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2,2′-OXO-1,1′-AZOBENZENE: MICROBIAL TRANSFORMATION OF RYE (*Secale cereale* L.) ALLELOCHEMICAL IN FIELD SOILS BY *Acinetobacter calcoaceticus:* III

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Abstract—*Acinetobacter calcoaceticus*, a gram-negative bacterium isolated from field soil, was found to be responsible for the biotransformation of 2(3H)-benzoxazolinone (BOA) to 2,2′-oxo-1,1′-azobenzene (AZOB). Experiments were conducted to evaluate the transformation of BOA to AZOB by this microbe in sterile and nonsterile soil. Transformation studies with soils inoculated with *A. calcoaceticus* indicated that the production of AZOB increased linearly with the concentration of BOA in sterile soil and showed a quadratic trend in nonsterile soils. This also indicated that all soil types studied for the transformation experiments might contain *A. calcoaceticus* capable of the conversion of benzoxazolinones.

Key Words—Allelopathy, 2,4-dihydroxy-1,4(2H)-benzoxazine-3-one, 2(3H)-benzoxazolinone, 2,2′-oxo-1,1′-azobenzene, *Acinetobacter calcoaceticus*.

INTRODUCTION

Rye is considered an important cover crop in Michigan, other parts of the United States, and around the world (Smeda and Weller, 1986; Smeda and Putnam,

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1988). Numerous allelochemicals with apparent weed control activity have been isolated and identified from rye (*Secale cereale* L.) herbage and found to inhibit lettuce (*Lactuca sativa* L.), redroot pigweed (*Amaranthus retroflexus* L.), and common lambsquarters (*Chenopodium album* L.) seed germination and root growth (Chou and Patrick, 1976; Patrick, 1955; Shilling et al., 1985). Barnes and Putnam (1986, 1987) and Barnes et al. (1987) isolated two hydroxamic acids, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and 2(3H)-benzoxazoline (BOA), from rye residues, compared their toxicity to phenyllactic acid and hydroxybutyric acid, the allelochemicals reported earlier from rye (Patrick, 1955; Shilling et al., 1985), and found that the hydroxamic acids, DIBOA and BOA, were the most toxic to both monocots and dicots. The benzoxazinones are thought to occur in plants as glucosides. Upon injury, they are released as DIBOA by glucosidase enzymes (Hofman and Hofmanova, 1968; Zungica et al., 1983). The quantities of these compounds in plants vary with the species, age, and the plant part used (Almeida, 1985; Barnes and Putnam, 1986; Tang et al., 1975; Virtanen et al., 1957). Recently, Nair et al. (1990) reported the production of an azoperoxide, 2,2′-oxo-1,1′-azobenzene (AZOB) in field soils incubated with BOA. The benzoxazinones produced by rye, DIBOA and BOA, were rapidly converted to AZOB in nonsterile bins over heat-sterilized soil, suggesting a microbial basis for the reaction. This new transformed allelochemical was more toxic to plants than its precursors, BOA or DIBOA (Hietala and Virtanen, 1960; Nair et al., 1990).

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). The microorganisms either release or produce phytotoxins directly from residues or use the residues as substrate in the production of bioactive compounds (Atlas and Bartha, 1987; Patrick, 1955; Proebsting and Gilmore, 1941). For example, the contribution of microorganisms in the production of toxins from peach root bark was investigated, and several soil-born microorganisms were found to degrade amygdalin, an allelochemical from the peach tree, to HCN and benzaldehyde (Patrick, 1955; Proebsting and Gilmore, 1941). These degradation products were shown to be the toxic allelochemicals present in the soil containing decayed peach tree roots. This paper describes the isolation and identification of a soil microbe capable of transforming benzoxazinone to the more toxic AZOB and the rate of transformation of BOA to AZOB by this microbe in some Michigan field soils.

METHODS AND MATERIALS

General Experimental. Ultraviolet (UV) and visible absorption spectral analysis were performed on a Gilford Response II spectrophotometer. BOA was obtained commercially from the Aldrich Chemical company, Milwaukee, Wis-

consin, and was uniformly mixed with soil using a mortar and pestle, before addition of water and vortexing (1 min). Column chromatography was performed using a J.T. Baker Flash Column (20 × 2.7 cm, 250 ml). The silica column used was Analtech (35–75 μm particle size, 60 Å pore size), and the flow rate was 3 ml/min, unless otherwise specified. All analytical TLC (250 μm) and preparative TLC (1500 μm) were done on Uniplates (Analtech, Inc., Newark, Delaware). Homogenization of microbial colonies was done in a Brinkman Polytron (CH-6010) blender for 5 min. Soil was sterilized by autoclaving (1 hr, 15 atm, 120°C) for four successive days, and distilled water and media used were autoclaved for 20 min.

Soil Collection. All soil collection sites were in the State of Michigan. Spinks loamy sand soil (Psammentic, Hapludalfs, sandy, mixed, mesic) was collected (November, 1987) from the Horticulture Teaching and Research Center field, located on Jolly Road in East Lansing, Michigan. Soil was passed through an aluminum sieve (2 × 12 mm opening) and stored in a cubic yard bin at room temperature (15–22°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 under the same conditions. The Kalamazoo Sandy loam soil was collected at the Clarksville Research Station located in Clarksville, the Oshtemo sandy loam was from the Sodus Horticulture Research Farm located in Sodus, and the Metea loam was collected from the Moore Seed Farm located in Elsie.

Preparation of Media. Yeast maltose glucose (YMG) agar (yeast extract, 4 g; maltose, 10 g; glucose, 4 g; bacto-agar 18 g/liter), potato dextrose agar (PDA, Difco) (39 g/liter), and Nz-Amine agar (Nz-Amine, ICN Biomedicals Inc., 3 g/liter, bacto-agar 18 g/liter) were used for growth and identification of soil microbes. Antibacterial plates were prepared by adding chloramphenicol to a final concentration of 100 μg/ml. Antifungal Nz-Amine agar plates were prepared by adding nystatin and cycloheximide at concentrations of 30 μg and 500 μg/ml, respectively.

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 hr). 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°C (96 hr), and extracted with MeOH (50 ml × 3). Biotransformation of BOA to AZOB by the soil extract containing microbes (1 ml) 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 10 different autoclaved test tubes, diluted to 10 ml each by sterile distilled water, and mixed well. Solutions from each tube (10 ul) were spread onto individual YMG plates. Plates at dilution of 10^{-6} showed the best separation of fungal and bacterial colonies. A total of nine individual colonies was 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 or antifungal compounds, respectively, and incubated (72 hr). Two fungi and one bacterium thus obtained were assayed for the transformation of BOA to AZOB.

Identification of Microbe. The bacterium that converted BOA to AZOB was further purified on antifungal Nz-Amine plates. Morphological and biochemical properties of the bacterium were determined with a Vitek 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) and distilled water (400 ml) and incubated (26°C, 96 hr) in four 2-liter Erlenmeyer flasks, as published earlier (Long et al., 1974). The soil was extracted with methanol (8 × 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 μm), and the solvent was removed by rotary evaporation. A preliminary TLC (toluene–ethylacetate, 5:4) of the crude extract indicated the presence of AZOB and unreacted BOA. 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 to produce dark brown crystals of AZOB (1.18 g).

Quantification of BOA and AZOB in Soil Extract. The samples were analyzed spectrophotometrically for BOA and AZOB. Standard curves for BOA and AZOB were prepared using known quantities of BOA (from 0.748 to 286.0 μg/100 ml in MeOH) and AZOB (from 1.51 to 96.402 μg/100 ml in MeOH) and by recording the absorbance at 273 and 432 nm, respectively. Standard solutions of each of the dried soil extracts stored at −20°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.

In Vitro Transformation Studies with Pure Cultures of A. calcoaceticus. Cultures of *A. calcoaceticus* were used from the stock cultures grown on YMG plates, and transformation of BOA to AZOB was carried out as reported earlier

(Nair et al., 1990). All treatments were incubated (26°C) in the dark for 1, 2, 4, 6, 12, and 24 days and extracted with MeOH (3 × 150 ml) and quantified for BOA and AZOB by UV spectroscopic procedure. Microbial populations were estimated by monitoring the colony-forming units (CFU) of the stock solution and were calculated to be 5.3×10^{10}.

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 *A. calcoaceticus* (5.3×10^{10} CFU, 1 ml) were added to the water and incubated (26°C) under shake conditions. The fermentation broth (96 hr old) was extracted with EtOAc (3 × 75 ml). Shake and nonshake flasks were extracted with EtOAc separately. The EtOAc layer from shake flasks was dried over anhydrous $MgSO_4$ and evaporated *in vacuo* (four days). 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 crystals of AZOB (46 mg).

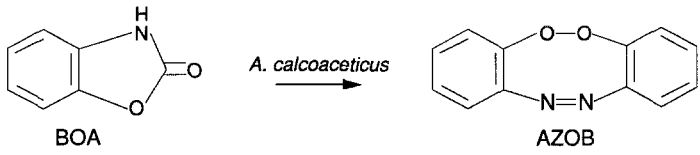
Microbial Transformation of BOA to AZOB. Experiments were conducted to study the rate of BOA transformation by the naturally occurring microbial population of *A. calcoaceticus* in field soil. BOA (30 mg each) was mixed with Spinks loamy sand (10 g each) containing distilled water (3.5 ml) in culture tubes (2.5 × 15.0 cm). Sterile foam 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°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 × 150 ml). The extracts were dried over anhydrous $MgSO_4$ and the solvent was removed *in vacuo*. All dried samples were stored at −20°C.

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

RESULTS AND DISCUSSION

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 transformation of BOA to AZOB (Scheme 1). 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. The organism grew well in a medium containing 0.4% peptone and



SCHEME 1. Transformation of BOA to AZOB in field soils using *A. calcoaceticus*.

0.02% tryptophan, under aerobic conditions, and 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 the microbe did not produce acid with raffinose, sorbitol, sucrose, inositol, adonitol, rhamnose, arabinose, or glucose. Moreover, the microbe did not hydrolyze esculin or *o*-nitrophenyl-β-D-galactopyranoside. Under aerobic conditions, this organism did not produce acid with glucose, lactose, maltose, mannitol, or xylose. Tests for the production of H_2S, urease, and plant indican were negative. The aforementioned properties are in conformity with those of *A. calcoaceticus* described in *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984).

Although analytical methods for benzoxazolines were reported earlier (Long et al., 1974; Nair et al., 1990; Virtanen et al., 1957), these methods are not convenient or quantitative for the determination of BOA and AZOB in soil extracts. Since BOA and AZOB gave characteristic UV–visible absorption maxima (Barnes et al., 1987; Nair et al., 1990), 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 273 and 432 nm, respectively. Standard curves were prepared separately by plotting each compound's concentration against its OD. Concentrations of AZOB and unreacted BOA in the various soil extracts were obtained directly from the standard curves.

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 portion of the AZOB isolated. The rate of production of AZOB in field soil was investigated under environmentally controlled conditions over a period of 24 days using sterile and nonsterile soils. In both cases, *A. calcoaceticus* cultures were added (5.3×10^{10}) to the soils previously incorporated with BOA. The quantities of AZOB production was proportional to the quantity of BOA avail-

able in the soil (Table 1). 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 (nonsterile) experiment showed the highest production of AZOB at day 3 (Table 1). The maximum yield of AZOB (9.7%) was observed during the incubation period of days 2–4 with an 85% decrease in BOA concentration. This yield is similar to the 9.1% reported by Nair et al. (1990).

The recovery of unreacted BOA from the treated soil was 33% of the total amount incubated. The remainder of the BOA already could have converted to AZOB or a portion could have decomposed to products that are not yet characterized. Furthermore, BOA might have decomposed to other products under mechanisms that 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. This indicated that the excess BOA might have functioned as an antimicrobial agent on the total microflora in the soil and prevented the soil microbes from utilizing BOA as a carbon or nitrogen source. Usually, microbial populations present in the soil are low in numbers but explode quickly when a suitable substrate becomes available (Atlas and Bartha, 1987). Therefore, having observed different trends for the BOA transformation in nonsterile field soil, it would seem logical that, given the appropriate time interval, a quadratic trend could be expected at both rates of BOA (early lag phase followed by linear phase). Rapid AZOB production detected in the different Michigan soils studied

TABLE 1. QUANTITIES OF BOA AND AZOB ISOLATED FROM STERILE SOIL INOCULATED WITH *A. calcoaceticus* AND NONSTERILE SOIL, BOTH INCUBATED WITH 0.1 mg OF BOA/g SOIL

	Days					
Compounds	1	2	4	6	12	24
Sterile soil						
BOA (μg)	331.9	279.2	229.9	253.5	193.7	191.7
AZOB (μg)	10.4	28.9	51.1	89.3	72.5	99.3
Nonsterile soil						
BOA (μg)	118.6	97.6	47.0	33.0	18.2	[a]
AZOB (μg)	11.2	32.6	33.8	13.5	3.1	[a]

[a] Not detectable levels.

suggested the presence of *A. calcoaceticus* capable of the conversion of BOA to AZOB (Figure 1 and 2).

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 the rate of production of AZOB under shake conditions appeared greater. Since the production of AZOB occurred readily in liquid cultures, it is not necessary to use soil for the transformation of BOA.

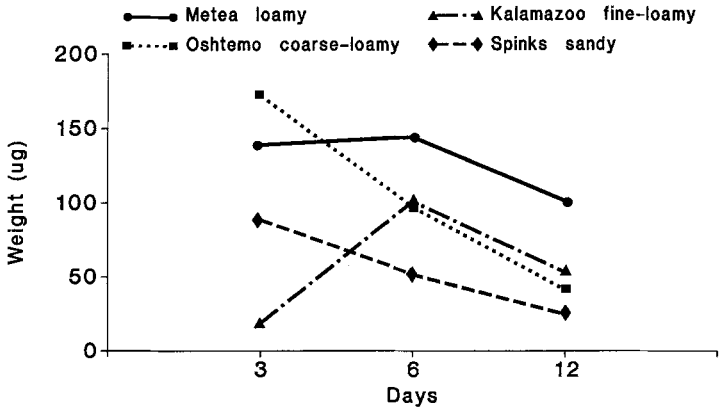


FIG. 1. Concentration of unreacted BOA present in the four Michigan soils used for transformation studies.

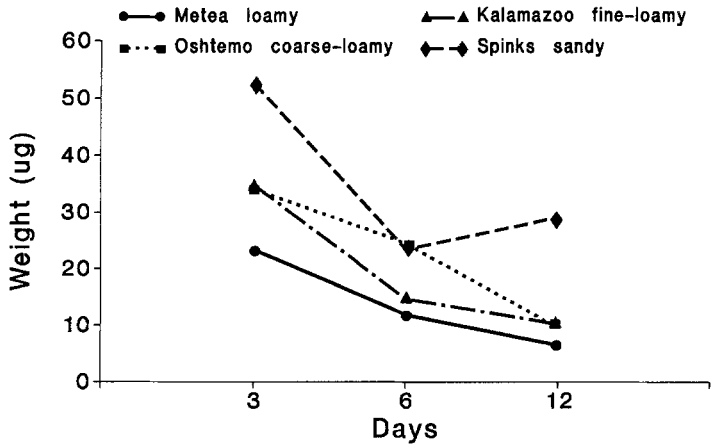


FIG. 2. Concentration of AZOB in four Michigan soils incubated with 0.1 mg of BOA per g of soil.

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. The experiments with BOA in nonsterile 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 (Table 1). These data support 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 nonsterile soil (Table 1) 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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