

Systemic Fungicidal Effect of Thiophanate Methyl on Verticillium Wilt of Cotton and Its Transformation to Methyl 2-Benzimidazolecarbamate in Cotton Plants

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ABSTRACT

Soil treatment with the systemic fungicide 1,2-bis(3-methoxycarbonyl-2-thioureido)benzene (thiophanate methyl or TPM) prevented foliar symptoms and vascular discoloration of cotton plants caused by subsequent stem inoculation with *Verticillium albo-atrum*. Foliar treatment with TPM was less effective against *Verticillium* wilt than treatment with the hydrochloride salt of methyl 2-benzimidazolecarbamate (MBC·HCl). When roots were allowed to take up TPM for 1 day, the foliage contained not only TPM but also another fungitoxic compound which was isolated and

identified as MBC. After 6 days, nearly all of the TPM had been converted to MBC. TPM was more rapidly converted to MBC in alkaline than in acidic solutions in vitro. The growth of *Verticillium* was inhibited more by MBC than by TPM, but more TPM than MBC was taken up by the foliage of cotton plants. However, MBC·HCl was taken up more readily than either MBC or TPM. Small amounts of TPM were detectable below the area of foliar application but only in the bark tissue.

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The antifungal spectrum of the systemic fungicide, 1,2-bis(3-methoxycarbonyl-1-thioureido)benzene, (thiophanate methyl or TPM) (Fig. 1) is similar to that of methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl). This is explainable since Selling et al. (9) found that TPM was transformed to the hydrolysis product of benomyl, methyl 2-benzimidazolecarbamate (MBC) (Fig. 1) in 50% aqueous acetic acid in the presence of copper acetate under reflux conditions. Vonk & Kaars Sijpesteijn (11) also reported that conversion to MBC was responsible for the fungitoxic effect of TPM. They also reported that the metabolic activity of fungi hastened the rate of conversion of TPM to MBC. Noguchi & Ohkuma (7), using ¹⁴C- and ³⁵S-labeled TPM, concluded that cleavage of bonds between carbon and sulfur in TPM occurred to produce MBC in plants and animals. Since MBC is the fungitoxic hydrolysis product of benomyl in aqueous solution and in plants (3, 8, 10), TPM represents a new source of this systemic compound.

We report here that TPM has a systemic fungitoxic effect against *Verticillium* wilt of cotton and is transformed to MBC (Fig. 1) in cotton plants.

MATERIALS AND METHODS.—*Inoculation and application of the fungicides.*—Cotton plants (*Gossypium hirsutum* L. variety 'SJ-1') were grown in the greenhouse in 10-cm diam (4-in) plastic pots containing either sterilized sand for root treatments or steamed soil (50% peat moss and 50% fine sandy loam) for foliar applications. Plants at 4 to 5 weeks of age were inoculated with a microsclerotial, defoliating isolate of *Verticillium albo-atrum* (V3H) either by stem puncture (5×10^4 spores/ml) with a 20-gauge hypodermic needle at the first node or by root drench with 100 ml of a spore suspension (10^5 spores/ml). TPM was used either as a 100% technical or a 70% wettable powder (Topsin M from Pennwalt Corp.). Concentrations of the chemicals were based on the active ingredient. Ten, 20, or 40 mg portions of TPM were suspended in 50 ml water and drenched on the soil in each pot.

TPM and a solution of MBC prepared in dilute HCl (1, 2) were sprayed on foliage. The wetting agent Triton X-100 (isooctyl phenyl polyethoxyethanol, 0.05%) was added to the MBC solution. Foliage, stems, and petioles were thoroughly sprayed and resprayed in the evening to retard drying as long as

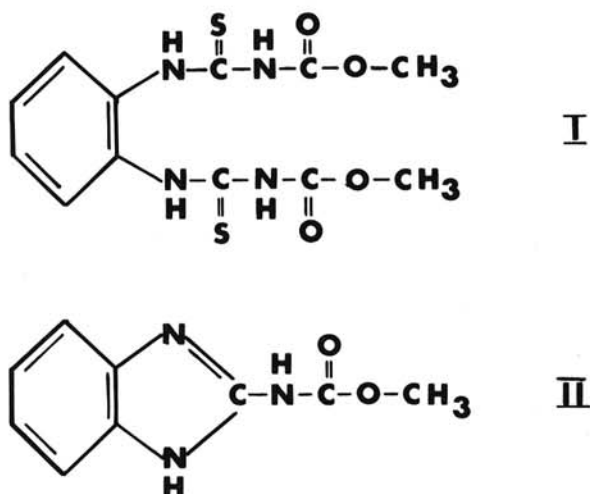


Fig. 1. Structural formulae of I) 1,2-bis(3-methoxycarbonyl thioureido)benzene (thiophanate methyl or TPM), and II) methyl 2-benzimidazolecarbamate (MBC).

possible. Paper towels were placed on the surface of the soil before spraying to prevent contamination of the soil by the fungicides.

At least four replicate plants were included in each treatment. Experiments were repeated two or three times.

Transformation of TPM to MBC.—TPM was converted to MBC by the addition of acetic acid and copper acetate as described by Selling et al. (9). The mass spectrum of the resultant white product was identical to that of MBC. The hydrochloride salt of MBC was prepared by dissolving MBC (2.5 g) in acetone (20 ml) and 0.34 N HCl (75 ml) and heating at 70 C in a waterbath. The solution was diluted with H₂O to 1 liter (2500 ppm MBC·HCl) (1, 2).

Bioassay.—To detect the systemic activity of the chemicals, leaf disks (10-mm diam) from leaves above the area of treatment were assayed for antifungal activity against *Verticillium* by the agar diffusion bioassay method (4). The diam of the zone of inhibition (ZI) indicated the presence of the fungitoxicant. To study downward translocation of TPM, stems were thoroughly sprayed while in a horizontal position except for the basal sections (15 cm) which were protected by wrapping with paper towels. After treatment the nontreated basal sections of the stems and roots were bioassayed for antifungal activity.

Isolation and characterization of the fungitoxicant from cotton plants.—TPM (1 g in 50 ml of water) was applied to each plant as a root drench. After 14 days the plant tissue was extracted by the acidic methanol method described by Sims et al. (9). The filtrate was evaporated under vacuum. The fungitoxic compound was recrystallized from hot methanol and characterized by mass spectrometry, ultraviolet spectrophotometry, and bioautography.

Rate of conversion of TPM to MBC in cotton

plants following root treatment and stem application.—To determine whether TPM itself or its conversion product MBC was taken up by roots and foliage and translocated in cotton plants, cotton plants were treated either with 20 mg of TPM/pot by root drench or with a foliar spray (15,000 µg/ml). At various times after treatment whole plants or xylem tissue from stems were cut in small pieces and homogenized in ethyl acetate containing 3.3% NH₄OH (28-30% NH₃). Following filtration the residue was washed with the same amount of ethyl acetate. The filtrate was divided and one part was used for detection of MBC and the other for TPM. To detect MBC (2, 5) the filtrate was made alkaline with 1.0 N NaOH (5 ml per 25 ml filtrate) and shaken in a separatory funnel for at least 3 min. After discarding the aqueous phase, the process was repeated with the same amount of 1.0 N NaOH. The organic phase was washed three times with small amounts of distilled water and extracted four times with 0.1 N HCl (1 ml per g of initial fresh weight of plant tissue). To each 5 ml of the HCl solution, 1 ml of 1.0 N NaOH, 1 ml of a salt solution (3.3% sodium acetate and 20% sodium chloride), and 1 ml of ethyl acetate were added and shaken for 3 min. The aqueous phase was discarded. After washing with water several times, the ethyl acetate fraction was extracted four times with 0.1 N HCl (0.5 ml per g fresh weight of plant tissue). The ultraviolet spectrum of the extract was obtained and compared at 275 and 281 nm to pure MBC in 0.1 N HCl saturated with ethyl acetate. The concentration of MBC in this fraction was designated as MBC-1.

The other part of the original ethyl acetate extract was evaporated under vacuum at 40 C. The residue was suspended in 50 ml of 50% aqueous acetic acid and copper acetate (25 mg/g fresh weight of plant tissue). The suspension was refluxed for 30 min and the pH adjusted to 7 with NH₄OH (28%). The fungicide was extracted with ethyl acetate (2 ml/g fresh weight of plant tissue) and MBC quantitated as above. The concentration of MBC in the second fraction was designated as MBC-2. The concentration of TPM was obtained by multiplying by 1.78. The amount of nonconverted TPM in plant tissue was calculated by difference.

Conversion of TPM to MBC in buffers at different pH values.—TPM (50 mg) was suspended in 50 ml of McIlvaine buffer at pH values ranging from 3-8 and shaken at 25 C. After an incubation period of 2 weeks, 10-ml samples were adjusted to pH 7 with 1 N NaOH and 0.5 N HCl, and the MBC concentration was determined chemically as above.

Bioautography.—Ethyl acetate extracts of TPM-treated plants were spotted on silica gel (HF 254, Brinkman Instruments, New York) thin-layer chromatography (TLC) plates and developed in chloroform:acetone (50:50, v/v). The dried plates were observed under 254 nm ultraviolet light, sprayed with suspensions of *Cladosporium cucumerinum* spores, and oversprayed with potato-dextrose agar (PDA) at 45-50 C to detect antifungal compounds. The plates were incubated in a moist chamber for 3 days at 25 C and the action of the fungitoxic

component was detected by inhibition of fungus growth.

In vitro test of fungitoxicity of MBC and TPM.—To test the fungitoxic effect of TPM and MBC against *Verticillium*, the fungicides were incorporated into potato-dextrose agar (PDA) at concentrations of 0, 10^{-4} M, 5×10^{-5} M, 5×10^{-6} M, and 10^{-6} M. After inoculation of the plates with a 5-mm mycelium and agar disk subsequent radial growth was recorded. There were four replicates per treatment.

Evaluation of symptoms.—The relative severity of wilt symptoms was rated in terms of foliar chlorosis (0 to 4, with a 4 rating indicating defoliation) and vascular browning. The foliar symptom index (FSI) is calculated:

$$\text{FSI} = \frac{\text{leaf symptom values}}{\text{number of leaves observed}} \times 100.$$

The vascular browning index (VBI), based upon the degree of discoloration of a cross-section of xylem tissue at each internode (rated 0 = white to 3 = completely brown) is calculated:

$$\text{VBI} = \frac{\text{vascular ratings}}{(\text{number of internodes rated}) \times 3} \times 100.$$

RESULTS.—*Root and foliar treatment with TPM and MBC.*—Both MBC and TPM prevented foliar and vascular discoloration symptoms due to *Verticillium* wilt (Table 1) when applied to cotton plants by root drench 2 days before inoculation by stem puncture. Neither TPM nor MBC appeared phytotoxic since there was no reduction of plant height. Bioassays of leaf disks at 23 days after treatment indicated that TPM itself or a conversion product of this chemical had systemic fungitoxic properties. However, only MBC was detected chemically in xylem tissue of plants at 21 days after treatment with either MBC or TPM.

Foliar treatment with MBC·HCl reduced the severity of foliar and vascular discoloration symptoms of *Verticillium* wilt to a greater degree than TPM (Table 2). MBC was detected in xylem tissue 19 days after the last treatment with either MBC·HCl ($9 \mu\text{g/g}$ tissue) or with TPM ($6 \mu\text{g/g}$ tissue).

In another experiment, TPM, MBC, and MBC·HCl were sprayed onto cotton plants three times. When xylem tissue beneath the treated area of the stem was bioassayed 8 days later, the largest zones of inhibition were from plants treated with MBC·HCl, followed by TPM, and then MBC (Table 3). Positive bioassays were also obtained from the bark but not xylem tissue in the nonsprayed stem section below the treated areas.

In vitro fungitoxicity of TPM.—The growth of *Verticillium albo-atrum* was inhibited more by MBC than TPM. TPM completely inhibited the growth of the fungus only at a concentration of 10^{-4} M, whereas MBC inhibited growth at 5×10^{-6} M. At 5×10^{-5} M, TPM reduced the radial growth by about half.

Isolation and identification of the fungitoxic material in plants following treatment with TPM.—To test the assumption that TPM was transformed to MBC in cotton plants, the fungitoxic compound isolated from plants at 10 days after treatment with TPM was characterized. The ultraviolet spectrum of the compound was identical with that of authentic MBC. The mass spectrum of the isolated compound was also identical to that of MBC [m/e (intensity as % of base peak): 191(96), 159(100), 146(28), 133(16), 132(32), 119(20), 118(20), 105(57), 90(20)]. The isolated fungitoxic compound also had the same *R_F* value of 0.48 as MBC on TLC plates.

Uptake of TPM by cotton roots and foliage and rate of transformation to MBC.—When plants were treated with TPM (10 mg) by root drench and stem xylem tissues analyzed after 1 day, the concentration of MBC was $3 \mu\text{g/g}$ and TPM $7 \mu\text{g/g}$; after 3 days MBC

TABLE 1. Effect of root applications with TPM^a and MBC^a 2 days before inoculation of cotton plants by stem puncture (5×10^4 spores/ml) on uptake of MBC and on control of *Verticillium* wilt 21 days later

Compound	Concentration (mg/pot)	Foliar symptom index ^b	Vascular discoloration index ^b	Leaf bioassay ZI (mm) ^c	MBC in xylem tissue ($\mu\text{g/g}$)	Plant height (cm)
Control noninoculated	0	0	0	0	0	90
Control inoculated	0	77	58	0	0	58
TPM inoculated	10	0	0	5	7	94
TPM inoculated	20	0	0	8	10	95
TPM inoculated	30	0	0	10	15	91
TPM inoculated	40	0	0	12	18	88
MBC inoculated	10	0	0	10	10	90
MBC inoculated	20	0	0	13	14	89

^a TPM = thiophanate methyl and MBC = methyl 2-benzimidazolecarbamate.

^b Foliar symptom index = $\frac{\text{Sum of leaf symptom values}}{\text{Number of leaves} \times 4} \times 100$, where symptom values range 0-4.

Vascular browning index = $\frac{\text{Sum of vascular ratings}}{\text{Number of internodes} \times 3} \times 100$, where vascular ratings range 0-3.

^c ZI = Zone of inhibition (diam, mm) of fungal growth around each leaf detected by the agar diffusion assay.

TABLE 2. Effect of foliar treatments with TPM^a (4,500 µg/ml equivalent to 2,500 µg/ml MBC) and MBC·HCl (2,500 µg/ml MBC) 2, 3, 4, and 5 days before inoculation by root drench on *Verticillium* wilt of cotton 19 days later

	Foliar symptom index ^b	Vascular discoloration index ^b	MBC detected in xylem tissue (µg/g)
Control noninoculated	0	0	0
Control inoculated	77	57	0
TPM inoculated	49	27	5
MBC·HCl inoculated	10	7	9

^a TPM = thiophanate methyl; MBC = methyl 2-benzimidazole carbamate.

^b Foliar symptom index = $\frac{\text{Sum of leaf symptom values}}{\text{Number of leaves} \times 4}$

× 100, where symptom values range 0-4.

Vascular browning index = $\frac{\text{Sum of vascular ratings}}{\text{Number of nodes} \times 3}$

× 100, where vascular ratings range 0-3.

was 16 µg/g and TPM was 8 µg/g; after 6 days MBC was 32 µg/g and TPM only 4 µg/g. The fungicides were more concentrated in the stem xylem tissue than in bark or foliage.

Two days after foliage and stems of plants were sprayed with TPM (15,000 µg/ml), the concentrations of chemicals recovered in the xylem tissue were TPM 8 µg/g of tissue and MBC 3 µg/g. Five days later TPM was 4 and MBC was 5 µg/g. Eleven days later TPM was 2 and MBC 4 µg/g.

Conversion of TPM to MBC at different pH values.—TPM was converted to MBC more rapidly at higher pH values in vitro. The concentrations of TPM (µg/ml) converted to MBC were pH 3 (0.3), pH 5 (0.7), pH 6 (5.0), pH 6.4 (10.0), pH 7 (12), and pH 8 (36).

TABLE 3. Uptake and movement of TPM^a (9,000 µg/ml), MBC^a (5,000 µg/ml) and MBC·HCl (5,000 µg/ml MBC) in stems of cotton plants sprayed 8, 9, and 10 days before sampling

Compound	Bioassay ZI ^b (mm)			
	Treated section		Nontreated section 10 cm below the treated area	
	bark	xylem	bark	xylem
MBC·HCl	25	14	5	0
MBC	23	3	5	0
TPM	25	6	7	0

^a TPM = thiophanate methyl and MBC = methyl 2-benzimidazole carbamate.

^b ZI = Zone of inhibition (diam, mm) of fungal growth around each leaf detected by the agar diffusion assay.

Water solubility of TPM.—The solubility of TPM in distilled water (pH 5.5) was 150-160 µg/ml at 25 C. Addition of the surfactant Triton X-100 (0.01 - 0.1%) did not increase the water solubility.

DISCUSSION.—TPM substantially reduced the severity of symptom expression in cotton plants inoculated with *Verticillium albo-atrum*. Although TPM was taken up by roots and translocated upward, a considerable amount of TPM was converted in plant tissues to the more fungitoxic MBC. This was proved by isolation and characterization of MBC from plants previously treated with TPM by root drench. Sims et al. (10) reported that benomyl was also converted to MBC in cotton plants.

The in vitro conversion of TPM to MBC is well documented. Selling et al. (9) reported detection of MBC when TPM was shaken in tap water for 5 days. Our data indicated that the rate of TPM conversion to MBC in vitro increased as a function of the pH, which confirms the data of Vonk & Kaars Sijpesteijn (11) and Matta & Gentile (6).

Most of the TPM applied to cotton stems by either root drench or foliar spray remained intact after 2 days, but increasing proportions of MBC appeared as a function of time. This indicated that TPM was taken up and translocated intact to the xylem tissue, and then underwent conversion to MBC. Noguchi & Ohkuma (7) also reported conversion of TPM to MBC in plant tissue.

TPM appeared to penetrate the bark tissue to a greater extent than MBC as shown by bioassay data. TPM applied to foliage and stems also induced a significant reduction in *Verticillium* wilt severity. Our previous work indicated that neither MBC nor benomyl (as Benlate) applied to foliage had any effect on *Verticillium* wilt. Similar to previous data for MBC·HCl and TBZ·HCl (1, 2), bioassays showed that only small amounts of MBC from either TPM or MBC were translocated downward in cotton stems and that the fungicides were present only in the bark and not in the xylem. Thus, TPM does not approach the desirable ideal of a downwardly translocated systemic fungicide.

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