THE MODE OF ACTION OF DIPHENAMID (N, N-DIMETHYL 2, 2-DIPHENYLACETAMIDE) IN PLANTS

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ABSTRACT

THE MODE OF ACTION OF DIPHENAMID
(N, N-DIMETHYL 2, 2-DIPHENYLACETAMIDE) IN PLANTS

by Charles D. Kesner

The growth of tomatoes (Lycopersicon esculentum Mill.) treated with diphenamid (N, N-dimethyl 2, 2-diphenylacetamide) was studied under controlled environment and field conditions. Under controlled environmental conditions the growth of tomato plants was equally enhanced by 0.001 to 1.0 ppm diphenamid in nutrient solution. One field study was conducted but this growth increase was not observed.

The growth of tomato plants was enhanced by low concentrations of filtrate from two fungal organisms; Trichoderma viride and Aspergillus candidus. High concentrations of the filtrate from these species inhibited the growth of tomatoes but this was overcome by the addition of diphenamid. Diphenamid also promoted the growth of these two fungi.

The fungal species T. viride and A. candidus metabolized diphenamid to MDA (N-methyl 2, 2-diphenylacetamide) and DA (2, 2-diphenylacetamide) within 48 hours. These are common nonpathogenic soil fungi and undoubtedly are important in the decomposition of diphenamid under field conditions.

The toxicity of diphenamid, MDA, and DA was determined on tomato and barnyard grass seedlings under sterile conditions. The two metabolites proved to be more toxic to both plant species than diphenamid. Diphenamid remained relatively inactive in sterilized

soil but was toxic in unsterilized soil or sterilized soil inoculated with fungi. This indicated that the phytotoxic moiety was not diphenamid, but one of its metabolites, probably the N-methyl derivative.

The rate of uptake and translocation of ^{3}H-diphenamid by tomato and barnyard grass plants reflected no differences between species. Both species absorbed and translocated maximum ^{3}H-diphenamid within 24 hours.

THE MODE OF ACTION OF DIPHENAMID

(N, N-DIMETHYL 2, 2-DIPHENYLACETAMIDE) IN PLANTS

By

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INTRODUCTION

Diphenamid (N, N-dimethyl 2, 2-diphenylacetamide) is a preemergence herbicide introduced in 1960. It is used commercially on several crops including tomatoes (Lycopersicon esculentum Mill.)*, peppers (Capsicum annum Linn.), strawberries (Fragaria virginiana Duch.), Irish potatoes (Solanum tuberosum Linn.), and ornamentals.

Yield increases from the application of diphenamid were observed the first season it was introduced as a commercial herbicide. This phenomenon was first observed on tomatoes by both growers and research workers. Similar observations were later reported for field beans (Phaseolus vulgaris Linn.), Sweet potatoes (Ipomoea batatas Poir.), and tobacco plants (Nicotiana tabacum Linn.).

None of these reports resulted from experimental work designed to show this enhancement, but were observations without actual yield data.

Diphenamid is absorbed through the roots of susceptible plants and shows little or no herbicidal activity when applied to the foliage (3). It controls several common seedling grasses such as barnyard grass (Echinochloa crusgalli L. Beauv.), crabgrass (Digitaria sanguinalis L. Scop.), goosegrass (Eleusine indica Gaertn.), and green and yellow foxtail (Setaria viridis and Setaria lutescens L. Beauv.). At higher rates of application it also controls several common broadleaf weed species as lambsquarter (Chenopodium album L.), pigweed

*All scientific names from Gray's Manual of Botany by M. L. Fernald. 8th Ed. 1950, American Book Company.

(Amaranthus retroflexus L.), and knotweed (Polygonum aviculare L.). It may be applied to resistant crop species either prior to their emergence or directly over the foliage of young plants, but must be applied before the weeds emerge.

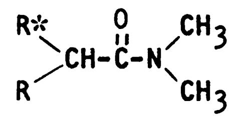
Little is known concerning the exact mechanism of action of any amide herbicide and there is only one published paper to date on the mode of diphenamid action.

The investigations in this thesis were designed to determine the effect of diphenamid on the growth of plants and factors within the environment affecting this relationship. The mechanism of diphenamid action was also studied in attempts to elucidate the nature of plant species tolerance and susceptibility to this herbicide.

REVIEW OF LITERATURE

Physical Properties.

Diphenamid, first described by Alder, Wright and Soper in 1960, (3, 67) has a molecular weight of 239.30 and the following structure:



It crystallizes in white prisms and is moderately soluble in acetone, dimethyl formamide, and phenyl cellosolve. The solubility in water is 260 ppm at 27^{o} C and the melting point is 134.5^{o} - 135.5^{o} C with slight decomposition at 210^{o} C. It has been reported that diphenamid is resistant to ultraviolet irradiation (23).

Mode of Action.

General:

Little is known concerning the mode of action for any of the amide herbicides. Jaworski (33) has postulated that CDAA (2-chloro-N, N-diallylacetamide) inhibits certain -SH containing enzymes that are involved in respiration. It has been reported (7) that dicryl (N-(3, 4-dichlorophenyl)-methacrylamide) will suppress catalase and peroxidase activity in cotton plants with the oxidation of peroxide to water and oxygen being inhibited.

A considerable amount of research has been conducted with maleic hydrazide (1,2,3,6-tetrahydro-3,6-dioxopyridazine) (MH). It has been

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*R = phenyl groups.

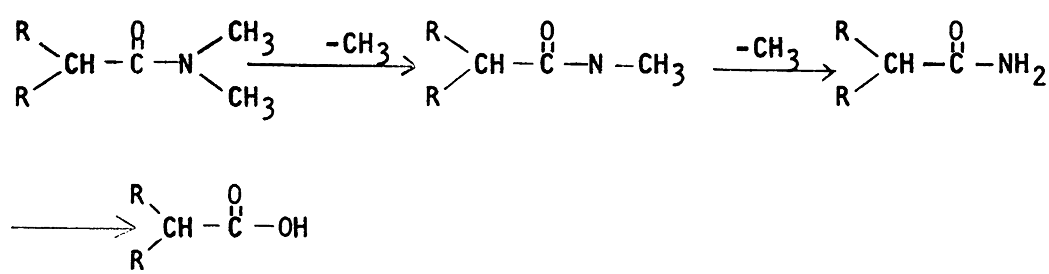
reported to inhibit diaphorase but not cytochrome oxidase (8). Mitosis is suppressed in a variety of plants (7), and chromosome breakage may occur in some meristematic tissue. This tissue becomes enlarged and cells mature rapidly rather than continuing normal division (15). Respiration is reduced in MH treated plants and it has been suggested that MH may compete for receptor sites on an enzyme involved in respiration (32).

Maleic hydrazide treated plants accumulate free amino acids. This has been explained as the result of continued photosynthesis with inhibited growth (56). Sucrose has also been reported to accumulate in MH treated plants (22, 51). Anti-auxin activity (40) and decreases in chlorophyll content of leaves of MH treated plants (12) have also been reported.

Diphenamid:

Diphenamid is absorbed through the roots of susceptible seedling plants and has practically no contact foliar activity. Where susceptible plants have not been completely killed by diphenamid, the root system is generally severely stunted (3). One paper has been published with a proposed mechanism of action for diphenamid. Lemin (39) used ^{14}C-diphenamid labeled in the carbonyl position to study its absorption, translocation, and metabolism in tomato seedlings. Seedlings were grown in Hoaglands nutrient solution containing 4.062×10^4 disintegrations per minute (dpm) per ml of ^{14}C-diphenamid or 5.5 ppm of radioactive diphenamid. Plants were harvested 6, 12, and 24 hours and 7 days after treatment. Benzene extracts were made at each sampling date and chromatographed. Six hours after treatment only diphenamid was detected but after 12 hours the N-methyl 2, 2-diphenylacetamide metabolite was detected and after 7 days the diphenylacetamide and diphenylacetic acid derivatives were also found in the tomato seedlings.

Lemin proposed the demethylation of the N, N-dimethyl 2, 2-diphenylacetamide to the N-methyl 2, 2-diphenylacetamide and further to the diphenylacetamide and diphenylacetic acid derivatives as the mechanism for resistance of tomato plants to this chemical. He found no detectible amounts of the original diphenamid in the plant extracts 21 days after treatment. From this he hypothesized that tomato seedlings were resistant to the herbicidal action of diphenamid because of their ability to convert it to the less phytotoxic demethylated derivatives. The following scheme illustrates the proposed metabolic pathway of diphenamid.



Lemin conducted no experiments to establish the phytotoxicity of these metabolites.

N-demethylation:

There have been reports of the N-demethylation of methylamines and methylamides by both plants and animals. Menzer and Casida (44) described the demethylation of Bidrin /3-(dimethoxyphosphinyloxy) -N, N-dimethyl-cis-crotonamide/ by plants as well as insects and mammals. Snap bean seedlings (Phaseolus vulgaris L.) of the cultivar Contender were treated with 200 ug of ^{32}P-Bidrin by injection into the stems. The material was rapidly translocated in the plants and persisted for several weeks.

The N-methyl-N-hydroxymethyl metabolite and the N-methyl metabolite were both found in the plant. The toxicity of Bidrin to both insects and mammals was increased upon successive N-demethylation.

McMahon (41) found that rat liver microsome fractions removed one methyl group from diphenamid and rabbit microsomes were able to demethylate both the N, N-dimethyl and the N-monomethyldiphenylacetamide. He also has found that N-methyl barbituates and related compounds can be demethylated by several mammalian liver microsomes.

Mammalian homogenates supplemented with DPN, AMP, and nicotinamide catalyzed the rearrangement of N, N-dimethyltyrosine oxide and N, N-dimethyltryptophan oxide to yield formaldehyde and secondary amines (11, 25, 26, 27). This system also catalyzed the oxidation of N, N-dimethyltryptamine to the corresponding N-oxide. The following scheme of demthylation is proposed by Fish, et al. (25, 26) with formaldehyde being the product formed.

$$R\text{-}N\text{-}(CH_3)_2 \rightarrow R\text{-}N\text{-}(CH_3)_2{:}O \longrightarrow R\text{-}N\text{-}(CH_3)\text{-}CH_2OH \longrightarrow R\text{-}NH\text{-}CH_3 + CH_2O$$

The N-demethylation of methylamines and methylamides seems to be quite common in both plant and animal systems. However, the question of whether the toxicity of these compounds is increased or decreased by successive demethylation is still not answered, particularly in plants.

Environmental Observations.

Several weed control research papers indicate that diphenamid must be altered in the soil before it becomes phytotoxic. Dickerson and Rahn (18) noted that diphenamid gave very poor control of barnyard grass under dry soil conditions and soil incorporation or added soil moisture tended to increase its effectiveness as a herbicide. Sheets, *et al*. (57) observed that four 0.6 cm increments of rainfall tended to decrease the phytotoxicity of diphenamid while the equivalent of one 2.5 cm rainfall did not reduce its phytotoxicity. They attributed this to a leaching phenomenon but it is possible that diphenamid may have been degraded to a phytotoxic and then a non-phytotoxic material under their conditions. They did not check for the presence of breakdown products but used a bioassay as the measure of phytotoxicity remaining after water applications.

Langer (37) noted that diphenamid applied to dry soil remains somewhat inactive and then becomes a highly active weed killer after moisture has been added. Davis, *et al*. (17) reported that irrigation tended to increase the phytotoxicity of diphenamid as measured by injury to ryegrass planted during this period. They also noted that shallow plowing or disking did not decrease its phytotoxicity. The phytotoxicity of diphenamid is only reduced after faily long periods

of moist conditions. A long period under these conditions would permit a series of metabolic detoxifications.

Cialone, et al. (14) applied diphenamid in combination with a petroleum mulch and obtained poor herbicidal activity. He proposed that under these conditions diphenamid was prevented from being "activated" by moisture during the critical weed seed germination period. LeBaron (38) also found that diphenamid herbicidal activity was usually improved by irrigation or rainfall after application.

Alder and Wright (4) reported that shallow cultivation of diphenamid treated strawberry fields did not destroy and may have even enhanced its effectiveness. They also noted some injury to tomato plants from diphenamid but this effect disappeared after 30 days.

These field observations substantiate the possibility of a chemical change in diphenamid by either water or soil microorganisms or both that results in a more phytotoxic material. Moisture is necessary for growth of soil microorganisms near the soil surface and the possibility of biological breakdown of diphenamid is increased by rainfall or irrigation.

Growth Enhancement.

Diphenamid has been tested as a herbicide on a host of plants including tobacco, ornamentals, tomatoes, peppers, flowers, onions (Allium cepa L.), strawberries, cole crops (Brassica sp. /Tourn./ L.), deciduous fruit species, sweet potatoes, soybeans (Glycine max Ell.), and field beans (1, 2, 6, 9, 16, 22, 24, 31, 34, 35, 42, 46, 47, 60). It is generally a good annual grass killer but also controls several broadleaf weed species. Weeds in the Solanaceae, Malvaceae and Cyperaceae families are resistant to diphenamid action.

Several herbicide researchers have observed increases in growth of resistant species where diphenamid was applied. These observations were seldom substantiated with actual data but reported as visual observations.

Jones (35), working with field beans in Canada in 1961, was one of the earliest researchers to report an increase in yield in his diphenamid plots which could not be accounted for by weed control alone. He reported that weed control was good at the 4 and 8 lb/A rate and that crop yields were higher in the diphenamid plots. Noll reported an increase in tomato yields where diphenamid was used in 1962 (46), in 1963 (47), and in 1964 (48). He stated that with weed control taken into consideration the diphenamid plots yielded better than any other treatment in both transplanted and direct seeded tomatoes. In 1964, yields from diphenamid plots averaged over 10 tons per acre, the untreated plots less than 2 tons per acre, and the best of the other herbicide plots 5 tons per acre.

Alder and Wright (4) also observed an increase in tomato yields from diphenamid in 1962 but attributed this increase to the excellent weed control attained. In this case the check was left uncultivated and comparisons are difficult to make.

Johnson and Amling (34) working with sweet potatoes in Alabama in 1963 reported that diphenamid gave satisfactory weed control at the 6 lb/A and increased the yield of sweet potatoes compared with the cultivated check. An increase in the yield of tobacco was also reported from diphenamid applications in North Carolina (22). At 4 and 6 lb/A the yield was increased by 5.3 and 13.6% respectively over cultivated check plots.

Riggleman, _et al_. (55) reported an increased sweet potato yield in their diphenamid plots in Maryland in 1963. The same year Riggleman, _et al_. (54) reported that tomato yields were increased in the diphenamid plots while the size of the fruit was not affected.

LeBaron (38) working in Virginia reported that tomato and Irish potato yields were almost always increased where diphenamid was used. Taylorson (60) reported significantly increased stands of direct seeded tomatoes in diphenamid treated plots in both 1964 and 1965. He suggested that diphenamid stimulated seed germination. He observed 9.8 plants per foot of row in check plots and 11.9 plants per foot of row in the diphenamid plots. In 1965 there were 16.9 and 19.8 plants per foot of row for check plots and diphenamid plots, respectively. He also reported an enhanced growth of tomato plants from diphenamid treatments in 1964. At a diphenamid application rate of 2.5 lb/A the average fresh weight per plant was 9.3 g while the check plants averaged 7.5 g per plant. He suggested that diphenamid enhanced both tomato seed germination and plant growth.

Smith (58) noted that tomato fruit matured more rapidly in plots treated with diphenamid. Treated plants matured their fruit an average of 2 days before untreated plants. Outstanding weed control in diphenamid plots along with increased yields were reported in several soybean plots in 1965 (61).

Soil Microorganisms.

General Discussion:

There are several classical examples of beneficial organisms in the soil such as nitrogen fixing bacteria, ammonification and nitrification bacteria, sulfur oxidizing bacteria, and those which oxidize and reduce iron and manganese (45). There are several known examples of antibiosis (one organism produces a condition inimical to the normal growth of another), symbiosis (two organisms benefit each other), synergism (activities of organisms in association results in changes not possible within either individual organism), and commensalism (an association where one organism is benefited while the other remains uneffected) (50).

One or all of these situations may occur in microorganism-plant-herbicide interrelationships. There is a great deal of evidence for herbicide breakdown by soil microorganisms and these metabolic processes may either result in detoxification or increased toxicity of the herbicide to the plant species involved. If microbial breakdown is involved, there is no immediate change in microbial population from the initial herbicide application. After a period of time the organisms metabolize the herbicide and this is parallelled by increasing numbers of soil microbes. This situation has been found typical for the breakdown of 2, 4-D in the soil (7). The most likely mechanism of this resulting soil microbial population enrichment involves the induction of adaptive enzymes which are produced only when the compound to be acted upon is present. The lag period in microbial buildup would correspond to the induction period of the adaptive processes by which the the herbicide-specific enzyme systems are synthesized. When these new enzymes are synthesized the microorganisms proliferate rapidly due to the presence of favorable herbicide substrate and lack of competition from unadaptive microbial species. This situation has been shown for certain bacteria (53). Thus a particular group or species of microorganisms which can attack a herbicide, and utilize it, will be greatly favored by its presence and will proliferate more rapidly than other competitive organisms.

Effect on Diphenamid:

Information on the persistance and metabolism of diphenamid in the soil is lacking but several researchers have proposed microbial breakdown. In 1966 Dubey, *et al.* (21) reported that diphenamid was detoxified more rapidly in soils of high organic matter than in soils low in organic matter. He attributed part of this detoxification to microbial action since organic matter is more suitable for microorganism growth. Dubey supported this idea with previous findings (19, 20) which indicated that diphenamid was more toxic to oat seedlings (*Avena sativa* L. Dubois) in sterilized soil than in nonsterilized soil. However, he chose the oat plant for bioassay after finding that it was extremely sensitive to diphenamid. These findings do not agree with reports which have shown diphenamid to be nonactive as a herbicide under situations inimical to organism growth.

Jones, *et al.* (36) reported in 1964 that silt loam soils in Kentucky showed a persistence of diphenamid residues at phytotoxic levels 10 to

11 months after field application even when applied at rates within the range needed for weed control.

Studies with the related amide compound CDAA (28, 49, 64) indicated a definite correlation between factors that favor microorganism growth and the detoxification of the herbicide. High organic matter in the soil and thus presumably high microbial activity rapidly detoxified CDAA as an herbicide.

<u>Summary</u>.

Information concerning the mode of action of diphenamid was lacking but demethylation of the molecule by tomato plants indicated one possible mechanism. The available data also indicated that the N-demethylation of methylamides is quite common in both plants and animals.

In general, the activity of soil applied diphenamid was increased by rainfall, irrigation and soil incorporation. The phytotoxicity was reduced only after fairly long periods of moist conditions. This indicated a period of metabolic detoxifications in the soil. Several field observations substantiated a chemical change in diphenamid by soil microorganisms, water or both of these factors.

Several herbicide researchers noted increases in crop yields where diphenamid was used. This was most often reported for tomatoes but several other crops were reported to respond in a similar manner. All reports, however, were primarily concerned with the weed control efficiency of diphenamid and these interesting effects were noted while collecting weed control data. There has been no work initiated to study specifically this enhancement.

There was considerable evidence for herbicidal breakdown by soil microorganisms. Diphenamid was reported to break down faster in soils of high organic matter than in soils of low organic matter but whether or not it becomes more or less phytotoxic during this process is not known.

MATERIALS AND METHODS

Special Abbreviations.

Diphenamid and its two successive plant metabolites were studied. The first metabolite, N-methyl 2, 2-diphenylacetamide, and the second metabolite, 2, 2-diphenylacetamide, will hereafter be referred to as MDA and DA.

Preparation of Stock Solutions.

Aqueous stock solutions of diphenamid were prepared at a concentration of 100 ppm by dissolving 100 mg of technical diphenamid in 1 liter of distilled water heated to 50° C and stirred for 1 hour. Final concentrations of solutions were made by serial dilution.

Analytical Procedures.

Plant tissues were dried in a forced air oven at 80° C. The dried tissues were weighed to the nearest mg on an analytical balance and ground through a 40 mesh screen in a Wiley Intermediate Mill. Samples were then analyzed for thirteen elements; nitrogen, phosphorus, potassium, calcium, magnesium, sodium, manganese, iron, copper, boron, zinc, molybdenum, and aluminum.* Samples were analyzed on an emission spectrograph for all elements except nitrogen and potassium. Nitrogen determinations were made by the standard Kjeldahl procedure and potassium was determined with a flame photometer.

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*Analyses made by Dr. A. L. Kenworthy, Plant Analysis Laboratory, Horticulture Department, Michigan State University.

Plant Screening Work.

Several species were initially tested to pick a suitable plant for future studies on growth enhancement. The first experiment was conducted using cucumber (cultivar Spartan Dawn), tomato (cultivar Heinz 1350), soybean (cultivar Chippewa), pigweed and lambsquarter. Seeds of these species were germinated in vermiculite, grown until the first true leaves appeared, transplanted into 10 cm clay pots containing number 7 Wausau quartz sand, and the pots placed in 13 cm plastic containers. The plants were grown under greenhouse conditions during August with day temperatures averaging 32^{o} C and night temperatures averaging 24^{o} C. The effect of diphenamid concentration on growth was determined by growing plants in logarithmic dilutions of diphenamid in half strength Hoaglands nutrient solution from 0.001 ppm to 1.0 ppm. A randomized block design with five replicates was utilized. The plants were watered each day with sufficient solution to fill the outside plastic dish to capacity. Six weeks after treatment the plants were harvested, roots and shoots separated, washed and dried. Growth was determined by dry weight measurements of both roots and shoots.

Tomato Plant Experiments.

Tomato was chosen as the plant for future work. Most subsequent experiments were conducted in a controlled environment chamber. All of the experiments in this study were conducted with a light intensity of 3500 ft-c under 28^{o} C day temperatures and 22^{o} C night temperatures with a 16 and 8 hr day and night period, respectively. Two other tests were conducted using a night temperature of 10° C and a day temperature of 15° C in the first and a constant 35° C in the second. These tests were designed to determine the effects if diphenamid on tomato plants under adverse as well as ideal conditions.

All tomato seeds were germinated in vermiculite and handled as described earlier. They were treated 4 days after transplanting with the concentrations of diphenamid previously used. This delay in treatment allowed the establishment of the plants prior to diphenamid applications. A randomized block design with 4 replicates was utilized.

One hundred ml of fresh solution was added to each pot per day for the first 2 weeks, 250 ml the second 2 weeks and 500 ml during the remaining time. This increase was necessary to maintain daily usage. Plants were watered through the top of the pot for the first week and through the bottom during the remaining period to avoid algal growth. Plants were generally harvested 5 to 6 weeks after treatment and handled in the manner previously described.

The Response of Tomatoes to Fungi.

Fungal contamination found in diphenamid treatments was taken to the Department of Botany and Plant Pathology for identification.* Two separate genera were identified as being present in about equal amounts. These were identified as *Aspergillus candidus* and *Trichoderma viride* (*lignorum*). There were also minute amounts of *Aspergillus niger* present. These fungi are of the form-class Deuteromycetes or Fungi-

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*The identification was made by Dr. E. S. Beneke, Michigan State University, East Lansing, Michigan.

Imperfecti (5). Therefore, there were 2 genera and 3 different species present in the nutrient cultures. These species are common non-pathogenic, saprophytic fungi found in nearly all soils and grow relatively well on meager substrates as long as moisture is available.

A preliminary test was designed to determine the effects of these organisms on tomato plants. The fungal treatments were prepared by macerating a sample of both fungi in a Waring blender with 100 ml of distilled water. Two ml of this solution was added to each 250 ml beaker containing the tomato plants. The beakers were aerated with stone diffusers under 3 psi air pressure. Plants and aeration tubes were held in place by the use of 1.3 cm thick styrofoam covers placed over the beakers with 1.3 cm holes drilled in them for the plant and the aeration tube. Cotton was then placed around the tomato plant to hold it stationary and prevent further contamination from the air. The plants were grown for 3 weeks, harvested, dried and weighed.

In the second experiment both _A. candidus_ and _T. viride_ were added to autoclaved nutrient solutions by means of a wire loop. Further contamination was inhibited by the use of styrofoam beaker covers and cotton plugs around the plants.

Culturing the fungi:

It was found that both species of fungi could be grown well on a potato dextrose agar medium containing 200 g of cooked and strained potatoes, 20 g of glucose, 20 g of agar, and sufficient water to bring the volume up to 1000 ml. This mixture was excellent for fungus growth but was not suitable for use in formulating nutrient solutions. An extract from the fungal organisms proved more desirable.

Several aqueous media were tested in an attempt to find a suitable means of rapidly culturing large amounts of fungi. A mixture of 0.1% KH_2PO_4, 0.6% $NaNO_3$, 0.05% $MgSO_4$, 1.5% $CaCO_3$ and 2.0% glucose per liter proved to be an excellent growing medium (43). This solution had a pH of 7.0. Aliquots of 200 ml of the above media per 250 ml Erlenmeyer flask were inoculated, put on a water bath shaker and grown at 28° C. Heavy mycellial growth of both organisms could be obtained in 3 or 4 days.

A mixture of half strength Hoagland solution, pH 6.2, using NH_4NO_3 as the nitrogen source, plus 0.02 M glucose was used under similar conditions. The growing conditions were identical to those described above. A dense growth of mycelium was produced by this system in 5 to 6 days and tomatoes grew well in this solution.

The fungi were allowed to grow for 1 week in this media, then the solution was filtered 5 times through a Seitz clarifying filter S-325041 with 5.0 u pore size. This was followed by 1 filtration through a 1.0 u pore size Seitz filter.

Filtrate applications to tomatoes:

Tomato seeds were surface disinfected with a 0.8% sodium hypochlorite solution for 20 minutes, and rinsed several times in autoclaved distilled water. They were transferred to autoclaved petri dishes containing 2 sheets of 90 mm Whatman No. 1 filter paper and 5 ml of distilled water. The seeds were germinated in an incubator at

26° C until the radicle was visible. Ten seeds were placed in autoclaved petri dishes containing the various treatments, put in a dark incubator at 26° C and left for 3 days. The effect of diphenamid concn on growth was determined by logarithmic dilutions of diphenamid in nutrient solution from 0.01 to 1.0 ppm and included a control. A randomized block design with 4 replicates was utilized. Fungal filtrate was added as either pure filtrate, a 1-10, 1-100, or 1-1000 dilution. Since the original fungal growing solutions contained 0.02 M glucose, this amount was added to all other solutions to eliminate the effect of the sugar on seedling growth. The radicles were measured after 3 days.

A. candidus treatments were applied to tomatoes growing as previously described and replicated 4 times. Tomatoes subjected to a 1-50 dilution of *A. candidus* filtrate for 24 hr were removed from the filtrate and placed back in nutrient solution. After 0, 1, 2, 4, and 8 days these plants were placed in 1.0 ppm diphenamid solution. One group of plants was grown for 15 and another 30 days after the first treatment. The reciprocal of this experiment was also conducted. Tomatoes were treated with 1.0 ppm diphenamid for 24 hr and placed in a solution containing *A. candidus* filtrate after 0, 1, 2, 4, and 8 days.

Diphenamid Metabolite Studies.

The influence of the MDA and DA metabolites of diphenamid on the growth of tomato and barnyard grass seedlings was studied. Tomato is resistant and barnyard grass susceptible to diphenamid injury.

Diphenamid and the 2 metabolites were applied to surface-disinfected tomato and barnyard grass seeds in sterile petri dishes as previously described. Each chemical was applied in 5 ml of sterile distilled water. Final concn of the chemicals were 0.1 ppm in the first experiment and 0.1, 0.5, 1.0, and 10 ppm in the second. Seeds were placed directly into the treated dishes and left in a dark incubator for 4 days at 26° C. Radicle and hypocotyl measurements were taken at the end of this period.

A third experiment was initiated to determine if MDA had an effective concentration range above which its phytotoxic effect was lost. This experiment was similar to the previous test except that the concentrations of both diphenamid and the MDA derivative were 0, 0.1, 1.0, 10, and 100 ppm.

Toxicity of diphenamid in soil:

Diphenamid and its MDA derivative were further tested on germinating tomato, German millet (*Setaria italica* L.), and barnyard grass seedlings grown in soil. A growth chamber was used with a 16 hr light period, 3500 ft-c intensity and a 28° C and 18° C day and night temperature. Sandy loam soil from the Michigan State University Horticultural farm was placed in 10 cm clay pots. Two thirds of the pots containing the soil were autoclaved under 15 psi for 3 hr and the remaining third was not sterilized. Upon removing the sterilized pots from the autoclave, they were placed immediately in large plastic bags to prevent contamination. The following day all pots were planted with surface disinfected seeds of the 3 plant species and treated with 300 ml of sterilized nutrient solution containing diphenamid at concentrations of 0.0, 1.0, 10, and 100 ppm. One half of the sterilized pots were inoculated with *A. candidus* and *T. viride* by pouring 10 ml of a suspension of spores and mycelium on the surface of the potted soil. All treatments were replicated 4 times.

The pots were watered with sterilized nutrient solution every 4 days for 3 weeks. They were removed from the chamber and growth of the grass plants was rated from 1 through 9. A rating of 1 indicated no injury and a rating of 9 complete grass kill. The effect of these treatments on the tomato plants was also noted.

In a second experiment under more rigorous conditions, 2 cm of soil was placed in petri dishes and 5 ml of water added to each dish. Two thirds of the dishes were autoclaved for 2 hr and the remaining third left unsterilized. Surface disinfected tomato and barnyard grass were placed on the surface of the soil in all the petri dishes and one third of the dishes left sterile, one third left sterile plus an inoculation of *T. viride*, and the other third left unsterilized. The treatments consisted of diphenamid and MDA at 0.0, 0.1, 1.0, 10, and 100 ppm. The petri dishes were placed in a dark incubator at 26^{o} C for 5 days. At the end of this period they were removed, closely inspected and rated.

Preparation of ^{3}H-Diphenamid.

Technical grade diphenamid of approximately 97% purity was furthere purified by the following procedure. Twenty ml of hot ethanol was supersaturated with technical diphenamid, the ethanol was then cooled in an ice bath until maximum recrystallization had occurred. The ethanol fraction which contained a yellow impurity was discarded.

This procedure was repeated 6 times or until a pure white crystalline compound was formed. This material was thoroughly dried and 1 g placed in a stoppered test tube for shipment.

The sample was tritiated by the Wilzback Technique* of exposure to carrier free tritium gas for 14 days. Labile tritium was removed by dissolving the ^{3}H-diphenamid in a hydroxylic solvent. The 1000 mg sample contained 150 mc or a specific activity of 0.15 mc/mg.

Six mg of the ^{3}H-diphenamid was dissolved in 3 ml of ethanol which resulted in a specific activity of 0.15 uc/ul. A 1 ul sample was placed in a scintillation vial with 15 ml of toluene-BBOT (2, 5-bis-/2-(5-tert-butylbenzoxazolyl)_7-thiophene) solution containing 4 g BBOT per liter of toluene. The sample was counted in a Tri-Carb Liquid Scintillation Spectrometer** at a window setting of 50-700 and a gain setting of 43%. The counting efficiency was 31%.

A 10 ul sample of ^{3}H-diphenamid was spotted on an Eastman type K301 silica gel chromogram sheet and developed to a 10 cm front in a mixture of benzene and ethanol (85-15 v/v). Under this system diphenamid has an R_f of 0.8 as determined in previous work using nonlabelled material. It was identified by both ultra violet light and a 10% ethanolic phosphomolybdic acid spray test. The R_f of the tritiated material as determined by counting procedures was also 0.8 but a considerable quantity of the activity remained at the origin. The compound

*Tracerlab, 1601 Trapelo Road, Waltham, Massachusetts.

**Packard Instrument Corp., 2200 Warrenville Road, Downers Grove, Illinois.

was, therefore, not considered pure since the 2 above tests showed diphenamid to be present at R_f 0.8 but not at the origin.

A 200 ul aliquot of the 0.15 uc/ul sample was spotted across the length of a 20 x 20 cm chromogram sheet 2 cm from the bottom and developed. A 1 cm strip was counted from the center of the sheet after development to make certain that ^{3}H-diphenamid was a R_f 0.8. A section of gel one half cm on either side of R_f 0.8 was removed by scraping, the gel placed in a graduated centrifuge tube and the ^{3}H-diphenamid eluted with 4 ml of ethanol. The ethanol-gel mixture was shaken for 5 minutes, centrifuged to remove the gel from the ethanol, the clear liquid portion poured off and used as the purified sample. Five ul of this sample was again spotted on a chromogram sheet and developed. All the activity of this material was at R_f 0.8.

The specific activity was determined by scraping the gel at R_f 0.8 from the chromogram into a 47 mm Millipore Filter Holder* and eluting with 200 ml of deionized distilled water at 20° C through a HAWP 047-00, HA 0.45 u size filter. None of the silica gel came through this filter. The 200 ml of water containing the ^{3}H-diphenamid was dried on a vacuum freeze-drier and the ^{3}H-diphenamid weighed on an analytical balance. The sample contained 6.5 mg which was dissolved in 1 ml of ethanol and 3 samples of 10 ul each were counted for activity and the cpm divided by the efficiency of 31% previously obtained to give the resultant specific activity in dpm. The specific activity of the purified sample was 0.114 mc/mg.

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*Millipore Filter Corp., Bedford, Massachusetts.

Preparation of a Quench Curve.

A quenched series of samples was prepared by grinding tomato seedlings in 2.0 ml of ethanol with a Kontes Tissue Grinder. A 0.5 ml aliquot was removed, which equalled 1.5 plants, and placed in a counting vial spiked with 10 ul containing 0.03 uc of ^{3}H-diphenamid and 15 ml of toluene-BBOT. A series of 1:1, 1:2, 1:4, and 1:8 dilutions of the extract was made and treated in the same manner. The samples were replicated 3 times and counted for 10 minutes and the data used to obtain a quench curve for the plant material. The same procedure was repeated using barnyard grass tissue with an identical quench curve resulting. Maximum counts in this system were obtained with a gain setting of 43%, a window setting of 50-700, and a 70-1000 window setting in the external standard channel. This system proved efficient and was utilized in all subsequent experiments.

Uptake and Translocation of ^{3}H-diphenamid.

Seedlings of tomato and barnyard grass were grown in quartz sand until cotyledon expansion. Two seedlings, 1 of each species, were transferred to aerated nutrient solutions in 50 ml beakers. The seedlings were suspended in the nutrient solution by a perforated foil covering with cotton plugs around the plants. The plants were grown with a day length of 16 hr and a temperature of 22° C. Forty eight hr after transplanting the solutions were replaced with nutrient solution containing 0.1 uc or 0.012 ppm of ^{3}H-diphenamid per beaker.

The plants were harvested after 4, 24, and 48 hr in the first experiment, and 4, 24, 48 and 72 hr in the second test. Roots and shoots were separated and the roots washed thoroughly in distilled water to remove any unabsorbed chemical. The plant parts were oven dried at 80° C for 24 hr and weighed.

Plant parts were extracted by grinding in 0.5 ml of ethanol in a tissue grinder. The extract was counted to determine the total amount of radioactivity per sample. This was done separately for roots and shoots to determine that retained in the root versus the amount translocated in the shoot.

Diphenamid Metabolism by Fungi.

Two samples each of *T. viride* and *A. candidus* cultured in 100 ml of liquid medium as described earlier were treated with 0.036 uc of ^{3}H-diphenamid in 150 ml Erlenmeyer flasks. One sample of each species was left for 4 hr and the other for 48 hr. At these times the diphenamid was extracted from the fungal solution by the following procedure.

1. The sample was placed in a 250 ml separatory funnel and 50 ml of chloroform added.
2. The mixture was shaken vigorously for 5 minutes and allowed to separate. The chloroform was drawn off and the procedure repeated.
3. The 100 ml of chloroform extract was centrifuged for 5 min.
4. The clear chloroform was removed and evaporated to 0.5 ml.
5. Twenty five ml of benzene was added to the residue remaining in the tube, shaken for 5 min and centrifuged.

6. The benzene was poured off and discarded.

7. Twenty five ml of ethanol was added, shaken and centrifuged.

8. The ethanol was poured off and evaporated to 0.5 ml.

Twenty ul aliquots of both the chloroform and ethanol soluble portions were spotted separately on a type K301R Eastman thin layer chromogram sheet with fluorescent indicator. The spots were placed 2 cm from the bottom and were kept separate by scoring the sheet from origin to front between the 2 spots and developed as previously described.

Twenty ul aliquots of both extracts were also spotted on separate chromogram sheets 2 cm from the bottom and 2 cm from the left margin. These were developed to a 12 cm front in benzene-ethanol (85-15 v/v) solution. The chromograms were dried and reference samples of diphenamid, MDA, and DA were placed along the left margin 2 cm from the bottom of the sheet. The sheet was then turned 90° and developed to a 12 cm front in a benzene-diethylamine solution (95-5 v/v).

The compounds were identified by ultra violet light, 10% ethanolic phosphomolybdic acid sprays, and by counting the radioactivity after removing strips from the chromatogram and placing these in scintillation vials.

Field Studies.

A field experiment was designed to determine if the enhancing effect of diphenamid on tomato plants could be achieved under field conditions. For data, see appendix.

RESULTS AND DISCUSSION

Plant Screening Work.

The dry weight of tomato, lambsquarter, and pigweed seedlings was increased by one or more of the diphenamid concn (Table 1). Tomato proved to be the most suitable test species because it germinated and grew rapidly, was easily transplanted and responded well to diphenamid. The two weed species did not attain appreciable size and were extremely sensitive to low diphenamid concn.

Table 1. The response of several species to various diphenamid concn.

Diphenamid concn (ppm)	Species: Tomato	Cucumber	Soybean	Lambsquarter	Pigweed
	Dry wt (g) 1/				
0.0	2.01 b	2.56 a	3.89 a	.66 b	.52 b
0.001	2.50 b	2.72 a	4.04 a	1.02 a	.77 b
0.01	--- *	2.57 a	4.14 a	.99 a	.90 a
0.1	2.68 a	2.70 a	3.70 a	.66 b	.78 b
1.0	2.12 b	1.59 b	4.06 a	.10 c	.03 c

1/ Numbers followed by unlike letters are significantly different at the 5% level.

*Plants mechanically injured and discarded.

The cucumber was not a satisfactory test species for growth chamber work, because of slow growth and production of a long vine. Soybeans grew rapidly but produced a fibrous stem which was difficult to grind for nutrient analysis.

Tomato Plant Experiments.

Tomato tests with diphenamid were conducted under 3 different environmental conditions. Each test consisted of the 5 treatments used in the previous test. The first experiment was set up on a greenhouse bench under an 11 hr day with day temperatures averaging 35° C and night temperatures averaging 24° C. The second experiment was conducted in a growth chamber with a light intensity of 3500 ft-c and a 12 hr day with a day temperature of 25° C and a night temperature of 20° C. The third experiment was also conducted in a growth chamber with identical daylength and light intensity but with a day temperature of 15° C and a night temperature of 10° C.

Neither plants grown on benches or in the growth chamber under low temperatures increased in growth from any of the concn of diphenamid. However, plants subjected to the more favorable growing conditions of a 20° C night and a 25° C day temperature did increase in dry wt with the various concn of diphenamid (Table 2, experiment 1). Weights of treated plants were all higher than the control plants but there was no difference between the diphenamid treatments. The enhancement that resulted from the diphenamid treatments was of equal magnitude over the range of diphenamid concn used in this experiment. The shoot/ratio of the plants did not change.

The above experiment was repeated using an 8 hr night at 18° C and a 16 hr day period of 28° C with a light intensity of 3500 ft-c. The results were essentially the same as the previous test. There was a difference in dry wt between the control plants and the diphenamid treated plants but no difference between treatments (Table 2, experiment 2). The shoot/root ratio did not change with treatments and fresh wt were not different.

Table 2. The increase in dry wt of tomato plants in response to diphenamid.

Diphenamid concn (ppm)	Dry wt (g) [1/] Experiment 1 wt/2 plants	Experiment 2 wt/plant
0.0	8.1 b	4.5 b
0.001	9.8 a	6.4 a
0.01	10.0 a	6.3 a
0.1	10.5 a	6.2 a
1.0	9.8 a	6.3 a

[1/] Numbers followed by unlike letters are significantly different at the 5% level.

Nutrient analysis of these plants revealed no differences between treatments with the exception of zinc which was much higher in diphenamid treated plants. The larger plants, of course, contained higher levels of nutrients but there was no difference in the amount per g of

dry wt for each element. Zinc content was tested further by rerunning the samples in the emission spectrograph at the plant analysis laboratory followed by running these samples on an atomic absorption unit.* Neither of these tests bore out the original findings of a high zinc content in diphenamid treated tomato plants.

The rates of diphenamid used in all previous long term experiments ranged from 0.001 ppm to 1.0 ppm. Analysis of variance of all test results indicated a significant increase in growth from the addition of diphenamid but no difference between rates. It was, therefore, necessary to test a wider range of diphenamid concn to find the lower and upper range of activity. Experiments were conducted with concn of 0.00001 ppm through 10 ppm. The results of these tests indicated that enhancement activity was lost below 0.001 ppm and the tomato plants were injured at the 10 ppm rate when subjected to this concn for more than 10-15 days. Thus, the concn previously applied were utilized in further experiments.

During the course of these experiments the diphenamid treated plants appeared to have thicker stems and it was thought that this phenomenon could have given the noted increases in dry wt measurements. Stem diameter measurements were made on the main stem 5 cm above the first lateral root. The measurements were made with a direct-reading caliper gauge graduated to 0.1 mm.**

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*Soil Science Department, Michigan State University, East Lansing, Michigan.

**Federal Products Corporation, Providence, Rhode Island. Model 49P - 172 - R1.

There was no difference between treatments except for the 10 ppm diphenamid rate which was smaller than other treatments (Table 3). This, however, was a result of plant damage at this concn.

Table 3. The stem diameter of tomato plants grown in diphenamid solutions.

Diphenamid concn (ppm)	Stem diameter (mm) 1/
0.0	6.6 a
0.00001	7.1 a
0.001	7.2 a
0.1	7.3 a
10.0	4.5 b

1/ Numbers followed by unlike letters are significantly different at the 1% level.

The Response of Tomatoes to Fungi.

During the course of the previous diphenamid experiments it was noted that certain fungal organisms appeared in the pots of diphenamid treated plants while the pots treated with nutrient solution did not become contaminated. Nutrient stock cultures from one experiment were saved for 2 weeks after the termination of the experiment and those containing diphenamid became heavily contaminated. The check solutions did not become visibly contaminated. The contamination increased as the concn of diphenamid increased. At the 10 ppm concn there was a dense mass of fungal growth both in the nutrient solution itself and on the inner sides of the carboy wall above the nutrient solution level. The fungal organisms produced both white and green fruiting bodies. The diphenamid was dissolved in water rather than an organic solvent indicating that the contamination could not have been induced by a residue of organic solvent in the solutions.

It became evident that it would be desirable to know the effects of these organisms, if any, on diphenamid or directly on the plants and what effect diphenamid might have on the organisms. A preliminary test was designed to determine the effects of these organisms on tomato plants.

Treatments were made by adding 2 ml of liquid from the fungal growing media, previously macerated in a Waring blender, to each 250 ml beaker containing tomato plants. Three treatments consisted of diphenamid in combination with the fungi and 1 treatment with nutrient solution and fungi only. The plants were allowed to grow for 3 weeks, harvested and weighed.

The dry wt of plants treated with fungi alone was greater than either control or fungi plus diphenamid treated plants (Table 4).

Table 4. The response of tomato plants to _A_. _candidus_, _T_. _viride_, and diphenamid.

Treatment	Diphenamid concn (ppm)	Dry wt (g) [1/]
None	0.0	0.47 c[2/]
Fungi	0.0	0.83 a
Fungi	0.01	0.62 b
Fungi	0.1	0.65 b
Fungi	1.0	0.63 b

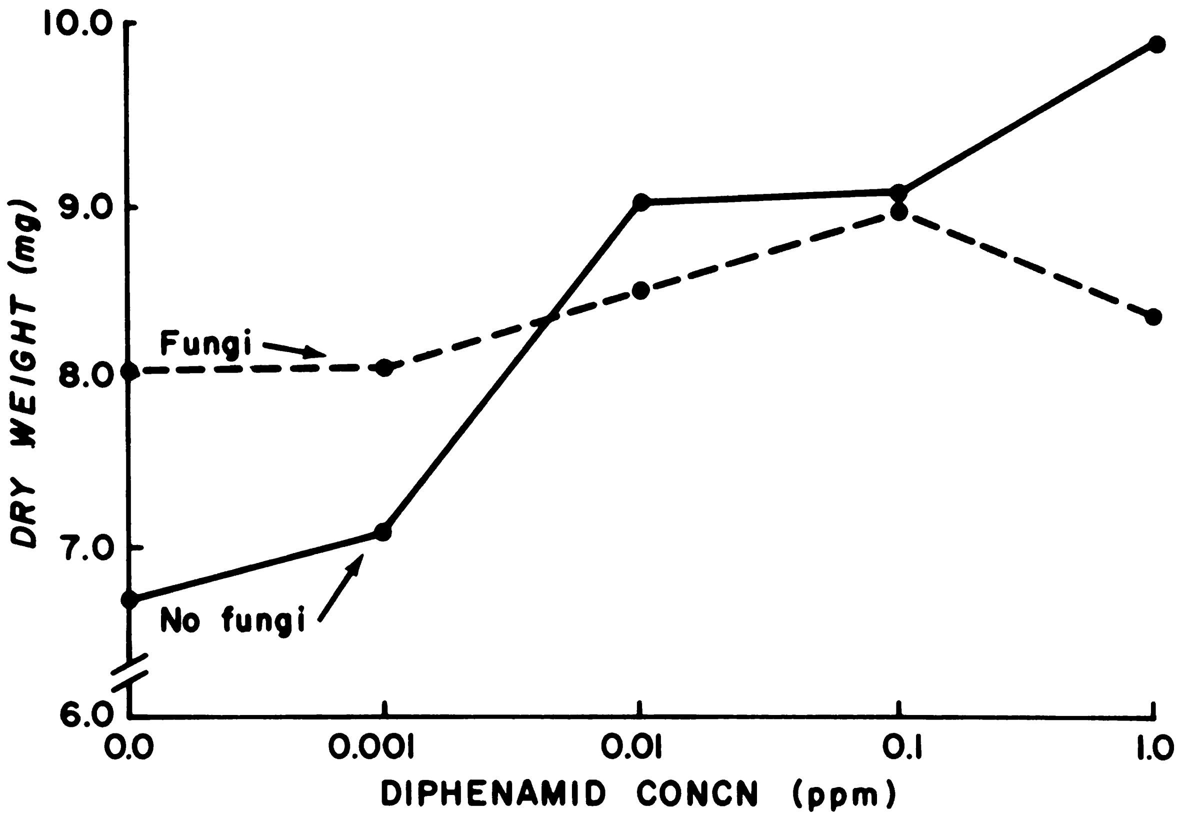
[1/]Average wt per plant.

[2/]Numbers followed by unlike letters are significantly different at the 5% level.

Another test was set up to separate the effects of the fungus from the effects of the diphenamid and vice versa. The treatments consisted of diphenamid concn from 0.001 to 1.0 ppm each with and without fungi. All fungal treatments were inoculated with both _A_. _candidus_ and _T_. _viride_. The plants were grown in aerated 250 ml beakers for 3 weeks, harvested, dried and weighed. An attempt was made to keep the treatments not inoculated with fungi as free from contamination as possible during the experiment.

There was an increase in the growth of plants when the fungus was added to the nutrient solution with no diphenamid present and this effect was increased by the presence of 0.1 ppm of diphenamid (Figure 1). Diphenamid itself also increased the growth of the plants at the 3 higher concn.

Figure 1. The growth of tomato plants treated with diphenamid, _A_. _candidus_ and _T_. _viride_. F value for the interaction fungi x diphenamid significant at the 5% level. Dry weights are per single 3 week old tomato plant.


10.0
9.0
8.0
7.0
6.0
DRY WEIGHT (mg)
Fungi
No fungi
0.0
0.001
0.01
0.1
1.0
DIPHENAMID CONCN (ppm)

Filtrate applications to tomatoes:

The effects of diphenamid and 2 fungal species were tested on germinating tomato seedlings. In the first experiment, filtrate from A. candidus and T. viride was used as the treatment rather than inoculating with the organisms themselves. Ten 3 day old seedlings were placed in each petri dish, covered, treated, and placed in a dark incubator at 26^{o} C for 3 days. Growth was determined by measuring the length of the radicles.

The treatments containing filtrate inhibited the growth of the tomato radicles. The radicles became enlarged with many branch roots (Table 5).

Table 5. The response of germinating tomato seedlings to A. candidus, T. viride, and diphenamid.

Treatment	Diphenamid concn (ppm)	Length of radicle (mm) [1/]
None	0.0	59.0 a
None	0.1	56.7 a
A. candidus filtrate	0.1	26.9 b
T. viride filtrate	0.1	16.9 c

[1/] Numbers followed by unlike letters are significantly different at the 5% level.

This data indicated that the filtrate was too concentrated and a more elaborate experiment was designed using dilutions of the fungal filtrates from 1-10 to 1-1000.

Ten surface disinfected pregerminated seeds were placed in each petri dish. Both A. candidus and T. viride species were used in this experiment. The seeds were placed in a dark incubator at 26° C for 3 days prior to measuring the radicles. The growth of A. candidus treated seedlings was increased over the control seedlings in most instances (Table 6). The 1-100 fungal dilution rate gave the most response with 0.1 and 1.0 ppm diphenamid while the 1-1000 rate had little effect.

Table 6. The response of tomato seedling radicles to A. candidus and diphenamid.

Filtrate ratio	Growth of radicle (mm) [1/] Diphenamid concn (ppm) 0.0	0.01	0.1	1.0
None	34 a	33 b	33 b	30 b
1-10	36 a	39 a	41 a	34 b
1-100	35 a	38 a	42 a	42 a
1-1000	34 a	33 b	38 a	35 b

[1/]Numbers followed by unlike letters are significantly different at the 5% level.

The seeds treated with the filtrate from T. viride responded the same except that the 1-10 dilution gave the best response while the effect was lost at the 1-100 and 1-1000 dilution (Table 7).

Table 7. The response of tomato seedling radicles to T. viride and diphenamid.

Filtrate ratio	Growth of radicle (mm) 1/			
	Diphenamid concn (ppm)			
	0.0	0.01	0.1	1.0
None	31 b	36 b	27 b	31 b
1-10	42 a	48 a	46 a	43 a
1-100	32 b	40 b	35 b	34 b
1-1000	35 b	36 b	31 b	33 b

1/ Numbers followed by unlike letters are significantly different at the 5% level.

This work was continued employing a wider range of diphenamid concn and filtrate from A. candidus. In this instance the filtrate was taken after 3 weeks of fungal growth rather than the usual 1 week. Only the A. candidus species was used in this experiment since both A. candidus and T. viride gave the same response in previous tests. The higher concn of filtrate was used along with higher concn of diphenamid in an attempt to pick out any interaction which might be occurring. Radicle growth measurements were taken 3 days after treatments were applied to the seedlings (Table 8).

Table 8. The response of tomato seedlings to A. candidus and diphenamid.

A. candidus filtrate ratio	Growth of radicle (mm) [1/]				
	Diphenamid concn (ppm)				
	0.0	0.01	0.1	1.0	10
None	31 a	36 a	51 a	51 a	54 a
1	11 b	31 a	54 a	53 a	49 a
1-10	16 b	17 b	16 c	36 b	57 a
1-100	16 b	21 a	20 b	55 a	57 a
1-1000	27 a	35 a	24 b	52 a	48 a

[1/] Numbers followed by unlike letters are significantly different at the 5% level.

The results of this experiment illustrated a relationship between diphenamid and the fungus. All filtrate treatments inhibited tomato growth but the addition of diphenamid completely overcame this inhibition at the higher rates. The inhibition was also overcome at low rates of diphenamid and high concn of filtrate but not at low concn of filtrate. Radicles which were inhibited were short, swollen, injured at the apex, and had a large number of lateral roots. However, when diphenamid overcame the inhibitory levels of filtrate, the roots were healthy with many root hairs and few lateral roots.

The previous tests indicated that possibly a toxin produced by the fungus or enzymes in the filtrate were rendered inactive by diphenamid.

A possible explanation for loss of inhibition at low diphenamid rates and high filtrate concn may have been that the higher concn of filtrate contained a higher concn of enzyme which was capable of demethylating all of the diphenamid in the solution and thus methylating and detoxifying more fungal toxin. At low filtrate concn, only a small amount of demethylating enzyme was present and less detoxification occurred. This will be explained further later in the thesis. This phenomenon also indicated a possible explanation for the rapid growth of these organisms in diphenamid cultures. Several fungal organisms are known to produce toxins which will inhibit the producing organism if the concn becomes sufficiently high (10). If diphenamid was detoxifying or removing this toxin as it was produced, the fungi would grow more rapidly.

A test utilizing tomato plants was initiated to further elucidate this protective effect of diphenamid. The tomato plants pretreated with A. candidus filtrate for 24 hr and placed back in 1.0 ppm diphenamid after the various time periods were in no case smaller than the control at either the 15 and 30 day harvest times (Table 9). There was no inhibitive effect from the filtrate after a 24 hr exposure.

Table 9. The response of tomato plants to 24 hr pretreatments with *A. candidus* and diphenamid followed by diphenamid and *A. candidus* applications.

		Dry wt (g) 1/			
		Harvests (days after treatment)			
		15		30	
		Pretreatment			
Treatment (days)	Diphenamid concn (ppm)	*A. candidus*	diphenamid	*A. candidus*	diphenamid
0	0.0	0.70	1.14	4.3	4.5 a
0	1.0	0.88	1.25	5.4	5.0 a
1	1.0	0.84	0.94	4.7	4.1 a
2	1.0	0.85	1.12	5.1	2.8 b
4	1.0	0.98	1.08	5.0	3.3 b
8	1.0	1.04	1.22	4.9	2.8 b

1/ Numbers followed by unlike letters are significantly different at the 5% level.

The dry wt of plants pretreated with diphenamid for 24 hr and placed in *A. candidus* filtrate at the various time periods was in no case smaller than the control at the 15 day harvest. However, at the 30 day harvest period, plants pretreated with diphenamid and exposed to the filtrate 2, 4, and 8 days later were inhibited in growth, indicating a protective effect of diphenamid which was lost when the plants were exposed to the filtrate 2 days after pretreatment and then grown for a 30 day period.

The growth of A. candidus and T. viride appeared to be enhanced by diphenamid. Several species of these 2 genera produce a highly antibacterial and antifungal antibiotic known as glyotoxin (10). T. viride produces the antibiotic viridin which has been shown to be quite highly toxic to plant pathogens such as damping-off fungi and wilt causing organisms (29). Thus, any stimulation of the growth of these organisms could affect the phytotoxicity of diphenamid as well as produce a beneficial secondary effect by their presence.

Timonin (63) studied the relationship and interaction of various fungi with cultivars of flax (Linum usitatissimum L.). He found that soil fungi were increased in number by the root exudate of the flax cultivars studied. Root exudate of Novelty cultivar, which is susceptible to Fusarium wilt, produced increased populations of this organism. Root exudate from Bison cultivar, which is resistant to Fusarium wilt, greatly enhanced the growth of Trichoderma species which have been shown by researchers to inhibit other microorganisms by the production of antibiotics (30, 65). Timonin suggested the possibility that the enhanced growth of Trichoderma species was a means of Fusarium wilt resistance in the Bison cultivar of flax.

Weindling (66) suggested that a buildup of this organism enhanced organic matter breakdown and thus nutrient availability to the plants since Trichoderma species are highly active in organic matter decomposition. These species also remain quite active under low soil moisture conditions which may help to release nutrients to the plants under stress conditions (62).

Processes of oxidation, reduction, hydrolysis and hydration may occur when herbicides are applied to the soil. Hartley (7) has shown hydrolysis to occur quite readily on the amide grouping of the phenylureas. This reaction is significant only under pH conditions usually outside the normal soil range. However, absorption of the material to acid soil colloids or the presence of certain soil microbes could greatly increase the probability of such reactions.

Raynor and Neilson-Jones (52) reported that some researchers consider the formation of mycorrhiza as being highly significant in relation to the nitrogen supply to higher plants and that this takes the form of readily available organic nitrogen compounds liberated by the fungal partner. They estimated that possibly 80% of the flowering plants develop mycorrhiza. If this is correct, there may be some doubt as to whether the applied inorganic soil nitrogen is always of direct and primary significance in the nutrition of these higher plants. Most plants will respond to inorganic nitrogen under sterile conditions but they rarely grow as rapidly.

Chesters and Street (13) reported that such observations direct attention to the possible importance of organic nitrogenous metabolites, vitamins, and auxins. Thus far, however, the evidence fails to completely establish the necessity of an external supply of any of these substances for optimum development and suggest that this field is still relatively unexplored and could be a fruitful field of research.

Chesters and Street did an experiment using lettuce (<u>Lactuca sativa</u>), cultivar May King, grown in sand culture and watered with nutrient solution plus various additivies. The treatments were as follows: 1) pure nutrient solution; 2) nutrient solution plus an oak leaf mould extract which had been decayed by bacteria and fungi such as the common organic matter decomposers of the Trichoderma and Aspergillus families; 3) nutrient solution plus casein and, 4) nutrient solution plus yeast extract. The leaf mould extract increased both dry and fresh wt of the plants over the control and over the other 2 treatments. Increased nitrogen uptake could have been responsible but was not found by the analyses, nor did these plants flower earlier which is an indication of high nitrogen. It was suggested that antibiotics may have been involved, and/or some growth enhancing factor but the authors did not hypothesize as to the exact mechanism.

Street (59) later continued the above experiments using 3 species of plants; radish (Raphanus sativus), oat and lettuce. He used the same treatment solutions as before plus an aqueous solution of bacterialized peat. He again increased the growth of the lettuce plants by the addition of the mould extract. Both the fresh and dry wt of the radish were increased by this treatment. Oat plant growth was not increased by any of the treatments although growth was not inhibited. Yeast extract produced a smaller but still significant stimulation of the growth of radish but was slightly deleterious to lettuce. Some of Street's pertinent data are shown below.

Lettuce - cultivar May King
Harvested after flowering had begun.

		control	mould extract	peat extract
Fresh wt	g	158.2	171.3	134.6
Dry wt	"	13.6	17.2	11.5
Fresh shoot	"	122.0	120.5	108.8
Dry shoot	"	10.0	12.6	9.5
Fresh root	"	36.2	50.8	25.8
Dry root	"	2.9	4.6	2.0

Radish - cultivar Turnip Red

		control	mould extract	peat extract	yeast extract
Fresh wt	g	3.75	5.03	3.96	4.26
Fresh shoot	"	1.26	1.77	1.36	1.44
Fresh root	"	1.88	2.60	1.95	2.13
Dry shoot	"	.10	.14	.11	.11
Dry root	"	.16	.20	.17	.18
F.S./F.R.	"	.51	.54	.52	.51
D.S./D.R.	"	.64	.68	.65	.64

The insensitivity of the monocotyledonary oat plants suggested an auxin effect from the leaf mould. The author also hypothesized this as the reason for the response of the dicotyledonary plants of lettuce and radish. Succulent dicotyledons are not only more sensitive to auxins but are apparently able to absorb, translocate and accumulate such hormones more rapidly than monocotyledons. Street uses the

effect of synthetic-auxin herbicides on these 2 types of plants as a comparison. He indicated that the effects he obtained simulated the results of applications of naphthalene acetamide at low concn but was unable to find adequate amounts of auxins to cause such a response. He also ran a series of tests using several synthetic-organic auxin materials in his water cultures and was not able to produce the enhancement effect obtained from the leaf mould extract. Therefore, it seemed improbable to him that his results were explainable on the basis of known growth-regulating substances.

An enhancement of plant growth from soil organisms similar to that observed in this research, therefore, has been observed and reported by several researchers.

Diphenamid Metabolite Studies.

This series of experiments was initiated to study the effect of diphenamid and its metabolites on susceptible and resistant plant species. In the first experiment diphenamid did not alter the growth of tomatoes (Table 10). In the MDA (N-methyl 2, 2-diphenylacetamide) and the DA (2, 2-diphenyl-acetamide) treatments the roots were shorter and produced more laterals. Tomato hypocotyls were also shorter in these 2 treatments. Barnyard grass roots were shorter from applications of 1.0 ppm of diphenamid. However, the MDA compound caused acute toxicity and dying at the root apex.

Table 10. The phytotoxicity of diphenamid, MDA, and DA to tomato and barnyard grass seedlings.

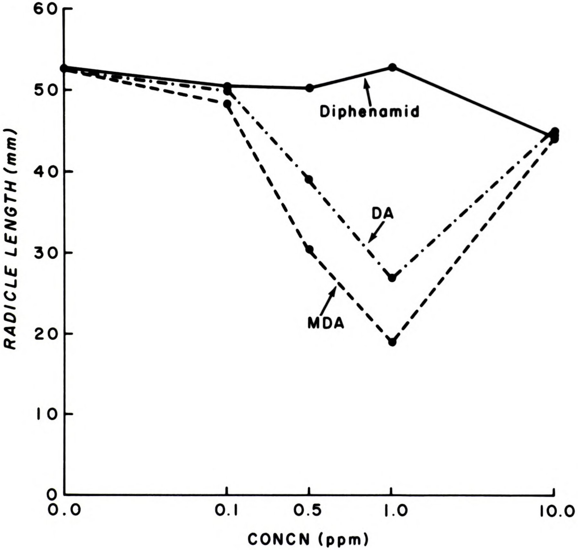
Species	Chemical	Concn (ppm)	Length (mm) [1/] Radicle	Hypocotyl
Tomato				
	Control	0.0	44.8 a	30.8 a
	Diphenamid	1.0	46.8 a	30.3 a
	MDA	1.0	25.5 b	16.7 b
	DA	1.0	16.2 c	12.3 b
Barnyard grass				
	Control	0.0	41.2 a	50.5 a
	Diphenamid	1.0	16.8 b	51.8 a
	MDA	1.0	4.3 c	23.9 b
	DA	1.0	10.9 b	23.4 b

[1/] Numbers followed by unlike letters are significantly different at the 5% level.

This indicated that under sterile conditions MDA was more toxic to barnyard grass seedlings than diphenamid. Another test was initiated using a concn range of these compounds on tomato and barnyard grass seedlings. In the study with tomatoes the roots were essentially not damaged by any of the diphenamid concn (Figure 2). Roots were long, slender, white, and showed little maturation and root hair development

Figure 2. The growth of tomato seedlings treated with diphenamid, MDA, and DA.

F value for the interaction rate x chemical significant at the 1% level.


60
50
40
30
20
10
0
RADICLE LENGTH (mm)
Diphenamid
DA
MDA
0.0
0.1
0.5
1.0
10.0
CONCN (ppm)

at the end of the 4 day growing period (Figure 3). Those treated with MDA had considerably shorter roots at the 1.0 ppm concn. At 10 ppm concn of both DA and MDA the roots were as long as those of the control plants (Figure 4). However, the roots were more mature than in diphenamid or control treatments and developed an extremely dense growth of root hairs. Thus, the absorbing surface of these roots was probably greater than that of control and diphenamid treated roots.

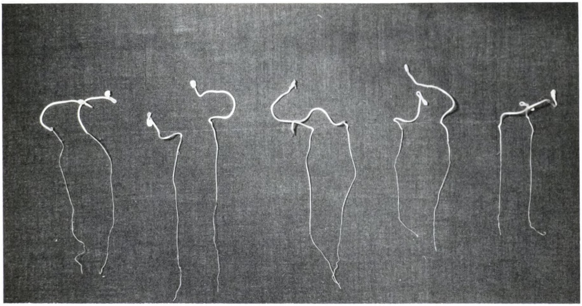
It has been reported (39) that diphenamid is more toxic to the tomato than its metabolites. This data does not substantiate these findings.

In the same experiment the tomato hypocotyl growth was analogous to the root growth. Diphenamid apparently did not affect hypocotyl growth whereas the 2 metabolites at both 0.5 and 1.0 ppm decreased growth by 50% of more (Table 11).

The growth of barnyard grass roots responded in a linear manner to diphenamid concn, decreasing as concn increased up to 10 ppm (Figures 5 and 6). The MDA decreased root growth at the 0.5 and 1.0 ppm concn and caused severe injury. These roots were less than 5 mm long, twisted, and dead at the apex (Figure 7). At 10 ppm of MDA roots were normal with no twisting or obvious injury at the apex. The DA compound produced a similar effect but gave less inhibition and injury at 0.5 and 1.0 ppm concn. MDA had a similar effect on the barnyard grass hypocotyls. They were shorter at 0.5 and 1.0 ppm and 10 ppm did not cause as much apparent injury (Table 11).

Figure 3. The response of tomato seedlings to various concn of diphenamid. Left to right: control, 0.1, 0.5, 1.0, and 10.0 ppm.

Figure 4. The response of tomato seedlings to various concn of MDA. Left to right: control, 0.1, 0.5, 1.0, and 10.0 ppm.



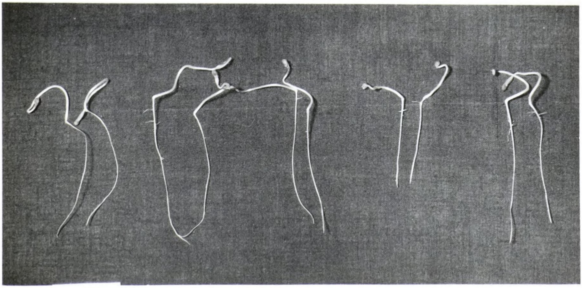
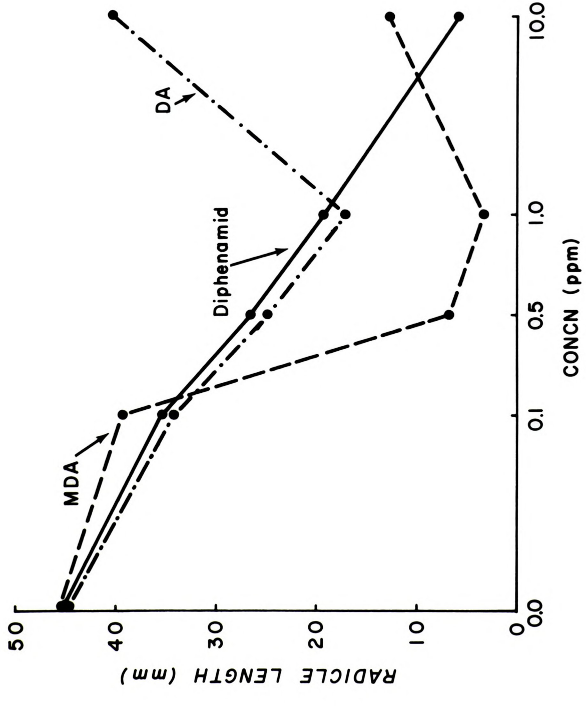


Figure 5. The effect of diphenamid, MDA, and DA on barnyard grass seedlings.

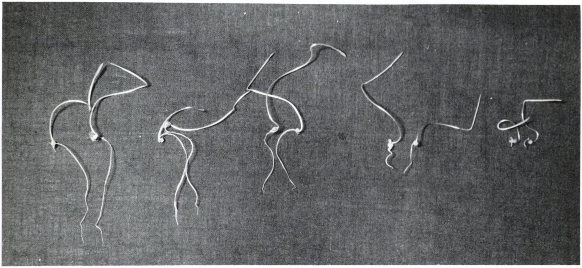
F value for the interaction rate x chemical significant at the 1% level.



RADICLE LENGTH (mm)
0
10
20
30
40
50
CONCN (ppm)
0.0
0.1
0.5
1.0
10.0
MDA
Diphenamid
DA

Figure 6. The response of barnyard grass seedlings to diphenamid. Left to right: control, 0.1, 0.5, 1.0, and 10.0 ppm.

Figure 7. The response of barnyard grass seedlings to MDA. Left to right: control, 0.1, 0.5, 1.0, and 10.0 ppm.



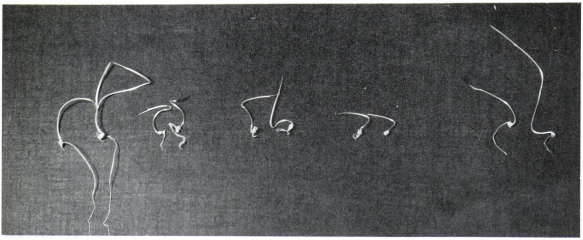


Table 11. The hypocotyl length of tomato and barnyard grass seedlings treated with diphenamid and 2 metabolites.

Chemical	Concn (ppm)	Hypocotyl length (mm) [1/]	
		Tomato	Barnyard grass
None	0.0	24.8 a	37.2 ab
Diphenamid	0.1	24.3 a	28.9 b
	0.5	24.0 a	30.3 b
	1.0	22.3 a	36.1 ab
	10.0	22.2 a	13.9 d
MDA	0.1	21.0 a	30.4 b
	0.5	16.0 c	15.9 d
	1.0	13.5 cd	9.2 d
	10.0	19.5 b	15.9 d
DA	0.1	21.4 a	42.7 a
	0.5	12.8 d	27.8 bc
	1.0	9.4 e	17.4 cd
	10.0	24.8 a	38.5 a

[1/] Numbers followed by unlike letters are significantly different at the 5% level.

In general, diphenamid was not as toxic to barnyard grass as MDA and had less effect on tomato at the range of concn studied, although even these metabolites were not highly toxic to tomato seedlings.

In the third experiment using higher concn of diphenamid and MDA, diphenamid did not cause acute toxicity to the tomato roots (Figure 8). However, the 100 ppm concn did inhibit root growth. Those treated with MDA were shorter at the 0.1 and 1.0 ppm concn but were not measurably injured by the higher 10 and 100 ppm concn. At the 100 ppm rate of MDA, the hypocotyls were necrotic. This may indicate that high concn of MDA were absorbed by the roots without injury but became toxic when translocated to the hypocotyl.

Barnyard grass roots responded the same in this experiment as in the previous one (Figure 9). The roots responded in a linear fashion to diphenamid concn, decreasing in length as the concn increased but with no twisting or necrosis of the tissue at any of the concn.

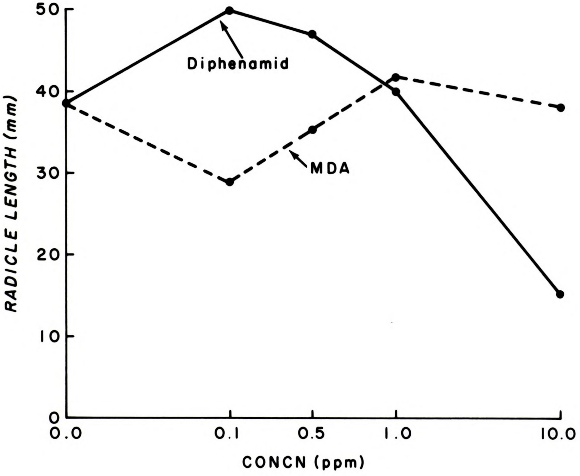
The MDA compound at the 0.1 and 1.0 ppm rate caused twisting and injury to the root apex. At 10 ppm roots were not injured or growth inhibited. However, at the 100 ppm rate the roots were shorter, twisted, and dead at the apex.

Toxicity of diphenamid in soil:

A study was conducted using soil to determine if the preceding response would occur in this environment. Two grass species, barnyard grass and German millet, responded identically to diphenamid applied to sterile, nonsterile, and sterile soil inoculated with fungi (Figures 10 and 11).

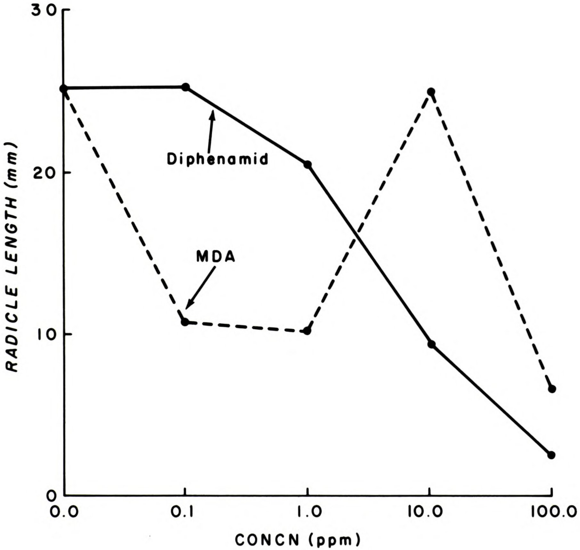
In unsterilized soil both grass species were severely injured by all concn of diphenamid. In sterilized soil inoculated with fungi severe injury resulted to both grasses at 10 and 100 ppm of diphenamid.

Figure 8. The response of tomato seedlings to diphenamid and MDA.


50
40
30
20
10
0
RADICLE LENGTH (mm)
Diphenamid
MDA
0.0
0.1
0.5
1.0
10.0
CONCN (ppm)

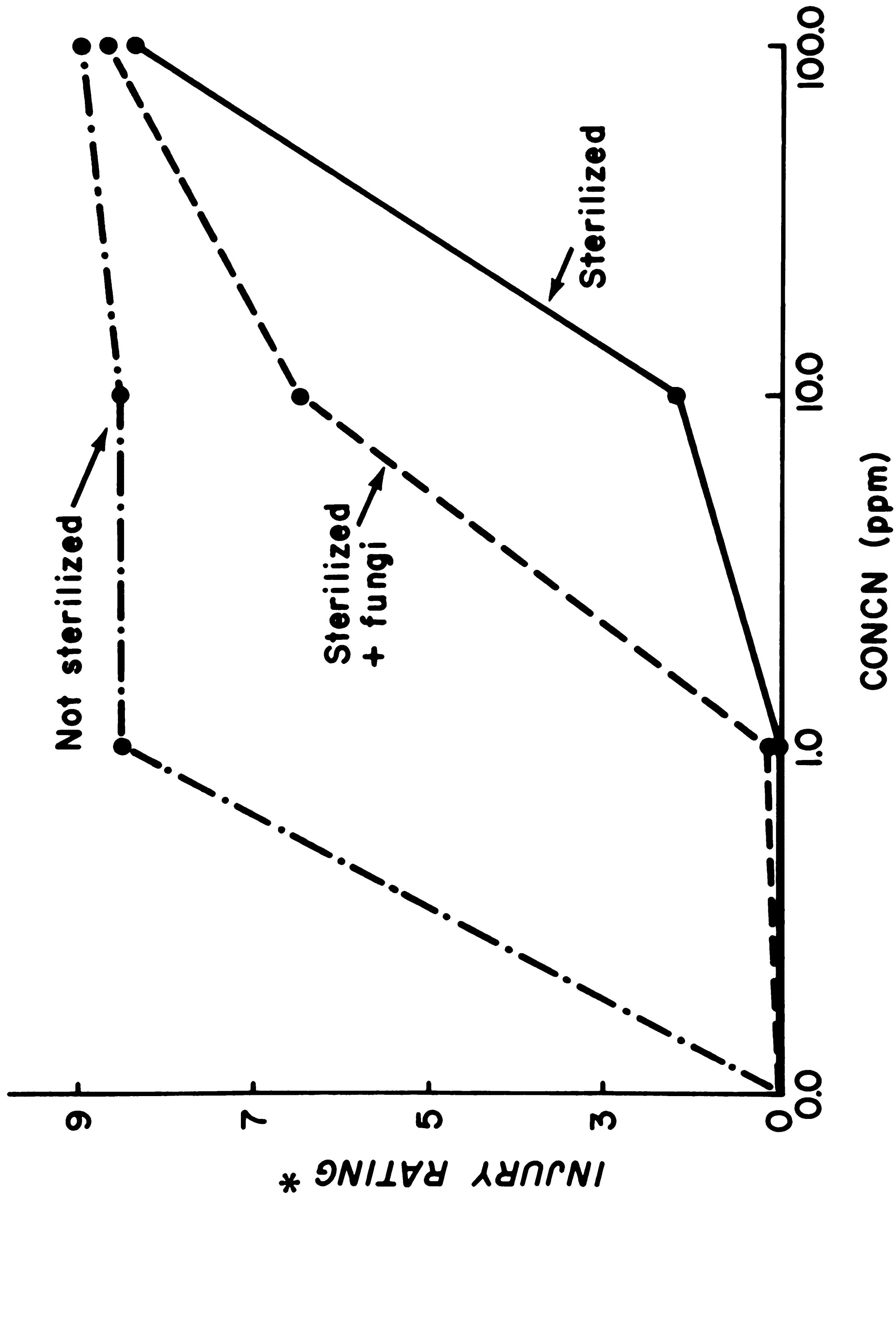
8

Figure 9. The growth of barnyard grass roots treated with diphenamid and MDA.
F value for the interaction rate x chemical significant at the 5% level.



30
20
10
0
RADICLE LENGTH (mm)
Diphenamid
MDA
0.0
0.1
1.0
10.0
100.0
CONCN (ppm)

Figure 10. Barnyard grass and German millet growth in sterile and nonsterile soil treated with diphenamid.



Not sterilized
Sterilized
+ fungi
Sterilized
INJURY RATING *
CONCN (ppm)
0.0
1.0
10.0
100.0
0
3
5
7
9

Figure 11. The growth of tomato and barnyard grass plants in diphenamid treated soil. Left to right: sterilized soil, sterilized soil plus fungi inoculation, and nonsterilized soil.

Whereas in sterile soil diphenamid only caused injury at 100 ppm.

Tomato seedlings were not injured in any of the treatments at the 2 lower rates but were stunted by the 100 ppm concn under all 3 conditions.

The grass species germinated in 10 ppm diphenamid treated non-sterile and sterile soil inoculated with fungi but became chlorotic and died when the plants were less than 1 cm tall. This indicated that diphenamid was not the toxic moiety responsible for the death of the grass plant species at concn up to 10 ppm. However, when diphenamid was placed in an environment where soil microorganisms were present, it apparently was altered to a more toxic compound.

In a similar experiment, conducted in petri dishes, roots and shoots of barnyard grass were closely examined and rated. None of the diphenamid treated seedlings in the sterilized soil were injured up to the 10 ppm rate (Table 12). Even at 100 ppm germination was excellent and plants were still a dark green color but the hypocotyls and radicles were approximately one-half the length of the control seedlings. Under these conditions the toxicity of diphenamid to grass plants was evident only at the 100 ppm rate.

The MDA metabolite severely injured barnyard grass seedlings at 10 and 100 ppm. Tomato seedlings were not affected by diphenamid rates up to 10 ppm but at 100 ppm they were slightly smaller than control seedlings. In the MDA metabolite treatments the tomato seedlings were not injured from any treatment except the 100 ppm concn.

Table 12. A comparison of the growth of barnyard grass seedlings in sterile and nonsterile soil treated with diphenamid and MDA. 1/

Concn (ppm)	Growth 2/ Sterile soil Diphenamid	MDA	Nonsterile soil Diphenamid	MDA
0.0	1.0 b	1.0 c	1.0 b	1.0 c
0.1	1.0 b	3.3 b	7.0 a	6.3 a
1.0	1.0 b	2.0 b	6.3 a	7.0 a
10.0	1.0 b	7.0 a	6.3 a	5.7 b
100.0	4.6 a	7.0 a	8.0 a	4.0 b

1/ Growth ratings: 1 = No injury, 9 = complete kill.

2/ Numbers followed by unlike letters are significantly different at the 5% level.

Diphenamid applied to nonsterile soil severely injured the grass seedlings at all rates. Tomato seedlings were smaller in nonsterile soil treatments but only appeared severely injured at the 100 ppm concn of diphenamid. The MDA metabolite severely injured barnyard grass seedlings at the 0.1 and 1.0 ppm rate but the injury was considerably less at the 10 and 100 ppm rate. This indicated a definite effective concn range for this chemical, above which, the toxic effect was less pronounced. Tomato plants were stunted at the 0.1 and 1.0 ppm rates but were not reduced in growth or injured by the higher concn.

Diphenamid treated sterilized soil inoculated with _T. viride_ affected the barnyard grass seedlings the same as the unsterilized soil treatment. Barnyard grass seedlings were injured at all diphenamid concn (Table 13).

Table 13. Barnyard grass seedling growth in sterilized soil treated with diphenamid and _T. viride_. 1/

Concn (ppm)	Growth 2/	
	Diphenamid	MDA
0.0	1.0 d	1.0 c
0.1	4.3 c	3.6 bc
1.0	8.0 a	5.1 b
10.0	6.0 b	7.0 a
100.0	8.1 a	7.0 a

1/Growth ratings: 1 = No injury, 9 = complete kill.

2/Numbers followed by unlike letters are significantly different at the 5% level.

Tomato plants were severely stunted only at the 100 ppm diphenamid concn. The MDA compound again injured the barnyard grass seedlings at all rates. However, the loss of toxicity at the 100 ppm rate did not become evident in this series of treatments. Tomato seedlings were about one half the size of control seedlings in all these treatments.

Uptake and Translocation of ^3H-Diphenamid.

Studies were initiated to determine whether there was a difference in the absorption of diphenamid between tomato and barnyard grass plants. The rate of uptake of diphenamid by the roots of barnyard grass and tomato indicated that both absorbed the chemical in large amounts after only 4 hr. After 24 hr, the amount of ^3H-diphenamid did not increase for either barnyard grass or tomato (Figure 12). This indicated no exclusion of diphenamid by the roots of the resistant tomato species. In fact, the tomato roots absorbed more diphenamid than the barnyard grass roots.

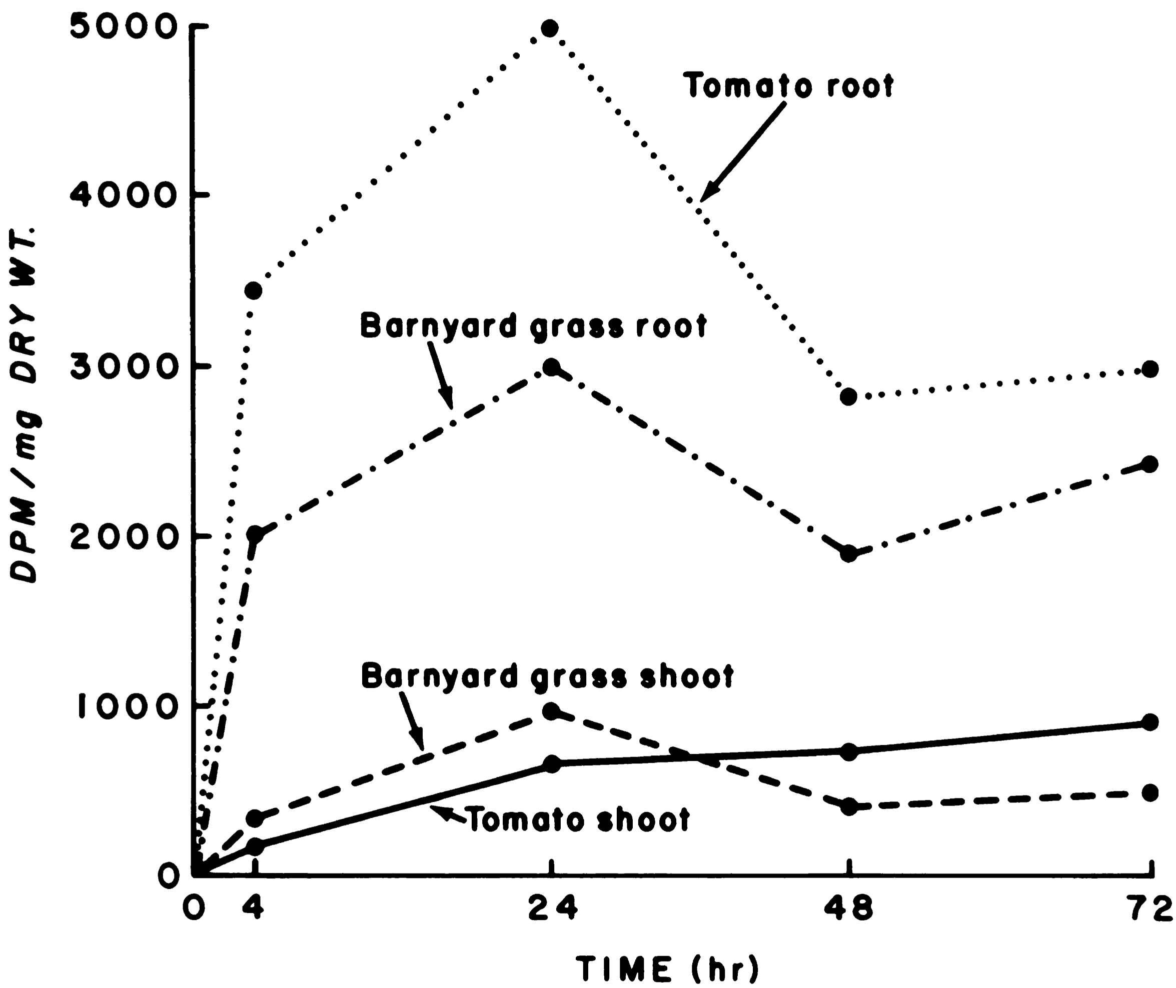
The rate of translocation of ^3H-diphenamid from root to shoot in the 2 species, estimated by measuring the radioactivity per unit of shoot wt at the various harvest times, again did not reflect any difference in absorption between the 2 species.

Diphenamid Metabolism by Fungi.

Both the chloroform and ethanol soluble extracts migrated to R_f 0.8 on the chromogram as did the ^3H-diphenamid reference spot when developed in a benzene-ethanol mixture. In this solvent system, the diphenamid appeared to be unchanged by the fungi. This same relationship held for both the 4 hr exposure and the 48 hr exposure to both organisms.

When the extracts were chromatographed first in benzene-ethanol and then at 90 degrees in benzene-diethylamine there was a definite separation. The extracts which had been exposed to the fungi for 4 hr contained a compound with the same R_f as the MDA reference spot or R_f

Figure 12. Uptake and translocation of ^{3}H-diphenamid by tomato and barnyard grass seedlings. Average of 2 experiments except for 72 hr observation.


5000
4000
3000
2000
1000
0
DPM/mg DRY WT.
Tomato root
Barnyard grass root
Barnyard grass shoot
Tomato shoot
0 4
24
48
72
TIME (hr)

0.53. There was also a spot corresponding to the diphenamid reference spot at R_f 0.64. The extracts from the 48 hr treatments had a larger spot corresponding to the MDA metabolite at R_f 0.53 and also detectable amounts at R_f 0.25 corresponding to the DA metabolite.

T. viride and *A. candidus*, therefore, begin to demethylate diphenamid within a very short time and the MDA metabolite can easily be detected within 4 hr. Demethylation continues and the DA can be detected after 48 hr of exposure to either fungal organism. Both the chloroform and ethanol extracts were identical in content but the chloroform extracts contained some fatty substances which were sometimes difficult to move from the origin on the chromogram. Further tests and other solvent systems produced identical results.

SUMMARY

The effect of diphenamid on the growth of tomatoes was studied under field and controlled environment conditions. Diphenamid enhanced the growth of tomato plants under optimum conditions in a controlled environment. The enhancement was of equal magnitude over a concn range from 0.001 to 1.0 ppm. Below this concn the enhancement effect was not evident and above it plants were injured. Spectrographic analyses revealed no nutrient element differences between diphenamid treated and control plants.

Diphenamid did not significantly alter tomato growth under field conditions. Environment conditions were not optimum, however, since rainfall was inadequate and temperatures were often above 33° C during the growing season.

Two fungal species, _Aspergillus candidus_ and _Trichoderma viride_, were found in diphenamid solutions not maintained under sterile conditions, while nutrient solutions containing no diphenamid were not visibly contaminated. Low concn of these organisms, or filtrates from them, increased the growth of tomatoes while high concn inhibited growth. The addition of diphenamid to the fungus overcame the inhibition with normal tomato growth resulting.

Both _A. candidus_ and _T. viride_ demethylated diphenamid to MDA (N-methyl 2, 2-diphenylacetamide) within 4 hr and further demethylated it to DA (2, 2-diphenylacetamide) within 48 hr. These fungi are important and common soil organic matter decomposers. They are considered

nonpathogenic, saprophytic organisms and are undoubtedly important in the metabolism of diphenamid under field conditions. *T. viride* also produces the antibiotic viridin which has been found to inhibit the growth of damping-off fungi and wilt causing organisms. Thus, any stimulation of the growth of these 2 fungi may affect the phytotoxicity of diphenamid as well as produce beneficial secondary effects.

The toxicity of diphenamid, MDA, and DA was studied on tomato and barnyard grass seedlings as representative resistant and susceptible plant species. Under sterile conditions, diphenamid did not injure tomato seedlings up to a concn of 10 ppm and only reduced growth at 100 ppm. Both MDA and DA reduced the growth of tomato seedlings at 0.5 and 1.0 ppm but did not cause acute toxicity. Barnyard grass seedlings responded in a linear manner to diphenamid concn, decreasing in growth as concn increased up to 100 ppm. MDA caused severe injury to barnyard grass seedlings at 0.1, 0.5, 1.0 and 100 ppm but acute toxicity was not evident at a concn of 10 ppm. This data indicated that MDA was more phytotoxic than diphenamid.

In sterilized soil diphenamid remained relatively inactive, but became phytotoxic under nonsterile conditions indicating that metabolism of diphenamid was necessary for it to become phytotoxic.

Future research is necessary to determine if diphenamid is demethylated to MDA and DA by the tomato plant (39). Such experiments should be conducted under sterile growing conditions to eliminate the possibility of microorganism demethylation and subsequent plant absorption and translocation.

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APPENDIXES

Appendix A. The effect of diphenamid on flowering.

Diphenamid formulation	Rate (lb/A)	Cluster count [1/] (Avg/20 plants)
Sprayed	0	20.0 a
	2	15.2 b
	4	15.6 b
	8	19.2 a
Granular	0	15.6 b
	2	14.6 b
	4	17.4 b
	8	19.6 a
Drench	0	16.6 b
	2	17.0 b
	4	19.4 a
	8	18.8 a

[1/] Numbers followed by unlike letters are significantly different at 5% level.

Appendix B. The effect of diphenamid on the stem diameter of tomato plants 4, 6 and 9 weeks after treatment.

Diphenamid formulation	Rate (lb/A)	Stem diameter (mm) [1/] weeks after treatment 4	6	9
Sprayed	0	8.6 b	18.0	21.2
	2	8.6 b	18.3	20.1
	4	9.1 b	18.8	19.7
	8	9.8 a	20.0	20.9
Granular	0	9.1 b	19.7	21.0
	2	9.5 b	20.2	20.4
	4	9.0 b	20.2	20.5
	8	9.1 b	19.4	20.7
Drench	0	9.3 b	18.9	20.6
	2	9.9 a	19.2	21.7
	4	9.5 b	19.3	19.7
	8	9.2 b	18.3	19.3

[1/] Numbers followed by unlike letters are significantly different at the 5% level.

Appendix C. The yield of tomato plants receiving different formulations and rates of diphenamid

Diphenamid formulation	Rate (lb/A)	Yield (lb/45 ft of row) [1/] first harvest	second harvest	total yield
Sprayed	0	81.5	214.6 b	296.1
	2	70.2	222.6 a	292.8
	4	68.6	190.0 b	248.6
	8	72.1	231.4 a	303.5
Granular	0	78.1	245.8 a	323.9
	2	78.7	234.4 a	313.1
	4	70.3	240.0 a	310.3
	8	91.1	227.0 a	318.1
Drench	0	71.9	247.2 a	319.1
	2	80.3	216.0 a	296.3
	4	76.6	240.8 a	317.4
	8	73.0	209.0 b	282.6

[1/] Numbers followed by unlike letters are significantly different at the 5% level.