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ISOLATION OF CORN SEMIOCHEMICALS ATTRACTIVE AND REPELLENT TO WESTERN CORN ROOTWORM[1] LARVAE

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Abstract—Dichloromethane extracts of germinating corn are significantly attractive to western corn rootworm larvae in choice tests with equal levels of carbon dioxide present on both sides of the choice. Two fractions that are significantly attractive and two fractions that are significantly repellent to larvae were isolated from these extracts of germinating corn by gas chromatography and silica gel chromatography. In a separate set of experiments, Porapak N was used to collect headspace volatiles from germinating corn; significantly more larvae were attracted to aliquots of these extracts in single-choice tests without added carbon dioxide present than to solvent controls.

Key Words—Coleoptera, Chrysomelidae, *Diabrotica virgifera virgifera*, semiochemical, kairomone, attractants, repellents, *Zea mays*, western corn rootworm.

INTRODUCTION

More acreage is treated with insecticide for *Diabrotica* spp. than for any other insect pest in the United States (Suguiyama and Carlson, 1985). An estimate of crop losses and treatment costs for corn rootworms is in the range of $1 billion per year (Metcalf, 1986). Because of their economic importance, the chemical

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ecology of adult *Diabrotica* has been well studied. The sex pheromones, as well as many attractants and feeding stimulants, have been identified for adult beetles (Guss et al., 1982; Chuman et al., 1987; Lampman and Metcalf, 1988; and references therein). However, it is the larval stage of most *Diabrotica* spp. that is damaging. The only compound known to have behavioral importance to any *Diabrotica* spp. larvae is carbon dioxide, which is an attractant for larvae of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988). WCR larvae are oligophagous and feed only on certain species of Poaceae (Gramineae) (Branson and Ortman, 1967, 1970). Because carbon dioxide is released by the roots of most plant species (Harris and Van Bavel, 1957), it does not appear to provide a basis for the ability of the larvae to distinguish the roots of host plants from the roots of nonhost plants. Hibbard and Bjostad (1988) collected corn seedling volatiles and demonstrated that these volatiles in conjunction with carbon dioxide were significantly more attractive to WCR larvae than the same level of carbon dioxide alone. The aim of the present study was to isolate these corn semiochemicals.

Two separate fractionation procedures were used to isolate the compounds of interest from the attractive crude extract. The first involved the use of silica gel column chromatography, which separates primarily on the basis of polarity. The second involved gas chromatography, using a column that separates primarily on the basis of molecular weight. A sequential fractionation scheme modified from Silverstein et al. (1967) then was used for isolating the individual semiochemicals. Behavioral choice-test bioassays were used to test the biological activity of crude extracts and individual fractions.

METHODS AND MATERIALS

Larvae and Corn Source. A nondiapausing strain of *D. virgifera virgifera* was obtained in June 1986 from the USDA-ARS laboratory in Brookings, South Dakota. The rootworm colony was maintained with the methods of Jackson (1986) as modified by Hibbard and Bjostad (1988). Second instars (6–7 days old and 5–8 mm long) were chosen for assays because preliminary work indicated that their behavior was similar to that of unfed, newly hatched first instars (the instar responsible for host location), and second instars were more robust for the large number of bioassays required. Dried, whole-kernel field corn (3090, Pioneer Hi-Bred International, Inc., Johnston, Iowa) not treated with fungicide or insecticide was washed and soaked (Hibbard and Bjostad, 1988), and corn seedlings [pre-stage 0 germinated seed (Hanway, 1966)] were removed after three to five days to obtain corn semiochemicals.

Bioassays with Porapak-Collected Corn Volatiles. Thermally conditioned Porapak N (1 1/2 g) was used as a solid adsorbent (Byrne et al., 1975) to collect

volatiles from approximately 500 g of corn seedlings. A vacuum pump (flow rate 6000 ml/min) pulled room air through the corn and Porapak N for 12 h. The Porapak N was extracted with 4 ml of dichloromethane, and aliquots of this solution (100 μl) were placed on rolls of filter paper (4 $\times$ 4 cm before rolling). The bioassay apparatus (diagrammed in Figure 1A) and procedures are described in detail in Hibbard and Bjostad (1988). Briefly, the solvent was

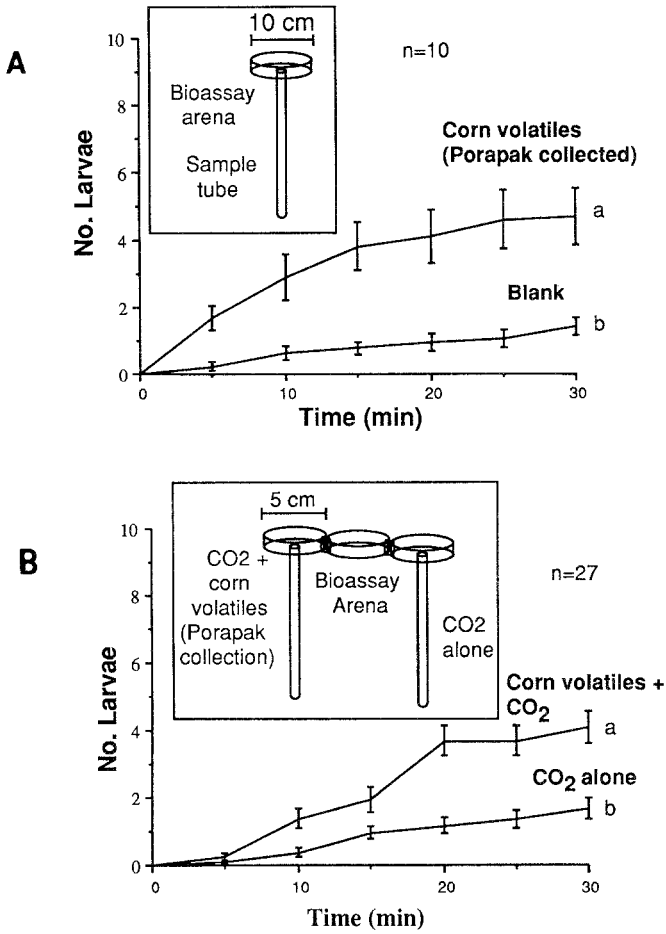

FIG. 1. (A) Single-choice tests with Porapak-collected corn volatiles and blank tubes. (B) Double-choice tests with Porapak-collected corn volatiles with added carbon dioxide on one side versus an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and diagrams of the bioassay arenas are shown. Standard error bars for each point are indicated. Different small letters represent significant differences ($P < 0.05$) within each graph.

allowed to evaporate, and the rolls of filter paper were placed in the bottom of a clean glass tube (35 cm × 12 mm) that was closed at the bottom. The tube was then connected to the bottom of a plastic Petri dish with a Teflon connector. No carbon dioxide was added. After a 5-min delay to allow for the establishment of a diffusion gradient, 10 larvae were added around the periphery of the Petri dish. Bioassays were conducted under dim lighting. The number of larvae that entered the tube was recorded at 5-min intervals for 30 min. Clean tubes were used as controls.

Compounds of smaller molecular weight (such as carbon dioxide) pass through Porapak (Byrne et al., 1975; Hibbard and Bjostad, 1988), so its use to recover volatile semiochemicals is limited to compounds of higher molecular weight. Because carbon dioxide is a known attractant for WCR larvae (Strnad et al., 1986; Hibbard and Bjostad, 1988), a double-tube choice-test (diagrammed in Figure 1B) was used to assess the attractiveness of carbon dioxide plus the higher-molecular-weight volatile components collected by Porapak to carbon dioxide alone. Equal amounts of carbon dioxide were introduced into both tubes to give a concentration of 5 mmol/mol at bioassay initiation and 1 mmol/mol after 30 min (Hibbard and Bjostad, 1988). The carbon dioxide concentration was measured with an infrared gas analyzer (IRGA, Beckmann model 865) that we interfaced with a Porapak N gas chromatograph column (3 mm × 2 m) as described in Hibbard and Bjostad (1988). A filter paper roll (5 cm diam.) was treated with an aliquot (100 μl) of Porapak-collected volatiles, and a second filter paper roll was treated with distilled dichloromethane as a control. The solvent was allowed to evaporate before placing the rolls in the two sides of the bioassay apparatus. After a 5-min delay, 10 larvae were placed in a small plastic Petri dish lid (40 mm diam. × 5 mm high), and this small lid was placed inside the center Petri dish of the bioassay apparatus. Bioassays were performed in dim lighting, and the number of larvae in each of the dishes was recorded every 5 min for 30 min.

Bioassays with Organic Solvent Extracts of Cryogenically Collected Corn Volatiles. Cryogenic collections of corn seedling volatiles are attractive to WCR larvae and were carried out as previously described (Hibbard and Bjostad, 1988). For these tests, however, 4-ml extracts (pentane or dichloromethane) of these collections were made by rinsing the tubes with solvent. The bioassay procedures were as in the Porapak experiments described above, except that glass wool served as the evaporative surface for the 4-ml collections instead of filter paper. Equal amounts of glass wool (0.10 g) and solvent were used on both sides of the choice test, but one side contained corn volatiles. Carbon dioxide concentrations were 5 mmol/mol at bioassay initiation on both sides of the choice test as described above. The number of larvae that entered either of the end dishes were recorded after 30 min and 60 min. Bioassays were performed in

the dark with a brief interruption after 30 min. New apparatuses were used for each treatment to minimize effects of contamination from the plastic Petri dishes.

Bioassays with Organic Solvent Extracts of Corn Seedlings. Approximately 80 g of moist corn seedlings [pre-stage 0 (Hanway, 1966), 3–5 days old] were extracted by placing them in a glass seed-holding tube (30 cm × 30 mm, tapering to 12 mm) for 3–6 hr and then dripping solvent (pentane or dichloromethane) through them until 4 ml of solution had been collected. Bioassays were as described above, but with new apparatuses to minimize effects of contamination of the plastic Petri dishes.

Silica Gel Column Chromatography. Organic extracts of corn seedlings (4 ml dichloromethane) were evaporated to 0.5 ml in a nitrogen stream. A 22.9-cm pipet (Pasteur type, Curtin Matheson Scientific, Inc.) was cut to 10.5 cm (the location where the pipet tapered) and plugged with glass wool. Silica gel (60–200 mesh, J.T. Baker Chemical Co., Phillipsburg, New Jersey) was transferred to the column (pipet) in dichloromethane until it was 3.5 cm from the top. Crude corn extract (0.5 ml) was then placed at the top of the column keeping the solvent level above the silica gel. Solvent was allowed to pass through the column (and corn extract), and fractions were collected. Because pentane extracts were not biologically active to WCR larvae, the initial solvent system used was 100% dichloromethane. Fractions 2, 3, 4, 5, and 6 were 4 ml each of 3%, 10%, 25%, 50%, and 100% diethyl ether in dichloromethane that had passed through the column. A small portion of each fraction from each replication was analyzed by gas chromatography. Bioassays were as described above, but with new apparatuses to minimize effects of contamination from the plastic Petri dishes.

Gas Chromatography. Organic solvent extracts of corn seedlings (4 ml dichloromethane) were evaporated to less than 10 μl with a nitrogen stream. The highly concentrated solution was separated with an OV-101 packed column (3% OV-101 on Gaschrom Q, mesh 100–120, Alltech Associates Inc., Applied Science Labs, Deerfield, Illinois) of glass tubing (1.83 m × 2 mm ID) in a Hewlett-Packard 5890 gas chromatograph temperature programmed from 60°C to 260°C at 10°C/min with a 1-min initial time and a final time of 9 min. With the detector and noncolumn gases turned off, fractions were collected with clean pipets that were snugly connected to the end of the column with a specially fitted Teflon connector. The collection efficiency of this system ranged from 70% to 85% with known compounds with similar retention times.

Because the attractive fraction from silica gel chromatography contained only one peak, we first isolated this fraction with gas chromatography by collecting during its retention time (16–17 min). The transfer pipets were rinsed with 200 μl of dichloromethane, and bioassays were as described above (with new apparatuses). As with the silica gel fractionation, a full set of fractions also

were collected and bioassayed. Fractions were collected every 3 min during 30 min GC run (all detectable peaks eluted during this time) for a total of 10 fractions. In a second set of GC collections, fractions were collected every minute during the retention time of the attractive fraction 8 (21–24 min) of the 3-min collections. One microliter of the 200-μl rinse of each fraction was analyzed by gas chromatography under the same conditions as the collection for both 3-min and 1-min collections to verify separation of the highly concentrated extract. Further fractionation of the repellent fractions in the 3-min collections were not performed because our primary interest was in host location. Bioassays were as described above, but with new apparatuses to minimize contamination from the plastic Petri dishes. When an isolation on a OV-101 packed column indicated only one peak present, the fraction also was injected onto a methyl silicone capillary column (30 m, RS-150, Supelco Inc., Bellefonte, Pennsylvania) to help determine if the peak had indeed been isolated to one compound.

Statistical Analysis. The statistical program MSTAT (Department of Crop and Soil Sciences, Michigan State University, East Lansing, Michigan 48824) was used for all computerized statistical calculations. Differences between the number of larvae in the two sides of the choice tests were used in ANOVAs for these paired data, while raw data were used for single-choice tests. All data were analyzed with one way analysis of variance (ANOVA). Ninety-five percent confidence intervals were then calculated using the error mean square from the ANOVA table (Snedecor and Cochran, 1980) for data sets with more than two means. Confidence intervals were calculated for fractionation experiments instead of multiple-range tests, because the only comparisons of biological relevance were the percent response of the larvae to zero (see Figures 4 and 5 below).

RESULTS

Significantly more ($P < 0.01$) WCR larvae were attracted to volatiles collected from corn seedlings on Porapak N than to sample tubes with ambient air in single-choice bioassays (Figure 1A). Porapak does not collect small compounds such as carbon dioxide (Byrne et al., 1975; Hibbard and Bjostad, 1988), so the level of carbon dioxide in the Porapak-collected volatile tubes was the same as that in ambient air (verified with infrared gas analysis). These data represent the first indication that carbon dioxide may not be a required component in a blend of corn semiochemicals that is attractive to WCR larvae.

When given a choice between Porapak-collected corn seedling volatiles and blank tubes with an equal concentration of carbon dioxide, significantly more ($P < 0.05$) WCR larvae chose the side with corn volatiles with added

carbon dioxide than the side with carbon dioxide alone (Figure 1B). The difference was statistically significant, but it was quite small compared to this difference when volatiles were collected cryogenically (see Hibbard and Bjostad, 1988).

There was no significant difference between the number of larvae attracted to the side with pentane extracts of cryogenic collections of corn volatiles (with added carbon dioxide) and the number of larvae on the side with an equal level of carbon dioxide alone (Figure 2A). Extracts of germinating corn with pentane also resulted in no significant differences in choice tests (Figure 2B). Pentane apparently does not extract the behaviorally active compounds. However, when dichloromethane was used, significantly more WCR larvae chose the side with extracts of both cryogenic corn collections (Figure 2C, $P < 0.01$) and extracts of germinating corn (Figure 2D, $P < 0.01$).

In choice-test bioassays from silica gel chromatography, fraction 2 (3% diethyl ether in dichloromethane) was significantly repellent ($P < 0.05$), and fraction 4 (25% diethyl ether in dichloromethane) was significantly attractive ($P < 0.05$) (Figure 3). Because GC analysis was performed on each fraction of each replication, an indication of the consistency of separation between individual fractionations was possible. A typical separation is shown in Figure 4. The attractive fraction 4 appeared to contain only one major component. Some variation was observed, and particular replicates in which fraction 5 was attractive contained more of the main component observed in GC analysis of fraction 4 than did other replicates of fraction 5.

Using gas chromatography, we were successful in isolating the attractive peak from silica gel fraction 4 (see Figure 7B below). The fraction was isolated using two different chemical techniques and was a single peak on OV-101 as well as on our methyl silicon capillary column, so we make the assumption that it is a single compound. The compound was significantly attractive ($P < 0.01$) in choice tests with equal levels of carbon dioxide on both sides of the test. Separations by gas chromatography with fractions collected every 3 min resulted in two fractions that were significantly repellent and one fraction that was significantly attractive (Figure 5). Fraction 9 (GC retention time 24–27 min) was significantly repellent, and GC retention times for the major components of this fraction correlated well with the GC retention times of the major components of the repellent fraction 2 of silica gel fractionation. Fraction 4 (GC retention time 9–12 min) was also significantly repellent ($P < 0.05$) and contained only one GC peak on OV-101 and our methyl silicone capillary column (see Figure 7A below), so a repellent was isolated in this 3-min GC collection. Fraction 8 (GC retention time 21–24 min) was significantly attractive in these choice tests, but contained several peaks.

Further fractionation by gas chromatography with fractions collected every

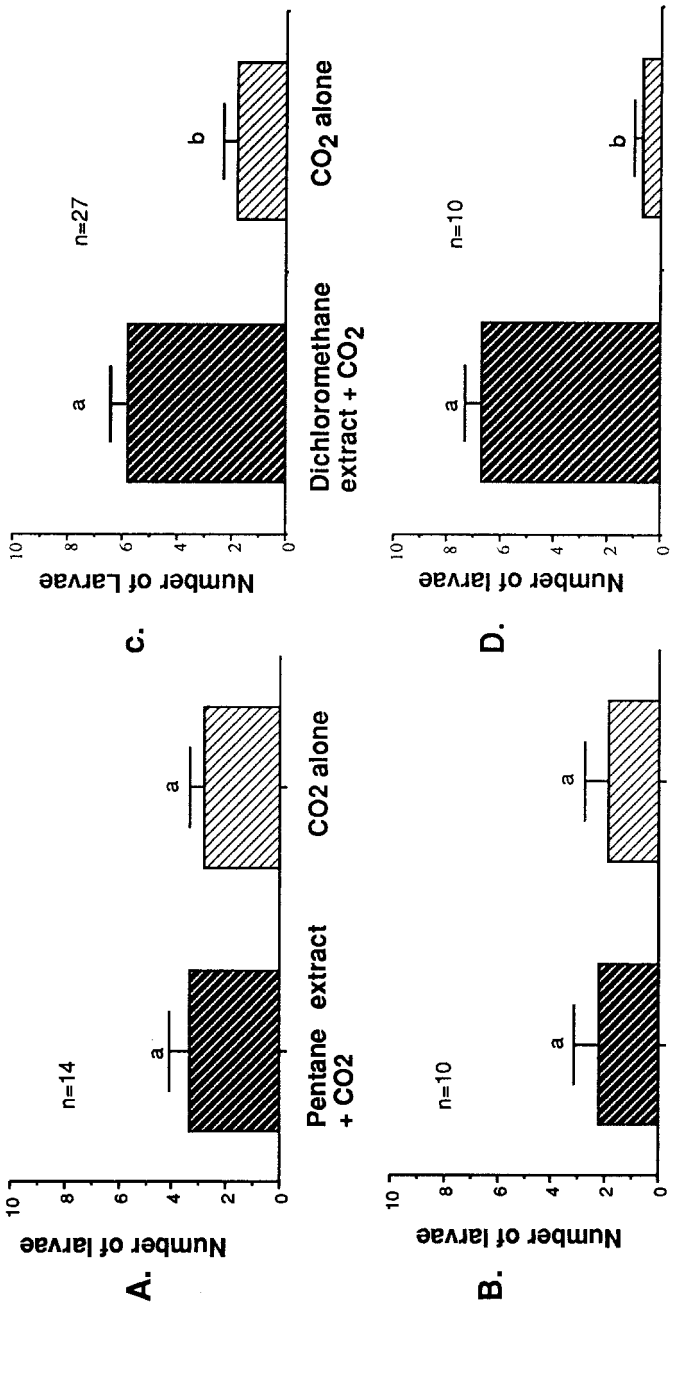

FIG. 2. Choice-test bioassays with carbon dioxide on both sides of pentane extracts of (A) cryogenic corn collections and (B) germinating corn, and dichloromethane extracts of (C) cryogenic corn collections and (D) germinating corn. The number of replicates are indicated by an "n," and error bars represent standard error. Significant differences ($P < 0.01$) within each graph are indicated by different lowercase letters.

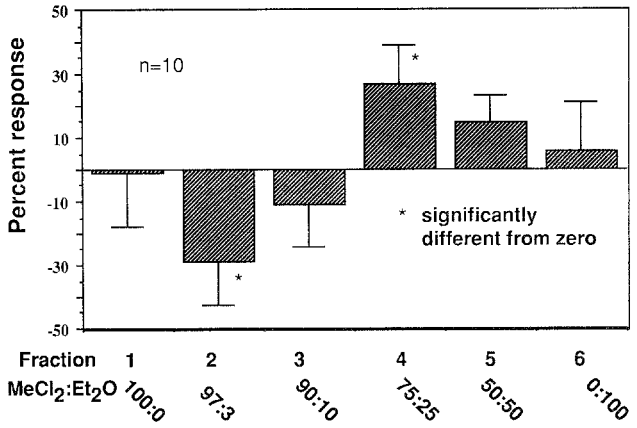

FIG. 3. Choice-test bioassays of silica gel column chromatography fractions of germinating corn extracts. A positive response indicates that more larvae chose the side with corn semiochemicals plus added carbon dioxide than the side with an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and error bars represent standard error. The only significant differences ($P < 0.05$) indicated are differences from zero, and are designated by an asterisk.

minute during the retention time of fraction 8 above resulted in one fraction (retention time 23–24 min) that was significantly attractive, while the other fractions were not significantly attractive or repellent (Figure 6). GC analysis of this last attractive fraction (retention time 23–24) indicated that at least two major components were present in this fraction (Figure 7C).

DISCUSSION

A number of corn root volatiles have been identified, but have not been tested behaviorally with WCR larvae. Buttery and Ling (1985) used Tenax to collect volatiles from the roots of young (60 cm high) and mature corn plants (2 m high). The principal volatiles recovered were sesquiterpene hydrocarbons: β-caryophyllene, longifolene, bazzanene (tentative), cyclosativene, α-ylangene, and a major hydrocarbon that was not identified. All of these compounds dissolve readily in dichloromethane and should elute with 100% dichloromethane in silica gel column chromatography. In the present study, pentane extracts and the 100% dichloromethane column chromatography fraction had no activity (Figure 3). We used corn seedling [pre-stage 0 germinated corn (Hanway, 1966)] extracts in which corn roots, kernels, and young leaves were present. Compounds previously identified from corn leaves include (Z)-3-hexenol, (Z)-3-

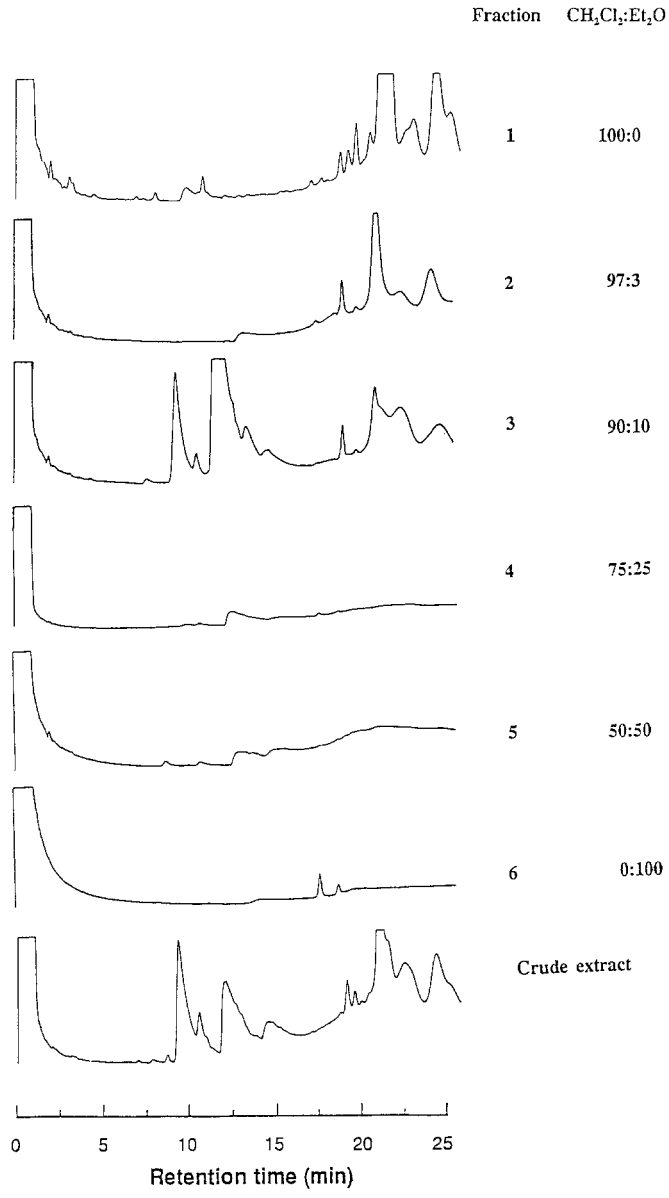

FIG. 4. GC traces on an OV-101 column of a silica gel separation of a dichloromethane extract of germinating corn. The ratio of dichloromethane to diethyl ether that eluted each fraction is given at the right.

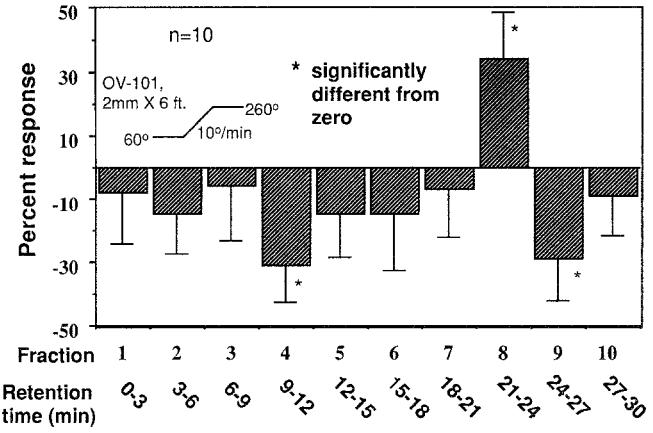

FIG. 5. Choice-test bioassays of gas chromatography fractions of germinating corn extracts. A positive response indicates that more larvae chose the side with corn semiochemicals plus added carbon dioxide than the side with an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and error bars represent standard error. The only significant differences ($P < 0.05$) indicated are differences from zero and are designated by an asterisk.

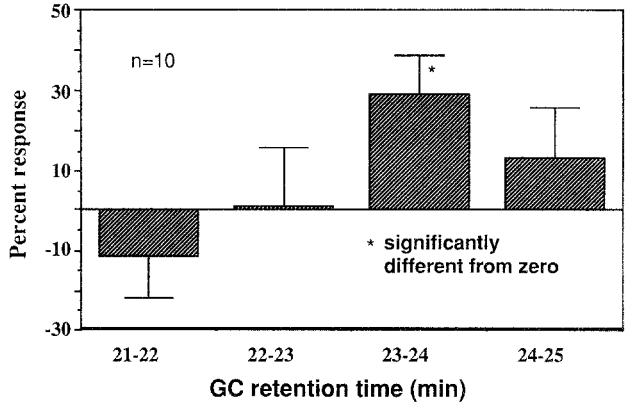

FIG. 6. Choice-test bioassays of gas chromatography fractions of germinating corn extracts. A positive response indicates that more larvae chose the side with corn semiochemicals plus added carbon dioxide than the side with an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and error bars represent standard error. The only significant differences ($P < 0.05$) indicated are differences from zero and are designated by an asterisk.

hexenyl acetate, (Z)-4-hepten-2-one, (Z)-4-hepten-2-ol, α-ylangene, cyclosativene (tentative), and caryophyllene (Buttery and Ling, 1984). Compounds previously identified from corn kernels include 2-nonan-ol, 2-heptan-ol, 4-hept-en-2-ol, 2-undecan-ol, 4-hept-en-1-ol, 4-hept-en-2-ol, 4-hept-en-2-one, α-ylangene, geranylacetone, β-ionone, and 2,4,7-decatrienal (Buttery et al., 1978). Most of the above corn leaf and kernel compounds would be soluble in pentane and also would be expected to elute with dichloromethane (the first fraction) in silica gel chromatography as well. Therefore, it appears that the attractive compounds from our experiments are not among these previously identified corn volatiles.

In the present study, one compound has been isolated (Figure 7B) that is attractive to WCR larvae in choice tests. As indicated by its elution from silica gel with 25% diethyl ether–75% dichloromethane, this compound is somewhat polar, which is consistent with our earlier observation that dichloromethane extracts are attractive and pentane extracts are not. One additional fraction [which contains at least two GC peaks (Figure 7C)] is also attractive in choice tests (Figure 6). One repellent has been isolated (Figure 7A), and another fraction [which contains at least three GC peaks (Figure 7D)] is repellent as well (Figure 5).

It may seem odd that compounds that are repellent to WCR larvae would be found in corn extracts, especially when the corn extract overall was found to be attractive. However, allelopathic chemicals that act as a defense against herbivores are produced by many plants, including corn. For example, Klun et al. (1967) showed that corn plants produce 2,4-dihydroxy-7-methoxy-(2H)-1,4,-benzoxazin-3(4H)-one (DIMBOA), which is a main factor in resistance of corn to first-generation European corn borer. Other classes of secondary plant compounds produced by *Zea* include alkaloids, cyanogenic glycosides, phenolics, and terpenes (Redak, 1987). All of these classes of compounds have been shown to deter herbivory in other systems. It may be that the repellent compounds we have isolated are secondary compounds produced by corn that are in some way toxic to WCR larvae.

All four of the active fractions in the present study were separated from crude dichloromethane extracts of germinating corn. Attempts were made to keep the concentration in fractionation experiments the same as that in experiments with the attractive crude extracts, but we have evidence that indicates that dosage is an important factor. The attractive compound isolated (Figure 7B) is the major peak in silica gel fraction 4 (Figure 4). However, in GC fractionations, the fraction with this retention time (fraction 6) was not attractive. GC analysis indicated that this peak was present in relatively high amounts in fraction 6 of the GC fractionation (a number of other compounds were also present). As shown in Figure 4, relatively high amounts also occurred in silica

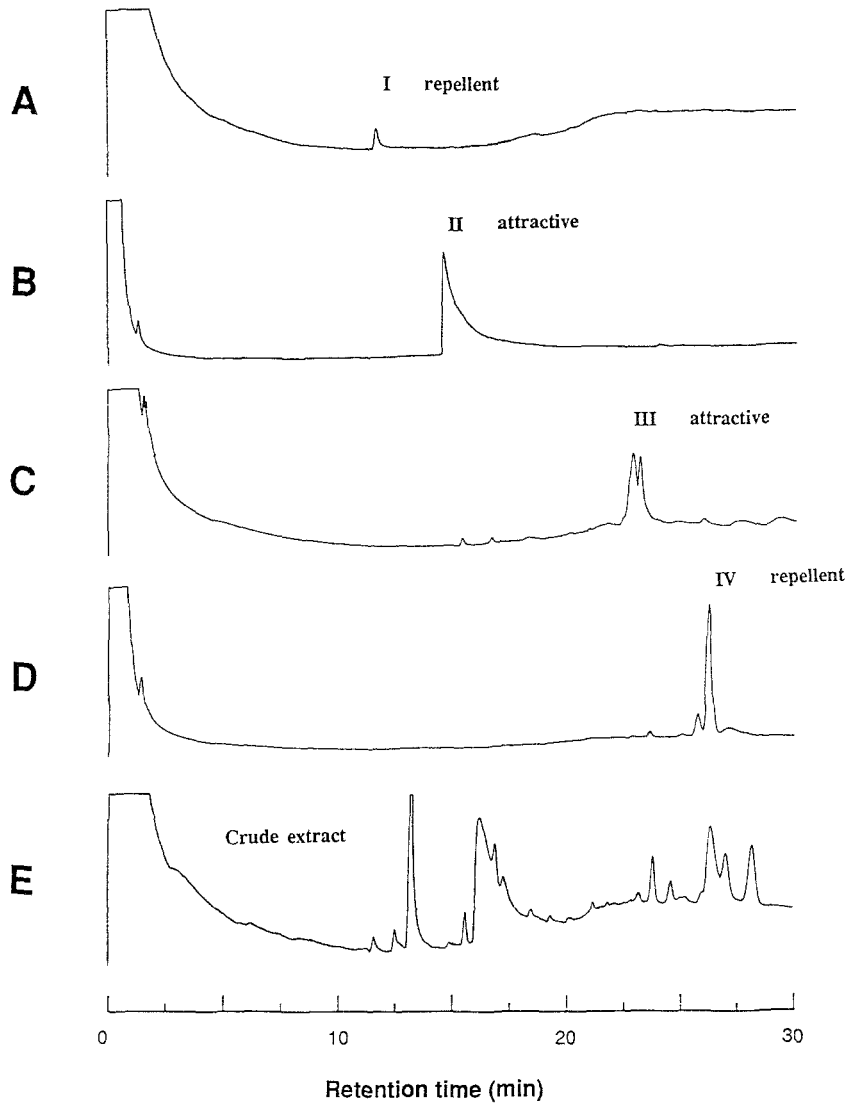

FIG. 7. GC traces of active fractions (doses of active fractions have been concentrated in relation to the crude extract). (A) Three-minute GC collection from a retention time of 9–12 min. (B) One-minute GC collection from a retention time of 16–17 min. The compound isolated was also the major component of silica gel fraction 4. (C) One-minute GC collection of retention time 23–24 min. (D) Three-minute GC collection from a retention time of 24–27 min. The major peaks present in this fraction also were present in silica gel fraction 2. (E) Dichloromethane crude extract of germinating corn.

gel fraction 3 (which was not attractive), while much lower amounts were found in silica gel fraction 4 (which was attractive). We believe that the compound isolated is not attractive in very high doses.

Porapak-collected corn seedling volatiles tested in combination with carbon dioxide were more attractive than a comparable amount of carbon dioxide alone, but the difference between the two sides of the choice test was much less than the difference between the two sides of the choice test in cryogenic corn collections in Hibbard and Bjostad (1988). This indicates that either Porapak N does not collect all of the attractive components from corn that a cryogenic collection does or that the concentration presented to the larvae in the Porapak experiment was not as close to the optimal concentration as was the concentration of these volatiles in the cryogenic collection experiment.

Because of its low molecular weight, carbon dioxide diffuses over greater distances in the soil atmosphere than volatile exudates of higher molecular weight (Green, 1971). Branson (1982) found that WCR larvae are attracted to the roots of nonhost as well as host plants. WCR larvae may be using carbon dioxide as a primary cue for longer distance orientation and more specific cues for close range orientation. Strnad and Dunn (1990) found that when unfed first-instar larvae contacted maize roots, they no longer were attracted to carbon dioxide, but continued localized searching.

Bioassays of Porapak extract in single-choice tests (Figure 1A) indicate that one or more corn semiochemicals are attractive to WCR larvae without carbon dioxide present. These data, in conjunction with the results of Hibbard and Bjostad (1989), suggest that it may be possible to incorporate these larger semiochemicals into insecticide formulations and attract WCR larvae to the insecticide to increase the efficacy of rootworm insecticides.

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