INFLUENCE OF THE HERBICIDE, CHLORAMBEN, ON SEVERITY OF ROOT ROT OF SOYBEAN CAUSED BY THIELAVIOPSIS BASICOLA

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ABSTRACT

INFLUENCE OF THE HERBICIDE, CHLORAMBEN,
ON SEVERITY OF ROOT ROT OF SOYBEAN
CAUSED BY THIELAVIOPSIS BASICOLA

By

May Lee

The herbicide chloramben (3-amino-2,5-dichlorobenzoic acid) applied at rates equivalent to recommended field rates, caused enhanced necrosis of root and hypocoytls of soybeans infected by *Thielaviopsis basicola*. In the greenhouse, soybeans planted in soil infested with the pathogen and treated with 2 mg chloramben per kg soil (3 lb/acre) had more severe symptoms than soybeans grown in infested soil without chloramben and fresh weight of roots and foliage was reduced. On a scale increasing from 0-6, the disease index usually was 4 for plants grown in soil with *T. basicola* only, and 6 for plants in soil with *T. basicola* plus chloramben. Soybeans grown in artificially infested soil in the field which had been treated with 3 lb chloramben per acre also were more severely diseased, had plant stand reduced by 22%, were stunted, and had seed yield reduced by 17%, as compared with soybeans grown in infested soil without herbicidal treatment.

Soybean varieties "Harosoy 63", "Corsoy" and "Hark" showed enhanced root rot in chloramben-treated infested soil, in the greenhouse. Both endoconidia and chlaymdospores of one isolate of the pathogen caused enhanced disease severity in infested soil treated with chloramben. Inocula consisting of mycelia with spores of four isolates of T. basicola collected from different localities in Michigan also enhanced disease in chloramben-treated soil. Spraying the herbicide on the surface of the soil in pots, or mixing it with the soil were equally effective in inducing more severe Thielaviopsis root rot.

Chloramben at 2, 10, 30 or 100 μg/ml did not stimulate the germination of chlamydospores or endoconidia in water or on soil. It had no effect on linear growth, dry weight or amount of sporulation of the pathogen in culture. Longevity of both types of propagules in soil was not affected by chloramben. The virulence of T. basicola was not altered after being cultured in nutrient media containing 2 μg chloramben/ml.

Other soil microflora did not play a role in disease enhancement, since chloramben-induced disease enhancement was expressed in pathogen-inoculated plants otherwise grown aseptically. Moreover, the fungal and actinomycetes population in chloramben-treated unsterilized soil was not significantly altered by the herbicide. Changes of bacterial populations were inconsistant.

Soybeans grown in the presence of chloramben (2 mg/kg soil) for one week then transplanted into T. basicola-infested soil lacking chloramben developed more severe symptoms than those first grown in untreated soil. Chloramben at 2 mg/kg soil, did not decrease the resistance of soybeans after penetration of the pathogen, since the cortex of soybeans injected with endoconidia developed lesions of similar length with or without chloramben treatment. Chlamydospores and endoconidia germinated 3 and 5 times more, respectively, in the rhizospheres of soybeans first grown aseptically for 7 days in a 2 μg chloramben/ml salt solution, than in rhizospheres of plants without chloramben treatment.

The root exudates of soybeans grown axenically in a dilute salt solution containing chloramben at 2 μg contained more than 5 times the amino acids and 3 times the electrolytes of those without the herbicide treatment. No significant difference in total fatty acids, carbohydrates and nucleic acids in root exudate was detected from plants untreated or treated with chloramben. The root exudates of soybeans grown with chloramben supported germination of 4-fold greater numbers of T. basicola endoconidia than did exudates from soybeans lacking chloramben-treatment. Soybeans inoculated with endoconidia suspended in a concentrated (16 X) exudate from chloramben-treated soybeans developed lesions more than twice as long as those treated with exudates from soybeans grown in the absence of chloramben. Casamino acids in an amount estimated to be similar to that exuded by soybeans under chloramben treatment, when added to sterile soil containing _T. basicola_, caused increased symptom severity on aseptically cultured soybeans as compared with soybeans not so treated.

It is concluded that chloramben stimulated the exudation of nutrients particularly amino acids from roots of soybeans which in turn increased the nutrient status of _T. basicola_ inoculum in the rhizosphere and that this resulted in increased severity of infection.

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ON SEVERITY OF ROOT ROT OF SOYBEAN
CAUSED BY THIELAVIOPSIS BASICOLA

By

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INTRODUCTION

The increased demand for plant protein has made soybeans one of the most rapidly expanding crops in Michigan and in the United States. Between 1948 and 1952 the average harvested acreage of soybeans in Michigan was 128,000. This had increased to 630,000 in 1974. The nation's annual soybean production increased from 846 million bushels in 1965 to 1.6 billion bushels in 1973 (56).

In Dickson's second edition of "Disease of Field Crops" published in 1956, he discussed 16 different diseases of soybeans. *Thielaviopsis basicola* as a pathogen infecting soybeans in the field was first reported by Lockwood, Yoder and Smith (28). The disease was found in Michigan causing necrosis of roots and hypocotyls of soybeans. The wide host range of *Thielaviopsis basicola*, and its wide geographical range suggest a widespread distribution of this pathogen. Its ability to produce chlamydospores allows the fungus to survive in soil for a long period of time.

Herbicides have been more and more widely applied to field crops in recent years, and are indispensable for production in mechanized farming. Chloramben is one of several herbicides used on soybeans. It is recommended for use in most soybean-producing states (11,30,31,35). The estimated usage of chloramben in Michigan is 20% of the total acreage of soybeans (W.B. Meggitt, Personal communication).

An increasing number of diseases is reported to occur more frequently or more severely after the application of herbicides (23). The purpose of this research was to determine if any of the herbicides commonly applied to soybeans could cause increased *T. basicola* root rot; to establish whether the herbicide-pathogen-soybean interaction resulting in disease enhancement is specific to certain soybean cultivars, isolates of the pathogen or environmental conditions; and to investigate the mechanism of disease enhancement.

LITERATURE REVIEW

Chloramben [3-amino-2,5-dichlorobenzoic acid] was first synthesized in 1957 by Amchem Products, Ambler, Pennsylvania. Its biological activity was discovered at the Amchem Research Farm and its patent was issued December 19, 1961. It has been extensively used on soybeans, vegetable crops and ornamentals in the United States (30,31) and other parts of the world (37).

Fate of chloramben in soybeans --- The absorption, translocation and molecular fate of chloramben in soybean, and physiological response of the plant were reviewed by Ashton and Crafts (3). Haskell and Rogers (16) soaked soybean and maize seeds in water for 12 to 72 hours before they were placed in radioactive chloramben for 3 hours. Soybean seeds presoaked for 12 hours absorbed chloramben readily throughout the seed in 3 hours, while maize seeds required 24 hours presoaking for penetration of chloramben into the embryo and 72 hours of presoaking for penetration throughtout the seed. Reider et al. (40) showed that the uptake of chloramben, linuron and other herbicides by dry soybean seed was directly proportional to the herbicide concentration in the soaking solution. Increase in temperature from 10 C to 30 C also increased the rate of absorption. The rate of uptake of chloramben by viable seeds was similar to that by liquid nitrogen-killed seeds. They also found that the absorption of chloramben continued after imbibition of water had stopped. Swan and Slife (48) showed that 32 days after preemmergent application of radioactive chloramben to soil, most of the radioactivity remained in the cotyledons and root system, and no significant amount of activity could be detected in the respired CO_2. Therefore, chloramben was not readily translocated nor broken down to CO_2 in the soybeans.

Colby (7) found that most of the chloramben in soybeans was present as N-glycosyl chloramben, which was later confirmed to be N-(3-carboxy-2,5-dichlorophenyl)-glucosylamine by infrared spectroscopy by Swanson et al. (49). This conjugate was found both in plants tolerant or susceptible to chloramben. Stroller and Wax (47) found that the log precent N-glucosyl chloramben in plants treated with radioactive chloramben was highly correlated with log I_{50} (chloramben concentration required to give 50% reduction of radicle extension); i.e., plants more tolerant to chloramben had higher percentages of N-glucosyl chloramben among the total radioactive compounds present in the plants. Decarboxylation of chloramben was reported by Freed at al. (12) to occur in soybean roots to a limited degree, and was not a major degradation pathway. A structurally unclear chloramben conjugate, chloramben-X, was also found in various treated plants, including soybeans.

Biochemical responses of plants to chloramben treatments --- Little is known about the mode of action of benzoic type herbicides except for their auxin-like properties. Chloramben did not inhibit or stimulate incorporation of

ATP into RNA, nor of leucine into protein, in hypocotyl sections of soybeans (33). Moreland and his coworkers (33) reported that chloramben had no effect on gibberellic acid-induced α-amylase synthesis in deembryonated barley half-seeds. Penner (37) found chloramben inhibition of α-amylase induction in intact barley seeds, and addition of gibberellic acid could not overcome this block. These results suggested that chloramben interfered with the normal function exerted by the embryo of aleurone cells signaling α-amylase production, and that secretion of gibberellic acid is merely one part of the function.

Mann and Pu (29) reported that chloramben at 1 to 20 mg/l stimulated 20 to 44% more incorporation of malonic acid into lipid by excised hypocoytls of Sesbania wexaltata than occurred in the untreated control. Other herbicides with auxin activity, such as 2,4-D, 2,4,5-T, and picloram were also stimulatory to lipid synthesis. Johnson and Jellum (22) tested the effects of herbicides on the chemical composition of soybean seeds, and found that chloramben did not affect the protein and oil content of soybean seeds. Chloramben was the strongest stimulator of upward force of emerging soybean seedlings among 19 herbicides tested (4).

Hubbeling and Chaudhary (21) reported a mutagenic effect of chloramben on Verticillium dahliae. After one week's incubation in 5% chloramben solution, one single spore culture out of fourteen survived. Subsequent single spore isolations of the survivor culture yielded isolates with varying degrees of pigmentation and virulence to one susceptible and three resistant tomato varieties. The most virulent was a black microsclerotial type producing much white mycelium; next was a totally black microsclerotial type, followed by a white mycelial type. A hyaline microsclerotial type proved to be the least pathogenic isolate. All these variants were less virulent than the original isolate, since they produced a less severe symptoms on the resistant tomato varieties than on the susceptible variety, while the original isolate induced equally severe disease on both resistant and susceptible hosts. The high concentration of chloramben used here is not normally encountered in the field. Soldatov et al. (44) found that chloramben applied at 4-6 kg/ha 3 days before sowing of sugar beets decreased soil catalase activity during the first 10 days after application, but later the enzyme activity was similar to that in untreated soil. Chloramben applied at 5 kg/ha insignificantly suppressed the growth of Azotobactor and pea root nodules. However, the biological activity of nitrogen-fixing bacteria was restored during the detoxification of the herbicide (43). There apparently are no other reports of changes in soil microbial activity caused by chloramben.

Effects of chloramben in plant disease development and on the permeability of cells or plant tissues have not been reported.

Influence of herbicides on disease development --- Recently, Katan and Eshel (23) reviewed the interactions between herbicides and plant pathogens. Four possible mechanisms whereby disease severity could be enhanced were identified. These were 1) direct stimulation of the pathogen, 2) increase in virulence of pathogen, 3) increase in host susceptibility, and 4) an indirect effect of pathogens through interaction with other affected microrganisms. A recent report by Percich and Lockwood (38) on increased populations of *Fusarium* and severity of pea root rot following atrazine treatment of soil is an example of the first type of mechanism. Incidence of pea root rot was three times, and of corn seedling blight was twice, that in nonamended soil, when the soil was amended with 30 μg/g atrazine and infested with *F. solani* f. sp. *pisi* or *F. roseum* f. sp. *cerealis* "Culmorum". Germination of macroconidia and subsequent chlamydospore formation of both fungi on soil were also increased by atrazine. Moreover, *Fusarium* populations in natural soil increased up to 4-fold after amendment with atrazine at 10, 30 and 100 μg/g, both in the laboratory and in the field.

Examples of unequivocal evidence of increases in virulence of the pathogen by herbicides are lacking. Hsia and Christensen (20) reported that wheat infected with washed spores and mycelia of Helminthosporium sativum grown in potato-dextrose broth containing 5,000 ppm 2,4-D had more severe root infection and lower green weight than those infected by the same fungus grown in the absence of 2,4-D. However, the unwashed inoculum grown in 2,4-D induced an even lower stand, heavier infection and lighter green weight than the washed inoculum. It was not clear whether the enhancement of disease was due to a change in virulence of the fungus or to predisposition possibly by the residual herbicide in the inoculum. However, the same authors showed that monosporous isolates of H. sativum developed sectors on PDA containing 5,000 ppm 2,4-D. Isolates from the sectors showed varying degrees of virulence, but none seemed obviously more virulent than the parent. This result is inconsistent with the hypothesis of increases of virulence by the herbicide.

Deep and Young (8) reported increased incidence of crown gall on Mazzard cherry (Prunus avium L.) when dichlone (2,3- dichloro- 1,4-naphthoquinone), a fungicide for control of Thielaviopsis and Chalaropis root infections was applied to the trees. Under dichlone treatment, young trees inoculated with an avirulent strain of Agrobacterium tumefacience were as heavily infected (38%) as those inoculated with a virulent strain under the same conditions, yet only 3% were infected by the avirulent strain without dichlone. Moreover, 27% of the trees became infected when treated with dichlone but not inoculated, and 11% of the trees were infected without any treatments. The authors speculated that dichlone suppressed the antagonists in the soil, but the disease enhancement could not be duplicated with captan when the avirulent strain was used. Whether the fungicide predisposed the plant, increased the virulence of the pathogen, or suppressed the antagonists was not determined. The virulence of the pathogen isolated from galls infected with the avirulent strain treated with dichlone was not tested.

Crop plants are more closely related to the target organisms of the herbicides than to the target organisms of other pesticides e.g., fungicides, nematocides, insecticides. Consequently, physiological effects on crop plants are more likely with herbicides than with other pesticides. The enhancement of disease in the host could be caused by morphological or physiological deviations which would increase the susceptibility of the host and/or facilitate the infection of the pathogen. For instance, enhanced root exudation as a result of herbicide treatments could stimulate the germination of pathogens and result in severe infection. Altman (2) found that Tillam [S-propyl butylethylthiocarbamate] and Pyramin [5-amino-4-chloro-2-phenyl-

3-(2H)pyridazinone] increased *Rhizoctonia* damping-off of sugar beets in steamed soil beyond that caused by *Rhizoctonia* alone. Glucose in exudates of roots was greater in plants grown in herbicide -treated soil than in soil not so treated. Lai and Semeniuk (25) showed that picloram at 500 ppm induced increased exudation of carbohydrates from corn seedlings in axenic culture. No increase in amino acid excretion was detected. It is interesting that picloram also stimulates lipid synthesis in hemp seedlings (29). Recently Wyse (60) found that EPTC [S-ethyl dipropylthiocarbamate] and chloramben at 10^{-5}M stimulated the exudation of amino acids from excised roots and hypocotyls of 6-day-old navy beans. Fusarium root rot of navy beans was enhanced by these herbicides, possibly by increased root exudation.

The survival and development of a microorganism depends not only on the physical environment, but also on the biological make-up of its surroundings, i.e. the antagonistic and synergistic interactions with other microflora or microfauna. Herbicide changes in the biological balance involving pathogens could either favor or inhibit the growth, multiplication and finally infection of the pathogen. In spite of reports of the lack of pronounced effects of herbicides on the gross population of soil microorganisms, e.g., picloram (55) and simazine (14), such herbicides may specifically inhibit certain groups or species of microflora while allowing the others to flourish, thus not altering the total population. A good example of a study of the specific effects of herbicides on interactions of microorganisms was the report on competitive colonization of paraquat-treated potato haulms by _Trichoderma veride_ and _Fusarium culmorum_ by Wilkinson and Lucas (59). Paraquat was sprayed on mature potato plants to desiccate the haulms before lifting, as a standard practice in the field. Paraquat-destroyed haulms were brought into the laboratory incubated in a mixture of sand-maize-meal cultures of _T. viride_ and _F. culmorum_ and unsterilized sand at 10 and 20 times the weight of inoculum. Disks were cut out from the potato leaves after incubation for 2, 7 and 14 days, surface sterilized and plated on acid PDA. Nearly 100% of untreated haulms were colonized by _T. viride_ but the ratios of _T. viride_ and _F. culmorum_ dropped to about 60/40 and 45/55 due to 5,000 and 10,000 ppm paraquat respectively. _T. viride_ is antagonistic to many important plant pathogens, such as _Fomes annosus_ (41), _Armillaria mellea_ (13), _Rhizoctonia solani_ (58) and _Sclerotium rolfsii_ (42). Although the experimental conditions did not simulate the complex species composition of microflora in nature, it provided a quantitative measurement of the competitive colonization under controlled conditions. The influence of an herbicide on the initial colonization of an organic substrate could change the population of the colonizing pathogen or that of its antagonists and the rate of decomposition of the substrate, which could in turn affect the persistence of the pathogen or its antagonists.

Effect of chemical compounds on *Thielaviopsis basicola* and disease development --- Linderman and Toussoun (26) found that benzoic, phenylacetic, 3-phenylpropionic and 4-phenylbutyric acids from decomposing plant residues in soil increased susceptibility of cotton to *Thielaviopsis* root rot. The chemicals did not stimulate germination of the fungus. Increased spore germination on root surfaces of plants treated with these chemical led to the speculation that changes in root exudation resulted in the increased activity of the fungus. Toussoun and Patrick (51) showed that extracts of decomposing plant residues enhanced root rot of beans caused by *Fusarium solani* f. sp. *phaseoli*, *Thielaviopsis basicola* and *Rhizoctonia solani*. Ninhydrin-positive substances and traces of sugars were exuded from the extract-treated area but not from water-treated area. The authors suggested that the alteration of cell permeability is one of the more important effects produced by toxic decomposition products. Toussoun et al. (52) reported that benzoic acid and phenylacetic acid were the major components of the extract of decomposing plant residue.

MATERIALS AND METHODS

The pathogen --- *Thielaviopsis basicola* (Berk. & Br.) Ferr. isolate 157, originally from Petersburg Township, Monroe Co., Michigan, was used unless stated otherwise. Isolates 170, 171 and 172 were obtained from Saginaw Co., Shiawassee Co., and Saginaw Co., respectively. Inoculum for greenhouse and laboratory experiments was grown for ten to fifty days at 24 C on potato-dextrose agar in petri dishes. For soil infestation for greenhouse experiments, the culture was blended with 100 ml of water before mixing with soil. Inoculum used in field experiments was grown on soybean-sand medium. Twenty g dry soybean seeds were chopped in a blender into a coarse meal and mixed with 1,000 g sand and 500 ml of water. The mixture was steamed for one hour, broken up, distributed into 4 one-liter flasks and autoclaved for 30 minutes.

The herbicides --- The herbicides used in the greenhouse and the field experiments are listed in Table 1 with their common names, trade names, chemical names, sources and concentrations applied. Commercial formulations were used in the greenhouse and field experiments. A stock solution of 10 mg active chloramben/ml was made by diluting the commercial formulation of chloramben, which is 2 lb. active chloramben/gal. with distilled water. It was stored at 4 C in the dark. A technical preparation of

Table 1. Herbicides, their sources and rates applied to greenhouse experiments.

Common names	Trade names	Chemical names	Sources	Conc. of commercial formulation (lb/gal.)	Rates applied (lb/acre)[a]
chloramben	Amiben	3-amino-2,5-dichlorobenzoic acid	Amchem Prod., Inc.	2	3
linuron	Lorox	3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea	E.I. duPont de Nemours & Co.	50% wp	2.5
alachlor	Lasso	2-chloro-2',6'-diethyl-N-(methoxy methyl)acetanilide	Monsanto Co.	4	2.5
DNBP	Dinoseb	2-sec-butyl-4,6-dinitro-phenol	Dow Chemical Co.	3	3
trifluralin	Treflan	α,α,α-trifluoro-2,6-dinitro N,N-dipropyl-p-toluidine	Eli Lilly & Co.	4	1.25
fluorodifen	Preforan	p-nitrophenyl-α,α,α,-trifluoro-2-nitro-p-tolyl ether	CIBA Corporation	3	4.5
metribuzin	Sencor	4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5(4H)-one	Chemago Corp.	50% wp	0.5
bentazon	Basagran	3-isopropyl-2,1,3-benzo-thiadiazinone-(4)-2,2-dioxide	Badische Anilin-& Soda-Fabrik AG	4	2

[a]Active ingredient

chloramben was used in laboratory experiments, such as in tests of spore germination in water and for application to axenically grown soybeans. Technical chloramben was dissolved into acetone to make stock solutions of 4,000 or 400 ppm which were stored at 4 C in the dark.

Greenhouse tests --- Soybean, Glycine max (L.) Merr., "Harosoy-63" was used as the host plant unless stated otherwise. Seeds were sown in a greenhouse mix consisting of steamed soil, peat and coarse sand (1:1:3,v/v/v) after herbicide application and/or fungal infestation. Chloramben was applied at the rate of 3 mg per 1400 g soil in a 10.5 cm (4 inch) diameter pot, which approximated the recommended field rate, 3 lb/acre or 3.34 kg/ha. The surface area of a pot is about 95 cm^2 and the depth 14 cm (5.5 inches). To assure an even distribution, the stock solution was diluted about 60 times with distilled water and atomized into the soil during hand-mixing in a pail. Routine watering was done by filling the saucers to prevent contamination by splashing. Since this method did not permit simulation of leaching of herbicides sprayed onto the soil surface, chloramben was incorporated into soil unless stated otherwise. Blended inoculum was then mixed with the soil by hand. Each pot of soil was infested with the equivalent of 1/4 agar culture. The soil was then placed in a plastic pot, watered, and ten to twelve seeds were planted 1 to 2 cm (0.5 inch) deep. The containers were punctured at the bottom to allow drainage, except when placed in controlled temperature water baths. Sufficient water was added daily to replenish the moisture lost through transpiration and evaporation. After 3 weeks, plants were removed from soil and evaluated for shoot and root growth and symptom development.

Disease symptoms were evaluated according to a 0 to 6 scale: 0, no disease symptoms; 1, isolated black flecks on roots or hypocotyls; 2, coalescence of flecks without girdling; 3, primary root or hypocotyl girdled by lesions, and the coalescent lesions shorter than 1 cm; 4, girdling lesions longer than 1 cm, and total lesion area less than 50% of the entire root system; 5, lesion area 50-90% of the entire root system; 6, lesion area covering 90-100% of root system and often completely dark charcoal colored. Each treatment was replicated four times in four different pots. Pot ratings were the mean of 8 to 10 individual plants, which seldom varied more than 2 units. The disease index was the mean of the replicate pot ratings. The fresh weight of plants in each pot was also recorded. Student's t-test was applied to all possible pairs of treatments. i.e., no treatment, chloramben alone, *T. basicola* alone and chloramben + *T. basicola*.

Field experiment --- The effect of chloramben on development of *Thielaviopsis* root rot was studied in a plot at the Michigan State University Farm. Rows 3.5 feet apart and 20 feet long were marked out, and a seed furrow about 2 inches deep was made in each row. About 30 g soybean seeds, "Harosoy-63", were sown uniformly by hand in each row on June 5, 1974. Treatments included rows treated with either chloramben or linuron alone, chloramben or linuron + T. basicola, T. basicola alone, and an untreated control. The treatment units were single rows, replicated 6 times in a latin square arrangement. Rows with T. basicola were infested with 40 g of a one-month old soybeanmeal-sand culture of the pathogen. Seed furrows were covered with soil after seeding and infestation. Commercial formulations of chloramben and linuron were diluted with water and sprayed with a hand-held CO_2-driven sprayer one day after planting. The sprayer were calibrated to deliver one quart of liquid at a pressure of 30 lb/in^2 in 70 seconds. The sprayer was moved steadily at 2 ft/sec over the rows, which received an equivalent of 3 lb chloramben/acre or 2.5 lb linuron/acre, the recommended rates. At emergence and at weekly intervals thereafter, all plants from a randomly chosen one-foot length of row were removed and examined for symptom development. Stand counts were made at 38 days, and plant height were measured 64 days after planting. Plants were harvested in mid-October, and seeds were threshed with a portable thresher and weighted.

Spore germination tests --- Endoconidia were removed from PDA culture tubes by adding sterile distilled water and agitating. They were then washed three times with sterile distilled water by centrifugation at 2 C. The final conidial pellet was resuspended in 5 tubes, each containing 10 ml sterile glass distilled water. Sufficient stock solution of chloramben was added to each of the four tubes of spore suspension to give final concentrations of 2, 10, 30 and 100 µg/ml chloramben. The fifth tube was left as a control. The same was repeated with acetone without chloramben at the highest concentration of 10 µg/ml. Approximately 60 µl of spore suspension were placed into the well of a depression slide, and were covered with a coverslip. The slides were examined under the microscope for germination. Two hundred spores in each of the three replicate wells were examined. All vessels and slides were acid-washed, thoroughly rinsed in distilled water and then autoclaved before use. Coverslips were autoclaved and care was taken not to handle the slips with fingers before sterilization. All procedures were carried out aseptically.

Except for spore preparation, germination of chlamydospores was tested in the same manner. Germination of one or more cells in a short chlamydospore chain was considered to indicate germination of that propagule. Germination of spores on chloramben-amended soil was tested with washed spores applied to smoothed soil plates and recovered on plastic film after incubation and staining.

Preparation of chlamydospores --- Many of the experiments required chlamydospores without the presence of endoconidia. A procedure was developed to obtain single cells or short chains of chlamydospores aseptically. *T. basicola* was streaked on enriched PDA (extract of 200 g potato, 5 g yeast extract, 30 g dextrose and 20g agar per liter) and incubated for at least 30 days until chains of chlamydospores were mature. A large portion of the endoconidia were removed by washing the surface of the culture three times with a stream of distilled water. The aerial mycelia bearing chlamydospores were then scraped from the agar with a spatula and were transferred to the glass vial of a motor-driven tissue homogenizer. After five minutes of grinding in an ice bath, the mycelia and chlamydospores were transferred to a 15 ml centrifuge tube and diluted with water to about 10 ml. The mixture was then centrifuged for brief periods repeatedly at slow speed using a table-top swinging bucket type centrifuge. For each centrifugation the rheostat was turned to approximately the mid-point for 25 seconds, which gave an average speed of 800 rpm during the last 5 seconds. The supernatant, consisting of a very large proportion of endoconidia, was decanted. The sediment was resuspended in 10 ml of water each time. After 8-10 such centrifugations, the sediment contained nearly 100% single-celled or short chains of chlamydospores.

Enumeration of the population of *T. basicola* in soil --- The most probable number method using carrot disks, was used to enumerate the population of *T. basicola* in soil. The procedure was similar to that described by Tsao and Canetta (54) except for the following modifications. A series of 10-fold dilutions up to 10^{-6} in distilled water were prepared from the equivalent of one gram oven-dry soil. One-tenth ml of each dilution except 10^{-1}, was placed on each of 10 freshly cut disks on moist filter paper enclosed in a 10 cm diameter glass petri dish. Each dilution was replicated in three separate dishes. Carrot disks of uniform surface area were sectioned ca. 8 mm thick from surface-sterilized carrot roots (24). After inoculation, the petri dishes were enclosed in a plastic bag to prevent desiccation and were examined for growth of *T. basicola* after 5 to 6 days. The number of disks having *T. basicola* was recorded. The most probable number of propagules was determined from a table prepared by Halvorson and Ziegler (15). The statistical significance of differences between two estimated values was tested by method of Cochran (5). Using 10 samples instead of 3 resulted in narrowing of confidence limits at the 95% level from 6.61 to 2.32, and reduction of standard error of the log of the population density from 0.34 to 0.18.

Enumeration of soil microorganisms --- The number of propagules of soil fungi, bacteria and actinomycetes was estimated by soil dilution plates techniques with selective media used by Farley and Lockwood (10). Soil suspension was prepared by adding the equivalent of 10 g oven-dry soil to 100 ml sterile 0.85% NaCl solution, followed by mixing with a wrist-action shaker for 20 minutes. A series of 10-fold dilutions was made up to 10^{-7}. Dilutions of 10^{-3} and 10^{-4} were used for enumerating fungi, and dilutions of 10^{-6} and 10^{-7} for bacteria and actinomycetes. Tergitol NPX, a detergent, was added to potato-dextrose agar (1,000 mg/l) to retard fungal growth. The medium was acidified with lactic acid at 30-50 mg/l after autoclaving, cooled to 42 C and mixed with 1 ml diluted soil suspension in a petri dish. Fungal colonies were counted after 5 days' incubation at 24 C. The bacterial colonies were enumerated after 3 to 5 days' incubation of 1 ml soil suspension with soil extract agar which contained 50 mg PCNB/l. The population of actinomycetes was estimated from 1 ml diluted soil suspension mixed in chitin agar after 10 days' incubation.

<u>Axenic culture of soybean plants</u>--- Soybean seedlings free from microorganisms were necessary for experiments in which nutrient levels in root exudates were monitored. The method for culturing axenic soybeans was developed after trying several conventional sterilization methods which often were phytotoxic. Soybean seeds with intact seed coats were surface-sterilized in 1,000 ppm sodium

hypochlorite for 15 minutes, then allowed to imbibe sterile water into which an air stream was continuously bubbled for 5 hours. This sterilization was not phytotoxic; however, it did not kill all the bacteria in the seed coats. Aeration enhanced uniformity of germination. Seeds were then surfaced-sterilized again in 1,000 ppm sodium hypochlorite for 15 minutes, then were rinsed in sterile distilled water. Sterile petri plates were lined with two pieces of 9 cm diameter autoclaved filter paper (Whatman No. 1) with folded parallel grooves and moistened with 5 ml water or 2 ppm chloramben solution. Surface-sterilized soybean seeds were placed in the parallel grooves of the filter paper with the hilum down. This moist chamber allowed sufficient water and air for germination. The grooves allowed the radicles to develop straight and the seedlings to be easy to handle. The petri dishes were enclosed in plastic bags and incubated at 24 C in the dark for 4 to 6 days, when the radicles were 3 to 6 cm long.

Axenically cultured soybean seedlings were incubated with radicles immersed in 1/10 X modified Hoagland solution (6) containing chloramben in 21 X 100 mm test tubes with stainless steel caps. Each tube contained 4 seedlings. Technical chloramben was added to sterilized distilled water at 2 μg/ml (10 μl) from a stock solution in acetone containing 4,000 μg chloramben/ml. Modified Hoagland solution was prepared in a 5X strength stock solution aseptically. The salt crystals were added aseptically and not subjected toheat to prevent precipitation. When plated on PDA, the solution was found to be free of micro-organisms. Chloramben-free acetone control was prepared by adding 10 µl acetone (final concentration 0.5 µl/ml) to the salt solution. Controls free of both acetone and chloramben were also prepared. Seedling holders were made from paper clips coated with paraffin to prevent rust or chemical interference by the metal. Holders were surface-sterilized in 1,000 ppm sodium hypochlorite before use. Seedlings were transferred to the holders with cotyledons being suspended above the liquid and radicles immersed.

Collection and analysis of root exudation --- After 36 hours' incubation, the holders with the supported seedlings were lifted from the original tube in a transfer hood with filtered air. The seedlings were rinsed with 2 ml aliquots of sterile distilled water 5 times to remove the herbicide, electrolytes and other material adhering to the root. Four seedlings were then placed in each of four 25 X 180 mm culture tubes containing 40 ml distilled water for 12 hours to collect root exudates. The seedlings were then placed once more in modified 1/10 X Hoagland solution with chloramben for 36 hours. When the plants were removed, the roots were washed once more, and the exudate collected. The incubation and exudation periods were alternately repeated for a total of four times.

The exudate solutions were measured for electrolyte concentration with a conductivity bridge, and were then concentrated with a flash evaporator at 60 C. Contamination was tested by allowing excess liquid to drop from the roots into a petri dish, then mixing with molten (42 C) PDA, and examining for the appearance of colonies after 2 days. The concentrated exudates were adjusted to equal volumes and were quantitatively analyzed for sugar, amino acids, fatty acids and nucleic acids. The anthrone reagent (34) was used to determine the total carbohydrate content, using glucose as a standard. Total amino acids and related compounds were determined with ninhydrin (32) using glycine as the standard. Ferric hydroxymate (39) was used to determine the total fatty acid content. The nucleic acid content of root exudate was determined by ultraviolet absorption at 260 nm.

Spore germination in the rhizospere --- Soybeans were germinated axenically in petri dishes containing 2 ppm chloramben solution for seven days. Chlamydospores were prepared as described before except that a similar number of endoconidia was retained in the suspension. Acid-washed glass slides were dipped in molten 2% water agar (45 C) and set horizontally, and the spore suspension was atomized onto the slides before the agar had solidified. It was then air-dried for 20 minutes. Each slide was then transfered to a petri dish with the side containing spores

upward, and each of five chloramben-treated and control soybean seedlings were placed onto separate slides with the radicle in contact with the agar film. Sieved, (2 mm) moistened (15-20%) Conover loam was added to conver the seedling and slide, filling the petri dish. The petri dish was then covered, taped to prevent shifting of the seedling and to ensure close contact of soil particles with the seedling and slide, and set at a slope (approximately 45°) with the long axis of the slide parallel to the slope. This was done to keep the roots growing along the slide during the 12 hour incubation period. After incubation, the petri dishes were inverted and the bottoms were carefully removed without disturbing the slides and seedlings. The outlines of radicles were traced on the back of the slides. The slides were then lifted carefully and laid with the spore side up in the air for 30 minutes. Most of the adhering soil particles were removed with a small camel's-hair brush. The cleaned slide was stained with cotton blue-lactol phenol for 10 minutes and rinsed by dripping in distilled water. Germination of approximately 700 spores of each kind within the marked outline area were counted on each slide.

RESULTS

Effect of herbicides on Thielaviopsis root rot --- Eight herbicides commercially used or with potential to be used for soybean crops were tested in the greenhouse for their effect on *Thielaviopsis* root rot development in artificially infested greenhouse mix or naturally infested soil collected from a soybean field in S. E. Michigan. The rates applied, which are within the range of recommended rates and resulting disease indices are listed in Table 2. Chloramben, alachlor, and DNBP significantly enhanced the symptoms of *T. basicola* on soybeans planted in both soils. Chloramben showed the greatest enhancement in the naturally infested soil. Linuron had no significant effect on symptom development, but the roots appeared lighter in color than those of control plants. Trifluralin, flurodifen, metribuzin and bentazon did not cause significant changes in disease development in either soil. No disease symptoms were detected on plants from any of the herbicide treatments in uninfested soil.

Effect of chloramben on Thielaviopsis root rot --- Chloramben increased *T. basicola* root rot of soybeans to a greater extent than other hebicides tested. Therefore, it was further investigated for its effects on development of infected plants such as fresh weights ahd shoot length, and for its effect on the disease under various conditions,

Table 2. The effect of herbicides on *Thielaviopsis basicola* root rot of soybeans planted in steamed greenhouse soil and in naturally infested soil in the greenhouse.

Treatment	Rate, lb/acre	Disease index[a]	
		Greenhouse mix	Naturally infested soil[b]
chloramben	3.0	6.0 A	4.8 A
alachlor	2.5	6.0 A	3.3 A
DNBP	3.0	5.9 A	3.6 A
linuron	2.5	4.3 B	1.0 B
trifluralin	1.25	5.0 B	1.9 B
fluorodifen	4.5	5.0 B	1.8 B
metribuzin	0.5	5.0 B	1.1 B
bentazon	2.0	5.4 B	2.1 B
untreated	0	4.5 B	1.1 B

[a]Mean of 4 replications, each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Analysis of variance was applied to all data in each column separately. Student's t-test was applied to all possible pairs of treatments. Means followed by different letters are significantly different, according to student's t-test (P = 0.05).

[b]The soil was collected from a soybean field in S.E. Michigan, and mixed with one-third volume of coarse sand before use.

such as different potting mixtures, different host varieties and isolates of the pathogen.

Soybean seeds were sown into T. basicola-infested chloramben-treated or untreated greenhouse mix or Conover loam in the greenhouse. After 3 weeks' incubation the plants were removed from soil, washed, weighed and graded for disease symptoms and for amount of stunting. Soybeans grown in either soil treated with chloramben again had more severe disease symptoms than those grown in soil without chloramben (Table 3, Figs. 1 and 2). Uninoculated plants showed no symptoms. Stunting of roots resulting from chloramben application was not considered in the disease rating. Either T. basicola or chloramben reduced the fresh weight of soybeans by nearly 10% in the greenhouse mix. However, chloramben + T. basicola reduced fresh weight of plants to nearly 50% of the no treatment control. Soybeans were compared with the mean height of no treatment plants and were rated 1, up to 10% shorter; 2 up to 20% shorter; 3 up to 30% shorter and so on. Chloramben + T. basicola plants were rated 3, while chloramben and T. basicola alone reduced the plants to 1 or 2, and 0.2, respectively. Similar results were obtained when the experiment was repeated.

Effect of chloramben and linuron on Thielaviopsis root rot of soybeans in the field --- Disease severity of soybeans grown in the field of chloramben-treated soil was increased over that of soybeans grown without chloramben

Table 3. The effect of chloramben on _Thielaviopsis basicola_ root rot of soybean in the greenhouse.[a]

Treatment	Disease Index[b]		Fresh Weight of plants(g)[b]		Stunting of shoots[b]	
	Greenhouse mix	Conover loam	Greenhouse mix	Conover loam	Greenhouse mix	Conover loam
No treatment	0.0 A	0.0 A	26.8 A	27.5 A	0.0 A	0.0 A
Chloramben	0.0 A	0.0 A	23.5 B	20.8 B	1.0 B	2.0 B
T. basicola	5.0 B	2.8 B	24.4 B	20.9 B	0.2 A	0.2 A
Chloramben + _T. basicola_	6.0 C	5.3 C	13.7 C	17.6 C	3.0 C	3.0 C

[a]Chloramben was applied to soil before seeding at the rate of 3 lb/acre.

[b]Mean of 4 replications each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Stunting index was based on a scale of decreasing shoot height from 0-6. As compared with the mean height of "no treatment" plants, soybean was rated 1 up to 10% shorter; 2, up to 20% shorter; 3, up to 30% shorter; 4, up to 40% shorter; 5, up to 50% shorter and 6, up to 60% shorter. Students's t-test was applied to all data in each column, separately. Means followed by different letters are significantly different (_P_ = 0.05).

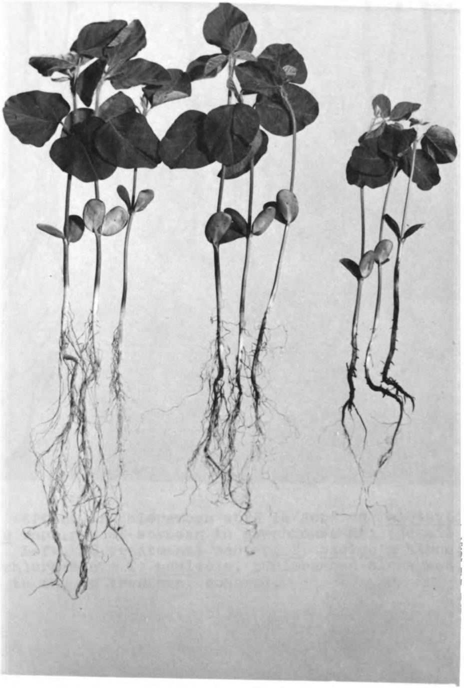

Fig. 1. Effect of chloramben at 3 lb/acre on Thielaviopsis basicola root rot of soybeans in greenhouse mix. Left, no treatment; center, T. basicola alone; right, chloramben + T. basicola. Chloramben alone was similar to the no treatment.

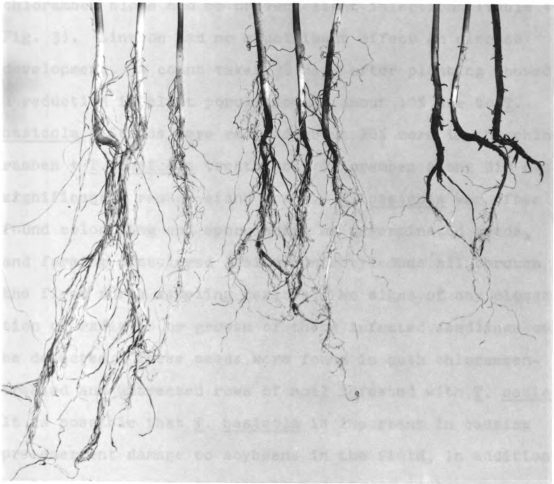

Fig. 2. Effect of chloramben at 3 lb/acre on Thielaviopsis basicola root rot of soybean in greenhouse mix (detail of roots). Left, no treatment; center, T. basicola alone; right, chloramben + T. basicola. Chloramben alone was similar to the no treatment control.

in all three sampling periods. No treatment controls or chloramben alone had no or very light infections (Table 4, Fig. 3). Linuron had no significant effect on disease development. A count taken 38 days after planting showed a reduction in plant population of about 10% due to *T. basicola*. Stands were reduced about 20% more in the chloramben + *T. basicola* treatment. Chloramben alone did not significantly reduce stand count. *T. basicola* was often found colonizing and sporulating on ungerminated seeds, and forming discolored lesions on cotyledons all through the first three sampling periods. No signs of any elongation of radicles or growth of these infected seedlings could be detected. These seeds were found in both chloramben-treated and untreated rows of soil infested with *T. basicola*. It is possible that *T. basicola* is important in causing pre-emergent damage to soybeans in the field, in addition to its pathogenesis to established stands (28).

Chloramben + *T. basicola* also decreased height and yield of soybeans when compared with the treatment of *T. basicola* alone (Table 4). Although the later was not significantly different in plant height when compared with no treatment or chloramben alone, its seed yield was 12 and 14% lower than the other two, respectively.

Table 4. Effect of chloramben on *Thielaviopsis basicola* root rot of soybean in the field.[e]

Treatment	Disease Index[a]			Stand count[b]		Plant Height[c] (inches)	Seed yield[d] (g)
	10 days	16 days	23 days	38 days	Harvest	64 days	Harvest
No treatment	0.1 A	0.5 A	0.5 A	127 A	140 A	18.5 A	1472 A
Chloramben	0.1 A	0.5 A	0.5 A	134 A	138 A	17.2 A	1503 A
T. basicola	1.8 B	2.4 B	2.2 B	114 B	110 B	16.9 A	1314 B
Chloramben + *T. basicola*	2.4 C	3.6 C	3.8 C	90 C	94 C	14.0 B	1283 C

[a]Disease symptoms were evaluated 10, 16 and 23 days after seeding. Each value is the mean of 6 replications of 40 to 50 plants from a randomly chosen one-foot length of row. Symptoms were rated on a scale of increasing symptom severity from 0-6.

[b]Stand count was made 38 days after seeding. Data are means of total number of plants in 6 replicated rows each 20 feet long.

[c]Plant height was measured 64 days after seeding. Data are means of 6 replications each of 10 plants randomly chosen from a 20-foot row. The height was measured from the soil line to the node of youngest unfolded leaf.

[d]Mean of 6 replications, each 20 feet long, except for 3 one-foot lengths removed due to previous sampling.

[e]Students's t-test was applied to each column of data separately and means followed by different letters are significantly different (*P*=0.05).

Fig. 3. Effect of chloramben at 3 lb/acre on _Thielaviopsis basicola_ root rot of soybean in the field. Right, no treatment; center, _T. basicola_ alone; left, chloramben + _T. basicola_. Chloramben alone was _similar_ to the no treatment control.

Enhancement of root rot symptoms by chloramben on 3 soybean varieties --- The soybean varieties "Corsoy" and "Hark" were compared with "Harosoy-63" for development of disease caused by T. basicola in chloramben-treated soil in the greenhouse. All three varieties showed typical symptoms in soil infested with T. basicola. Severity was enhanced by application of chloramben (Table 5). The experiment was repeated and similar results were obtained.

Effect of chloramben on disease severity with different isolates of the pathogen --- Four isolates of T. basicola from different localities were tested in the greenhouse for their virulence in soil treated with chloramben. All the isolates were pathogenic to soybean. Disease induced by all isolates was increased in soil treated with chloramben (Table 6).

Effect of chloramben on the severity of disease using chlamydospores or endoconidia as inoculum --- Endoconidia or chlamydospores of isolate 157 of T. basicola were used as inocula in chloramben-treated or untreated soil in the greenhouse. Endoconidia were applied at the rate of 6×10^7 spores per pot (4×10^4 spore/g soil) and chlamydospores at 3×10^6 propagules per pot (2×10^3 propagules/g soil). Soybeans grown in chloramben-treated soil developed much more severe disease than controls with either form of inoculum (Table 7).

Table 5. Effect of chloramben on severity of *Thielaviopsis basicola* root rot in three varieties of soybean[a]

Treatment	Disease index[b]		
	Harosoy-63	Corsoy	Hark
No treatment	0.0 A	0.0 A	0.0 A
Chloramben	0.0 A	0.0 A	0.0 A
T. basicola	3.4 B	3.7 B	3.4 B
Chloramben + *T. basicola*	4.8 C	5.1 C	5.5 C

[a]Chloramben was applied to soil before seeding at the rate of 3 lb/acre.

[b]Mean of 4 replications each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Student's t-test was applied to each column of data separately, and means followed by different letters are significantly different (*P* = 0.05).

Table 6. Effect of chloramben on severity of disease induced by different isolates of *Thielaviopsis basicola*[a]

Treatment	Disease index[b] Isolate 157	Isolate 170	Isolate 171	Isolate 172
No treatment	0.0 A	0.0 A	0.0 A	0.0 A
Chloramben	0.0 A	0.0 A	0.0 A	0.0 A
T. basicola	5.4 B	5.2 B	5.2 B	5.0 B
Chloramben + *T. basicola*	6.0 C	5.8 C	5.8 C	5.6 C

[a]Chloramben was applied to soil before seeding at the rate of 3 lb/acre. Isolates 157, 170, 171 and 172 of *T. basicola* were originally obtained from soybean fields in different localities in Michigan.

[b]Mean of 4 replications, each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Student's t-test was applied to each column of data separately, and means followed by different letters were significantly different (*P* = 0.05). There was no difference in the virulence among isolates, with or without chloramben application, as determined by analysis of variance.

Table 7. Effect of chloramben on severity of disease using endoconidia or chlamydospore as inoculum.[a]

Treatment	Disease index[b] Endoconidia	Disease index[b] Chlamydospores
No treatment	0.0 A	0.0 A
Chloramben	0.0 A	0.0 A
T. basicola	3.9 B	4.0 B
Chloramben + *T. basicola*	5.0 C	5.3 C

[a]Chloramben was applied to soil before seeding at the rate of 3 lb/acre.

[b]Mean of 4 replications each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Student's t-test was applied to each column of data separately, and means followed by different letters were significantly different (*P* = 0.05).

Effect of rate of chloramben and application methods on disease development --- Chloramben applied at half of the usual rate, 1.5 mg/pot (ca. 1.5 lb/acre) did not result in enhanced disease development. Disease indices were 5.3 for this treatment and the *T. basicola* alone. In the same experiment the disease index for 3.0 mg chloramben/pot in the presence of *T. basicola* was greater than that of the *T. basicola* control according to the t-test ($P = 0.05$).

Chloramben was either incorporated into soil as in the usual greenhouse experimental treatments, or an equal amount was sprayed with an atomizer onto the soil surface after sowing to simulate field practice. The chloramben solution used for spraying was diluted to 5 ml per pot so the volume of liquid received per unit surface area was close to that of a field application. One hundred ml of water was added to the saucer under each pot daily except that the same amount of water was sprinkled onto the soil surface to simulate natural rainfall once every six days. Application of chloramben by spraying enhanced disease to a similar level as application by incorporation into soil (Table 8). Similar results were obtained in another experiment.

Effect of soil temperature on the development of Thielaviopsis root rot of soybeans in chloramben-treated soil --- Pots of soybeans in soil treated with chloramben

Table 8. Effect of mode of application of chloramben on *Thielaviopsis basicola* root rot of soybeans.

Treatment	Disease index[a] Spray application[b]	Soil incorporation[c]
No treatment	0.0 A	0.0 A
Chloramben	0.0 A	0.0 A
T. basicola	3.3 B	3.3 B
Chloramben + *T. basicola*	5.2 C	5.5 C

[a]Mean of 4 replications each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Student's t-test was applied to each column of data separately, and means followed by different letters were significantly different (*P* = 0.05).

[b]Five ml of a suspension containing 0.6 mg chloramben/ml was atomized onto the soil surface in each pot after seeds were sown.

[c]Twenty ml of a suspension containing 0.15 mg chloramben/ml was atomized onto soil (ca 1400 g) with mixing. The soil was then potted and seeded.

and/or T. basicola, were set in temperature-regulated water baths at 14, 18, 22 and 26 C. The lower temperatures, 14 and 18 C, were most favorable for disease development (Table 9). However, at 14 C, the plants germinated and grew much slower. Even so, chloramben enhanced disease at all temperatures.

Possibility of direct stimulation of germination and growth of T. basicola by chloramben --- The possibility that the pathogen was directly stimulated by chloramben in the soil and that this would account for enhanced disease, was investigated. The criteria used to test the possible stimulation were the germination of spores in soil and in water, mycelial growth as linear extension on agar media and dry weight, and the sporulation and longevity in soil of the pathogen in the presence of chloramben.

Neither endoconidia nor chlamydospores germinated more frequently when incubated in technical chloramben solution than in water or acetone solution. Endoconidia germinated less than 1% and chlamydospores about 10% in water, acetone (10 μl/ml water) solution, and in all concentrations (2, 10, 30 and 100 μg/ml) of chloramben. Similar results were obtained in repeated experiments. Analysis of variance showed no significant differences with either spore type.

Neither endoconidia nor chlamydospores showed increased germination during incubation on Conover loam

Table 9. Effect of soil temperature on the development of Thielaviopsis root rot of soybeans in chloramben-treated soil[a]

Treatment	Disease index[b] 14 C	18 C	22 C	26 C
No Treatment	0.0 A	0.0 A	0.0 A	0.0 A
Chloramben	0.0 A	0.0 A	0.0 A	0.0 A
T. basicola	5.3 B	4.8 B	4.2 B	1.5 B
Chloramben + *T. basicola*	6.0 C	6.0 C	5.3 C	2.5 C

[a]Pots of soybeans with the various treatments were set in temperature-regulating water baths at 14, 18, 22 and 26 C. Chloramben was applied to soil before seeding at the rate of 3 lb/acre.

[b]Mean of 4 replications, each containing 8-10 plants. Disease index was based on a scale of increasing symptom severity from 0-6. Student's t-test was applied to each column of data separately, and means followed by different letters were significantly different. (*P* = 0.05).

amended with chloramben as compared with that on non-amended soil. Endoconidial germination on natural soil was nearly zero regardless of the chloramben amendment; yet germination was 70% on sterilized soil. About 10% of the chlamydospores germinated on soil with or without chloramben, and no difference was detected with analysis of variance. Growth of _T. basicola_ was measured in 0.1 strength PDA containing 0, 1, 3, 10, 30 or 100 ppm chloramben. The center of the petri dish was inoculated with a disk cut with a cork borer from the edge of a young colony of _T. basicola_. Plates were incubated at 24 C. Colony diameters were measured daily from the third to the 10th day. No significant differences in colony diameters were detected. No sectoring was observed in any treatment during the experiment.

Dry weight and sporulation were measured in colonies of _T. basicola_ in liquid culture. Five flasks containing 50 ml modified potato-dextrose broth (per liter: extract of 200 g potato, 30 g dextrose and 5 g yeast extract) were supplemented with technical chloramben at 2 μg/ml. The other five flasks did not contain chloramben and were used as controls. One-tenth ml of an endoconidial suspension was added to each flask, and the cultures were incubated at 24 C for 21 days. The mycelia in each flask were fragmented in a Waring blender, and four 2 ml samples were taken for dry weight determination by drying at 70 C overnight in pre-weighed pans. Sporulation was determined by counting

the endoconidia in samples of the fragmented tissue with a haemocytometer. Mycelia grown in chloramben-amended or control media had 10^6 to 10^7 endoconidia per ml. Mean dry weight of mycelia from the media containing chloramben was 48.9 mg and that of the controls was 49.2 mg. No significant differences in sporulation or dry weight were detected between the chloramben treatments and controls when paired t-tests were applied to the data.

The possibility that chloramben caused the spores of T. basicola to survive longer or to multiply in the soil was tested by comparing the populations of the pathogen in soil with or without chloramben treatment. Washed endoconidia or chlamydospores were mixed into 120 g sieved Conover loam at 10^5 and 1.5 X 10^4 spores/g soil, respectively. A chloramben solution was atomized into half of the quantity of each infested soil to give a final concentration of 3 µg/g; this was incubated in three 6 cm diameter petri dishes (20 g each) at 18 C. The remaining soil was treated in the same manner except that the chloramben solution was replaced with water. Population of T. basicola determined after 1, 6 and 16 days' incubation showed no change due to chloramben amendment. The populations stayed within the confidence limits of either treatment throughout the incubation period. The two types of propagules gradually declined in numbers at a similar rate.

Possibility of increased virulence of *T. basicola* due to chloramben --- The effect of chloramben on virulence of *T. basicola* was tested by growing the pathogen in modified potato-dextrose broth amended with chloramben. The washed and fragmented mycelia were mixed (3 ml/pot) into vermiculite into which soybean seeds were sown. Pots were replicated 3 times and all pots were kept in a greenhouse for 4 weeks. Disease index was the same (4.4) for plants grown in vermiculite infested with *T. basicola* cultured in chloramben-amended media as with the fungus grown without chloramben.

From the above experiments it was concluded that chloramben did not affect the germination, growth, sporulation, survivial in soil or virulence of *T. basicola*.

Effect of chloramben on microbial populations in soil --- The possibility that chloramben altered the microbial population which in turn influenced disease development was investigated. Conover loam (ca. 1500 g) was moistened with an aqueous suspension of chloramben until the soil moisture content was about 12% and the concentration of chloramben was 2 μg/g soil (3 lb/acre). Control soil contained 12% moisture, but no chloramben. Two additional treatments included glucose added to the chloramben solution or to water to make a concentration of 0.1% (1.5 g/1500 g soil) to provide an energy source for the microorganisms. The soils were incubated in

closed plastic bags at 24 C. Total populations of fungi, bacteria, and actinomycetes were determined one and three weeks after treatment with the dilution plate method using selective media. The same experiment was repeated once. Fungal and actinomycete populations were not affected by chloramben application, as compared with their controls. The bacterial population was increased by chloramben in the first experiment,but was decreased in the second experiment. Thus, the data were inconclusive regarding changes in bacterial populations. Glucose amendment increased the population of all microbial groups slightly as compared with treatments lacking glucose amendment, but no differential effects due to chloramben on fungi, bacteria and actinomycetes were revealed.

An experiment was designed to bypass the influence of other microorganisms by testing whether chloramben would increase the disease in axenically-grown plants. Soybean seedlings grown for 8 days in the presence or absence of chloramben were transferred to autoclaved soil (15 g) in each of 32 test tubes (16 X 120 mm). Two ml of washed endoconidial suspension (5 X 10^4 spores/ml) were transferred into each test tube with a sterilized pipette. Thirty μg chloramben (2 μg/g soil) from a stock solution in acetone were added to half of the tubes containing chloramben-treated seedlings. The tubes containing seedlings that had no chloramben treatment received water instead of the chloramben suspension. All procedures were done aseptically. The tubes were incubated for 14 days at 24 C about 6 inches below continuous fluorescent light (6.3×10^3 lux). After incubation, 2 ml of sterilized water were transferred into each tube, the tube was shaken, and 1-2 ml of the soil suspension that did not settle was poured immediately into a petri dish in which molten PDA (42 C) was mixed. Colonies observed after 2 and 5 days were *T. basicola* only. The average disease index of 16 control plants was 3.8 and that of 16 chloramben-treated plants was 5.7. Student's t-test indicated the two treatments differed significantly (P=0.05). The same experiment was repeated with similar results.

The result indicated that, in the absence of other microorganisms, chloramben increased disease to a similar extend as it did in the greenhouse and in the field. Consequently, other microroganisms did not play a major causal role in disease enhancement.

Predisposition of soybeans to Thielaviopsis root rot by chloramben --- Since chloramben did not stimulate the pathogen directly nor influence microbial activities that could indirectly result in more severe disease, the effect of chloramben on the host was studied by allowing the herbicide and the host to interact before inoculation. Soybeans were grown in chloramben-treated greenhouse mix (2 mg/kg soil) for seven days, washed and transplanted into T. basicola-infested soil with no chloramben. The roots were examined 2 weeks after transplanting. The disease index of such plants was 4.0 as compared with 2.8 for plants transplanted into infested soil from soil without chloramben. Student's t-test indicated the two treatments were significantly different (P=0.05). This experiment indicated that soybeans became more susceptible when exposed to chloramben without the presence of the pathogen, and that the effect persisted when chloramben was removed. The experiment was repeated with similar results.

The nature of the predisposition of soybeans to Thielaviopsis root rot by chloramben was investigated by determining changes in spore germination in the rhizosphere, chemical composition of root exudates and alteration of host resistance.

Lesion development on chloramben-treated soybeans by T. basicola introduced under the epidermis --- The enhanced susceptibility of soybeans brought about by chloramben could be due to decreased host resistence. To differentiate this possibility from any change in the external environment of the root, such as altered root exudation, approximately 5 µl of an endoconidia suspension containing 300 spores/µl, were injected into the cortex of the hypocotyls of 9-day-old soybeans grown in chloramben-amended greenhouse mix or greenhouse mix without chloramben in the greenhouse. An alcohol-sterilized microsyringe was used to deliver the spore suspension to soybeans removed from the soil. The inoculated soybeans were replanted into their original pots and incubated 2 weeks in a controlled temperature water bath at 18 C. The mean lesion lengths for 41 chloramben-treated and 37 untreated soybeans were 1.9 and 2.0cm respectively. The two figures were not significantly different when compared by the t-test. Plants injected with sterile water or those uninjected showed no symptoms. The experiment was repeated with similar results.

The experiment indicated that chloramben treatment did not alter host resistance after penetration of the fungus.

Spore germination in soybean rhizospheres --- An average of 39% of chlamydospores and 17% of endoconidia germinated in the rhizospheres of chloramben-treated plants, whereas 14% of chlamydospores and 4% of endoconidia germinated in rhizospheres of untreated soybeans. The results were significantly different (*P*=0.05) for both kinds of spores. The experiment was repeated with similar results. The experiment indicated that chloramben had caused quantitative or qualitative changes in the nutrient level of the rhizosphere. A study of root exudates followed.

Root exudation --- Since spores germinated more frequently in rhizospheres of chloramben-treated soybeans, the chemical nature of the root exudates of plants grown aseptically in a salt solution in the presence or absence of chloramben (2 µg/ml) was investigated. The data were based on four replications, each of which was the pooled contents of four test tubes. Chloramben-treated seedlings exuded significantly more amino acids and electrolytes than seedlings exposed to either acetone or water in measurements made on the 4 th, 6th, 8th and 19th days (Figs. 4 and 5). Chloramben had no significant effect on the overall exudation of carbohydrates, fatty acids and nucleic acids. (Figs. 6, 7 and 8). However, the amount of fatty acids in exudates collected on the 8th day was significantly higher than that in the controls. Exudation of amino acids, carbohydrates and fatty acids was more abundant in the 8th and 10th days' collection than in earlier collections. The total material exuded by plants cultured in chloramben in excess of that exuded by plants cultured in the absence of chloramben were as follows: 440% of amino acids, 105% for electrolytes, 23% for fatty acids and 32% for mucleic acids (Fig. 9). The increases in exudates from chloramben-treated soybeans over acetone controls were 268% for amino acids, 113% for electrolytes and insignificant amounts of carbohydrates, fatty acids, and nucleic acids.

<u>Germination of endoconidia in root exudates of chloramben-treated soybeans</u> --- Washed endoconidia were suspended in sterile distilled water and 50 µl of this suspension were placed in each well of a sterilized depression slide.

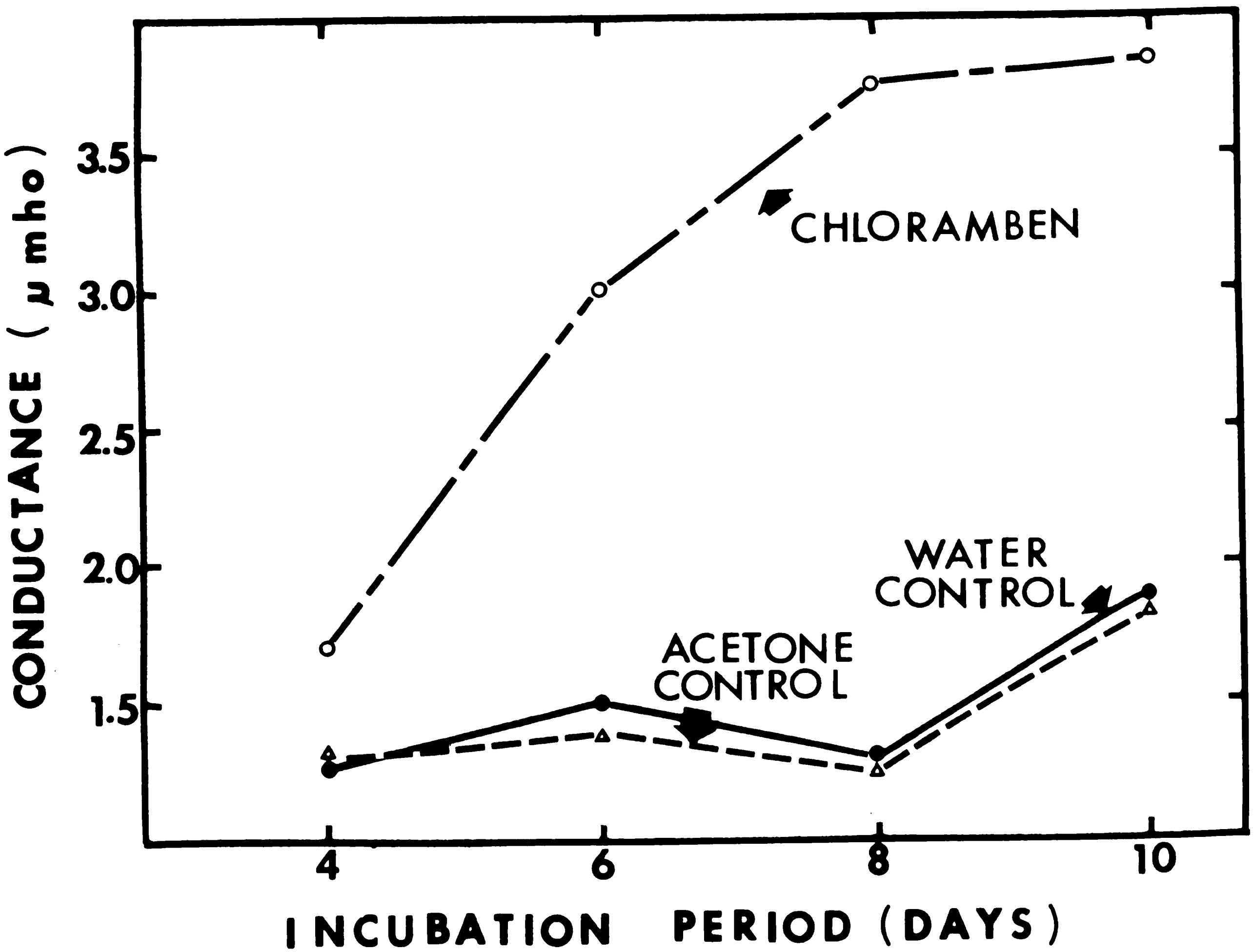

Fig. 4. Electrolytes exuded in a 12 hour period by one soybean seedling (mean of 64 seedlings) cultured aseptically in a dilute salt solution containing 2 µg chloramben/ml, acetone in an amount equivalent to that in the chloramben treatment, or in the salt solution alone (water control).

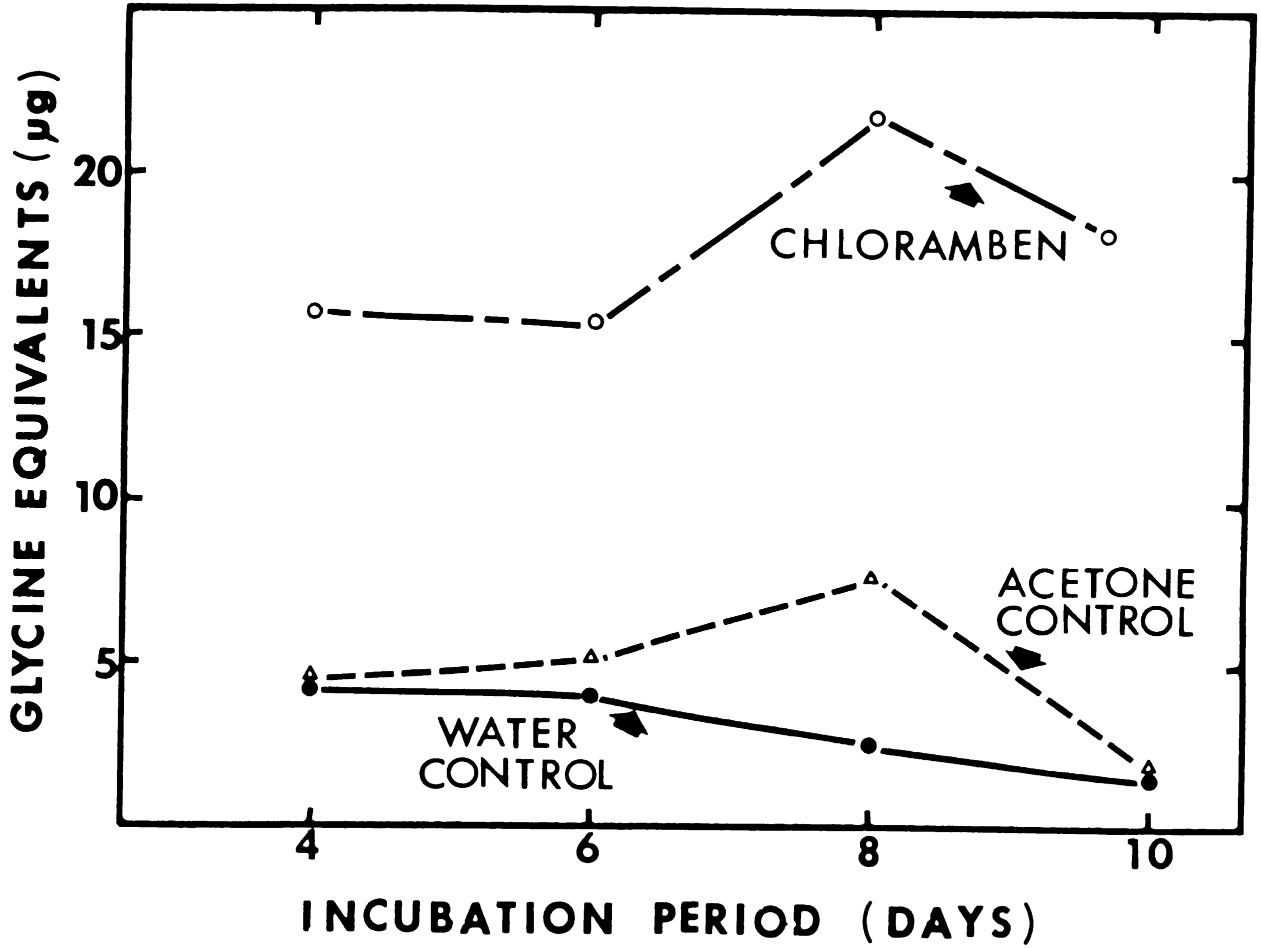

Fig. 5. Amino acids exuded in a 12 hour period by one soybean seedling (mean of 64 seedlings) cultured aseptically in a dilute salt solution containing 2 µg chloramben/ml, acetone in an amount equivalent to that in the chloramben treatment, or in the salt solution alone (water control).

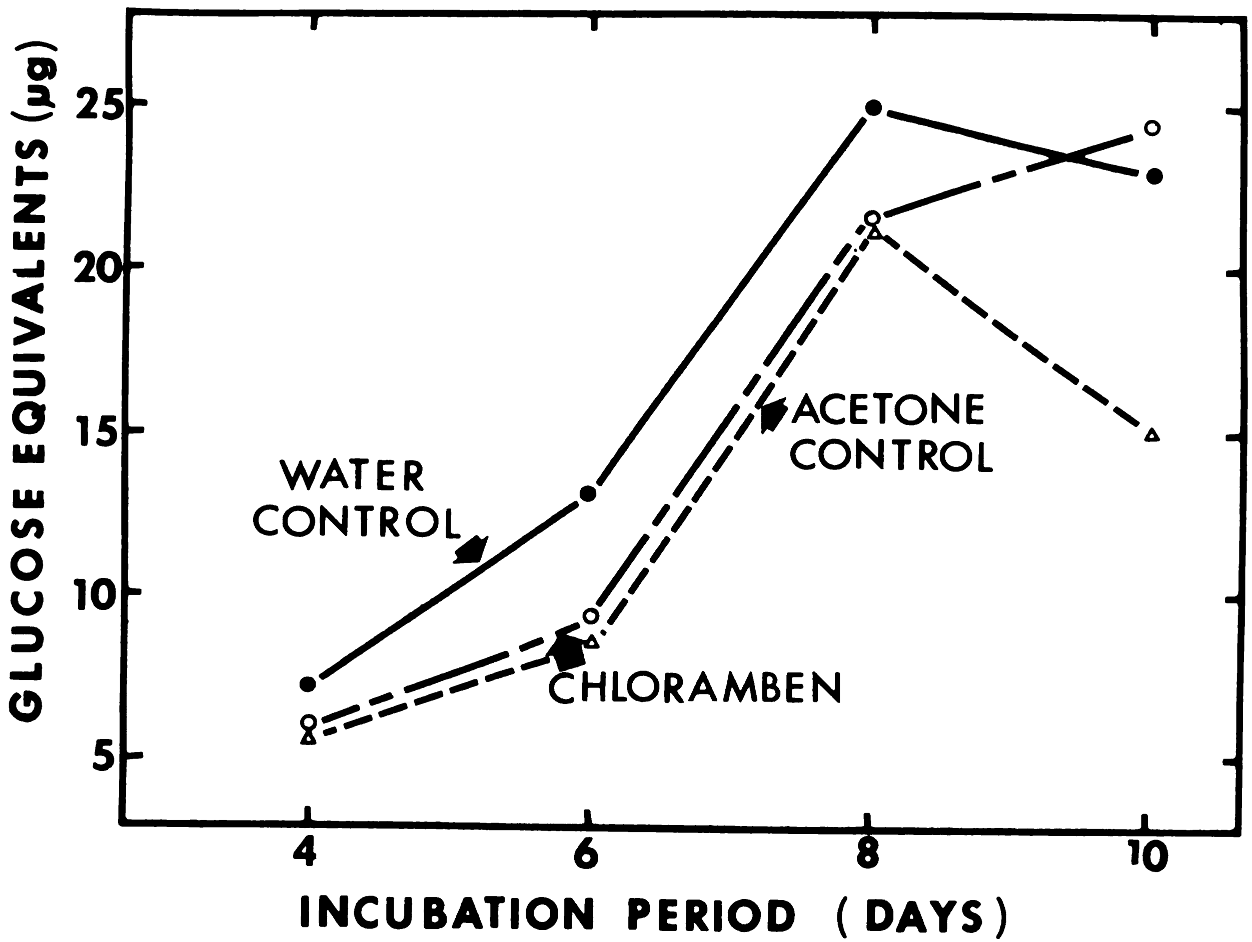

Fig. 6. Carbohydrates exuded in a 12 hour period by one soybean seedling (mean of 64 seedlings) cultured aseptically in a dilute salt solution containing 2 µg chloramben/ml, acetone in an amount equivalent to that in the chloramben treatment, or in the salt solution alone (water control).

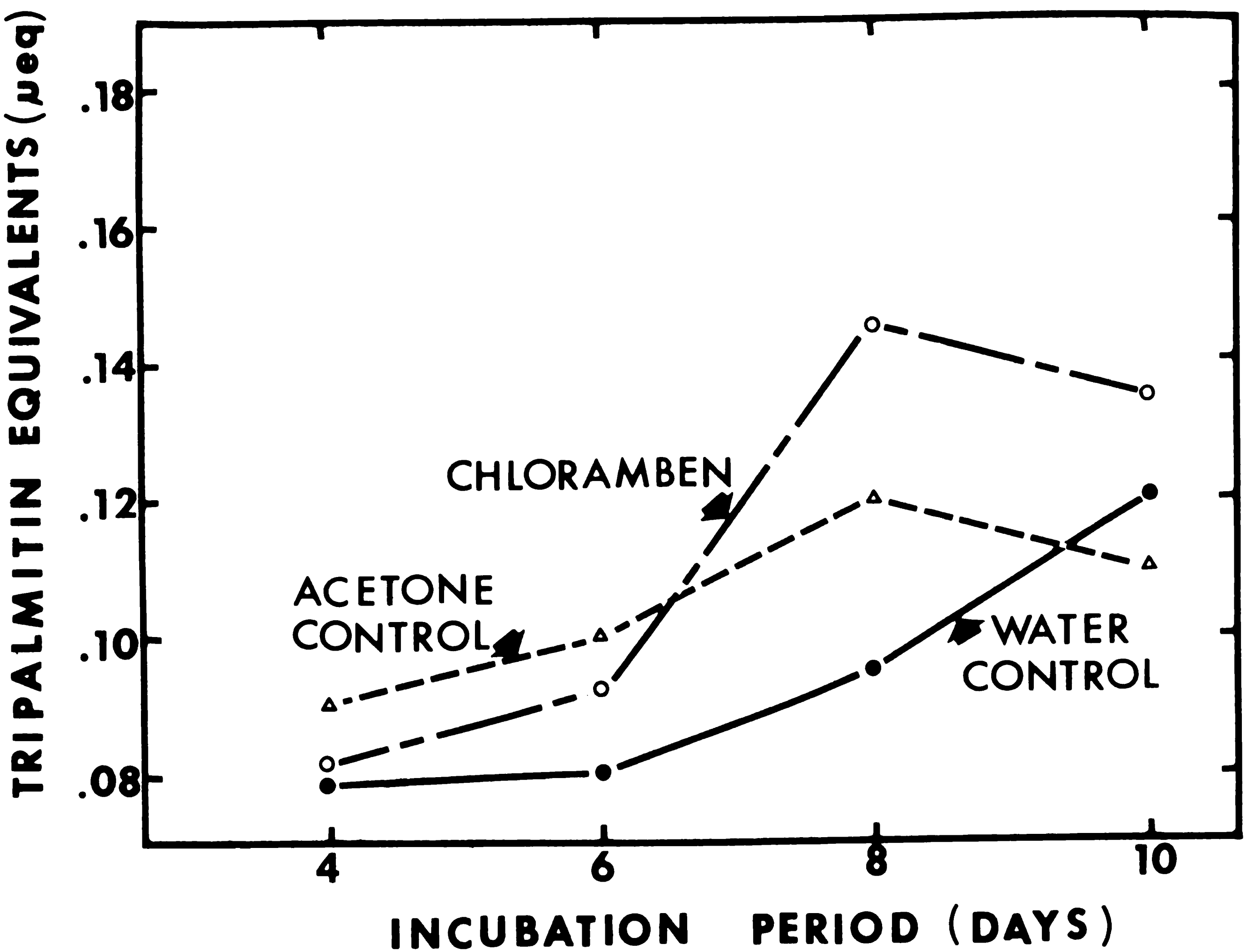

Fig. 7. Fatty acids exuded in a 12 hour period by one soybean seedling (mean of 64 seedlings) cultured aseptically in a dilute salt solution containing 2 µg chloramben/ml, acetone in an amount equivalent to that in the chloramben treatment, or in the salt solution alone (water control).

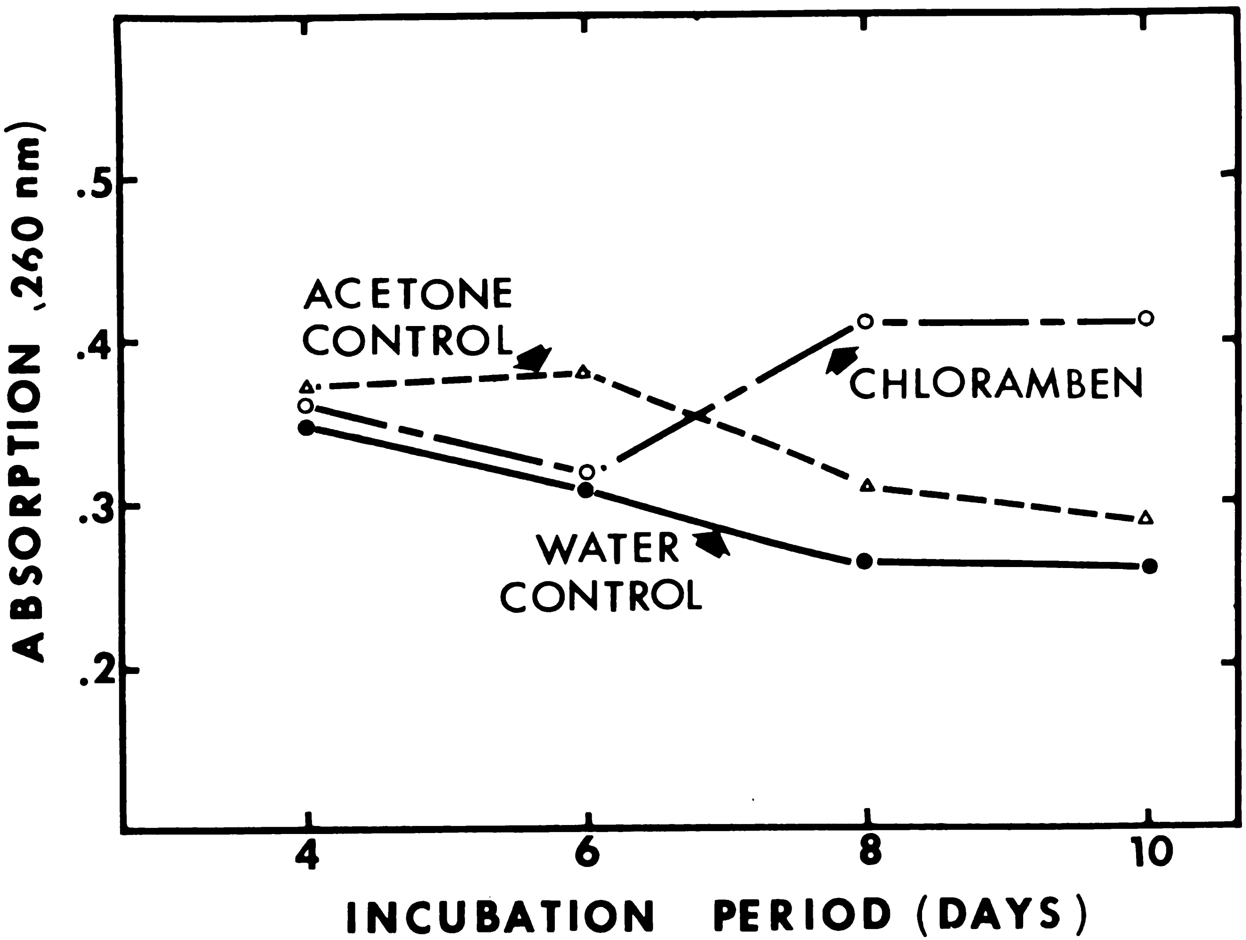

Fig. 8. Nucleic acids exuded in a 12 hour period by one soybean seedling (mean of 64 seedlings) cultured aseptically in a dilute salt solution containing 2 μg chloramben/ml, acetone in an amount equivalent to that in the chloramben treatment, or in the salt solution alone (water control).

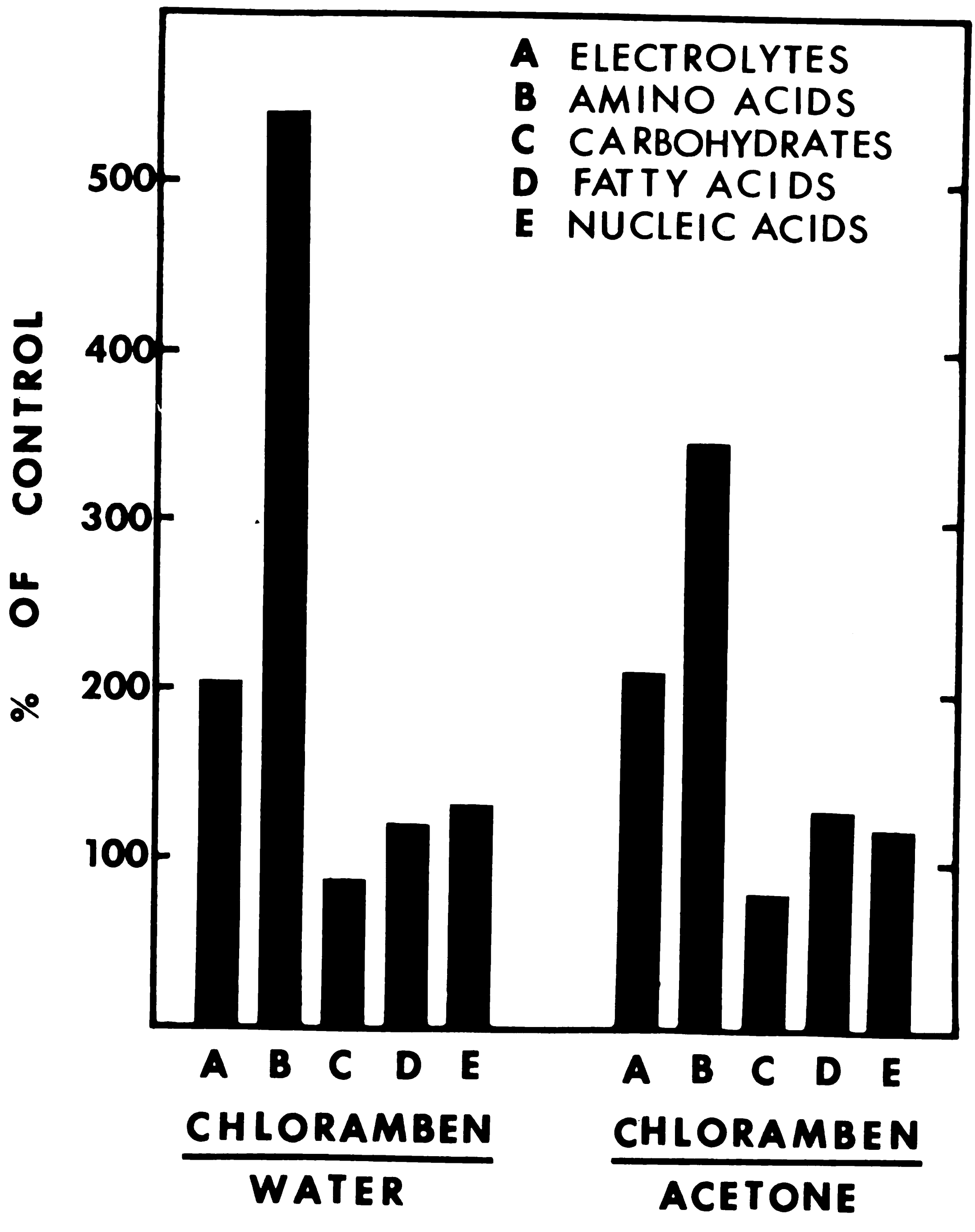

Fig. 9. Effect of chloramben on quantities of electrolytes, amino acids, carbohydrates, fatty acids and nucleic acids exuded by roots of soybean seedlings. Exudates were collected during four, 12-hour periods over 10 days from seedlings cultured aseptically in a dilute salt solution containing 2 μg chloramben/ml, acetone in an amount equivalent to that in the chloramben treatment, or in the salt solution alone (water control).

Concentrated (0.25 of the original volume) root exudate collected from soybeans cultured aseptically in the presence or absence of chloramben were filtered through Millipore filters (0.22 μm) and 10 μl of each of the filtrates were added aseptically to each of 6 wells. The wells were then covered with sterilized coverslips and the slides were incubated in a moist petri dish for 22 hours at 24 C. Two hundred spores per well were then examined for germination. The exudate from chloramben-treated soybeans supported a mean of 20% germination, whereas the exudate from water and acetone controls supported an average of 5% germination. None of the spores germinated in water alone. The experiment was repeated once with similar results.

Effect of root exudates from chloramben-treated soybeans on Thielaviopsis lesion development --- Root exudates from soybeans grown aseptically in chloramben solution (2 μg/ml), acetone of the same concentration as in the chloramben solution, or without either, were concentrated 4 X. An equal volume of concentrated exudate was mixed with a washed endoconidial suspension. Five μl of each of these mixtures were applied to the hypocotyls of each of 20 axenically grown 6 day-old soybean seedlings, kept moist in petri dishes. The inoculated plants were incubated at 24 C under fluorescent light (12 hours/day) for 7 days. The mean length of lesions was 20.8 mm for seedlings receiving exudates from chloramben-treated soybeans. By contrast, hypocotyles receiving exudates from untreated and acetone-treated soybeans, and water alone, had lesion lengths of 8.1, 8.4 and 4.2 mm, respectively. The experiment was repeated with similar results.

Enhancement of root rot severity by Casamino acids --- To verify that amino acids are the ingredient of the root exudate mainly responsible for the increased severity of symptoms, vitamin-free Casamino acids (Difco) were added to tubes of sterilized soil (approximately 12 g) containing an axenically grown soybean seedling and endoconidia of *T. basicola*. The tubes were incubated at 24 C for 21 days. Since the mean quantity of amino acids exuded during four 12 hours periods over 10 days by a single soybean seedling cultered with chloramben was approximately 71 μg, the amount of Casamino acid used was 200 μg per tube. This was a conservative estimate of that exuded during an incubation period for 21 days. The mean disease index of 18 plants in tubes supplemented with Casamino acids was 2.7 and that of the inoculated control without Casamino acids was 2.0. The difference was significant at the 5% level. No disease symptoms were detected on the uninoculated control. Plant grown at the same time in chloramben-amended soil (2 μg/g soil) infested with *T. basicola* also had an average of disease index of 2.7, whereas controls without chloramben had a disease index of 2.0. Similar results were obtained when the experiment was repeated. The results indicated that amino acids are a component in the root exudates important in the increased infection.

DISCUSSION

Soybeans grown in the greenhouse and in the field in soil containing herbicide chloramben had more severe symptoms of *Thielaviopsis* root rot than those grown in soil without chloramben treatment. Enhancement of the disease was found to be caused by increased nutrient levels in root exudates from soybean grown in the presence of chloramben. Possibilities of disease enhancement due to the direct stimulation of the pathogen, to increased virulence of the pathogen, or to population changes of other soil microorganisms were ruled out.

Chloramben-induced enhancement of *Thielaviopsis* root rot of soybean was shown in the greenhouse using different soybean cultivars, various isolates of the pathogen, and with endoconidia or chlamydospores as inoculum. It occurred over a range of soil temperatures from 14 to 26 C. Applications of the herbicide to soil by spraying the soil surface or by incorporation into the soil both resulted in increased disease. Disease enhancement was also shown when soybeans were planted in naturally-infested soil brought from the field, then treated with chloramben in the greenhouse. The possible economic importance of this effect was shown in a field experiment, where chloramben plus *T. basicola* significantly reduced plant stand, height and seed yield as compared with plants grown in the presence of the pathogen alone. The wide host range and geographical distribution, and long survival time of the pathogen in soil suggest that chloramben be avoided when there is evidence of the presence of fungus in the field.

Chloramben did not stimulate germination of endoconidia or chlamydospores of *T. basicola* in water or on soil, and did not increase mycelial growth or sporulation. Moreover, population changes following infestation of soil with endoconidia or chlamydospores of *T. basicola* were not altered due to chloramben amendment. These results are in contrast to those of Percich and Lockwood (38) on the enhancement of *Fusarium* root rot of pea and corn by atrazine (38). Atrazine increased the population of the *Fusaria* in the soil up to 10-fold. It increased the germination of macroconidia and subsequent chlamydospore formation of *Fusarium solani* f. sp. *pisi* and *F. roseum* f. sp. *cerealis* "*Culmorum*" on soil.

T. basicola grown in the presence of chloramben was not more virulent than when grown in the absence of chemical. No sectors were observed when the fungus was grown on chloramben-amended media, indicating that mutation rate was not increased. Chloramben and 2,4-D were reported to induce variants of *Verticillium dahliae* (21) and *Helminthosporium sativum* (20) in cultures, but none of the variants was more virulent than the original isolate. The rates applied (a 5% solution of chloramben and 5,000 ppm 2,4-D) were far beyond the rates used in the field and in this research.

Herbicides that inhibit antagonists of the pathogen may cause increase in disease (23). Chloramben increased Thielaviopsis root rot in axenically cultured soybeans to an extend similar to that occurring in soil. Moreover, soil treatment with 3.3 kg/ha chloramben caused no significant displacements in populations of actinomycetes and fungi in soil. Consequently, chloramben-enhanced disease did not appear to involve inhibition of other organisms.

The study of total microbial populations is useful as a preliminary step to search for drastic population shifts, yet it may not be very helpful in understanding interactions which are more specific. Therefore, the lack of detectable population changes does not imply that its influence on other organisms was negligible. Soldatov et al. (44) reported that chloramben at 4-6 kg/ha was one of several herbicides that decreased soil catalase activity during the first 10 days after application. Later, the enzyme activity returned to the level of untreated soil. Catalase is one of the enzymes responsible for detoxification of hydrogen peroxide, which is speculated to play a key role in microbial community composition (24,50). If catalase-producing microorganisms were suppressed in the present work, this population shift does not seem to be crucial to disease enhancement.

Soybeans first grown in soil amended with chloramben, then transplanted into T. basicola-infested soil without chloramben, showed more severe disease than those grown in soil without the chemical and similarly transplanted. This experiment indicated that the soybean itself was the element in the system that was altered by the herbicide.

The nature of the predisposition was sought first by determination of whether the change affected pre- or post-penetration stages. Since lesion development was not different when the inoculum was injected into the cortex of plants in chloramben-treated or untreated soil, chloramben apparently did not influence the post-penetration defense mechanisms in the host. Therefore, the response of the pathogen in the rhizosphere of chloramben-treated soybeans was studied. Chlamydospores and endoconidia germinated two or four times more frequently in the rhizospheres of chloramben-treated soybean seedlings than in those of untreated seedlings, indicating that nutrient levels in the rhizosphere were raised due to chloramben treatment of the host. Application of exudates from chloramben-treated plants to seedlings inoculated with T. basicola endoconidia resulted in development of much longer lesions than occurred with exudates from control plants.

Chemical analyses of exudates from soybeans showed large increases in amino acids from chloramben-treated seedlings. Moreover, the addition of Casamino acids to T. basicola infested soil caused the soybeans grown therein to develop more severe symptoms than those without amendment. These experiments indicated that increased exudation of amino acids is the cause of the enhancement of the disease.

Wyse (60) found similar effects of chloramben and EPTC on excretion of amino acids from excised hypocotyls and roots of navy beans. The application of EPTC to soil enhanced *Fusarium* root rot of navy beans. Toussoun and Patrick (51) reported that ether extracts of decomposing plant materials, later found to contain benzoic acid, caused increased exudation of ninhydrin-positive substances from bean stems, and increased the pathogenesis of *T. basicola*, *Fusarium solani* f. sp. *phaseoli* and *Rhizoctonia solani*. Linderman and Toussoun (26) showed that ether extracts of decomposing barley did not stimulate the germination of *T. basicola* when applied to non-sterilized soil, but germination of chlamydospores of *T. basicola* was stimulated on root surfaces of cotton seedlings treated with these ether extracts. Chloramben itself [3-amino, 2,5 dichloro benzoic acid] is a substituted benzoic acid; its enhancement of *Thielaviopsis* root rot seems to share the same mechanism as its analogue, benzoic acid. These related researches support the hypothesis that chloramben stimulated the exudation of amino acids which in turn increased the inoculum potential of the pathogen resulting in increased severity of *T. basicola* root rot in soybeans.

Papavizas and Kovacs (36) reported that germination of chlamydospores and endoconidia of T. basicola was increased in soil amended with unsaturated fatty acids, soybean lecithin, and was partially increased by soybean protein. Although the total fatty acids exuded by soybeans treated with chloramben was 23% higher than those exuded by the control seedlings, only on the 8th day, were the fatty acids exuded from chloramben-treated soybeans significantly higher than in control plants. It is possible that during that short period of increased fatty acid levels, the inoculum potential was further increased.

Little is known about the mode of action of substituted benzoic acid-type herbicides except for their auxin-like properties in promoting proliferation of plant tissue. These herbicides, e.g.,2,4-D have been shown to interfere with the metabolism of nucleic acids. However, according to Moreland et al. (33), chloramben does not stimulate or inhibit RNA and protein synthesis, and therefore does not share this mode of action with the phenoxy-type herbicides. The increase in electrolytes lost from roots of soybeans treated with chloramben indicated that chloramben altered the permeability of cell membranes. Whether the permeability change is a cause of herbicidal activity merits investigation.

It is concluded that chloramben stimulated the exudation of nutrients, particularly amino acids, from roots of soybeans, which in turn increased the nutrient status of *T. basicola* inocula in the rhizosphere resulting in increased severity of infection.

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