Uptake of Two Systemic Fungicides and Their Breakdown Products by Soybean Seeds

B. L. Kirkpatrick and J. B. Sinclair

Graduate Research Fellow and Professor, respectively, Department of Plant Pathology, University of Illinois, Urbana 61801. Senior author's present address: Department of Botany, University of Illinois, Urbana 61801.

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ABSTRACT

The growth of *Macrophomina phaseolina (Rhizoctonia bataticola)* on potato-dextrose agar (PDA) containing 5, 10, or 50 μg/ml of the systemic fungicides BD 18654 [methyl (1-(((5-cyanopentyl)amino)carbonyl)-1H-benzimidazole-2-yl) carbamate] and TM [thiophanate-methyl = 1,2,-bis-(3-methoxy carbonyl 1-2-thioureido)benzene] was significantly less than controls. Thin-layer chromatography detected BD 18654 and a breakdown product in fresh aqueous solutions; the former but not the latter was heat-stable. *M. phaseolina* and *Penicillium atrovenetum* were sensitive to both compounds in vitro. TM was stable for 1 wk in an aqueous solution, but yielded a breakdown compound, possibly methyl 2-benzimidazole carbamate (MBC), during autoclaving. *M. phaseolina* was sensitive to both of these compounds, but *P. atrovenetum* was sensitive to only MBC.

The four compounds, BD 18654 and its breakdown product, and TM and its breakdown product (MBC), were absorbed by germinating soybean seeds within 4 hr. Charcoal rot was not controlled if seeds were treated with either BD 18654 or TM at 5 and 10 g/kg.

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Macrophomina phaseolina Tassi Goid [*M. phaseoli* (Maubl.) Ashby] [*Rhizoctonia bataticola* (Taub.) Butler] causes charcoal rot of soybean [*Glycine max* (L.) Merr.] (22) and reduces yields in the U.S. (17) and India (11). Charcoal rot has been considered a minor disease of soybeans in Illinois (4), but is observed with increased frequency (L. E. Gray and J. B. Sinclair, *unpublished observations*). The systemic fungicides benomyl, carboxin, and chloroneb were shown to be taken up by germinating soybean seeds (19, 20). The uptake and translocation of benomyl, oxycarboxin, thiabendazole, and chloroneb by soybean seedlings was reported (6, 7). Control of charcoal rot by systemic fungicides may be possible.

This study describes the sensitivity of *M. phaseolina* to two experimental systemic fungicides and their breakdown products in vitro and the uptake of the four compounds by germinating soybean seeds. Studies on the uptake and translocation of these compounds by soybean seedlings were discussed (12).

MATERIALS AND METHODS.—*Materials*.—Certified 'Amsoy' soybean seeds were used throughout this study. An isolate of *M. phaseolina* was recovered from an Illinois soybean field cropped in soybeans for 6 consecutive yr. An isolate of *Penicillium atrovenetum* (G. Smith) was used for bioassays. Stock

cultures of both fungi were maintained on Difco potato-dextrose agar (PDA) at room temperature (25 ± 3 C).

M. phaseolina inoculum was produced by placing a 6-mm diam agar disk (cut with a sterile corkborer) from a 4- to 7-day-old stock culture in each of several culture plates containing 15 ml of soybean-seed extract (SSE) broth and incubating for 24 hr at 35 C. SSE was prepared by boiling 35 g of soybean seeds in 500 ml of distilled water for 10 min, and filtering the broth through cheesecloth. The filtrate was diluted to 1 liter with distilled water, 10 g dextrose was added and the medium autoclaved (121 C for 15 min).

The systemic fungicides were: BD 18654 methyl (1-(((5-cyanopentyl)amino)carbonyl)-1H-benzimidazole-2-yl) carbamate = Chemagro's Bay Dam 18654 50 WP); TM (thiophanate-methyl = 1,2-bis-(3-methoxycarbonyl-2-thioureido)benzene = Pennwalt's Topsin-M 70 WP); and benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate = duPont's Benlate 50 WP).

In vitro studies.—The sensitivity of *M. phaseolina* and *P. atrovenetum* to BD 18654 and TM in vitro was studied using a modification of Borum and Sinclair's method (3). Concns of 5, 10, and 50 μg/ml of each fungicide were prepared in sterile distilled water and mixed with PDA either immediately (fresh) after 1 wk of storage at room temperature, or autoclaved and used immediately. PDA without fungicides served as controls.

Agar plugs (6-mm diam) were cut from 7-day-old PDA cultures of *M. phaseolina* and placed in the center of each of eight culture plates per treatment. The mean diam of growth was recorded after 60 hr at 30 C. A spore suspension of *P. atrovenetum* was prepared in sterile distilled water from conidia scraped from the surface of 3- to 4-wk-old PDA cultures and added to 20 ml of cooled, unsolidified PDA in culture dishes to give approximately 150,000 conidia/ml. Agar disks (8 mm) were cut from culture plates with or without one of the fungicide concns and placed in the center of each plate containing *P. atrovenetum*. The mean diam of inhibition zones were recorded after 12 hr at 8 C plus 60 hr at room temperature.

The fresh autoclaved and nonautoclaved stock solutions were subjected to thin-layer chromatographic (TLC) analysis to determine heat stability and presence of breakdown products. Fifty ml from each aqueous solution was extracted separately by shaking with 10 ml of chloroform about 1 min, then centrifuging at 3,500 *g* for 2 min, and finally collecting the chloroform layer. Ten μl from each chloroform extract plus those from the stock solutions in chloroform were spotted separately on chromatographic sheets (Eastman No. 6061 without indicator). Sheets were developed for 13 to 14 cm in a chloroform: acetone (6:1, v/v) ascending solvent system. Areas on the chromatograms were bioassayed by placing them for 3 hr silica-side down in sterilized enamel trays containing PDA supplemented with either 3 g/liter of homogenized *M. phaseolina* SSE cultures including mycelium and sclerotia, or *P. atrovenetum* spores (130,000/ml agar). The R_f values of inhibition zones were measured after 48 hr at 30 C.

Seed uptake.—The uptake of BD 18654, TM, or benomyl by germinating soybean seed treated at 0.5 g/100 g seed was studied using a modification technique of Allam et al. (1). Seeds were shaken with the fungicides in a plastic bag. Ten treated or nontreated seeds were placed in separate sterile, culture plates containing 10 layers of sterile absorbent paper wetted with 10 ml of sterile, distilled water. The experiment was done twice at 20, 25, and 30 C. After 4 or 8 hr under conditions favorable for germination, 10 seeds were removed, rinsed twice in sterile, distilled water and blotted dry. The seed coats were removed and the seeds rinsed again, blotted dry, sealed in plastic bags, and stored at −15 C for at least 24 hr before bioassay.

For bioassay, each group of seeds without seedcoats was homogenized with 10 ml of distilled water in a VirTis blender for 2 min at high speed. The homogenates were rinsed from the blender with 2 ml distilled water, filtered individually through cheesecloth into separate test tubes and autoclaved. Two ml of each homogenate were pipetted into each of three culture plates to which 8 ml of warm PDA was added. After solidification, a 6-mm plug from a 1-wk-old culture of *M. phaseolina* was placed in the center of each dish and incubated at 30 C. Colony diam were measured when controls reached ~50 mm (about 30 hr).

The uptake and translocation of the fungicides were studied in seedlings planted in 181 × 265 × 51 cm plastic trays filled with 2,500 cc of vermiculite (Terralite) moistened with 1,200 ml of tap water and grown in an Isco growth chamber programmed for 25 C, with a wet-bulb depression of 7 C and a 14-hr day at 43,040 ± 2,152 lx (4,000 ± 200 ft-c). Two 5-day-old seedlings were transplanted to 475-ml (16-oz) plastic (Styrofoam) cups filled with 400 ml vermiculite, wetted with 300 ml tap water and kept uniformly moist with 100 ml drenches of tap water. Seedlings grew normally.

RESULTS AND DISCUSSION.—*In vitro studies.*—*M. phaseolina* and *P. atrovenetum* differed in sensitivity to BD 18654 and TM in vitro. There was a significantly greater inhibition of growth of *M. phaseolina* with each increase in concn of either fungicide (Table 1). Concns of freshly prepared and 1-wk-old nonautoclaved BD 18654 solutions produced significantly greater inhibition than corresponding concns of TM. Inhibition zones of *P. atrovenetum* significantly increased with increase in BD 18654 concns. There was no significant difference between inhibition by fresh and 1-wk-old, nonautoclaved solutions of TM. Autoclaved solutions of BD 18654, however, produced significantly greater zones of inhibition than the corresponding concns of TM. The fungicides and their breakdown products appeared to be fungistatic.

Chromatographs showed that two zones of inhibition were produced by both the nonautoclaved-fresh solution of BD 18654 and the

TABLE 1. The in vitro growth of *Macrophomina phaseoli* and inhibition of *Penicillium atrovenetum* after 60 hr at 30 C on potato-dextrose agar containing solutions of BD 18654 or thiophanate-methyl prepared in sterile water and used immediately or after 1 wk of storage at room temperature (25 ± 3 C) or prepared in water, autoclaved (121 C for 15 min) and used immediately

Organism	Average[w] treatment		Colony diameter or zone of inhibition by solutions added to the medium		
	Chemical[x]	Concn (μg/ml)	Nonautoclaved fresh	Nonautoclaved 1-week-old	Autoclaved fresh
M. phaseoli	None (control)	–	85.0	85.0	85.0
	BD 18654	5	12.4 c[y]	15.8 c	17.1 e
		10	10.5 b	13.0 b	13.0 b
		50	6.4 a	7.2 a	10.4 b
	Thiophanate-methyl	5	18.8 e	20.0 e	17.0 e
		10	15.6 d	17.2 d	13.9 d
		50	12.5 c	13.2 b	9.6 a
P. atrovenetum	None (control)	–	8.0[z]	8.0	8.0
	BD 18654	5	20.2 a	17.6 a	16.4 b
		10	28.8 b	30.4 b	30.5 d
		50	36.9 c	50.1 c	51.6 f
	Thiophanate-methyl	5	8.0	8.0	9.6 a
		10	8.0	8.0	20.5 c
		50	8.0	8.0	38.0 e

[w] Average of eight replicates.

[x] BD 18654 = methyl (1-(((5-cyanopentyl)amino)carbonyl)-1H-benzimidazole-2-yl)carbamate. Thiophanate-methyl = 1,2-bis-(3 methoxycarbonyl-2-thioureido)benzene.

[y] Duncan's multiple range test. Means with same letter in each column are not significantly different at the 1.0% level. Means of 85.0 were not included in the analysis because of no variation.

[z] A value of 8 represents zero activity.

autoclaved-fresh solution of TM. Bioassay of chromatographs showed that both test organisms were sensitive to the heat-stable BD 18654 and the heat-labile breakdown product in fresh aqueous solutions. The mean R_f values for BD 18654 solutions, using *M. phaseolina* and *P. atrovenetum*, respectively, were: nonautoclaved-fresh, 0.35 + 0.74, and 0.35 + 0.75; autoclaved-fresh, 0.35 and 0.34; and chloroform, 0.34 and 0.35. TM was stable for 1 wk in aqueous solution, but lost its toxicity during autoclaving (Table 1). No inhibition zones were formed against *P. atrovenetum* from either nonautoclaved-fresh or chloroform solutions of TM. The mean R_f values for fungitoxic materials in TM solutions using *M. phaseolina* and *P. atrovenetum*, respectively, were: nonautoclaved-fresh solution, 0.66 and 0.0; autoclaved-fresh solution, 0.33 + 0.68 and 0.33; and chloroform, 0.67 and 0.0.

In a separate experiment, chloroform extracts of 4-wk-old aqueous solutions of benomyl were compared to the TM breakdown product by TLC analysis. Two spots detected by reagents were observed for the benomyl solution. The R_f of the TM breakdown product was similar to that expected for methyl benzimidazole carbamate (MBC) from benomyl (14, 15). In an analytical method described by Pennwalt Corporation (2), a TM metabolite was produced identical to MBC. This and other evidence (18, 21) showed that TM apparently breaks down into MBC. *M. phaseolina* was sensitive to both TM and MBC, while *P. atrovenetum* was sensitive to only MBC.

Seed uptake.—The mean growth rate of *M. phaseolina* on all plates containing extracts from treated seeds was lower than controls except extracts of seeds treated with BD 18654 at 20 C for 4 hr (Table 2). The mean diam of *M. phaseolina* colonies growing on plates with extracts from seed treated with TM and incubated at 20 C for 4 or 8 hr were significantly less than those from benomyl-treated seeds, but not at 25 or 30 C (Table 2). The mean diam of *M. phaseolina* on extracts from BD 18654-treated seeds was significantly greater at all temperatures and for both incubation periods than those on extracts from benomyl- and TM-treated seeds, except at 30 C for 8 hr. At 30 C, all mean diam were not significantly different.

Bioassay of seed extracts indicated that BD 18654, TM, and benomyl [or its breakdown product, MBC (5, 14, 17)] were absorbed within 4 hr at 20, 25, and 30 C. This is the first report to show the uptake of any systemic fungicide by any seed within 4 hr. Allam et al. (1) showed uptake of carboxin by cottonseeds within 12 hr, and Thapliyal et al. (19) reported uptake of benomyl and chloroneb by soybean seeds within 12 hr and carboxin within 24 hr.

Longer periods of incubation and higher temperatures generally resulted in greater uptake of the fungitoxicants. The time-uptake correlation was

TABLE 2. Growth of *Macrophomina phaseolina* on potato-dextrose agar containing extracts of soybean seed nontreated (control) or treated with fungicides at 5 g active ingredient/kg seed and incubated for 4 or 8 hr at 20, 25, or 30 C

Incubation time (hr)	Chemical	Mean colony diameter (mm)[y] 20 C	25 C	30 C
4	None (control)	50.0	50.0	50.0
	Benomyl	20.5 d	16.0 b	15.5 b
	BD 18654	50.0	25.3 d	18.8 c
	Thiophanate-methyl	19.7 c	16.3 b	15.3 b
8	None (control)	50.0	50.0	50.0
	Benomyl	19.0 b	15.2 a	14.1 a
	BD 18654	29.1 e	19.2 e	14.3 a
	Thiophanate-methyl	17.1 a	15.5 a	14.0 a

[y] Benomyl = methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate. BD 18654 = methyl (1-(((5-cyanopentyl) amino)carbonyl)-1H-benzimidazole-2-yl)-carbamate. Thiophanate-methyl = 1,2-bis-(3-methoxycarbonyl-2-thioureido)benzene.

[z] Average of six replicates. Duncan's multiple range test. Means with same letter in a column are not significantly different at the 5% level. Means of 50 mm were not included in the analysis because of no variation.

similar to that reported by Allam et al. (1). If uptake of fungitoxicants is directly related to rate and duration of germination, then the longest possible exposure at the optimum for germination (13) should have the greatest uptake of fungitoxicants. This was the case when seeds were incubated at 30 C for 8 hr.

Charcoal rot control.—There was no control of charcoal rot on seedlings grown from any of the treated seed. The percent diseased seedlings for each treatment, respectively was: control, 0; inoculated control, 88; BD 18654 at 5 and 10 g/kg, 72 and 62; and TM at 5 and 10 g/kg, 75 and 62. Seedlings whose roots were exposed for 24 hr to BD 18654 and TM were protected against infection by *M. phaseolina* when inoculated 1 and 3 days after treatment, but not after 7 days (12). One-wk-old seedlings treated with drenches of the two fungicides and inoculated 12 hr later, developed no symptoms after 7 days (12). The two fungicides were taken up by roots and translocated to above-ground tissues and controlled charcoal rot. This was the first report showing systemic distribution of BD 18654 in any plant. There are many reports on the effective control of plant diseases by TM (9, 10, 23). Hardison (8) refers to TM as a systemic fungicide, but provided only circumstantial evidence. The control exhibited by TM presumably was due to its MBC breakdown product.

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