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RESPONSE OF CUCUMBERS (Cucumis sativus L.), SNAP BEANS (Phaseolus vulgaris L.), and WEEDS TO COVER CROP RESIDUES AND RYE (Secale cereale L.) ALLELOCHEMICALS

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William Robert Chase

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William Robert Chase

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ABSTRACT

RESPONSE OF CUCUMBERS (*Cucumis sativa* L.), SNAP BEANS (*Phaseolus vulgaris* L.), AND WEEDS TO COVER CROP RESIDUES AND RYE (*Secale cereale* L.) ALLELOCHEMICALS

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Experiments were conducted in 1988 and 1989 to determine the effectiveness of spring planted oats (*Avena sativa* L.), spring barley (*Hordeum vulgare* L.), and rye for weed control in cucumbers and snap beans. Although rye produced 20 to 64% less biomass than oats or barley, its residues provided superior weed suppression (up to 79%) compared to no residues. Cucumber and snap bean yields were increased in the presence of the residues in 1988 (a dry year) and slightly reduced by the residues in 1989 (a wet year).

Microbial transformation of 2(3H)-benzoxazolinone (BOA) an allelochemical from rye to 2,2'-oxo-1,1'azobenzene (AZOB) occurred rapidly in four Michigan soils. The soil microbe capable of this transformation was isolated and identified as *Acinetobacter calcoacetius*. AZOB, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and BOA toxicities were assessed singly and in combination on cucumbers, snap beans, and weed indicators. AZOB was generally more toxic to weeds and less toxic to the crops than DIBOA and BOA.

DEDICATION

This thesis is dedicated to my wife, Kathy, for her love, patience, and unceasing understanding; and to my sons, Nathan and Nicky for providing a reference point and enduring throughout these stressful years. Finally to Dr. Alan R. Putnam for introducing me to allelopathy and providing me guidance when he would rather have been at the end of a fishing pole in Montana.

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LITERATURE REVIEW

Introduction

At present, there is an increasing concern over monitoring agriculture's input and management practices. Since the Environmental Protection Agency's (EPA)'s 1984 report to Congress stating that 90% of the States have identified agriculture as being a nonpoint source of pollution, the public has been more vocal about what they are willing to accept as suitable agricultural practices.

On the other hand, the desire for an increase in crop productivity has lead farmers to use more inputs such as energy, nutrients, and pesticide inputs in their practices which in turn may adversely impact the natural environment. Increasing energy inputs is usually correlated with using more tillage practices. This exposes the land to increasing sediment runoff which carries nutrients and pesticides into the water systems. Phipps and Crossian (1986) estimated that between 50 to 70% of all nutrients reaching surface waters are principally nitrogen and phosphorous that originate from agricultural land. A 7-year study conducted by the National Stream Quality Accounting Network and the National Water Quality Surveillance Systems evaluated 116 stations for nitrate concentrations and reported 23% to have increasing nitrogen levels. They associated these increases directly with agricultural activity.

In 1987, the United States Department of Agriculture reviewed EPA's priority pesticides to identify pesticides with possible water pollution qualities according to their usage, amount applied each year, and a description of their mobility in the soil. Of the pesticides

listed, the majority (88%) were herbicides which were labeled "leacher" by water and therefore had the potential to enter the water system. Hallberg (1987) found that in Iowa and Minnesota the herbicides detected at the highest levels were those applied to the largest agriculture acres and were leachable herbicides in the triazine and acetanilide classes. Hall et al. (1989) also reported detecting simazine (6-chloro-N-N'-diethyl-1,3,5-triazine-2,4-diamine) and atrazine (6-chloro-N-ethyl-N'-(methylethyl)-1,3,5-triazine-2,4-diamine) at low levels but was unable to detect metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methylethyl)acetamide) and cyanazine (2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2methylpropanenitrile) in any wells. However, he also noted that under no-tillage (NT) conditions herbicide leaching through a well-drained soil matrix was more pronounced. One could speculate that with increased public concern for groundwater contamination coupled with the data available on nutrient leaching and pesticide contamination, safer methods for food production will be demanded by the public.

Conservation tillage

During the "dust bowl" era of the 1930's, public opinion was awaken to develop ways to reduce soil erosion caused by wind at the time of planting on exposed dry soils. With the formation of the Soil Conservation Service in 1935 and the increased knowledge of ways to conserve soil loss, NT was evaluated as an alternative cropping system. Several workers have reported comparisons of conventional tillage (CT) methods with NT methods. However in the early studies, there was a problem in that there was no definitive description of NT. For example, Webster's strict definition would be no cultivation of the

land whatsoever whereas Young (1982) defines NT as "not disturbing more than 10% of the surface area and having a protective residue uniformly distributed over the soil surface the year around". In Hall et al. (1989), NT refers to an untilled soil and has no mention of a residue being present, whereas Knavel et al. (1977 and 1986) and Blevin et al. (1971) established their NT experiments in either planted ryegrass or naturally occurring bluegrass. Still another modification is planting into existing stubble after the harvest of the previous crop such as wheat (Mascianica et al. 1986). More recently the planned introduction of cover crop residue into the crop management scheme has provided an additional approach to NT management (Putnam and Defrank, 1983; Smeda and Weller 1986). This introduction of a cover crop residue fits under the scheme put forth by Young (1982).

With the recurrence of "dust bowls days" in the 1950's and 1980's there has been renewed interest in using no-tillage to reduce erosion from wind while at the same time improving water infiltration and soil organic matter. It is difficult to measure soil losses due to water erosion because it can vary with the soil type, slope of the land, and the amount of cover present on the soil surface. For example, Young (1982) found with conventional tillage when the slope of the land increased from 3 to 12%, soil losses increased from 3.8 to 27.7 tons per acre per year. In contrast, a no-tillage system with 90% ground cover on similar slopes lost only 0.3 to 2.4 tons per acre per year. Likewise, Hayes (1982) found that on a 5% slope soil loss could be reduced by 12 tons per acre per year by adding one ton per acre of plant residue. Knowing that 1 ton of soil loss per acre is equal to 1/150 of an inch of soil per year and that using plant residues in a

cropping system can greatly reduce erosion has made NT with plant residue an attractive alternative for conservation-minded farmers.

Additional advantages from NT are improved water infiltration and soil organic matter. Blevin et al. (1971) reported a 4 to 8% increase in water content in the top 8 cm of soil when comparing no-tillage to conventional tillage practices over a two year time period. Glenn and Welker (1989) evaluated four soil management systems; a herbicide strip, cultivated strip, mowed sod, and killed sod and reported that the sod treatments captured more rainfall than did the herbicide and cultivated strips. This was attributed to the sod reducing the amount of runoff and thus increasing the amount of water infiltrating the soil. This finding led to renewed interest in how increasing water content of the soil would affect soil temperature. Wall and Stobbe (1984) showed that there was an average soil temperature decrease of $2^{o}C$ in the top 5 cm in NT compared to CT with a surface residue. Also Derpsch et al. (1986) compared four tillage systems for soil temperature during the daytime and ranked them from the highest soil temperature to the lowest at 3 and 6 cm depths. His rankings were in the order of CT > chisel plow > NT > and permanent cover. Kladivko et al. (1986) summarized the effects of conservation tillage as improved soil water content, lower soil temperature, more organic matter, more water-stable aggregates near the surface, and higher bulk densities when compared to CT systems.

A specialized approach within NT systems has been the use of plant residues of cereal grain cover crops. These residues can be beneficial for improving soil properties but they have also been proven to be beneficial in interfering with weed growth. Muller (1969) defined the

components of plant interference to include competition for physical factors such as light, water, and nutrients and also included indirect effects by the release of breakdown products or chemicals derived from decaying residues. Molisch (1937) had previously called the latter allelopathy. It is important to understand what mechanisms of interference are acting to predict impacts on crop growth. Putnam and Duke (1974) and Fuerst and Putnam (1983) proposed a set protocol to separate the competitive and allelopathic components.

Several books and reports describe the advantages and disadvantages of NT for agronomic and horticultural crops (Phillips and Young, 1973; Young, 1982; and Hayes, 1982). Perennial horticultural crops lend themselves well to this cultural method (Putnam and Lacy, 1977; Putnam et al., 1983). However, annual vegetable crops have a wide range of growing conditions and varying seed sizes which has presented unique challenges for adapting them to NT cover crop systems. As Putnam and Defrank (1983) generalized, usually large seeded crops like corn (Zea mays L.), cucumber (Cucumis sativus L.), pea (Pisum sativum L.), and snap bean (Phaseolus vulgaris L.) will have an equal or positive yield response when planted into spring cover crops of barley (Hordeum vulgare L.), oats (Avena sativa L.), rye (Secale cereale L.), sorghum (Sorghum bicolor L. Monench.), sudangrass (Sorghum bicolor sudanense (Piper) Hitchc.) and wheat (Triticum aestivum L.) when compared to bareground . Small seeded vegetables like lettuce (Lactuca sativa L.), cabbage (Brassica oleracea L.) and carrot (Daucus carota L.), generally showed a negative response, while tomato (Lycopersicon esculentum Mill.) varied from test to test. This could be due to the fact that larger seeded crops are planted deeper away

from the residues whereas small seeded crops are planted shallow in close proximity to the toxic residues. Putnam and Defrank (1983) confirmed their findings by planting cucumber, pea, and snap beans into spring planted covers with no significant difference in yield when compared to a conventional tillage system.

Effects of Tillage Systems on Warm - Season Vegetables

There have been numerous studies conducted with cucumber, snap bean, lima bean (Phaseolus lunatus L.), sweet corn, summer squash (Cucurbita pepo L.), transplanted tomato and transplanted pepper (Capsicum annuum L.) to further define their needs for light, water, and nutrients in NT systems. Both Williams et al. (1973) and Teasdale et al. (1983) evaluated snap bean row spacings from 12.7 to 91 cm and found that as the row spacings were decreased the yield of the snap beans increased and resulted in decreased weed growth. Teasdale et al. (1983) concluded that as the rate of canopy closure increases fresh weed weight decreases by 50 to 67% due to differential shading. Teasdale suggests his maximum yields and best weed reduction was obtained within the 15 to 36 cm row spacing whereas wider row spacings did not intercept enough of the light allowing more earlier-season weed growth. Their results suggested that for sweet corn, snap bean, and onion (Allium cepa L.) there is a 2 to 3 week weed-free period needed after crop emergence to prevent yield losses due to weeds. Mascianica et al. (1986) double cropped snap beans after harvesting a wheat crop and studied the effects that stubble heights of 8, 15, 23, 30,and 38 cm had on the crop morphology and yields. His results suggested that the shading by 30 and 38 cm-high wheat stubble reduced the yields of the crop due to shading caused by a reduction of incident light on the

surface of the soil. He also noted that all stubble heights resulted in etiolation of plant internodes and that stubble heights of 8 to 23 cm increased the total yields of snap beans. He also concluded that stubble heights of 15 to 30 cm enhanced the harvest efficiency without affecting the total yield. This was possible because the interference of the stubble caused an increase in the elongation of the plant internodes thereby causing the pod clusters to move upward thus decreasing pod losses during mechanical harvesting. Mascianica et al. (1986) also proposed that the stubble provided the snap bean plants with support thus reducing lodging. Mullins et al. (1988) conducted experiments to study the effect of cropping systems on snap bean pod length. His experiments evaluated snap beans planted with 2 row spacings of 0.46 and 0.92 m and in plots with 3 tillage treatments of CT, NT, and rotary strip tillage. He concluded from the results that none of the parameters studied had any effect on pod length and therefore thought pod length to be strictly an individual cultivar trait uninfluenced by environment. He also observed poor weed control when a winter wheat cover crop was removed prior to planting, particularly in the wider row spacing. This might suggest that the type of interference the mulch provided was shading the soil which would have been minimal to the crop in the 2-row spacing.

Generally under NT with cover crops there has been improved moisture content of the soil. Mascianica et al. (1986) suggested that the snap bean plant requirement for water is 180 mm per year and that the growth and pod yields are similar under CT vs NT with adequate water present. It is when there is a shortage of water which can occur in a drier growing season that the NT systems have greater yields.

Knavel et al. (1977) concurs that there is a greater yield in plant dry matter in the NT compared to CT in drier years but that CT has equal or greater yields in wetter growing seasons. Peterson et al. (1986) also observed that NT treatments generally have wetter and cooler soils in the spring thus reducing yields due to limited early growth and development.

Nutrient availablity to the crop has been shown to be affected by different tillage systems. Mullins et al. (1980) concluded that tillage did not affect the pH of the soil in the top 10 cm but NT treatments had higher pH's in the top 10 to 15 cm depth. Generally he found under CT systems that the macro-nutrients nitrogen (N), phosphorous (P), potassum (K), and calcium (Ca) were distributed deeper in the soil but plant uptake was unchanged when comparing CT and NT. Mullin et al. (1980) found that P and K levels in the soil were not affected by tillage at the 0 to 5 cm depth but were affected at depths greater than 10 cm. However, tillage system had no effect on plant uptake of P and K at any level. Knavel et al. (1977) reported that N, P, K, and Ca availablity depended on the crop (cucumber, sweet corn, tomato, pepper), nutrient, tillage system, and time of sampling. For example, sidedressing with N caused an increase of N in the crop from the first to second sampling date in NT cucumber, tomato, and corn. Where there were significant differences between the N content of the crops due to the cropping systems, it was found to be higher in the CT system. When N differences were found later in the season, it could possibly be related to soil moisture changes as reported by Larson et al. (1970). Skarphol et al. (1987) evaluated snap bean response to N in NT and CT systems using legume and cereal grain cover crops in 1984

and 1985. His CT treatments consisted of cover crop residues being incorporated into the soil prior to planting. In 1985, he detected less nitrate loss possibly because of less leaching and denitrification and also due to a shorter interval between incorporation of crop residues prior to planting. He also reported a 14% greater yield in the NT vs CT treatments for both years and attributed that to an increase in soil moisture levels. There was no advantage of applying nitrogen when a legume cover crop was used like hairy vetch (Vicia villosa Roth.) or Austrian winter-pea (Pisum sativum ssp. arvense L. Poir.). Knavel et al. (1977) reported that NT crops of cucumber, sweet corn, tomato, and pepper contain equal or greater levels of P when compared to CT-grown crops. He and Blevins et al. (1971) offered the explanation that there is usually greater P uptake in NT systems due to an increase in soil moisture levels. Knavel et al. (1977) found K levels in general were not influenced by cultural practices but in some cases varied in cucumbers and peppers depending on sampling date and cropping systems. He also found Ca uptake varied among the four different crops tested, but Ca content was generally lower in NT early in the season and equal to or greater than that in CT crops by the last date sampled. Therefore, nutrient availability can vary with the soil microclimate, tillage system, crops, and time interval sampled in the growing season.

With the complexity of competitive interaction taking place involving light, water levels, and nutrient availability it is difficult to differentiate the responses that are strictly competitive or allelopathic in field evaluations. Therefore, to determine the differences, one needs controlled experiments in the greenhouse or

laboratory.

Allelopathy

In more recent years, research has focused on the chemical basis of plant interference called allelopathy. Allelopathy was first observed as early as 1832 by de Candolle when he reported "soil sickness" that reduced the productivity of the crops. Since then several articles and books by Tukey (1969) , Rice (1971), Putnam and Duke (1978), Rice (1984), Putnam and Tang (1986), and Putnam (1988) have further detailed the development of allelopathy from being an unexplained occurrence to its current status as a science. Generally it has been acknowledged that Molisch (1937) first coined the term allelopathy to mean a "biochemical interaction between all types of plants including microorganisms " (producing either a detrimental or beneficial response). Microbial influences may be the result of phytotoxins produced by microorganisms themselves or the release of phytotoxins when microorganisms degrade plant residues. Allelochemicals are therefore compounds that are produced by higher plants or microorganisms that can affect the growth of other plants. Whittaker and Feeny (1971) and Rice (1984) further modified Molisch's definition of allelopathy to mean the overall mediation of one plant's growth by another through chemical means. A major obstacle in proving these allelochemical responses has centered on removing the competitive component from plant-plant interactions and focusing only on isolation of allelochemicals.

The diversity of plants has made it difficult to establish a set protocol for the proof of allelopathy. Fuerst and Putnam (1983) have developed a set of criteria to prove in a stepwise manner whether or

not an allelopathic response is present. Initially the symptoms of interference must be identified in the natural state. The next step is to develop supportive assays that can examine factors which might influence the primary source of toxicity, the method of release, and the role of microorganisms in the expression of toxicity. To help support and define the allelochemical response, it is useful to evaluate several indicator species and record the injury symptoms. Other major steps in the protocol call for chemical isolation and identification. Also, it is important to study the release and movement and to quantify the toxin as a proof that the allelochemical response is present. This can be confirmed by adding the toxin back into natural systems and monitoring plant reaction. Drost and Doll (1980) suggested the importance of confirming the activity of the isolated chemicals by further testing in field experiments. This protocol is very expensive, time-consuming and may require cooperation between scientific disciplines. It is difficult to experimentally provide a clear cut analogy to what the actual reactions are in natural ecosystems.

Allelochemicals are present in virtually all plants and can be found in an array of tissues such as leaves, flowers, fruits, buds, seeds, stems, and roots (Putnam and DeFrank, 1983). Putnam (1988) indicated that even though natural product chemists may isolate and identify new compounds from plants and microorganisms, it is important to confirm if they are present and released in sufficient quantities to have an allelopathic effect. Tang et al. (1986) has outlined a trapping technique using a live plant in its habitat to collect root exuduates and to evaluate their allelochemical activity. His system

uses a healthy plant growing in either a basaltic rock or silica sand medium, continuously circulating nutrient solution and trapping of chemicals on nonionic resins.

Crop and Weed Response to Allelochemicals

Ruiz-sifre and Ries (1983) planted sweet corn into 49 day old sorghum residues which had been divided into treatments of roots, shoots, and whole plants and compared them to bareground. Of these treatments, the sorghum shoots showed a 15 to 45% stimulation of corn seedlings whereas the root residues showed a 20 to 30% inhibition of seedlings for the first seven weeks, after which growth was essentially equal to the bareground regime. This perhaps indicated that after death of the plant there is a release or breakdown of plant material to form stimulators or inhibitors. In other research, Barnes and Putnam (1983, 1986) evaluated spring planted rye compared to bareground and recorded a 94% reduction in weed biomass. Barnes further partitioned 35 day-old rye into root and shoots and found shoots to be about twice as inhibitory on germination of lettuce and barnyardgrass (Echinochloa crus-galli (L.)Beauv.) as the roots. Tang et al. (1975) had previously evaluated rye for the presence of 2(3H)-benzoxazolinone (BOA) and found 8 times more BOA in the shoots than in the roots. Barnes et al. (1987) further partitioned the rye herbage into water soluble and insoluble fractions and found the soluble fraction to be inhibitory to garden cress (Lepidium sativum L.). This was in agreement with several other investigators who have reported the aqueous fraction of different herbages to be active on germination (Harper and Lynch, 1982; Guenzi and McCalla, 1967; Weston and Putnam 1986). Barnes et al. (1987) proceeded further and partitioned the aqueous rye herbage fraction into

acetone, hexane, ethylacetate, diethyl ether, and chloroform fractions and reported the diethyl ether fraction to be two to ten times more active than the other organic fractions. In the diethyl ether fraction, there were two active toxins isolated, 2,4-dihydroxy-1,4(2H) benzoxazin-3-one (DIBOA) and BOA, polar and non-polar compounds, respectively. Zungica et al. (1983) had previously evaluated rye for the presence of DIBOA and found it to be present in both cultivated and wild accessions. He also found that both hydroxamic acids, DIMBOA and DIBOA, were present in higher concentrations in the younger leaves and reached their highest concentration after four days. Argandona et al. (1980, 1981) evaluated rye for hydroxamic acids and found them to be in highest concentrations from 10-16 days after germination. Nair et al. (1990) compared two different allelochemicals derived from rye (DIBOA, BOA) and the microbial transformation product of BOA, 2,2'-oxo-1,1'-azobenzene (AZOB), and found corn and soybean (Glycine max L. Merrill) crops to be unaffected while barnyardgrass, redroot pigweed (Amaranthus retroflexus L.), velvetleaf (Abutilon theophrasti Medik.), garden cress, and tomato were highly sensitive to AZOB at concentrations of 5 to 200 ug/ml. Barnes et al. (1987) found dicot species in petri dish assays to be 30% more sensitive to rye allelochemicals than monocots.

Putnam (1984) postulated that there can be a positive, neutral or negative effect of allelochemicals depending on plant species. Ideally it would be beneficial to be able to harness the positive effects for crop growth while still maintaining the negative effects on a particular pest (e.g. selectivity). Barnes and Putnam (1987) indicated that in a NT rye cover cropping system there tends to be greater activity against weeds near the surface and therefore one possible

selectivity scheme would be to place the seeds of a crop below the active rye residue levels. Putnam and DeFrank (1983) used 7 different spring planted cover crops prior to planting cucumbers, sweet corn, peas, and snap beans and monitored good germination, growth, and yield of these vegetable crops. The same parameters indicated poor response of direct seeded cabbage and lettuce in the cover crop residues while seeded tomatoes and carrots were also damaged by some residues. Almeida (1985) tested 7 different winter cover crop mulches for fresh biomass production and found large variations. In addition, the cover crops lupine (Lupinus albus L.), wheat, rape (Brassica napus L.), radish (Raphanus sativus), and oats had total weed biomasses of 300 to 1350 g/m^2 with 83 to 100% being grasses, whereas rye and triticale (X Triticosecale Wittmack.) allowed weed biomasses of 500 and 1100 g/m^2 respectively with 64 to 77% being broadleaves. This suggested that different cover crops are selective in their suppression of weed growth.

Another possible selectivity mode would be to incorporate the residue thoroughly with the soil or to plow down the residue. Christian et al. (1985) compared the effects of the depth of straw incorporation of three cover crops, wheat, barley and oats, on seedling establishment. Using a control plot where surface mulch was removed by burning, he determined that burying the residue 20 cm deep resulted in a 29% reduction in seedling establishment caused by the cover crop residues. All of these studies suggested that selectivity may be achieved by variations in seed size, species type, depth of planting, and by the choice of a cover crop.

Some workers have attempted correlations between the time

intervals of plant decomposition and phytotoxicity. Almeida (1985) found that although there was decomposition with time, the residue cover that had the greatest biomass throughout the growing season also produced the greatest reduction in weed biomass. His ranking from least to most weed biomass were oats < rye < rape < triticale < wheat < and < lupine when evaluated at 118 days after desiccation. Smeda and Putnam (1988) evaluated residues of fall-planted covers of barley, rye, and wheat desiccated in the spring. They observed that 17 days after cover crop desiccation, all three covers similarly reduced weed biomass by 80-90% when compared to a bareground treatment. At 71 days after desiccation the bareground and barley plots had the same weed biomass, while wheat and rye still exhibited a 51 to 73% reduction in weed biomass. Srivastava et al. (1986) evaluated the activity of rotting wheat straw water extracts and found that the greatest reduction (35-45%) in wheat germination occurred between 8 and 15 days. He related this toxicity to allelochemical components of the wheat straw because the treatments of sterile and non-sterile extracts had essentially the same results. Kimber (1973) compared rye straw under sterile verses nonsterile conditions as well as green verses rotting straws. He found extracts of four day old green straw to be more toxic than the rotted straw which strongly inhibited wheat growth. Guenzi et al. (1967) monitored wheat seedling germination following treatments of water soluble extracts of wheat, oats, sorghum and corn residues and found that wheat and oat residues contained no water-soluble toxins after 8 weeks while corn and sorghum residues appeared to release toxic components for up to 22 weeks. Harper and Lynch (1981) determined by gravimetric analysis the major components of undegraded oat straw to be

7.3% water soluble materials, 35.5% hemicellulose, 42.1% cellulose, and 13.5% lignin. They concluded that although there were seasonal changes in straw decomposition, hemicellulose and cellulose appear to be degraded at a fairly constant rate during any time frame measured. They also observed that acetic acid, the most active allelochemical, was produced under anaerobic conditions and it's concentration declined progressively during straw decomposition. The course of decline in production of acetic acid parallelled the loss of dry weight of hemicellulose and cellulose. They also concluded that the production of acetic acid occurred until 21.5% of the straw remained as polysaccharides. But in practice, no phytotoxic accumulations occurred in the natural ecosystems once 50% of these compounds had been decomposed which usually occurred within 50 days following desiccation. Therefore as stated by Putnam and Duke (1978) and Rice (1984), if the time interval is long enough, toxins can be liberated by residues either by decomposition or by leaching with water to eliminate any weed or crop growth reductions.

Role of Soil Microbes in Allelopathy

Allelochemicals can be released by such processes as volatilization, root exudation, leaching, microbial transformation, and decomposition of plant residues (Chapman and Lynch, 1983; Rice, 1984; and Putnam, 1984). More recently, the involvement of microorganisms to release phytotoxins from residues or their role in using the residue as a substrate to produce secondary compounds has been studied (Nair et al. 1990). One of the early scenarios involved the removal of old peach trees from orchards and unsuccessful attempts to reestablish a new peach planting. Proebsting and Gilmore (1941) postulated that upon

tree removal, either the roots left in the soil or breakdown products from the roots were toxic to new trees. They found that combining peach roots with the soil and planting peach seedlings into the mixture caused severe inhibition. They proposed that the chemical amygdalin, an allelochemical derived from the peach root bark was responsible for the toxicity. However when tested by itself for peach seedling injury, no activity was observed. But with the addition of the enzyme emulsin which catalyzes the hydrolysis of amygdalin to glucose, benzaldehyde, and hydrogen cyanide peach seedlings exhibited severe injury. Patrick (1955) examined the involvement of microorganisms in the production of toxic compounds from peach root bark and isolated many microorganisms from the soil capable of hydrolyzing amygdalin. 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 degradation of amygdalin.

Patrick and Koch (1958) conducted tests on the effects of aqueous extracts of decaying residues of timothy (Phleum pratense L.), rye, corn, and tobacco (Nicotina tabacum L.) on respiration, oxygen uptake, and growth of tobacco seedlings. They proposed a correlation between acidity and toxicity of the decomposing aqueous extracts indicating that as the residues decompose, the aqueous portion becomes more acidic. However, they found that if the pH of the extracts were adjusted to neutrality, the toxicity to tobacco seedlings was retained without much change. Also they compared the toxicity of extracts of decomposed residues to extracts of non-decomposed residues mixed with soil and confirmed the role of microbes in the toxicity. It appeared that the plants themselves contained no compounds inhibitory to

respiration of tobacco seedlings, but that toxic compounds were formed during microbial degradation of the residues. Chapman and Lynch (1983) also studied microbial effects on the toxicity of decomposing plant residues to barley root growth and concluded that the inhibition was primarly from the chemical inhibitors.

In general, the total soil microbial population is estimated to be in the range of 10^6 to 10^9 microorganisms per gram of soil (Atlas and Bartha 1987). Of the microorganisms found in the soil, bacteria and fungi make up about 90 to 95% of the population. Mishra and Kanaujia (1968) indicated that studies of microbial populations in soil have been done on a seasonal or monthly basis since the fluxes of the population can change rapidly. Tiwari et al. (1987) indicated that wetting of the soil increases the dissolution of soluble organic matter and will reflect a rapid increase of bacterial populations during the first 24 hours. Cochran et al. (1977) generally found no toxicity from straw during periods of low moisture but within one week after rainfall, lentil and wheat straws significantly inhibited root elongation of wheat seedlings. He also observed that production of toxins from straws was irregular and generally was greater in wet weather with air temperatures in the range of 0 to 15^{o}C. He postulated that during dry periods there could be storage of toxins in the plant tissue which are released when soil moisture levels increased. Mishra's and Cochran's results possibly indicate that with higher moisture levels there is increased microbial activity resulting in the production of more phytotoxins.

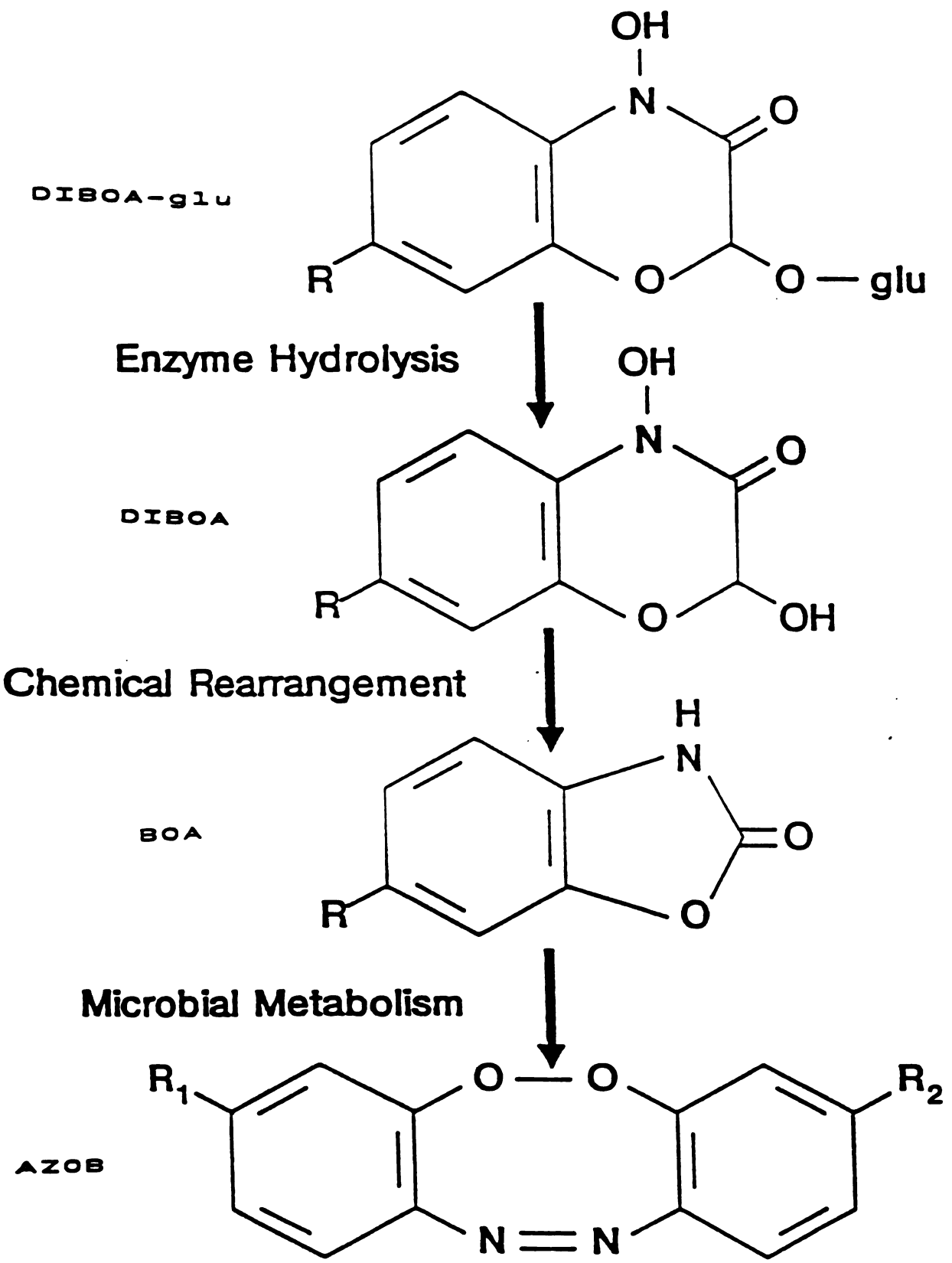
Compounds Implicated in Rye Toxicity

As indicated earlier, the allelochemicals present in plants are

numerous and diverse in their chemical structures. Chou and Patrick (1976) identified nine compounds in decaying rye residues which included vanillic, ferulic, phenylacetic, 4-phenylbutryic, p-coumaric, p-hydroxybenzoic, salicylic, o-coumaric acids, and salicylaldehyde. These compounds were all found to be inhibitory to germination in lettuce seed bioassays. Shilling et al. (1985) isolated phenyllactic acid and hydroxybutyric acid from field grown rye residues and found them to be inhibitory to redroot pigweed and common lambsquarters (Chenopodium album L.) root growth at 2 mM concentrations. Barnes et al. (1987) isolated two hydroxamic acids, DIBOA and BOA from rye residues, and compared their toxicity to phenyllactic acid and hydoxybutyric acid. Three monocotyledons and dicotyledons were assayed at concentrations ranging from 67 to 267 ppm and monitored for germination number, root, and shoot lengths. Among the four allelochemicals tested there was variation in response of the three species in the parameters measured. DIBOA and BOA were the most toxic of the four chemicals to root growth of large crabgrass (Digitaria sanguinalis (L.)Scop.), barnyardgrass, proso millet (Panicum miliaceum L.), redroot pigweed, tomato, and lettuce at concentrations of 67 to 267 ppm. Queirolo et al. (1981) and Barnes (1981) have suggested that the benzoxazinones are inhibitory to photophosphorylation in the chloroplasts which interferes with electron transport. This is similar to polycyclic urea herbicide activity which in turn is related to the H-N-C=O grouping (Moreland and Hill (1963)). BOA contains this chemical grouping and DIBOA has it in a reduced form. Therefore, chlorosis was a symptom of injury by rye residues on several indicators (Barnes and Putnam 1983).

Virtanen et al. (1957) isolated the cyclic hydroxamic acid, BOA, from four day-old rye seedlings. It was extracted by boiling the plant tissue with water and extracting the aqueous portion with diethyl ether. His unsuccessful attempts to extract BOA from fresh tissue suggested the presence of a precursor in the tissue. By boiling the tissue he was able to inactivate all enzymes in the tissue followed by the extraction of the glucoside precursor, GDIBOA. (Scheme 1). By treating GDIBOA with a hydrolytic enzyme he was able to obtain the hydrolysis products glusose and a second precursor, DIBOA. Hofman and Hofmanova (1969) were able to extract the glucoside precursor from intact plant tissue using liquid nitrogen. Upon injury to the plant the glucosidases were released which caused rapid hydrolysis of the glucoside to DIBOA. Similar scenarios for a methoxylated DIBOA have been reported to occur in corn, wheat, Job's Tear (Coix lachrymas jobi), and bears breech (Acanthis mollis L.) (Loomis et al., 1957; Koyama et al., 1956; Wolf et al., 1985). These are the only known instances of 1,4 benzoxazinone production in higher plants although their production by microoganisms is fairly common (Tipton et al., 1967).

Nair et al. (1990) reported the in vitro production of the azoperoxide, 2,2,'oxo-1,1'-azobenzone (AZOB), when BOA was mixed with soil. He also showed the benzoxazinones produced by rye, DIBOA and BOA, could rapidly undergo microbial transformation to AZOB, (Scheme 1). Although intact rye tissue has not been shown to produce AZOB by itself, the addition of either benzoxazinone to the soil in sufficient



Scheme 1. Scheme for formation of AZOB proposed by Nair et al. (1990)

quantities has shown rapid production of AZOB. DIBOA and BOA were less active than AZOB at concentrations of 67 to 266 ppm on garden cress and barnyardgrass radical elongation. However, AZOB was 8 to 10 times more herbicidally active than DIBOA and BOA when tested against several indicator species at 67 ppm.

Detection of Benzoxazinones and AZOB

DIBOA, BOA, and AZOB can be detected by thin layer chromatography (TLC) and spray reagents and quantitative UV-visible spectroscropy (Hietala and Virtanen, 1960; Nair et al., 1990). These compounds gave different R_f values on TLC plates with $CHCl_3$-MeOH (6:1) or CH_2Cl_2-EtOAc (9:1) solvent systems and were detected using spray reagents such as ferric chloride ($FeCl_3$) for DIBOA or ceric sulfate ($CeSO_4$) for BOA (Nair et al., 1990).

The objectives of this study were to 1) determine the compatibility of spring planted cover crops to cucumbers and snap beans, 2) to assess response of these crops and weeds to rye allelochemicals, 3) to quantify the rate of transformation of benzoxazinones, and 4) to isolate a soil microoraganism responsible for the biotransformation of BOA to AZOB.

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Chapter 2

FIELD AND GREENHOUSE RESPONSE OF CUCUMBERS AND SNAP BEANS TO COVER CROP RESIDUES

ABSTRACT

Plant residues (cover crops) have shown promise as a method of reducing synthetic chemical inputs for weed control. Oats (Avena sativa L.), spring barley (Hordeum vulgare L.), and rye (Secale cereale L.) were spring planted and allowed to grow for 37 to 45 days before chemical desiccation with glyphosate, N-(phosphonomethyl)glycine, followed by planting of cucumbers (Cucumis sativus L.) and snap beans (Phaseolus vulgaris L.). In 1988 and 1989, rye was the most effective residue reducing weed biomass by 46 to 79% at 35 to 42 days after planting although it produced 20 to 64% less biomass than the other two cover crops. In 1988, a drier and warmer year, both vegetables produced 28 to 59% higher yields with residues compared to bareground, while in 1989, a wetter and cooler year, the crops grown in residues showed a reduction in yield of 7 to 33%.

Environmently controlled experiments compared the placement of rye and poplar excelsior (Populus tremuloides L.) residues placed at the soil surface or 1.3, 2.6, and 3.9 cm depths. Cucumbers and snap beans planted at 1.3 cm exhibited a 15 to 64% yield reduction depending on the residue rate and placement. Leaching of rye residues with and without microbial degradation for four and eight days removed inhibitory activity against cucumber. However, rye residues that were not leached provided a 64% reduction in cucumber germination and 68 to 70% reduction in seedling biomass when compared to poplar excelsior.

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Chapter 2

INTRODUCTION

No-tillage (NT) has shown promise as a method of reducing synthetic inputs into cropping schemes. The use of plant residues (cover crops) in NT systems has been beneficial in reducing erosion, improving soil moisture content, and interfering with weed growth. Young (1982) and Hayes (1982) reduced soil erosion by utilizing both NT and a cover crop with plant residues of 1 ton per acre. The reduction in soil erosion can be attributed to soil cover which acts to absorb some of the forces from the rainfall and to impede surface water runoff that carries soil. Blevins et al. (1971) and Glenn et al. (1989) generally found that covering the soil surface increased moisture content in the top 8 cm and attributed this to either improved water infiltration or reduced water runoff. Cover crops also insulate the soil and prevent water evaporation. Various investigators reported improved yields in drier years when decomposing plant residues were present (Mascianca et al., 1986; Knavel et al., 1977; and Ruiz-sifre and Ries, 1983). Peterson et al. (1986) reported that soil in NT plots was generally cooler and wetter in the spring. Wall and Stobbe (1984) attributed plant growth differences to soil maxima temperatures throughout the growing season to be an average decrease of about 2^{o}C with the maximum occurring at about 1300 hours daily.

Another beneficial result from plant residues has been their ability to interfere with weed growth. Interference includes competition for physical factors such as light, water, and nutrients plus chemical effects from the decaying plant material called

allelopathy (Muller, 1969). Putnam and Defrank (1983) and Barnes and Putnam (1983) have attempted to duplicate the physical component of interference in field settings with the introduction of poplar (Populus tremuloides L.) excelsior (PE) mulch which degrades slowly. When comparing the standard check (bareground) to PE there is usually a higher weed biomass reported in the bareground. Several cover crop residues however, have reduced weed growth more than the PE.

Williams et al. (1973) and Teasdale and Frank (1983) evaluated different snap bean row spacings from 13 to 91 cm and generalized that as the row spacing decreased, the yield of the snap beans increased while weed growth decreased. Teasdale's results showed a 50 to 70% weed biomass reduction due to the shading with the best results achieved at 15 and 36 cm spacings. His results on corn, snap beans, and onions indicated the importance of a two to three week weed-free period after crop emergence to prevent yield losses.

Mascianica et al. (1986) evaluated the shading that could occur from residues when planting into a wheat stubble. He concluded that stubble heights of 30 to 38 cm reduced crop yield due to the reduction in light recieved by the emerging crop. Although yield reductions occurred as stubble height increased, fruit quality improved and a higher percentage of the fruit was recovered at harvest.

Putnam and Defrank (1983) generalized that large seeded crops, i.e. sweet corn (Zea mays L.), cucumber, peas (Pisum sativum L.), and snap beans, had an equal or enhanced yield when planted into spring planted cover crops of barley (Hordeum vulgare, oats (Avena sativa L.), rye (Secale cerale L.), sorghum (Sorghum biocolor L.), sudangrass (Sorghum biocolor sudanense (Piper) Hitchc.), and wheat (Triticum

aestivium L.) compared to bareground. Conversely, yields decreased for small seeded crops like cabbage (Brassica oleraceae L.) and carrots (Daucus carota L.). Possible reasons for greater tolerance of large seeded crops could be more vigorous seedlings able to withstand more variablity in placement of the seed, or deeper planting which places seed away from the toxic residues.

Plant parts vary in the potential to produce allelochemicals (Putnam 1988). For example, Barnes and Putnam (1986) reported a 94% reduction in weed biomass for spring planted rye compared to bareground. Barnes partitioned 35-day-old rye into roots and shoots and found shoot residues and extracts to be twice as active as those from roots on lettuce and barnyardgrass germination. Furthermore, several investigators performed aqueous partitioning of grass herbages into water soluble and insoluble fractions and found more activity in the soluble fractions (Barnes et al., 1987; Harper and Lynch, 1982; Guenzi et al., 1967; Weston et al., 1986).

Several workers have correlated the time interval of cover crop decomposition and resulting phytotoxicity. Almeida (1985) found covers that had the greatest biomass throughout the growing season also provided the greatest reduction of weed biomass. His ranking of weed biomass suppression 118 days after desiccation was in the order oats > rye > rape (Brassica nupus L.) > triticale (X Triticosecale Whittmack.) > wheat > lupine (Lupinus albus L.). Smeda and Putnam (1988) evaluated fall planted covers of barley, rye, and wheat for weed control following desiccation in the spring. At 17 days after cover crop desiccation, all three residues were statistically equal exhibiting between 80 to 90% reduction in weed biomass when compared to a

bareground treatment. At 71 days after cover crop desiccation, the bareground and barley residue had the same weed biomass while wheat and rye still exhibited 51 to 73% weed biomass reductions. Similarly, Stivastarva et al. (1986) evaluated water extracts of rotting wheat straw and found the greatest reduction in wheat germination (35 to 45%) after straw had rotted for 8 and 15 days. He attributed this toxicity to allelochemical components of the wheat straw since the sterile and non-sterile extracts had essentially the same results. Kimber (1973) compared rye straw under sterile versus nonsterile conditions and green versus rotting straws. He found cold extracts of four-day-old green straw to have the greatest inhibition when compared to rotted straw. Guenzi et al. (1967) monitored water soluble extracts of wheat, oats, sorghum and corn residues on wheat seedling germination. He found wheat and oat residues contained no toxic water-soluble toxins after 8 weeks but corn and sorghum residues were observed to have active extracts for up to 22 weeks. Harper and Lynch (1982) determined that seasonal changes in oat straw decomposition rates occurred but that the presence of active allelochemicals parallels the decomposition of plant residues.

In Michigan and much of the temperate zone, there is a three to five week period when soils lay fallow prior to planting warm season vegetables. Cucumbers and snap beans are generally planted after June 1 when soil temperatures reach 15 to 16^{o}C and night temperatures stay above 10^{o}C. This provides an opportunity to utilize cover crops to reduce soil erosion and early weed populations. The objective of this study was to evaluate the response of cucumber and snap beans to three spring planted cover crops, and to monitor reductions in weed biomass

obtained with these cover crop residues.

MATERIALS and METHODS

FIELD EXPERIMENTS. Experiments with five identical treatments were conducted in 1988 and 1989 at the Horticulture Research Center, E. Lansing, MI, on a Capac loam (Aeric Ochraqualfs, fine-loamy, mixed) soil with 0 to 3% slope. Appendix 1 provides the soil analyses from samples taken at the time of cover crop planting in early May of each year. 'Heritage' oats, 'Browers' spring barley, and 'Wheeler' rye were planted at rates of 143, 133, and 152 $kg \cdot ha^{-1}$, respectively using a three-point-hitch, mounted (1.8 meter wide) Moore Unidrill. A control mulch, PE, was also included for a comparison of the physical impacts that a mulch would produce. PE was applied at a rate of 3300 $kg \cdot ha^{-1}$ within two days after vegetable crop planting. Immediately after PE application, a green plastic netting with square openings of 2.5 X 5.5 cm was laid on top of the mulch to secure it. For comparable purposes, cover crop treatments were compared to conventional tillage and a slow degrading mulch of poplar excelsior.

1988. On May 13, plots of 4.5 X 6.0 meters were arranged in a randomized complete block design having 4 blocks and planted with the three cover crops. At 37 days after planting (DAP), all treatments received glyphosate at 1.5 $kg \cdot ha^{-1}$ and seven days later paraquat, 1,1'-dimethyl-4,4'-bipyridinium ion, plus X-77 at 1.1 $kg \cdot ha^{-1}$ plus 0.5% by volume/volume (v/v). On June 23, 'Flurry (85%) plus Sumpter (15%)' cucumbers and 'Bush Blue Lake 47' snap beans were planted using a Heath Universal Vacuum Precision Seed Planter. Two rows of each crop were planted 60 cm apart with seed placement for cucumbers being 1.2 cm and

snapbean 0.8 cm within the row. PE was laid 1 DAP and secured with the netting. Nitrogen fertilizer (55 $kg \cdot ha^{-1}$) in the form of ammonium nitrate was applied to the soil surface 14 DAP with a hand held applicator and irrigated with 0.6 cm of water. Throughout the growing season, random square meter measurements of weed number and total dry weight shoot biomass for cover crops and weeds were harvested at 21, 32, and 42 DAP. Cucumbers were destructively harvested on two dates (44 and 48 DAP), while snapbeans were harvested at 69 DAP. For each crop, fruit and plant weights were recorded.

1989. On May 2, plots (6.0 X 6.0 m) were laid out in a Latin Square Design with 5 blocks and planted with the same cover crops and vegetables as in 1988. The plots were sprayed 45 DAP with glyphosate at 1.2 $kg \cdot ha^{-1}$. On June 26, cucumbers and snap beans were planted using the same planter, row spacing, seed spacing, and cultivars as in 1988 except one additional row of each crop was planted. Nitrogen fertilizer (55 $kg \cdot ha^{-1}$) was applied to the soil surface at 13 DAP in the same manner as in 1988. Soil cores were taken 3 DAP using a round golf course borer (16.2 cm) at a depth of 10 cm to determine soil moisture content gravimetrically. Soil temperatures were taken at time intervals of 11, 12, and 15 DAP at a depth of 6.8 cm with a WEATHERtronics soil thermometer (model 4466). Random square meter measurements for cover crop shoot biomass were harvested at 10, 41, and 81 DAP and total shoot biomass and number of weeds at 35 and 81 DAP. On August 1 and 2 the main plots (cover crops) were split into weeded vs nonweeded. On August 14 all snap bean plots received an additional hand weeding. Plant and fruit yields were obtained 47 DAP for cucumber and 81 DAP for snap beans.

CONTROLLED ATMOSPHERE EXPERIMENTS: Experiments to study the effects of placement and decomposition of rye residues on cucumbers and snap beans where conducted in Conviron (model CMP3244) growth chambers. Growth conditions in the chambers were 26/23^{o}C, 50% relative humidity, 380 $uDm^{-2}s^{-1}$ light intensity, and a 16-hour photoperiod. Repeated experiments were completed with identical treatments arranged in a randomized complete block design with 3 blocks. Data was analyzed using analysis of vaiance and means were separated using the LSD test.

Fall-planted Rye residue ('Wheeler', 225 day-old, 4 to 8 leaf, 6 to 11 tillers, 8 to 16 inches in height) stored as whole plants (-20 ^{o}C) was lyophilized and ground through a Wiley Mill with a screen size of 2 mm. Rye and poplar excelsior (5 and 10 g) were placed at intervals of 1.3 cm on the surface of 12.5 X 18.5 cm rectanglar styrofoam flats. Spinks loamy sand (Psammentic Hapludalfs, sandy, mixed, mesic) was added volumetrically using 200 ml of soil for each 1.3 cm of depth added. 'Flurry' cucumber and 'Bush Blue Lake 47' snap bean were planted at a 1.3 cm depth in a configuration with 2 rows of 4 seeds per crop. After planting, all flats were placed in 15.5 X 22.5 cm aluminum pans filled with 300 ml of water. The duration of the experiments was 14 days during which the flats were maintained with daily surface watering of 100 ml for the first 7 days and 150 ml for the remaining 7 days. Crops were harvested at the soil surface using a razor blade to cut the hypocotyl. Plant numbers and total dry weights of each crop were recorded.

Rate and placement of residue: Rye residue at rates of 5 and 10 grams was compared to 10 grams of poplar excelsior placed at 0, 1.3, 2.6, and 3.9 cm depths. A no-residue treatment was also included to use as a

comparison to the mulch treatments. The data from three duplicate experiments were combined for analysis.

Leaching of residues Duplicate studies were conducted in the same environmental setting as previously mentioned using a randomized complete block design having 3 blocks. Residues were leached by adding silica sand (50 g) to the bottom of 230 ml styrofoam cups, punching 4 holes for drainage, adding 50 grams of silica sand, and then placing the residues on top. Microbial decomposition was studied by mixing 10 grams of Spinks loamy sand soil to one set of residue treatments. Water (200 ml) was added daily to leach the residue for 4 or 8 days. Residues were then air dried for 24 hours at 30^{o}C and reground to allow uniform distribution over the cucumber seeds which had been planted 1.3 cm deep. Plants were allowed to grow for 14 days, then harvested by cutting the plants at soil level. Germination numbers and total weights were recorded for each treatment. Data from duplicate experiments were combined for the analysis of variance.

RESULTS and DISCUSSION

Field Experiments. As indicated by the temperature and precipitation information for the two years (Appendix 2), 1988 had 66% less rainfall than 1989 and 26% more growing degree days (GDD). This data may explain some of the differences in total biomass of weeds as well as vegetable yields obtained during the two seasons. In 1988, there was little weed biomass production at 21 DAP for any of the treatments (Table 1). However by 42 or 35 DAP, in both years, weed biomass was significantly reduced when plant residues were compared to no residue. For both years, the early season weed biomass appeared to be reduced more with cover crop residues than with PE indicating that they were

TABLE 1: Total Weed Biomass as Influenced by Cover Crops and Time for 1988 and 1989

cover crop	DAP	1988[1]		1989		
		21	42	35[2]	81[3]	
					weeded	nonweeded
		($G \cdot M^{-2}$)				
Oats		4.0	91.3	71.8	10.5	296.9
S. Barley		.7	126.5	74.4	7.4	284.1
Rye		.9	69.3	64.4	7.7	383.9
Poplar Excelsior		4.1	235.0	82.6	29.8	199.6
None		5.2	324.8	118.4	3.3	243.7
LSD (.05)		4.5	187.8	37.1		
	Mean[4]				11.8	281.6

1 - F value for cover crops vs none significant at P=0.05.
2 - F value for cover crops vs none significant at P=0.01.
3 - F value for cover crop effects at 81 DAP are NS.
4 - F value for main effect of weeded vs non-weeded is significant at P=0.01.

exhibiting both competitive and allelopathic responses.

Weed biomass increases rapidly with time and as indicated by Smeda and Putnam (1988). The suppression of weeds by cover crop residues decreased between 17 and 71 days after desiccation depending on conditions. In both years residue treatments significantly reduced the total weed biomass at 21 to 42 days after planting. This agrees with previous results of Putnam and Defrank, 1983; Barnes and Putnam, 1986; and Smeda and Weller 1986. The difference between treatments and years could be related to the different weed spectra, environmental conditions, and possible concentrations of allelochemicals present from residues. In both years, common purslane was the predominant weed species, representing 77 to 78% of the population in 1988 and 64 to 89% in 1989 depending on the time sampled. In 1988, other predominante weeds included redroot and prostrate pigweeds (16%) and witchgrass (29%). In 1989, 15% of the population were miscellaneous weed species with redroot pigweed (7%) being second in abundance.

With 1989 having more rainfall, it seems likely that allelochemicals would be leached faster from the residues over a shorter time interval. Also in 1988, the comparison of PE with cover crop residues at 42 DAP indicated weed biomass was the highest with the former treatment. This suggests that the cover crop residues are supressing weeds other than by a competitive manner, suggesting allelopathy. When comparing the total weed biomass production at 81 days in 1989 there was excessive weed biomass in all of the residue treatments, none of which differed from the control. The effect of the cover crop had been lost probably because the toxins had been leached from the residues.

Total decomposition of the cover crops, at 42 or 81 DAP, was between 62 to 81% in both years while PE decomposed only about 15% over the duration of the experiments (Table 2). The order from least to greatest biomass of cover crops was rye < oats < spring barley < PE. The fact that weed suppression was not related to cover crop biomass in 1988 provides indirect evidence for allelopathy since the cover having the least biomass had the greatest weed-suppressing activity for both years.

The yield data of both plants and fruit over the two seasons indicated several important diferences. Total cucumber plant weight in 1988 for all treatments was less than half that recorded for 1989 (Table 3). Generally higher fruit yields were recorded in 1988. Lower cucumber plant weights in 1988 could be explained by the higher temperatures and dryer conditions early in the growing season which stressed plant growth. Still the plants in the residues were able to recover and outproduce those in the bareground checks. The higher fruit yield in 1988, compared to 1989, could be attributed either to the plant residue retaining more moisture during the early part of the 1988 season or the enhanced phytotoxicity of the residues exhibited during the early damp part of the 1989 season. These conditions could have liberated more water soluble allelochemicals that could have affected crop growth.

Snap bean plant yields were similar for both years but fruit yields in 1989 were 60% of those for 1988 in the bareground plots. This difference could suggest that the plant residues in 1988 provided a better growing environment by providing improved moisture earlier in the growing season, while in 1989 there was excessive moisture during

Table 2: Decomposition of Cover Crops During 1988 and 1989.[1]

	1988			1989		
Cover crop	(DAP) 21	32	42	10	41	86
	($G \cdot M^{-2}$)					
Oats	153.6	141.5	58.0	211.4	133.4	49.3
S. Barley	184.0	216.5	119.8	194.4	139.2	70.3
Rye	129.4	74.8	42.2	143.2	112.0	27.0
Poplar Excelsior	335.1	297.5	284.0	326.8	221.6	270.8
LSD (.05)		25.1			24.8	

1 - dry weight of shoot biomass only.

TABLE 3: Cucumber and Snap Bean Yields as Influenced by Cover Crops and Weeding in 1988 and 1989.

A. Cucumbers

		Plant			Fruit	
		1988	1989[1]		1988[3]	1989[3]
Cover crops	(DAP)	48	47		48	47
			Weeded	Nonweeded		
		($G \cdot M^{-1}$)			($T \cdot ha^{-1}$)	
Oats		72.4	154.3	112.5	13.5	10.7
S. Barley		81.3	159.2	96.9	11.6	8.9
Rye		59.8	125.9	84.9	13.0	8.9
Poplar Excelsior		73.8	180.9	124.7	14.9	6.3
None		59.2	238.7	204.1	7.7	13.2
LSD (.05)		NS			NS	
		Mean[2]	171.8	124.6		1.2

1 - F value for cover crop effects are NS.
2 - F value for main effect of weeded vs nonweeded is significant at P=.01.
3 - F value for cover crops vs none is significant at P=0.01.

TABLE 3: Cucumber and Snap Bean Yields as Influenced by Cover Crops and Weeding in 1988 and 1989.

B. Snap Bean

	Plant			Fruit		
	1988	1989[1]		1988	1989[1]	
Cover Crop (DAP)	86	81		86[3]	81	
		Weed	Nonweed		Weeded	Nonweed
	($G \cdot M^{-1}$)			($T \cdot ha^{-1}$)		
Oats	197.2	188.3	197.2	9.4	2.7	1.6
S. Barley	215.4	156.6	215.4	9.0	2.3	1.8
Rye	218.9	143.6	218.9	13.9	2.1	1.2
Poplar Excelsior	205.2	194.5	205.5	13.3	2.8	2.4
None	151.4	198.9	151.5	6.5	2.9	2.0
LSD (.05)	66.8			4.4		
		Mean[2] 176.4	197.7		Mean[2] 2.6	1.8

1 - F value for cover crop effects are NS.
2 - F value for main effect of weeded vs nonweeded is significant at P=.01.
3 - F value for cover crops vs none is significant at P=0.01.

planting time. This higher moisture level not only could have caused more allelochemicals to be released in a shorter time, but may also have caused lower soil temperatures unfavorable for bean growth.

In 1989, cucumber yield decreased 19 to 48% when cover crop residues were compared to the bareground treatments. This might be explained by the residues causing either too moist a condition or temperatures less than ideal during the early season (See Table 4).

The snap bean yield results for 1988 and 1989 show the same reversals. It appears that the beans and cucumbers responded similarly to conditions in soil moisture and temperature which might explain the reduced yields in 1989. In 1989, temperatures were as much as 4^{o}C higher in bareground plots while moisture levels were up to 18% higher in the residue plots (Table 4). If the temperatures were limiting for cucumber and snap bean growth, they could delay germination of the plants which would have increased the exposure to toxic components from the residues. Also the soils under higher moisture conditions might have been firmer and less friable restricting emergence of dicot crops. Early stand data for both cucumber and snap bean in 1989 reflected poorer germination or emergence caused by the residues when compared to bareground (Table 5). This can be seen when one examines the progression of plant populations from 11 to 81 DAP. Early stands of snap beans appeared to be affected less by the residues than the cucumbers, although snap bean plant weights were about the same for both years. Fruit yield was greatly reduced in 1989 either due to poor fruit set or possibly not enough heat units over the same time frame to produce yields equal to those of 1988.

Controlled Environment Experiments: When evaluating the placement of

TABLE 4: 1989 Temperature and Soil Moisture Comparisons Under Different Cover Crop Systems.

Cover Crop	(DAP) 3	11	12	15
	(%H20)	----------(°C)[1]----------		
Oats	12.6	29.6	32.1	31.6
S. Barley	11.6	29.2	29.7	31.0
Rye	13.3	30.8	31.1	31.6
Poplar Excelsior	11.0	30.7	32.6	31.5
None	10.9	32.3	36.0	31.0
LSD (.05)	1.7	.9	1.2	NS

1 = Soil temperatures taken between 1300 and 1400 hours at 6.8 cm.

TABLE 5: 1988 and 1989 Cucumber and Snap Bean Plant Stands as Influenced by Cover Crop Residue.

A. Cucumber Plant Stand

	1988		1989[1]			
Cover crop (DAP)	44	48	11	15	52	
					Weed	Nonweed
	(NO. $\cdot M^{-2}$)					
Oats	3.3	4.4	3.6	3.8	5.4	3.9
S. Barley	4.4	4.4	3.5	3.0	5.6	4.7
Rye	4.8	4.1	3.5	2.9	5.0	4.2
Poplar Excelsior	7.2	5.3	2.7	3.1	5.9	4.5
None	6.0	6.0	6.6	6.5	8.9	7.9
LSD(.05)	3.0	2.8	1.5	1.9	Mean[2] 6.2	5.0

1 - F value for cover crop effects and the interaction are NS.
2 - F value for main effect of weeded vs nonweeded is significant at P = .01

TABLE 5: 1988 and 1989 Cucumber and Snap Bean Plant Stands as Infulenced by Cover Crop Residue.

B. Snap Bean Plant Stand

	1988	1989[1]			
Cover Crop	(DAP) 88	11	15	81	
				Weed	Nonweed
	(NO.·M^{-2})				
Oats	8.5	5.7	7.3	10.7	8.8
S. Barley	8.7	5.5	6.6	8.9	8.9
Rye	8.3	4.8	6.7	9.6	7.7
Poplar Excelsior	9.6	7.3	8.1	11.2	10.1
None	6.9	9.5	10.0	11.4	10.5
LSD (.05)	2.9	2.4	1.7		
Mean[2]				10.4	9.2

1 - F value for cover crop effects and the interaction are NS.
2 - F value for main effect of weeded vs nonweeded is significant at P = .01

residue in order to eliminate crop injury, the residues generally need to be positioned at a distance of 1.3 cm or greater from the seed to eliminate toxicity (Tables 6 and 7). When rye or poplar excelsior residues were placed on the soil surface or 1.3 cm depth with the seeds, snap bean emergence was reduced compared to when the residues were placed at 2.6 or 3.9 cm depths. However, emergence in all residue treatments was better than that acheived with bareground. Reduced snap bean seed germination might be attributed to fluxes in soil moisture whereas with the residue present the soil moisture content would be more constant.

Snap bean seedling weights were reduced only when the rye and poplar excelsior were placed on the soil surface at the 10 gram rate. Shoots were more sensitive than roots to the placement of residues when comparing the 0 and 1.3 cm depth when compared to the 2.6 and 3.9 cm treatments. Cucumber germination was reduced when rye residues were placed with seeds at both the 5 and 10 gram rate, while both rates either at the soil surface or at 1.3 cm depth significantly reduced the growth of cucumber seedlings. This suggests that a certain level of rye is needed to liberate enough toxins to reduce cucumber growth. At the 5 gram rate the residue must be in close contact with the seed to produce this effect. Also, with the soil surface treatment being the most active, and shoots being the most sensitive, one might predict that residues placed on the surface would have the greatest impact on germinating seeds.

Rye and PE residues (+ or - microbes) were compared 4 and 8 days following leaching. Non-leached rye residues exhibited a 61 to 64% reduction in cucumber germination and a 66 to 71% reduction in

TABLE 6: Snap Bean Germination and Growth as Influenced by Placement of Rye and Poplar Excelsior Residues[1]

	Rye		PE	Rye		PE
depth (cm)	5 g	10 g	10 g	5 g	10 g	10 g
	(NO.·POT^{-1})			(Total mg·POT^{-1})		
0	2.1	2.2	3.2	549	567	754
1.3	4.3	5.1	6.3	901	974	1230
2.6	3.3	5.8	4.7	771	1233	872
3.9	4.1	4.8		837	1027	
no residue	1.8			313		
LSD (.05)		1.7			393	

1 - Data represent means from three experiments each having three blocks.

Table 7: Cucumber Germination and Growth as Influenced by Placement of Rye and Poplar Excelsior Residues[1].

	Rye		PE	Rye		PE
depth (cm)	5 g	10 g	10 g	5 g	10 g	10 g
	(NO.·POT^{-1})			(Total mg·POT^{-1})		
0	4.9	2.4	6.6	322	136	379
1.3	4.3	3.7	7.3	332	257	485
2.6	6.1	5.7	7.8	479	413	564
3.9	7.1	7.8		530	770	
no residue	7.3			543		
LSD (.05)		1.46			118	

1 - Data represent means from three experiments each having three blocks.

seedling growth when compared to the PE control mulch or residues leached for 4 to 8 days (Table 8). This suggests that compounds toxic to cucumbers are present in nonleached rye and could be released rapidly upon injury of the plant. The rapid reduction in toxicity of the leached residues suggests that these compounds are relatively water soluble. Snap bean assays were variable and inconclusive either due to nonuniform seed germination or a poor growing environment.

Conclusions: There are several environmental factors including moisture, temperature and desiccation time which can influence the activity of decomposing plant residues. As these results and others suggest, there appears to be a time interval of about 25 days after desiccation when residues can greatly reduce weed growth (Barnes and Putnam, 1983; Smeda and Putnam, 1988). Harper and Lynch (1981) suggested that there are seasonal rate changes in straw decomposition, and under anaerobic or high moisture situations greater phytotoxic activity occurs. This data suggested that after the plant residues are decomposed to 35 to 70% of the orginal weight, no weed suppressing activity remains. As observed in 1989, and previously reported (Glenn and Welker, 1989; Blevins et al., 1971) plots with residues had higher soil moisture contents which could have caused toxins to be liberated quickly. Since 1988 was a drier year, the release of phytotoxins could have been delayed under low moisture conditions and thus remained at a toxic level over a longer period of time (Cochran et al., 1977). The environmental differences which occurred during the two years of our studies could help explain the difference in crop and weed response to allelochemicals. For example, temperatures are generally lowered under NT system (Wall and Stobbe, 1984; Derpsch et al., 1986; Peterson et

Table 8: Cucumber germination and seedling growth as influenced by periods of leaching and microbial degradation of rye or poplar excelsior residues.

Days	Rye [1]	PE	Rye	PE
	---(NO.·Pot^{-1})---		----(mg·Pot^{-1})----	
0	2.8	7.5	82	259
4	7.8	7.8	272	257
8	7.1	7.5	240	273
LSD (.05)	0.94		53	

1 - Since F value for + or - microbes was NS, data points represents mean of combined data. All other interactions were NS.

al., 1986) which could explain the slower germination of seeds and lower yields that were obtained in 1989. The greater sensitivity of shoots to cover crops under slower germination conditions could also have contributed to the reduced yields in 1989. The mechanisms of interference occurring under field experiments are difficult to separate (Fuerst and Putnam, 1983). The cover crops used here have been reported to have chemical activity, but in the field, this undoubtedly interacts with other competitive components of interference. Alemeida (1985) related the greatest chemical activity of plant residues with those that had the greatest biomass production. In contrast, our results indicated that the residue with the most activity was rye which also had the least residue biomass. Therefore, Alemeida's results may have reflected light, water, or nutrient competition effects more than allelopathy.

The majority of weeds present in these studies were broadleaved species. Alemeida (1985) suggested different residues can be selective for either grass or broadleaved weeds. Barnes and Putnam (1986), and Shilling et al. (1985) have reported rye residues to be more active on dicot than monocot species. This occurred in our 1988 research (a drier year), while in 1989 rye was the only residue producing a significant reduction of dicot weeds compared to a bareground control. Knowing that dicots are more sensitive to residues than monocots makes it imperative to test dicot crops for safety under a variety of environments. Results of Putnam and Defrank (1983) and these results indicate that larger seeded crops are less effected by residues. This could be because larger seedlings are more vigorous or because of the placement of the seed below the greatest allelochemical concentration

zones. When Christian et al. (1985) removed the residues from the surface by burning, he removed 29% of the toxicity observed on the crop was also removed.

The reduction in both snap bean and cucumber weight when the residues were placed on the surface can be explained by light interference as well as allelopathy and can be separated to some extent by the use of PE. These results indicate that shoots are more sensitive than roots to rye and the zone of greatest crop activity is in the upper 1.3 cm of soil. This also explains the reduction in weed biomass with cover crop residues compared to PE since the majority of weed seeds germinate in this 1.3 cm zone.

As Putnam and Duke (1978) and Rice (1984) indicated, if the time interval is long enough, toxins can be liberated from residues and either be decomposed or leached to eliminate any weed and crop reductions. Harper and Lynch (1982), Alemeida (1985), Smeda and Putnam (1988), Srivastava et al. (1986), Guenzi et al. (1967) have all reported residue phytotoxicity at different time intervals. It is apparent that this time interval difference is related to the different crop microclimates affecting leaching and the decomposition of the residues. Ruize-sifre and Ries (1983), Knavel et al. (1977), Mascianica et al. (1986), Teasdale and Frank (1983), and Williams et al. (1973) have all shown positive responses to plant residues when growing NT snap beans under normal to dry growing conditions. If a vigorous crop can be established and quickly produce a canopy to reduce the light penetrating to the soil surface, further weed reductions are apparent. Tiwari et al. (1987) suggested that under drier conditions there is little toxic substance release from plant material. If this

is the case, the reduction in weed biomass earlier in the season can be attributed more to shading while the later response is due to the allelochemicals being released. Also in wetter growing seasons, residues have the potential to cause poorer crop germination, reduced growth and lower yields either by modifying soil conditions or releasing more phytotoxins which in turn inhibit crop growth.

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CHAPTER 3

Identification of the Soil Microbe Responsible for the Transformation of BOA to AZOB and quantification of AZOB in Field Soils

ABSTRACT

Nutrient agar plates enriched with fungicidal and bactericidal compounds were used to isolate and identify a soil microbe responsible for the biotransformation of 2(3H)-benzoxazolinone (BOA) to 2,2'-oxo-1,1'-azobenzene (AZOB). Experiments were conducted to evaluate the transformation process of BOA to AZOB in the presence of this microbe under sterile and non-sterile conditions. The quantification of unreacted BOA and AZOB in field soil was achieved by UV-visible spectroscopic methods. The yield of AZOB isolated from field soil showed a 3 fold increase over reported values. Transformation studies with soils inoculated with Acinetobacter calcoacetius indicated that the production of AZOB increased linearly with the concentration of BOA in sterile soils and showed a quadratic trend in non-sterile soils. This also established that all soil types studied for the transformation experiments contained either Acinetobacter calcoacetius or another organism capable of the conversion of BOA to AZOB. Conversion of BOA to AZOB occurred rapidly in all four soils studied.

CHAPTER 3

INTRODUCTION

Numerous allelochemicals have been isolated and identified from rye (Secale cereale L.) herbage. Chou and Patrick (1976) isolated nine compounds from decaying rye residues and characterized them to be vanillic, ferulic, phenyllacetic, 4-phenylbutyric, P-coumaric, P-hydroxybenzoic, salicylic, O-coumaric acids, and salicylaldehyde. All nine compounds were found to inhibit lettuce (Lactuca sativa L.) seed germination. Shilling et al. (1985) isolated phenyllactic acid and hydroxybutyric acid from field grown rye and observed the inhibitory activity on redroot pigweed (Amaranthus retroflexus L.) and common lambsquarters (Chenopodium album L.) for germination and root growth. Barnes et al. (1987) isolated two hydroxamic acids, 2,4-dihydroxy-1,4(2H)-beenzoxazin-3-one (DIBOA) and 2(3H)-benzoxazoline (BOA), from rye residues, compared their toxicity to phenyllactic acid and hydroxybutyric acid and found that the hydroxamic acids, DIBOA and BOA, exhibited the highest toxicity to both monocots and dicots.

Virtanen et al. (1957) reported the presence of BOA in 4-day old rye seedlings. The benzoxazinones are proposed to occur in plants as glucosides and upon injury are released to DIBOA by glucosidase enzymes (Hofman and Hofmanova, 1968; Zungica et al., 1983). The quantities of benzoxazinones in the plant have been shown to vary with the species (Zungica et al., 1983), age (Argandona et al., 1981) and depend on the part of the plant used (Tang et al., 1975; Barnes et al., 1987). More recently, Nair et al. (1990) reported the production of an azoperoxide, 2,2',oxo-1,1'-azobenzene (AZOB) in field soils incubated with BOA.

They also showed that the benzoxazinones produced by rye, DIBOA and BOA, could rapidly undergo microbial transformation to AZOB. This new allelochemical AZOB exhibited more inhibition to garden cress (Lepidium sativum L.) (a dicot) root elongation at all rates studied (67 to 250 ppm) whereas for barnyardgrass (Echinochloa crus-galli L.) (a monocot) more inhibitory activity occurred at 67 ppm. The higher rates produced toxicity on barnyardgrass similar to the two benzoxazinones.

Allelochemicals can be released into the environment by processes such as volatilization, root exudation, leaching, microbial transformation, and release of toxins from decomposition of plant residues (Chapman and Lynch, 1983; Rice, 1984; Putnam, 1984). The involvement of microorganisms to either release or produce phytotoxins from residues or use the residue as a substrate to produce bioactive compounds has been well documented. For example, Proebsting and Gilmore (1941) reported that with soil microbes and peach tree roots present there was severe inhibition in growth of young peach trees planted in the peach tree roots-soil mixture. Initially they suspected that the chemical amygdalin leached from the tree roots and was responsible for this inhibition. However applying this compound to the soil did not inhibit the growth of peach trees. Later, Patrick (1955) examined the involvement of microorganisms in the production of toxicity from peach root bark and isolated several microorganisms capable of degrading amygdalin in the soil. Based on these tests, he proposed the toxic effects of peach root bark in the soil were caused by hydrogen cyanide and benzaldehyde produced by microbial decomposition of amygdalin.

Investigation of the crops containing benzoxazolinones for their potential germicidal, insecticidal, and herbicidal activity involved quantification of these compounds and studies of their transformation in the environment. Quantification of hydroxamic acids in plant material or soil was achieved earlier by four methods; tracing C^{14} isotopes, direct measurement of benzoxazolinone at a specific wavelength, spectrofluorometry, and by the use of colorimetric reagents such as $FeCl_3$ or $CeSO_4$ (Long et al., 1974; Barnes et al, 1987). However, the spectroscopic measurement of benzoxazolinones and azoperoxide is a convenient method to quantify these compounds in plant or soil extracts. By monitoring the optical density (OD) of the benzoxazolinones or azoperoxide, at their λ max in MeOH solutions gives accurate concentrations of these compounds in soil or plant extracts.

Rye is considered an important cover crop in Michigan, other parts of the United States, and around the world (Putnam et al., 1984; Smeda and Weller, 1986). Allelopathic effects and weed control activity of rye residues were attributed partially to DIBOA and BOA (Barnes et al., 1987). Discovery of AZOB in the field soil containing BOA and its enhanced allelopathic activity over BOA and DIBOA was additional chemical support for the strong allelopathic activity demonstrated by rye residue (Nair et al., 1990). Therefore, further research was conducted on the transformation of BOA to AZOB in field soils. The objectives of this study were a) to isolate and identify the soil microbes capable of transforming BOA to AZOB and b) to study the rate of transformation of benzoxazolinones by this soil microbe in Michigan field soils.

MATERIALS AND METHODS

General experimental: Ultraviolet (UV) and visible absorption spectral analysis were performed on a Gilford Response II ultraviolet spectrophotometer. BOA was obtained commercially from the Aldrich Chemical company, Milwaukee, Wisconsin and was uniformly mixed with soil using a mortor and pestle before adding water, and vortexing (1 min) on a Scientific Products S8220 Deluxe Mixer at 50 cycles per second. Column chromatography was performed using a J.T. Baker Flash Column (20 X 2.7 cm, 250 ml). The silica column used was (Analtech sovent, 35 to 75 micron particle size, 60 Angstrom pore size) and the flow rate was 3 ml/min unless otherwise specified. All TLC (250 micron) and preparative TLC were done on Anspec plates (1500 micron). Homogenization of microbial colonies was done on a Brinkman Polytron (CH-6010) blender for five minutes.

Soil collection. Spinks loamy sand soil (Psammentic, Hapludalfs, sandy, mixed, mesic) was collected (November, 1987) from the Horticulture Research Center field, located on Jolly road in East Lansing, Michigan. Soil was stored in a cubic yard bin at room temperature (15-22 ^{o}C) for one year prior to use. Three other soils, Kalamazoo sandy loam (Typic Hapludalfs, fine-loamy, mixed, mesic), Metea loam (Arenic Hapludalfs, loamy, mixed, mesic) and Oshtemo sandy loam (Typic Hapludalfs, coarse-loamy, mixed, mesic) were collected in October and November of 1988 and stored in the same conditions previously mentioned. The Kalamazoo Sandy loam soil was collected at the Clarksville Research Station located in Clarksville, MI, Oshtemo sandy loam from the Sodus Horticulture Research Farm located in Sodus, MI, and the Metea loam was collected from the Moore Seed Farm located

in Elsie, MI. Each of these three sites had two adjacent sampling areas, one with a gramineae crop grown the same year and another with no crop for two consecutive years. All soils were seived using an aluminium sieve (2 X 12 mm opening) prior to conducting the experiments.

Sterilization of soil: Spinks loamy sand soil was sterilized by weighing soil into Erlenmeyer flasks and autoclaving (1 h, 15 atmosphere, 120 ^{o}C) for 4 successive days. Distilled water used for the experiments was sterilized by autoclaving (20 min., 120 ^{o}C) in 250 ml erlenmeyer flasks (100 ml) or in 50 ml test tubes, each containing 10 ml.

Preparation of media: Yeast maltose glucose (YMG) agar, potato dextrose agar (PDA), and Nz-Amine agar were used for growth and identification of soil microbes. YMG was prepared by mixing yeast (4 g), maltose (10 g), glucose (4 g), and bacto-agar (18 g) in distilled water (1 L) and dispensed into 2 L erlenmeyer flasks (250 ml each) and autoclaved (20 min, 120 ^{o}C at 15 atmosphere). The cooled media (50 - 55 ^{o}C, 20 ml) was poured into petri dishes (100 X 15 mm) and allowed to settle at room temperature. The same procedure was repeated for PDA agar (PDA 39 g/L) and Nz-Amine agar (Nz-Amine agar 3 g/L, bactoagar 18 g/L) plates. All plates were stored at room temperature.

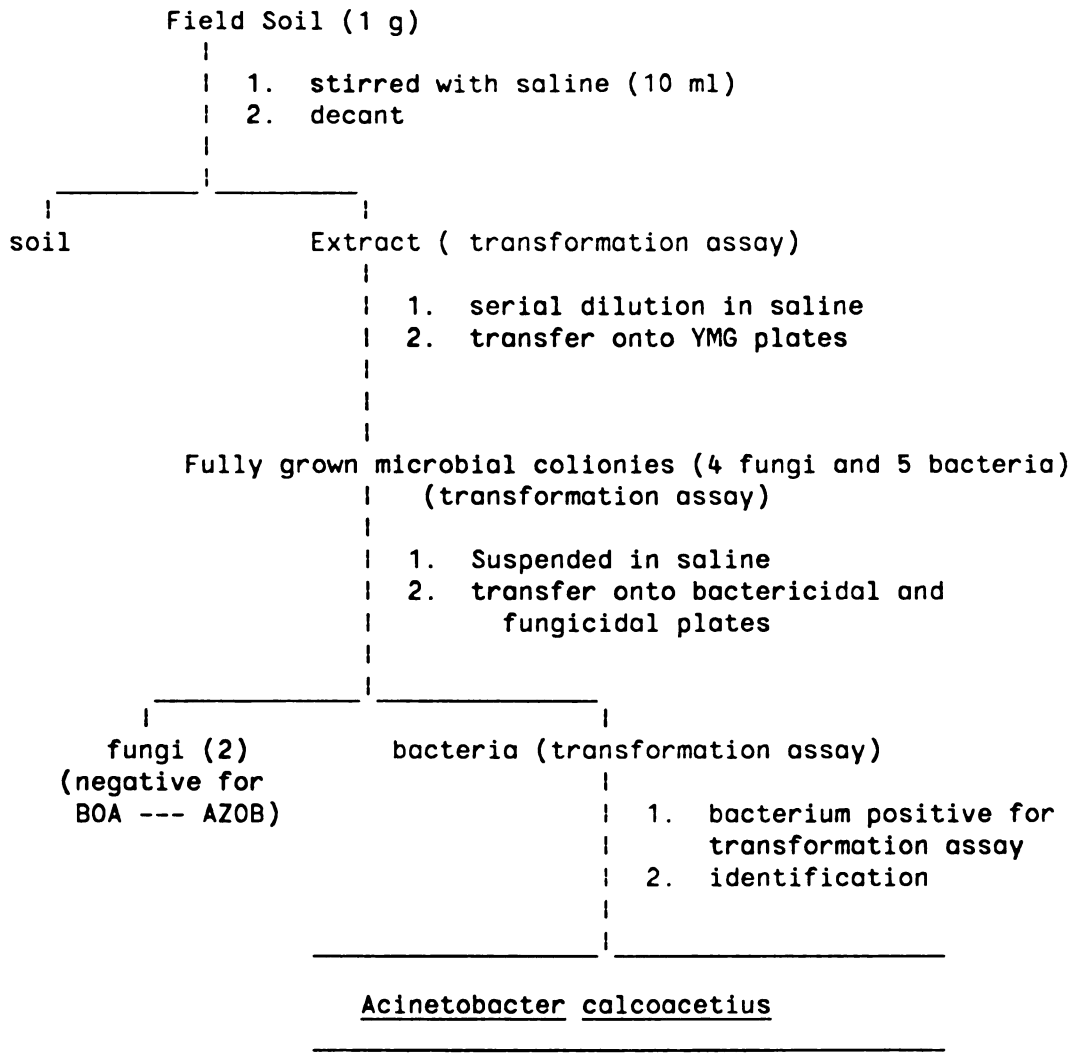
Antibacterial plates were prepared by dissolving PDA in distilled water and autoclaved as above. After cooling the medium to 50 - 55 ^{o}C, a solution of chloramphenicol (100 mg dissolved in 10 ml ethanol) was added and thoroughly mixed. Plates were then poured and stored at room temperature.

Antifungal Nz-Amine agar plates were prepared according to the

procedure described for antibacterial plates with the exception that Nystation (0.03 g dissolved in 1 ml DMSO) and cycloheximide (0.5 g dissolved in 10 ml acetone) were added to the cooling Nz-Amine agar. Plates were poured and stored at room temperature.

Isolation of soil microbes: Spinks loamy sand soil (1 g) was suspended in sterile physiological saline (9 ml) (0.85% NaCl in distilled water, w/v), vortexed (15 min) and allowed to settle (1 h). This soil extract was tested for in vitro transformation of BOA to AZOB by incubating BOA (10 mg) with sterile soil (100 g), according to the procedure published earlier (Nair et al., 1990). Cultures were allowed to incubate in the dark at 26 ^{o}C (96 h), and extracted with MeOH (50 ml X 3). Biotransformation of BOA to AZOB by the soil extract was verified by TLC comparison with the standard AZOB and by UV-visible spectroscopy.

Separation of total soil microbes into bacteria and fungi. Serial 10-fold dilutions of the supernatant/soil extract was prepared in sterile distilled water. Soil extracts (1 ml each) were pipetted into ten different autoclaved test tubes, diluted to 10 ml each by sterile distilled water and mixed well. Solutions from each tube (10 ul) were lawned seperately onto YMG plates. Plates at dilution of 10^{-6} showed the best separation of fungal and bacterial colonies. A total of 9 individual colonies were further transferred onto YMG plates separately and assayed with BOA for the production of AZOB. Colonies found to be positive for transformation of BOA to AZOB were transferred to Nz-Amine agar and PDA plates enriched with antibacterial and antifungal compounds, respectively, and incubated (72 h). Two fungi and one bacterium thus obtained were assayed for the transformation of BOA to AZOB. All fungi were found to be inefficient in the transformation



Scheme 1. Isolation, purification, and identification of a soil microbe responsible for BOA transformation to AZOB.

of BOA to AZOB however the bacterium was able to convert BOA to AZOB.

Identification of the microbe: The bacterium which converted BOA to AZOB was further purified on antifungal Nz-Amine plates. Morphological and biochemical properties of the bacterium were performed on a Viteck Auto Microbic System using Vitek GNT gram negative identification cards.

Spray reagents for TLC detection. DIBOA was detected on thin-layer plates with ferric chloride ($FeCl_3$) spray reagent consisting of 5% $FeCl_3$ in 95% ethanol, acidified with concentrated HCl. A spray reagent consisting of 1% ceric sulfate ($CeSO_4$) in concentrated H_2SO_4 was used to detect BOA.

Production and extraction of AZOB from soil. A commercial sample of BOA (4 g) was mixed thoroughly with Spinks loamy sand (4 kg) with distilled water (400 ml) and incubated (26 ^oC, 96 h) in 4 X 2 L Erlenmeyer flasks. The soil was extracted with methanol (8 X 500 ml) and filtered through a Buchner funnel using filter paper (Whatman No. 1). The extract was further filtered through a sintered glass filter (fine, 4 - 5 micron) and the solvent was removed by rotary evaporation. A preliminary TLC (toluene: ethylacetate, 5:4) of the crude extract indicated the presence of unreacted BOA and AZOB. Separation of BOA from AZOB was achieved by flash column chromatography (260 g, toluene: ethylacetate, 5:4). The orange band eluted was collected and dried in vacuo affording dark brown crystals of AZOB (1.18 g).

Production of AZOB in liquid media. Baffle-bottomed Erlenmeyer flasks (500 ml) containing distilled water (100 ml) were autoclaved and cooled to room temperature. BOA (80 mg) and freshly homogenized colonies of Acinetobacter calcoacetius (5.3×10^{10} CFU, 1 ml) were added to the

water and incubated (26 ^{o}C) under shake and non-shake conditions. The fermentation broth (96 h old) was extracted with EtOAc (3 X 75 ml). Shake and non-shake flasks were extracted with EtOAc separately. The EtOAc layer from shake flasks was dried over anhydrous $MgSO_4$ and evaporated *in vacuo* (4 day). Purification of AZOB from BOA by TLC (toluene: ethylacetate, 5:4) gave an orange band, free from BOA, and was eluted with $CHCl_3$:MeOH (1:1) and dried *in vacuo* affording pure cyrstals of AZOB (46 mg). A similar procedure was followed for the non-shake flasks but the yield of AZOB was not calculated.

Microbial transformation of BOA to AZOB: Experiments were conducted to study the rate of BOA transformation by the naturally occurring microbial population of *Acinetobacter calcoacetius* in field soil. BOA (3 and 30 mg each) was mixed with Spinks loamy sand (30 g each) containing distilled water (3.5 ml) in culture tubes (2.5 X 15.0 cm). Sterile styrofoam plugs were used to cap the tubes which were then wrapped with aluminum foil for the duration of the experiment. Culture tubes were placed in the dark and incubated (26 ^{o}C) for periods of 1, 2, 4, 6, 12, and 24 days. At the end of the incubation period, the soil was extracted with MeOH (3 X 150 ml). The extracts were dried over anhydrous $MgSO_4$ and the solvent was removed *in vacuo*. All dried samples were stored at -20 ^{o}C.

Quantification of BOA and AZOB in the soil extract: The samples were analyzed spectrophotometrically for BOA and AZOB. UV-visible spectra of BOA and AZOB in MeOH gave λ maximum at 273 and 432 nm, respectively (Figures 1 and 2). In equal mixtures of BOA/AZOB, the maxima were still distinct (Figure 3). Standard curves for BOA and AZOB were prepared using known quantities of BOA (from 0.748 to 286.0 ug/100 ml

in MeOH) and AZOB (from 1.51 to 96.402 ug/100 ml in MeOH) and by recording the absorbance at 273 and 432 nm, respectively, for BOA and AZOB.

Standard solutions of each of the dried soil extracts stored at -20 ^{o}C were prepared by dissolving the extract in MeOH in a 100 ml volumetric flask. The absorbance was recorded for each solution. Concentrations of BOA and AZOB in a given sample were obtained from the standard curve (Figures 4 and 5).

In Vitro transformation studies with pure cultures of Acinetobacter calcoacetius: Cultures of Acinetobacter Calcoacetius for transformation studies were prepared from the stock cultures grown on YMG plates and kept at 4^{o}C. The cultures used in each test were prepared by transferring a stored culture onto YMG plates followed by incubation (72 h) before use.

Acinetobacter calcoacetius colonies from eleven fully grown plates were pooled and suspended in 110 ml of sterile distilled water. A portion of this stock solution (3.5 ml) was then added to each treatment of BOA (3 mg) in sterile Spinks loamy sand (30 g) that was placed in culture tubes (2.5 X 15.0 cm). All treatments were incubated (26 ^{o}C) in the dark for 1, 2, 4, 6, 12, and 24 days and extracted with MeOH (3 X 150 ml). These soil extracts were quantified for BOA and AZOB by the spectroscopic procedure described earlier.

Microbial populations were estimated by monitoring the colony forming units (CFU) of the stock solution. Ten-fold serial dilutions were made in sterilized distilled water by transferring 1 ml of the stock culture to 9 ml of distilled water. After uniform mixing, 100 ul of each dilutions were placed onto YMG plates, sealed with parafilm and

incubated (26 ^{o}C, 24 h). The CFU of the stock solution was calculated to be 5.3 X 10^{10}.

Statistical methods. Data obtained in duplicated transformation studies were subjected to analysis of variance and means were compared with the least significant different (LSD) test.

RESULTS and DISCUSSION

Bacterium isolation: Initial purification of soil extracts containing total microbial isolates were positive for the conversion of BOA to AZOB. Partial purification of the total isolates into bacteria and fungi afforded one bacterium and two fungi positive for the tranformation of BOA to AZOB. However, final purification of the fungi on bactericidal plates did not show transformation of BOA and hence, they were not investigated further.

The microbe capable of transforming BOA to AZOB in field soil was identified as a gram negative rod-shaped bacterium with a tendency to form coccoid bodies and grew well in a medium containing 0.4% peptone and 0.02% tryptophan, under aerobic conditions. This organism was capable of using malonate as the sole source of carbon. Decarboxylase tests of the bacterium were negative with lysine, ornithine and arginine. Glucose was not fermented in the presence of specific inhibitors, DP300 and p-coumaric and did not produce acid with raffinose, sorbitol, sucrose, inositol, adonitol, rhamnose, arabinose, or glucose. Also, it did not hydrolyze esculin or o-nitrophenyl-B-D-galactopyranoside. Under aerobic conditions, it did not produce acid with glucose, lactose, maltose, mannitol or xylose. Tests for the production of H_2S, urease and plant indican were negative with this organism. The aforementioned properties are in conformity with those

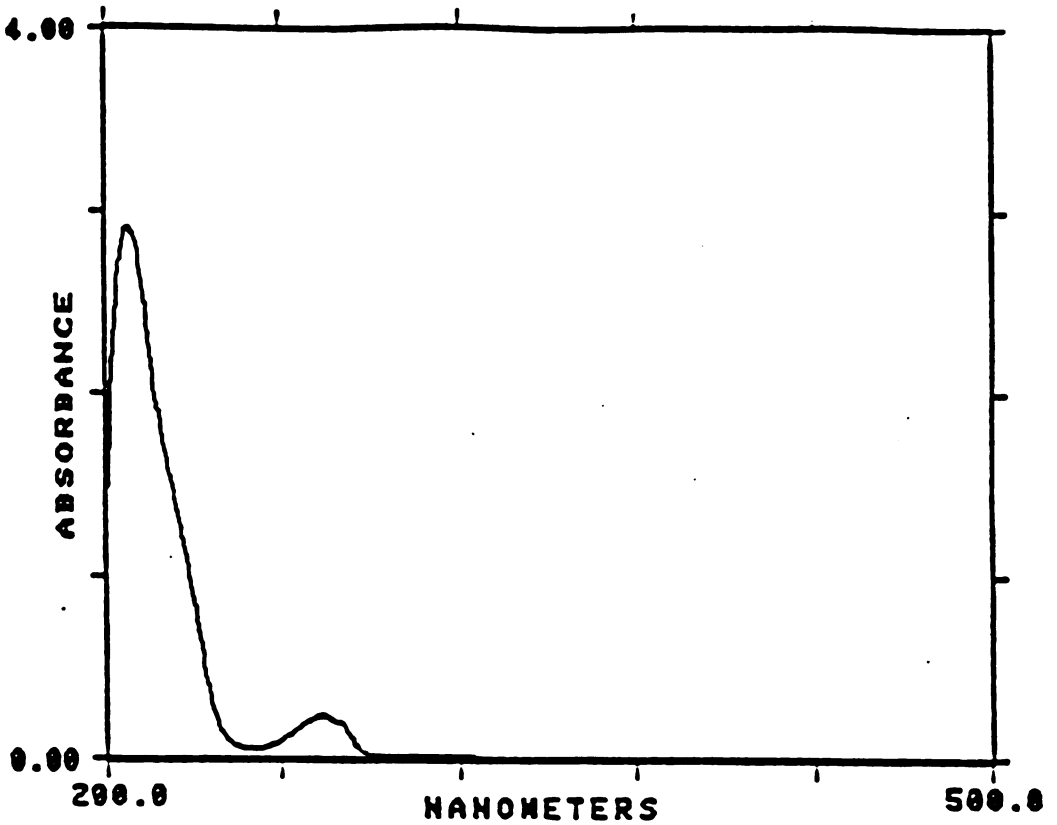
of Acinetobacter calcoacetius described in Bergey's manual of systematic Bacteriology (1984).

Spectra analysis of BOA and AZOB. Even though analysis of benzoxazolines were reported earlier (Virtanen and Hietala, 1957; Long et al., 1974; Nair et al., 1990), these methods are not convenient or efficient to quantify BOA and AZOB present in soil extracts. The UV-visible spectra of BOA (Figure 1) and AZOB (Figure 2) gave distinct absorption maxima at 273 and 432 nm. Since BOA and AZOB gave characteristic UV-visible absorption maxima, it is easy to calculate the concentrations of these compounds from their mixtures by monitoring the optical density (OD). This was confirmed from the concentration curves obtained for BOA and AZOB where OD was read at 273 and 432 nm, respectivitly. Standard curves were prepared separately by plotting each compound's concentration against its OD. The curve obtained from the mixtures of BOA and AZOB were identical to the curve generated by plotting concentrations of pure compounds against its OD (Figure 3). Concentrations of AZOB and unreacted BOA in the various soil extracts were obtained directly from the standard curve (Figures 4 and 5).

Microbial transformation. The percentages of AZOB isolated from in vitro transformation experiments was greater than previously reported (Nair et al., 1990). The modified extraction procedure of AZOB from soil gave yields three times greater (29.8% vs 9.1%) than previously reported values (Nair et al., 1990). This could be attributed to the new solvent system employed for the separation of AZOB from BOA. In the reported procedure (Nair et al., 1990), HCOOH and NH_4OH were used as part of the developing solvent system and these may have degraded a

Figure 1. UV spectrum of 2,3-benoxazolinone (BOA) in MeOH

Figure 2. UV spectrum of 2,2',oxo-1,1'-azobenzene (AZOB) in MeOH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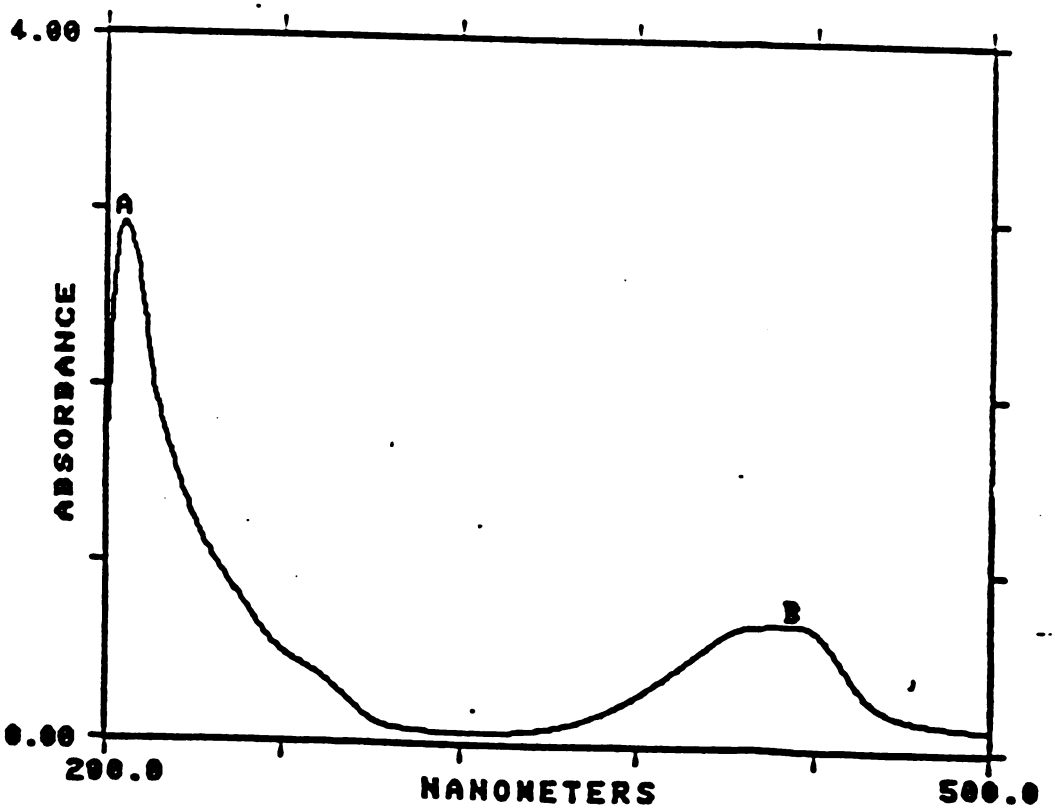
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Figure 3. UV spectrum of a mixture of BOA and AZOB (1:1) in MeOH

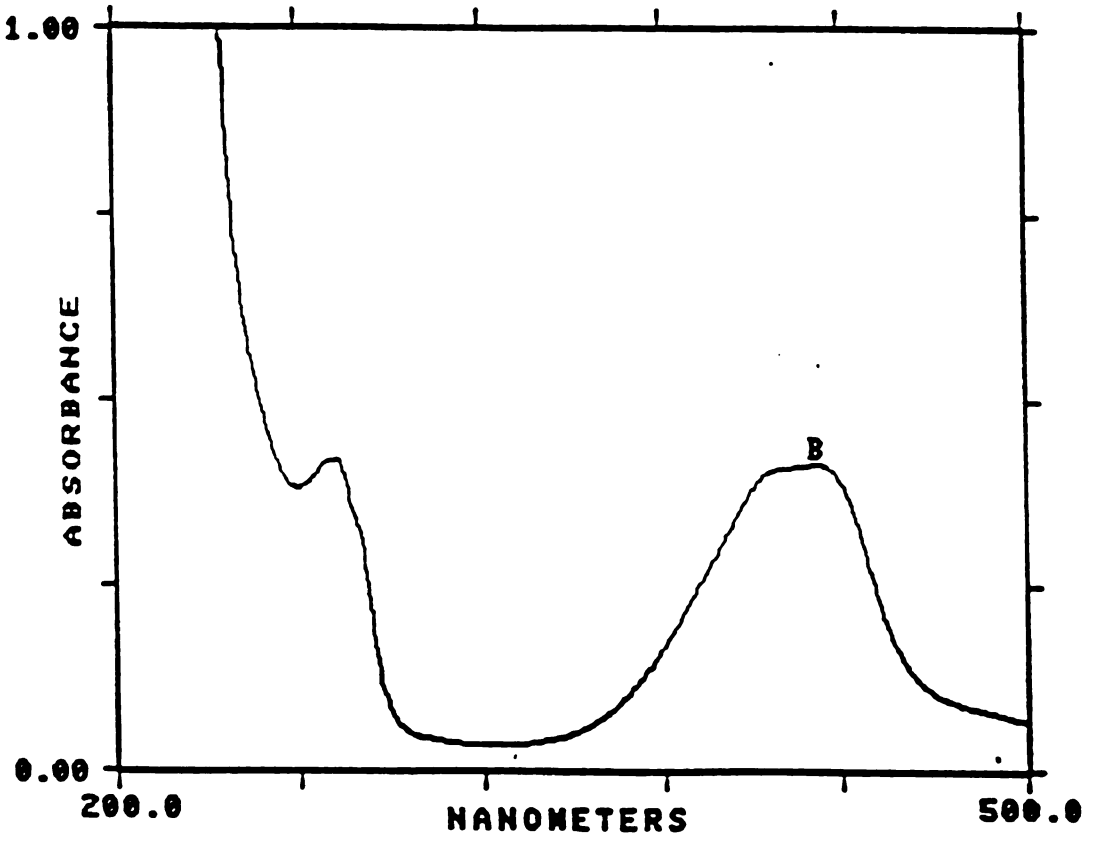
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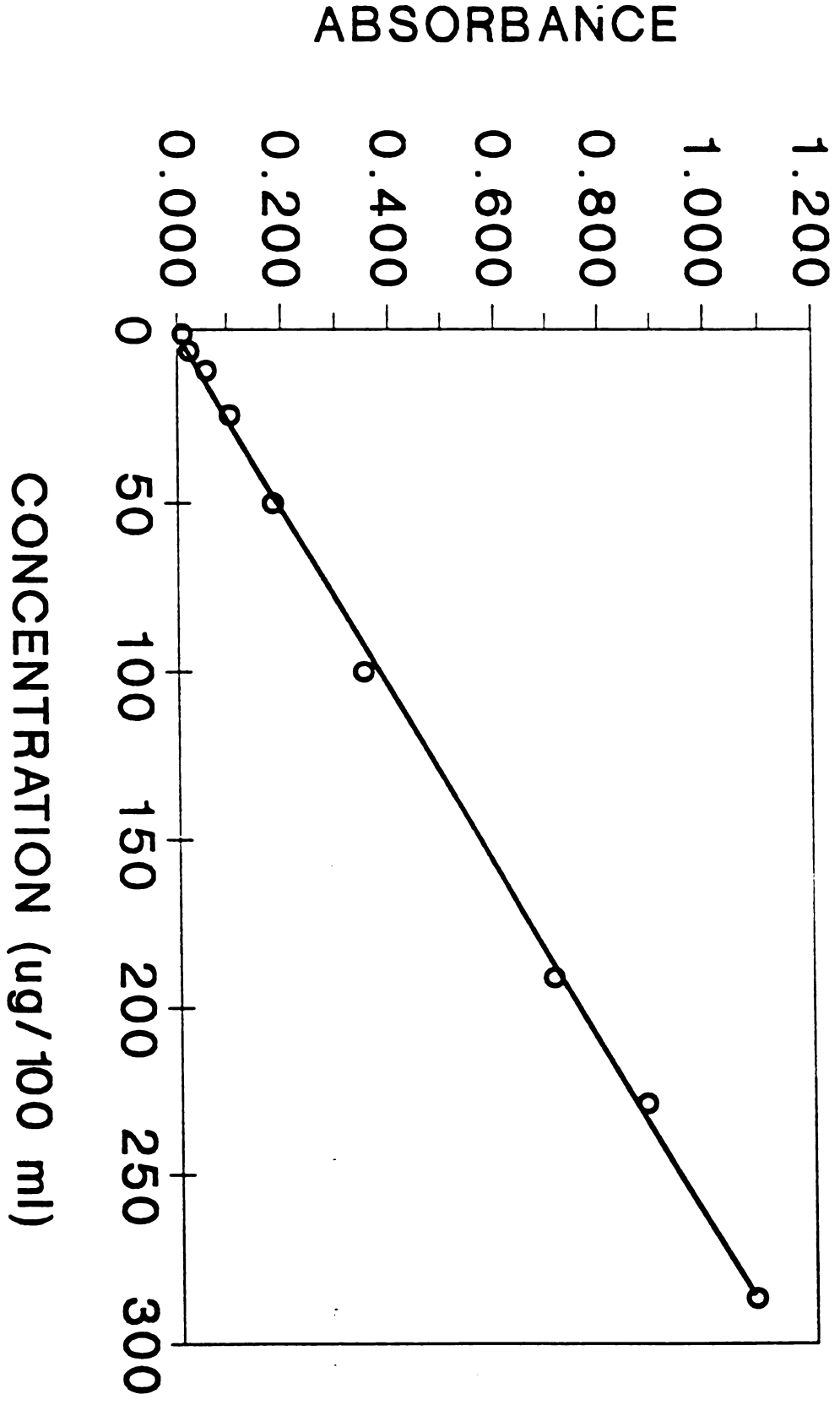
Figure 4. UV absorbance standard curve for varying concentrations of BOA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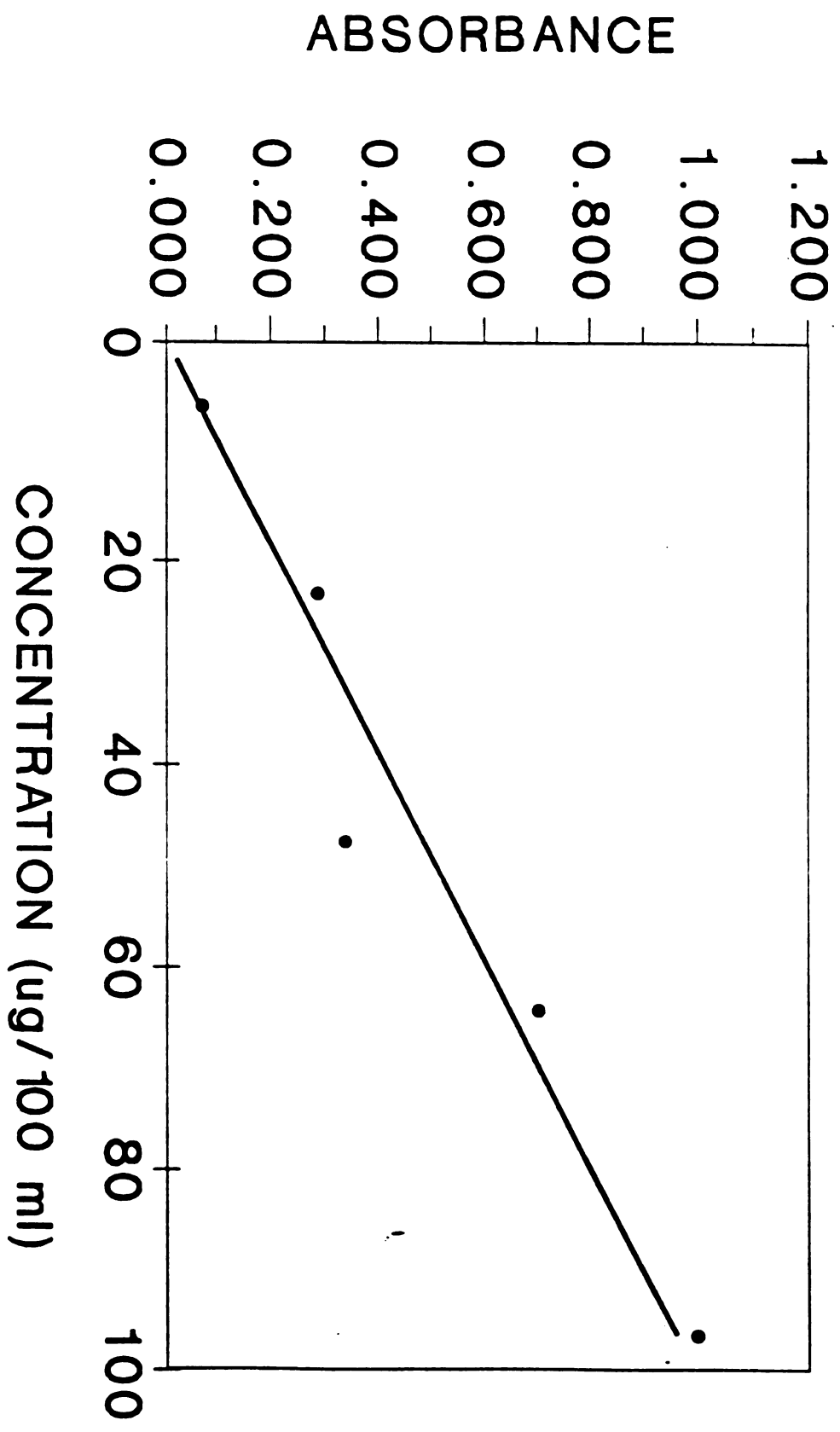
250
300
CONCENTRATION (ug/100 ml)

Figure 5. UV absorbance standard curve for varying concentration of AZOB


ABSORBANCE
0.000
0.200
0.400
0.600
0.800
1.000
1.200
CONCENTRATION (ug/100 ml)
0
20
40
60
80
100

portion of the AZOB isolated. The rate of production of AZOB in field soil was investigated under environmently controlled conditions over a period of 24 days using sterile and non-sterile soils. In both cases, Acinetobacter calcoacetius cultures were added (5.3×10^{10}) to the soils previously incorporated with BOA. The rate of production of AZOB was linearly related to the quantity of BOA available in the soil (Figure 6). At the end of day 12, only 7% AZOB was detected with a loss of 12% BOA. However, the 0.1 mg of BOA/g soil (non-sterile) experiment showed a quadratic trend in the production of AZOB (Figure 7). Maximum yield of AZOB (9.7 %) was observed during the incubation period of days 2 to 4 with an 85% decrease in BOA concentration. This is simliar to the 9.1% reported by Nair et al. (1990).

The recovery of BOA from the soil was 33% of the total amount incubated. The remainder of the BOA could have already converted to AZOB or a portion could have decomposed to products that are not yet characterized. Also, BOA might have decomposed to other products under mechanisms which are not yet known. However, a possible mechanism for the production of AZOB from BOA, monomethoxy AZOB (MAZOB) from MBOA and dimethoxy AZOB (DIMAZOB) from MBOA was described earlier by Nair et al. (1990). When the concentration of BOA in field soil was raised to 1.0 mg/g soil, the AZOB production increased linearly to the concentration of BOA (Figure 8). This indicated that the excess BOA had an impact on the total microflora in the soil. Usually, microbial populations present in the soil are low in numbers but explode quickly when a suitable substrate becomes available (Atalas and Bartha, 1987). Therefore having observed different trends for the BOA transformation in non-sterile field soil, it would seem logical that given the

Figure 6. Rate of transformation of BOA to AZOB in sterile soil incubated with Acinetobacter calcoacetius. Concentration of BOA was 0.1 mg/g soil.

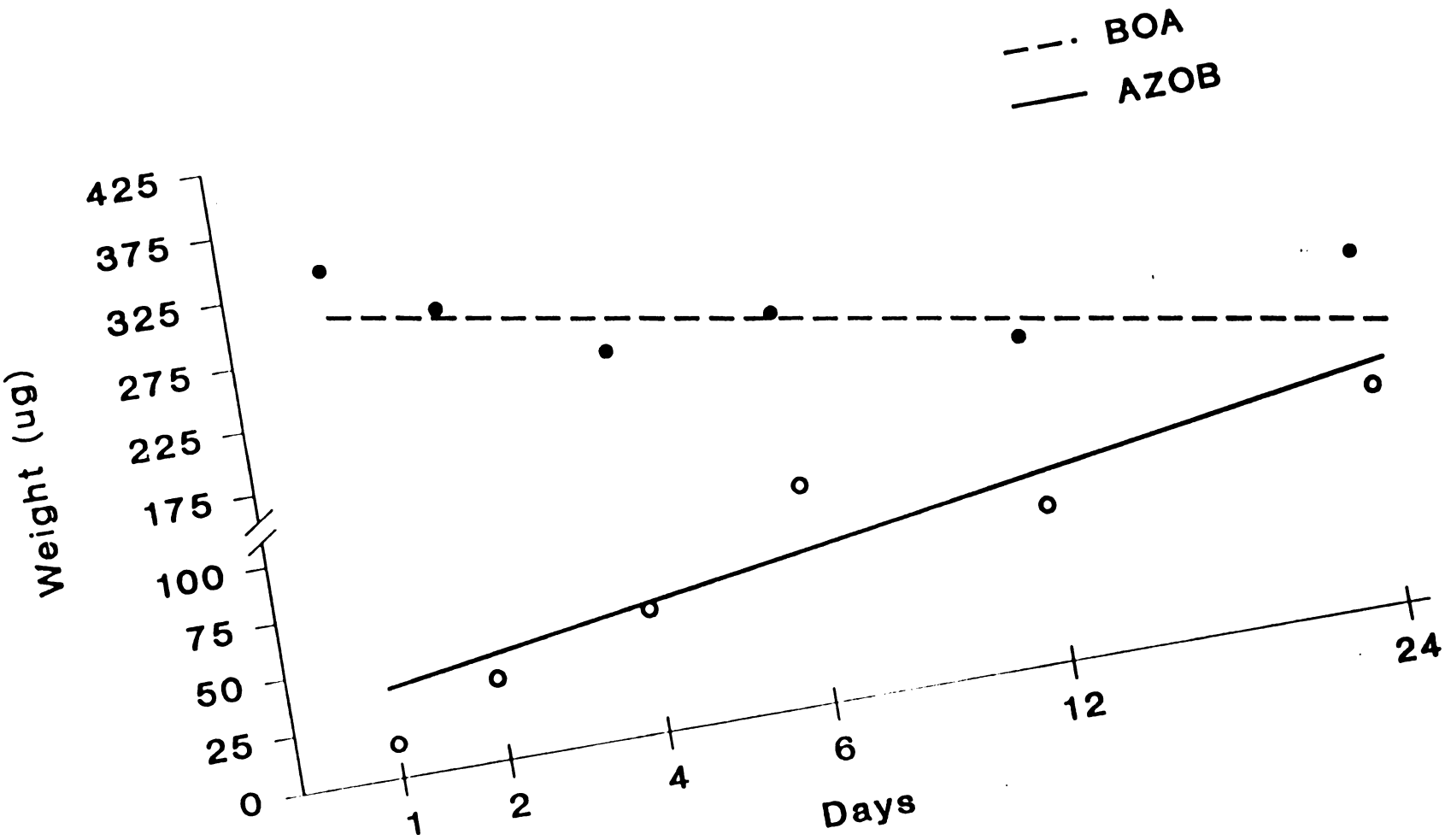

BOA
AZOB
Weight (ug)
425
375
325
275
225
175
100
75
50
25
0
1
2
4
6
12
24
Days

Figure 7. Rate of transformation of BOA to AZOB in non-sterile soil incubated with a BOA concentration 0.1 mg/g of soil.

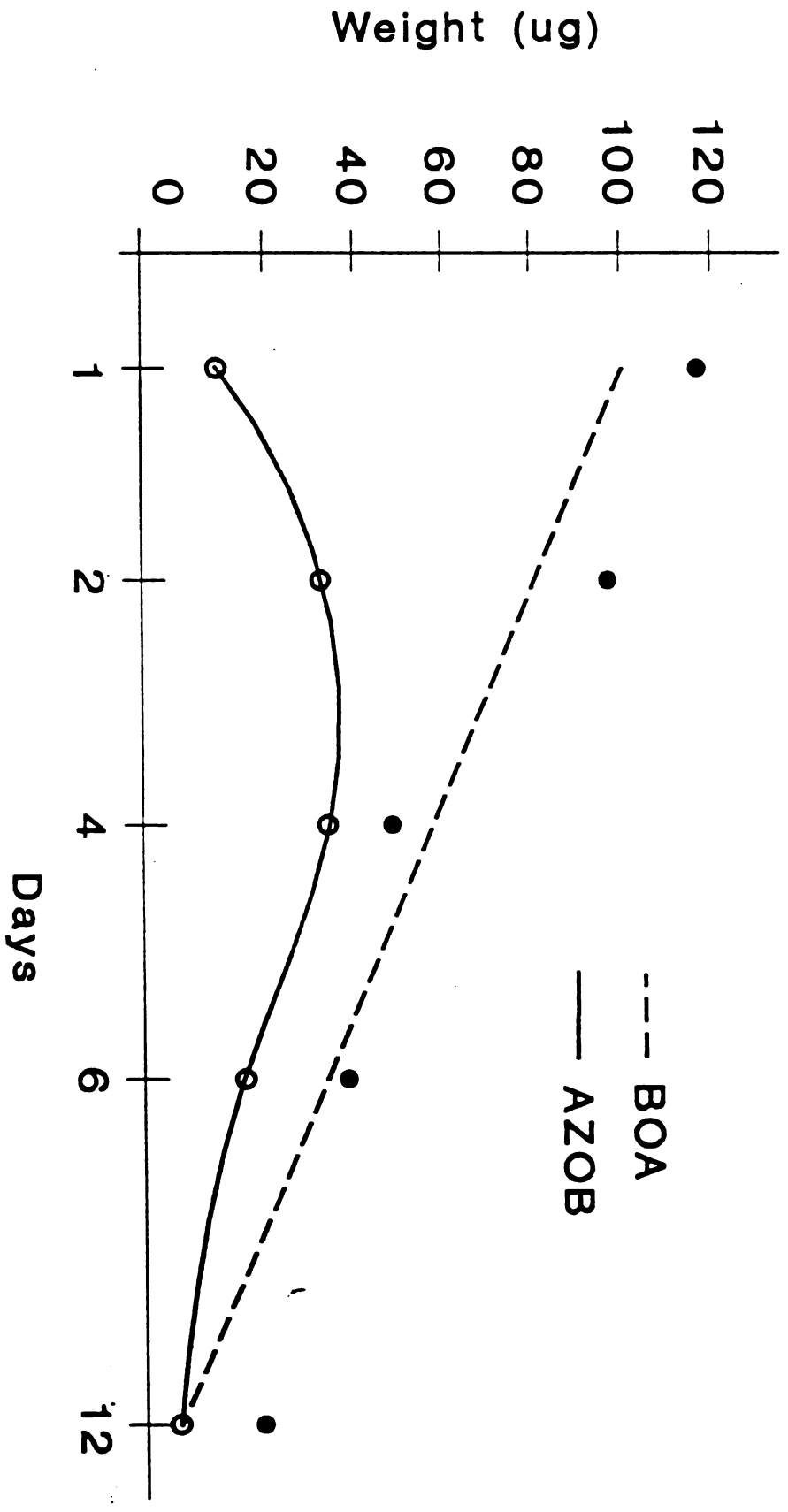
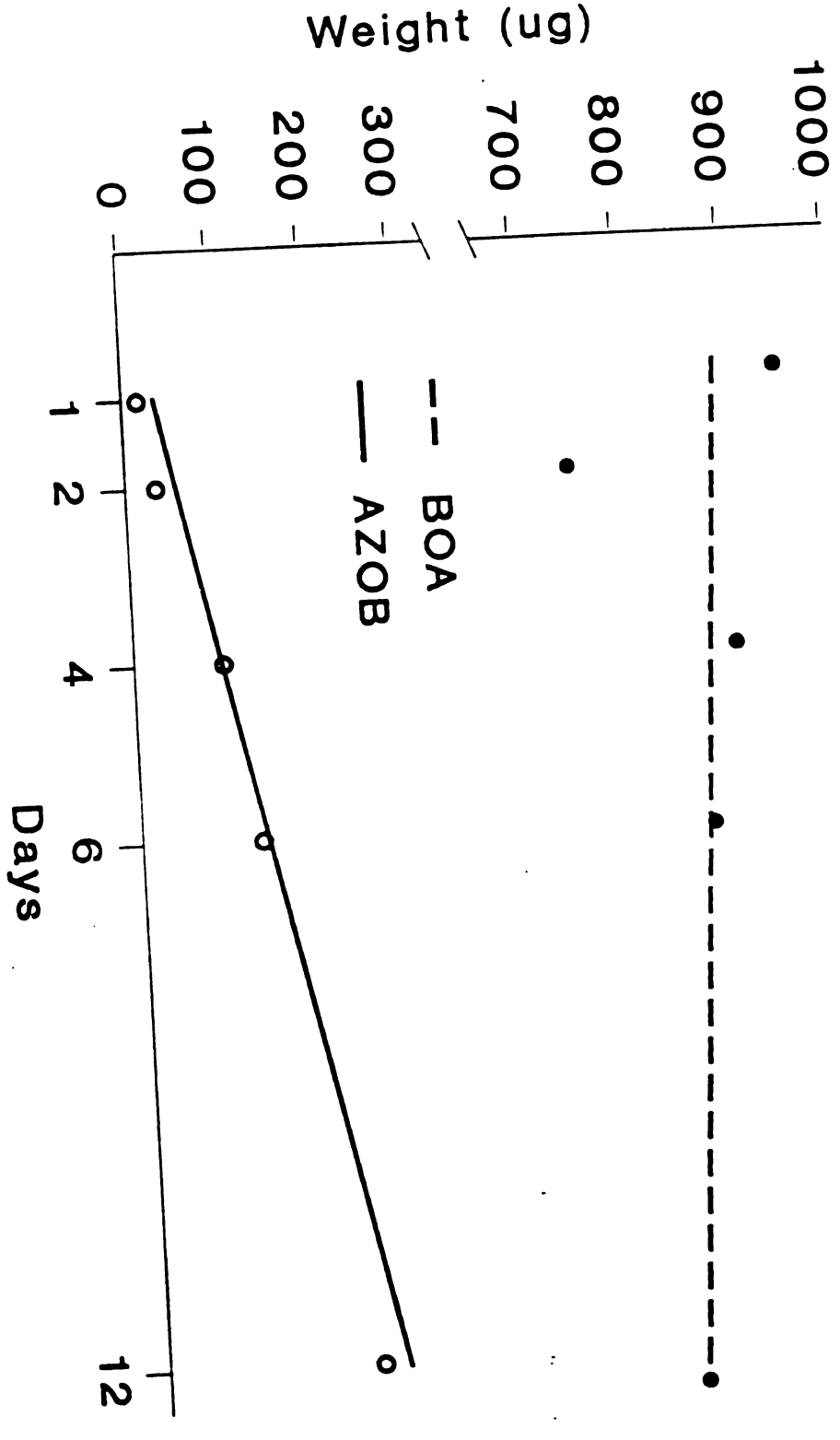
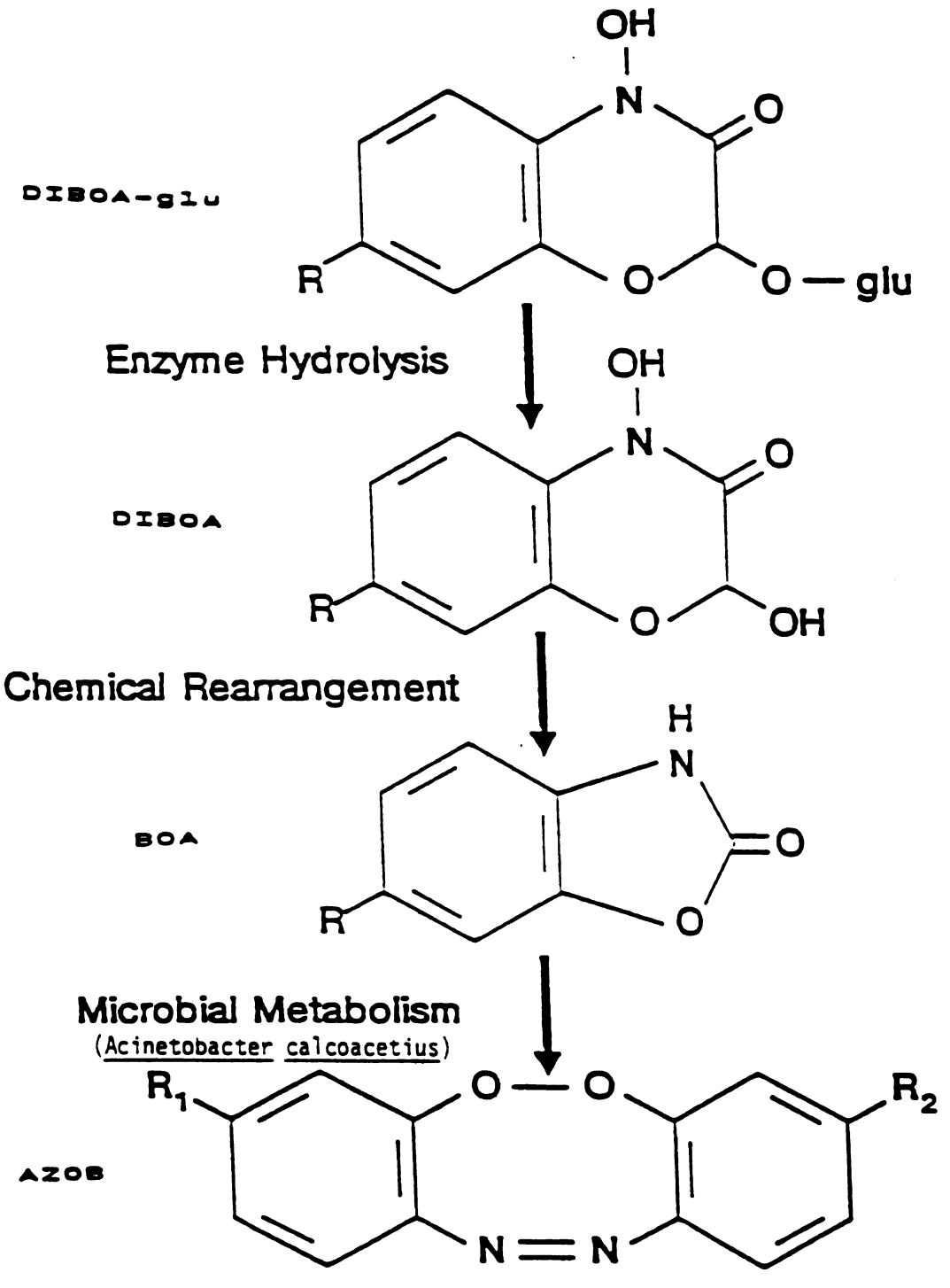

Weight (ug)
0
20
40
60
80
100
120
Days
1
2
4
6
12
--- BOA
—— AZOB

Figure 8. Rate of transformation of BOA to AZOB in non-sterile soil incubated with a BOA concentration of 1.0 mg/g of soil.


Weight (ug)
0
100
200
300
700
800
900
1000
Days
1
2
4
6
12
-- BOA
— AZOB



Scheme 2: Proposed scheme for formation of AZOB with Acinetobacter calcoacetius.

appropriate time interval a quadratic trend could be expected at both rates of BOA (early lag phase followed by linear phase).

Rapid AZOB production did occur in the different Michigan soils tested and would suggest the presence of Acinetobacter calcoacetius (Scheme 2) or another similiar organism capable of the conversion of BOA to AZOB (Figures 9 and 10). Analysis of various soil parameters did show differences in mechanical makeup, fertility, organic matter and cation exchange capacity but did not correlate with the transformation process (Appendix 3). Whether or not a gramineae crop was previouly grown did not affect the conversion.

AZOB was produced in large quantities in aqueous shake cultures for bioassay experiments. This method was convenient, cost effective, and less time consuming. Extraction of AZOB and BOA by EtOAc from aqueous media was easily performed, and even though the concentration of AZOB in the non-shake conditions was not evaluated, the rate of production of AZOB under shake conditions appeared greater. Since the production of AZOB occurred readily in liquid cultures it is not neccessary to use soil for the transformation of BOA. Even with a lower yield from the liquid medium (24%) when compared to the soil (30.8%), water was a more attractive transformation medium for large scale in vitro production of AZOB.

Conclusions: The experiments with BOA in non-sterile soil also indicated that the AZOB produced might have been used by other soil microbe(s) as a substrate thus explaining the quadratic trend observed in Figure 7. This data supports the difficulty encountered in the detection of AZOB from the soil incubated with rye tissue. The concentration of AZOB started to decline in the experiment with lower

Figure 9. AZOB concentration in 4 Michigan soils incubated with 0.1 mg BOA/g soil.

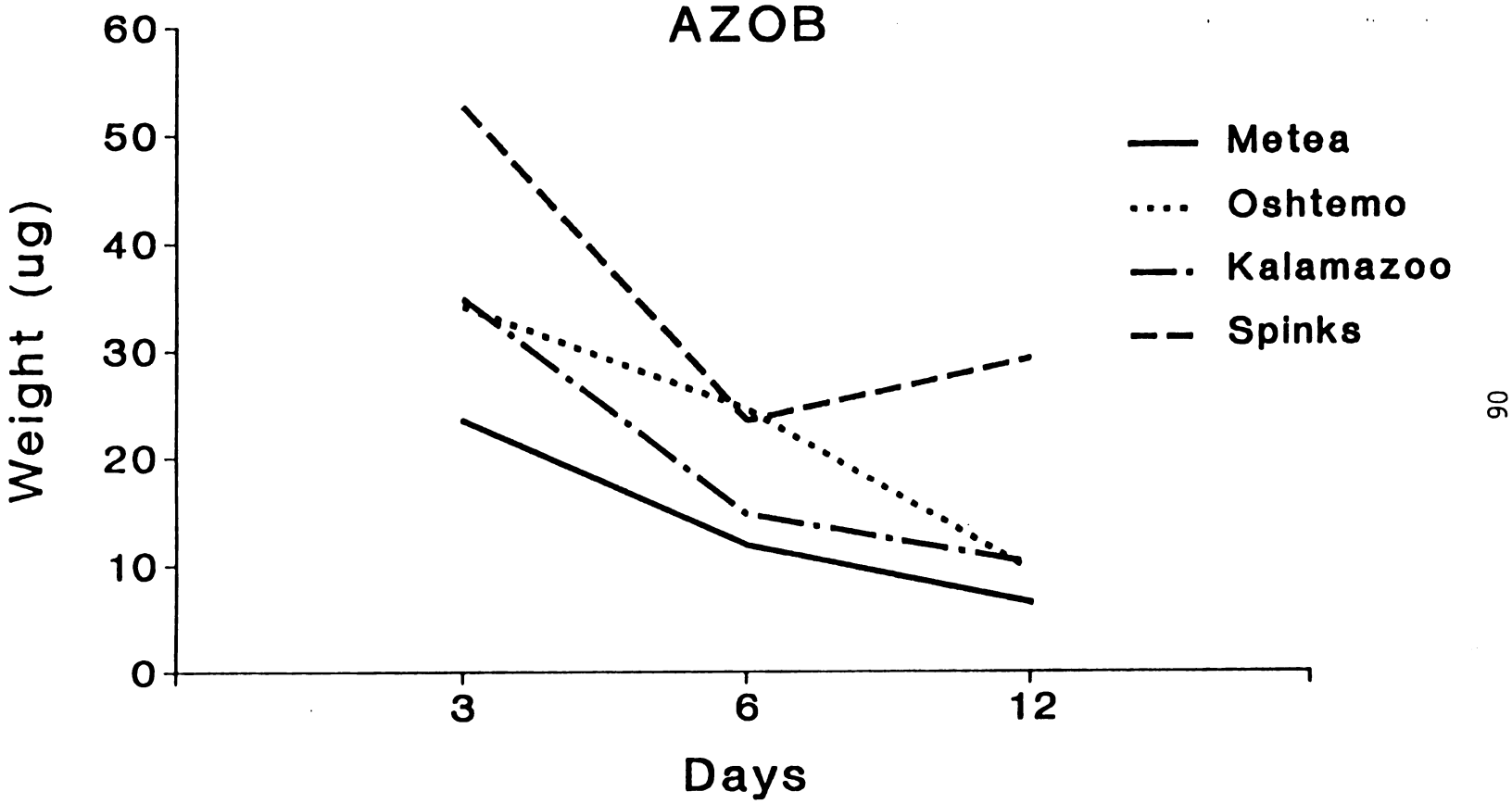
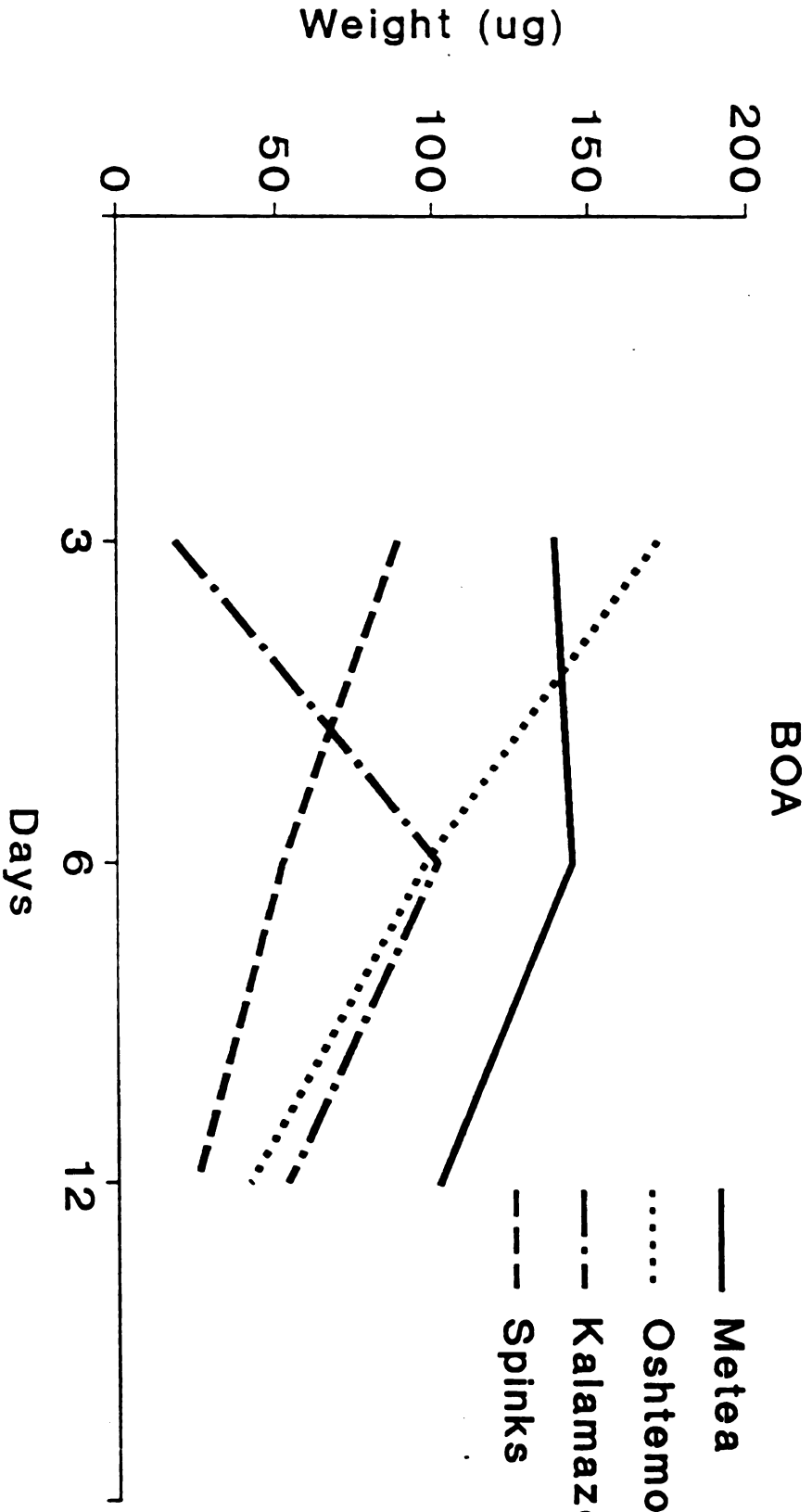

AZOB
Weight (ug)
60
50
40
30
20
10
0
3
6
12
Days
Metea
Oshtemo
Kalamazoo
Spinks

Figure 10. BOA concentrations in 4 Michigan soils incubated with 0.1 mg BOA/g soil.


Weight (ug)
200
150
100
50
0
3
6
12
Days
BOA
Metea
Oshtemo
Kalamazoo
Spinks

concentrations of BOA (Figure 7) by the end of day 4. Similar situations in the field can be envisioned where BOA leached from the rye residues is undergoing rapid transformation to AZOB. This experiment also suggested that the accumulation of AZOB will be difficult to detect in field soils with or without rye residues.

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CHAPTER 4

Relative Toxicity of Rye (*Secale cereale* L.) Allelochemicals to Weeds and Vegetable Crops

ABSTRACT

Three allelochemicals from rye or its breakdown products were evaluated for activity on garden cress (*Lepidum sativum* L.), barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.), cucumber (*Cucumis sativus* L.), and snap bean (*Phaseolus vulgaris* L.). 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA), 2(3H)-benzoxazolinone (BOA), and 2,2'-oxo-1,1'-azobenzene (AZOB) were all applied singly at 50, 100, and 200 ppm and in two and three way combinations each at 50 and 100 ppm.

AZOB at 100 and 200 ppm produced 38 to 49% more inhibition than DIBOA while combinations of BOA/DIBOA which contained AZOB at 100 ppm had 54 to 90% more inhibition when compared to DIBOA/BOA combinations. All combinations were slightly antagonistic to barnyardgrass while several combinations caused a synergistic response to garden cress germination and growth. Cucumbers and snap beans exhibited both types of responses depending on the allelochemical combination and application rate. Plant-produced benzoxazinones were more inhibitory to crops than weeds. Therefore, improved selectivity to cucumbers and snap beans would be expected if there was rapid transformation of the benzoxazinones to the microbially produced AZOB.

INTRODUCTION

Rye (Secale cereale L.) is an annual cereal grain that has shown potential as a cover crop in a variety of cropping systems (Putnam and Defrank, 1983; Barnes and Putnam, 1986), because it has been found to reduce weed biomass, improve soil aggregation, reduce soil erosion and enhance water penetration and retention (Blevins et al., 1971; Young, 1982). With the renewed interest in lowering energy inputs, reduced tillage systems employing cover crops will undoubtedly be an important tool of the future. Maintenance of residues on the soil surface will be inherent to these reduced tillage systems. Residues can interact with weeds or crops to potentially reduce the growth of the plants. This interference can arise from competition for essential plant growth requirements such as light, water, and nutrients (Knavel et al, 1977; Teasdale et al., 1983; Mascianica et al., 1986; Skarphol et al., 1987; Glen and Welker, 1989) or from allelopathic chemicals released into the environment from decaying plant residues (Shilling et al., 1985; Barnes et al., 1987; Nair et al., 1990).

Allelopathy is defined as one plant chemically interfering with the germination, growth, or development of another and has been implicated in numerous plant interactions (Muller, 1966; Whittaker and Feeny, 1971; Barnes et al., 1986). Proof of allelopathy requires detection of small quantities of biologically active compounds. Even though natural product chemists are able to characterize new plant compounds, rigorous testing is required to determine their allelopathic potential (Putnam 1988).

Although there have been numerous studies on toxicity of rye

residues (Kimber, 1973; Chou and Patrick, 1976; Putnam and Defrank, 1983; Shilling et al., 1985; Barnes and Putnam, 1986; Barnes et al., 1987), no one has offered a complete explaination of interference reported by these plant residues. There have been several attempts to isolate and identify the many compounds within rye showing biological activity. Virtanen (1957) isolated a cyclic hydroxamic acid, 2(3H)-benzoxazoline (BOA), from four-day old rye seedlings. He reported BOA as being responsible for the chemical resistance of rye to Fusarium infection. Tang et al. (1975) further quantified BOA present in rye and found 8 times more BOA in the shoots than in the roots. Chou and Patrick (1976) identified nine compounds from decaying rye tissue and found them applied individually to be inhibitory to lettuce seed germination. Zungica et al (1983) have evaluated rye for two hydroxamic acids, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and BOA, and found them commonly occurring in both cultivated and wild accessions. Shilling et al. (1985) isolated phenyllactic acid (PLA) and hydroxybutyric acid (HBA) from field grown rye and found each allelochemical inhibited redroot pigweed (Amaranthus retroflexus L.), common lambsquarters (Chenopodium album L.), and common ragweed (Ambrosia artemisiifolia L.) growth at concentrations of 2 mM. Barnes et al. (1987) compared the activity of four rye compounds PLA, HBA, DIBOA, and BOA and reported DIBOA and BOA to be the most toxic to large crabgrass (Digitaria sanguinalis (L.) Scop.), barnyardgrass (Echinochloa crus-galli (L.) Beauv.), proso millet (Panicum miliaceum L.), redroot pigweed, tomato (Lycopersicon esculentum Mill.), and lettuce (Lactuca sativa L.) root growth at concentrations of 67 to 250 ppm.

Nair et al. (1990) isolated a microbially produced 2,2'-oxo-1,1'-azobenzone from the soil enriched with BOA (Chapter 1, page 21, Scheme 1) and showed that DIBOA, BOA, and AZOB were inhibitory to barnyardgrass and garden cress (Lepidum sativum L.) at concentrations of 67 to 250 ppm. In these tests, AZOB was more biologically active than BOA and DIBOA.

Although there have been numerous compounds isolated and identified from rye tissue, it is difficult to predict the active concentration of any one compound at any one time as it occurs in nature. It is possible that they might occur either alone or in numerous combinations under various environmental and plant conditions. Since the hydroxamic acids, BOA and DIBOA, and their AZOB breakdown product exhibited the most activity, the objective of this study was to evaluate these three known rye allelochemicals singly and in combinations for their activity on weeds, cucumbers and snap beans.

MATERIALS AND METHODS

Spray Reagents for TLC Detection. DIBOA was detected on thin-layer plates with ferric chloride ($FeCl_3$) spray reactant consisting of 5% $FeCl_3$ in 95% ethanol, acidified with concentrated HCl. A spray reagent consisting of 1% ceric sulfate ($CeSO_4$) in concentrated H_2SO_4 was used to detect BOA.

SOURCE OF ALLELOCHEMICALS. BOA was obtained from Aldrich Chemical company and AZOB was produced from BOA according to the procedure described in Chapter 3, page 63.

Extraction of DIBOA. Seedlings of rye ('Wheeler', 11 days old, 2.2 kg) were homogenized in a Waring blender with distilled water (4.5 L), and kept at room temperature (1 h) to ensure enzymatic hydrolysis of the

DIBOA-glucoside to DIBOA. After filtering through cheese cloth, the filtrate was heated in a water bath until the temperature of the extract reached 70 ^{o}C. It was then cooled immediately in an ice bath. Coagulated components were removed by vacuum filtration using filter paper (Whatman No. 1). Filtrate was then lyophilized at 5 ^{o}C and stored at -20 ^{o}C until further separation.

Lyophilized rye extract (121.91 g) was stirred with HCl in MeOH (1.5 L, pH 1.0) for 2 hours at room temperature. The mixture was then filtered through a sintered glass filter (fine, 4-5 A^{o}) and the filtrate dried <u>in vacuo</u>. This crude residue was extracted with $CHCl_3$ (3 X 100 ml) and the organic extract was evaporated to dryness. The oily residue (35.3 g) was redissolved in $CHCl_3$ and precipitated with hexane (500 ml) and filtered. This procedure was repeated twice and the combined $CHCl_3$-hexane extract was dried <u>in vacuo</u> (12.84 g). A TLC analysis of this crude product confirmed the presence of DIBOA. Further purification of DIBOA from the above partially purified mixture was carried out by vacuum liquid chromatography. A slurry of column silica (40-60 mesh size, 260 g) in $CHCl_3$-MeOH (4:1) was packed under vacuum in a sintered glass filter (medium, 10-15 A) and washed with $CHCl_3$-MeOH (4:1, 300 ml). The partially purified gum containing DIBOA (12.84 g) in $CHCl_3$-MeOH (4:1, 30 ml) was applied on the silica under vacuum and eluted with the same solvent system. Four fractions were collected in 250 ml portions and analyzed by TLC. Fractions 2 and 3, positive for DIBOA by $FeCl_3$ spray, were combined and evaporated to dryness (10.94 g). This was purified again by flash column chromatography (silica, $CHCl_3$-MeOH 4:1 v/v). The initial fraction (30 ml) was discarded and upon TLC analysis of fractions 1-21 (each 5 ml)

were found to be negative for DIBOA. The fractions 22-29 (each 10 ml), monitored by TLC with $FeCl_3$ spray, were found to contain the largest quantity of DIBOA. Removal of solvent at reduced pressure afforded a brown solid (2.43 g). Final purification of DIBOA was achieved by TLC (silica, $CHCl_3$-MeOH, 4:1) and the resulting product recrystallized from $CHCl_3$-MeOH mixtures, and gave pale yellow crystals of DIBOA (187 mg).

BIOASSAY: Activity of three known compounds associated with rye (DIBOA, BOA, and AZOB) were assayed on two weed indicators (garden cress and barnyardgrass) and two crops (cucumber (Cucumus sativus L.) and snap bean (Phaseolus vulgarus L.)). Growth parameters recorded were seed germination, and root and shoot lengths. All three compounds were applied alone at 50, 100, and 200 ppm and in two and in three way combinations each applied at 50 and 100 ppm. The experimental design was a randomized complete block design with 4 replications.

Stock solutions ($1mg \cdot ml^{-1}$ methanol) of purified compounds were applied to filter paper (Whatman's No. 1) which lined the petri dishes. Pure methanol was also included as a control. After application of the compounds on the filter paper, the solvent was allowed to evaporate (2 to 3 hrs.) in the dark before seed placement. In the snap bean assays, the solvent was allowed to evaporate for an additional 6 to 8 hrs prior to planting.

Weed seeds (15) were uniformly distributed on filter paper (4.25 cm) in petri dishes (60 X 15 mm) after which distilled water (1.5 ml) was added. Garden cress and barnyardgrass seeds were then incubated for 72 and 96 hrs respectively. 'Flurry' cucumber seeds (5) were uniformly distributed on filter paper (7 cm) in petri dishes (100 X 15 mm) and then distilled water (2 ml) was added. Cucumbers were then

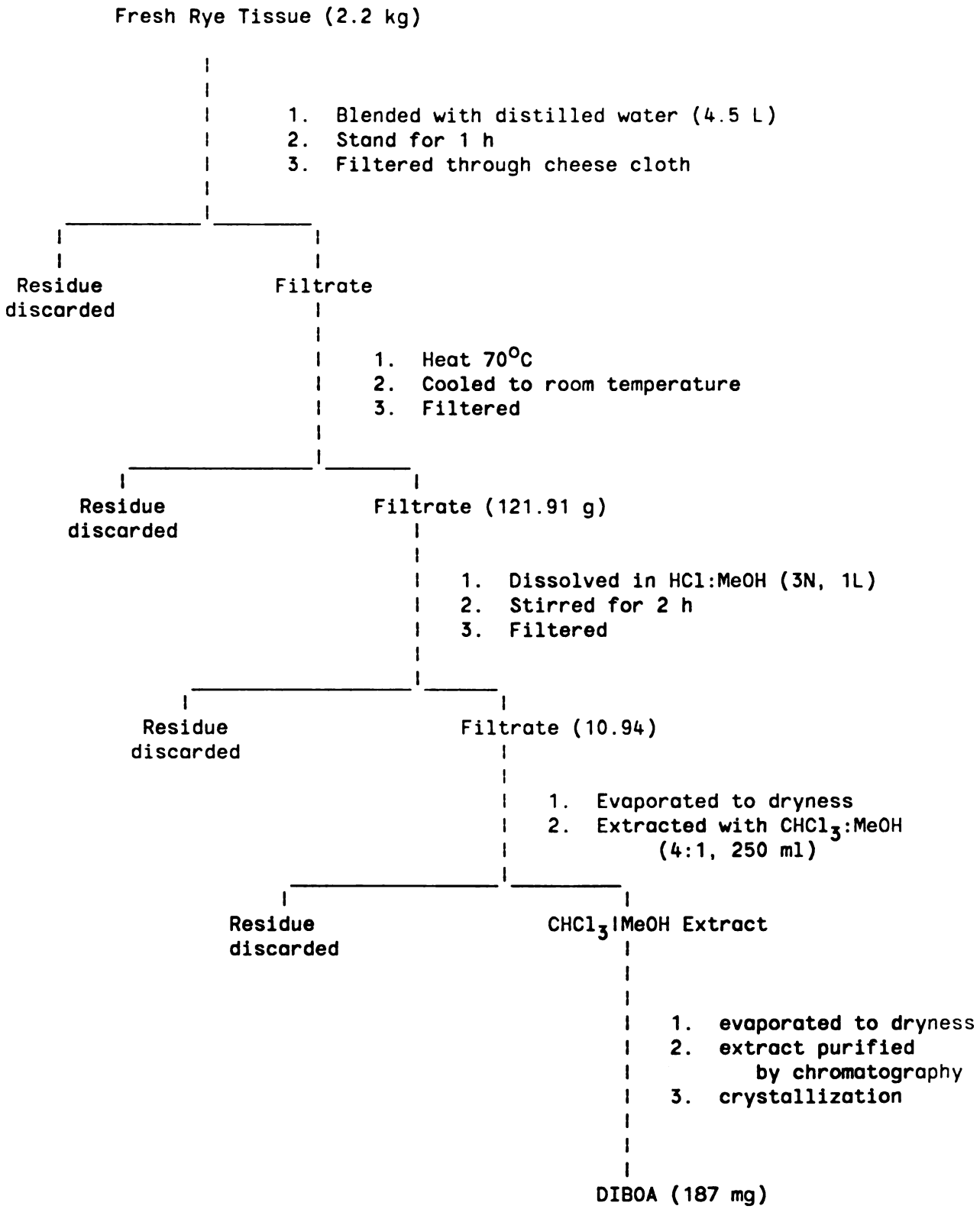
incubated for 120 hrs. 'Bush Blue Lake 47' snap bean seeds (4) were uniformly distributed on seed germination paper (5 X 5 cm) in Magenta boxes (6 X 6 X 9 cm) after which distilled water (5 ml) was added. A cellulose sponge (3.0 X 3.8 X 6.2 cm) saturated with distilled water was suspended from the sides of the box before attaching the lids. Each container was individually sealed with parafilm (American Can Co.) and incubated for 216 hrs.

Incubation of bioassay. After placing the lids onto the containers, they were randomly placed on trays (36 X 57 cm) that were lined with damp brown paper toweling. Each tray held one to two blocks and was covered with a clear plastic bag before placing into the incubator at 26 ^{o}C for the duration of the study.

Statistical methods. Data obtained in bioassays were subjected to analysis of variance and means were compared with the least significant difference (LSD) test. Tables represent the means from duplicate experiments. Analysis for synergism or antagonism of combination treatments were conducted according to Colby's methods (1967).

RESULT AND DISCUSSION

DIBOA quantification: Since DIBOA was not available commercially, it was neccessary to isolate it from field or greenhouse grown rye tissue. The modification of DIBOA extraction procedure (Scheme 1) gave a higher yield of DIBOA than previously reported (Nair et al., 1990). The major difference in the extraction step was the treatment of lyophilized extract with HCl in MeOH. Barnes et al. (1986) quantified hydroxamic acids by colorimetric methods and obtained a 27 fold increase of DIBOA in greenhouse grown rye shoots over field grown. Our yields were lower



Scheme 1. Extraction of DIBOA from 11 day-old greenhouse grown rye

than Barnes et al. (1986) but were four-fold higher than Nair et al. (1990). However we did not process all the positive DIBOA containing fractions. This DIBOA along with BOA and AZOB was used for evaluating allelopathic activity of these compounds under field and greenhouse conditions.

Relative biological activity of allelochemicals: Of the three chemicals tested on barnyardgrass, AZOB was the most active, reducing both root and shoot growth at all three application rates (Table 1). Combinations of the compounds containing AZOB showed 41 to 67% more activity on roots and 20 to 33% more activity on shoots when compared to combinations of DIBOA/BOA at the same concentrations. The combinations showed slightly less activity when compared to expected values by Colby's methods. This would suggest that these compounds are antagonistic when they are applied together.

For garden cress, all parameters measured were inhibited by the application of AZOB (Tables 2 and 3). Germination was reduced by 3 to 4-fold by combinations containing AZOB/BOA each at 100 ppm. In addition, AZOB or BOA applied singly showed 2 to 4-fold more activity on both shoot and root growth when compared to DIBOA. Shoot and root growth was severely inhibited with combinations of AZOB/BOA or AZOB alone at 100 ppm. In the above mentioned combinations, the observed values were always less than the expected, suggesting synergism. The order of activity on garden cress was AZOB > BOA > DIBOA which is in agreement with Nair et al. (1990).

For cucumbers, DIBOA was more inhibitory to both root and shoot growth than either BOA or AZOB when applied singly (Table 4). When

Table 1. Barnyardgrass root and shoot response to DIBOA, BOA, and AZOB applied singly and in combinations.

compound	(ppm)	Root 50	Root 100	Root 200	Shoot 50	Shoot 100	Shoot 200
		(% of control)					
DIBOA		83	75	60	94	92	76
BOA		101	91	82	86	86	82
AZOB		50	28	38	79	65	79
DIBOA & BOA[1]		90 (84)	73 (68)		100 (81)	89 (79)	
DIBOA & AZOB		46 (42)	24 (21)		80 (71)	60 (60)	
BOA & AZOB		53 (50)	31 (26)		72 (68)	61 (56)	
DIBOA, BOA & AZOB		52 (42)	26 (19)		67 (64)	61 (51)	
LSD (.05)			13			14	

( ) = expected value - $E = D^1 \times B^1 / 100$

E = expected value, D_1 and B_1 are growth as a percent-of-control of allelochemicals

1 - all combinations were each applied singly at the given rate

Table 2: Garden Cress germination response to DIBOA, BOA, and AZOB applied singly and in combinations.

compound	(ppm)	Germination 50	100	200
		------(% of control)-------		
DIBOA		101	102	102
BOA		106	105	95
AZOB		100	94	97
DIBOA & BOA[1]		107 (107)	104 (107)	
DIBOA & AZOB		93 (101)	71 (96)	
BOA & AZOB		90 (106)	24 (96)	
DIBOA, BOA & AZOB		92 (107)	17 (101)	
LSD (.05)			10	

( ) = expected value calculated by $E = D^1 \times B^1 / 100$. See pg 105.

1 - all combinations were each applied singly at the given rate

Table 3. Garden cress root and shoot response to DIBOA, BOA, and AZOB applied singly and in combinations.

	Root			Shoot		
compound (ppm)	50	100	200	50	100	200
	(% of control)					
DIBOA	77	90	65	100	86	83
BOA	82	60	52	74	61	47
AZOB	74	56	33	56	34	42
DIBOA & BOA[1]	98 (63)	77 (54)		86 (74)	64 (52)	
DIBOA & AZOB	93 (57)	35 (50)		71 (56)	19 (29)	
BOA & AZOB	50 (61)	13 (34)		34 (41)	6 (21)	
DIBOA, BOA & AZOB	51 (47)	8 (30)		32 (41)	5 (18)	
LSD (.05)		23			21	

( ) = expected value calculated by $E = D^1 \times B^1 / 100$. See pg 105.

1 - all combinations were each applied singly at the given rate

Table 4. Cucumber root and shoot response to DIBOA, BOA and AZOB applied singly and in combinations.

compound (ppm)	Root			Shoot		
	50	100	200	50	100	200
	(% of control)					
DIBOA	91	55	31	98	66	30
BOA	87	76	56	83	52	47
AZOB	85	79	82	82	63	76
DIBOA & BOA[1]	53 (79)	39 (42)		41 (81)	43 (34)	
DIBOA & AZOB	76 (77)	51 (43)		86 (80)	61 (42)	
BOA & AZOB	79 (74)	70 (60)		61 (68)	59 (33)	
DIBOA, BOA & AZOB	65 (67)	47 (33)		61 (67)	44 (21)	
LSD (.05)		17			32	

( ) = expected value calculated by $E = D^1 \times B^1 / 100$. See pg 105.

1 - all combinations were each applied singly at the given rate

applied singly at 50 ppm, none of the chemicals reduced root or shoot growth, but when concentrations were 100 ppm or greater there were significant reductions in growth. Again, DIBOA, BOA and all combinations were more active with increasing concentrations. The DIBOA/BOA combinations were 14 to 44% more inhibitory than the combinations of BOA/AZOB and appeared to be synergistic while the BOA/AZOB combinations appeared to have antagonistic effects. This suggested that the most toxic compounds to cucumbers were ones found in the rye herbage and not the microbially produced AZOB. If rapid conversion of these compounds did occur, one could expect improved cucumber crop safety in the presence of decomposing plant material.

In the snap bean assay, there was more variation perhaps due to seed variability or more intricate experimental design. Overall, shoot growth appeared more sensitive than roots with DIBOA and BOA showing a rate response with increasing concentrations (Table 5). For combinations with 50 ppm of each compound, only slight synergistic activity was seen, while at 100 ppm, antagonism was evident. Applications of BOA at 100 and 200 ppm and combinations of BOA/AZOB showed the greatest inhibition. Both large seeded dicot crops were more affected by DIBOA/BOA and BOA/AZOB combinations. From previous work (Putnam and Defrank, 1983 and Skarphol et al., 1987) both cucumber and snap beans have been shown to respond favorably to cover crop residues. A possible explanation is that the large seed crops are located too deep to be influenced by the residues, or alternately the concentrations of these chemicals are too low to affect the emergence of the crops. The effect seen on small seeded weeds such as cress and barnyardgrass could come from rapid degradation of the benzoxazinones

Table 5: Snap bean root and shoot response to DIBOA, BOA, and AZOB applied singly and in combinations.

compound	(ppm)	Root 50	Root 100	Root 200	Shoot 50	Shoot 100	Shoot 200
		(% of control)					
DIBOA		102	91	107	95	79	72
BOA		104	64	50	60	56	24
AZOB		108	83	94	85	66	68
DIBOA & BOA[1]		84 (106)	79 (54)		92 (57)	55 (44)	
DIBOA & AZOB		80 (110)	101 (76)		61 (81)	50 (52)	
BOA & AZOB		96 (112)	75 (53)		29 (51)	44 (37)	
DIBOA, BOA & AZOB		80 (114)	66 (48)		74 (48)	51 (29)	
LSD (.05)			32			38	

( ) = expected value calculated by $E = D^1 \times B^1 1 / 100$. See pg 105.

1 - all combination each were applied singly at the given rate

from the injured plant material and the more active microbially produced AZOB. Also the smaller seeds are located closer to the soil surface where higher concentrations of these compounds might exist.

Conclusions: The benzoxazinones have been reported in cereal grains and corn plants (Virtanen et al. 1957). In addition, the recent isolation of the microbially produced AZOB may help to account for a portion of the 88% of inhibition by rye residues that Barnes et al. (1986) could not account for. Our results show that both small seeded species were strongly inhibited by AZOB and garden cress by BOA as well. These results agree with Barnes et al. (1986) and Nair et al. (1990) where they reported BOA to be more active on dicot and small seeded crop species. For monocots, there has been allelochemical activity reported with DIBOA. The finding that AZOB has herbicidal potential against most small seeded species gives hope that microbially produced allelochemicals would be useful in weed control.

In general, when comparing the response of the different growth parameters, root and shoot inhibition were more sensitive indicators of allelopathic activity than germination inhibition. Only in the case of garden cress was germination a positive indicator of activity. Neither of the large seeded crops nor barnyardgrass showed significant germination inhibition. This indicated that the mode of action for barnyardgrass, cucumber, and snap bean was not on germination processes but rather on early plant development processes. This suggests the expected field response would be reduced plant growth and not reduction in number of plants.

Our results not only suggest that these compounds are selectively allelopathic, but indicated the relative quantities of these compounds

are important in determining the expected outcome. Earlier bioassays with these compounds used small seeded species whereas ours compared larger seeded crops. It appears that selectivity can be achieved based on seed size and seed placement. This same principle has allowed the selective use of synthetic herbicides for decades. The difficulty with allelochemicals is to predict or quantify what might be present at any particular time in the field.

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APPENDIX 1

Analysis of soil at Time of Planting for 1988 and 1989

year	P	K	Ca	Mg	Zn	Mn	pH	CEC[1]	O.M.	sand	silt	clay
	(lbs/acre)				(ppm)					(%)		
1988	200	312	2103	285	1.9	14.3	6.0	12	2.5	65.3	20.0	14.7
1989	200	244	1853	223	1.4	10.5	6.0	11	1.9	65.5	25.3	8.9

1 = me / 100g

APPENDIX 2:
Bi-Monthly Temperature and Precipitation information for 1988 and 1989.

Month	day no.	1988	1989	1988 Rainfall	1988 Irrigation	1989 Rainfall	1989 Irrigation
		--(GDD)[1]---		----------(inches)----------			
May	1-15	121.0	-38.5	.54[A]	.75	.47[B]	-
	16-31	228.5	188.0	.07		3.99	
June	1-15	247.5	240.5	.12	1.00	1.83	
	16-30	298.5	267.5	.04[C]	2.75	1.44[D]	.75
July	1-15	375.5	338.0	.08	1.75	.26	.75
	16-31	418.5	337.0	2.32	.50	1.56	
August	1-15	473.0	287.5	1.47	1.25	4.63	
	16-31	260.5	280.0	2.35		2.26	
September	1-15	257.5	225.0	.80		5.40	
Totals		2680.5	2125.5	7.79	8.00	21.84	1.50

1 = units calculated on a 50° Farenheight base temperature

Cover crop planting dates: A= 5/13/88 B= 5/02/89
Vegetable planting dates: C= 6/23/88 D= 6/26/89

Appendix 3. Soil Laboratory Analysis of Field Soils

Class/Type	Gramineae	Sand	Silt	Clay	pH	OM(%)	CEC	P	K	Ca	Mn
Kalamazoo sandy loam	no	64.6	19.0	16.4	6.7	1.91	7	91	444	1684	448
	yes	49.6	35.0	15.4	7.0	2.41	6	171	391	1684	328
Metea loam	no	69.6	14.0	16.4	7.3	5.52	13	100	269	3733	701
	yes	71.3	9.4	19.4	7.4	7.50	13	100	194	3733	800
Oshtemo sandy loam	no	88.6	5.0	6.4	6.5	0.62	2	193	76	720	94
	yes	88.6	7.4	6.4	6.1	1.00	2	253	152	720	94
Spinks loamy sand	yes	87.6	6.0	6.4	7.5	1.22	4	171	84	1120	168