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MICROBIAL TRANSFORMATION OF RYE (<u>Secale</u> <u>cereale</u> L.)
RESIDUES: A SOURCE OF POTENT ALLELOCHEMICALS

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MICROBIAL TRANSFORMATION OF RYE (Secale cereale L.) RESIDUES: A SOURCE OF POTENT ALLELOCHEMICALS

By

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ABSTRACT

MICROBIAL TRANSFORMATION OF RYE (Secale cereale L.) RESIDUES: A SOURCE OF POTENT ALLELOCHEMICALS

By

Curt James Whitenack

Stable diazoperoxides were isolated from soil and characterized after in vitro microbial degradation of the benzoxazinones, the allelochemicals previously reported from rye. 2,3-benzoxazolinone (BOA) was shown to be transformed in the soil to 2,2'-oxo-1,1'-azobenzene (Compound 4), while 6-methoxy-2,3-benzoxazolinone (MBOA) resulted in production of mono- and dimethoxy analogs of the above compound. Leaching experiments with rye residue in vitro showed the presence of both BOA and 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) in the soil, but failed to detect any of the above compounds. Bioassays of the compounds showed strong herbicidal activity, 8-10 times more toxic than the previously reported allelochemicals, the benzoxazinones. These results may explain an additional source of toxicity from rye residues that has not been accounted for by the benzoxazinones. Nematicidal assays indicated that the LC_{50} of Compound 4 was between 1 and 10 ppm. 100% mortality was observed after four hours exposure to 10 ppm.

Dedication

This work is dedicated to my Aunt Caroline, and my mother, Lois, who made it possible in so many ways, and also to Brenda, because without her guidance, I would probably still be wondering if I could get into graduate school.

ACKNOWLEDGEMENTS

After nearly four years in Michigan, it occurs to me that I have recieved a great deal of help, from a lot of people, and while it is not possible to recognize all of them, I would like to mention the major players. I would like to thank the members of my advisory committee, Dr. Stanley Ries, Dr. James Miller, and especially my major professor, Dr. Alan Putnam, for their advice and guidance, and my "unofficial" committee member, Dr. Muralee Nair, for his extroardinary patience and teaching ability. Without their input, there would be no thesis. I would also like to thank Jackie Schartzer, for her assistance in the preparation of this manuscript. And finally, I would like to thank Brenda for her encouragement and belief, and my friends here in Michigan, who kept me sane and were always willing to help blow off steam. There's only one thing to say to you guys:

"What a long, strange trip it's been."

- The Grateful Dead

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LITERATURE REVIEW

INTRODUCTION

In recent years, increased public concern over the environmental impact of pesticide use has caused a re-evaluation of the place of synthetic chemicals in agricultural pest control. Even chemicals such as atrazine and alachlor, once regarded as relatively safe, are being scrutinized because of their presence in ground water. Others, such as aldrin, dieldrin, and DDT, are still detectable in Great Lakes fish, even though the use of these chemicals was banned several years ago. It seems obvious that the public will not allow continued use of synthetic crop protection chemicals at current levels, thus suitable alternatives should be developed.

An attractive alternative to synthetic chemicals is the use of natural products from higher plants or microorganisms. Duke (1986) lists several advantages to the development of natural products as crop protection chemicals. First, because many biologically active compounds are produced by pathogens, these compounds may have a built-in specificity, allowing the development of highly selective pesticides. Natural products also may provide a basis for new chemical groups of pesticides. Specifically, the synthetic chemist can use the molecular structure of the natural product as a "template" to develop analogs that affect a specific site of action. This may be especially helpful in controlling resistant weeds with altered target sites. Finally, since chemicals of plant and microbial origin consist primarily of carbon, hydrogen, oxygen, and nitrogen, and have been present in the environment for many years, it is likely that microorganisms have evolved that can easily degrade them. In contrast, many synthetic compounds, such as the halogenated hydrocarbons, have extremely long half-lives in the soil.

Natural products also have disadvantages. They may be extremely toxic to mammals. For example, Botulinum toxin, a natural product of _Clostridium botulinum_, is among the most toxic chemicals known. Scale-up from laboratory to pilot plant to full production may be prohibitively expensive and time consuming. There are, however, numerous examples where natural products have been used successfully for agricultural pest control.

A classic example of the use of a natural compound to control a pest is the use of pyrethroid insecticides. Since ancient times, the powdered flowers of _Chrysanthemum cinerariaefolium_ Vis. (_Pyrethrum cinerariaefolium_ Trev.) have been used as an insecticide (Matsui and Yamamoto, 1971). The insecticidal components are called pyrethrins, and while they were once used extensively in the field, their use is now limited mainly to control of domestic insects. Originally, the crushed flowers were extracted with an organic solvent to partially purify the pyrethrins, but when this became too costly, the toxins were characterized and produced synthetically.

Microbial Pesticides. Microbially produced antibiotics have been used in human medicine for many years, but several also have applications in the management of plant pathogens. Some are registered for agricultural use in Japan, the most widely used being streptomycin, for the control of various bacterial diseases of fruit trees and vegetables. Blasticidin S and kasugamycin, registered in 1962 and 1965, respectively, are used to control rice blast, a fungal infection of rice (*Oryza sativa*) (Misato, 1982).

Microbial phytotoxins are an attractive potential source of new herbicides and new herbicide chemistry. Cycloheximide, a glutarimide antibiotic produced by several *Streptomyces* species, is reported to control barnyardgrass (*Echinochloa crusgalli* L.) and pond weed (*Potamegeton* sp.) in paddy-grown rice without adversely affecting the rice seedlings (Sekizawa and Takematsu, 1982).

Anisomycin, a metabolite of a *Streptomyces* sp., is toxic to barnyardgrass and crabgrass species (*Digitaria* sp.), but has no effect on tomato (*Lycopersicon sculentum* Mill.) seedlings (Yamada, et. al., 1974). Using the structure of anisomycin as a starting point, Japanese chemists developed the synthetic herbicide methoxyphenone, now used commercially in Japan to control barnyardgrass in rice.

Bialaphos (2-amino-4-methylphophinoyl-butylyl)-alanylalanine), a new microbial herbicide produced by *Streptomyces viridochromogenes* is related to the commercial herbicide glyphosate. While it is slower acting than paraquat, it is substantially faster than glyphosate (Sekizawa and Takematsu, 1982).

Heisey and Putnam (1987) examined the herbicidal properties of geldanamycin and nigericin, two antibiotics produced by *Streptomyces hygroscopicus*. Geldanamycin is a member of the ansamycin group of antibiotics and has strong phytotoxic activity in vitro. In petri dish germination bioassays, the authors reported inhibition of cress (*Lepidium sativum* L.) radicle elongation to 50% of control at 1-2 ppm.

Nigericin, a polyether antibiotic, showed equal activity.

Mishra, et. al. (1988) screened 906 microbial isolates for phytotoxic properties. These included 796 actinomycete isolates, 70 fungi, and 40 non-actinomycete bacteria. Of all the isolates tested, 72, or about 8% caused significant inhibition of radicle elongation in cress germination bioassays. The most frequently active genera were *Streptomyces* and the novel actinomycete *Actinoplanes*. Of these genera, 18% and 13%, respectively, were active. Specific phytotoxins isolated in this study were reported by Heisey, et. al. (1988). This work indicates that microbial phytotoxins are fairly widespread in nature, and that continued screening in this area could lead to the development of new herbicides.

ALLELOPATHY

Metabolites of higher plants also exhibit phytotoxic properties, as evidenced by the numerous reports of the adverse effects of both living plants, and decaying plant residues on crops. This influence may be the result of phytotoxins leaching directly from the residues or production of phytotoxins by microorganisms associated with the residues. Allelochemicals are compounds that are produced by higher plants or microorganisms and that affect the growth of other plants. The overall mediation of one plant's growth by another through chemical means is known as allelopathy (Whittaker and Feeny, 1971; Rice, 1974)

Cubbon (1925) reported that a rye (*Secale cereale* L.) crop, grown simultaneously with a grape crop, reduced the grape's growth relative to a control. Nutrient and water supply were carefully monitored to ensure that these factors did not become limiting. The high nutrient level of the soil and abundant water supply suggested that competition was not the cause of the reduced grape growth. Rather, a chemical agent produced by the rye caused this effect (Cubbon, 1925).

Phytotoxins released by the microbial breakdown of plant residues can be leached into the soil and influence the germination and growth of susceptible plants. For many years, the problem of establishing peach trees on the site of an old peach orchard was attributed to pathogens, nutrient deficiencies, or spray residues, none of which seemed to completely explain the cause. Proebsting and Gilmore (1939) investigated the hypothesis that after tree removal, either the roots left in the soil, or their breakdown products were toxic to the new trees. They found that when they combined peach roots with soil and planted peach seedlings in this soil, there was a severe inhibition of seedling root growth. Eventually, they refined this to show that the majority of the toxicity was contained in the root bark. They noted that when peach bark is heated with water, it gives off the odor of benzaldehyde. The authors took this to indicate the presence of amygdalin, a cyanogenic β-glycoside. To test the possibility that amygdalin was the toxic agent, amygdalin was added to soil in which a peach seedling was grown. This treatment caused no injury, indicating thst amygdalin itself is not toxic. If a trace of emulsin, an enzyme that catalyzes the hydrolysis of amygdalin to glucose, benzaldehyde, and hydrogen cyanide was added along with amygdalin, severe injury resulted. Patrick (1955) examined the involvement of microorganisms in the production of toxicity from peach root bark and found that many microorganisms capable of hydrolyzing amygdalin could easily be isolated from the soil. Based on this, he proposed that the toxic effects of peach root bark in the soil were caused by hydrogen cyanide and benzaldehyde produced by microbial hydrolysis of amygdalin.

Patrick and Koch (1958) examined the effects of aqueous extracts of decaying residues of timothy (Phleum pratense L.), rye, corn (Zea mays L.), and tobacco (Nicotiana tabacum L.) on the respiration, oxygen uptake, and growth of tobacco seedlings. They found that as the residues decomposed, the aqueous extracts became more acidic, and that there was a high correlation between extract acidity and toxicity. The most highly toxic extracts were always acidic. When the pH of toxic extracts was adjusted to a more neutral range, toxicity decreased only slightly, suggesting that the inhibition of tobacco seedling respiration was caused by toxic substances, rather than the acidity of the extract alone. Additionally, they compared the toxicity of extracts of decomposed residues to extracts made by mixing macerated tissue with soil and extracting before decompostion could occur. In all cases, they found no toxicity unless microbial degradation of the residues had occurred. They concluded that the plants themselves contained no compounds inhibitory to the respiration of tobacco seedlings, but all toxic compounds were formed during the microbial degradation of the residues.

Phytotoxic chemicals produced by higher plants that are contained in the intact plants may be released upon disruption of the tissue during the decomposition of the residues. Chou and Patrick (1976) studied the phytotoxic compounds released during the degradation of corn and rye residues. Plant material was combined with soil and allowed to decompose for up to 30 days. In the aqueous extracts of decomposing corn residue, they detected eighteen phytotoxic compounds, mostly low molecular weight organic acids such as benzoic, phenylacetic,and 4-phenylbutyric acids, along with cinnamic acid derivatives such as ferulic, caffeic, o-coumaric, p-coumaric, trans-cinnamic, and vanillic acid. The cinnamic acid derivatives are frequently implicated in allelopathy by many species, and are one of the fundamental groups of allelochemicals (Rice, 1984).

Weston and Putnam (1986) showed that aqueous extracts of quackgrass (Agropyron repens L. Beauv.) severely inhibited radicle elongation in alfalfa (Medicago sativa L.), cress (Lepidium sativum L.), soybeans (Glycine max (L.) Merr.), and navy beans (Phaseolus vulgaris L.). These extracts did not affect the growth of Rhizobia commonly associated with legumes, indicating that inhibition of legume growth by quackgrass was not the result of inhibition of the growth of the rhizobial symbiont. Subsequently, a flavone (tricin) was isolated that was particularly inhibitory to root growth, specifically the formation of root hairs (Weston, et al, 1987). Although this compound also had no affect on rhizobia, the point of infection in the legume-rhizobium symbiosis is the root hair. Therefore, inhibition of root hair formation results in inhibition of the symbiosis.

Barnes and Putnam (1983, 1986) examined the allelopathic potential of residues and aqueous extracts of rye. Comparisons between a bare ground control and a living cover of spring planted rye showed a 94% reduction in total weed biomass under the rye. When residues were planted in the spring and killed after 40 days, there was a 69% reduction in total weed biomass, relative to a bare ground control. To separate the physical and chemical components, comparisons were also made to a control consisting of a poplar (Populus tremuloides L.) excelsior cover. The poplar excelsior simulates the physical effects produced by the mulch, such as shading, moisture retention, etc. In this case, the killed residue reduced weed biomass 32% relative to the poplar excelsior control, indicating that there was a chemical component to the weed supression. Later work (Barnes et al., 1987) implicated the benzoxazinones, cyclic hydroxamic acids produced by cereal crops, as the toxic agents in allelopathy by rye.

BENZOXAZINONES

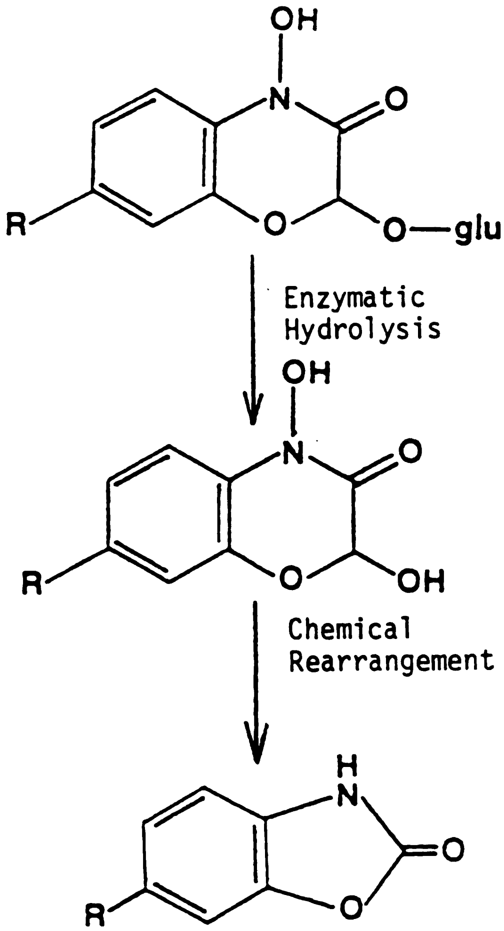
Chemistry. In 1955, Virtanen, et. al. isolated a cyclic hydroxamic acid, or benzoxazinone, from rye seedlings. Chemical analysis identified the compound as 2,3-benzoxazolinone (BOA). The same authors later isolated 6-methoxy-2,3-benzoxazolinone (MBOA) from wheat and corn (Virtanen et al., 1957) simultaneously with Loomis (1957). These compounds were isolated and purified based on their biological activity against fungi (*Fusarium nivale*) and European corn borer (*Ostrinia nubilalis*). When the structures were confirmed, the authors first believed they had isolated and identified the active principle. Virtanen, however, noted that while the benzoxazolinone could be extracted from crushed tissue with boiling water, direct extraction of the crushed tissue with diethyl ether failed to extract any benzoxazolinone. This suggested the presence of a precursor in the tissue. To isolate this precursor, intact tissue was boiled to inactivate all enzymes prior to extraction. Using this method, they isolated a glucoside precursor, 2-O-glucosyl-1,4(2H)-benzoxazin-3-one (GDIBOA) from rye, and a methoxylated analog, 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one (GDIMBOA) from corn and wheat. Treatment of the glucoside with a crude enzyme preparation from rye caused hydrolysis to glucose and a second precursor, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) in rye and 7-methoxy-2,4-dihydroxy-1,4(2H)-benzoxazin-3-one

(DIMBOA) in corn and wheat (Virtanen and Hietala, 1960) (Hietala and Virtanen, 1960). Hofman and Hofmanova (1969) showed that when intact tissue was carefully extracted, using liquid nitrogen as a fixative to prevent all enzyme activity, only the glucoside derivatives were extracted. This indicated that there was no free aglycone or benzoxazolinone in living, intact tissue. Upon injury to the plant, B-glucosidases are released that rapidly hydrolyze the glucoside to the aglycone, which in turn decomposes in water to form the benzoxazolinone (Figure 1). Naturally occurring benzoxazinones have also been reported from two additional plants. Job's tears (Coix lachryma jobi) contains methoxylated derivatives (Koyama, 1955) and bears breech (Acanthis mollis L.) contains GDIBOA in the seeds (Wolf et al. 1985). These are the only known instances of 1,4-benzoxazinone production in higher plants, although production by microorganisms is fairly common (Tipton, et. al., 1967).

Biological Activity of Benzoxazinones

Activity against insects. Loomis (1957) investigated the resistance of corn to the European corn borer (Ostrinia nubilalis). At that time, it was established that different cultivars of corn had varying degrees of susceptibility to corn borer attack. Despite years of research aimed at developing a highly resistant cultivar, no one was able to clearly define the mechanism of resistance. Loomis succeeded in isolating and purifying a compound he termed "resistance factor A" (RFA). Incorporation of RFA in an artificial diet completely inhibited the growth of the corn borer larvae. Chemical analysis indicated that RFA was, in fact, MBOA. Virtanen felt that, because the derivative present

Figure 1: Chemical breakdown scheme proposed by Virtanen and Hietala (1960) for benzoxazinone compounds produced by cereal crops.


OH
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OH
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Rearrangement
H
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Figure 1

in intact tissue is the glucoside (GDIMBOA), it would be highly unlikely that the borer would be exposed to the benzoxazolinone. Based on the rapidity of the enzymatic hydrolysis of the glucoside upon injury to the tissue, it is more likely that the feeding insect would be exposed to the aglucone (DIMBOA). To differentiate between the possible derivatives, Klun and Brindley (1966) attempted to correlate the MBOA content of 11 inbred lines of corn with the observed resistance of those lines in the field to corn borer attack. They found that a linear relationship existed between MBOA recovered from 100 g of tissue and resistance to attack. The most highly resistant lines had approximately ten times as much MBOA in the tissue as the most highly susceptible lines. However, because MBOA is directly derived from DIMBOA, and ultimately from GDIMBOA, the amount of MBOA present is actually a measure of the DIMBOA or GDIMBOA initially present. Therefore, a strong correlation between MBOA concentrations and resistance can only be interpreted as a correlation between generic benzoxazinone concentrations and resistance. To attribute a greater level of importance to a single derivative, the differential toxicities must be evaluated. When the authors bioassayed MBOA against corn borer larvae, they found that it significantly decreased pupation of the larvae. They were unable to show any toxicity or increased mortality caused by MBOA, reinforcing the idea that the active compound was actually DIMBOA. Klun, Tipton, and Brindley (1967) investigated this possibility by examining the activity of purified DIMBOA in an artificial diet. They found an inhibition of pupation similar to that caused by MBOA. They also found that DIMBOA caused a 25% mortality rate.

Long and co-workers (1977) examined the role of DIMBOA in the resistance of corn to the corn leaf aphid (Rhopalosiphum maidis Fitch.). Incorporation of DIMBOA into the diet at a concentration of 0.5 mg/ 1.0 g of diet resulted in 20.8% mortality. These authors also showed an inverse linear relationship between the benzoxazinone concentration and the resistance to aphid infestation in the field. Subsequent analysis of the chemical basis for this resistance by Beck (1983) strengthens the idea that the dominant chemical factor in determining resistance is the benzoxazinone content of the plant. These authors examined the relationship between resistance and the concentrations of hydroxamic acids, total phenols, and orthodihydroxyphenol concentrations in corn tassels. They were unable to establish any significant correlation to resistance other than that involving hydroxamic acids.

Argandona (1980) compared the resistance of wheat, rye, and barley to infestation by the cereal aphid (Metopolophium dirhodum). Six days after applying aphids to plants, researchers recorded the extent of infestation and extracted and quantified the benzoxazinones. Barley, the most severely infested species, contained no detectable benzoxazinones. The three lines of wheat included in the study had intermediate levels of infestation. Rye exhibited the highest degree of resistance to infestation and the highest concentration of benzoxazinones. To confirm the role of DIMBOA in resistance to this aphid, the authors immersed freshly cut barley leaves in a solution of DIMBOA and allowed the leaves to take up the solution. The leaves were exposed to aphids. After five days, the aphids were counted to determine the extent of infestation, and the DIMBOA was quantified. Again, they found a very high coefficient of determination (0.94) between the quantity of DIMBOA present and the degree of aphid infestation. Additionally, as the plants aged, the concentration of benzoxazinones decreased, as did their resistance to infestation. When aphids were fed an artificial diet containing DIMBOA or MBOA, significant mortality resulted, but the concentration of MBOA required to kill 50% of the population (LD50) was 15 times the concentration of DIMBOA required for the same effect. Concentrations of 500 ppm deter the feeding activity of both European corn borer and African army worm (Spodoptera exempta) in leaf disk feeding bioassays (Kubo and Kamikawa, 1983).

Anti-microbial activity. Virtanen (1957) first isolated 2,3-benzoxazolinone from rye seedlings as an anti-Fusarium factor. His observations of the difference in resistance of rye varieties to infection by Fusarium nivale led to an investigation of the possibility that rye contained a chemical factor that conferred resistance to infection. Extraction and purification of the active substance yielded 2,3-benzoxazolinone. Bioassays of BOA against Fusarium nivale indicated that BOA was especially active in vitro, causing complete inhibition of fungal growth at concentrations above 500 ppm. Further bioassays showed BOA to inhibit Penicillium roquefortii and Sclerotinia trifoliorum.

El Naghy and Linko (1962) investigated the role of benzoxazinones in the resistance of wheat to stem rust and demonstrated an inverse linear relationship between the concentration of GDIMBOA and extent of injury. The most strongly resistant cultivars had high concentrations of GDIMBOA, while the least resistant cultivars had barely detectable levels of GDIMBOA. Hypersensitivity is a common characteristic of resistant cultivars. Upon infection, host cells in the immediate area of infection die rapidly, resulting in small areas of necrotic tissue. The rust pathogen (Puccinia graminis), being an obligate intracellular parasite, is unable to spread beyond the dead cells. Both the aglycone and the benzoxazolinone caused a high degree of phytotoxicity at the levels at which the purified compounds were inhibitory to the rust. Based on this, the authors proposed that the mechanism of resistance depends on this phytotoxicity. Upon infection by the pathogen, tissue damage results in the release of B-glucosidases that rapidly cleave the glycoside, releasing the phytotoxic aglycone. The aglycone causes the death of host cells at the infection loci, preventing the spread of the parasite. The aglycone also inhibits germination of the uredospores. Later work (Knott and Kumar, 1972) cast serious doubt on the dominance of GDIMBOA in resistance of wheat to stem rust. While these authors demonstrated a strong relationship between GDIMBOA concentration and resistance for extremely sensitive and extremely resistant varieties, the relationship did not hold at all for the varieties with intermediate resistance. Therefore, while it appears that the benzoxazinones are involved in resistance to stem rust, there are clearly other, possibly more important, mechanisms involved.

A stronger case exists for the involvement of benzoxazinones in the resistance of corn to Helminthosporium turcicum, the causal agent of northern corn leaf blight. Corn has two main chromosomal loci that are involved in resistance to H. turcicum. The Ht locus is a single dominant gene conferring resistance to infection. The Bx locus is also a single, dominant gene that determines production of benzoxazinones. Couture (1971) compared corn varieties with a bxbx, or benzoxazinone-deficient genotype to those with a BxBx, or benzoxazinone-normal genotype, with dominant and recessive Ht genotypes, to assess the relative importance of the two loci. In all cases, the benzoxazinone deficient genotype was the most susceptible to infection, regardless of the Ht genotype, indicating the importance of the benzoxazinones in this resistance. Bioassays of DIMBOA on germinating spores of *H. turcicum* indicate a very high level of fungitoxic activity. Concentrations above 6 ppm almost completely inhibited spore germination. Spores that germinated had shorter germ tubes than controls. These results indicate that DIMBOA has two main effects on *H. turcicum* infection. First, it inhibits germination of fungal spores, and secondly, it restricts the mycelial growth of any spores that do germinate. Long (1975, 1978) demonstrated a significant linear correlation between DIMBOA concentration and resistance to *H. turcicum*.

Detoxification of s-triazines. Tolerance of corn to the pre-emergence herbicide simazine (2-chloro-4,6-bis(ethylamino)-s-triazine) may be based, in part, on the nonenzymatic dechlorination of simazine to form 2-hydroxysimazine. Hamilton and Moreland (1961) used radiolabelling techniques to show this conversion in vivo, and recover hydroxysimazine from the plant tissue. They also demonstrated the mediation of this reaction in vitro by both GDIMBOA and DIMBOA. Monitoring the reaction by ultraviolet absorption showed that the hydroxamic acid was not degraded or altered in the course of the reaction, indicating that it plays a catalytic role in the process. Later work by Hamilton (1964) indicated that while the benzoxazinone-catalyzed degradation of s-triazines may by involved in resistance, it is not the dominant factor. Hamilton demonstrated a linear relationship between benzoxazinone content and ability of excised roots of several cereal crops to degrade simazine. Sorghum, however, contains no benzoxazinones, yet is more tolerant than wheat or rye. Palmer and Grogan (1964) compared two lines of corn that are essentially isogenic, except for a single recessive gene that rendered one sensitive to atrazine and simazine. Although both lines contained GDIMBOA, the resistant line contained nearly 2.5 times a much at each age tested. The sensitive line converted more atrazine per unit weight than the resistant line, suggesting that the resistance is based on a different mechanism than benzoxazinone-catalyzed dechlorination.

Phytotoxic Activity. The first report of phytotoxic activity by benzoxazinone derivatives was by Virtanen (1957), when he noted that 2,3-benzoxazolinone (BOA) inhibited the germination of oat seeds at a rate of 0.5 to 1.0 mg per seed. El Naghy (1962) also noted that BOA exhibited phytotoxic activity on wheat, as did the aglucone, DIBOA, in the hypersensitivity reaction to Puccinia graminis infection.

The most thorough investigation of the phytotoxic properties of the benzoxazinones produced by rye was conducted by Barnes, et al (1987a & b). The greatest phytotoxic activity contained in aqueous extracts of rye herbage was associated with two compounds later identified as DIBOA and BOA. Of the two compounds, DIBOA exhibited a higher degree of toxicity in petri dish bioassays. Inhibition of cress (Lepidium sativum L.) root elongation to 50% of control was achieved with DIBOA at a concentration of 0.37 mM, while a concentration of 1.05 mM was required for the same effect with BOA. Additional bioassays demonstrated the phytotoxic effects of DIBOA and BOA on a wide range of monocotyledons and dicotyledons, including tomato, barnyardgrass, proso millet (Panicum miliaceum L.), large crabgrass (Digitaria sanguinalis L. Scop.), redroot pigweed (Amaranthus retroflexus L.). In general, dicotyledonous species were approximately 30% more sensitive to these compounds than the monocotyledononous species tested.

Benzoxazinones are thought to cause phytotoxicity by inhibiting photophosphorylation in the chloroplast (Quierolo, et al, 1981). Barnes showed that DIBOA at 7.5×10^{-5} M and BOA at 1.0×10^{-3} M caused a 50% inhibition of chlorophyll production by the green alga Chlamydomonas rheinhardtii. This observation provides support for the idea that the site of action of the benzoxazinones is the chloroplast, specifically, chlorophyll production.

Although the benzoxazinone derivatives tested are highly phytotoxic to most plants tested, Barnes calculated that only about 12% of the potential toxicity of the aqueous extract was present in the benzoxazinone fraction (Barnes and Putnam, 1987), suggesting the presence of additional phytotoxins. Clearly, some of this additional activity is caused by cinnamic acid derivatives and volatile acids and aldehydes (Chou and Patrick, 1976). The toxicity of rye residues in the field appears to be the result of this combination of toxicants, but a detailed study of the toxic constituents of the leachate from rye residues has not been conducted. An additional source of toxins is from microbial metabolic activity on compounds in the leachate. The great diversity of soil microorganisms presents limitless possibilities for metabolism of rye-produced compounds, either attenuating or enhancing the total toxicity.

The objectives of this study were to assess the effects of microbial transformation on benzoxazinones produced by rye, to isolate and identify any such transformation products, and to determine their biological activity.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF PRODUCTS FROM MICROBIAL OXIDATION OF BENZOXAZINONES IN CEREAL CROPS

ABSTRACT

Compound 4, 2,2'-oxo-1,1'-azobenzene, was isolated and characterized from soil supplemented with 2,3-benzoxazolinone (BOA). A parallel experiment with 6-methoxy-2,3-benzoxazolinone (MBOA) yielded Compound 4, as well as it's monomethoxy- and dimethoxy- derivatives, Compounds 5, and 6, respectively. These compounds were produced only in the presence of soil microorganisms, via possible intermediates, I and II, which may dimerize, or react with the parent molecule to form the final products. In the case of MBOA, it was shown that the demethoxylation precedes the oxidation step. Although BOA and DIBOA were leached out of the rye residues, there were no detectable amounts of Compounds 4, 5, or 6 in the soil. When BOA was mixed with soil and rye residue, either under field conditions or *in vitro*, no 2,2'-oxo-1,1'-azobenzene was detected. Levels of free BOA in the soil were greatly reduced by incubation.

INTRODUCTION

Once an allelopathic system has been described in nature, it is desirable to elucidate the chemical processes involved. Fuerst and Putnam (1983) outlined four criteria, paralleling Koch's postulates, for proof of allelopathy. First, the adverse reaction of the recipient must be demonstrated and quantified, followed by isolation and purification of the chemical agent responsible for the inhibition and repeated demonstration of the inhibition using the pure compound(s). Finally, the compound should be recovered from the recipient's local environment. An investigation of the allelopathic activity of rye (_Secale_ _cereale_ L.) by Barnes, et. al. (1987) confirmed that rye is highly allelopathic and implicated 2,3-benzoxazolinone (BOA), and 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) as the primary allelochemicals produced by rye. Experiments to detect BOA and DIBOA in the soil were not conducted. Once in the soil system, the benzoxazinones would be susceptible to microbial transformation by various soil microbes. For the benzoxazinones to be involved in long term allelopathic activity, they must be sufficiently resistant to such microbial transformations. Alternatively, if the parent compounds are metabolized, it is concievable that biologically active metabolites may be involved in the overal process of allelopathy by rye residues. The chemistry of the benzoxazinones is well documented. Originally isolated from rye by Virtanen (1957), BOA was subsequently shown to be the third step in a chemical breakdown scheme initiated upon injury to the plant (Virtanen and Hietala, 1960). Hofman and Hofmanova (1969) showed that when rye foliage was carefully extracted, so that no enzyme activity was allowed to take place, only the glucosyl conjugate of DIBOA was isolated. Earlier work (Honkanen and Virtanen, 1961) had clearly established the reaction of DIBOA to BOA. BOA is the most logical compound to choose for studies on the fate of benzoxazinones in the soil since it is the most likely to be present in the soil. Patrick and Koch (1958) conducted a study of the toxic substances arising as a result of microbial decomposition of plant residues, including rye. They found that unless decomposition of the residues occurred, no toxic substances were present in the soil extract. If, however, decomposition occurred, the extract of the soil-residue mixture was extremely toxic to the respiration, oxygen uptake and growth of tobacco seedlings. They did not determine whether the toxins were of plant or microbial origin.

The objective of this work was to assess the impact of soil microbes on benzoxazinones, isolate and identify any bio-transformation products, and to monitor the levels of BOA and DIBOA in both residues and underlying soil over a period of time.

MATERIALS & METHODS

Experimental. Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) analyses were performed on a Varian XL-300 spectrometer, 300 MHz for proton and 75 MHz for carbon. Electron impact mass spectral (EI-MS) analysis was done on a Hewlett Packard model 5895 quadrupole mass spectrometer at 70 eV. Chemical ionization (methane) (CI-MS) and High Resolution (HRMS) mass spectra were generated on a

Jeol model HX-110 mass spectrometer. Ultraviolet (UV) absorption analyses were performed on a Gilford Response II ultraviolet spectrophotometer and infrared (IR) spectra, on a Perkin Elmer model 1170 FTIR spectrophotometer. Melting points were determined on a Thomas model 40 Micro Hot Stage apparatus and are uncorrected. HPLC analysis was performed on a Waters Radial Pak 8 mm ID x 10 cm, radially compressed C18 column (Waters Assoc., Div. of Millipore, Inc. Milford, MA), using KH_2PO_4 (0.01 M, pH 3.0)-acetonitrile (80:20 v/v). Unless specified, flash chromatography was performed on a silica gel column (Merck silica gel G, grade 60) and thin layer chromatography, on silica gel plates (Merck silica gel G F-254, 0.250 mm layer). 2,3-benzoxazolinone (BOA), Compound 1, was obtained commercially from the Aldrich Chemical Co., Milwaukee WI. Soil used was a Spinks loamy sand (Psammentric, hapludalf, sandy, mixed, mesic), collected from a field site at Michigan State University for use in the greenhouse, and stored in large bins under dry conditions for approximately one year. Soil was sterilized when necessary by autoclaving (1 hr on three successive days).

Spray reagents for TLC detection. DIBOA was detected on thin layer plates with a spray reagent consisting of 5% $FeCl_3$ in 95% ethanol, acidified with conc. HCl. A spray reagent consisting of 1% ceric sulfate in conc. H_2SO_4 was used to detect BOA and MBOA.

Isolation of 6-methoxy-2,3-benzoxazolinone (MBOA), Compound 2. Ten-day-old maize seedlings ('Pioneer 3737') were harvested and frozen at -20°C overnight (592 g). The thawed plant material was homogenized in a Waring blender with distilled water (1.4 L). This was kept at room temperature (1 hr) to ensure hydrolysis of the glucoside, 2-O-glucosyl-

7-methoxy-1,4-benzoxazin-3-one (GDIMBOA) to 7-methoxy-2,4-dihydroxy-1,4-benzoxazin-3-one (DIMBOA) and refluxed (2 hr), to cause the conversion of DIMBOA to MBOA. After cooling, the extract was strained through cheesecloth and the filtrate was acidified with conc. HCl (pH 1.0). The acidified extract was filtered through Whatman #4 and extracted with diethyl ether (5 x 300 ml). The ether fraction was washed once with distilled water (300 ml), dried over anhydrous $MgSO_4$ and the solvent was removed in vacuo. The crude extract thus obtained (720 mg) was initially purified by flash column chromatography ($CHCl_3$-MeOH 5:1) followed by TLC check with ceric sulfate detection. The fractions positive to ceric sulfate were pooled and the solvent was removed in vacuo. This partially purified fraction (451.0 mg) was further chromatographed by TLC ($CHCl_3$-MeOH 6:1). The ceric sulfate positive band was removed, eluted ($CHCl_3$-MeOH 2:1), and the solvent was removed by rotary evaporation. The colorless compound (129.8 mg) thus obtained was recrystallized from hexane-acetone (-20°C), yielding needle-like crystals (70.8 mg), Compound **2.** mp 144-148°C, UV (MeOH) 232.5 nm (9420), 290.0 nm (5050), IR (KBr) 3200, 1790, 1638, 1500, 1318, 1210, 1140, 1100, 1025, 970 cm^{-1}, ^1H-NMR (CD_3OD) d 3.56 (3H, s, OCH_3), 7.80 (1H, d, J = 8.2 Hz), 7.65 (1H, d, J=2 Hz), 7.58 (1H, d, J = 8.7 Hz), EI-MS, m/z: 165 (100, M^+), 150 (80).

Isolation of 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA), Compound 3. Seedlings of rye ('Wheeler', 25-days-old) were lyophilized at 15° C (101.5 g), homogenized in a Waring blender with distilled water (1 L) and kept at room temperature (1 hr) to ensure enzymatic hydrolysis of the glucoside 2-O-glucosyl-1,4-benzoxazin-3-one (GDIBOA) to 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). After filtering through cheesecloth, the filtrate was heated in a water bath until the temperature of the extract reached 70°C. It was then cooled immediately in an ice bath. Coagulated components were removed by vacuum filtration using Whatman # 1 filter paper. The filtrate was acidified with conc. HCl (pH 1.0) and extracted with diethyl ether (4 x 300 ml). The ether fraction was washed once with distilled water (300 ml), and the solvent was removed <u>in vacuo</u>. The crude extract thus obtained (355.8 mg) was initially purified by column chromatography ($CHCl_3$-MeOH 4:1), followed by TLC check with ferric sulfate detection. The fractions positive to ferric sulfate were pooled and the solvent was removed at reduced pressure. This partially purified fraction (187.2 mg) was further chromatographed by TLC ($CHCl_3$-MeOH 6:1). The ferric sulfate positive band was eluted ($CHCl_3$-MeOH 2:1) and removal of the solvent by rotary evaporation afforded a pale yellow residue (89.2 mg). This residue was titurated with diethyl ether and filtered through a sintered glass filter (fine). Recrystallization from ether-cyclohexane afforded colorless needles (22.4 mg) of Compound 3, mp 151-155°C, IR (KBr) 3400, 3200, 2380, 1670, 1590, 1280, 1220, 1040, 750 cm^{-1}, ^1H -NMR (CD_3OD) d 5.70 (1H, s), 7.35 (1H, dd, J = 1.0, 8.0 Hz), 7.10 (3H, m), HRMS, m/z: 181.0476 ($C_8H_7O_4N$, M^+)

Isolation of 2,2'-oxo-1,1'-azobenzene, Compound 4. Compound 1, 2,3-benzoxazolinone, (20 mg) was mixed thoroughly with soil (50 g) and the mixture was transferred to a 250 ml erlenmeyer flask. Distilled water (5.0 ml) was added to the above and incubated at 26°C (10 days). Soil for sterile controls was autoclaved. BOA and filter-sterilized (0.22-um membrane) water (5.0 ml) were added aseptically to the soil, and the system was incubated at 26°C (10 days).

The control and experimental soil as described above, were extracted with methanol (4 x 100 ml each). The methanol extract of the control soil was pale yellow, while the experimental extract was intensely orange. Both extracts were filtered, separately, through a sintered glass filter (fine) and the solvent was removed by rotary evaporation. A preliminary TLC analysis of the extracts indicated the presence of an orange band, only in the experimental soil. This orange compound (19.8 mg) was purified by TLC, ($CHCl_3$-MeOH-HCOOH 20:1:1). Repeated purification was carried out by TLC (toluene-EtOAc-NH_4OH 5:4:1) until the resulting dark red compound (1.8 mg) gave only one spot by TLC. Recrystallization from hexane-acetone (-20°C) yielded Compound 4 as orange-red needles, mp. 223-228°C (decomp.), UV (MeOH) 237 nm (E=28268), 432 nm (E=23711), IR (KBr pellet) 760, 1574, 1587 cm^{-1}, ^1H -NMR (DMSO) d 7.75 (2H, d, J = 8.4 Hz, H-6, H-6'), 7.55 (6H, m, H-3, -4, -5 and H-3', -4', -5'), ^{13}C -NMR (DMSO) d 99.87 (d, x2), 104.86 (d, x2), 117.25 (d, x2), 126.60 (d, x2), 129.19 (s, x2), 130.34 (s, x2), EI-MS, m/z: 212 (100, M^+), 185 (60), 184 (33); CI-MS, m/z: 212 (100, M^+), 185 (28) 184 (18); HRMS, m/z: 212.0588 ($C_{12}H_8N_2O_2$, M^+); FAB-MS (+) m/z: 213 (M^+ + H)

A similar experiment with compound 3, DIBOA, was carried out as in the case of compound 1 and the work up and purification yielded two compounds that by analysis proved to be BOA and Compound 4, as well as unreacted Compound 3.

Isolation of Compound 4 from Field Soil to Which BOA was Added. A commercial sample of Compound 1 (1.0 g) was mixed with the top 1/2" of soil in a small plot (4 ft^2) at the location from which soil used for <u>in vitro</u> conversion of BOA to Compound 4 was obtained. A soil sample

(390 g) was taken from the center of the plot (7 days) and extracted with methanol as above (105.5 mg). Removal of the solvent at reduced pressure gave an orange residue. Analysis of this extract by TLC confirmed the presence of Compound **4**. A control plot of the same size and location, but not supplemented with BOA was also extracted for comparison, and showed no benzoxazinones or diazoperoxides present.

Isolation of 4-methoxy-2,2'-oxo-1,1'-azobenzene Compound 5, and 4,4'-dimethoxy-2,2'-oxo-1,1'-azobenzene, Compound 6. The experiment with Compound **2** (MBOA) was carried out as in the case of compound 1 and work- up and purification yielded a single component. Recrystallization from hexane-acetone afforded Compound **5** as red-orange needles, mp 218 - 220°C (decomp.); UV (MeOH) 231 nm (E = 23591), 450 nm (E = 21122); IR: 2860, 2830, 1650, ^{1}H -NMR (DMSO) d 3.70 (3H, s, OMe), 7.74 (1H, d, J = 8.4 Hz), 7.64 (1H, d, J = 8.8 Hz), 7.52 (3H, m), 7.11 (1H, J = 2 Hz), 7.01 (1H, J = 6 Hz), EI-MS m/z: 242 (100, M^{+}); FAB-MS m/z: 243 (M^{+})

A small amount of 4,4'-dimethoxy-2,2'-oxo-1,1'-azobenzene, Compound **6**, was detected by both EI- and FAB-MS at 272 (M^{+}) and 273 (M^{+} + H), respectively.

An additional experiment was carried out in which a 1:1 mixture of Compound **1** and Compound **2** was added to soil and incubated as above. Work-up, purification and analysis by EI-MS gave compounds **4**, **5**, and **6**. EI-MS m/z: 212 (72%), 242 (26%), and 272 (1.5%).

Microbial Ecology of Transformation Reaction. Several attempts were made to isolate the organism(s) responsible for the biotransformation of BOA. Soil (1 g) was mixed with physiological saline (0.85% NaCl, 9 ml) and vortexed. Serial dilutions (1:10) were then plated on NZ-amine agar (NZ-amine A, 3g; agar 18 g, distilled water 1L). The plates were sealed in a polyethylene bag and incubated at 25°C. After 72 hr, individual colonies were removed and inoculated into screw-capped test tubes containing sterile soil (5 g), and BOA (2 mg). These were wet with sterile distilled water (0.5 ml) and incubated as above.

Leaching Experiments with Rye Residue. Rye seedlings ('Wheeler', 35-days-old) were harvested at the soil level (900 g), placed on a flat (25 cm x 50 cm) of soil and watered over the top at 72 hr intervals. Residue and underlying soil samples were taken at 0, 1, 2, 3, 6, 10, 17, and 27 days, respectively. Methanol extracts (4 x 100 ml) of the soil samples were stored at -20°C. The lyophilized residue samples were homogenized in a Waring blender with distilled water (300 ml), kept at room temperature (1 hour), and strained through cheesecloth. The pH of the extract was adjusted to 1.0 with conc. HCl. The acidified extract was filtered through Whatman #1 under vacuum and extracted with diethyl ether (4 x 100 ml). The solvent was removed in vacuo and the dried samples were stored at -20°C.

Several experiments were also conducted in which lyophilized rye residue (10 g) was cut into 2 cm pieces and mixed into dry soil (1 kg) with distilled water (100 ml). Three such systems were set up in 2 L beakers, each comprising a sample, that was extracted at 24, 72, and 240 hr respectively. Soil and residue were separated after drying at 45°C (1 hr). Methanol extraction of soil (3 x 500 ml) and removal of the solvent yielded dry samples that were stored at -20°C. The lyophilized residue was homogenized in a Waring blender with distilled water (200 ml), kept at room temperature (1 hr), strained through cheesecloth and acidified with conc. HCl (pH 1.0). The extracts were processed as above and stored at -20 °C. All samples were analyzed simultaneously. Preliminary analysis of the samples was carried out by TLC ($CHCl_3$-MeOH 5:1). DIBOA and BOA were detected with the ferric chloride and ceric sulfate spray reagents, respectively. Samples that did not contain BOA or DIBOA by TLC were not analyzed further. Samples containing BOA or DIBOA were partially purified by TLC (CHCl3-MeOH 5:1) and analyzed by HPLC. Both compounds were quantified respective to external standards by monitoring at 230 nm.

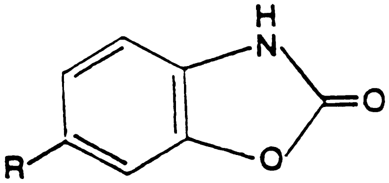
An additional experiment was conducted in which BOA (10 mg), rye tissue (5 g fresh), and distilled water (2.5 ml) were added to soil (25 g) and incubated at 26 °C (10 days). An identical system, lacking residues, was used as a control.

RESULTS AND DISCUSSION

Compound **4**, 2,2'-oxo-1,1'-azobenzene, isolated as a biotransformation product of 2,3-benzoxazolinone (BOA) (Figure 1) from non-sterile soil, gave the molecular formula of $C_{12}H_8N_2O_2$ by HRMS. the doublet at 7.75 ppm, integrated for 2 protons was assigned to the 6, and 6' hydrogens ortho to the nitrogen in the aromatic ring. The only other signal observed in the H NMR spectrum was a multiplet at 7.55 ppm, integrated for 6 protons. The ^{13}C -NMR spectrum of compound **4** gave only 6 signals for 12 carbon atoms, indicating a symmetrical structure for this molecule. The strong IR absorption at 760 cm^{-1}, indicative of an aromatic system with four adjacent hydrogens, was in full agreement with the NMR data and confirmed the proposed structure (Figure 2).

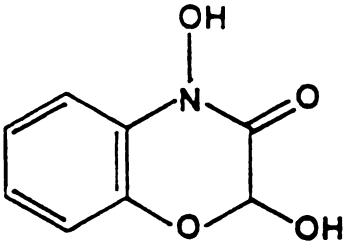
Incorporation of DIBOA, compound 3, into soil also resulted in the

Figure 1: Structure of parent benzoxazinones, compounds 1, 2, and 3.



R = H = Compound 1 = 2,3-Benzoxazolinone (BOA)

R = OMe = Compound 2 = 6-methoxy-2,3-benzoxazolinone (MBOA)

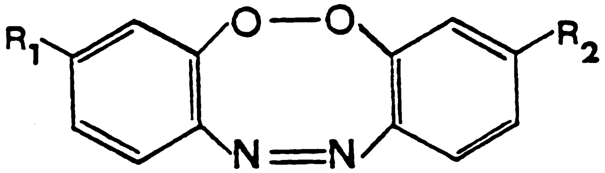


Compound 3

2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA)

Figure 1

Figure 2: Proposed structures of compounds **4**, **5**, and **6**, arising from microbial oxidation of 2,3-benzoxazolinone in the soil.



$R_1 = R_2 = H$ = Compound 4

$R_1 = H$, $R_2 = OMe$ = Compound 5

$R_1 = R_2 = OMe$ = Compound 6

Figure 2

formation of compound **4**. Addition of methoxylated BOA, compound **2**, gave compounds 5 and 6, characterized by spectral methods (Figure 2). It is interesting to note that the most abundant product of MBOA transformation is compound **5** (95%), with compound **6** present as a minor species, as evident by mass spectral data. Trace amounts of compound **4** were also present in the above mixture.

Formation of compound **4** in the soil could be the result of an intermediate (I, Figure 3), produced enzymatically from BOA, which in turn reacts with another molecule of BOA. Alternatively, this intermediate could undergo dimerization to afford compound **4**. In the case of MBOA, the major product was compound **5**, a monomethoxy analog of Compound **4**. This indicated that prior to the enzymatic oxidation leading to intermediate II (Figure 3), there is a demethoxylation step involved. That is, an enzymatic demethoxylation, followed by enzymatic oxidation would also yield intermediate I, which in turn would react with MBOA, to give compound **5**. The dimethoxy derivative, compound **6**, should result from the reaction of intermediate II with MBOA, or from dimerization of intermediate II. The experiment with equal amounts of BOA and MBOA gave compound **4** as the major product (72%), compound **5** (26%), and compound **6** in very small quantity (1.5%). Therefore, it is evident that intermediate II is less abundant than intermediate I, and that the demethoxylation occurs prior to the enzymatic oxidation. Based on these results, it is possible to extend the breakdown scheme for benzoxazinones proposed by Virtanen et. al. (1960) (Figure 4).

Addition of BOA or MBOA into sterile soil did not produce compounds **4**, **5**, or **6**, suggesting that these compounds are produced by soil microbes. A saline extract of the soil, to isolate the total microbial population was added to soil containing BOA, and resulted in

Figure 3: Proposed structures of intermediates I and II in the formation of compounds **4**, **5**, and **6**

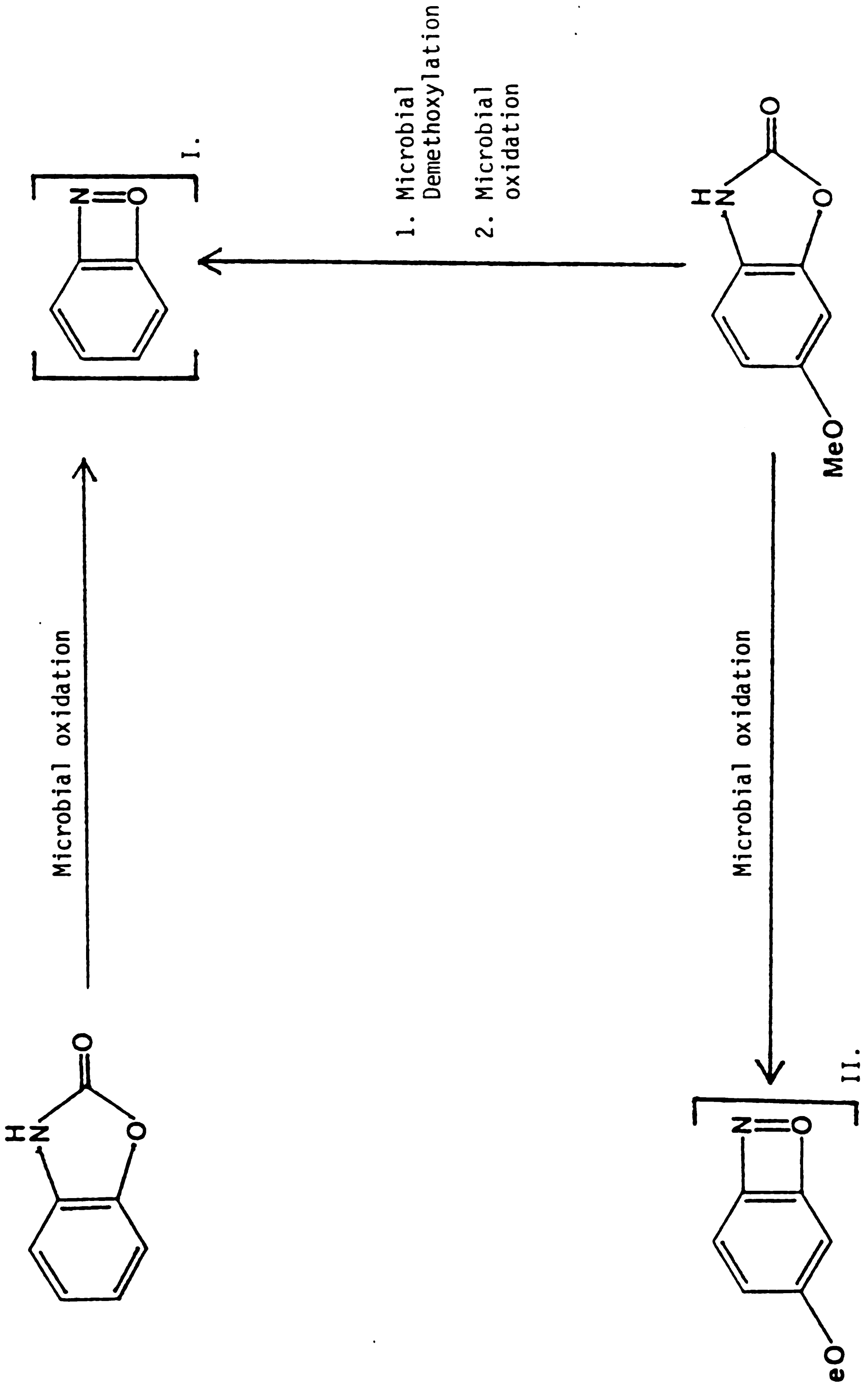
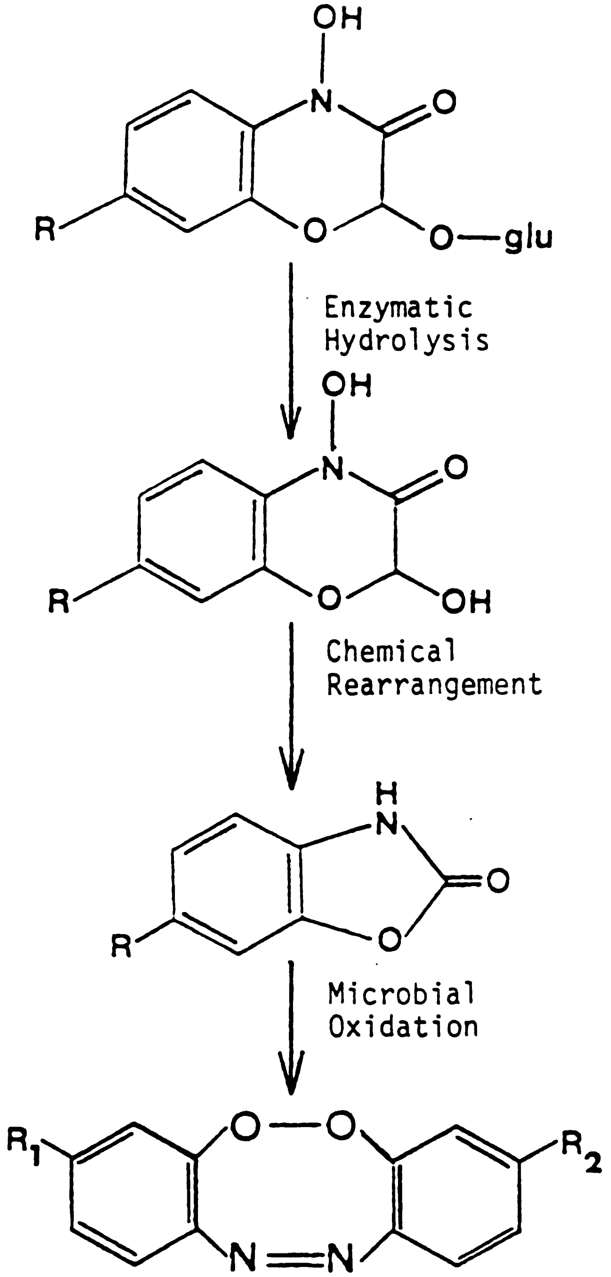

Microbial oxidation
I.
1. Microbial Demethoxylation
2. Microbial oxidation
MeO
Microbial oxidation
II.
H
N
O

Figure 3.

Figure 4: Chemical breakdown scheme of Virtanen amended to include Compounds **4**, **5**, and **6**.



R = H, OMe
$R_1 = R_2 = H$
$R_1 = H, R_2 = OMe$
$R_1 = R_2 = OMe$

Figure 4

production of compound 4, after incubation for 7 days.

Experiments to isolate the organism(s) responsible for the transformation were inconclusive. None of the colonies isolated were capable of transforming BOA. It appears as if the transformation reaction does not provide a selective advantage to those microbes capable of carrying it out. That is, the reaction is probably not an energy yielding reaction, but perhaps instead, is catalyzed by an extracellular oxidase enzyme. Production of compound 4 was also demonstrated in the field, using commercial BOA, indicating that these organisms are present in the environment. Although trace amounts of BOA and DIBOA were isolated from the soil in laboratory leaching experiments using rye residue, Compound 4 was apparently not in this soil. In order to verify this result, an in vitro experiment with rye residue and BOA was conducted and the products were quantified. Only trace amounts of compound 4 were observed by TLC from the soil containing BOA and fresh rye residue. It was also interesting to note that most of the added BOA in the residue sample (92%) had disappeared. The control sample containing no residue, produced the normal amount of compound 4 as mentioned earlier, yet contained approximately 9.7 times as much of the added BOA as did the residue sample. These results indicated that either some enzymatic inhibitors had leached from the rye residue into the soil, or that the leachate had killed the microbe(s) responsible for the conversion. Since the microbes responsible for the production of compounds 4, 5, and 6 have not yet been identified, it is difficult to argue that the leachate has killed these organisms. Chemical reaction between BOA and the leachate is a possible explanation for the diminished production of compound 4, as well as the disappearance of most of the added BOA. The above results were repeated in large scale experiments as well.

Our results suggest a role for soil microorganisms in the overall process of allelopathy by rye. The primary allelochemicals produced by rye, BOA and DIBOA, have been shown to be present in the soil by in vitro leaching experiments. Once in the soil, these compounds undergo microbial transformation, resulting in the diazoperoxides, Compounds **4**, **5**, and **6**. Compound **4** was not detected in field soil, or in the soil of in vitro experiments with rye residue. However, the biological activity of Compound **4** (Chapter 3) clearly demonstrates the potential role of these compounds in allelopathy by rye residues.

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CHAPTER 3

BIOLOGICAL ACTIVITY OF 2,2'-OXO-1,1'-AZOBENZENE, AN ALLELOCHEMICAL ARISING THROUGH MICROBIAL OXIDATION OF 2,3-BENZOXAZOLINONE (BOA)

ABSTRACT

Compound 4, 2,2'-oxo-1,1'-azobenzene, an allelochemical from rye, isolated previously and shown to be a product of microbial oxidation of 2,3-benzoxazolinone, was evaluated in terms of it's biological activity. The compound exhibited strong phytotoxic activity. Root elongation of tomato (*Lycopersicon esculentum* Mill.) was strongly inhibited (IC_{50} = 5-7 ug/ml), as were barnyardgrass (*Echinochloa crusgalli* L.), redroot pigweed (*Amaranthus retroflexus* L.), velvetleaf (*Abutilon theophrasti* L.), and garden cress (*Lepidium sativum* L.). Radicle elongation of rye (*Secale cereale* L.) was promoted 59% relative to control at 6.25 ug/1.5 ml. Proso millet (*Panicum milaceum* L.) radicle elongation was promoted 66% relative to control at 6.25 ug/1.5 ml, but was inhibited 90% at 200 ug/1.5 ml. In comparative assays using cress and barnyardgrass, Compound 4 was shown to be 7.5 times as toxic as BOA, and 10 times as toxic as DIBOA. Antifungal assays indicated no activity against *Aspergillus flavus*, *Rhizoctonia solani*, and *Fusarium oxysporum* f. sp. *asparagi*. Insecticidal activity was evaluated with mosquito larvae, *Aedes triseriatus* Say) and showed limited activity at 200 ppm. The compound showed strong nematicidal activity (*Panagrellus redivivus*) with 100% mortality after 24 hours at 10 ppm.

INTRODUCTION

Benzoxazinones are reportedly involved in several of the chemical defense systems of cereal crops. Originally isolated by Virtanen (1957) as chemical factors in the resistance of rye to Fusarium infection, these compounds were eventually implicated in the resistance of corn to Helminthosporium turcicum infection (Couture, 1971), and in the resistance of wheat to stem rust (El Naghy and Linko, 1962). In addition to antifungal activity, benzoxazinones were shown to be involved in the resistance of corn to attack by european corn borer (Loomis, 1957), corn leaf aphid (Long et. al., 1977) and cereal leaf beetle (Argandona, et. al., 1980). Recently, Barnes, et. al. (1987) demonstrated the allelopathic potential of benzoxazinones produced by rye, but they were unable to account for all of the potential toxicity with benzoxazinones alone. Apparently, these compounds did not account for all of the observed activity.

The wide range of biological activity, along with the inability of previous authors to account for all of the observed toxicity in terms of the benzoxazinones, led to the investigation of the possibility that the metabolites arising from microbial metabolism of the parent benzoxazinones might also lend activity. A stable diazoperoxide was isolated from soil in laboratory experiments on the microbial metabolism of benzoxazinones (Chapter 2). The obvious question is whether this compound, or related diazoperoxides, represent additional components to the above mechanisms of resistance to disease, insects and plant interference, or are merely inactive by-products. Production of biologically active microbial metabolites would establish a microbial component to the overall process of allelopathy by rye. The primary allelochemicals produced by the plant lead to additional allelochemicals derived from microbial metabolism of the primary compounds. The fact that these compounds arise through metabolism by microbes that dwell in the soil raises the question of their potential involvement in resistance to pests that dwell in the soil. These would include fungal and bacterial pathogens, insect pests, and parasitic nematodes, as well as competitive plants. In response to these questions, bioassays were conducted in the above areas, to ascertain herbicidal, antimicrobial, insecticidal, and nematicidal properties of the diazoperoxide.

MATERIALS AND METHODS

Herbicide Bioassays. Phytotoxic activity was evaluated in terms of seedling growth inhibition. Stock solutions (1 mg/ml methanol) of purified compounds were applied to Whatman #1 filter paper in plastic petri dishes (60-mm x 15-mm) to obtain doses ranging from 6.25 to 200 ug/dish. Pure methanol was also added to a control plate. The plates were left open to allow the solvent to evaporate completely (20 min). Indicator seeds (10, unless specified) were then placed on the filter paper and distilled water (1.5 ml) was added. The plates were wrapped with parafilm to prevent drying and incubated in the dark (26°C, for 72 hr, unless specified). After incubation, seedling root length was measured and compared to the control. The experiment was designed as a randomized complete block, with three replicates. Indicators included representative weeds barnyardgrass (*Echinochloa crusgalli* L. Beauv.), redroot pigweed (*Amaranthus retroflexus* L.), velvetleaf (*Abutilon theophrasti* Medic.), proso millet (*Panicum milaceum* L.), common purslane (*Portulaca oleracea* L.) and crops corn (*Zea mays* L. 'Pioneer 3784'), soybean (*Glycine max* L. 'Corsoy'), tomato (*Lycopersicon esculentum* Mill. 'Lafayette'), garden cress (*Lepidium sativum* L.), rye (*Secale cereale* L. 'Wheeler'), and cucumber (*Cucumis sativum* L. 'Calypso') .

Seed Treatments. Some weed seeds, because of poor germination, were scarified to increase their germination to levels that could be used in bioassays. Incubation at higher temperatures was also helpful in breaking dormancy. Redroot pigweed seeds were scarified in conc. H_2SO_4 for 3 min followed by a 30 min rinse in running distilled water. The bioassay was then performed with 20 seeds per dish incubated 96 hr at 35^{o}C. Velvetleaf seeds were scarified in conc. H_2SO_4 for 30 min followed by a 30 min rinse in running distilled water. Immediately prior to setting up the bioassay, seeds were surface sterilized with sodium hypochlorite (15% Clorox, 20 min). Velvetleaf seeds were incubated at 35^{o}C for 72 hr.

Fungal Bioassays. Fungal bioassays were performed on plates of YMG agar (yeast extract 4 g, malt extract 10 g, glucose 4 g, agar 20 g, distilled water 1 L). Indicator fungi were grown on plates of the same medium, or on potato dextrose agar (Difco). Bioassay plates were inoculated by cutting a small square out of the fungal lawn on the growth plate. This inoculum was placed in a sterile test tube with 5 ml of sterile physiological saline (0.85% NaCl in distilled water) and vo

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vortexed for 2 min. An aliquot of this suspension (1.0 ml) was added to a plate of YMG and spread with a flame-sterilized glass rod. After the liquid had been absorbed into the medium, sterile filter paper disks (6.35 mm dia, BBL) were placed on the surface of the medium. A standard solution of the compound (20 ug/ul DMSO) was added to the disk to obtain doses of 200 or 100 ug. Pure DMSO was added to another disk as a control. The plates were incubated in an inverted position at 26°C and were evaluated after 24 hr. Zones of inhibition around the filter paper disks were measured and compared to the control. Fungi tested were Aspergillus flavus, Rhizoctonia solani, and Fusarium oxysporum f. sp. asparagi.

Insecticidal Assays. Insecticidal activity was evaluated with mosquito larvae (Aedes triseriatus Say, "Walton" strain) reared in our laboratory. Eggs obtained from Dr. Richard Merritt, Michigan State University, Dept. of Entomology, were placed in distilled water that had been sparged with nitrogen gas (5 min) to stimulate larval emergence. After emergence, the larvae were fed desiccated liver powder for one week and the bioassay was conducted on 3-4 instar larvae. The bioassay was conducted in glass test tubes (10-mm x 75-mm). Five larvae were transferred to the tube with distilled water (1 ml). Ten ul of a stock solution of the purified compound (20 ug/ul DMSO) was added to the first tube to obtain a concentration of 200 ug/ul. Serial dilutions (1:10) afforded concentrations of 20, 2.0 0.2, and 0.02 ug/ml. Ten ul of pure DMSO was added to one tube as a control. All doses were evaluated in two replications. Larvae were observed for mortality after 0.25, 0.5, 1, 2, 4, 24, 48, and 72 hours, and percent mortality was calcuated for each dose, and analyzed as the mean of the replications for each treatment.

Nematicidal Activity. Nematicidal activity was evaluated using the free-living nematode Panagrellus redivivus. Tarjan (1955) evaluated several species of nematodes, both free-living and plant parasitic, and concluded that P. redivivus was representative of all those tested, and therefore was suitable for use in screening potential nematicides. Nematodes were reared in Dr. James Miller's laboratory, Michigan State University Dept. of Entomology, in 250 ml erlenmeyer flasks containing 5 ml of A-9 medium (Warren, et. al., 1955). An aliquot (0.25 ml) was removed from the nematode culture and added to fresh, sterile A-9 broth (4.75 ml). A portion of the diluted suspension (50 ul) was added to each well of a flat bottomed, 96-well tissue culture plate. This volume contained 30-45 nematodes. The purified compound was added in DMSO to obtain concentrations of 500, 100, 10, and 1.0 ug/ml, without exceeding 5% DMSO. These treatments were compared to a negative control (blank), a 5% DMSO control, and a positive control of 500 ug/ul valinomycin, a potent nematicide (Patterson and Wright, 1970). Treatments were observed with a dissecting microscope (375 X) after 4, 24, 48, and 96 hours. Percent mortality was calculated as the mean of three replications at each dose.

Statistical Methods. Data obtained in bioassays were analyzed by analysis of variance. Treatment means were compared on the basis of LSD, and also by non-orthogonal comparisons to the control mean, to determine level of significance.

RESULTS AND DISCUSSION

Herbicide Bioassays. Barnes, et. al. (1987) isolated 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and 2,3-benzoxazolinone (BOA) from rye herbage and demonstrated that they were the most active allelochemicals produced by rye, but was unable to account for all of the toxicity in terms of the benzoxazinones, indicating the presence of other inhibitory compounds. The microbial production of biologically active metabolites in the soil could provide a possible explanation for this observation. In order to determine the allelopathic potential of the metabolites arising from microbial oxidation of BOA and MBOA, they were bioassayed against a wide range of monocotyledonous and dicotyledonous crops and weeds. The metabolite from BOA, 2,2'-oxo-1,1'-azobenzene, was bioassayed more extensively than the methoxylated derivative from MBOA. Because BOA is an important breakdown product from rye and is available commercially in high purity, more bioassays were performed with that compound than with the methoxylated analog.

Barnes, et. al. (1987) found dicotyledonous species to be approximately 30% more sensitive to BOA and DIBOA than were monocotyledonous species. Therefore, an effort was made to assess the relative response of monocotyledons and dicotyledons to the metabolite arising from these compounds. Initial assays, conducted with garden cress and barnyardgrass as indicators, showed a high degree of toxicity to radicle elongation of both species (Figures 1 & 2). Having established that this compound is highly toxic to cress and

Figure 1: Response of garden cress seedling root elongation to increasing concentrations of compound **4** in laboratory petri dish germination bioassays. IC_{50} = 18 ug/ml.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05

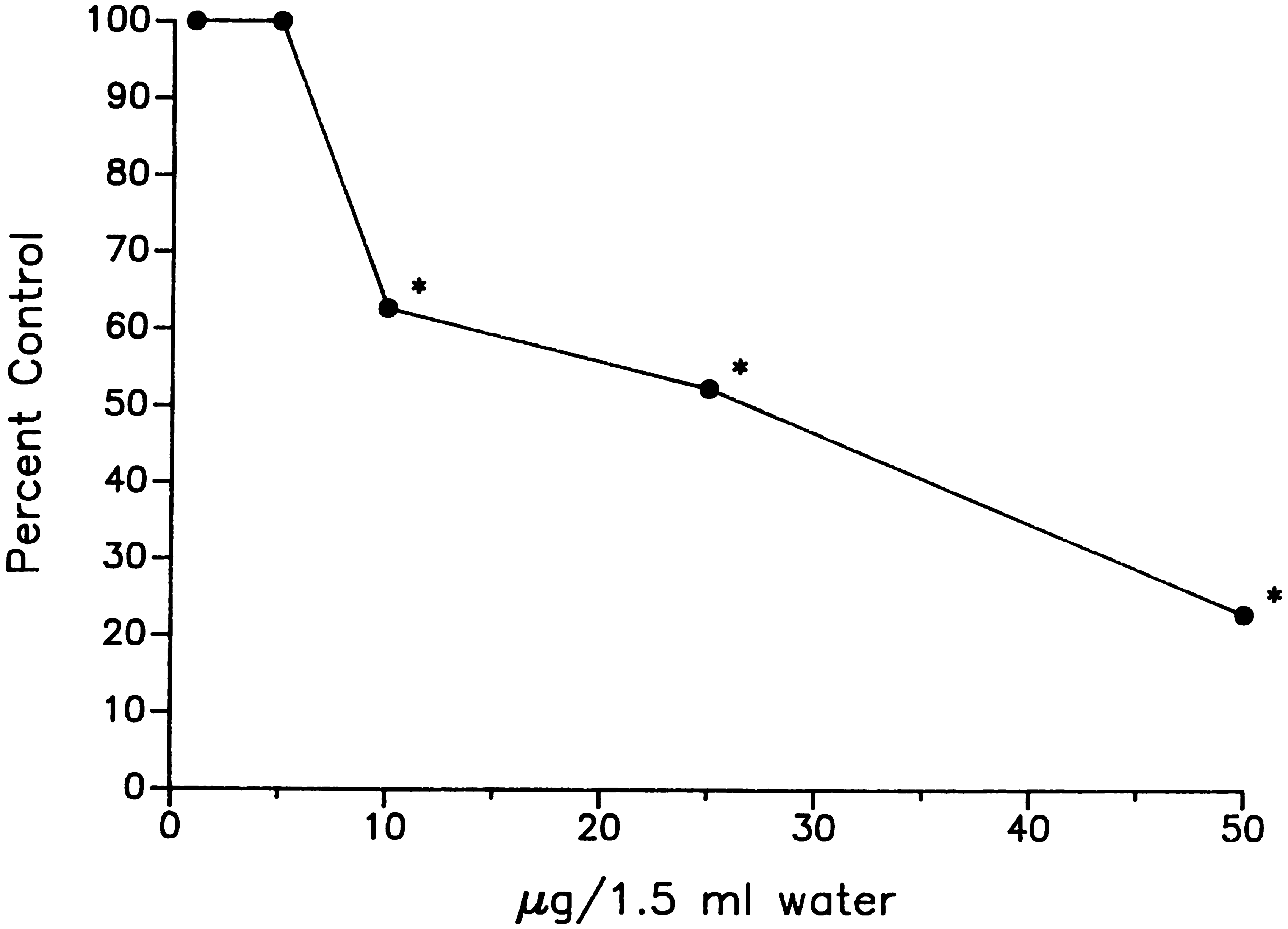
Percent Control
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
μg/1.5 ml water
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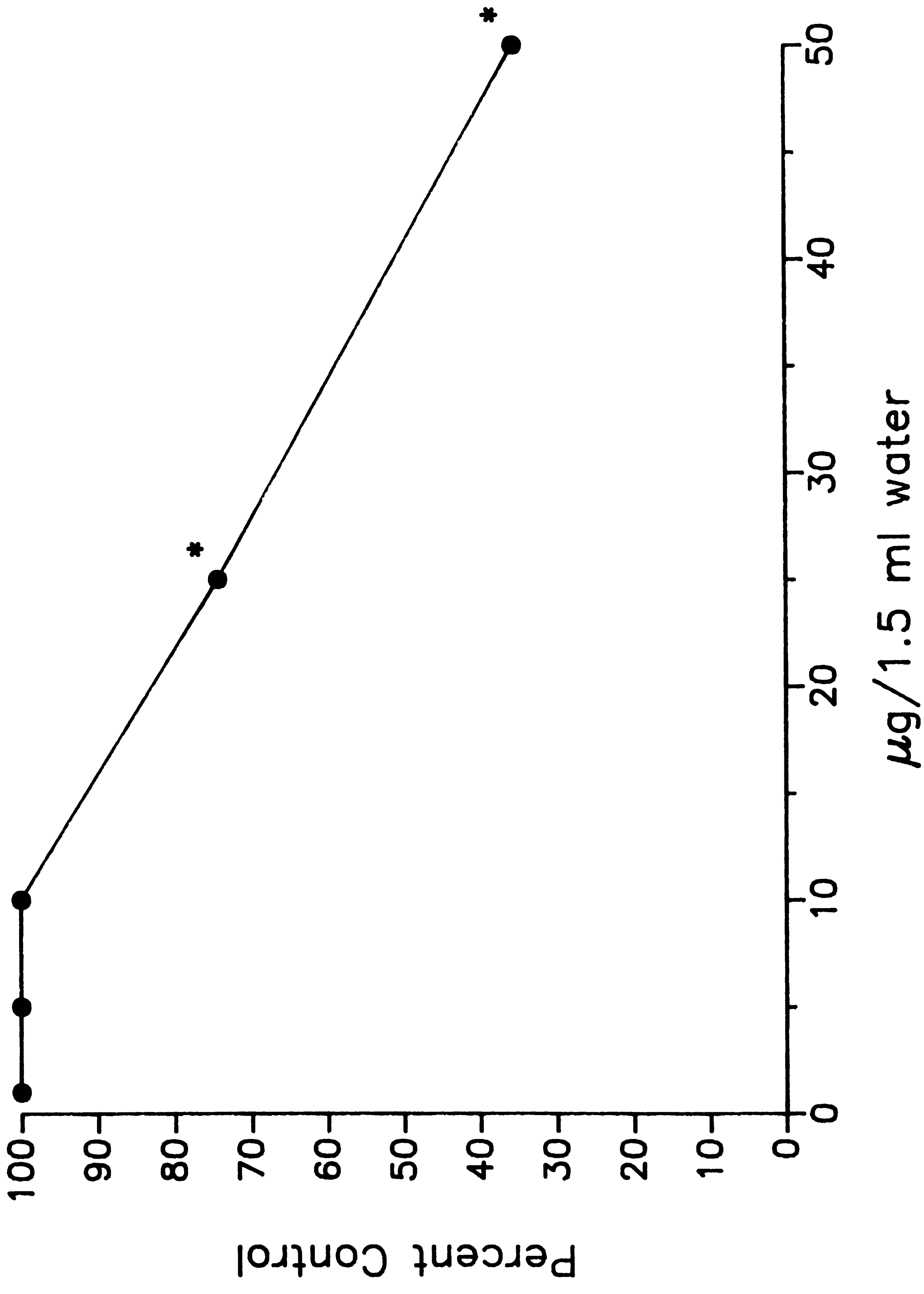
Figure 2: Response of barnyardgrass seedlings to increasing concentrations of compound 4 in petri dish bioassy, relative to untreated control. IC_{50} = 27 ug/ml.

** = Significantly different from control by LSD at 0.01

* = Significantly different from control by LSD at 0.05


Percent Control
100
90
80
70
60
50
40
30
20
10
0
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10
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40
50
µg/1.5 ml water
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barnyardgrass (IC_{50} = 25-35 ug/1.5 ml), they were next bioassayed against a wide range of crops and weeds, to determine the scope of activity and selectivity. In general, there were no broad differences observed between monocotyledons and dicotyledons. One striking difference is the response of rye and proso millet. These two monocotyledonous indicators were the only indicators tested that showed stimulation of radicle elongation. Rye growth, as measured by radicle elongation, was promoted at all doses tested, while proso millet radicle elongation was promoted at low doses and inhibited at high doses (Figures 3 & 4). Other monocots tested, such as barnyardgrass and corn, exhibited no positive response, indicating that this is not simply a difference between monocots and dicots. Possibly, in the case of rye, it is a response developed through natural selection. The presence of rye residues in the field might expose the seedlings to rye leachate. Constant exposure to this compound could select for those plants that are able to survive, and ultimately, those that are positively influenced. This suggests a complex ecological interaction between the soil microbes and the rye plants, in which leachate from dead rye residues selects for a microbial community that is beneficial to subsequent generations of rye. Although we were unable to demonstrate the presence of the metabolite in the soil from greenhouse leaching experiments, different environmental factors may be involved in the field. In general, the differences in the bioassays appear to be between crops and weeds, with the large-seeded crops such as corn and soybean being unaffected, while all weeds tested, except common purslane, were highly sensitive to 2,2'-oxo-1,1'-azobenzene. Dose-response assays allowed the calculation of IC_{50} values for each sensitive indicator. Among the crop species tested, corn, cucumber,

Figure 3: Promotion of rye seedling root elongation by Compound 4 in petri dish germination bioassay relative to untreated control.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05

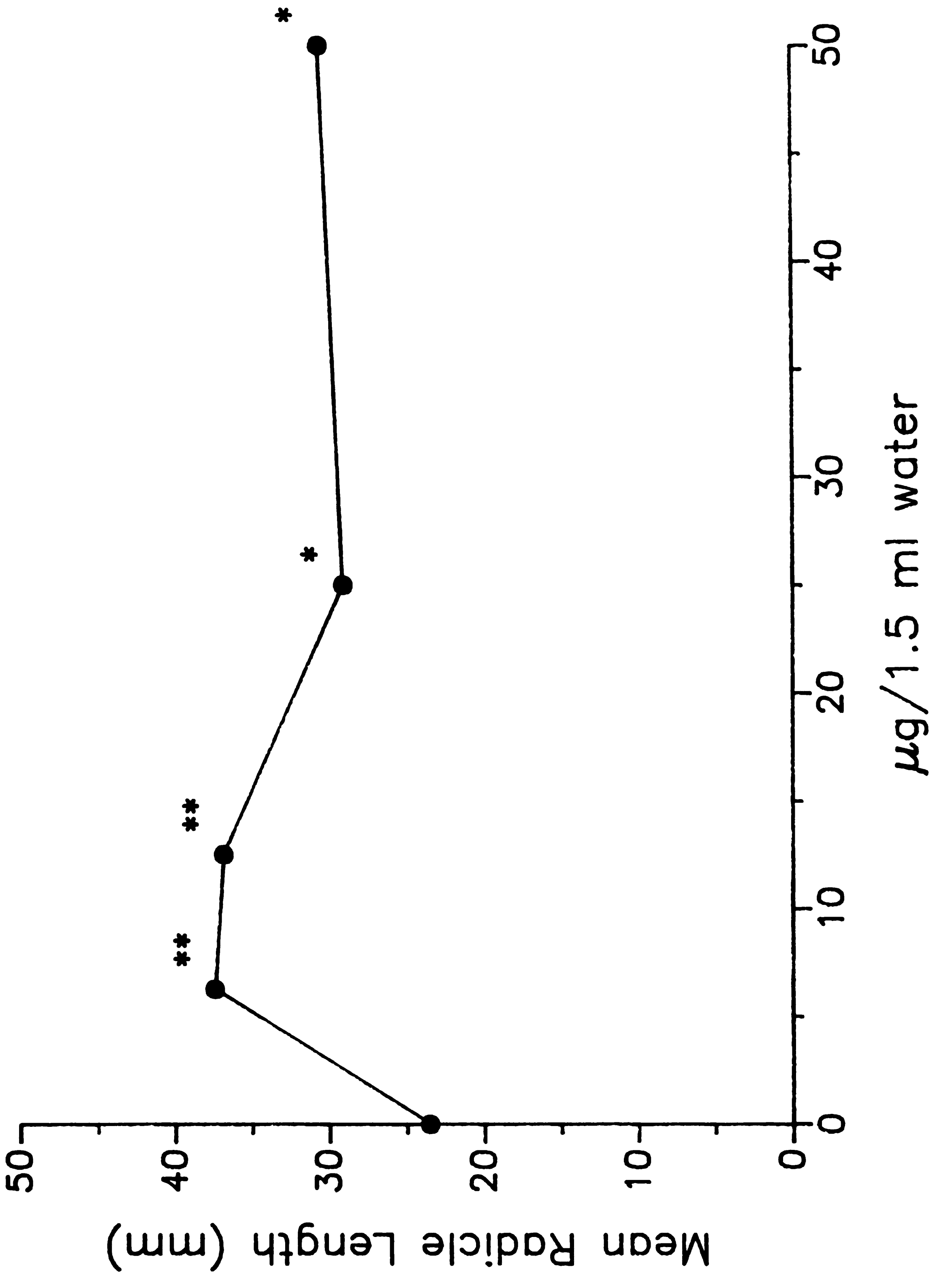
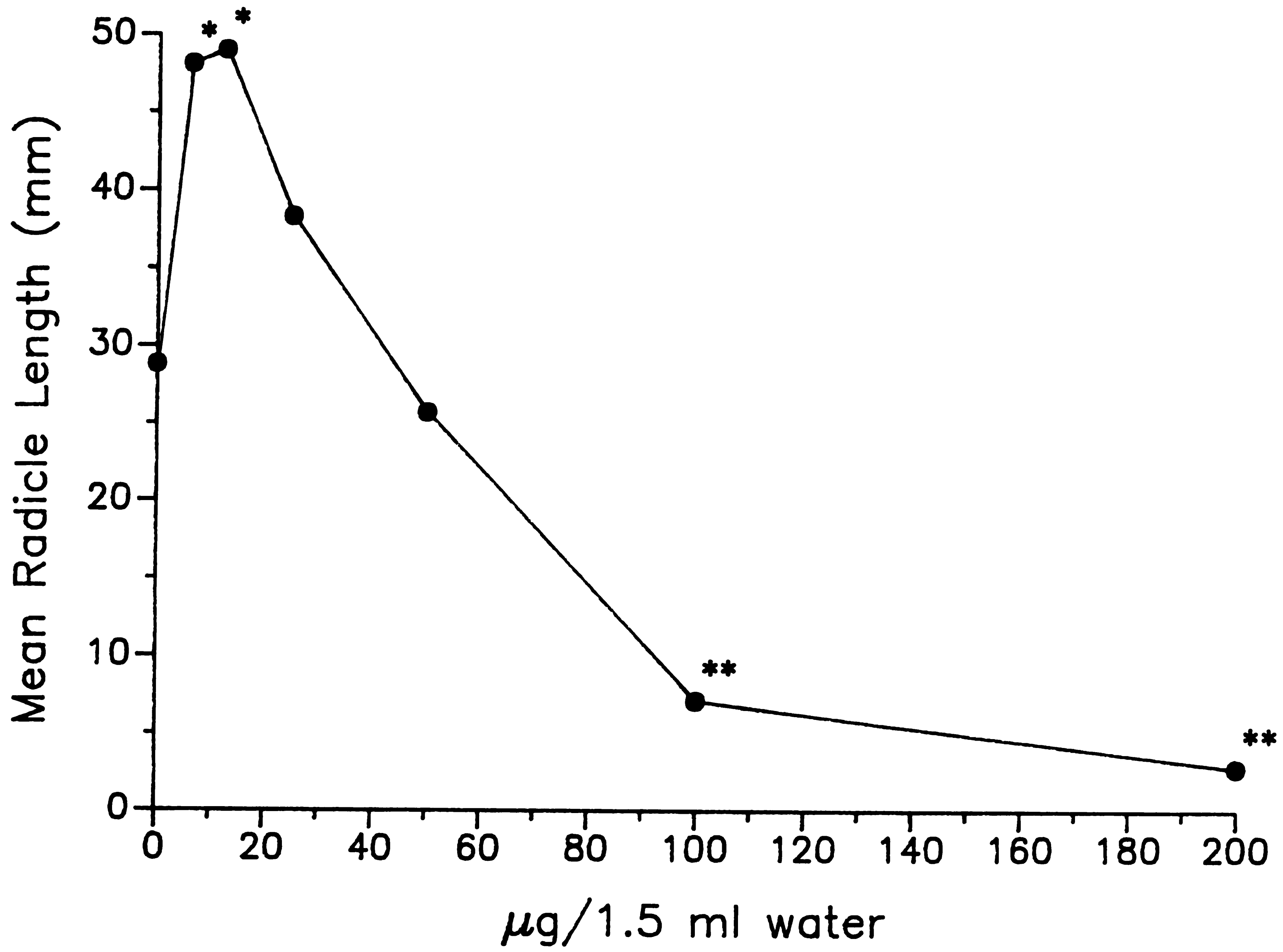

Mean Radicle Length (mm)
0
10
20
30
40
50
μg/1.5 ml water
0
10
20
30
40
50
**
**
*
*

Figure 4: Response of proso millet seedling root elongation to Compound 4 in petri dish germination bioassays relative to untreated control.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05


Mean Radicle Length (mm)
0
10
20
30
40
50
*
*
**
**
0
20
40
60
80
100
120
140
160
180
200
μg/1.5 ml water

and soybean were unaffected, while rye radicle elongation was promoted. The only sensitive crop tested, tomato, was also more sensitive than any of the weeds tested with an IC_{50} value of approximately 5-7 ug/ml (Figure 5). At higher doses, the root apex appeared blackened and necrotic. This might explain why rye residues have been reported to inhibit the growth of tomato seedlings in the field (Putnam, et. al., 1983). Bioassays using weeds as indicators suggest a high degree of non-specific activity, both against monocots and dicots. The dicots that were sensitive to 2,2'-oxo-1,1'-azobenzene included redroot pigweed, IC_{50} = 16 ug/ml (Figure 6), and velvetleaf, IC_{50} = 48 ug/ml, (Figure 7). Common purslane was not significantly affected by any dose tested. Proso millet was significantly promoted at the doses of 6.25 and 12.5, but unaffected at 25 or 50, and significantly inhibited at 100 and 200 ug/ 1.5 ml.

Cress and barnyardgrass were used to compare the toxicity of Compound 4 to that of the parent compounds BOA and DIBOA. Fifty percent inhibition of cress radicle elongation was achieved with BOA at a concentration of 200 ug/ml, and with DIBOA at a concentration of 133 ug/ml. In contrast, 18 ug/ml of Compound 4 caused the same level of inhibition (Figure 8). This corresponds to an increase in activity by a factor of 11 for BOA and 7.5 for DIBOA. Shoot elongation of cress was inhibited to 50% of control at the same concentrations. The barnyardgrass root elongation IC_{50}'s for BOA and DIBOA were both greater than 250 ug/ml, while 27 ug/ml 2,2'-oxo-1,1'-azobenzene caused 50% inhibition relative to control (Figure 9), a 10-fold increase in activity. Interestingly, the compound had no effect on barnyardgrass shoot elongation at any doses tested. For this reason, all subsequent comparisons of activity against different indicators were based on root

Figure 5: Inhibition of tomato seedling root growth by Compound 4 in petri dish germination bioassays relative to untreated control. IC_{50} = 5-7 ug/ml.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05

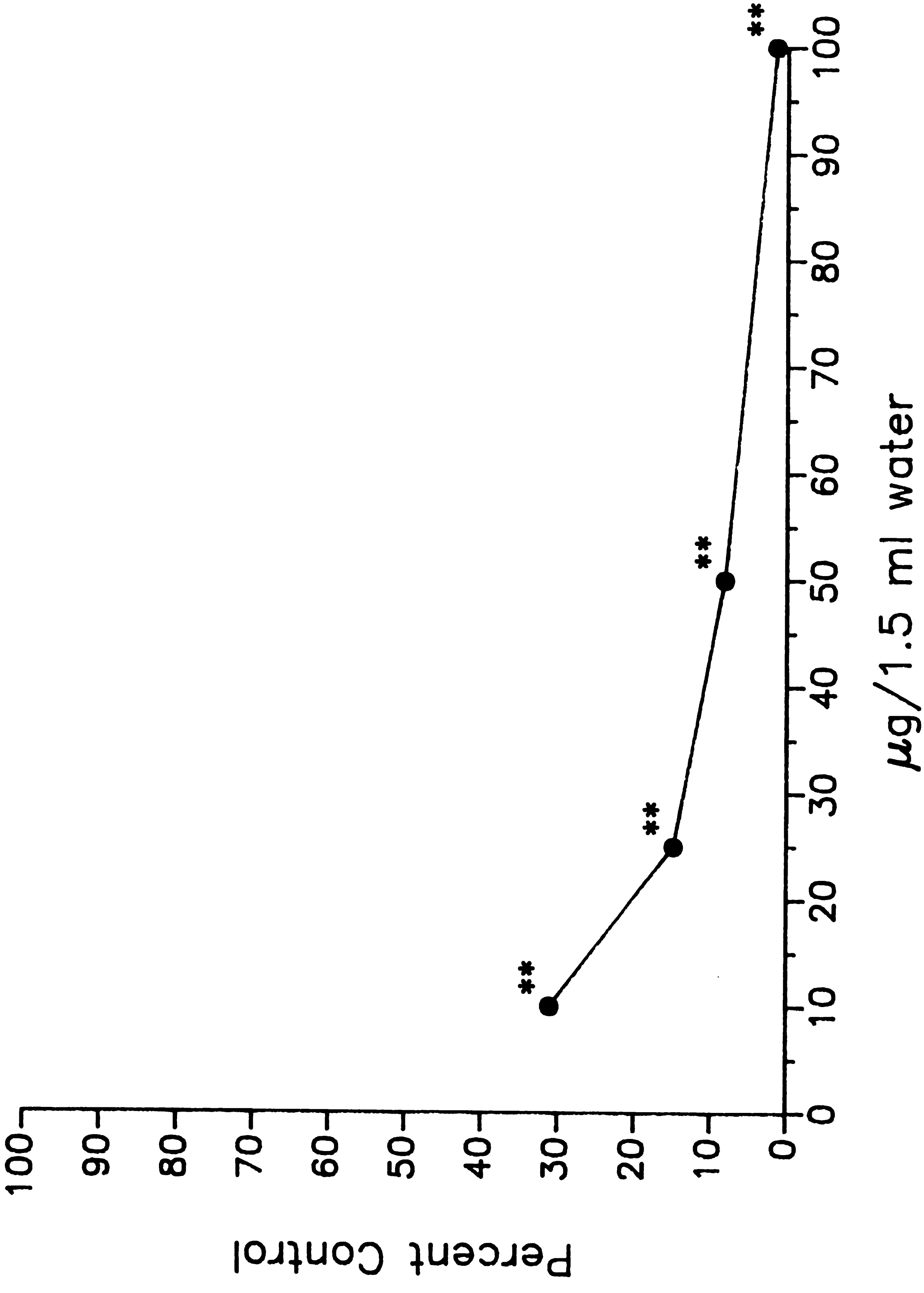
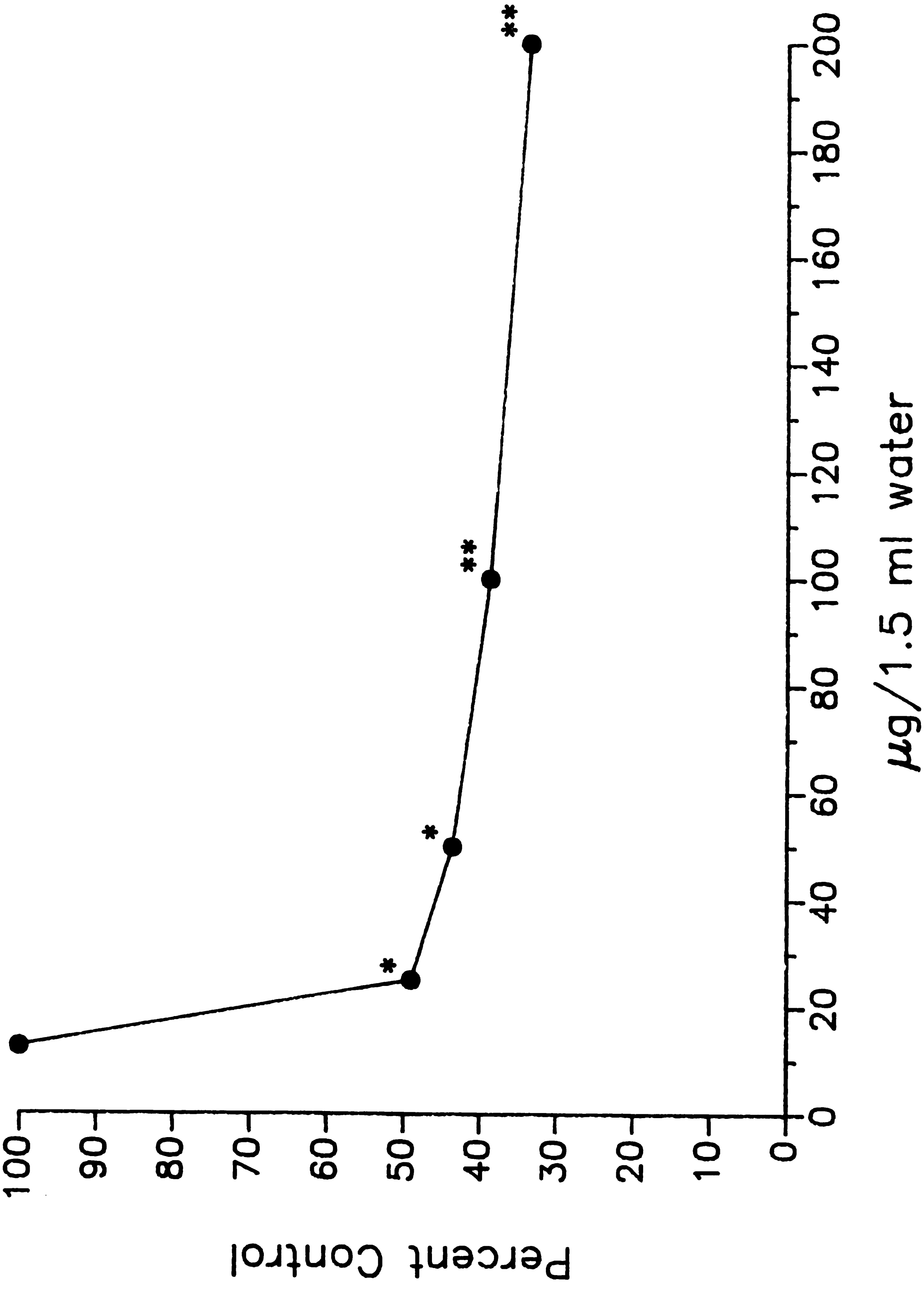

Percent Control
0
10
20
30
40
50
60
70
80
90
100
μg/1.5 ml water
0
10
20
30
40
50
60
70
80
90
100
**
**
**
**

Figure 6: Inhibition of redroot pigweed seedling root elongation by compound 4 in petri dish germination bioassays relative to untreated control. IC_{50} = 16 ug/ml.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05


Percent Control
100
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80
70
60
50
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30
20
10
0
0
20
40
60
80
100
120
140
160
180
200
μg/1.5 ml water
*
*
**
**

Figure 7: Inhibition of velvetleaf seedling root elongation by compound 4 in petri dish germination bioassays relative to untreated control. IC_{50} = 48 ug/ml.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05

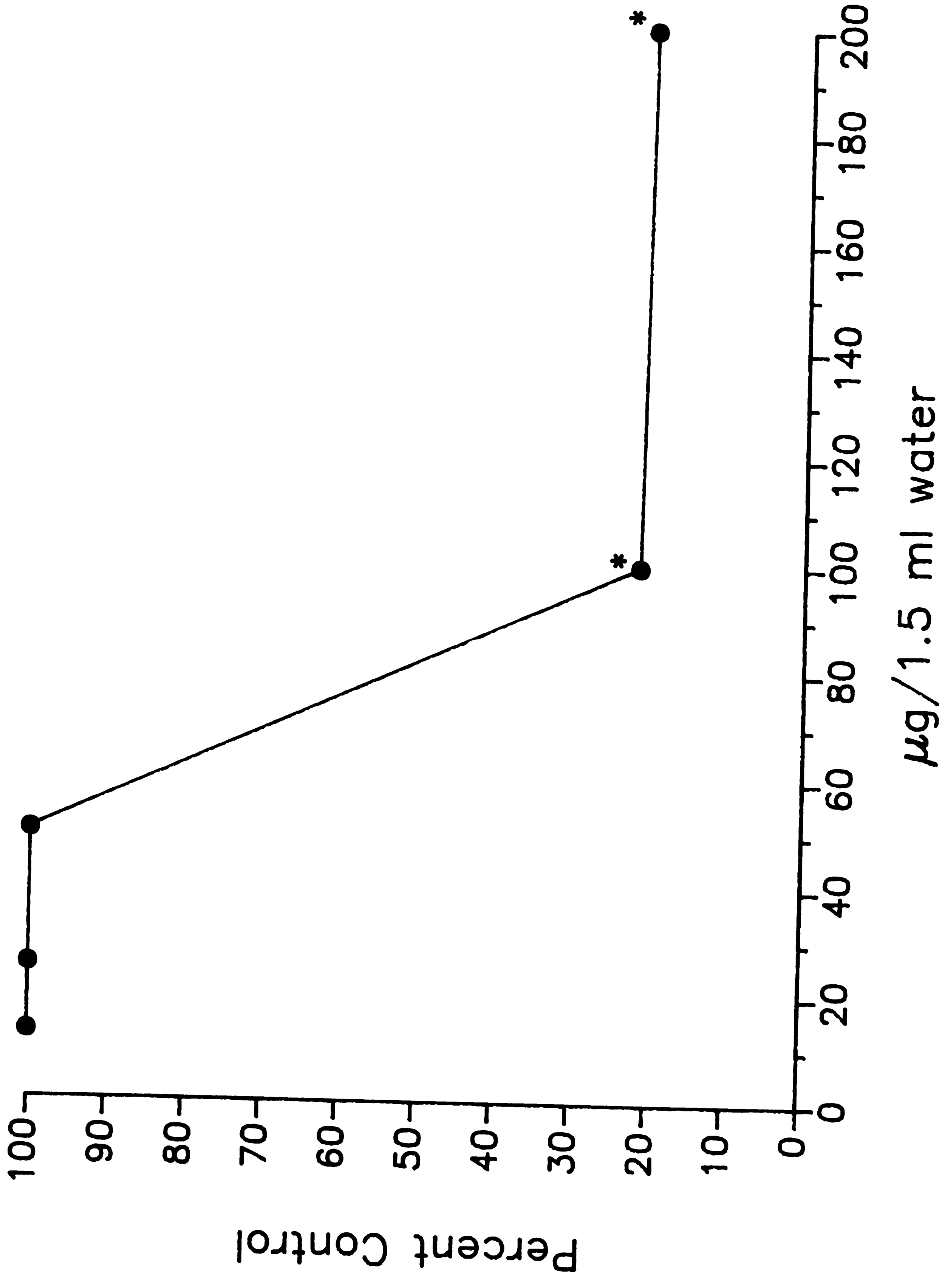

Percent Control
0
10
20
30
40
50
60
70
80
90
100
µg/1.5 ml water
0
20
40
60
80
100
120
140
160
180
200
*
*

Figure 8: Relative toxicities of Compound 4 and the parent compounds BOA and DIBOA in petri dish germination bioassays against garden cress relative to untreated control. IC_{50}: BOA = 200 ug/ml, DIBOA = 133 ug/ml, Compound 4 = 18 ug/ml.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05

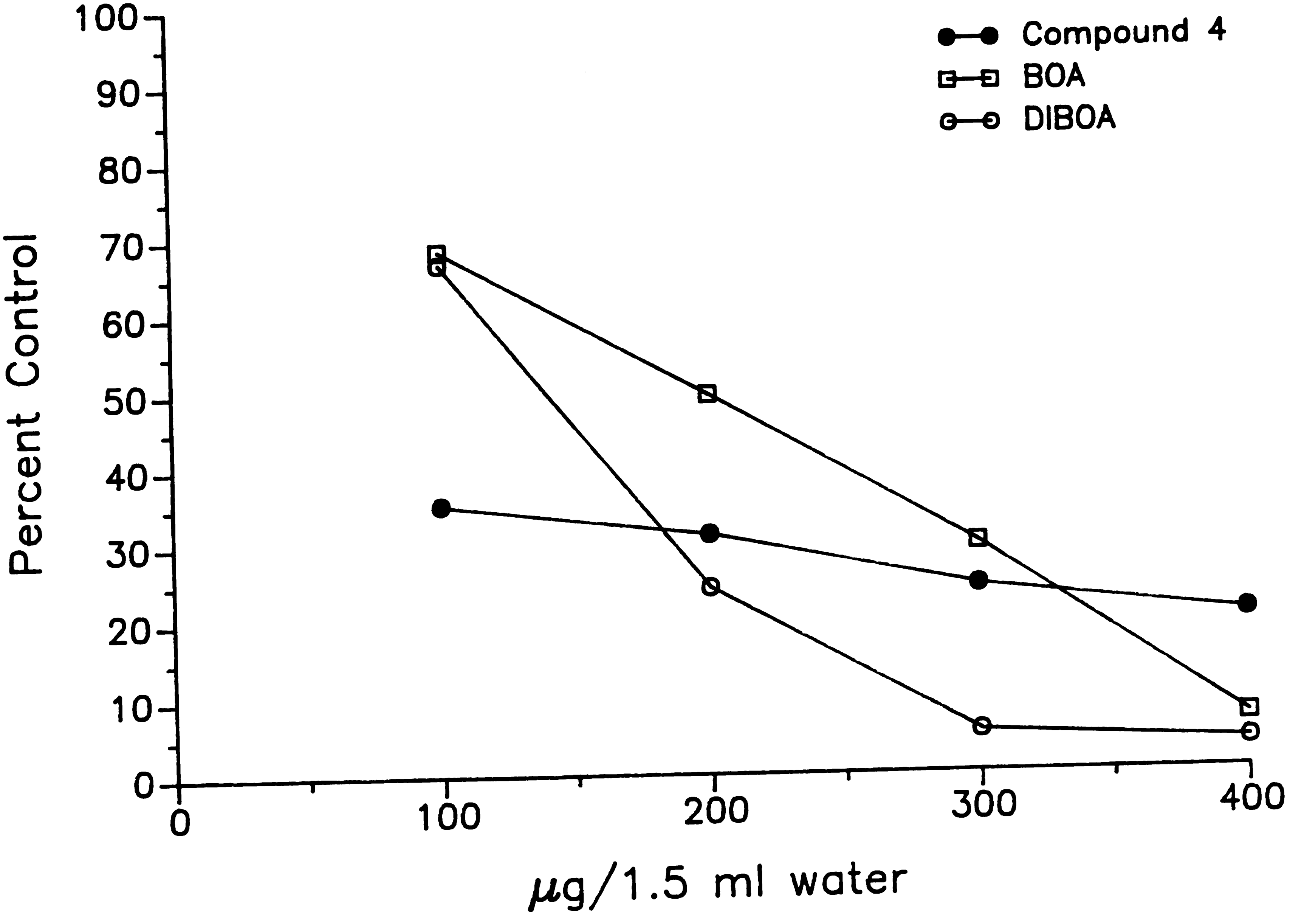
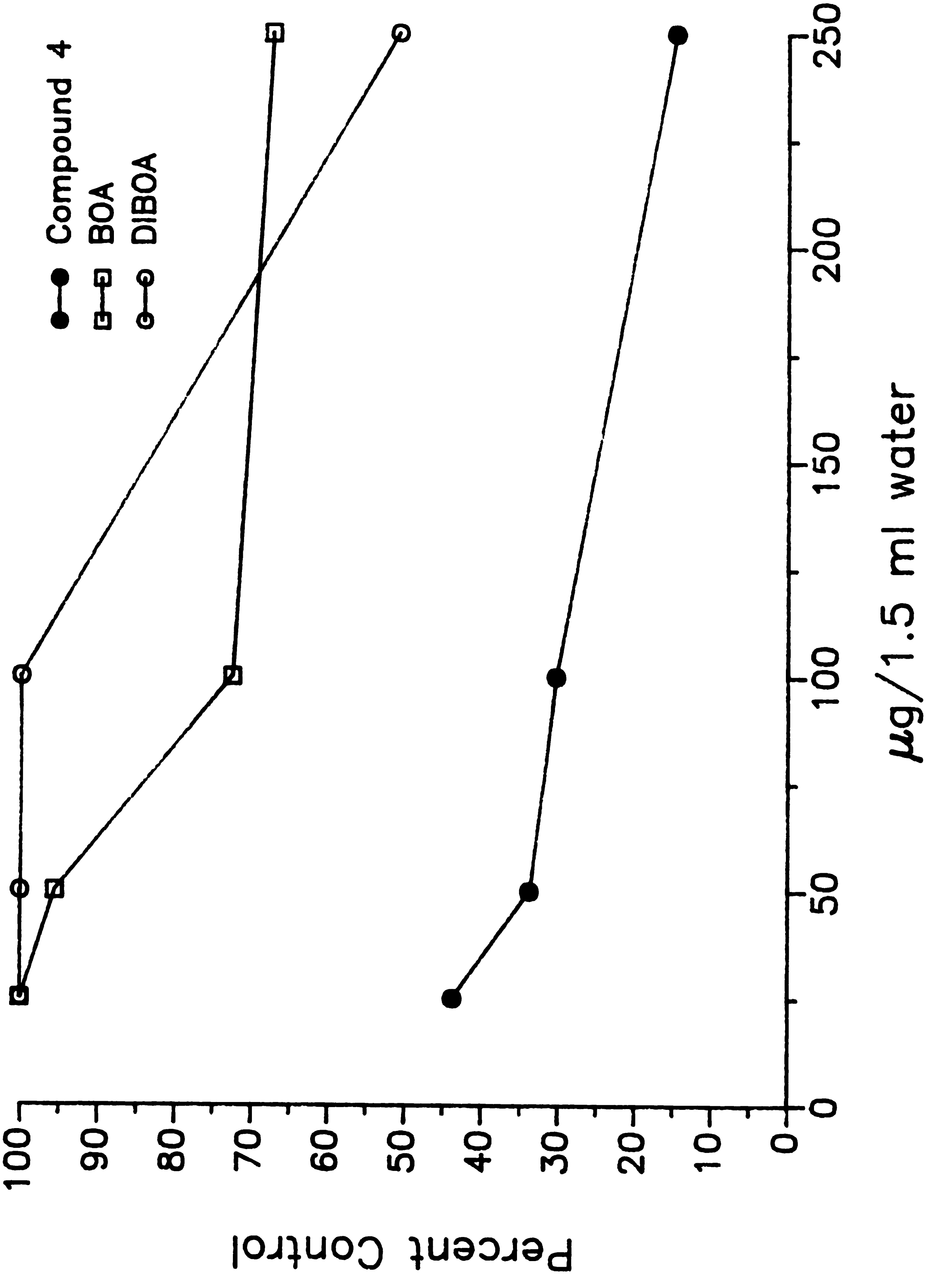

Compound 4
BOA
DIBOA
Percent Control
0
10
20
30
40
50
60
70
80
90
100
0
100
200
300
400
μg/1.5 ml water

Figure 9: Relative toxicities of compound 4 and the parent compounds BOA and DIBOA against barnyardgrass root elongation in petri dish germination bioassays. IC_{50}: BOA and DIBOA 250 ug/ml, compound 4 = 27 ug/ml.
** = Significantly different from control by LSD at 0.01
* = Significantly different from control by LSD at 0.05


Compound 4
BOA
DIBOA
Percent Control
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
μg/1.5 ml water

elongation. The observation that the metabolite is much more phytotoxic than the parent compounds is important, because of the inability of previous studies to account for all of the potential toxicity of rye residues in terms of the benzoxazinones. Metabolism of the parent compounds to highly active diazoperoxides may explain this observation. Although the compounds may be produced slowly and in small amounts, their greater toxicity allows them to be considered in the overall process of allelopathy by rye residues.

Anti-fungal Bioassays. Benzoxazinones have been reported to have a wide spectrum of anti-fungal activity. Virtanen (1957) first isolated BOA based on it's activity against *Fusarium nivale*, an important pathogen of rye, and subsequently established that it was also toxic to *Penicillium roquefortii*, and *Sclerotinia trifoliorum*. El Naghy and Linko (1962) demonstrated that the glucoside of DIMBOA was involved in the resistance of wheat to stem rust (*Puccinia graminis*). Couture, et. al. (1971), implicated DIMBOA and MBOA in resistance of corn to infection by *Helminthosporium turcicum*, the causal agent of northern corn leaf blight. This activity of the parent compounds leads to the question of whether or not the metabolites possess a similar type of activity. To assess the antifungal activity of Compound **4**, it was bioassayed against three common fungi: *Aspergillus Flavus*, *Fusarium oxysporum* f. sp. *asparagi*, and *Rhizoctonia solani*. The filter paper disk method somewhat restricts diffusion of the compound. Therefore, close to the disk, spores are exposed to a very high concentration of the compound. No inhibition was observed, however for any of the fungi tested. Based on the slow rate of conversion observed in flasks in the laboratory, concentrations present in the field could be expected to be much lower than those tested.

Insecticidal Assays. Loomis (1957) isolated a compound from corn that he termed "resistance factor A" (RFA), based on it's activity against European corn borer larvae. Chemical analysis indicated that RFA was actually MBOA. Later work by other authors (Klun and Brindley, 1966; Klun et. al. 1967) further established the involvement of both MBOA and DIMBOA in the resistance of corn to attack by borers. Argandona et. al. (1980) and Beck et. al. (1983) demonstrated the role of MBOA and DIMBOA in resistance of corn to the corn leaf aphid. Based on these observations of insecticidal activity by benzoxazinones, the potential for insecticidal activity by Compound **4** was investigated using mosquito larvae as indicators. Mosquito larvae are sensitive indicators of broad spectrum insecticidal activity. A comparison of several agricultural insect pests, using commercial insecticides, indicated that mosquito larvae are representative and sensitive enough to be used in a screening program to detect insecticidal compounds (Mishra et. al., 1987). In bioassays of Compound **4**, larval mortality was observed at 200 ppm in less than 4 hours. At a dose of 20 ppm, while no mortality was observed, larval motility was reduced.

Nematicidal Assays. The greatest potential ecological impact of toxic metabolites from benzoxazinones would be on organisms that live in the soil, since they would be directly exposed. Soil dwelling insects and nematodes are examples of pests that would be affected. In bioassays using Panagrellus redivivus as an indicator, 2,2'-oxo-1,1'-azobenzene was shown to have a high degree of toxicity to nematodes. At 10 ppm, 100% mortality was observed after 24 hours. No effects were observed at the next lowest dose, 1 ppm, indicating that the LD_{50} is within this range. This rate is on the order of valinomycin and other potent nematicides, indicating that nematodes are extremely sensitive to Compound 4. As with the phytotoxic properties of Compound 4, this high rate of toxicity toward nematodes is important in view of the potentially low doses attainable in the environment through natural processes.

Conclusions. This work implies a microbial role in the overall process of allelopathy by rye. Specifically, it appears that the potential toxicity of the allelochemicals produced by rye is increased by microbial metabolism to form new compounds that have greater toxicity than the parent compounds. The transformation product of BOA exhibited strong phytotoxic, insecticidal and nematicidal activity. Fungal bioassays indicated no activity against those fungi tested. Bacterial toxicity of this compound has yet to be investigated. The potent toxicity of Compound 4 towards plants and nematodes, shown in *in vitro* assays indicates that these compounds might be active in the soil. While weed control benefits from a rye cover crop are known, nematicidal properties have not previously been reported. Additional work needs to be done to determine if an ecological function exists for these compounds. An interesting possibility is that compounds from rye leachates, possibly BOA, exert selective pressures that favor the growth of the microbe or microbes that are responsible for the metabolism of benzoxazinones. The antimicrobial activity of BOA suggests that the bio-transformation organism must be resistant to BOA. This fact alone seems to suggest that there is some selective pressure exerted in the soil when BOA is present. This selection mediated by rye leachate may have an ecological function, since the biotransformation product, 2,2'-oxo-1,1'-azobenzene, was shown to promote the growth of rye, when measured by radicle elongation.

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