Dichloromethane: Nonaqueous Vehicle for Systemic Fungicides in Soybean Seeds

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ABSTRACT

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Fungicide activity was detected in dormant soybean (*Glycine max* 'Wells') seeds after soaking for 0.5, 1.5, 4, or 24 hours in 25, 50, 100, 200, 400, 800, or 1,600 μg/ml methyl 2-benzimidazolecarbamate (MBC) or [2-(4'-thiazolyl)benzimidazole] (thiabendazole) in dichloromethane (methylene chloride) (DCM), but not when captan, thiram, and carboxin were used. Carboxin, but not captan or thiram, lost fungicidal activity when mixed with DCM. Dichloromethane facilitated the movement of MBC and thiabendazole into dormant soybean seeds in the absence of water. Zones of inhibition in agar plates around treated seeds increased in size with increased concentration of fungicide and soaking time. Soybean (cultivars Hill and Wells) seeds treated with MBC in DCM and thiabendazole in DCM had decreased incidence of internally-borne fungi (*Phomopsis* spp.), higher germination in vitro, and emergence in vermiculite and soil than control seeds treated with DCM alone. Dichloromethane appeared to have some antifungal activity.

Additional key words: seed treatment, *Pencillium expansum*.

Ellis et al. (2) reported that captan and thiram diffused into soybean [*Glycine max* (L.) Merr.] seed coats in the presence of water, but did not penetrate the cotyledons of treated seeds, whereas benomyl a systemic fungicide, moved through the seed coat and into the cotyledons. Other workers reported the movement of systemic fungicides into soybean seeds in the presence of water (3, 4, 9). These fungicides presumably diffuse into seeds along with imbibition water (2). The use of water on soybean seeds, in any but very small quantitities, causes a loosening and slipping of the seed coat and stimulates germination.

Meyer and Mayer (6) reported that anhydrous dichloromethane (DCM) could be used as a carrier to introduce chemicals into seeds in the absence of water without apparent effects on germination and respiration. The ability of DCM to introduce chemicals into dry seeds presumably is related to its miscibility with polar and apolar solvents and limited solubility in water (5, 6). Royse et al. (8) reported that antibiotic activity could be detected in the seed coat and cotyledons of soybean seeds soaked in a mixture of potassium penicillin G in DCM. They found that the mixture had no effect on germination. We report on the uptake of two systemic fungicides in DCM by dormant soybean seeds and their effect on internally-borne fungi and seed germination.

MATERIALS AND METHODS

Soybean seeds (cultivar Wells) were treated by soaking in a 500 μg/ml mixture of either captan [N-((trichloromethyl)thio)-4-cyclohexene-1,2-dicarboximide, Captan 80 WP, Stauffer Chemical Co. Mt. View, California]; thiram [bis-(dimethylthiocarbamoyl) disulfide, Thylate 65 WP, E. I. duPont de Nemours and Co., Inc., Wilmington, Delaware]; carboxin [5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide, Vitavax 65 WP, Uniroyal Chemical, Naugtuck, Connecticut]; thiabendazole [2-(4-thiazolyl)benzimidazole, Mertect 50 WP, Merck and Co., Inc., Rahway, New Jersey]; or MBC (methyl 2-benzimidazolecarbamate, E. I. duPont de Nemours and Co., Inc. in DCM [dichloromethane (methylene chloride), Fisher Scientific Co., Fairlawn, New Jersey]. One-hundred grams of seeds were soaked in each mixture and in DCM alone for 24 hours, and then air-dried for 1 hour.

Fungicide activity was measured using Gibco potato-dextrose agar (PDA) seeded with spores of *Penicillium expansum* Link. A 50-ml spore suspension of approximately 550,000 spores/ml, calculated from spectrophotometer measurements, was added to 1,000 ml of liquid PDA (50 C). A thin layer (5 ml) of this bioassay medium (*P. expansum* agar) (PEA) was syringed into 9-cm culture plates (1), which were stored at 4 C until used.

Ten seeds from each treatment group were washed for 1 minute in running distilled water. The seed coats then were aseptically removed and one cotyledon from each embryo was plated on the PEA. Remaining cotyledons were washed for an additional 1 minute in running distilled water, then plated on the PEA. Plates were incubated at 4 C for 18 hours, then at 30 C for 48 hours (1). Washed, but nontreated seeds, and seeds soaked in DCM alone served as controls. Zones of inhibition were measured from the edge of the plated tissue to the widest point of the zone. The experiment was done four times.

To determine the effect of DCM on fungicide activity, 12.7-mm paper assay disks (Schleicher and Schuell, Inc., Keene, New Hampshire) were dipped separately into 500 μg/ml mixtures of each test fungicide in DCM or in

water. Assay disks dipped in DCM and water alone served as controls. Ten disks from each treatment and controls were air dried for 4 hours then plated on PEA. Zones of inhibition were measured as previously described. The experiment was done four times.

Fungicides MBC and TBZ were the only ones which moved into the cotyledons in the presence of DCM. Therefore, MBC and TBZ were the only fungicides used in further experiments.

In a second experiment, approximately 500 g of Wells soybean seeds were nontreated or treated with one of the following concentrations of either thiabendazole or MBC in DCM: 25, 50, 100, 200, 400, 800, or 1,600 μg/ml. Seeds were soaked in any one mixture for 0.5, 1.5, 4, or 24 hours and allowed to air dry. Seeds soaked in DCM without fungicide served as controls. Whole seeds were washed for 1 minute after treatment with running distilled water and cut in half at right angles to the embryo axis before being plated cut-side downward on the PEA medium. All plates were incubated and zones of inhibition were measured as previously described. There were three replications of 10 seeds per treatment, per concentration, and per time of assay. All data were analyzed using an analysis of variance.

In a third experiment, 10 g of dry seeds were placed in a plastic bag containing either 0.5 g of MBC or thiabendazole and shaken or soaked in 400 μg/ml MBC in DCM or thiabendazole in DCM. After 2 hours the seeds were washed for 1 minute in running distilled water. The seed coats from 50 seeds from each treatment and a nontreated control and one cotyledon from each seed were plated on PEA. The second cotyledon was washed again for 1 minute and plated on PEA. All seed parts were assayed for fungicidal activity as previously described.

To detect the presence of MBC in treated seeds, 25 g of seeds were soaked for 4 hours in a 400 μg/ml mixture of MBC in DCM, washed as previously described, and ground in 200 ml of chloroform in a mortar and pestle. The same method was used to detect the presence of thiabendazole in treated seeds, except that seeds were ground in acetone. These mixtures were centrifuged at 3,500 *g* for 15 minutes. The supernatant solutions were filtered through two layers of Whatman No. 1 filter paper three times, and reduced in volume to approximately 10 ml with a rotating flash evaporator under vacuum. Nontreated seeds treated in the same way served as controls. Twenty microliters of each of the following were spotted separately on Eastman 6061 (silica gel thin-layer chromatographic plates without fluorescent indicator) (TLC): (i) extracts from seeds treated with MBC in DCM and nontreated seeds, and (ii) MBC stock solution (1,000 mg/ml in acetone). Each treatment was chromatogrammed three times in either of two solvent systems. Solvent fronts were run from 12 to 14 cm for

TABLE 1. Inhibition of *Penicillium expansum* on potato-dextrose agar plates by soybean (*Glycine max*) seed halves treated with various concentrations of methyl 2-benzimidazolecarbamate (MBC) or [2-(4′-thiazolyl)benzimidazole] (thiabendazole) (TBZ) in dichloromethane (DCM)

Concentration (μg/ml)	Inhibition zones (mm) around seeds soaked for time in hours[a]							
	0.5		1.5		4		24	
	MBC	TBZ	MBC	TBZ	MBC	TBZ	MBC	TBZ
25	2.9	0.0	4.3	2.6	5.2	4.3	6.6	6.4
50	4.7	2.0	5.7	3.6	7.0	6.0	8.3	7.5
100	7.1	3.0	9.3	4.8	10.9	7.2	12.4	7.7
200	9.0	3.8	10.1	6.0	11.5	7.9	13.3	9.3
400	10.9	5.4	11.5	7.7	12.3	9.8	14.7	11.9
800	11.8	7.5	13.5	10.0	14.6	11.8	15.3	13.7
1,600	13.5	10.2	14.6	12.0	15.6	13.5	17.0	15.6

	MBC	TBZ
LSD ($P=0.05$)=	0.46	0.31
LSD ($P=0.01$)=	0.66	0.92

[a]Means based on 10 seeds in each of three replications. Control seeds soaked in DCM alone produced no zones of inhibition, for any treatment time.

TABLE 2. Germination, total fungi, and *Phomopsis* spp. at 25 C, and emergence in vermiculite and soil in the greenhouse from soybean (*Glycine max*) seeds of two cultivars soaked for 5 hours in 800 μg/ml methyl 2-benzimidazole carbamate (MBC) or [2-(4′-thiazolyl)benzimidazole] (thiabendazole) (TBZ) in dichloromethane (DCM)

Treatment	Percent means for cultivar[a]									
	Hill					Wells				
	PG	TF	PHO	EV	ES	PG	TF	PHO	EV	ES
MBC in DCM	93	8	5	95	86	91	10	1	94	87
TBZ in DCM	93	10	5	96	83	91	8	1	92	86
DCM	63	31	26	72	57	80	24	4	76	72
Control	60	90	33	66	52	74	48	8	75	65
LSD ($P = 0.05$)	8	6	6	7	6	3	10	3	9	6
LSD ($P = 0.01$)	12	9	9	11	9	5	14	4	13	9

[a]Based on three replications of 100 seeds per replication and treatment. PG = percent germination; TF = total fungi; PHO = *Phomopsis* spp.; EV = emergence in vermiculite; and ES = emergence in soil.

MBC-spotted plates using either ethyl acetate plus chloroform (60:40, v/v) or ethyl acetate, chloroform plus acetic acid (1:1:0.04, v/v); and for thiabendazole-spotted plates, acetone, or ethyl acetate. All plates were developed using the bioautographic technique of Peterson et al. (7). Plates were dried then sprayed with warm liquid PEA. The plates were placed on moist paper towels and incubated at 30 C for 48 hours. Zones of inhibition appeared as clear areas in developed plates.

To measure the effects of the various treatments on the seed germination and internally-borne fungi, two soybean seed lots known to contain a high level of seed-borne fungi and low germination were used. A seed lot of 'Hill' produced in 1974 at Mississippi State University had 55% germination with 25 and 87% internally-borne *Phomopsis* spp. and total fungi, respectively. A seed lot of soybean cultivar Wells produced in 1974 at the University of Illinois' South Farm had 74% germination and 8 and 42% *Phomopsis* spp. and total fungi, respectively. Seeds from each lot were soaked either in 800 μg/ml MBC in DCM or thiabendazole in DCM, or in DCM alone for 5 hours and allowed to air dry. Nontreated seeds served as controls. Four days later seeds from both lots from each treatment were surface sterilized by soaking in a 0.25% sodium hypochlorite solution for 4 minutes, in 80% ethanol for 2 minutes, and finally rinsed in sterile distilled water. Three replications of 100 seeds per treatment for both seed lots were either plated onto PDA or planted in flats of vermiculite (Terralite). Three replications of 100 nonsurface-sterilized seeds of both lots per treatment were planted in flats of nonautoclaved field soil. The percentage germination and occurrence of fungi of seeds in culture plants were recorded at 7 days at 25 C. The percent emergence was recorded for seeds in flats after 15 days in a greenhouse. The data were analyzed by analysis of variance.

RESULTS AND DISCUSSION

Zones of inhibition were produced around all washed and nonwashed cotyledons from seeds soaked in MBC in DCM and TBZ in DCM only. Washed and nonwashed cotyledons from seeds soaked in captan, thiram, and carboxin in DCM or in DCM alone produced no zones of inhibition. All assay disks dipped in mixtures of each fungicide and water produced zones of inhibition. Assay disks dipped in water alone or in DCM alone produced no zones of inhibition. All assay disks dipped in mixtures of captan, thiram, MBC, and TBZ in DCM produced zones of inhibition. Assay disks dipped in carboxin in DCM produced no zones of inhibition. Carboxin apparently lost its fungicidal activity in the presence of DCM. Dichloromethane appeared to have no effect on the fungicidal activity of captan or thiram; however, these compounds did not move into the seed in the presence of DCM. No zones of inhibition were observed around washed and nonwashed cotyledons from seeds covered with dry MBC or TBZ and then washed; therefore, it appears that DCM facilitated the movement of MBC and TBZ through the seed coat of soybeans, and into the cotyledons in the absence of water.

Zones of inhibition around soybean seeds treated with MBC in DCM and thiabendazole in DCM increased in size with increase in concentration and exposure time (Table 1). No zones of inhibition were produced around seeds soaked in DCM alone for all treatment times. Zones of inhibition with identical R_f values were noted on all bioautographs spotted with either MBC alone or from extracts of seeds treated with MBC in DCM, and on all bioautographs spotted with either thiabendazole alone or from extracts of seeds treated with thiabendazole in DCM in all solvent systems. No zones of inhibition were noted for extracts of nontreated seeds. Seeds treated with MBC in DCM and thiabendazole in DCM had significantly higher germination in vitro and emergence in vermiculite and soil and a lower total number of kinds of fungi than did seeds that were treated with DCM alone or nontreated (Table 2). Seeds treated with DCM alone had significantly higher germination and less total fungi than nontreated seeds. There were no significant differences between fungicide treatments for either cultivar.

Treating dormant soybean seeds with certain fungicides in DCM provides a method of introducing the fungicides into seed tissue before water imbibition and germination begins. The method has been used to introduce penicillin into soybean seeds (8). Dichloromethane has no adverse effect on germination and appears to be somewhat antifungal. The method could be used to increase effectiveness of seed treatments; reduce losses due to poor germination and seed decays due to internally-borne microorganisms; and insure germination of breeders' seed and seeds with a high percentage of internally-borne microorganisms. The treatment of dry dormant soybeans also may be beneficial when long periods of storage are required, which could lead to seed infection by storage fungi.

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