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6-METHOXY-2-BENZOXAZOLINONE: A SEMIOCHEMICAL FOR HOST LOCATION BY WESTERN CORN ROOTWORM LARVAE

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Abstract—A bioassay-driven sequential fractionation scheme was used to isolate all portions of a crude dichloromethane corn seedling extract behaviorally active to larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. 6-Methoxy-2-benzoxazolinone (MBOA) was identified as one of the most important components of an attractive crude corn extract. MBOA was found on or in the intact root tissues by injecting an extract of undamaged roots onto an HPLC immediately after extraction. MBOA was demonstrated to be volatile and functions as a semiochemical in conjunction with carbon dioxide in host location by western corn rootworm larvae, which are oligophagous on the roots of maize and several other species of grasses. Because MBOA occurs almost exclusively in maize and other grasses, it offers a simple way for the larvae to distinguish possible hosts from non-hosts. MBOA has previously been reported as a chemical defense against other insect species. This is the first report in grasses of a secondary compound that is toxic or a deterrent to nonadapted insect herbivores but that is used as a semiochemical in host location by a specialist insect species.

Key Words—*Diabrotica virgifera virgifera*, 6-methoxy-2-benzoxazolinone, hydroxamic acids, semiochemical, attractants, western corn rootworm, host location, Coleoptera, Chrysomelidae, *Zea mays*, kairomone.

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a serious pest of maize (*Zea mays* L.) throughout much of the maize-growing regions of

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North America. Larval damage to maize roots results in reduced plant height, reduced yield, and lodging of maize, which interferes with harvesting (Branson et al., 1980). An estimate of crop losses and treatment costs for corn rootworms is in the range of $1 billion annually (Metcalf, 1986).

Unlike host location in many other herbivorous insect species, western corn rootworm adults do not lay their eggs on a host plant. The eggs are laid in the soil in late fall, and the larvae emerge the following spring (Krysan and Miller, 1986). Host location is then carried out underground by the neonate larvae, which are oligophagous on the roots of a few species of grasses (Branson and Ortman, 1967, 1970). The larvae can distinguish the roots of maize from the roots of broad-leaved plants on the basis of olfactory cues. In choice test bioassays with cut roots, significantly more western corn rootworm larvae were attracted to the roots of maize than to the roots of soybean, squash, or sunflower (Branson, 1982).

We previously found that cryogenic collections of volatiles from germinating maize seeds were attractive to western corn rootworm larvae in laboratory behavioral bioassays (Hibbard and Bjostad, 1988, 1989). One of the attractive compounds is carbon dioxide, which is attractive to western corn rootworm larvae (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988) and many other soil organisms (Jones and Coaker, 1977; Doane et al., 1975; Pline and Dusenbery, 1987; and references therein). Carbon dioxide is released into the soil by the roots of maize and many other plant species (Massimino et al., 1980), but because it is a general plant metabolite, it does not appear to provide a basis for selective attraction of western corn rootworm larvae to maize over broad-leaved plants. We also found in choice test bioassays that western corn rootworm larvae rely on chemical cues in addition to carbon dioxide to locate maize plants. In choice test bioassays with equal levels of carbon dioxide on both sides of the choice (verified by infrared gas analysis), larvae chose the maize volatile source significantly more often than the source with carbon dioxide alone (Hibbard and Bjostad, 1988). We used a sequential fractionation scheme modified from Silverstein et al. (1967) to isolate several behaviorally active compounds (Hibbard and Bjostad, 1990). We now report the chemical identification of a volatile secondary maize metabolite that western corn rootworm larvae use in orienting to their host.

METHODS AND MATERIALS

Larvae and Maize Source. A nondiapausing strain of *D. virgifera virgifera* was obtained in June 1986 from the USDA-ARS laboratory in Brookings, South Dakota, and was maintained with the methods of Jackson (1986) as modified by Hibbard and Bjostad (1988). Second-instar larvae (5–7 days old and 5–8 mm

long) were chosen for bioassays because initial bioassays indicated that they behaved similarly to first-instar larvae (the instar that must locate a host), and second-instar larvae were more robust for the large numbers of bioassays required. To prevent possible effects from previous testing, larvae were not reused in the bioassays. Dried whole kernels of maize (3090, Pioneer Hi-Bred International, Inc., Johnston, Iowa), not treated with insecticide or fungicide, were washed and soaked (Hibbard and Bjostad, 1988), and maize seedlings [pre-stage 0 germinated seed (Hanway, 1966)] were removed after 3 to 5 days to obtain maize semiochemicals.

Extraction and Isolation of Behaviorally Active Compound. Approximately 80 g of moist maize seedlings was extracted by placing them in a glass seed-holding tube (30 cm × 30 mm, tapering to 12 mm) for 3 to 6 hr and subsequently dripping dichloromethane through them until 4 ml of solution had been collected. The dichloromethane maize extract was then separated into fractions by column chromatography on 60- to 200-mesh silica gel (J.T. Baker Chemical Co., Phillpsburg, New Jersey), eluted batchwise with mixtures of dichloromethane and diethyl ether (100:0, 97:3, 90:10, 75:25, 50:50, and 0:100). The most active fraction in choice test bioassays with equal concentrations of carbon dioxide in both choices was the 75:25 fraction. The major compound in this fraction was isolated by gas chromatography as described by Hibbard and Bjostad (1990). A 2-mm-ID × 1.83-m 3% OV-101 (100/120 mesh) on Chromosorb Q (Alltech Associates, Inc., Deerfield, Illinois) packed column was temperature programmed at 60°C for 1 min, then 10°C/min to a final temperature of 260°C, in a Hewlett-Packard 5890 gas chromatograph. The 4-ml extract was concentrated to 8 μl under a nitrogen stream and injected onto the gas chromatograph. With the detector and noncolumn gases turned off, the fraction with a retention time of 16–17 min (the retention time on the major component in the 75:25 silica gel fraction) was collected with clean pipettes that were snugly connected to the end of the column with a specially fitted Teflon connector (collection efficiency, 70–85%). The collection was repeated 10 times with fresh extract, and the collection pipette was rinsed with the same 300 μl of solvent each time.

Bioassays with Purified Maize Volatile. Fifteen-microliter aliquots (approximately 28 g equiv of 3-day-old corn seedlings) of the 300-μl sample of purified maize volatile in dichloromethane were placed on 0.1 g of glass wool, and an equal amount of dichloromethane was placed on another piece of glass wool. After the solvent had evaporated, the pieces of glass wool were tested in choice test bioassays with equal levels of carbon dioxide (4 mmol/mol at bioassay onset) on both sides of the choice (verified with infrared gas analysis) as described by Hibbard and Bjostad (1990). Ten larvae were placed in the center dish of the choice test (see Hibbard and Bjostad, 1990), and the number of larvae that had reached either of the end dishes was recorded after 1 hr. Bio-

assays were conducted in the dark at room temperature and were replicated 15 times.

Chemical Analysis. Solid probe electron-impact (EI, 70 eV) and chemical ionization (CI, NH_4^+) mass spectra of the purified maize volatile were obtained with a VG, Inc., mass spectrometer (Model MM-16F). A ^1H NMR spectrum of the purified maize volatile in $CDCl_3$ was obtained with a Bruker AM500 (500 MHz). A diffuse reflectance infrared spectrum was obtained on a Nicolet 60SX Fourier transform spectrometer. A synthetic sample of 6-methoxy-2-benzoxazolinone (MBOA) was obtained (donation from Frank Stermitz, Chemistry Department, Colorado State University, and later from Lancaster Synthesis Ltd., Windham, New Hampshire) and was also analyzed by EI-MS, CI-MS, NMR, and IR as described above. A sample of synthetic MBOA was analyzed by coinjection with the purified maize volatile using an RSL-150, 30-m × 0.33-mm (ID) methyl silicone capillary column programmed at 60°C for 1 min, then 10°C/min to a final temperature of 240°C, in a Hewlett Packard 5890 gas chromatograph. The injection port was set at 240°C and the detector was set at 250°C.

Behavioral Bioassays with Synthetic MBOA. Serial dilutions of synthetic MBOA (Lancaster Synthesis Ltd., Windham, New Hampshire) were prepared and 700 ng of synthetic MBOA was tested in choice test bioassays as described above, with equal amounts of carbon dioxide on both sides of the choice. Seven hundred nanograms was chosen, because the amount of MBOA present in the bioassays of the purified maize volatile was determined to be 700 ng. The number of larvae that were found in either side of the choice test was recorded after 1 hr and the experiment was replicated eight times.

MBOA on Corn Seedlings. The primary naturally occurring precursor to MBOA in corn is DIMBOA-glucoside (Wahlroos and Virtanen, 1959). Hofman and Hofmanová (1971) later corroborated this conclusion, but the limits of detectability of the aglucones in these early papers were not given. Recently, Zúñiga and Massardo (1991) detected a significant amount of the aglucone in the intact tissues of wheat despite inhibiting the β-glucosidase with liquid nitrogen and methanol. They also found only the aglucone in 2-day-old wheat and in undifferentiated wheat cell cultures. Because the limits of detection of the aglucones in corn were not given in the early manuscripts, we wished to test specifically for free MBOA.

Untreated corn kernels were washed and soaked as described above and were placed on moist germination paper for three days. The major root of the germinated seed was then quickly dipped into a 4-ml solution of dichloromethane. A total of 70 germinated seeds was extracted over a time period of 5 min. A portion of the extract (300 μl of 4 ml) was concentrated to 50 μl in a nitrogen stream (which took approximately 1 min) and was immediately injected onto a 25 cm × 4.6-mm, 5-μm Econosphere C-18 HPLC column with a Kratos

Spectroflow UV absorbance detector set at a wavelength of 290 nm. Flow through the system was held constant at 1 ml/min. Operating conditions were as follows: mobile phase, solvents A (20 mmol phosphoric acid, pH 2.3) and B (methanol)—0–5 min 0% B isocratic; 5–20 min 0% B–50% B linear gradient; 20–25 min 50% B–100% B linear gradient; 25–27 min 100% B isocratic, 27–30 min 100% B–0% B linear gradient. In addition to injection onto the HPLC, the same amount of the "quick dip" extract was also injected onto an RSL-150 nonpolar methyl silicone capillary GC column (30 m × 0.25-μm ID × 0.25-μm film thickness). Temperature conditions were as described above. As with the HPLC, the crude extract was also coinjected with synthetic MBOA under the same conditions.

MBOA Volatility. MBOA is a high-melting solid [melting point, 158°C (Chen and Chen, 1976)], but other high-melting solids such as camphor [melting point, 180°C (Weast and Astle, 1981)] have appreciable volatility under ambient conditions. It has been questioned whether MBOA is volatile at room temperature. In order to address this question, we conducted three types of experiments. First, because retention time on a methyl silicone GC column is highly correlated with vapor pressure ($r^2 = 0.724$) (Heath and Tumlinson, 1986), we injected synthetic MBOA and a series of known lepidopteran pheromone components. We used our RSL-150 methyl silicone capillary column with the conditions described above. Second, we extracted MBOA-formulated clay granules after being exposed to room air in a layer of granules one granule thick for periods of 0, 1, 2, 4, 8, and 16 days. Each sample consisted of 0.313 g of clay granules that had previously been formulated with 1600 μg of MBOA/g of clay granules. The samples were extracted with 0.5 ml of dichloromethane and 1 μl was injected onto the RLS-150 capillary column under the conditions described above. The experiment was replicated three times. In a third experiment, volatilized MBOA was collected in a cold trap for analysis. Ten milligrams of synthetic MBOA was placed in a 6.35-mm Teflon tube and held in place by 7.6 cm of tightly packed glass wool. One end of the tube was then connected with Swagelock fittings to a nitrogen tank with a two-stage regulator, and the other end of the tube was connected to a loop of Teflon tubing that was bathed in liquid nitrogen. Glass wool (7.6 cm) was placed in the bottom of the loop in the liquid nitrogen as a condensation site. The nitrogen flow rate through the system was held constant at 10 ml/min. After 26 hr, the tube was removed from the liquid nitrogen and extracted with 4 ml of dichloromethane. One milliliter of the extract was concentrated to 50 μl and injected onto the HPLC system as described above.

Statistical Analysis. The statistical package SAS (SAS Institute, 1990) was used in all data analysis. Proc ANOVA was used alone for tests with only two means, and with Duncan's (1955) multiple-range test for analysis with more than two means.

RESULTS

Behavioral Bioassays with the Purified Maize Volatile. The purified maize volatile was significantly attractive ($P < 0.01$) in choice tests with equal levels of carbon dioxide on both sides of the choice (Figure 1A).

EI and CI Mass Spectra. The EI mass spectrum of the purified maize volatile included peaks at *m/z* 165 (100), 150 (63), 136 (4), 122 (11), 109 (16), 106 (34), and 80 (15). The CI mass spectrum included peaks at 183 (100, M + NH_4), 166 (33, M + 1), 165 (50), 153 (2), 139 (2), 124 (5), 106 (4). The EI and CI mass spectra of the purified maize volatile indicated that the M^+ ion

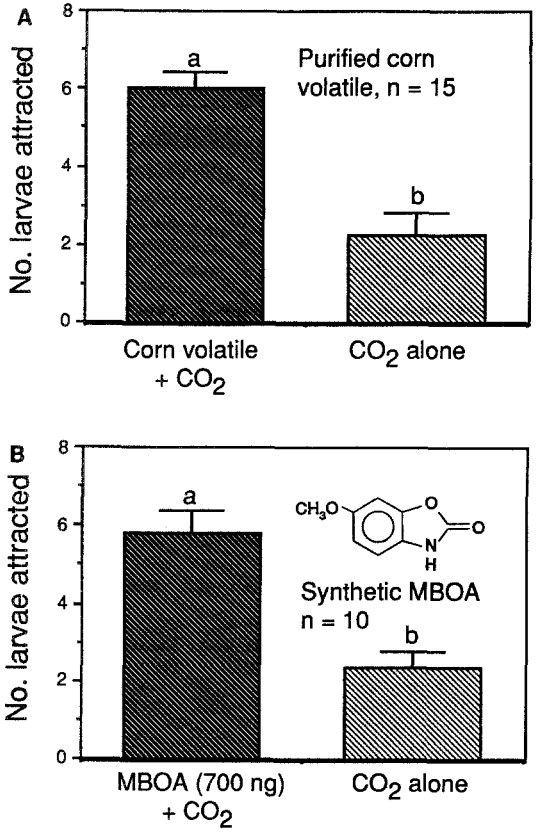

FIG. 1. Choice test bioassays of (A) purified maize volatile and (B) synthetic 6-methoxy-2-benzoxazolinone (MBOA) with equal levels of carbon dioxide on both sides. Treatment means labeled with different lowercase letters were significantly different ($P < 0.01$) according to a one-way analysis of variance (ANOVA). Error bars indicate the standard error of the mean (negative portions not shown).

is *m/z* 165. The odd molecular weight was consistent with the presence of a nitrogen atom in the molecule.

NMR Spectrum. The ^{1}H NMR spectrum of the compound is shown in Figure 2. The singlet at δ 3.89 (3H) indicated a methoxy group. The doublets at δ 6.92 (1H) and δ 6.82 (1H) and the double doublet at δ 6.69 (1H) indicated three aromatic hydrogens, with two of the hydrogens adjacent on the aromatic ring. The singlet at δ 8.19 (1H) indicated a proton on nitrogen.

IR Spectrum. The IR spectrum of the purified maize volatile indicated absorptions at 3150, 1770, and 1680 cm^{-1}, which was consistent with N—H, C=O, and C—N, respectively.

Identification. On the basis of the MS, NMR, and IR spectra of the natural and synthetic compound, the structure was determined to be 6-methoxy-2-benzoxazolinone (MBOA). The retention times of synthetic MBOA and the purified maize volatile on an RSL-150 capillary gas chromatograph column were both 14.3 min and this was verified by coinjection. The spectra from EI-MS, CI-MS, NMR, and IR of synthetic 6-methoxy-2-benzoxazolinone were in full agreement with the spectra of the isolated maize volatile.

Behavioral Bioassays with Synthetic MBOA. Synthetic MBOA was significantly attractive ($P < 0.01$) to western corn rootworm larvae at the dose present

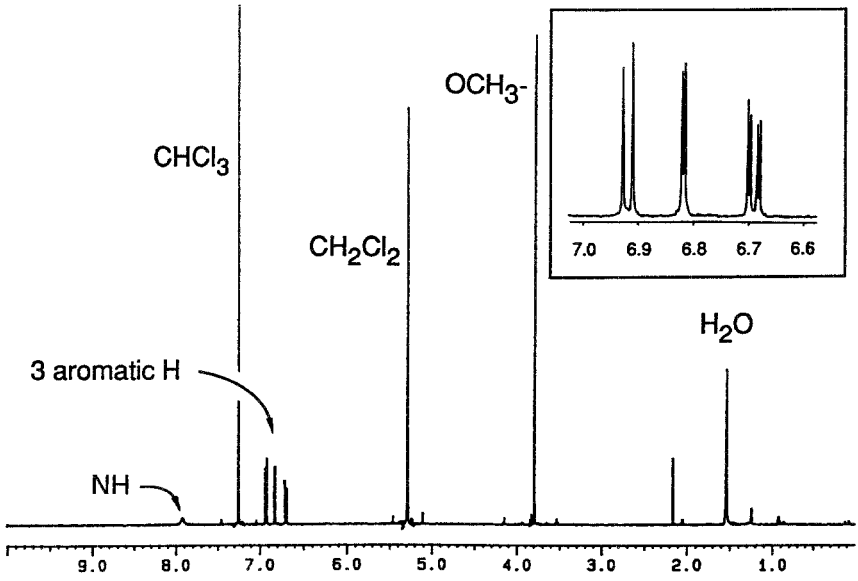

FIG. 2. ^{1}H NMR spectrum of the purified maize volatile. Inset: Magnification of the three aromatic protons.

in the purified maize volatile (700 ng) on glass wool in choice test bioassays with equal levels of carbon dioxide on both sides of the choice (Figure 1B).

MBOA on Corn Seedlings. Corn root extracts were analyzed using HPLC and GC within 8 min of the extraction of the first corn roots and within 3 min of the extraction of the last corn roots. Using HPLC, 98.7 ng of MBOA/g of 3-day-old corn roots (fresh weight) was found. MBOA was one of the largest peaks in the GC chromatogram, accounting for over 30% of the detected peak area. Over 8 μg of MBOA/g of 3-day-old corn roots (fresh weight) was found by GC with this "quick dip" method. The difference between the two methods can be explained by thermal degradation of MBOA derivable compounds in the injection port of the GC (240°C).

MBOA Volatility. The retention time of MBOA under the described GC conditions was 14.04 min. The retention time of (Z)-7-dodecenyl acetate was 12.49 min; (Z)-11-tetradecenyl acetate, 14.76 min; hexadecyl acetate, 16.74 min; and octadecyl acetate, 18.63 min. Since each of these compounds is known to be a volatile pheromone component for various lepidopteran species, and since retention time on nonpolar methyl silicone columns correlate well with vapor pressure (Heath and Tumlinson, 1986), the retention time of MBOA on our methyl silicone column supports the assertion that it is volatile. In the MBOA volatilization experiment, an average of 298 $\pm$ 35 μg of MBOA was extracted from 0.333 g of clay granules at time 0, 165 $\pm$ 10 μg after 1 day, 135 $\pm$ 20 μg after 2 days, 87 $\pm$ 5 μg after 4 days, and 20 $\pm$ 5 μg after 8 days. After 16 days, no detectable MBOA was found without concentration of the sample. In the cold-trapping experiment of synthetic MBOA crystals, over 40 ng of MBOA was recovered from 26 hr of passing a nitrogen stream over 10 mg of MBOA at a rate of 10 ml/min.

DISCUSSION

We have used a bioassay-driven sequential fractionation scheme to isolate behaviorally active portions of a crude dichloromethane corn seedling extract, and we have identified 6-methoxy-2-benzoxazolinone (MBOA) as one of the most important components of an attractive crude corn extract. Choice test bioassays with equal levels of carbon dioxide of both sides of the choice were used with the crude extract, each fraction of two separate types of separations, the purified natural compound, and the synthetic of our identification. MBOA is the first compound—other than carbon dioxide—identified from corn roots as behaviorally active to western corn rootworm larvae, one of the most important insect pests in the North America.

It was previously believed that MBOA is not an *in vivo* constituent of maize tissue but is produced through the decomposition of a glucoside that occurs in

the maize plant, 2-*O*-glucosyl-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (Wahlroos and Virtanen, 1959). When the plant is injured, the glucoside is enzymatically hydrolyzed, releasing the aglucone 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which is subsequently degraded to MBOA (Willard and Penner, 1976). Recently, however, the lack of aglucones in grass tissue has been questioned. Zúñiga and Massardo (1991) concluded that a significant amount of aglucones are present in the intact tissue of wheat. In the present study, we found 98.7 ng of MBOA/g of 3-day-old corn root (fresh weight) by quickly dipping the roots of undamaged corn seedlings in dichloromethane and immediately injecting the sample onto the HPLC. The enzymatic conversion of DIMBOA-glucoside to DIMBOA takes 30 min at room temperature (Lyons et al., 1988). The conversion of DIMBOA to MBOA has a half-life of 5.3 hr at 28°C and pH 6.75 (Woodward et al., 1978a). Since our injection was made within 12 min of the first corn root extracted, our data support the assertion that MBOA is present on or in the tissues of 3-day-old germinated corn.

Many plant species contain specific allelochemicals that provide an effective toxic defense against herbivorous insects (Waller, 1987). Despite this, even a plant species that is chemically well defended may be attacked successfully by a few specialist insect species that not only withstand a particular plant toxin, but use it as a semiochemical to locate and recognize their plant host (Waller, 1987; Harborne, 1988; Rosenthal and Janzen, 1982). No examples of this have been reported for insect herbivores of maize, one of the principal agricultural plant species worldwide. We now report that an important toxin produced by maize and other grasses, 6-methoxy-2-benzoxazolinone (MBOA), is used as a semiochemical for host location by western corn rootworm larvae.

The toxic and antifeedant effects of MBOA have been documented for several insect species, including the European corn borer, *Ostrinia nubilalis* (Hübner) (Klun and Brindley, 1966; Campos et al., 1988; Nicollier et al., 1982), the southwestern corn borer, *Diatraea grandiosella* Dyar (Nicollier et al., 1982), the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Nicollier et al., 1982), the cereal aphid, *Metopolophium dirhodum* (Walker) (Argandoña et al., 1980), the silkworm, *Bombyx mori* (L.) (Kubo and Kamikawa, 1983), the dried fruit beetle, *Carpophilus hemipterus* (L.) (Dowd, 1990), *Spodoptera exempta* (Walker) (Kubo and Kamikawa, 1983), the German cockroach, *Blatella germanica* (L.), and the southern armyworm, *Spodoptera eridana* (Cramer) (Beck and Stauffer, 1957), as well as bacteria (Nicollier et al., 1982; Beck and Stauffer, 1957), and fungi (Beck and Stauffer, 1957; Wahlroos and Virtanen, 1958; Beck and Smissman, 1961). However, MBOA is not toxic to western corn rootworm larvae (Abou-Fakhr et al., 1992).

DIMBOA, the precursor to MBOA, is generally considered a more potent toxin and is toxic to fungi (Long et al., 1975; Guthrie et al., 1985), and bacteria (Woodward et al., 1978b; Corcuera et al., 1978), the European corn borer (Klun

et al., 1967; Reed et al., 1972; Robinson et al., 1982; Campos et al., 1989), the greenbug, *Schizaphis graminum* (Rondani) (Argandoña et al., 1983), other aphids (Beck et al., 1983; Bohidar et al., 1986), and western corn rootworm larvae (Xie et al., 1990). Techniques have been developed that can quantify the amounts of known hydroxamic acids from corn roots (Xie et al., 1991a). The toxicity of DIMBOA to western corn rootworm larvae may be relevant to the isolation of strains of maize that have an antibiosis effect on the insect since quite high amounts of DIMBOA equivalents have been found in the cortex tissues of maize roots (Xie et al., 1991b). DIMBOA acts as a feeding deterrent at high concentrations (Argandoña et al., 1983; Corcuera et al., 1985). DIMBOA is an inducible chemical defense in maize, increasing significantly in concentration after feeding by larvae of *Sesamia nonagrioides* (Lefebvre) (Gutierrez et al., 1988). For more comprehensive reviews of the biology and chemistry of cyclic hydroxamic acids, see Willard and Penner (1976) and Niemeyer (1988).

Hydroxamic acids such as MBOA are well suited as semiochemicals for host location by a soil insect. MBOA has sufficient vapor pressure to diffuse in air, yet it is appreciably water soluble as well. MBOA is volatile under ambient conditions despite its relatively high melting point (158°C) and crystalline structure at room temperature, just as other volatile compounds such as camphor (m.p. 180°C) have similar melting points and crystalline structure at room temperature. In the present paper we demonstrated that MBOA is a volatile compound with vapor pressure comparable to that of many insect pheromones. These characteristics may allow it to move effectively through the soil under a wide range of soil moisture conditions. Hydroxamic acids have been documented in 10 genera of the Gramineae, but there are only two reports outside the Gramineae (Niemeyer, 1988). MBOA provides a simple way for western corn rootworm larvae to limit their orientation to grasses rather than to plants in general. Western corn rootworm larvae are not infallible in their location of appropriate hosts, in that they will orient to many nonhost grass species as well as they do to maize (Branson, 1982), perhaps because MBOA or related compounds are common to both. Larvae are attracted to the roots of sorghum and feed on them, despite the fact that sorghum roots contain cyanogenic glycosides that quickly kill western corn rootworm larvae (Branson et al., 1969; Strnad and Dunn, 1990).

MBOA and carbon dioxide are not the only compounds involved in host selection by western corn rootworm larvae. We have isolated an additional attractive fraction and two repellent fractions by GC separations of dichloromethane extracts of germinating maize (Hibbard and Bjostad, 1990). It is possible that the repellent fractions include compounds that are toxic to western corn rootworm larvae. These behaviorally active fractions are currently under

investigation and may be additional compounds used in discrimination of host plants from nonhosts.

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