# Detection of the Systemic Fungicide, Thiabendazole, in Cotton Plants and Soil by Chemical Analysis and Bioassay

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ABSTRACT

Thiabendazole, 2-(4-thiazolyl)benzimidazole, translocated from roots in treated soil to stems but was not always detected in leaves by the agar diffusion bioassay method. By analysis of plants utilizing ethyl acetate as extractant, thiabendazole was detected in roots, stems, and leaves, but the concentrations in upper parts of the plants were much less than in lower parts. By analysis of treated soil utilizing hot methanol, HCl, and ethyl acetate, 77% recovery of thiabendazole was obtained. A similar recovery was obtained by a bioassay method in which crude extracts of thiabendazole from soil were diluted in dimethylsulfoxide (DMSO) and delivered to a paper assay disc on agar. When soil from a treated field (35-40 ppm) was assayed by the DMSO extraction method, only a trace of Thiabendazole was detected after 2 months in the field. Phytopathology 61:964-967.

*Additional key words:* fungitoxicity, translocation, xylem, *Verticillium albo-atrum, Verticillium* wilt of cotton.

Thiabendazole, 2-(4-thiazolyl)benzimidazole, is systemic and fungicidal (7). Control of *Verticillium* wilt in cotton plants inoculated by stem puncture has been obtained by soil treatments with thiabendazole (4). The purpose of this study was to follow the movement of thiabendazole by bioassay in cotton plants from roots to stems and leaves after treatment of soil. To determine whether the fungitoxic substance, detectable by an agar diffusion bioassay method, was thiabendazole, plant tissue which received thiabendazole via roots was analyzed by an extraction and purification method and measured by spectrophotofluorometry. Quantitative analysis of thiabendazole in soil by chemical and by bioassay means was also compared.

METHODS.—Methods of growing Deltapine Smooth Leaf cotton plants, inoculation of stems with a defoliating isolate of *Verticillium albo-atrum* Reinke & Berth. (V3H), and preparation of inoculum were reported previously (1, 4).

Thiabendazole supplied as 60% active wettable powder from Merck Chemical Division, Rahway, N.J., was applied as a drench to the potting medium after making four holes (each ca. 9 ml in volume) in the sand about 1 inch away from the plant. Concentration is expressed as active thiabendazole per pot (w/w).

For the agar diffusion bioassay, 10 ml of potato-dextrose agar (PDA) or PDA-S (amended with streptomycin, 30 μg/ml) were poured in plastic petri dishes 85 mm in diam. A suspension of thiabendazole (0.1 ml) was pipetted on a paper disc (12.7 mm-diam) which was subsequently placed in the center of each dish. Plates of PDA-S were sprayed with spores (ca. 5-6 × $10^6$ ml) 24 hr after placement of the fungicide (1). About 48-72 hr later, presence of TBZ was indicated by a zone of inhibition of fungal growth (ZI). The area of the ZI minus the area of the original disc is expressed in $mm^2$. The procedure for the detection of a fungitoxic compound in plant tissue was basically the same as the in vitro agar diffusion test. Whole plant parts (stems or leaves with petioles) were surface-disinfested in a 0.5% sodium hypochlorite solution for 5 min prior to freezing at −10 C for 24 hr. Leaf sections (9 mm-diam) and stem sections (1 cm long) were placed on PDA-S plates for 24 hr at ambient temperature to allow the fungitoxic compound to diffuse from the plant tissue. The plates were sprayed with a suspension of spores. Since the ZI from treated plant material was not necessarily amenable for quantitative data and the area of the plant tissue was irregular, the area of ZI expressed as $mm^2$ includes the tissue plated.

An analysis was made for thiabendazole in plant tissue utilizing spectrophotofluorometry by Merck Chemical Division. Eight plants/treatment were divided into upper leaves, lower leaves, upper stems, lower stems, and roots. The small amount of soil which was retained by the root hairs was included in the root sample. Dried samples (1.0 g) were finely divided in a Waring Blendor and submerged in a salt solution (3.3% sodium acetate and 20.0% sodium chloride) and extracted with three 10-ml portions of ethyl acetate. The combined extracts were washed with 1 N NaOH and then with distilled water. Five-ml portions of 0.1 N HCl were shaken with the washed extracts for 10 min, and the acid phase was transferred to a solution containing 1 ml 0.1 N NaOH, 5 ml of the sodium acetate-sodium chloride solution (above) and 25 ml ethyl acetate, and shaken for 10 min. The ethyl acetate phase was shaken 10 min in a centrifuge tube with 2 ml 0.1 N HCl. The final acid layer was analyzed on an Aminco-Bowman spectrophotofluorometer having the excitation wavelength set at 300 nm and the emission at 360 nm.

*Analysis of thiabendazole in soil.*—The soil used in the extraction experiments was a Hanford sandy loam, pH 7.6, from Tulare County, Calif. An aliquot of the soil was air-dried and passed through a 2-mm screen. A 100-g sample served as a nontreated check, while another 100-g sample was treated with 10 mg of pure TBZ and mixed well after addition of 50 ml deionized

water. The soil samples were air-dried, screened, and mixed again. Each of the samples was subdivided into three replications of 25 g each for extraction of TBZ and analysis by two different methods.

The extraction procedure was a modification of a method suggested by B. W. Greenwald, Merck Chemical Division. Both treated and nontreated soil samples were simultaneously extracted for 72 hr in a Soxhlet extractor, using hot methanol as the solvent. The crude extracts were removed, cooled, and brought to a volume of 250 ml with methanol.

For purification, 15-ml aliquots were removed from each of the crude methanol extracts and evaporated to dryness in centrifuge tubes in a water bath at 100 C. To the dried material, 15 ml of a buffer (3.3% anhydrous sodium acetate adjusted to pH 4.5 with HCl) and 15 ml of ethyl acetate was added and shaken for 5 min. After centrifugation for 5 min, the ethyl acetate layer was transferred to a clean centrifuge tube and shaken with 10 ml of 0.1 N HCl for 5 min.

The absorbance value of the final acid extract from soil was measured in a 1-cm cell on an ultraviolet light spectrophotometer at 302 nm. Since the ultraviolet absorbance was directly proportional to the concentration of thiabendazole, the concentration per g of soil was calculated from a graph constructed with these data.

To compare the percentage recovery of TBZ by the soil bioassay method with that of the ultraviolet spectrophotometric method, a 2-ml aliquot was removed from each of the crude methanol extracts of soil prior to purification (as above) and evaporated to dryness in test tubes in a water bath at 100 C. The dried material was dissolved in 2 ml of dimethylsulfoxide (DMSO) and bioassayed against spores of *Verticillium albo-atrum* using the agar diffusion method. To paper discs, 0.1 ml of the following materials was applied: (i) the DMSO solvent (check); (ii) the extracted material from nontreated soil; and (iii) the extracted material from the treated soil. The mean areas of the zones of inhibition of *V. albo-atrum* were calculated for each of the treatments for each of three replications.

RESULTS.—*Fungitoxicity of thiabendazole in vitro.*—The effects of several concentrations of thiabendazole on *V. albo-atrum* were compared using the paper disc agar diffusion test. The minimal concentration at which a ZI could be detected in treated plates was 0.3 μg (Fig. 1). The area of the zone of inhibition was directly proportional to the concentration of thiabendazole. Although nearly all spores on the agar in the ZI germinated, growth of the germ tubes ceased. When germinated spores were transferred to a fresh medium, they resumed growth, indicating that thiabendazole was fungistatic and not fungicidal.

*Detection of thiabendazole in plants.*—To determine if a systemic fungitoxic substance (presumed to be thiabendazole) could be detected in plants, four treated plants (50 mg thiabendazole/800 g sand in 50 ml water was applied to the root zone by drenching) and four nontreated plants were bioassayed 42 days after treatment. A ZI (113-452 mm$^2$) was noted around the basal stem sections, but not from sections collected higher than 10 cm above the base. When the xylem cylinder and the bark of the basal sections were bioassayed separately, the ZI around the bark tissue was larger (314 mm$^2$) than that around the xylem tissue (150 mm$^2$). This suggested that the fungitoxic substance not only moved upward in xylem, but perhaps also laterally to phloem or parenchyma tissue in the bark. No ZI was detected in nontreated plants.

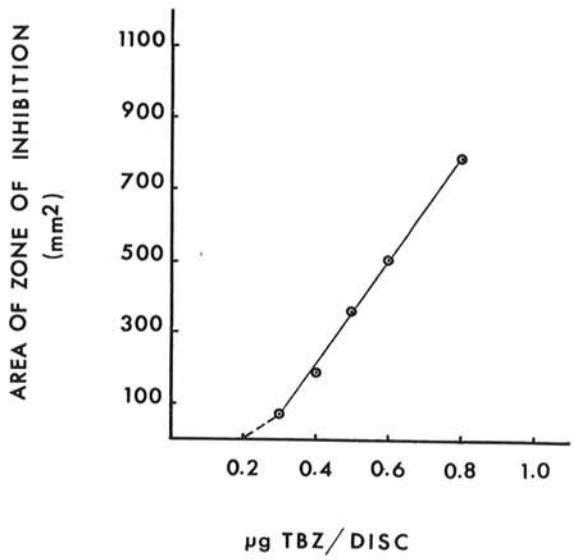


**Fig. 1.** The effect of varying concentrations of thiabendazole applied to paper discs (in 0.1 ml) on agar plates on inhibition of *Verticillium albo-atrum*. A spore suspension ($10^8$/ml) was sprayed on the medium 24 hr after applying the fungicide to the paper disc in the center of the plate.

In a similar experiment, basal stem sections from four treated and four nontreated plants were bioassayed at intervals of 1-34 days. On the first day after treatment with thiabendazole (50 mg/800 g sand), there was a ZI of 113 mm$^2$ around the xylem tissue, but none around the bark tissue in basal stem sections. Three days later, the ZI around the xylem tissue was 907 mm$^2$ and 78 mm$^2$ around the bark tissue. Six days after treatment, the ZI was almost as great around the bark sections (452 mm$^2$) as around xylem sections (615 mm$^2$). However, 34 days after treatment, the ZI around xylem sections was 113 mm$^2$ compared to 390 mm$^2$ around bark tissue. There was no ZI around sections from nontreated plants.

In another experiment, 1-month-old cotton plants growing in 6-inch-square pots (1,600 g sand) were treated with thiabendazole (50 mg/pots). The roots, stems, and leaves of four replicate plants were bioassayed periodically over a 44-day period of time after treatment (Table 1). In bioassays of leaves from the fourth node 5 days after treatment, a ZI of 283 mm$^2$ was recorded. In subsequent bioassays of leaves 8, 13, 21, and 44 days after treatment, no ZI was detected. On the 21st day, there was a ZI from bark tissue but a smaller ZI from xylem tissue. After termination of the experiment, cotton plants were again planted in the same pots and the stems and leaves bioassayed. Since

TABLE 1. Detection of thiabendazole at different times by bioassay of stems and leaves of cotton plants growing in sand amended with two different concentrations of the fungicide when 1 month old[a]

| Rate/pot | Time after treatment (days) | Area of zone of inhibition in plates seeded with *Verticillium* | | |
|---|---|---|---|---|
| | | Leaf | Stem | |
| | | | Xylem | Bark |
| *mg.* | | $mm^2$ | $mm^2$ | $mm^2$ |
| 50 | 5 | 283 | 172 | 393 |
| | 8 | 0 | 277 | 283 |
| | 21 | 0 | 37 | 486 |
| | 28 | 0 | 44 | 376 |
| | 35 | 0 | 0 | 78 |
| | 44 | 0 | 0 | 0 |
| 40 | 1 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 |
| | 3 | 0 | 123 | 33 |
| | 4 | 0 | 57 | 189 |
| | 5 | 0 | 7 | 118 |
| | 10 | 233 | 707 | 817 |
| | 11 | 267 | 707 | 962 |
| | 15 | 0 | 380 | 767 |
| | 27 | 0 | 123 | 541 |
| | 40 | 0 | 0 | 0 |

[a] At each time after treatment, four plants, at the 50-mg/pot (1,600 g of sand) rate, and two plants, at the 40-mg rate, were sacrificed and bioassayed by plating a 10-mm leaf disc and a section (1 cm long) of the stem, separated into xylem and bark, on potato-dextrose agar amended with streptomycin which was seeded subsequently (24 hr later) with spores of *Verticillium* to detect a zone of inhibition of growth (ZI). The ZI for leaves excludes the area of the plant material and the ZI for stems includes it.

no ZI around stems or leaves was detected, it appeared that the active thiabendazole in the sand was depleted.

In another experiment, 1-month-old plants in 4-inch pots of a coarse sandy loam (800 g) were each treated with 50 ml of a suspension containing 40 mg of TBZ. On each day, two plants were harvested and a disc of plant tissue from an upper leaf and a lower leaf and a section of the basal stem about 1 cm in length (separated into xylem and bark) were bioassayed. A ZI occurred around xylem and bark tissue on the 3rd day, but not around leaf discs until the 10th and 11th day. Subsequently, thiabendazole was not detected again (Table 1).

To determine whether thiabendazole per se could be detected in the leaves, stem, and roots, plants in soil in which TBZ (100 ppm, w/w) was mixed immediately prior to planting were inoculated by stem puncture ($5 \times 10^3$ spores/ml). After disease evaluation indicated that the onset of *Verticillium* wilt had been prevented, the plants were sent to Merck Chemical Division for chemical analysis for TBZ by spectrophotofluorometry.

This analysis (Table 2) indicated that the highest concentration of thiabendazole was in the roots and the lowest concentration in the shoots. These data complemented the bioassay data and confirmed that the translocated fungitoxic substance was thiabendazole. Uptake by roots and subsequent translocation of thiabendazole occurred, but the greatest amount remained in the roots and lower stems. In studies with $^{14}C$ thiabendazole, similar data were also obtained (1, and M-C. Wang, D. C. Erwin, J. J. Sims, N. T. Keen, & D. E. Borum, *unpublished data*).

TABLE 2. Concentration of thiabendazole in various parts of cotton plants grown in sand amended with thiabendazole[a] and later inoculated with *Verticillium albo-atrum*

| Tissue | Thiabendazole (mg/kg)[b] | |
|---|---|---|
| | Treated plants | Control plants |
| Upper leaves | 11.8 | 0.67 |
| Lower leaves | 10.7 | 0.31 |
| Upper stem | 10.5 | 0.29 |
| Lower stem | 20.4 | 0.8 |
| Roots | 146.0 | 0.13 |

[a] Thiabendazole applied to sand at 100 ppm (w/w) immediately prior to planting. Analysis by spectrophotofluorometry (courtesy Merck Chemical Div., Rahway, N.J.). The low background values for control plants were considered to be due to interference by certain compounds from plants.

[b] Plants were inoculated by stem puncture with $5 \times 10^3$ conidia/ml 12 days after treatment. All inoculated nontreated plants showed symptoms 50 days after treatment with a mean vascular discoloration index of 4.8 (0 = no discoloration, 5 = xylem tissue completely brown). Control plants showed no external or vascular symptoms, and treated plants showed no external symptoms and a vascular discoloration index of only 0.8.

*Detection of thiabendazole in soil.*—When an extract from soil that was treated with thiabendazole (0.1 mg/g soil) was assayed by ultraviolet spectrophotometry, the final corrected absorbance value 0.995 (value for thiabendazole-treated soil minus control) corresponded with the absorbance value of a solution containing 7.70 μg thiabendazole/ml in 0.1 N HCl. Since the maximum theoretical yield from the treated soil should be 10.0 μg thiabendazole/ml in 0.1 N HCl, the efficiency of recovery from soil was estimated to be 77%.

When extracts were bioassayed, the ZI for the methanolic extract in DMSO from the treated soil was 749 $mm^2$ which corresponded with a ZI induced by a solution of 0.77 μg thiabendazole/disc (Fig. 1). Since a maximum theoretical yield of thiabendazole from the treated soil should have given a reading comparable to 1.0 μg thiabendazole/disc by this method, we concluded that the efficiency of recovery was 77%, the same value obtained by spectrophotometry. No ZI was observed from the DMSO solvent check or from the DMSO extracted material from the nontreated soil.

To determine if the extract that was bioassayed was thiabendazole, the soil extract in 0.1 N HCl was scanned on an ultraviolet light spectrophotometer. The peak (302 nm) and the absorbance curve were the same as those obtained for a solution of reference thiabendazole (10 ppm) prepared in 0.1 N HCl.

Another set of experiments was conducted to determine whether thiabendazole could be detected by the bioassay method after a crude extraction with DMSO. Thiabendazole (10 mg/100 g soil, w/w) was thoroughly mixed with a Hanford sandy loam soil by screening and remixing. A 25-g aliquot of soil was extracted by stirring in 100 ml DMSO for 1 hr and then filtered. The

soil was washed twice with 50-ml aliquots of DMSO and the volume of filtrate increased to 250 ml.

Bioassays were made by the agar diffusion method on paper discs. The average ZI (minus the area of the disc) for the filtrate from the thiabendazole-treated soil was 562 $mm^2$, a value which corresponded to about 64% of the maximum expected recovery (Fig. 1).

TBZ was applied to Hesperia sandy loam soil in a vertical band 20 inches deep behind a chisel to study its effects on *Verticillium* wilt in the field (2) (USDA Cotton Research Station, Shafter, Calif.). The estimated concentration applied in the treated area was 35-40 ppm. When a sample from each of three replications was assayed 2 months after application using the DMSO extraction method, one of the three samples contained only 0.7 ppm thiabendazole and the others none. It appeared that persistence of thiabendazole in field soil was short.

DISCUSSION.—Hine et al. (5) reported that thiabendazole was detectable in a soil bioassayed directly on PDA against *Penicillium expansum* up to 12 weeks but not at 18 weeks. He also indicated that TBZ was shorter-lived in soil than the related compound benomyl. This agrees with our data from field application (2).

It is significant that thiabendazole not only translocates from the roots to the stems of cotton plants but also can be detected in the bark tissue. Since downward translocation in phloem tissue would be a desirable attribute of a systemic fungicide, we have looked for evidence of downward translocation by use of $^{14}C$ thiabendazole (1) and by bioassay methods but have detected only upward movement. Recently, Peterson & Edgington (6) noted a ZI associated with bark tissue from bean stems which received benomyl via roots. However, when they separated the bark from the xylem by means of a glassine paper impervious to the movement of solutes, no benomyl was detected. They concluded that benomyl diffused laterally from xylem to the bark.

Why thiabendazole could not be bioassayed readily from leaf discs except at certain times early in the uptake period of cotton plants is not readily explainable, since the data in Table 2 indicated that chemically extractable TBZ was present in leaves. Based on our unpublished work with $^{14}C$ thiabendazole, the possibility exists that TBZ binds with normal plant material. It would be reasonable to assume that TBZ bound to higher molecular wt products might not diffuse in agar.

Although both thiabendazole and benomyl exerted a similar degree of toxicity in our agar diffusion tests (a minimal inhibitory concentration is 0.3 μg/paper disc [0.1 ml]) benomyl was the most efficient systemic fungicide. This is apparently due to its ability to translocate more rapidly and extensively in the plant than thiabendazole. Peterson & Edgington (6) reported that benomyl applied via roots accumulates at the margins of leaves. Our radioautograms from $^{14}C$ thiabendazole studies indicated that the greatest concentration was in the xylem vessels and not in the leaf margins (1, *unpublished data*). More research on the chemical and physical factors affecting translocation of systemic compounds is thus needed in the study of synthesis and modification of systemic fungicides for their maximal agricultural use.

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