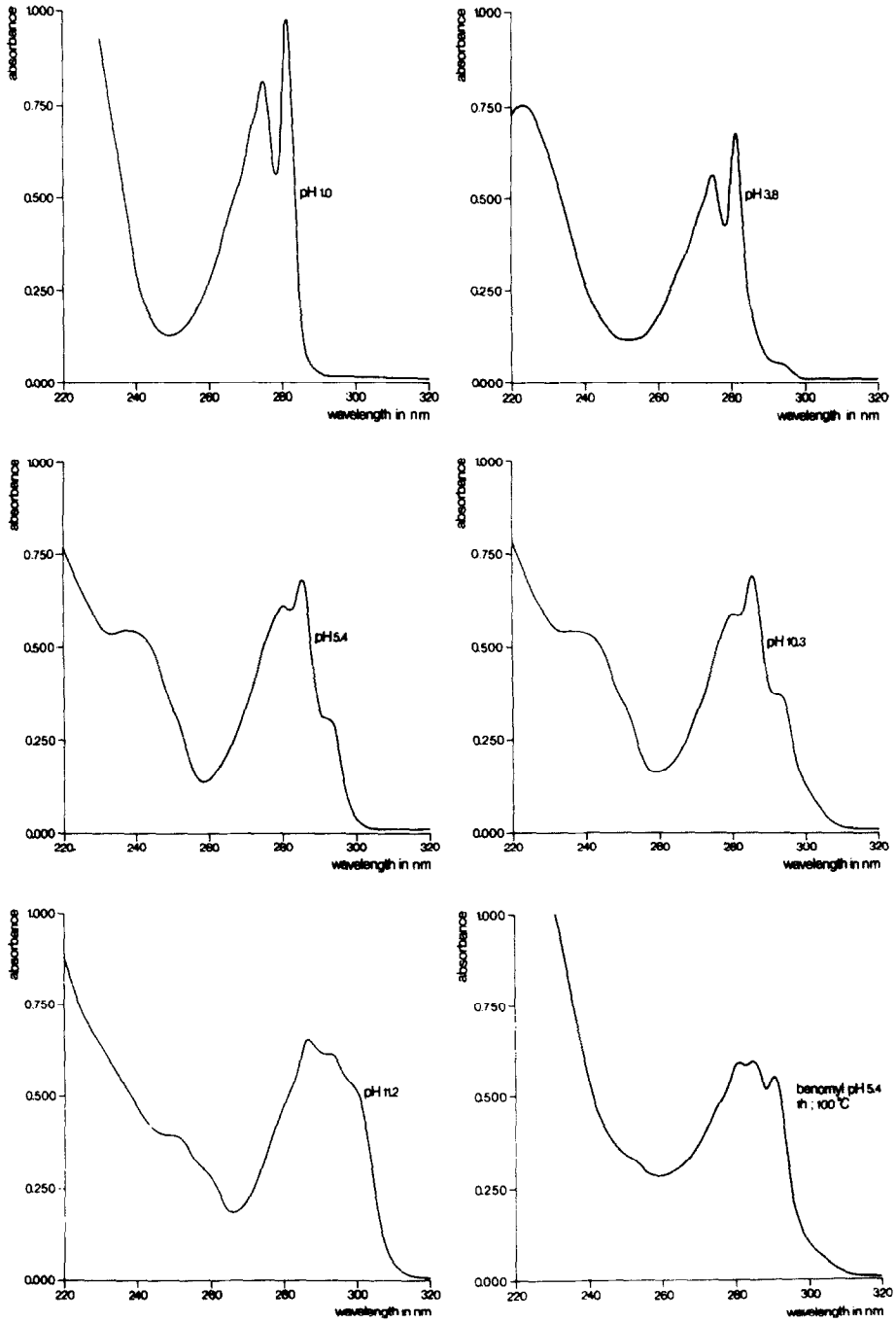

FIG. 2. *Ultraviolet absorption spectra of BCM at different pH's. Aliquots of a 0.05% (w/v) ethanolic solution of BCM were diluted (1:50) with distilled water adjusted to pH as indicated. For comparison, the absorption spectrum of a boiled (1 h; 100°C) aqueous solution (pH 5.4) of benomyl (technical grade) has been given (diluted, not purified, and not readjusted to exactly pH 5.4 before measuring).*

TABLE 1

Spectral characteristics of BCM as affected by pH

	λ_{max}											
BCM pH 1.0					274	(15566)[a]	281	(18718)				
BCM pH 3.8	223	(14420)			275	(10753)	281	(12988)				
BCM pH 5.4			238	(10390)			280	(11670)	285	(13026)	292	(5864)[b]
BCM pH 10.3			237	(10390)			280	(11212)	285	(13217)	292	(7086)[b]
BCM pH 11.2				251 (7544)					286	(12510)	292	(11804)
Benomyl 1 h; 100°C pH 5.4							281		285		291	

	λ_{min}						shoulder					
BCM pH 1.0	249			278								
BCM pH 3.8	252			278							292	
BCM pH 5.4		258			282		291[b]					
BCM pH 10.3		258			282		291[b]					
BCM pH 11.2			266				291	259	280		299	
Benomyl 1 h; 100°C pH 5.4		258			282	288						

[a] Molar extinction ϵ given in parentheses.

[b] Pronounced shoulder.

the present article are based on the active ingredients.

Experiments in vitro. As is shown in Fig. 2, uv absorption by BCM strongly depends on pH (see, however, also Discussion). For convenience, the characteristic minima and maxima at several pH-values have been summarized in Table 1. Therefore, the position of minima and maxima of ultraviolet spectra was used as an indication of the presence of BCM[2] in experiments on the effect of pH and temperature on the rate of transformation of benomyl and thiophanates. The conversion rates were estimated by recording the spectra (Beckman DB-G spectrophotometer) of buffered 400 μM solutions (McIlvaine buffer pH 4.1, 6.1, 8.0 and Tris–HCl buffer pH 9.1, respectively), stored at room temperature for 2, 21, 45, and 122 h, respectively, and of aqueous 400 μM solutions (pH 5.4), boiled under reflux for 1 h. Before recording the spectra all solutions were diluted with water to give a final concentration of 50 μM. For the thiophanates, the disappearance of characteristic maxima at 260–262 nm was taken as additional evidence for their decomposition.

The same solutions as mentioned previously were examined using a thin-layer chromatographic bioassay (14), with either *Cladosporium cucumerinum*, *Penicillium expansum* or *P. frequentans* as the test organisms; in this case, the buffered solutions were bioassayed after incubation for 4, 24, 50, and 128 h, respectively.

Cross-resistance of benomyl-resistant fungal strains against the thiophanates was also examined with the thin-layer chromatographic bioassay. Aliquots of variously treated solutions of all four fungicides were spotted on silica gel thin-layer plates, and these, after having been run in ethyl acetate, were developed by spraying with the resistant strains mentioned before. Other plates which served as "controls," were

[2] Or, in the case of thiophanate, of BCE. Although, strictly, this is not entirely justified, the uv spectral characteristics of BCM have also been used for the identification of BCE.

sprayed with benomyl-sensitive strains, to detect fungitoxic spots.

Experiments in vivo. One- to two-week-old seedlings of barley, bean, Chinese aster, cucumber, and pea, grown in sand or garden soil, were immersed with their roots in freshly prepared aqueous 400 μM solutions (pH 5.4) of the four fungicides examined; seedlings of barley and bean were also placed in identical solutions which had been boiled under reflux for 1 h. After 2 days, the aerial parts (subdivided into several parts, if necessary) of some of the plants were weighed and put in the deep-freeze until further analysis. Other plants were potted in garden soil, after the roots had been thoroughly washed with distilled water; at various intervals two to four plants were harvested, and treated as above.

The plant material was ground with sand in a mortar and extracted with 96% ethanol; after centrifuging, the pellet was resuspended in 96% ethanol and again extracted and centrifuged. The two supernatants were added together and made up to a volume (in milliliters) equivalent to the fresh weight (in grams) of the extracted sample. Identical aliquots of each extract were spotted and chromatographed; the inhibition zones, which became visible 2 days after spraying the chromatograms with the test fungus, were compared with those produced by a series of known amounts of BCM. In this way, not only could the amount of fungitoxicant in each sample be measured exactly but at the same time the sizes of the various fungitoxic spots on the thin-layer chromatograms were comparable directly. Usually, the test organism was *Cladosporium cucumerinum*; with this fungus amounts of fungitoxicant as low as 0.01 μg could be assayed accurately.

Protection of fungicide-treated plants against various pathogens was examined by inoculating them with suitable pathogenic fungi (see above) 14 days after the treatment, the inoculation being repeated weekly, unless otherwise indicated.

RESULTS

Influence of pH and temperature on rate of transformation. Results of spectrophotometric analyses on the influence of pH on the rate of conversion have been summarized in Table 2. They show, that, at room temperature, conversion of benomyl and NF 48 was almost independent of pH. With this method conversion of thiophanate and thiophanate-methyl, however, could only be demonstrated at pH 9.1. Even then, the rate of conversion of thiophanate seemed rather slow. In general, these results agree with those of Vonk and Kaars Sijpesteijn (6), who studied the transformation of thiophanates *in vitro*.

Parallel thin-layer chromatographic bioassays of the same solutions revealed a more extensive decomposition of the parent compounds and formation of larger amounts of BCM (respectively, BCE). This will be largely due to continued transformation of the parent compounds during the running of the chromatograms, as was substantiated by two-dimensional chromatography. In addition to the usual fungitoxic spots at the origin[3] and at the R_f values of the parent compounds and of BCM (respectively, BCE), a fourth one with R_f value 0.64 (solvent: ethyl acetate) was revealed for thiophanate-methyl. Thiophanate also produced a small fourth spot with a similar R_f value (Fig. 3). It should be understood, that the presence of an inhibition zone is not necessarily due to a fungitoxic substance that moved as such. It may have been produced on the spot from a nonfungitoxic precursor. Because of the continued transformation of the parent com-

[3] Part of BCM (respectively, BCE) seemed to be present in a nonmoving (ionized?) form; chromatographic analysis showed it to be in equilibrium with "normal" BCM (respectively, BCE).

TABLE 2

Effect of pH on the rate of transformation of benomyl and thiophanates (based on spectral data)

pH	Benomyl				Thiophanate				Thiophanate-methyl				NF 48			
	Incubation time (h)															
	2	21	45	122	2	21	45	122	2	21	45	122	2	21	45	122
4.1	−[a]	+	++	++	−	−	−	−	−	−	−	−	−	+	++	++
6.1	−	+	++	++	−	−	−	−	−	−	−	−	−	+	++	++
8.0	−	+	++	++	−	−	−	−	−	−	−	−	−	+	++	++
9.1	+	++	++	+++	−	+	+	+	−	+	++	++	−	+	++	++

[a] Characteristic maxima or shoulders of BCM absent (−), weak (+), distinct (++) or very pronounced (+++).

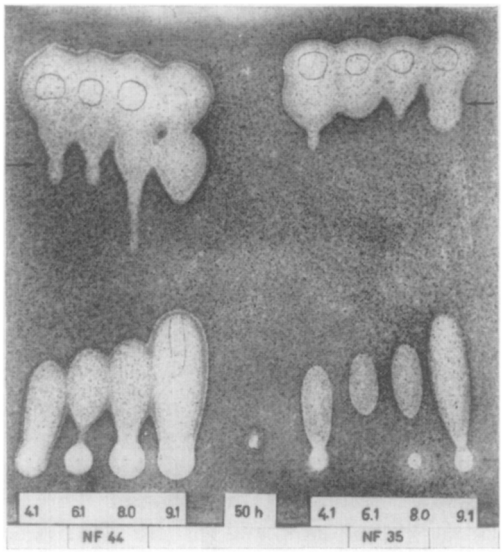

FIG. 3. *Thin-layer chromatograms indicating transformation of thiophanate-methyl (NF 44; left) and thiophanate (NF 35; right) as influenced by pH (McIlvaine buffer pH 4.1, 6.1, and 8.0; Tris-HCl buffer pH 9.1); incubation time 50 h. Especially note the intermediates (arrows), most distinctly visible in the case of thiophanate-methyl (NF 44), pH 9.1.*

pounds and their conversion products to BCM (respectively, BCE) during incubation of the chromatograms after they have been sprayed with the test fungus, BCM (respectively, BCE) might even be the only actual fungitoxic compound present at each spot. BCM, or its ethyl analog, should then be the "common antifungal principle" of benomyl and the thiophanates as was suggested earlier by Vonk and Kaars Sijpesteijn (6).

The rate of conversion of benomyl and thiophanates was also greatly influenced by temperature. At room temperature, in aqueous solutions (pH 5.4) of 2500 mg benomyl/liter (= 8621 μM), even at pH 10.0, it took several days for complete transformation. Heat sterilization for 20–30 min or boiling under reflux for 1 h, however, was sufficient to convert benomyl to BCM almost completely, even at these high concentrations. Similarly, conversion of thiophanates was greatly enhanced at high temperatures: in boiled aqueous 400 μM solutions of thiophanate and thiophanate-methyl only a little of the parent compound was left; with NF 48, on the other hand, transformation appeared to be quantitative.

In short, the transformation rates of benomyl and thiophanates depend on physical factors like pH and temperature. In 400 μM solutions at room temperature, these transformation rates (T) decreased in the order: $T_{\text{benomyl}} > T_{\text{NF 48}} > T_{\text{thiophanate-methyl}} > T_{\text{thiophanate}}$, with the latter two compounds only being transformed at high pH (9.1).

Cross-resistance of benomyl-resistant fungal strains. When thin-layer chromatograms of

variously treated (fresh or aged, heated or unheated, neutral or alkaline, aqueous or buffered) solutions of benomyl or thiophanates were developed by spraying them with any of the benomyl-resistant strains listed under Materials and Methods not one fungitoxic zone was observed. The benomyl-sensitive strains, however, proved to be sensitive to this fungicide, to the thiophanates and to all conversion products of the parent compounds (Fig. 4). Hence, the cross-resistance of benomyl-resistant strains to thiophanates is almost certainly due to resistance to BCM (respectively, BCE). In an analogous way, sensitivity to these fungicides merely means sensitivity to BCM (respectively, BCE). Thus, these results again support the hypothesis, that BCM (respectively, BCE) is the actual fungitoxic principle. These results confirm those of Bollen (2, 13) and Bollen and Scholten (12) who, using growth inhibition on agar media as a criterion for fungitoxic activity, also concluded that benomyl-resistant strains of *Botrytis cinerea*, *Penicillium brevicompactum* and *P. corymbiferum* displayed cross-resistance to, among others, thiophanate and thiophanate-methyl.

Uptake, transport and conversion of benomyl and thiophanates in plants. In benomyl-treated plants, BCM is also considered to be the actual fungitoxic principle, this compound being the only one consistently found in plant extracts (10, 15–18). Therefore, it might be assumed at first, that the effectiveness *in vivo* of those compounds which produce BCM, when administered via the roots, will be identical, or at least will reflect the rate of conversion of the parent compounds *in vitro*. Preliminary experiments proved this hypothesis to be incorrect: for instance, benomyl protected cucumber plants against powdery mildew for many weeks, even though the plants

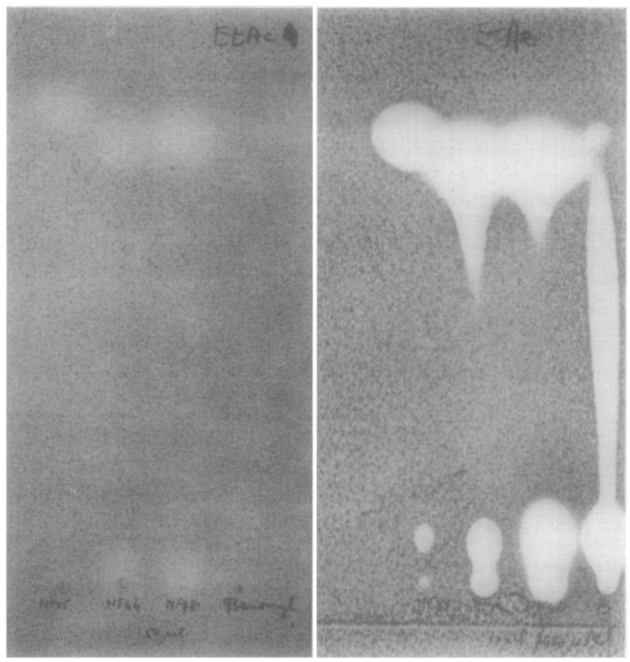

FIG. 4. *Thin-layer chromatogram indicating insensitivity ("cross-resistance") of a benomyl-resistant strain of* Penicillium brevicompactum *(left) and sensitivity of a "normal" strain (right) of the same species, respectively, for benomyl and thiophanates and their conversion products (from left to right: thiophanate (NF 35), thiophanate-methyl (NF 44), NF 48, benomyl).*

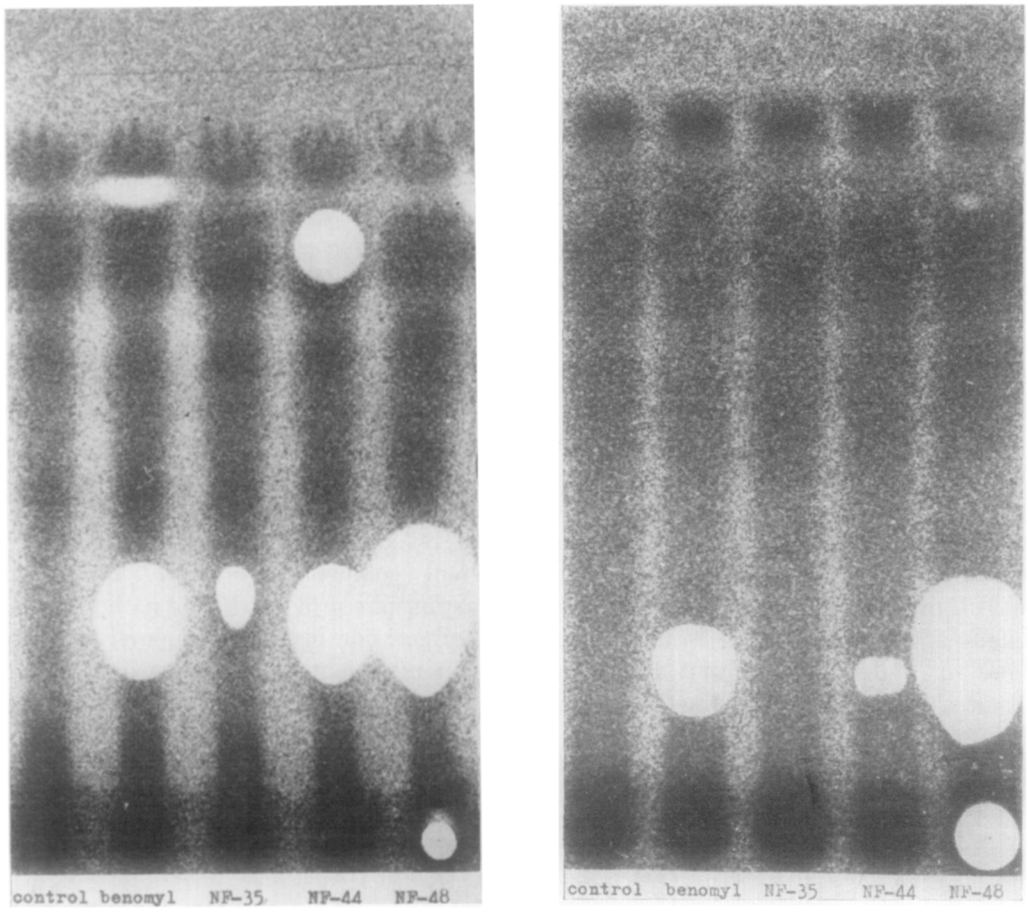

FIG. 5. *Thin-layer chromatograms of ethanolic extracts of aboveground plant parts of (left) pea (extracted immediately after fungicide treatment) and (right) cucumber (extracted 21 days after fungicide treatment); from left to right: untreated, treated with benomyl, thiophanate (NF 35), thiophanate-methyl (NF 44), and NF 48, respectively. Note the fungitoxic spot at the R_f value of the parent compound in the case of thiophanate-methyl-treated pea plants.*

were allowed to take up the fungicide from aqueous solutions for only 2 days. NF 48, on the other hand, under similar conditions appeared to be far less active than could be expected from its ready transformation to BCM. Thus, protection of plants by benomyl can not just be attributed to conversion to BCM. Therefore, uptake, transport and conversion of benomyl and thiophanates in plants were examined more closely.

Ethanolic extracts of aerial parts of seedlings of barley, Chinese aster, cucumber, and pea, analysed either immediately (0 days) or 21 days after the plants had taken up the fungicides from freshly prepared aqueous 400 μM solutions (pH 5.4) for 2 days, contained almost exclusively BCM (or BCE) as the fungitoxicant. Only extracts of barley and pea showed small fungitoxic spots with the same R_f values as the parent compounds in the 0-days extracts (Fig. 5). From the size of the fungitoxic spots, the following order for the concentration (C)[4] of the fungitoxicant in the

[4] That is the "over-all-concentration," based on fresh weight of plant material.

aerial parts was deduced: $C_{NF\ 48} > C_{benomyl} > C_{thiophanate\text{-}methyl} > C_{thiophanate}$.[5] This sequence neither reflected the order of the transformation rates *in vitro* nor that of the effectiveness *in vivo*. On account of these data, two conclusions seemed justified: (a) not only BCM (respectively, BCE) is taken up by the plant, but also the parent compounds or conversion products other than BCM (respectively, BCE); (b) the over-all-concentration of fungitoxicant in the plant does not reflect the degree of protection.

From the literature it is known, that the distribution of BCM within plants after administration of benomyl is not homogeneous and depends greatly upon mode and duration of application, concentrations used, etc. (16–26). Since all these conditions were kept constant in our experiments, our data seemed to suggest, that the final distribution of BCM over the plants also depended on the chemical nature of the parent compounds, i.e., each fungicide, whether producing BCM or BCE, should give a specific distribution pattern of the fungitoxicant within the plant.

In order to test this hypothesis, the aerial parts of fungicide-treated bean plants were subdivided, at "zero" time, into stem and primary leaves, and, 15 days after they had been transferred from the fungicide solutions to garden soil, into stem parts plus primary leaves, and first, second, and third trifoliate leaves, respectively. Subsequently, all parts were extracted and the extracts analysed separately. At zero time, concentrations of fungitoxicant in stems and primary leaves were almost equal for benomyl, thiophanate, and thiophanate-methyl; NF 48, however, gave a concentration of fungitoxicant, that was distinctly higher in the primary leaves than in the stems. However, much more significant results were obtained with those plants which, during the 15 days after transplanting had unfolded the first, second, and third trifoliate leaves. As is shown in Fig. 6, in benomyl-treated plants a gradual concentration gradient of fungitoxicant was found, extending to the third trifoliate leaf. In NF 48-treated plants there was a much steeper concentration gradient, with concentrations of fungitoxicant in the primary leaves exceeding those in comparable leaves of benomyl-treated plants, whereas in the

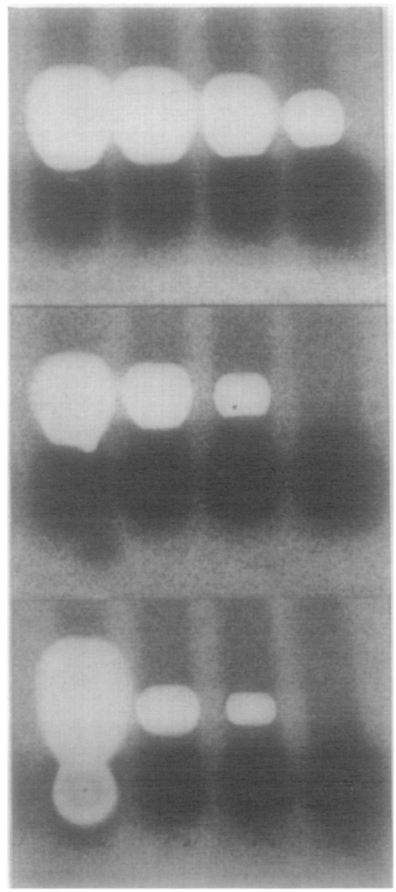

FIG. 6. *Thin-layer chromatograms (lower halves) of ethanolic extracts of (from left to right) primary, and first, second, and third trifoliate leaves (plus adjacent internodium), respectively, of bean plants, extracted 15 days after treatment with (from top to bottom) benomyl, thiophanate-methyl, and NF 48, respectively.*

[5] Because of the lower intrinsic fungitoxicity of BCE, in fact, thiophanate can not be directly compared with the other fungicides.

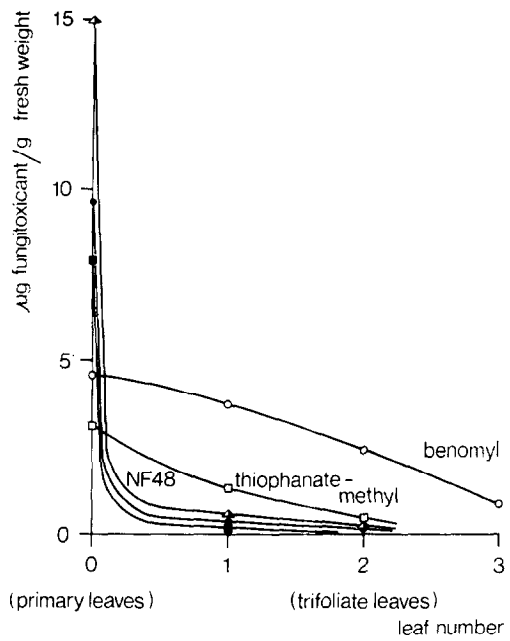

FIG. 7. *Concentration gradients of fungitoxicant (BCM) in bean plants, treated with fresh* (○, □, △) *and boiled (1 h, 100°)* (●, ■, ▲) *solutions of benomyl, thiophanate-methyl, and NF 48, respectively.*

first leaves concentrations were already lower than in the third leaves of the latter plants. In the third leaves of NF 48-treated plants no fungitoxicant could be detected at all. Thiophanate-methyl-treated plants took an intermediate position, with a gradual concentration gradient, which only extended to the second trifoliate leaves.[6] Thus, it seemed that, although BCM is the actual fungitoxic compound, it is not the only and perhaps not even the most essential factor which determines the systemic fungicidal character of benomyl and the thiophanates.

To substantiate this assumption, barley and bean plants were treated with fresh and boiled aqueous 400 μM solutions of benomyl, thiophanate-methyl, and NF 48, respectively. The results obtained with plants, treated with the fresh solutions of the fungicides, confirmed those of earlier experiments. They showed a difference in distribution pattern of the fungitoxicant depending on the parent compounds applied. However, in plants treated with the boiled solutions, about the same concentration gradient was found in all instances irrespective of the nature of the parent compound; moreover, this gradient was similar to the concentration gradient found in plants, treated with fresh solutions of NF 48 (cf. Fig. 7, in which all data on concentration gradients have been summarized graphically). Hence, it appears that various compounds, which produce BCM, nevertheless exhibit different behaviour within the plant.

The conclusions drawn so far have been entirely corroborated by the results of concurrent experiments on the effectiveness (E) of these fungicides *in vivo*. From Tables 3 and 4 it is obvious that the effectiveness against powdery mildew of barley (*Erysiphe graminis* f. sp. *hordei*) and pea (*Erysiphe*

TABLE 3

Effectiveness of benomyl and thiophanates against powdery mildew in pea plants (for details see text)

Treatment[a]	Time of evaluation in days after first inoculation[b]		
	22	27	35
Water (control)	5[c]	5	5
Benomyl	0	0	0
Thiophanate	4	4	4
Thiophanate-methyl	2	2	2
NF 48	2	3	3

[a] Plants immersed with their roots in aqueous 400 μM solutions for 2 days.

[b] Plants inoculated 14 days after fungicide treatment; inoculation repeated weekly.

[c] 0: no symptoms; 5: infection level like that of control.

[6] Because of the lower intrinsic fungitoxicity of BCE, no fungitoxicant could be detected in the upper part of the plants, when the extracts of thiophanate-treated plants were prepared at the same dilution as in the other treatments. Therefore, no further experiments with thiophanate will be discussed.

pisi) decreased in the order: $E_{\text{benomyl}} > E_{\text{thiophanate-methyl}} > E_{\text{NF 48}} > E_{\text{thiophanate}}$. This sequence thus reflects the distribution pattern of BCM (respectively, BCE) originating from the parent compounds within the plants: the more homogeneous this distribution, the more effective is the parent compound as a systemic fungicide. In barley plants, treated with any of the thiophanates, not only the degree of infection with powdery mildew, but also the distribution of the mycelium over the leaves differed from the untreated controls: the distal leaf halves of older leaves remained entirely free from powdery mildew, especially in the case of NF 48-treated plants; infected younger leaves, however, showed a uniform distribution of powdery mildew like the untreated controls. Comparable results were obtained with cucumber plants infected with powdery mildew (*Sphaerotheca fuliginea*); again, the effectiveness of the fungicides decreased in the order $E_{\text{benomyl}} > E_{\text{thiophanate-methyl}} > E_{\text{NF 48}} > E_{\text{thiophanate}}$. Upon treatment with thiophanates, the margins of older leaves remained virtually free from powdery mildew but the younger leaves became mildewed all over. Details of the latter experiment have been published elsewhere (27).

All four fungicides equally suppressed cucumber scab in 2-week-old cucumber seedlings, which had been inoculated with *Cladosporium cucumerinum* immediately after the fungicide treatment. Apparently, in all cases enough BCM (respectively, BCE) was taken up and transported to the cotelydons and still expanding first leaves to give complete protection. On the contrary, no protection at all was found by any of the fungicides tested in Chinese aster plants which, 14 days after the treatments had been discontinued, were inoculated with *Phytophthora cryptogea*. From its insensitivity to benomyl, or rather BCM, such a result was highly predictable (3).

TABLE 4

Effectiveness of benomyl and thiophanates against powdery mildew in barley plants (for details see text)

Treatment[a]	Time of evaluation in days after first inoculation[b]			
	7	14	21	35
Water (control)	5[c]	5	5	5
Benomyl	0	0	0	0
Thiophanate	3	5	4	4
Thiophanate-methyl	1	1	2	2
NF 48	3	4	4	4

[a], [b], [c] Explanation of footnotes as for Table 3.

TABLE 5

Effectiveness of unheated and heated (1 h, 100°C) aqueous 400 μM solutions (pH 5.4) of benomyl and thiophanates against powdery mildew in barley plants (for details see text)

Treatment[a]	Time of evaluation in days after first inoculation[b]			
	Unheated		Heated	
	20	29	20	29
Water (control)	5[c]	5	5	5
Benomyl	0	1	4	4
Thiophanate	5	4	4	3
Thiophanate-methyl	0	2	3	4
NF 48	1	3	3	3

[a] Plants immersed with their roots in solutions as indicated for 2 days.

[b], [c] Explanation of footnotes as for Table 3.

The hypothesis that the distribution pattern of BCM within plants is of paramount importance in their protection against fungal attack was strongly supported by the failure of boiled solutions of benomyl, thiophanate, thiophanate-methyl, and NF 48 to protect barley plants against powdery mildew (Table 5). As has been stated before, in such solutions no appreciable quantities of the parent compounds are left, all being transformed almost quantitatively to BCM (respectively, BCE). Because BCM will have been quite rapidly transported to the leaves

present at the time of treatment, when the plants were still in the seedling stage, the younger leaves, that expanded during the 29 days after the treatment, remained entirely unprotected.

DISCUSSION

In aqueous solutions, benomyl (9–11) as well as the thiophanates (5–8) are readily converted to BCM (respectively, BCE). With benomyl and NF 48 this transformation merely means hydrolysis with splitting off of the butylcarbamoyl side chain or cyclization with production of H_2S, respectively (Fig. 1). In the case of thiophanate-methyl our thin-layer chromatographic data revealed an intermediate [Fig. 3; cf. (28)], with R_f value 0.64 in ethyl acetate. After the running of the chromatogram this substance most likely was further degraded to BCM, thus causing an inhibition zone upon spraying the silica gel plate with the test organism. On account of the results of the experiments *in vivo*, to be discussed later, it is tempting to suggest that this intermediate represents a "benomyl analog," which differs from benomyl only in the nature of the side chain at N-1 in the benzimidazole nucleus, and emerges by direct ring closure of the side chains in thiophanate-methyl. In fact, such a direct cyclization step giving rise to an N-1 substituted BCM has also been postulated by Noguchi *et al.* (1970, personal communication), which could precede a further (stepwise) hydrolytic degradation of the side chain. In the case of thiophanate an intermediate was also detected (Fig. 3), presumably the ethoxy analog of the intermediate found with thiophanate-methyl.

The rate of transformation *in vitro* of benomyl and thiophanates to BCM (respectively, BCE) is influenced by pH (6, 28) (see also Table 2) and temperature. Since the ultraviolet absorption spectrum of BCM is also pH-dependent (Fig. 2, Table 1), evaluation, on the basis of spectral data, of the effect of pH on this rate of transformation is most accurate when the fungicide solutions are adjusted to the same pH before measuring. This is the more important since Duineveld and Beijersbergen (29) found extensive reduction of fungicidal activity on heating benomyl solutions (50% wettable powder), adjusted to pH 8.5, for 1 h. Contrary to heated neutral (pH 7.0) benomyl solutions, where no loss of activity was observed, the heated alkaline solutions showed ca. 60% loss in activity. This loss might be ascribed to a further degradation of the BCM formed to a nonfungicidal compound, e.g., aminobenzimidazole. Ultraviolet absorption spectra of aqueous solutions of BCM, adjusted to pH-values of up to 11.2 (Fig. 2, Table 1) and incubated for 6 days at room temperature, however, not only appeared to be unchanged, but acidification of these solutions to pH 3.8 revealed spectral characteristics completely identical to those of a freshly prepared aqueous solution of that pH. From these and other observations the inference may be made that at room temperature BCM is not further degraded at any pH between 1.0 and 11.2, Nor did boiling of solutions of benomyl and thiophanates in distilled water, pH 5.4, for 1 h cause any appreciable breakdown of BCM (respectively, BCE). Thus, inactivation of BCM (and, by analogy, most probably also BCE) apparently only takes place upon heating at alkaline pH.

Uptake via the roots and upward translocation of benomyl has been studied in a number of plant species, with different bioassay methods and variable experimental conditions [see in particular (16, 18, 19, 21)]. Independent of plant species, but depending on the mode of application of benomyl, a concentration gradient of fungitoxicant was observed, with initially highest concentrations in the basal parts

of the plants (roots, stems). With time, however, this concentration gradient gradually changed, and fungitoxicant was found to accumulate in tips and margins of leaves, probably transpiration being the primary cause of fungicide movement. When the supply of benomyl to the roots was discontinued by transferring plants to fresh soil, the roots and stems rapidly became depleted, fungitoxicant being then found exclusively in the leaves, and after some time even only in the leaf margins. From these observations, the idea can be put forward, that the distribution pattern of BCM within plants is determined to a great extent by the duration of application and the concentrations of benomyl administered. According to this view short-term treatment and low dosages would cause a more rapid depletion of the roots and stems with accumulation in the leaf margins than long-term treatment and high dosages.

The results of our experiments *in vivo* can be reconciled with this idea quite readily. In all of our experiments, the treated plants were exposed to equimolar concentrations of the investigated fungicides for only 2 days and then transferred to fungicide-free garden soil. Therefore, a quite rapid depletion of the roots and stems, and accumulation of fungitoxicant in the leaves, especially the leaf tips and margins, could be expected. Protection against fungal diseases would then be confined to older leaves present at the time of treatment, particularly to leaf tips and margins. This situation was actually found for NF 48-treated plants, and in all cases, where heated aqueous solutions of the three BCM-producing fungicides were used, i.e., where BCM was the exclusive or prevalent fungicide in the aqueous solutions applied. Thus, even though heating, especially of thiophanates (28), causes an appreciable increase of fungicidal activity, this was not reflected in an effective over-all protection of plants. The depletion of the roots and stems even seemed to take place so rapidly, that hardly any fungitoxicant accumulated in the younger leaves, which expanded after the treatment was discontinued. However, in thiophanate-methyl-treated plants and in particular in benomyl-treated plants the experimental periods chosen (from 15 up to 35 days) evidently were not sufficient to cause complete depletion of the roots and stems, at the dosages used, so that BCM in these cases continued to be transported to the leaves which expanded after the treatment period. Thus, treatment with thiophanate-methyl and benomyl caused a much more gradual release of fungitoxicant into the aerial parts. Consequently, protection was much more uniform and longer lasting (Tables 3–5).

Benomyl is BCM substituted at the N-1 position in the benzimidazole nucleus, whereas thiophanate-methyl, most likely, produces an intermediate which is another N-1 substituted BCM. This suggests, that N-1 substitution is responsible for the retention of the parent compound, or, in the case of thiophanate-methyl, the intermediate product, in the roots and the gradual release of BCM to the other plant parts. A significant retention in the roots of the closely related fungicide 2-(4′-thiazolyl) benzimidazole has been reported recently (30).

The nature of this retention is not yet known; it could be due to adsorption of the N-1 substituted compounds onto the outer root surface. Uptake of these compounds followed by rapid "immobilization" within the roots might constitute another explanation. There is some evidence, although not substantial, in favour of the latter view: only immediately after uptake could the parent fungicides be detected in some instances in aerial parts (Fig. 5); moreover, plant tissues are known to be able to "activate" the thiophanates with production of BCM and BCE (28). However, usually, BCM (respectively, BCE)

can be assumed to be the only fungitoxic compound present in the stems and leaves. This is supported by extensive experimental evidence; here, our finding, that bleeding sap of tomato plants, after uptake of the fungicides via the roots, contained exclusively BCM (respectively, BCE) is especially worth mentioning.

Thus, benomyl and to a somewhat lesser extent thiophanate-methyl seem to be excellent systemic fungicides not only because their transformation product BCM possesses a high intrinsic fungitoxicity, but also because of their "innate" retention in (or on) the roots which release a constant supply of BCM to protect the growing plant.

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