DISSERTATION

ATTRACTION OF WESTERN CORN ROOTWORM AND SUBTERRANEAN TERMITES TO CARBON DIOXIDE WITH IMPLICATIONS FOR PEST MANAGEMENT

Submitted by

Elisa Jo Bernklau

Department of Bioagricultural Sciences and Pest Management

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300 North Zeeb Road
P.O. Box 1346
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ELISA JO BERNKLAU ENTITLED ATTRACTION OF WESTERN CORN ROOTWORM AND SUBTERRANEAN TERMITES TO CARBON DIOXIDE WITH IMPLICATIONS FOR PEST MANAGEMENT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Carol A. Ishmael

D. A. Peterson

Ben C. Kondratieff

L. B. Bjostad

Advisor

Thomas O. Holtzer

Department Head

ABSTRACT OF DISSERTATION

ATTRACTION OF WESTERN CORN ROOTWORM AND SUBTERRANEAN TERMITES TO CARBON DIOXIDE WITH IMPLICATIONS FOR PEST MANAGEMENT

Carbon dioxide (CO_2) is an attractant for larvae of the western corn rootworm (WCR), *Diabrotia virgifera virgifera* LeConte, a major pest of corn in the United States. CO_2 is important to soil-dwelling termites of economic importance, but its exact role has not been determined.

Elevated concentrations of CO_2 prevented neonate WCR larvae from locating corn roots in soil bioassays. Larvae were attracted away from corn by gaseous CO_2 pumped into the soil and by non-toxic CO_2-emitting materials mixed into the soil. Test materials produced CO_2 concentrations between 15.8 and 18.5 mmol/mol (compared to 1.7 - 2.6 mmol/mol in controls) and this was sufficient to prevent larvae from locating corn roots. In field trials, CO_2-generating treatments resulted in root ratings that were significantly lower than for control plants.

In feeding bioassays, neonate WCR larvae fed vigorously on paper disks treated with liquid pressed from corn roots or with an acetone extract of corn, but not on disks treated with distilled water. Amounts of the insecticide thiamethoxam required for 50% mortality of WCR larvae were reduced 100-fold when extracts of germinating corn were used to entice the neonate larvae to feed on it.

Granules of spent grain, processed corn cob, and clay, coated with corn extract and thiamethoxam, provided better control of WCR larvae than a standard rate of

Counter 20CR insecticide. The attractant CO_2 improved insecticide efficacy, and CO_2 plus feeding stimulants caused even greater enhancement of insecticides than CO_2 or feeding stimulants alone. In field trials, corn treated with CO_2-generating granules containing feeding stimulants and insecticide had significantly less larval feeding damage than untreated control plants.

In behavioral bioassays *Reticulitermes flavipes, R. tibialis*, and *R. virginicus* were attracted to CO_2 concentrations between 5 and 50 mmol/mol. In field tests, stations containing CO_2-generating baits attracted termites away from wooden fence posts. Discovery time was shorter and visitation rates were higher for the CO_2-baited stations. This is the first report of attraction to CO_2 for any termite species.

Elisa Jo Bernklau
Bioagricultural Sciences
& Pest Management Department
Colorado State University
Fort Collins, CO 80523
Summer 2003

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CHAPTER 1

INTRODUCTION

Carbon dioxide (CO_2) is attractive to a number of soil-dwelling insects (Klingler 1957, 1966, Doane et al. 1975, Jones and Coaker 1977), including larvae of corn rootworm beetles, *Diabrotica* spp. (Jones and Coaker 1977, Strnad et al. 1986, MacDonald and Ellis 1990, Jewett and Bjostad 1996, Bernklau and Bjostad 1998a). Rootworm larvae feed on the roots of growing corn plants, causing reduced plant heights, reduced root systems and plant lodging that ultimately results in reduced crop yields (Branson et al. 1980). One species, the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, costs U.S. growers in excess of $1 billion annually in control costs and crop losses (Metcalf 1986).

Sven Strnad (1986) first demonstrated that CO_2 is attractive to western corn rootworm (WCR) larvae. More recently, it was demonstrated that CO_2 is the only volatile compound produced by corn roots that attracts WCR larvae, and that larvae can be attracted away from corn by a higher concentration of CO_2 alone (Bernklau and Bjostad 1998b). In addition, larvae can detect differences in CO_2 concentration as small as 12% (Bernklau and Bjostad 1998a).

These studies suggest that CO_2 may have implications for control of WCR larvae. In the field, less than 0.1% of pesticides may reach the target pest (Pimentel and Levitan 1986, Pike et al. 1995, Paoletti and Pimentel 2002). The remaining 99.9% may pose a risk to human health, wildlife, beneficial insects, invertebrates and microbes in the soil (Ripper 1956, Pimentel and Levitan 1986). The use of attractants may improve the efficiency of insecticide delivery and reduce the amount of insecticide needed to obtain pest control. Baits that contain attractants are currently used for

control of WCR adults (Tollefson 1998, Pingel et al. 2001) and it may be possible to use CO_2 as an attractant in a soil-applied bait for rootworm larvae.

The studies undertaken for this degree were based on a firm foundation of research with regard to CO_2 and rootworm larvae. A logical evolution for this body of work was to develop the basic concept (CO_2 attraction) into practical applications for control or WCR larvae. A second direction was to investigate the possibility that CO_2 may be attractive to other soil insects of economic importance. For example, subterranean termites live and forage in the soil and CO_2 may serve as a behavioral cue for these insects.

The hypotheses of my dissertation research were: 1) that CO_2 can be used to disrupt the host location behavior of WCR larvae in soil, 2) that it is practical to use CO_2-generating sources for the control of WCR larvae in commercial agriculture, 3) that additional semiochemicals can be used in conjunction with CO_2 for rootworm control and 4) that subterranean termites are attracted to CO_2.

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CHAPTER 2

LITERATURE REVIEW

Carbon Dioxide and Insects

Introduction

Much of what is currently known about the effects of carbon dioxide on insect biology, behavior and physiology was summarized in a comprehensive review published in 1989 (Nicolas and Sillans 1989). Recent studies focus on the practical uses of CO_2 in attracting or controlling insects, the identification of new sources of carbon dioxide, the behavioral effects of CO_2 on adult and larval insects and the effects of increasing atmospheric CO_2 on insects and insect populations.

Acute Exposure of Insects to Carbon Dioxide

Carbon dioxide is commonly used by entomologists to immobilize insects for study, but exposure to high levels of CO_2 may have lethal or sub-lethal effects. High concentrations have been shown to suffocate insects in an enclosed environment (Mulrooney and Adams 1998). In studies with *Drosophila*, Barron (2000) found that CO_2 used to anaesthetize flies significantly increased copulation latency, and therefore he does not recommend using CO_2 in behavioral studies. Similarly, anaesthetizing with CO_2 may increase the activity of *D. melanogaster* for several hours (Vale 1980). A short exposure to a dose of pure CO_2 causes changes in worker honeybee behavior that negatively impact the colony (Ribbands 1950, Heran 1952, Marden and Rinderer 1980). For *Locusta migratoria*, CO_2 anaesthesia reduces aggregation behavior (Nicolas 1979). Acute CO_2 exposure has been shown to inhibit drinking and feeding in house crickets (Woodring et al. 1978), to reduce locomotion in *L. migratoria* larvae (Nicolas 1978) and to delay mating in the cotton leafhopper (Kumar and Saxena 1978).

Carbon Dioxide as a Fumigant for Pest Control

Carbon dioxide can be used as a fumigant for control of stored grain pests and as such, is considered to be a non-residual treatment (Mann et al. 1999, Donahaye 2000). CO_2 tested as a fumigant in conjunction with low levels of phosphine provided effective control of all life stages of *Rhyzopertha dominica* (F.) (Martinazzo et al. 2000). Martinazzo (2000) reported that eggs and adult insects were the susceptible life stages and that atmospheres containing 21% CO_2 in combination with 0.50 or 0.75 g m(-3) phosphine were the most effective treatments. Using a mix of oxygen and 60% carbon dioxide, Hashem (2000) was able to control the resistant pupal stage of *S. paniceum*, and found the gas to be a promising method for controlling insect pests of stored medicinal products. In a similar study, CO_2 fumigation effectively controlled newly-hatched apple maggot larvae and longer exposures reduced survival of the egg stage as well (Agnello et al. 2002).

Choi (1997) suggests using carbon dioxide foam to control insects in agricultural environments or in greenhouses and in homes. An aqueous foam, which has been used in agriculture as a carrier for herbicides, might act to immobilize insects which would then be suffocated by the carbon dioxide and the gelatin-protein foam solution.

Use of Carbon Dioxide by Adult Insects for Host-Location

Olfaction is used in conjunction with other senses (primarily vision) by blood-sucking and biting insects to locate their vertebrate hosts. As a major component in the exhalent of vertebrates, carbon dioxide serves as one behavioral cue for these

adult insects (Allan et al. 1987, Nicolas and Sillans 1989). Female mosquitoes in search of a blood meal required for reproduction respond to a variety of volatile emanations from vertebrate hosts that include fatty acids, lactic acid and CO_2 (Takken 1999). Of these cues, carbon dioxide is the compound used most by mosquitoes to locate their prey (Hoyle 1960, Takken 1999).

Stable flies use CO_2, lactic acid and other human odors, in combination with visual stimuli, to find their prey (Warnes and Finlayson 1985b, Warnes and Finlayson 1985a, Warnes and Finlayson 1986, Raul and Carlson 2000). Traps baited with CO_2-emitting yeast cultures were shown to attract and capture adult triatome bugs, including *Triatoma infestans* Klug, the primary carrier of Chagas disease (Lorenzo et al. 1998). Carbon dioxide is also used for host location by tsetse flies (Glossinidae) that transmit sleeping sickness (Vale 1980). Flies in the families Tabanidae (Thornhill and Hays 1972, Nicolas and Sillans 1989) and Simuliidae (Sutcliffe 1986, Nicolas and Sillans 1989) respond to CO_2 and some non-biting flies may also exhibit an attraction to the gas (Vale 1980, Nicolas and Sillans 1989).

Human breath normally contains approximately 4.5% carbon dioxide (Nicolas 1979), compared to the ambient air with 0.035% (Nicolas 1979). Flies respond to a general increase in CO_2 concentration as opposed to any specific concentration (Vale 1980, Nicolas and Sillans 1989). Simulidae use the olfactory cues of carbon dioxide and other body odors in combination with visual cues of motion, color, size and shape to locate a suitable host (Gillies 1972, 1974, Gillies and Wilkes 1982). For Culicidae, and Simuliidae, specific cues are different for long range, midrange and close range attraction to a vertebrate host. Host-specific odors are used for long range orientation, but when closing in, these insects key on host odors in combination with carbon dioxide (Bursell 1987). It is in close range orientation and landing that the visual cues become more important.

As an attractant, carbon dioxide has been incorporated into devices used for trapping mosquitoes and biting flies (Gillies 1974, Vale and Hall 1985, Kline 1999). One current product, the Mosquito Magnet® (American Biophysics Corporation, East Greenwich, Rhode Island) attracts and kills mosquitoes by mimicking a human host with a combination of CO_2, heat, moisture and octenol (Kline 1998, 1999). Brenner and Pierce (1991) found that traps baited with distillers grain, which has been shown to produce CO_2 (Bernklau et al. 1999), were effective in attracting cockroaches.

Carbon Dioxide in the Soil Environment

The soil atmosphere obtains carbon dioxide from the respiration of plant roots and soil-dwelling organisms, and by the breakdown of organic material by microorganisms (Clinton and Vose 1999, Frank et al. 2002). In well-aerated soils, CO_2 is constantly being exchanged with oxygen and released into the atmosphere. Compared to atmospheric CO_2, which contains approximately 0.035% carbon dioxide, surface soil CO_2 levels vary from 0.3% in well-aerated soil to as high as 10% in poorly drained soils (Brady 1990). Natural factors that directly affect the movement of air, and the subsequent levels of carbon dioxide and oxygen within the soil, include the soil type, soil texture, amount of organic material and the soil depth. These factors are in turn influenced by specific plant and animal inhabitants, moisture, and weather, as well as by human manipulations related to cropping (Brady 1990).

Carbon dioxide produced by the respiration of plant roots causes chemical reactions in the soil atmosphere that benefit the plant. Under normal conditions, CO_2 given off by roots reacts with soil water to form carbonic acid ($CO_2 + H_2O \rightarrow H_2CO_3$) which dissociates H^+ protons into the soil water. These protons exchange with nutri-

ent cations (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}) adsorbed on clay particles in the soil, leaving the nutrient cations available in the soil water for uptake by plant roots (Tan 2000). In addition, root-respired CO_2 and the subsequent acidification of the rhizosphere may serve as a cue for beneficial rhizosphere bacteria to colonize sites along the roots (Glass and Siddiqi 1981, Bashan and Levanony 1989).

The carbon cycle and CO_2 are of particular interest to soil scientists because carbon is commonly considered to be "the focal point of energy transformations" (Brady 1990). Carbon is introduced to the soil in the form of organic wastes, residues and manure from live and dead animals and plants, and as underground residues from plants and soil-dwelling organisms. During decomposition by soil organisms, carbon from the organic debris is transformed and maintained in the soil as stable molecules of humus, or released as carbon dioxide (Tan 2000). As a result, levels of soil CO_2 are largely dependent upon the amount of organic material in the soil. Interactions over time between the climate, topography, organic life, and parent material cause organic carbon levels in a particular soil to reach and maintain an equilibrium (Stevenson 1986). Short-term fluctuations in CO_2 levels occur as a result of seasonal changes and environmental factors (Brady 1990).

Attraction of Soil-Dwelling Insects to Carbon Dioxide.

It is advantageous for polyphagous insects with a moderate-to-broad host range to respond to non-specific primary plant metabolites such as CO_2, rather than to host-specific secondary compounds (Klingler 1958, Nicolas and Sillans 1989). As a primary metabolite of respiration by plant roots, CO_2 serves as a host-location cue for soil-dwelling insects. Carbon dioxide alone is attractive to a number of soil invertebrates, including insect larvae (Klingler 1957, 1958, 1959, Paim and Beckel 1964, Klingler 1965, 1966, Stadler 1971, 1972, Meeking et al. 1974, Doane et al. 1975,

Jones and Coaker 1977, 1979), insect adults (Paim and Beckel 1963), mites (Moursi 1962, 1970), chilopods (Moursi 1970), nematodes (Johnson and Viglierchio 1961, Klingler 1961, 1963, 1965, Gaugler et al. 1980, Prot 1980, Dusenbery 1987, Pline and Dusenbery 1987, Robinson 1995) and bacteria (Scher et al. 1985).

Larvae of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte), a major pest of corn (*Zea mays*) in the United States (Krysan and Miller 1986) are phytophagous, soil-dwelling insects that feed on the roots of growing corn plants. Sven Strnad (Strnad et al. 1986) first reported that WCR are attracted to CO_2 and this attraction has also been demonstrated by other investigators (Hibbard and Bjostad 1988, MacDonald and Ellis 1990, Jewett and Bjostad 1996, Bernklau and Bjostad 1998a). The attraction of western corn rootworm larvae to corn roots is caused by CO_2 alone, and no other volatile chemical cues are involved (Bernklau and Bjostad 1998b). Upon hatching, these tiny (2 mm) larvae must crawl through the soil to locate the roots of a host plant. Survival and health of the larvae is dependent upon their ability to locate a suitable host plant within 24 hours of hatching (Strnad and Bergman 1987, Branson 1989) and larvae may travel up to 1 meter through the soil to locate corn roots (Short and Luedtke 1970). Larvae orient towards a CO_2 gradient emanating from the host plant and this "guide" eliminates the need for random searching.

WCR larvae are attracted to CO_2 concentrations as low as 1.25 mmol per mol (0.1%) and are able to detect differences in concentration as small as 12% (Bernklau and Bjostad 1998a). Jewett and Bjostad (1996) showed that dichloromethane is attractive to *Diabrotica* larvae, apparently because the structure of dichloromethane mimics CO_2 in its interaction with larval chemoreceptors.

It may be possible to use CO_2 to attract soil organisms (insects, nematodes and mites) away from their host plants or to confuse them so they are unable to locate host plants. One practical source of CO_2 is carbonated water which, when used to irrigate

soil, has been shown to enrich the soil and increase the health and production of certain crops (Mauney and Hendrix 1988, Novero et al. 1991, Ariezo et al. 1993, Enoch and Olesen 1993, Stoffella et al. 1995). CO_2 sources might be used to attract soil-dwelling organisms to pesticide granules or to pellets containing a biocontrol agent (Meyer and Huettel 1993, Kim and Youn 2000). In laboratory studies with soil-dwelling nematodes, the volume of CO_2 required to attract *M. incognita* and *R. reniformis* was 4-40 cm^3, which can be produced with 10-100 mg of HCO_3 (Robinson, 1995). Under field conditions, sufficient CO_2 gradients might be produced by granules of potassium bicarbonate, co-formulated with an acid and a pesticide that are broadcast or incorporated into the soil. In laboratory studies, Robinson (1995) determined that the optimal rate of CO_2 to attract soil nematodes is similar to the amount of CO_2 produced by less than 1 milligram of baker's yeast. Bernklau and Bjostad (1998b) found that 0.1 g of yeast produced 23 mmol per mol (2.3%) CO_2 and this was sufficient to attract WCR larvae away from volatile collections from germinating corn, which also contained 6 mmol per mol (0.6%) CO_2.

Other soil-dwelling insect larvae that use CO_2 in host location include *Otiorrhynchus sulcatus* F, *Melolontha* spp. and *Agriotes* spp. (Klingler 1957) and the carrot fly larvae, *Psila rosae* (Jones and Coaker 1977, 1979). Wireworm larvae are able to detect CO_2 concentration increases of 0.002-0.003% (Klingler 1966, Doane et al. 1975).

Elevated Atmospheric Carbon Dioxide

Levels of carbon dioxide in the earth's atmosphere have been increasing in modern times, chiefly as a result of burning fossil fuels and deforestation (Pandey

2002). Levels have risen from 0.029% at the beginning of the 20th century to 0.036% in 2000, and they are estimated to reach as high as 0.06% in the next millenium (Revelle 1982, Tan 2000). Numerous studies are being conducted in an attempt to predict the effects of elevated atmospheric CO_2 on plant life and the subsequent indirect effects on herbivores.

Each year 15% of carbon obtained from atmospheric CO_2 is used by plants in photosynthesis and that same amount is returned to the atmosphere from "plant respiration, and the decomposition of soil organic matter and plant litter" (Amthor 1995). Higher plants are generally positively affected by increases in CO_2. One common response seen in higher plants is an accelerated rate of photosynthesis, which is often expressed in crop plants as increased growth, greater biomass and greater economic yields (Rogers et al. 1994, Romanova et al. 2002). In addition, plants tend to better tolerate environmental stresses such as drought, extreme temperatures, salinity and pollution under elevated CO_2 conditions (Rogers et al. 1994). Not all plants respond the same to increased CO_2, and some negative effects have been reported (Rogers et al. 1994). However, studies have shown no decrease in crop quality as a result of elevated CO_2, and have in most cases, shown increased crop production (Rogers et al. 1994).

In studies of grasslands, elevated CO_2 caused changes in the root system and resulted in an increase in root biomass, root length, density and number of roots (Stirling et al. 1998, Arnone et al. 2000). Wheat cultivars exhibited an increased tolerance to drought stress in an atmosphere with double the ambient CO_2 concentration (Lin and Wang 2002). In similar studies, sugar beets responded to increases in CO_2 with greater leaf area and more root dry weight and radishes exhibited an increase in growth and net production (Overdieck 1996).

Environmental scientists are concerned about the direct effects of elevated CO_2 on hardwood trees and the forest ecosystem. Scots pine (*Pinus sylvestris* L.) have been shown to acclimate readily to elevated atmospheric CO_2 (400 mu mol mol (-1)) and (in these studies) the overall biomass of trees exposed to high CO_2 was 55% higher than of trees in normal atmospheres (Jach et al. 2000). Similar results have been observed for Sitka spruce (*Picea sitchensis*) (Murray et al. 2000) and Aspen (*Populus tremuloides*) (Pregitzer et al. 2000). In these three studies, trees responded to elevated CO_2 with an increase in root biomass exhibited by longer roots, greater root diameter, or an increase in the number of fine roots. Mangrove trees responded to enriched CO_2 with higher root to shoot ratios, increased biomass, increased total leaf area and increased relative growth rates. In addition, the "CO_2-treated" trees reached reproductive maturity a full two years earlier than normal (Farnsworth et al. 1996).

There is not a consensus among environmental experts with regard to acclimation or long term effects of high CO_2 on forest trees or grassland environments. In a review of forest field studies, Norby et al (1999) points out the variability in results obtained from seedling studies and emphasizes that it is not prudent to predict changes in the dynamic environment of the forest soil based on observations from a small number of short-term studies. In addition, results obtained from seedling studies may not predict the effects on other tree stages when size, age, health and environmental factors come into play (Saxe et al. 1998).

Effects of Elevated Atmospheric Carbon Dioxide on Insect Herbivores.

Changes in plant physiology may have direct or indirect effects on insects. Newman et al. (1999) observed smaller populations of bird cherry-oat aphids in CO_2 rich atmospheres. Lincoln et al. (1984) reported higher carbohydrate, reduced nitrogen and protein content for plants grown in CO_2-enriched environmental chambers, but this food source held less nutritional value for *Pseudoplusia includens,* one of its

insect herbivores, (Lincoln et al. 1984). In a study by Agrell (2000) an enriched CO_2 atmosphere resulted in decreased water content, increased starch and reduced foliar nitrogen in three tree species. Production of secondary plant compounds (phenolic glycosides in aspen and condensed tannins in birch and maple) increased. Effects of these physiological plant changes on the insect herbivore, *Orgyia leucostigma* included a reduction in survival, increased development time and reduced pupal mass (Agrell et al. 2000). The effect of high CO_2 concentrations on increased production of secondary plant compounds has also been reported for thyme (thymol and aromatic phenol) and essential oils (monoterpenes, piperitonone oxide and imonene) from mint (Tisserat and vaughn 2001).

Effects of Elevated Atmospheric Carbon Dioxide on the Soil Environment.

In addition to root respiration, carbon dioxide in the soil is provided by the breakdown of organic carbon material, root biomass and microbial populations and the concentration varies as a result of changes in moisture content and temperature (Clinton and Vose 1999, Frank et al. 2002).

In grassland studies increased atmospheric CO_2 resulted a shift in the root density within the soil profile with a significantly greater proportion of roots growing in the upper 0-6 cm of soil than under normal conditions (Arnone et al. 2000). An overall increase in root mass (higher root yields) has been observed in response to CO_2 enrichment in several environmental studies and VanGinkel et al (1996) found that the rate of organic decomposition in the soil decreased under elevated soil CO_2 levels. These effects could cause a greater amount of organic material to be present in the soil environment, resulting in higher carbon storage (VanGinkel et al. 1996).

The net effect of these predicted changes is an increase in C deposited in the soil (Amthor 1995). In general, plants are positively affected by higher CO_2 levels in the soil. The use of carbonated water to irrigate soil has been shown to enrich the soil and

increase the health and production of crops (Mauney and Hendrix 1988, Novero et al. 1991, Ariezo et al. 1993, Enoch and Olesen 1993, Stoffella et al. 1995).

To date, studies have not been conducted specifically to determine or predict the effects of increased soil CO_2 on soil-dwelling arthropods. It is logical to assume that some insects will be negatively impacted, while others will adapt to the change.

Natural Occurrence of Elevated Carbon Dioxide in Insect Habitats.

Some insects have already adapted and thrive in environments that are naturally enriched with CO_2 or in which CO_2 levels may periodically flux. The low metabolic rate of troglobites may be the result of high CO_2 concentrations in the cave environment, which may be twice that found at the surface (Howarth 1983, Nicolas and Sillans 1989). Similarly, adult wood-boring beetles that overwinter under bark, where CO_2 levels are increased, are found to have more lactic acid in the hemolymph (Pasche and Zachariassen 1973).

Higher levels of CO_2 are naturally found in the habitats of some social insects. CO_2 concentrations in termite nests (Macrotermitinae) have been measured at 0.3-5% (Cook 1932, Peakin and Josens 1978, Zimmerman et al. 1986) and beehives may have levels as high as 6% (Buhler et al. 1983, Nicolas and Sillans 1989). Pests of stored grain, including lepidopterans and coleopterans survive well in grain bins with CO_2 concentrations as high as 2% (Sinha et al. 1986, Nicolas and Sillans 1989).

The termite *Macrotermes natalensis* has been reported to rebuild porous walls when the CO_2 concentration in the nest increases to 2% (Ruelle 1964). In nests of honey bees where CO_2 concentrations cycle in response to temperature changes, flight traffic in and out of the nest is at its peak when CO_2 concentrations are at the lowest levels (Dietlein 1985, Nicolas and Sillans 1989) and increases in CO_2 may stimulate fanning behavior (Seeley 1974). Juvenile hormone levels in honey bees are controlled by the combination of CO_2 concentration and temperature (Buhler et al. 1983).

Pockets of elevated CO_2 occur naturally when insects are concentrated within a small area in an enclosed space. This has been shown in museums where valuable objects are infected by destructive insects. Koestler et al (2000) propose the use of infrared spectroscopy to detect insects inside or around museum artifacts and art. Using FTIR, they were able to detect changes in CO2 concentration as low as 0.09 ppm and were able to attribute these changes to the presence of insects in works of art comprised of fabric, wood or feathers that are most commonly attacked (Koestler et al. 2000). With this method, insects such as termites, carpet beetles and silverfish might be detected before any visible signs of their activity occur.

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CHAPTER 3

DISRUPTION OF HOST LOCATION OF WESTERN CORN ROOTWORM LARVAE (COLEOPTERA: CHRYSOMELIDAE) WITH CARBON DIOXIDE

Introduction

Larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, a major pest of corn (*Zea mays* L.) in the United States (Krysan and Miller 1986, Metcalf 1986, Pike et al. 1995) use carbon dioxide (CO_2) to locate the roots of growing corn plants on which they feed. Strnad et al. (1986) first reported that WCR are attracted to CO_2, and this attraction has also been demonstrated by other investigators (Hibbard and Bjostad 1988, MacDonald and Ellis 1990, Jewett 1995, Bernklau and Bjostad 1998a, b). In a glass bead bioassay apparatus, larvae were attracted to the higher concentration of CO_2 (Bernklau and Bjostad 1998a), and they were able to detect differences as small as 12%. An extensive series of experiments demonstrated that the attraction of western corn rootworm larvae to corn roots is caused by CO_2 alone, and that no other volatile chemical cues are involved (Bernklau and Bjostad 1998b). In addition, larvae were attracted away from corn by a higher concentration of carbon dioxide alone, and both organic and inorganic formulations were used as sources of CO_2. In one experiment in which baker's yeast was used as an organic source of CO_2, larvae were attracted away from corn volatiles and moved towards the CO_2-generating yeast despite metabolic byproducts produced by the yeast (Bernklau and Bjostad 1998b).

The previous studies were conducted using a glass bead bioassay apparatus that was developed especially for neonate WCR larvae (Bernklau and Bjostad 1998a). The bioassay device accommodates the positive geotropic tendency of the larvae and provides thigmotactic cues to the larvae, and a syringe pump allows slow, accurate delivery of volatile cues into the choice-test apparatus. In the current study, CO_2 was tested in soil bioassays with neonate WCR larvae to test the hypothesis that sufficient levels

of CO_2 will disrupt the host location behavior of WCR larvae in the soil. In addition, we tested a variety of CO_2-generating sources for behavioral effects.

Materials and Methods

Insects. Western corn rootworm larvae were obtained from a colony that has been reared in our laboratory since 1986 (non-diapausing strain, originally obtained from J. Jackson, USDA-ARS Laboratory, Brookings, South Dakota). Additional insects were purchased from Lee French Agricultural Enterprises (Lamberton, Minnesota) and added from time to time to maintain genetic variability. The insects were reared on corn plants grown in soil using methods described by Jackson (Jackson 1985) and modified by Hibbard and Bjostad (1988).

Soil. Soil was obtained from a local agricultural research farm (ARDEC, Agricultural Research Demonstration and Education Center, Fort Collins, Colorado) whose history was known, and where no corn had been grown or chemicals applied for at least 5 years. The soil was characterized as having a clay loam texture, containing 1.5% organic matter, and having good filtration (Soil, Water and Plant Testing Lab, Soil and Crop Sciences Dept. and Cooperative Extension, Colorado State University). For bioassays, the soil was adjusted to contain 20% moisture (by weight) for optimum larval movement (MacDonald and Ellis 1990).

GC-MS-SIM Analysis of CO_2. Mass spectrometry was used to determine CO_2 concentrations in the soil for each bioassay. A Hewlett-Packard Series II 5890 gas chromatograph interfaced with a Hewlett-Packard 5971 mass selective detector was operated in selected ion monitoring mode (SIM) for m/z 44 with a methyl silicone capillary column (30 m x 0.32 mm ID, RSL-150, Alltech, Inc., Deerfield, Illi-

nois). A standard 10 millimoles per mole concentration of CO_2 (a 300 ml glass bottle into which 3 ml of CO_2 were injected) was used to calculate the CO_2 concentrations of the unknown samples.

Measuring CO_2 in Soil.

Small Soil Tubs. A jig to measure CO_2 was constructed from foamboard (Hunt Corporation, Philadelphia, Pennsylvania, cut 12 cm x 11 cm x 10 mm). Six holes (1 mm diam) were drilled through the foamboard and a piece of glass tubing (5 cm long x 1 mm ID) was inserted through each hole. A piece of metal wire (5.3 cm) was inserted into each glass tube so that the wire projected 3 mm from the end of the glass tube. The jig was inserted, wires first, 4 cm into the soil. The wire plugs were removed from the glass tubes, leaving a 3 mm gap in the soil just below the end of each glass tube. The needle of a 10-µl Hamilton syringe was inserted into each glass tube so that it projected 1 mm into the gap, and a 5-µl sample of soil headspace was removed for analysis.

Large Soil Tubs. A jig to measure CO_2 was constructed from foamboard (cut 3 cm x 3 cm x 10 mm). A single hole (1 mm diameter) was drilled through the foamboard and a piece of glass tubing (5 cm long x 1 mm ID) was inserted through the hole. For each large soil tub, three jigs were inserted into the soil, one at each end of the tub and one in the center section.

Insect Recovery from Bioassay Tubs.

From Soil - Flotation Method. For some experiments, a plastic partition was used to divide the soil in the plastic tub into equal parts. Soil from each part was scooped into a separate round plastic tub and water was added. The larvae (whose density is less than that of the soil) were removed and counted as they floated to the top of the water.

From Corn - Flotation Method. To recover insects feeding on the outside of the corn roots, the corn plant was removed from the soil and placed in a separate plastic tub and water was added. Larvae were removed as they floated to the top.

From Corn - Clarifying Method. The roots of the germinating corn seed were clarified by soaking the seed in a 50% Clorox solution (Clorox Company, Oakland, California) for 30 minutes. Larvae could then be seen inside the roots under a dissecting microscope.

From Corn - Funnel Method. For later experiments, a more convenient technique, based on a Berlese funnel, was developed to recover larvae. Berlese-like devices were made from two plastic cups (Solo cups, Solo Cup Co., Urbana, Illinois). The bottoms were removed from both cups. One cup was inserted into the other cup while holding a square (15 cm x 15 cm) of plastic insect screen to the bottom of the inner cup, thereby stretching the screen near the bottom, between the two cups. A receptacle made from the bottom cut from one of the cups was filled halfway with water and placed beneath the Berlese funnel. A corn plant containing larvae was removed from the soil and gently placed in the funnel. Over a period of 2 days, as the plant and roots slowly dried, larvae crawled out of the roots and fell into the water below. The white interior of the receptacle allowed floating larvae to be seen and counted.

Small Soil Tub Bioassay with Gaseous CO_2. CO_2 gas was pumped into one end of a tub containing soil in an attempt to attract western corn rootworm larvae away from germinating corn.

Soil Tub Preparation. To remove fungal spores, untreated, dried corn seeds (*Zea mays*, hybrid Pioneer 3055, provided courtesy of Gary D. Lawrance, Pioneer Hi-Bred International, Johnston, Iowa) were soaked for 24 h in soapy water (1 drop of Ivory Dishwashing Liquid, Procter & Gamble, Cincinnati, Ohio, per liter of water),

and rinsed thoroughly with water. Soil containing 20% moisture (by weight) was prepared by adding water to the dry soil (described previously) and stirring vigorously. A plastic tub (15 cm x 8 cm x 4 cm high, Rubbermaid, Wooster, Ohio) was filled to the top edge with approximately 300 g of the moist soil. A single soaked corn seed was planted in the soil at one end of the tub, 2 cm from one end and 2 cm deep. The soil tub was placed in a larger plastic tub (28 x 18 x 12 cm) filled 1 cm full with water. The tub was covered loosely to allow diffusion of air and to maintain a humid environment. After 2 days the tubs were removed from the larger chamber and used for larval bioassays.

Bioassay Procedure. Soil tubs were prepared as described above. When the corn was 2 days old, a hole (0.5 cm) was drilled in the center of the tub wall at the distal end (opposite end from where the corn was planted), and a 60-ml polyethylene syringe containing 100% CO_2 was connected to the tub with Tygon tubing inserted through the hole. The syringe was clamped onto a syringe pump that was adjusted to provide a flow of 1 ml/min. Controls were set up as described and were connected to a syringe containing ambient air. After 15 minutes of pumping, CO_2 concentrations in the soil were measured using GC-MS-SIM (described previously) to verify that a gradient of CO_2 had been established in the soil. Neonate larvae (30 larvae, less than 24 hours old) were removed with a fine camelhair brush from a tub containing eggs in soil and were placed on a moistened piece of filter paper (1 x 1 cm). A slight depression was made in the soil in the middle of a bioassay tub and the paper was laid gently upside down in the depression and covered lightly with soil. The pump was run for 6 h and the polyethylene syringes were replaced every hour. After 6 h, the tubs were disconnected from the pump. The soil was divided into 3 parts (the corn side, the middle and the pump side), and larvae were recovered from the soil and the outside of the corn roots using the flotation method (described previously). Larvae were recov-

ered from inside the corn roots using the clarifying method (described previously). The experiment was repeated 8 times.

Statistical Analysis. T-tests were conducted to compare the number of larvae recovered from each section of the control (ambient air) tubs with the larvae recovered from the same section of the CO_2 tubs using Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts) with $\alpha = 0.05$.

Soil Bioassays with CO_2-Generating Materials. A series of bioassays were conducted to determine whether host location of WCR larvae could be disrupted with CO_2-generating materials. Bioassays were conducted using the small soil tubs with a single corn seed planted in one end (described above).

Effervescing Tablet Bioassay. When the corn was 2 days old, 2 tablets (Fizzies, Premiere Innovations, Pacific Palisades, California, 5 g each) were broken into 6-8 pieces and stirred gently throughout the soil in the tub. Fizzies are manufactured from an equimolar mixture of sodium bicarbonate and citric acid with Nutrasweet™ artificial sweetener and artificial flavoring. The "punch" flavored tablets were used for all experiments because they dissolved more slowly than the other tablets. The CO_2 concentrations were measured, one hour after introducing the tablets, using the method described to confirm that the tablets were reacting with moisture in the soil and were producing CO_2. Newly hatched larvae (30) were introduced into the soil 2 cm from the distal end of the tub. Control tubs contained no treatment. After 24 h, larvae were recovered from the soil on the corn side of the tub (one-half), from the soil on the distal end (one half), and from the outside of the corn root using the flotation method (described previously). Larvae were recovered from inside the corn plant using the clarifying method (described previously). The experiment was repeated 8 times.

Sucrose Pellet Bioassay. Bioassay tubs were prepared with a single germinating corn seed as described above. Sucrose pellets (1 g, white cake décors, Signature

Brands, LLC, Ocala, Florida) were mixed throughout the soil before planting the corn seed. When the corn seed was 2 days old, newly-hatched larvae (30) were introduced into the soil 2 cm from the distal end of the tub. Control tubs contained no treatment. After 24 h, larvae were recovered from the soil on the corn side of the tub (one-half), from the soil on the distal end (one half), and from the outside of the corn root using the flotation method (described previously). Larvae were recovered from inside the corn plant using the clarifying method (described previously). The experiment was repeated 5 times.

Yeast Bioassay. Bioassay tubs were prepared with a single germinating corn seed as described above. Baker's yeast (1 g, Burns Philp Food, Inc., Fenton, Missouri) was mixed throughout the soil before planting the corn seed. When the corn seed was 2 days old, newly-hatched larvae (30) were introduced into the soil 2 cm from the distal end of the tub. Control tubs contained no treatment. After 24 h, larvae were recovered from the soil on the corn side of the tub (one-half), from the soil on the distal end (one half), and from the outside of the corn root using the flotation method (described previously). Larvae were recovered from inside the corn plant using the clarifying method (described previously). The experiment was repeated 5 times.

Statistical Analysis. For each experiment, t-tests were conducted to compare the number of larvae recovered from each section of the control tub with the number of larvae recovered from the same section of the treated tub using Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts) with $\alpha = 0.05$.

Natural Products Bioassay. A number of organic substances were tested as a source of carbon dioxide using a variation of the small tub soil bioassay. Bioassay tubs were prepared with a single germinating corn seed as described above. At the time the corn seed was planted, a treatment (1 g) was placed in a narrow trench dug in the soil approximately 2 cm from the distal end of the tub (opposite the corn seed) and

1 cm deep. When the corn seed was 2 days old, 30 neonate larvae were introduced into the center of the tub. Control tubs contained no treatment. After 8 hours, larvae were recovered from the corn plant using the funnel method (described previously). The following treatments were tested: ground roots from dried 3-d-old germinated corn seeds, ground seed from dried germinated corn, ground germinated corn seed, crushed corn seed (not germinated), malted barley powder (Beer Beer & More Beer, Concord, California), whole malted barley (Beer Beer & More Beer, Concord, California), ground malted barley, ground roots from dried 5-d-old germinated wheat, corn meal (Quaker Oats Co., Chicago, Illinois), wheat flour (Pillsbury Inc., Minneapolis, Minnesota), dried spent grain (New Belgium Brewery, Ft. Collins, Colorado), distillers grain (obtained from Dr. Jarosz, Food Science Department, Colorado State University), artificial pollen (Dadant and Sons, Inc., Hamilton, Illinois), crushed puffed rice cereal (Malt-O-Meal Co., Minneapolis, Minnesota), and crushed puffed wheat cereal (Malt-O-Meal Co., Minneapolis, Minnesota). The experiment was repeated 10 times.

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Dunnett's test was used for *a posteriori* comparisons with a control, with $\alpha = 0.05$ (Winer 1971).

Large Tub Soil Bioassays with Yeast Granules. Bioassays were conducted in a plastic tub (40 x18 x 8 cm) that had 4 pinholes drilled into the bottom corner edge on each side to allow CO_2 equilibration. The tub was filled to within 2 cm of the top with sieved (5 mm mesh) soil containing 20% moisture. A single corn seed was planted at one end of the plastic tub, 1 cm from the far end and approximately 3 cm deep and the lid was securely placed on the tub. Candidate CO_2-generating materials were mixed into the soil at the time of planting. Control tubs contained no added materials.

The CO_2 was measured in the soil (described previously) prior to introducing larvae into the tub.

When the corn was 2 d old, 30 neonate larvae were introduced into the bioassay tub as described previously. The bioassay tubs were left undisturbed for 24 h, after which larvae were recovered from the corn plant using the recovery funnels (described above).

Yeast granules were prepared by mixing baker's yeast (3 g, Fleischmanns, Burns Philp Food, Inc., Fenton, Missouri), corn syrup (10 g, Best Foods, Englewood Cliffs, New Jersey) nutrient agar mix (1 g, YPD Broth, Fisher Scientific, Pittsburgh, Pennsylvania) and corn meal (10 g, Quaker Oats Co., Chicago, Illinois). The dry ingredients were placed in a food processor and the liquid (corn syrup and nutrient agar) was slowly added with the processor set on a slow (chopping) setting. The granules were spread onto waxed paper and allowed to dry. For each experiment, treatments of 6 g or 18 g of the dried granules were tested. In one test, the granules were mixed evenly throughout the entire tub. In a second test, the granules were placed in a trench 5 cm deep and 2 cm from the distal end of the tub (opposite side from the corn). In the third test, the granules were placed in a trench approximately 4 cm from the distal end of the tub, and the larvae were introduced in the center of the tub, between the corn plant and the trench of yeast granules.

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Fisher's LSD test was used for all *a posteriori* comparisons, with $\alpha = 0.05$.

Field Trials. Field tests were conducted in 1997 and 1998 at a cornfield located at the Agricultural Research, Demonstration and Education Center (Block 1080, ARDEC, Colorado State University, Fort Collins, Colorado). The site had been planted with continuous corn for at least 5 years.

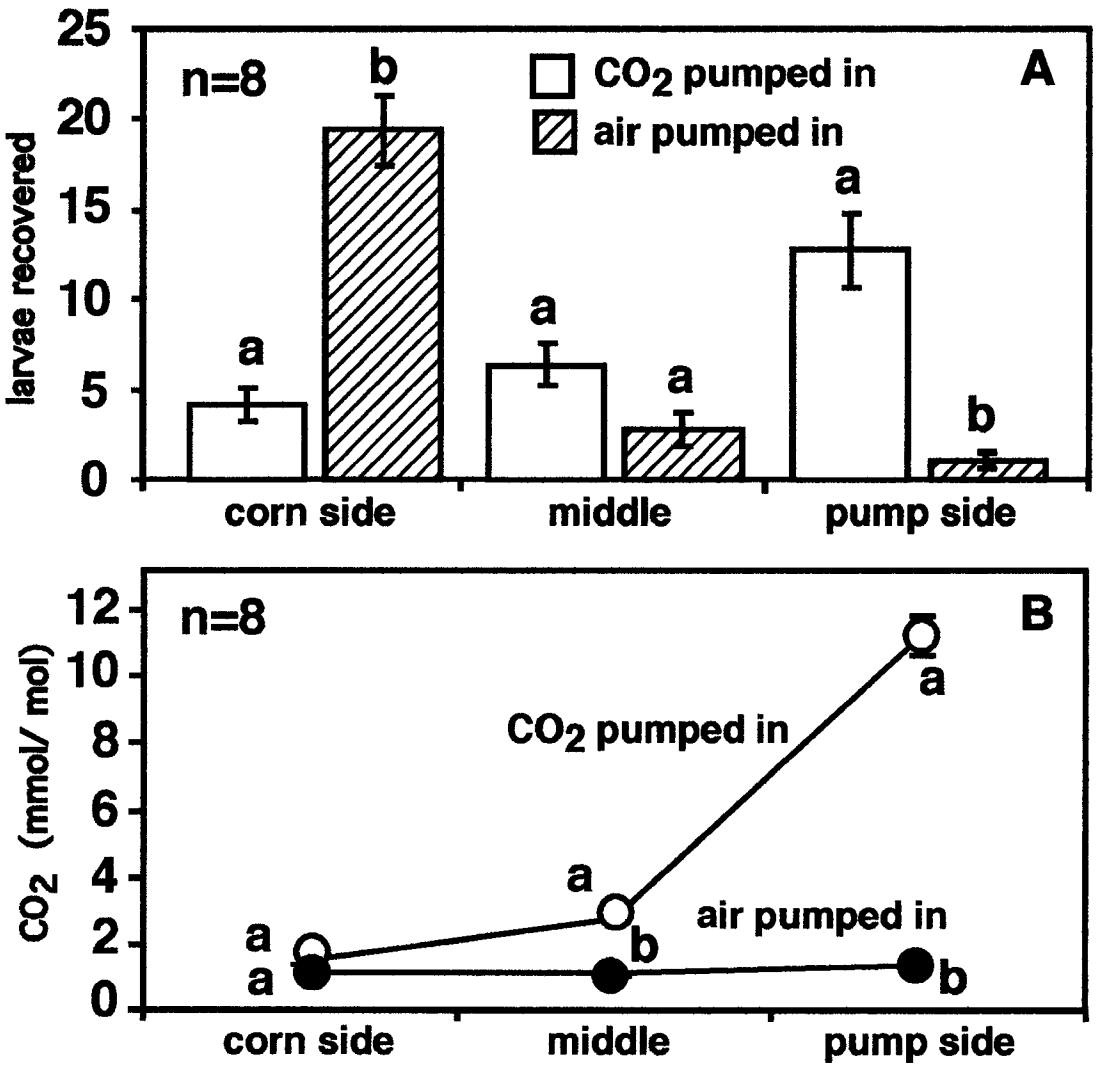

Fig. 3.1. (A) Larvae recovered from tub with CO_2 or air (control) pumped into the distal end. (B) CO_2 concentration in tubs with CO_2 or air pumped into the distal end. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

In 1997, treatments were placed at planting time in a trench dug 10 cm deep and 10 cm to each side of the corn row. The following treatments were tested in 1997: baker's yeast (10 g per m of corn row), yeast nutrient granules (prepared as described previously) at a rate of 10 g per m of corn row, yeast nutrient pellets (granular material pressed into cylindrical pellets measuring 1.5 cm high x 1.5 cm diameter, 3 g each) at a rate of 39.5 pellets per m of corn row, effervescing tablets (Fizzies, Premiere Innovations, Pacific Palisades, California) at 19 tablets per m of corn row and sucrose (table sugar) at 10 g per meter of corn row. Plots (5 m long) were arranged in a Latin square design (Winer 1971) with a total of 12 replicates for each treatment. Root ratings were conducted in mid July. The feeding damage for each plant was evaluated using the Iowa 1-6 scale (Hills and Peters 1971), in which a rating of 1 indicates no visible rootworm feeding scars and a rating of 6 indicates at least three nodes of roots destroyed.

In 1998, granular treatments (less than 4 mm diameter) were placed 10 cm deep and 10 cm on either side of the corn seed using a cone planter (modified 2-row Maximerge planter, John Deere, Inc. Moline, Illinois) on a 6400 John Deere tractor (John Deere, Inc. Moline, Illinois). Larger materials (more than 5 mm diameter) were placed by hand in trenches hoed 10 cm deep and 10 cm on either side of the corn. An insecticide treatment of Counter 20CR (American Cyanamid, Mount Olive, New Jersey) was applied at a rate of 513 mg granules per meter of corn row as a positive control. The Counter 20CR insecticide was placed in a standard T-band pattern over the corn row using modified Winter-Stieger meters (Read Brothers, Inc., Henry, Illinois). Treatments included clean cracked corn (Dynagro brand purchased at Country General farm store) at 27 g and 82 g per m of corn row, corn gluten feed (obtained from Dr. Jarosz, Food Science Department, Colorado State University) at 82 g per m of corn row, distillers grain (Dr. Jarosz) at 82 g per m of corn row, dried spent grain

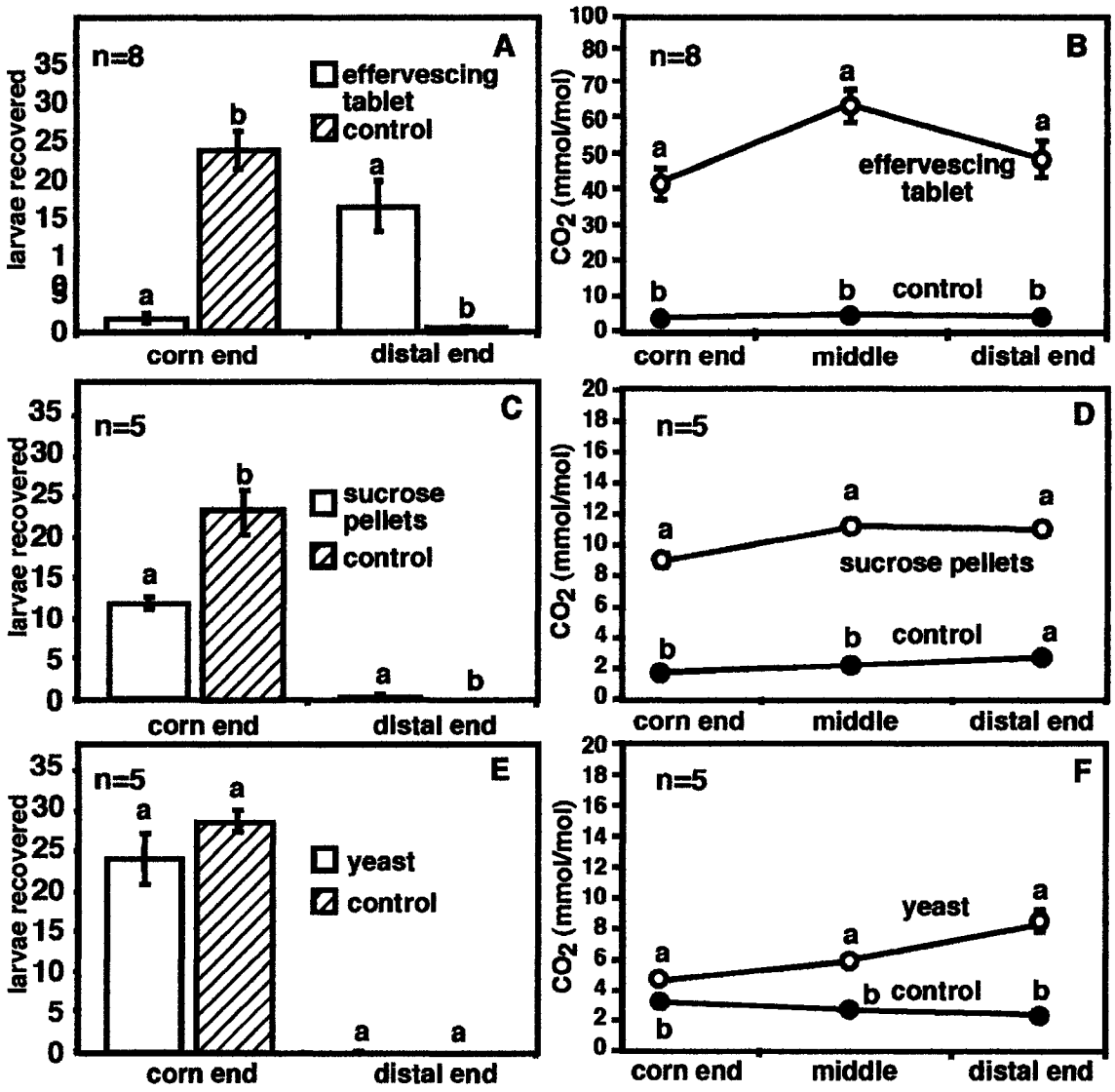

Fig. 3.2. (A) Larvae recovered from corn and from the distal end of tubs with effervescing tablets and untreated tubs. (B) CO_2 concentration in treated (effervescing tablets) and control tubs. (C) Larvae recovered from corn and from the distal end of tubs with sucrose pellets and untreated tubs. (D) CO_2 concentration in treated (sucrose pellets) and control tubs. (E) Larvae recovered from corn and from the distal end of tubs with baker's yeast and untreated tubs. (F) CO_2 concentration in treated (baker's yeast) and control tubs. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

(obtained from a local micro-brewery) at 82 g per m of corn row, malted barley (Beer Beer & More Beer, Concord, California) at 82 g per m of corn row, dried ground germinated corn seed (prepared in the laboratory) at 82 g per m of corn row, effervescing tablets (Fizzies, described previously) at 19 tablets per m of corn row and yeast granules (prepared as described previously) applied at a rate of 82 g per m of corn row.

Inorganic CO_2-generating compounds were obtained from Church & Dwight, Inc. (Princeton, New Jersey). These included: ammonium bicarbonate (compacted chunk form, 4 cm long, variable thickness) applied at 27 g and 82 g per m of corn row, granular ammonium bicarbonate (approximately 4 mm diameter) at 27 g and 82 g per m of corn row, milled ammonium bicarbonate (approximately 3 mm diameter) at 27 g and 82 g per m of corn row, prilled urea (3 mm diameter) (82 g per m of corn row) and 50:50 prilled urea:ammonium bicarbonate (3 mm diameter) at 27 g per m of corn row. All treatments were applied at corn planting time. The plots were 12.5 m long and were arranged in a randomized complete block pattern with a minimum of 6 replicates for each treatment. Roots were evaluated in mid-July using the Iowa 1-6 scale (Hills and Peters 1971).

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Dunnett's test was used for *a posteriori* comparisons with a control, with $\alpha = 0.05$ (Winer 1971).

Results

Small Soil Tub Bioassay with Gaseous CO_2. Significantly fewer larvae ($F=46.21$, $df=15$, $p < 0.05$) were recovered from the corn seed and soil around the corn roots when CO_2 gas was pumped into the soil tub apparatus (4.13 ± 0.90) than from tubs treated with ambient air (19.25 ± 1.98) (Fig. 3.1A). The CO_2 concentration in the

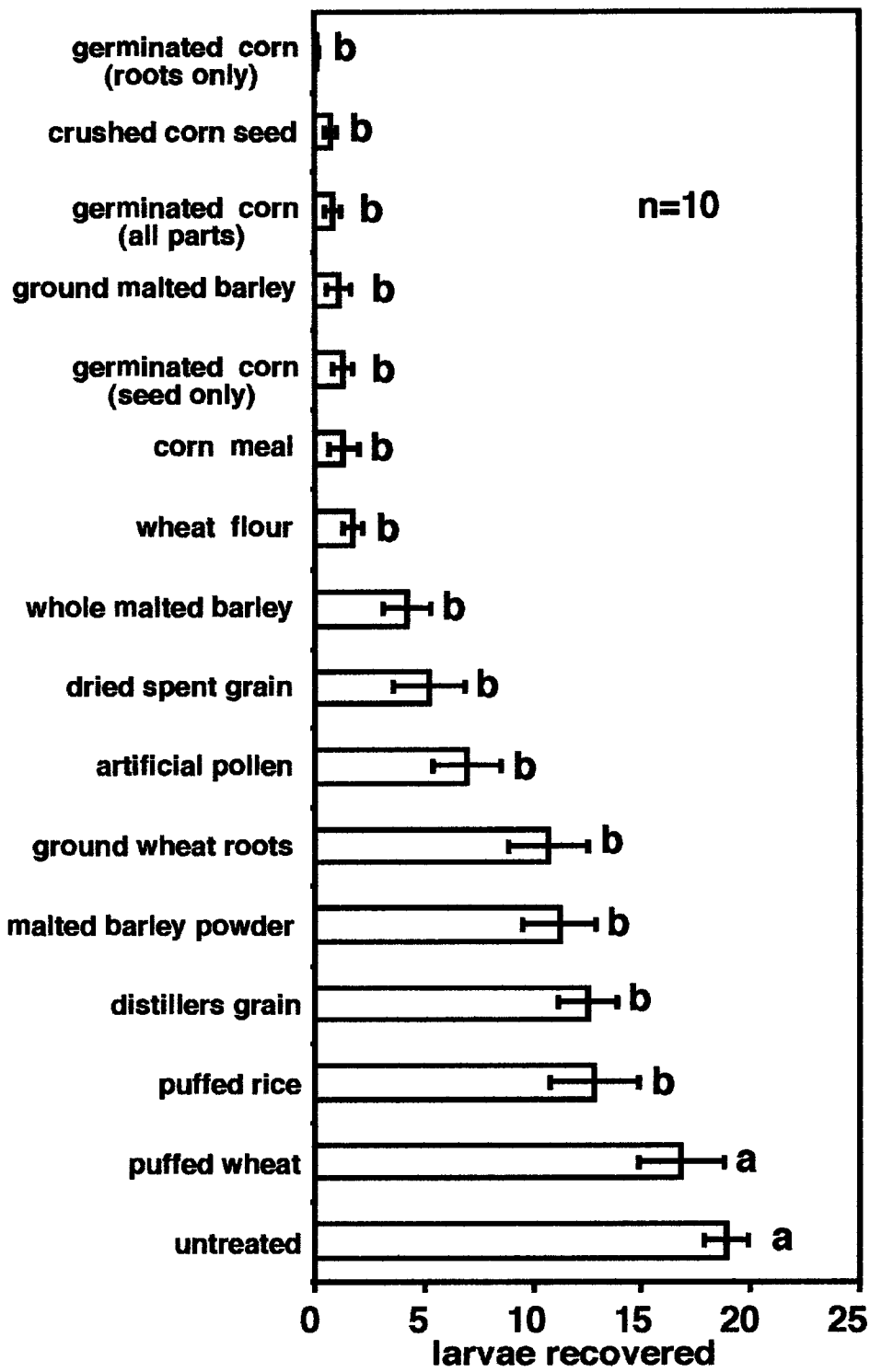

Fig. 3.3. Larvae recovered from corn in soil tubs with natural products as sources of CO_2. Significant differences (Dunnett's test, $p < 0.05$) are indicated by different lower case letters. Bars represent standard errors.

soil into which CO_2 was pumped was 1.36 ± 0.03 mmol/mol (corn end), 2.72 ± 0.1 mmol/mol (middle) and 11.28 ± 0.47 mmol/mol (CO_2 end) mmol/mol (Fig. 3.1B). In the control tub (ambient air), the CO_2 concentration was 1.06 ± 0.03 mmol/mol (corn end), 1.06 ± 0.02 mmol/mol (middle) and 1.30 ± 0.03 mmol/mol (air end) (Fig. 3.1B).

Soil Bioassays with CO_2-Generating Materials.

Effervescing Tablet Bioassay. Significantly fewer larvae ($F=84.12$, $df=15$, $p < 0.05$) were recovered from the corn seed and soil around the corn roots when the soil was treated with effervescing tablets (1.86 ± 0.59) than from untreated tubs (23.80 ± 2.42) (Fig. 3.2A). The CO_2 concentration in the center of the tablet-treated soil tub was 63.06 ± 5.22 mmol/mol and the CO_2 concentration in the center of the untreated soil tub was 1.24 ± 0.12 mmol/mol (Fig. 3.2B).

Sucrose Pellet Bioassay. Significantly fewer larvae ($F=20.80$, $df=9$, $p < 0.05$) were recovered from the corn seed and soil around the corn roots when the soil was treated with sucrose pellets (11.20 ± 0.58) than from untreated tubs (21.60 ± 2.20) (Fig. 3.2C). The CO_2 concentration in the middle of the treated tub was 11.25 mmol/mol and the CO_2 concentration in the middle of the untreated tub was 2.14 mmol/mol (Fig. 3.2D).

Baker's Yeast Bioassay. There was no significant difference ($F=1.60$, $df=9$, $p < 0.05$) in the number of larvae recovered from the corn seed and soil around the corn roots when the soil was treated with baker's yeast (24.20 ± 3.18) than from untreated tubs (28.60 ± 1.40) (Fig. 3.2E). For both the treated and untreated tubs, no larvae were recovered from the far end. The CO_2 concentration in the center of the treated soil tub was 8.63 ± 0.69 mmol/mol and the CO_2 concentration in the center of the untreated tub was 2.47 ± 0.12 mmol/mol (Fig. 3.2F).

Natural Products Bioassay. Significantly fewer larvae ($F=26.76$, $df=159$, $p < 0.05$) were recovered from the corn for every treatment than from the corn in the

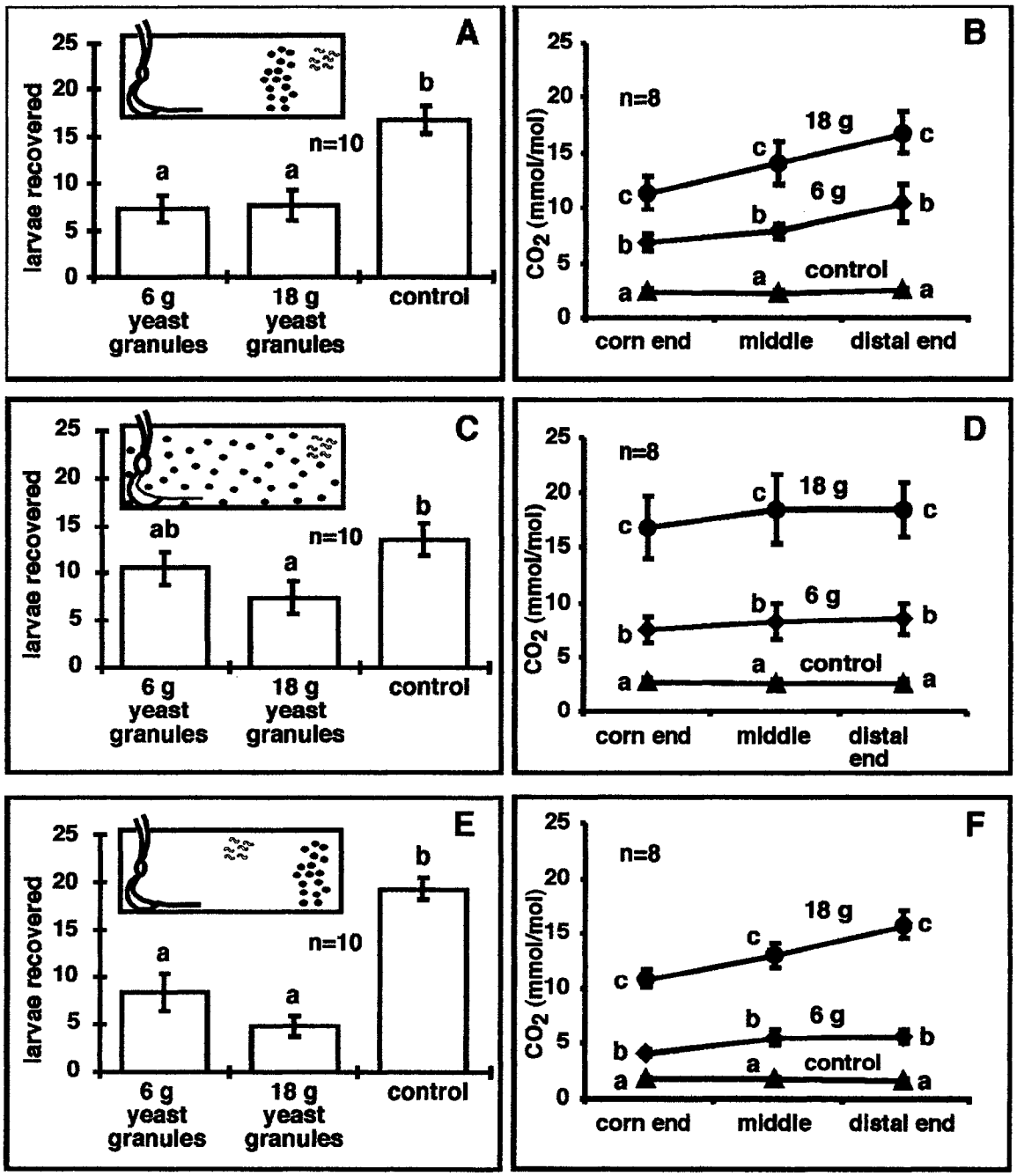

Fig. 3.4. Soil tests with different placements of yeast as a source of CO_2. (A) Larvae recovered from corn with yeast granules in a trench in soil tub. (B) CO_2 concentration in tubs with yeast granules in a trench. (C) Larvae recovered from corn with yeast granules mixed throughout soil tub. (D) CO_2 concentration in tubs with yeast granules mixed throughout. (E) Larvae recovered from corn with yeast granules in a trench and larvae introduced in middle of tub. (F) CO_2 concentration in tubs with yeast granules in a trench and larvae in the middle. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

untreated tubs, (18.89 ± 0.97 larvae) except for the puffed wheat treatment (Fig. 3.3). The fewest larvae were recovered from the tubs treated with the dried roots of germinated corn seed (0.10 ± 0.10 larvae), dried germinated corn seed (seed only) (1.30 ± 0.45 larvae), ground germinated corn seed (all parts) (0.80 ± 0.36 larvae), crushed corn seed (0.70 ± 0.26 larvae), and corn meal (1.30 ± 0.67 larvae).

Large Tub Soil Bioassays with Yeast Granules. When the yeast granules were placed in a trench, significantly fewer larvae ($F=6.79$, $df=34$, $p < 0.05$) were recovered from the corn in the treated tubs (16.80 ± 1.51) than in the untreated tubs for 6 g (7.30 ± 1.50) and 18 g (7.70 ± 1.68) of yeast granules (Fig. 3.4A). There was no significant difference in larvae recovered from corn for the two doses of granules. The CO_2 concentration in the untreated control tubs was significantly ($p < 0.05$) lower than the concentration in the treated tubs (for 6 g and 18 g of granules) at all three points measured (corn side $F=20.29$, $df=23$, $p < 0.05$; middle $F=24.61$, $df=23$, $p < 0.05$; distal end $F=23.45$, $df=23$, $p < 0.05$). The CO_2 concentration in tubs containing 18 g granules was significantly higher (at all three points measured) than in tubs with 6 g granules (Fig. 3.4B).

When the yeast granules were mixed throughout the soil, significantly more larvae ($F=3.95$, $df=35$, $p < 0.05$) were recovered from the corn in the control tubs (13.57 ± 1.76) than in the tubs containing 18 g of granules (7.40 ± 1.70) (Fig. 3.4C). The number of larvae recovered from tubs with the 6 g of treatment (10.54 ± 1.72) was not significantly different ($p > 0.5$) from either the control or the 18 g treatment. The CO_2 concentration in the control tubs was significantly ($p < 0.05$) lower than the concentration in the treated tubs (for 6 g and 18 g of granules) at all three points measured (corn side $F=16.38$, $df=23$, $p < 0.05$; middle $F=16.80$, $df=23$, $p < 0.05$; distal end $F=22.37$, $df=23$, $p < 0.05$). The CO_2 concentration in tubs containing 18 g granules

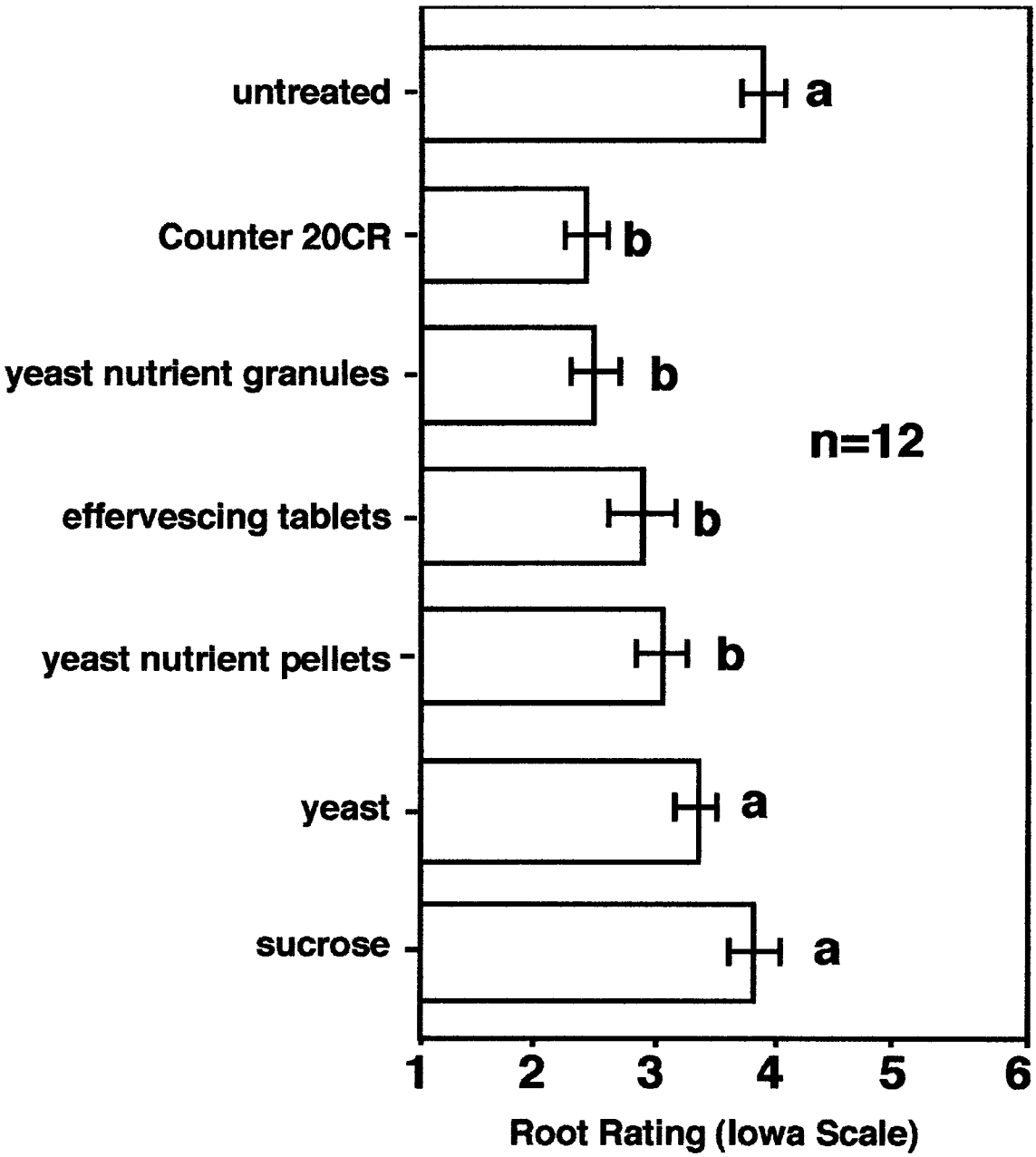

Fig. 3.5. Root ratings (Iowa 1-6 scale) from 1997 field trials. Significant differences (Dunnett's test, $p < 0.05$) between the treatments and the untreated control are indicated by different lower case letters. Bars represent standard errors.

was significantly higher (at all three points measured) than the concentration in tubs with 6 g granules (Fig. 3.4D).

When the yeast granules were placed in a trench and larvae were introduced in the center of the tub, significantly more larvae ($F=23.98$, $df=27$, $p < 0.05$) were recovered from the corn in the control tubs (19.37 ± 1.19) than in the tubs containing 6 g (8.40 ± 1.93) or 18 g (4.80 ± 1.08) of granules (Fig. 3.4E). There was no significant difference between the two doses of granules. The CO_2 concentration in the control tubs was significantly ($p < 0.05$) lower that the concentration in the treated tubs (for 6 g and 18 g of granules) at all three points measured (corn side 92.58, $df=23$, $p < 0.05$; middle $F=57.31$, $df=23$, $p < 0.05$; distal end $F=81.16$, $df=23$, $p < 0.05$). The CO_2 concentration in tubs containing 18 g granules was significantly higher (at all three points measured) than the concentration in tubs with 6 g granules (Fig. 3.4F).

Field Trials. In 1997, 4 treatments produced root ratings that were significantly ($F=11.32$, $df=83$, $p < 0.05$) lower than the untreated control, (3.90 ± 0.25) (Fig. 3.5). These included Counter 20CR (2.49 ± 0.24), yeast nutrient granules (2.51 ± 0.23), effervescing tablets (2.85 ± 0.33), and yeast nutrient pellets (3.15 ± 0.25).

In 1998, nine treatments produced root ratings that were significantly ($F=2.63$, $df=259$, $p < 0.05$) lower than the untreated control, (3.30 ± 0.19) (Fig. 3.6). These included: Counter 20CR (2.27 ± 0.13), compacted ammonium bicarbonate at 82 g per m of corn row (2.42 ± 0.14), ground germinated corn (2.44 ± 0.14), milled ammonium bicarbonate at 82 g per m of corn row, (2.48 ± 0.12), compact ammonium bicarbonate at 27 g per m of corn row (2.50 ± 0.14), urea/ammonium bicarbonate (2.50 ± 0.14), distillers grain (2.60 ± 0.18), granular ammonium bicarbonate at 82 g per m of corn row (2.60 ± 0.33) and granular ammonium bicarbonate at 82 g per m of corn row (2.67 ± 0.18).

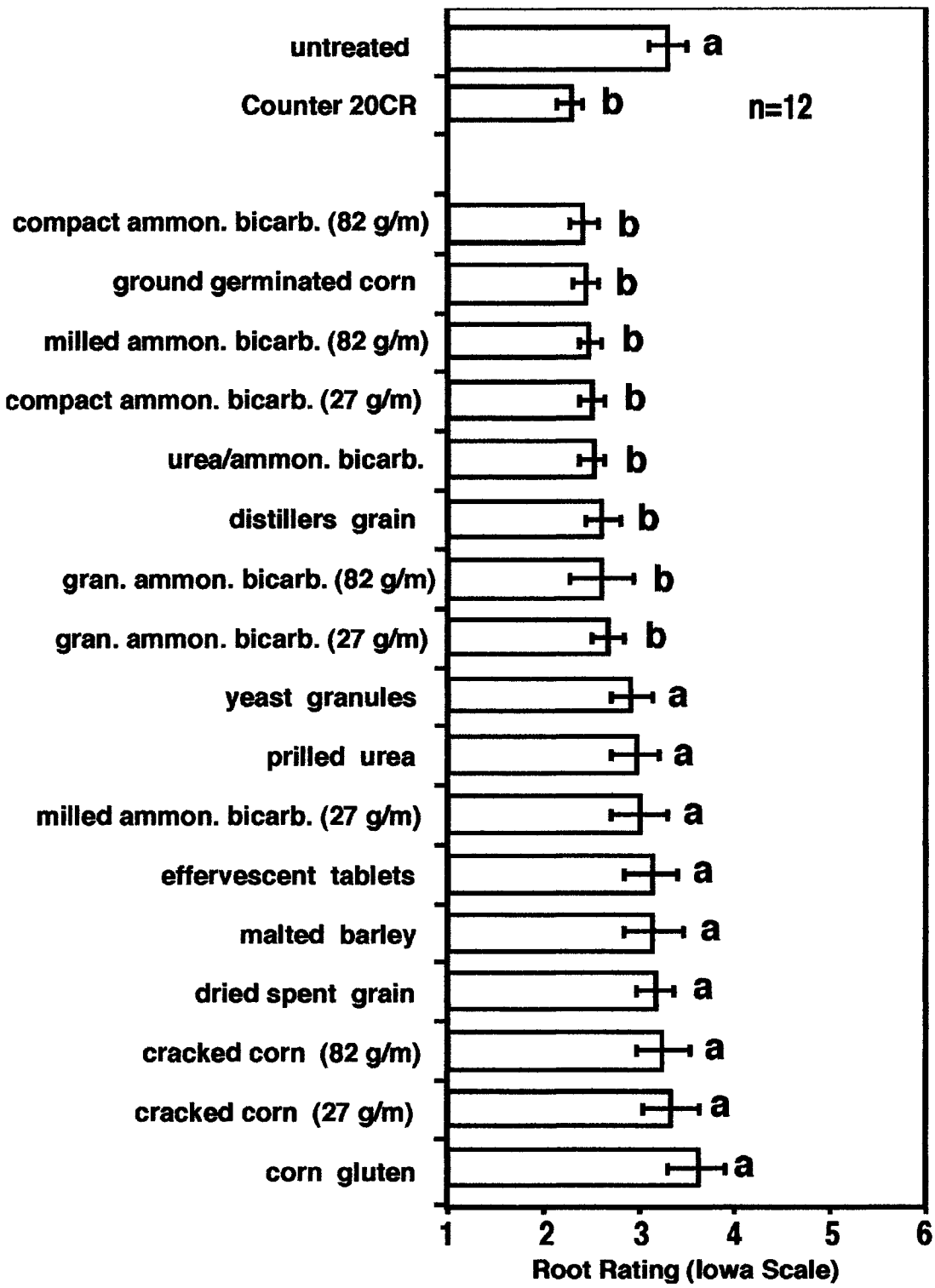

Fig. 3.6. Root ratings (Iowa 1-6 scale) from 1998 field trials. Significant differences (Dunnett's test, $p < 0.05$) between the treatments and the untreated control are indicated by different lower case letters. Bars represent standard errors.

Discussion

In soil tub tests with the gaseous CO_2, larvae were attracted to a gradient of CO_2 in a soil environment. This behavior is consistent with previous reports regarding larval behavior in an olfactometer (Strnad et al. 1986) and in soil (Strnad and Dunn 1990). In the current tests, larvae moved away from a germinating corn seed towards an artificial source of CO_2. In a glass bead bioassay (Bernklau and Bjostad 1998a) larvae were attracted away from corn volatiles by a concentration of CO_2 alone that was twice that produced by the corn. CO_2 levels in soil around 6-day-old germinating corn plants was measured at 4.36 mmol/mol (Bernklau and Bjostad 1998b). In the current large tub soil bioassays, the CO_2 concentration at the high end of the gradient was greater than 11 mmol/mol, a level sufficient to attract the larvae away from a single germinating corn seed.

Larval host location was disrupted by an overall increase in the CO_2 concentration in the soil. Two treatments, effervescing tablets and pellets of sucrose, were effective in preventing larvae from locating the corn plant, but the behavioral mechanism is not clear. The overall increase in CO_2 concentration throughout the tub may have confused the larvae and prevented them from locating and following the CO_2 gradient produced by the corn roots. Alternatively, the larvae may have spent their time following the individual gradients to each piece of CO_2-generating material and, in the process, either ignored or failed to encounter the smaller gradient produced by the corn roots.

The baker's yeast was the only treatment that did not prevent larvae from locating the corn within 24 hours, despite the sufficiently high levels of CO_2 produced by the yeast. It is possible that the larvae were repelled by metabolic products produced by the yeast and, therefore avoided the yeast particles in the soil. Alternatively,

the life of the yeast and its subsequent CO_2 production may have been shortened because suitable nutrients were not available in the soil. For further experiments, baker's yeast was combined with nutrients and substrate materials into a granular form in order to extend its activity.

In the larger soil tub bioassay, yeast granules prevented larvae from locating the corn plant. When the granules were evenly mixed throughout the soil, the lower dose (6 g) was not effective despite the elevated concentrations of CO_2 in the soil (7.49 mmol/mol, in the center of the tub). A higher dose (18 g) produced concentrations of 18.56 mmol/mol (center of the tub), and this was sufficient to keep larvae from the corn. Fewer larvae were able to locate the corn when the yeast granules were concentrated in a trench at one end of the soil tub. This placement was effective when the larvae were introduced between the corn and the treatment, and when the larvae were placed on the far side of the treatment. The concentration of CO_2 in the region of concentrated yeast granules was 15.79 mmol/mol (Fig. 4B) to 16.80 mmol/mol (Fig. 4F) with 18 g of granules and 5.69 mmol/mol (Fig. 4F) to 10.42 mmol/mol (Fig. 4B) with 6 g granules. These levels of CO_2 were sufficient to attract larvae away from the corn.

Organic and inorganic sources of CO_2 reduced damage to corn roots in field trials, but the inorganic formulations composed of ammonium bicarbonate and/or urea disintegrated quickly in continuous moist soil in the field. Ammonium bicarbonate, which decomposes to produce ammonia and carbon dioxide, is widely used as a fertilizer in China and other developing countries (Bouwman et al. 2002). It is less desirable as a fertilizer in the United States because of its rapid decomposition and subsequent high ammonia volatilization loss (Zhang et al. 1997, Bouwman et al. 2002, Roelcke et al. 2002). In preliminary lab tests, these materials produced immediate bursts of CO_2 in the soil, but did not maintain high levels for more than a few days.

The organic formulations produced CO_2 for 2 weeks, making them the best candidates for field treatments. Based on our own field surveys, WCR larval hatch generally begins during the first week of June in Colorado and continues to the end of July, with a peak around late June. Based on this information, a CO_2-generating formulation applied at planting time (late April to mid May) would need to produce sufficient amounts of carbon dioxide for a period of 8-9 weeks to provide adequate control of western corn rootworm larvae. One method of extending the length of time for CO_2 production might be to co-encapsulate yeast and a nutrient substrate with calcium alginate (Robinson 1995) or with κ-carrageenan (Wijffels et al. 1991, Cheong et al. 1993).

Rootworm larvae move best through loam and silty clay soil (MacDonald and Ellis 1990) and have been shown to survive best in soils that are uncompacted and have greater pore continuity (Ellsbury et al. 1994). CO_2-generating compounds may be more effective against larvae in sandy to silty soils because these types of soils allow better diffusion of gases than compacted clay soils. The soil in the test site fields was characterized as having a clay loam to sandy-clay texture (Soil, Water and Plant Testing Lab, Soil and Crop Sciences and Cooperative Extension, Colorado State University) that would be conducive to larval movement and survival (MacDonald and Ellis 1990) and that would allow sufficient, if not optimal, diffusion of CO_2. The longevity of the CO_2-generating compounds would also be dependent on soil moisture content and the amount of organic material in the soil. The laboratory soil bioassays were conducted in soil with a relatively high soil moisture content (20% by weight) to allow for optimum larval movement (Strnad and Bergman 1987, MacDonald and Ellis 1990). The soil used in the current study contained approximately 1.5% organic material, which facilitated the production of adequate levels of CO_2. It is likely that

placing the same materials placed in soils with more organic content would result in higher initial CO_2 levels but a reduced length of activity.

The tests with CO_2-generating compounds in soil demonstrate that it is possible to attract WCR larvae away from their host plants or to confuse the insects so that they are unable to locate roots of a host plant. CO_2 sources might also be used to attract WCR larvae, or other soil-dwelling organisms to pesticide granules or to pellets containing a biocontrol agent, as suggested by previous work (Kim and Riggs 1992, Meyer and Huettel 1993, Robinson 1995). Alternatively, CO_2 might be used as the attractive component of a toxic bait for WCR larvae in the same way that feeding stimulants are incorporated into adult baits currently in use for areawide management programs (Tollefson 1998, Pingel et al. 2001).

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CHAPTER 4

INSECTICIDE ENHANCEMENT WITH FEEDING STIMULANTS IN CORN FOR WESTERN CORN ROOTWORM LARVAE (COLEOPTERA: CHRYSOMELIDAE)

Introduction

The larval stage of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is the most economically- significant lifestage of the insect (Pike et al. 1995), costing U.S. growers over $1 billion annually in crop losses and control costs (Metcalf 1986). WCR larvae have limited exposure to insecticides because they are either moving in the soil, or they are feeding inside the plant roots. WCR larvae are oligophagous, soil-dwelling insects that are attracted to the roots of both host and non-host plants (Branson and Ortman 1967, 1970). The larvae can survive to adulthood on a number of grass species including barley, foxtail, wheat, various wheatgrasses, Johnsongrass, rye, rice millet, fescue and ryegrass (Branson and Ortman 1967, 1970). Rootworm larvae are attracted to carbon dioxide (CO_2) (Strnad et al. 1986, Hibbard and Bjostad 1988, Jewett and Bjostad 1996, Bernklau and Bjostad 1998a, b), which is produced by corn roots (Harris and Van Bavel 1957, Massimino et al. 1980, Desjardins 1985, Labouriau and Jose 1987), but larvae are not attracted to other volatile compounds produced by corn (Bernklau and Bjostad 1998b).

Although little is known about the chemical basis for the feeding preferences of WCR larvae, lab studies revealed that southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber) larvae prefer bitter cucurbit roots over non-bitter roots, and corn roots over bitter cucurbit roots (Deheer and Tallamy 1991, Eben et al. 1997). Corn rootworm larvae are typically reared using germinating corn as the food source (Rimando et al. 1966, Skelton and Hunter 1970, Sutter et al. 1971, Jackson 1985). Sutter et al. (1971) successfully reared southern corn rootworm larvae on an artificial diet containing agar and wheat germ. Rose and McCabe (1973) improved this diet with the addition of sorbic acid, KOH, formalin, corn oil, tetracycline, peni-

cillin G, bacitracin, streptomycin and methyl paraben. Both diets were modifications of the Vanderzant-Adksson wheat germ diet developed for pink bollworm (Adkisson et al. 1960). Marrone et al. (Marrone et al. 1985) enhanced larval growth by altering the concentrations of sugar, oil and antimicrobials in the diet. More recently, Pleau et al. (Pleau et al. 2002) assessed and adjusted individual constituents of the Marrone diet to optimize the growth and development of western corn rootworm larvae. The most significant changes in the diet included the removal of formalin, the addition of plant adjuvant and an adjustment of the dietary pH to 9.0.

Bitter cucurbitacins are known to elicit feeding in diabroticite adults (Derr et al. 1964, Chambliss and Jones 1966, Sharma and Hall 1971, Howe et al. 1976, Rhodes et al. 1980, Metcalf et al. 1981, Metcalf et al. 1982, Mullin et al. 1991, Eben et al. 1997, DeMilo et al. 1998, Cosse and Baker 1999, Schroder et al. 2001), and have been implemented in trapping and control strategies for western corn rootworm (Lance and Sutter 1992, Lance 1993, Behle 2001). Cucurbitacins may provide rootworm beetles with protection against soil-borne pathogens (fungi), and recent studies suggest that the protection may be due to bacteria associated with the cucurbits (Martin and Schroder 2000).

Pollens are a source of non-cucurbitacin feeding stimulants for adult rootworms. In feeding studies, western corn rootworm adults preferred sweet corn and winter squash pollen over the pollen of sunflower or Canada goldenrod (Hollister and Mullin 1998, 1999). Amino acid profiles revealed 5 prominent amino acids in the preferred pollens. In feeding tests, mixtures of these amino acids elicited phagostimulation in rootworm adults (Hollister and Mullin 1999). In a screening test of 52 different amino acids, L-alanine, L-serine and beta-alanine elicited the most feeding by rootworm adults (Kim and Mullin 1998). Lipids, amides and flavonol from sunflower pollen have been identified as additional phagostimulatory compounds (Lin and Mullin 1999).

In preliminary studies, solvent extracts of germinating corn elicited strong feeding by western corn rootworm larvae. Larval feeding stimulants have implications for control of WCR and specifically towards the development of an insecticide bait similar to those currently used against rootworm adults. In the current study, feeding stimulants were combined with an insecticide (thiamethoxam) in behavioral bioassays with neonate WCR larvae to test the hypothesis that such combinations would be superior to insecticide alone.

Materials and Methods

Insects. Western corn rootworm larvae were obtained from a colony that has been reared in our laboratory since 1986 (non-diapausing strain, originally obtained from J. Jackson, USDA-ARS Laboratory, Brookings, South Dakota). Additional insects were purchased from Lee French Agricultural Enterprises (Lamberton, Minnesota) and added from time to time to maintain genetic variability. The insects were reared on corn plants grown in soil using methods described by Jackson (1985) and modified by Hibbard and Bjostad (1988).

Feeding Bioassays. Each bioassay apparatus was made from a plastic petri dish (5 cm diameter). A ring of blotter paper (Anchor Paper, Minneapolis, Minnesota, cut 1 cm wide and 5 cm in diameter) was placed inside the inverted lid of the petri dish. After moistening the blotter paper ring with distilled water, the petri dish bottom was placed lightly on top of the paper. The ring of blotter paper provided a 1 mm gap between the stacked halves of the petri dish.

Disks of filter paper (Whatman No. 2, Cat. No. 1004-090, Springfield Mill, Maidstone, Kent, England, 1.2 cm diameter) were washed by agitating them in distilled water for 8 minutes, then air dried. Treatments (described below) were applied

to the disks and the disks were air-dried for 24 hours. For bioassays, a treated, dried disk of filter paper was placed in the center of the arena and moistened with 25 microliters of distilled water. Ten neonate larvae (less than 24 h old) were removed from a tub containing eggs in soil with a fine, camelhair brush and were placed in the center of the moistened disk, and the arena cover was replaced. The larvae were observed under a compound microscope for 30 minutes. The number of larvae on the paper and the number of larvae feeding were recorded every 5 minutes. A larva feeding on the filter paper disk could be identified by behaviors similar to those exhibited by larvae tunneling into corn roots: head angled sharply downward toward the paper, back and forth motion of the mandibles on the paper, and side-to-side motion of the head in a tearing motion on the paper.

Preparation of Concentrated Extract of Corn. In preliminary experiments with various extracts of germinating corn, a concentrated acetone extract elicited the best feeding from WCR larvae. To remove fungal spores, untreated, dried corn seeds (*Zea mays*, cv 3055 provided courtesy of Gary D. Lawrance, Pioneer Hi-Bred International, Inc., Johnston, Iowa) were soaked for 24 h in soapy water (1 drop of Ivory Dishwashing Liquid, Procter & Gamble, Cincinnati, Ohio, per liter of water), and rinsed thoroughly with water. The washed seeds were germinated 3 d on blotter paper (Steel Blue, Anchor Paper Company, St. Paul, Minnesota) in a closed polyethylene tub (30 x 15 x 8 cm), where the plants typically reached a shoot length of 1 cm and a root length of 6 cm. The 3-d-old germinating corn seeds (70 g wet wt) were soaked in a round-bottom flask filled with 300 ml acetone. After 4 days the liquid was filtered through filter paper (Whatman No. 2, Cat No. 1004-090, Springfield Mill, Maidstone, Kent, England) and most of the acetone was removed using a rotary evaporator, leaving an extract that was concentrated 8-fold by volume.

Concentrated Extract of Corn vs. Fresh Corn Liquid. Three treatments were tested in the feeding bioassay: distilled water, concentrated extract of corn and liquid pressed from corn roots. For the distilled water (control) treatment, 25 microliters of distilled water was applied to each disk and the disks were allowed to air dry. For the concentrated extract of corn, 25 microliters was applied to the disks and allowed to air dry. For liquid from roots, the roots of 3-d-old germinating corn (4 roots, approximately 4 cm long) were gently crushed onto the filter paper feeding disk with a metal spatula until the disk was saturated with the liquid. Corn root residue was gently scraped off and the paper was allowed to dry. For bioassays, the disks were re-wetted with distilled water (25 microliters). To avoid the possibility of larvae feeding on corn particles, all disks were inverted and placed in the arena with the treated side down before being re-wetted with distilled water. The number of larvae on the paper and the number of larvae feeding were recorded every 5 minutes for 30 minutes. Tests were conducted with 14 replications of each treatment.

Concentrated Corn Extract Plus Insecticide. Six dilutions of thiamethoxam insecticide (provided by Novartis, Inc., Basel, Switzerland) were prepared in water. The 6 individual solutions contained 0, 0.001, 0.01, 0.1, 1 or 10 parts per million (ppm) insecticide. The rate of thiamethoxam recommended for use against rootworm larvae was 1 ppm (Novartis, Inc., personal communication). Filter paper disks were treated with 25 microliters of concentrated corn extract and air-dried. Control disks were treated with 25 microliters of distilled water and air-dried. For bioassays, a disk was placed face down in the bioassay arena and re-moistened with 25 microliters of an insecticide solution. Feeding bioassays were conducted as described previously and the number of larvae on the paper, the number of feeding larvae and the number of dead larvae were recorded every 5 min for 30 min. A larva was counted as dead if it was lying limp on its side or if it did not move upon being prodded gently with a metal

probe. The 6 insecticide solutions were tested on filter paper disks that had been treated with concentrated corn extract and on control disks that had been treated only with distilled water. Tests were conducted with a minimum of 10 replications for each treatment.

Larval Survival. At the completion of the 30-minute feeding bioassay, the 10 larvae were gently removed from the filter paper disk using a camelhair brush and placed on the roots of two 3-d-old germinating corn seeds. The seeds were placed on moist filter paper in a covered plastic petri dish (5 cm diameter). After 24 hours, the seeds were removed from the petri dish and larvae were recovered using a modified Berlese technique. This procedure allowed long-term insecticide mortality to be detected, because larvae perish quickly without food.

Insect Recovery. Berlese-like devices were made from two plastic cups (Solo cups, Solo Cup Co., Urbana, Illinois). The bottoms were removed from both cups, and a square (15 cm x 15 cm) of plastic insect screen was stretched between the bottoms of the two cups, providing a taut screen surface near the bottom. A receptacle was made from the bottom of one of the cups, filled halfway with water and placed beneath the Berlese funnel. A germinating corn seed containing larvae was removed from the petri dish and gently placed in the funnel. Over a period of 2 days, as the plant and roots slowly dried, larvae crawled out of the roots and fell through the screen and floated on the surface of the water where they could be seen and counted. In preliminary tests, more than 90% of the larvae in a corn plant were recovered with this technique.

Statistical Analysis.

30-Minute Feeding Bioassays. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Fisher's LSD

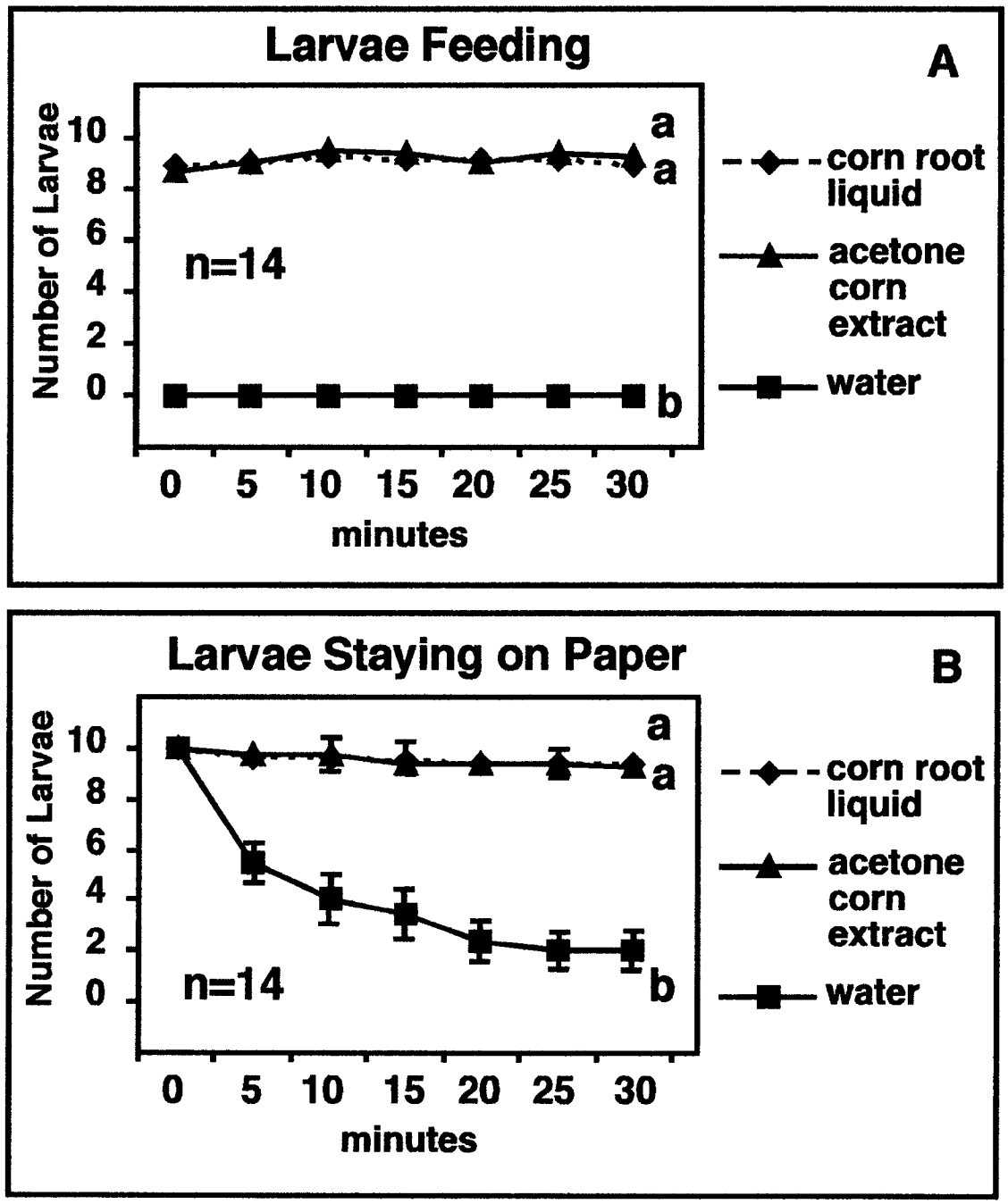

Fig. 4.1. (A) Neonate rootworm larvae feeding on treated filter paper disks. (B) Neonate rootworm larvae staying on treated filter paper disks. Mean separations were conducted at 30 minutes. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

test was used for all *a posteriori* comparisons, with $\alpha = 0.05$. For feeding bioassays, analysis was conducted for the final (30 minute) observations.

Larval Survival. T-tests were conducted to compare results from insecticide on corn extract-treated disks and insecticide on water-treated disks for each insecticide dose using Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts) with $\alpha = 0.05$.

Results

Solvent Extract of Corn vs. Fresh Corn Extract. In 30 minute feeding bioassays, significantly more larvae ($F=586.08$, $df=41$, $p < 0.05$) fed on disks treated with corn acetone extract (9.29 ± 0.19) or corn root liquid (9.33 ± 0.28) than on the water-treated disks (0.00 ± 0.00) (Fig. 4.1A). There was no significant difference in feeding between the acetone extract and the corn root liquid. Significantly more larvae ($F=100.93$, $df=41$, $p < 0.05$) were present on the paper after 30 minutes for the acetone extract-treated disks (9.29 ± 0.19) and the corn root liquid-treated disks (9.00 ± 0.33) than for the water-treated disks (2.00 ± 0.65) (Fig. 4.1B), but there was no significant difference between the two corn treatments.

Corn Extract plus Insecticide. For corn extract-treated disks, the 0.1, 1 and 10 ppm concentrations of thiamethoxam insecticide elicited significantly less feeding ($F=133.76$, $df=65$, $p < 0.05$) after 30 minutes than the three lower doses (Fig. 4.2A). The lowest concentration that caused reduced feeding by larvae was 0.1 ppm (7.70 ± 0.54). For water-treated disks, there were no significant differences ($F=1.85$, $df=63$, $p < 0.05$) among the insecticide treatments for the number of larvae feeding (Fig. 4.2B).

For corn extract-treated disks, there were no significant differences ($F=0.48$, $df=65$, $p= 0.05$) among the treatments for the number of larvae staying on the paper (Fig. 4.3A). For the water-treated disks, significantly more larvae ($F=4.64$, $df=63$, $p <$

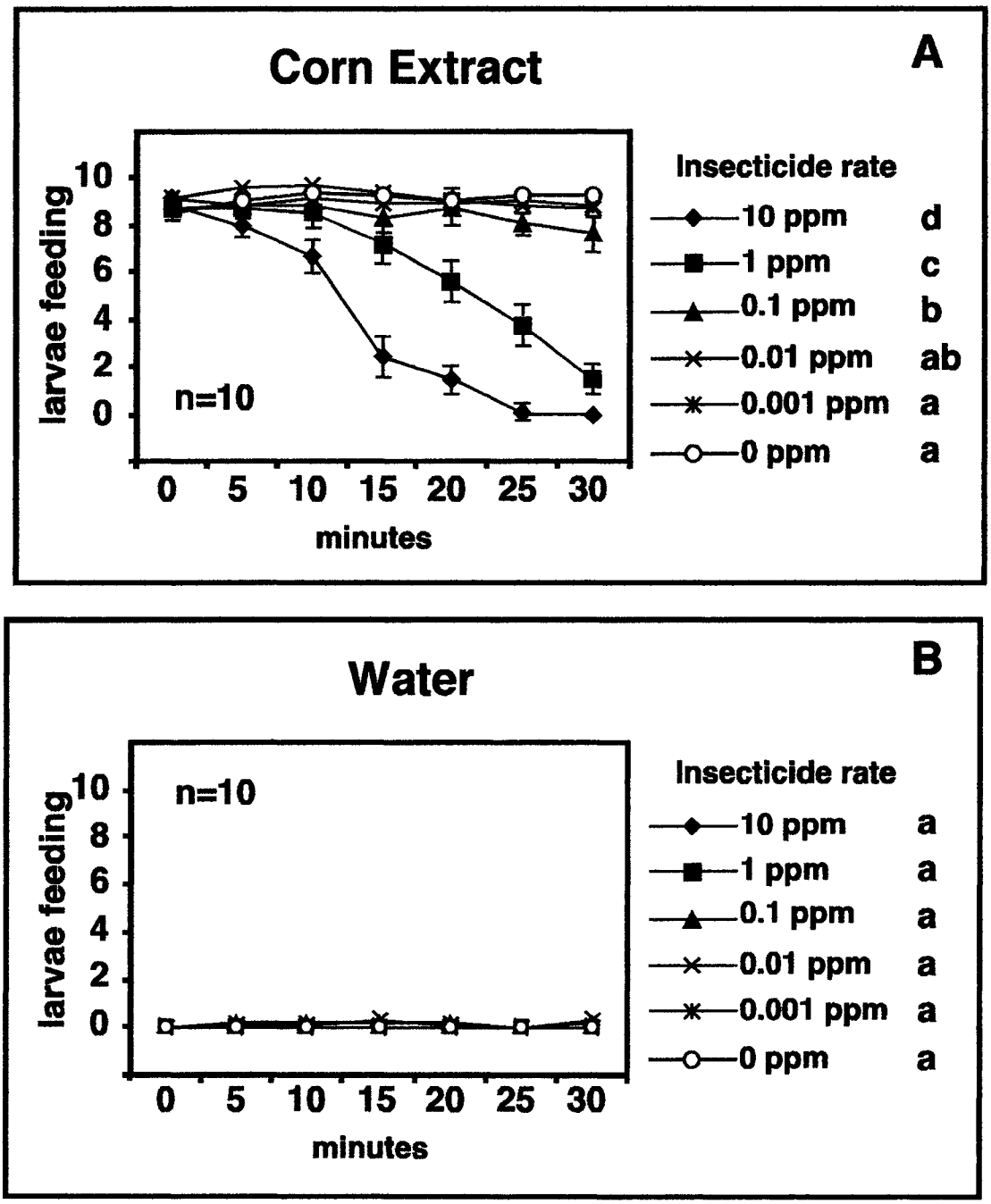

Fig. 4.2. (A) Neonate rootworm larvae feeding on filter paper disks treated with thiamethoxam and with corn extract. (B) Neonate rootworm larvae feeding on filter paper disks treated with thiamethoxam and water. Mean separations were conducted at 30 minutes. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

0.05) remained on the paper for the 10, 1, 0.1 and 0.01 ppm concentrations of insecticide than for the 0.001 and 0 ppm concentrations (Fig. 4.3B).

For corn extract-treated disks, significantly more larvae died in the 30 minute feeding period for the 1 ppm (7.20 ± 0.44) and 10 ppm (9.50 ± 0.22) concentrations of insecticide ($F=207.52$, $df=65$, $p < 0.05$) than for the lower 4 doses (Fig. 4.4A). The lowest concentration that caused significantly more larval mortality was 1 ppm (7.20 ± 0.44). For water-treated disks, the 10 ppm concentration resulted in significantly more larval mortality (3.80 ± 1.06) than the other insecticide concentrations ($F=9.98$, $df=65$, $p < 0.05$) (Fig. 4.4B).

Larval Survival. For the 1 and 10 ppm concentrations of insecticide, significantly greater larval mortality was observed immediately after the 30 minute feeding test on corn extract-treated disks than on water-treated disks (Fig. 4.5A). For 0, 0.001, 0.01 and 0.1 ppm concentrations of insecticide, there was no significant difference ($p < 0.05$) in larval mortality immediately after feeding for corn extract-treated disks than for water-treated disks (Fig. 4.5A). After 24 hours, significantly greater larval mortality was observed for the 0.01, 0.1, 1 and 10 ppm concentrations of insecticide on corn extract-treated disks than for the same concentrations on water-treated disks (Fig. 4.5B). The greatest difference between the corn extract and water-treated disks was observed with 1 ppm of insecticide. For the 1 ppm insecticide on corn extract-treated disks, larval survival was 28.00 ± 4.42% after 30 minutes, and, for the 1 ppm insecticide on water-treated disks, larval survival was 88.00 ± 5.54%. For the 1 ppm insecticide on corn extract-treated disks, larval survival after 24 hours was 4.00 ± 2.21% and, for the 1.0 ppm insecticide on water-treated disks, larval survival after 24 hours was 44.00 ± 5.2%. The lowest concentration of insecticide on corn extract-treated disks that caused at least 50% larval mortality after 24 hours was 0.01 ppm

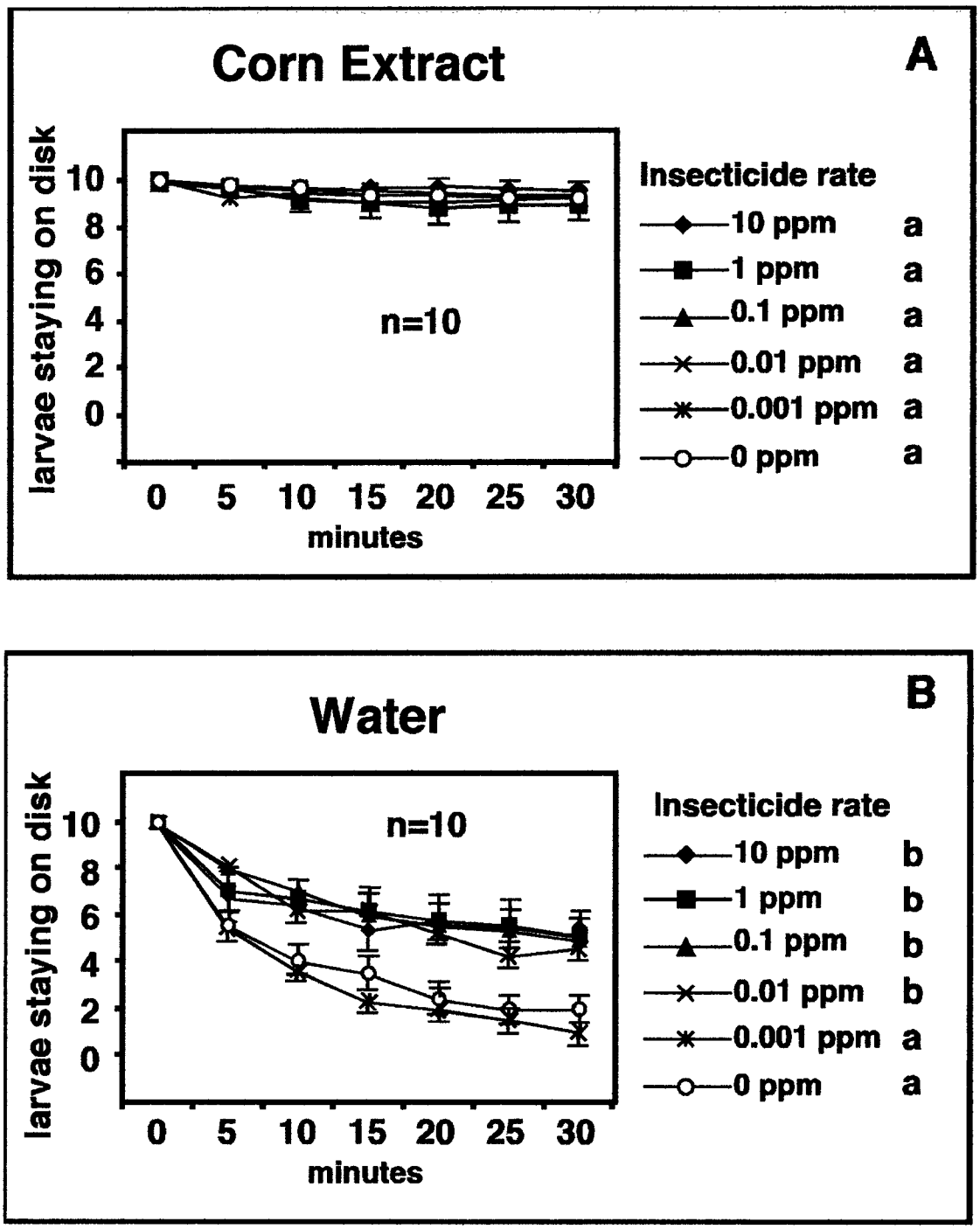

Fig. 4.3. (A) Neonate rootworm larvae staying on filter paper disks treated with thiamethoxam and with corn extract. (B) Neonate rootworm larvae staying on filter paper disks treated with thiamethoxam and water. Mean separations were conducted at 30 minutes. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

(35.00 ± 7.79%) and the lowest concentration of insecticide on water-treated disks that caused at least 50% larval mortality after 24 hours was 1.0 ppm (4.00 ± 5.21%).

Discussion

Neonate western corn rootworm larvae fed vigorously on paper disks saturated with the liquid pressed from germinating corn roots and on disks saturated with a concentrated extract of germinating corn. Larvae on the corn root liquid-treated disks and corn extract-treated disks displayed the following feeding behaviors: angling of the head down towards the paper, back and forth motion of the mandibles on the paper, and side-to-side motion of the head in a tearing motion on the paper. Once larvae began feeding, they showed no inclination to leave the corn extract-treated disks. In contrast, during control tests with water, most of the larvae quickly left the paper disks. The few larvae that remained on the disks were observed wandering slowly around the paper and they did not demonstrate any feeding behaviors.

In further feeding tests, the efficacy of an insecticide (thiamethoxam) was improved by the addition of the feeding stimulants (concentrated corn extract). For the 10 ppm concentration of thiamethoxam on corn extract-treated disks, 100% larval mortality was observed after 30 minutes of exposure. Only 22% mortality occurred when the insecticide was tested at the same concentration on water-treated disks. An LD50 (lowest concentration to result in at least 50% mortality) for thiamethoxam was determined from the larval survival 24 hours after exposure (during the feeding test). When the feeding stimulants were not present, the LD50 was 1 ppm, which is the recommended rate of the insecticide for rootworm larvae, and when the feeding stimulants were present the LD50 was 0.01 ppm (100-fold difference).

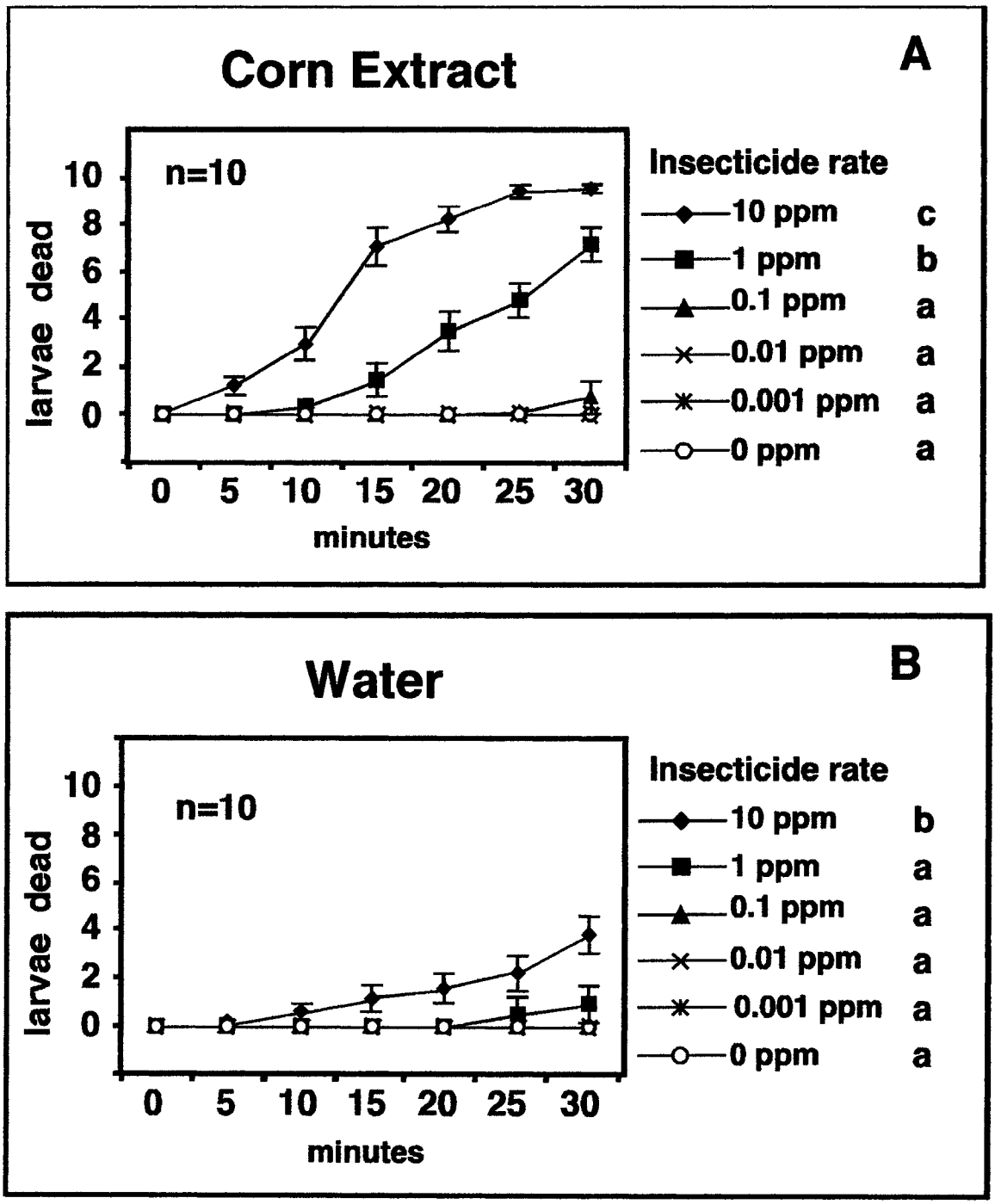

Fig. 4.4. (A) Neonate rootworm larvae dead on filter paper disks treated with thiamethoxam and with corn extract. (B) Neonate rootworm larvae dead on filter paper disks treated with thiamethoxam and water. Mean separations were conducted at 30 minutes. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

The insecticide thiamethoxam used in this study is a second generation neonicotinoid insecticide (Schroeder and Dumbleton 2001). Thiamethoxam (Cruiser,® Syngenta Crop Protection, Basel, Switzerland) received EPA approval a in 1999 for use as a seed treatment on corn, rape, wheat, sorghum, potatoes, canola, etc. for the control of a variety of soil insects including rootworm, wireworm, thrips, hessian fly, and seedcorn maggot (Schroeder and Dumbleton 2001, Steffey 2002). In the current study, thiamethoxam did not diminish the feeding stimulant effect of the corn extract. Larvae fed vigorously for the full 30 minutes on the 0, 0.001, 0.01 and 0.1 ppm concentrations of the insecticide when corn extract was present. For the two higher doses of thiamethoxam (10 ppm and 1 ppm), a reduction in the number of larvae feeding was observed on corn extract-treated disks after 15 minutes. This reduced feeding was not due to larvae leaving the paper, but rather to larval mortality after feeding briefly on the high doses of the insecticide. Very little feeding occurred on thiamethoxam when corn extract was not present. Larvae left water-treated disks with 0.001 ppm thiamethoxam (lowest dose) at the same rate that they left water-treated disks with no thiamethoxam (0 ppm). Larvae were slower to leave the water-treated disks with the 4 higher thiamethoxam concentrations, and this was likely due to the toxic effect of the insecticide. Larvae showed no preference for feeding on liquid from corn roots compared to feeding on the concentrated corn extract, indicating that all the compounds required to elicit strong larval feeding were extracted by acetone and were stable when dried in air for 24 hours.

The results of these behavioral bioassays suggest using feeding stimulants to improve insecticide activity against WCR larvae in the field. It may be possible to incorporate feeding stimulants with a larval attractant into granules to attract and kill rootworm larvae. Strnad et al. (1986) first reported that WCR are attracted to CO_2, and this attraction has also been demonstrated by other investigators (Hibbard and

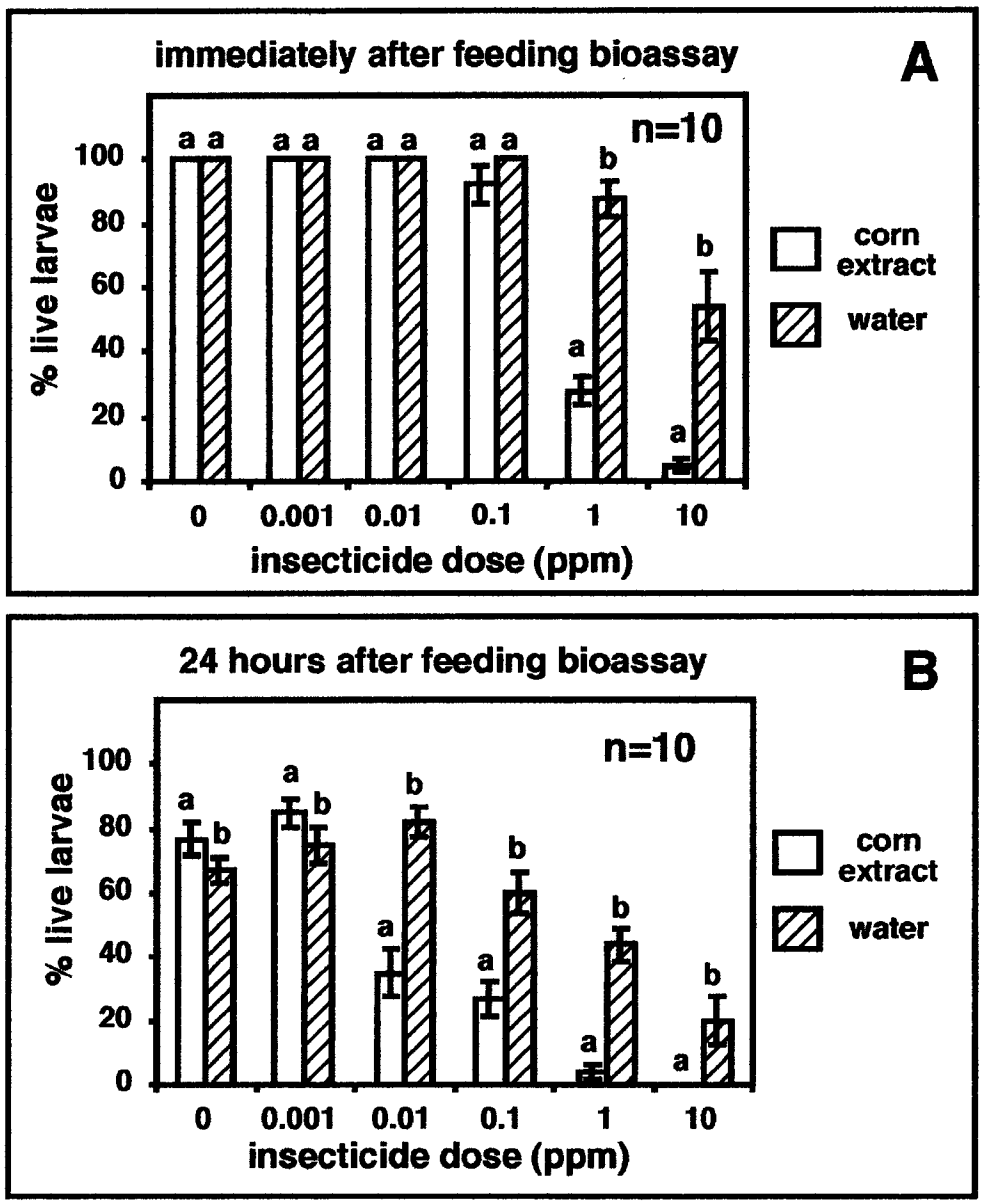

Fig. 4.5. (A) Percent larvae alive after 30 minutes on filter paper disks treated with thiamethoxam and corn extract or treated with thiamethoxam and water. Significant differences ($p < 0.05$) between the corn extract and water treatments for each dose of thiamethoxam are indicated by different lower case letters (0 ppm, t=0, df=29; 0.001 ppm, t=0, df=19; 0.01 ppm, t=0, df=19; 0.1 ppm, t=1.71, df=19; 1 ppm, t=71.68, df=19; 10 ppm, t=21.19, df=19). (B) Percent larvae alive 24 hours after feeding on filter paper disks treated with thiamethoxam and corn extract or treated with thiamethoxam and water. Significant differences ($p < 0.05$) between the corn extract and water treatments for each dose of thiamethoxam are indicated by different lower case letters (0 ppm, t=0.24, df=29; 0.001 ppm, t=2.19, df=19; 0.01 ppm, t=27.57, df=19; 0.1 ppm, t=16.30, df=19; 1ppm, t=50.00, df=19; 10 ppm, t=6.92, df=19). Bars represent standard errors.

Bjostad 1988, MacDonald and Ellis 1990, Jewett 1995, Bernklau and Bjostad 1998a, b). Larvae are able to detect differences in CO_2 as small as 12.5% and, when given a choice of concentrations, they are always attracted to the higher concentration (Bernklau and Bjostad 1998a). In addition, larvae can be attracted away from respiring corn roots by a higher concentration of carbon dioxide alone (Bernklau and Bjostad 1998b). Calcium alginate co-encapsulation is used for storage and dispersal of microorganisms (Cheong et al. 1993, Vives et al. 1993), and it has the potential to be used in a variety of applications. Starch granules (Lewis et al. 1995, McGuire and Shasha 1995) and k-carrageenan encapsulation (Wijffels et al. 1991, Cheong et al. 1993) have been used as formulations for microbial pesticides. It may be possible to apply these or similar technologies to the development of a soil-applied granular bait for control of WCR larvae.

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CHAPTER 5

CARBON DIOXIDE AND FEEDING STIMULANTS COMBINED WITH INSECTICIDES FOR CONTROL OF WESTERN CORN ROOTWORM LARVAE (COLEOPTERA: CHRYSOMELIDAE)

Introduction

In crop situations, less than 0.1% of pesticides may reach the target pest (Pimentel and Levitan 1986, Pike et al. 1995, Paoletti and Pimentel 2002). The remaining 99.9% can become a concern to human health or the environment and may have detrimental effects on beneficial insects, invertebrates and microbes in the soil (Ripper 1956, Pimentel and Levitan 1986). The use of attractants and feeding stimulants may improve the efficiency of insecticide delivery.

Baits that contain feeding stimulants and/or attractants are currently used for control of adult western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) (Tollefson 1998, Pingel et al. 2001), a major pest of corn in the United States (Krysan and Miller 1986). WCR larvae feed on the roots of growing corn plants and cost U.S. growers over $1 billion each year in control costs and crop losses (Metcalf 1986). Treatments for rootworm larvae are expensive and time consuming and they are not always effective (Behle 2001, Pingel et al. 2001, Schroder et al. 2001). Area-wide management programs that target the adult stage utilize toxic baits that have been shown to reduce egg laying and these may provide an alternative to broadcast insecticide applications (Behle 2001, Pingel et al. 2001, Schroder et al. 2001). One commercial bait is comprised of a cucurbitacin feeding stimulant with 0.3% carbaryl insecticide, and a non-pheromonal volatile attractant on a bran-based dry carrier (Lance and Sutter 1992). The addition of cucurbitacins improved the efficacy of a water-soluble bait containing a dye toxin in laboratory and field cage studies (Schroder et al. 1998).

WCR larvae are attracted to carbon dioxide (CO_2) (Strnad et al. 1986, Hibbard and Bjostad 1988, MacDonald and Ellis 1990, Jewett and Bjostad 1996, Bernklau and Bjostad 1998a) emitted by the roots of growing corn (Harris and Van Bavel 1957, Massimino et al. 1980, Desjardins 1985, Labouriau and Jose 1987). The attraction of

WCR larvae to corn roots is caused by CO_2 alone, and no other volatile chemical cues are involved (Bernklau and Bjostad 1998b). It may be possible to use CO_2-generating granules to disrupt WCR larval host location and prevent the insects from locating growing corn roots in soil. One approach would be to incorporate an attractant, feeding stimulants and insecticide into granules to be applied in the soil. By attracting larvae directly to the treatment and enticing them to feed on the insecticide, the amount of insecticide required for larval control might be reduced. CO_2-generating granules containing feeding stimulants and insecticide were used in soil bioassays and as field treatments to test our hypothesis that a feeding stimulant/attractant combination would provide better control of rootworm larvae than the feeding stimulant or attractant alone.

Materials and Methods

Insects. WCR larvae were obtained from a colony that has been reared in our laboratory since 1986 (non-diapausing strain, originally obtained from J. Jackson, USDA-ARS Laboratory, Brookings, South Dakota). Additional insects were purchased from Lee French Agricultural Enterprises (Lamberton, Minnesota) and added from time to time to maintain genetic variability. The insects were reared on corn plants grown in soil using methods described by Jackson (Jackson 1985) and modified by Hibbard and Bjostad (1988).

Soil Bioassays. Bioassays were conducted in soil-filled plastic tubs (40 x 18 x 8 cm, Rubbermaid, Wooster, Ohio), each with 6 pinholes drilled into the wall at the bottom edge of each side to prevent CO_2 from accumulating in the bottom. Soil was obtained from a local agricultural research farm (ARDEC, Agricultural Research Demonstration and Education Center, Fort Collins, Colorado) whose history was known, and where no corn had been grown or chemicals applied for at least 5 years. The soil

was characterized as having a clay loam texture, containing 1.5% organic matter and having good filtration. For bioassays, soil was modified to contain 20% moisture (by weight) for optimum larval movement (Strnad and Bergman 1987, MacDonald and Ellis 1990). Moist soil was prepared by sieving dry soil (5 mm mesh) and adding water slowly while stirring vigorously. A tub was filled to within 2 cm of the top with 1 kg of the prepared soil. A single corn seed (*Zea mays*, cv 3055, Pioneer Hi-Bred International, Inc., Johnston, Iowa) was planted at one end of the plastic tub (2 cm from the far end and 3 cm deep) and the lid was placed loosely on the tub (not air-tight). Candidate CO_2-generating materials were mixed into the soil at the time of planting as described below. Control tubs contained no added materials. When the corn was 4-5 d old, 30 neonate (less than 24 h old) larvae were collected from a tub containing eggs in soil with a camelhair brush. Larvae were placed on a moistened square of filter paper (Whatman No. 2, Cat. No. 1004-090, Springfield Mill, Maidstone, Kent, England, cut 2 cm x 2 cm). The paper was transferred to the bioassay container by digging a depression in the soil (1 cm deep), inverting the filter paper, placing it into the depression, and covering the paper gently with soil. The bioassay tubs were left undisturbed for 24 h, after which larvae were recovered from the corn plant as described below.

Insect Recovery. Berlese-like devices were made from two plastic cups (Solo cups, Solo Cup Co., Urbana, Illinois). The bottoms were cut from both cups, one at 6 cm from the bottom and the other at 2 cm from the bottom. The shorter cup was inserted into the longer cup while a square (15 cm x 15 cm) of plastic insect screen was stretched between the cut edges of the two cups, providing a taut screen surface near the bottom. A receptacle was made of the bottom cut from one cup, filled half-way with water and placed beneath the Berlese funnel. A corn plant containing larvae was removed from the soil and gently placed in the funnel. As the plant and roots

slowly dried over a period of 2 days, larvae crawled out of the roots and fell through the screen and into the water below. The white interior of the receptacle allowed floating larvae to be seen and removed from the water. In preliminary tests, more than 90% of the larvae in a corn plant were recovered using this technique.

GC-MS-SIM Analysis of CO_2. Mass spectrometry was used to determine CO_2 concentrations in the soil. A Hewlett-Packard Series II 5890 gas chromatograph interfaced with a Hewlett-Packard 5971 mass selective detector was operated in selected ion monitoring mode (SIM) for m/z 44 with a methyl silicone capillary column (30 m x 0.32 mm ID, RSL-150, Alltech, Inc., Deerfield, Illinois). A 10 millimoles per mole standard concentration of CO_2 (a 300 ml glass bottle into which 3 ml of CO_2 were injected) was used to calculate the CO_2 concentrations of the unknown samples. A jig to measure CO_2 was constructed from foamboard (Hunt Corporation, Philadelphia, Pennsylvania, cut 3 cm x 3 cm x 10 mm). A single hole (1 mm diameter) was drilled through the foamboard and a piece of glass tubing (5 cm long x 1 mm ID) was inserted through the hole. A piece of metal wire (5.3 cm long) was inserted into each glass tube so that the wire projected 3 mm from the end of the glass tube. The jig was inserted, wire first, 4 cm into the soil. The wire plug was removed from the glass tubes, leaving a 3 mm gap in the soil just below the end of the glass tube. The needle of a 10-µl Hamilton syringe was inserted into the glass tube so that it projected 1 mm into the gap, and a 5-µl sample of soil headspace was removed for analysis. For each bioassay, a jig was inserted into the soil in the center of the tub and the CO_2 was measured just prior to introducing the larvae.

Insecticides. Insecticides were obtained either as granules or in solution for preparation of unique granular treatments. Terbufos (40%, American Cyanamid, Mount Olive, New Jersey) and fipronil (98%, Aventis, Kansas City, Missouri) were obtained in solution and diluted in acetone. Tefluthrin was extracted in acetone from Force 3G

granules (Novartis, Basel, Switzerland) and concentrated under a nitrogen stream. Liquid carbaryl (Ortho Sevin, Chevron Chemical Co., San Ramon, California) was purchased from a local Kmart store. Thiamethoxam was provided by Novartis, Inc. (Basel, Switzerland) in a 40% aqueous solution.

Bioassays with Terbufos-Treated Yeast Granules.

Yeast Granule Preparation. Yeast granules were prepared by mixing 3 g bakers yeast (Fleischmanns, Burns Philp Food, Inc., Fenton, Missouri), 10 g corn syrup (Best Foods Div, CPC International, Inc., Englewood Cliffs, New Jersey) 1 g nutrient agar mix (YPD Broth, Sigma Aldrich, St. Louis, Missouri) and 10 g corn meal (Quaker Oats Co., Chicago, Illinois). The dry ingredients were placed in a food processor and the liquid (corn syrup and nutrient agar) was slowly added with the processor set on a slow (chopping) setting. For each batch, a defined amount of 40% terbufos solution was added to the liquid to produce yeast granules that contained either 0%, 1% or 5% terbufos. The granules were spread onto waxed paper and allowed to dry.

Yeast Granule Bioassays. For soil bioassays, yeast granules were mixed evenly throughout the soil in the tub just prior to planting the corn seed. Separate tests were conducted with 1 g of yeast granules containing 0% terbufos (0 mg ai per kg soil), 1% terbufos (10 mg ai per kg soil) or 5% terbufos (50 mg ai per kg soil). The experiment was repeated using 3 g of yeast granules containing 0% terbufos (0 mg ai per kg soil), 1% terbufos (30 mg ai per kg soil) or 5% terbufos (150 mg ai per kg soil). Control tubs contained untreated soil and positive controls were conducted using Counter 15G insecticide granules (American Cyanamid, Mount Olive, New Jersey) at a rate equivalent to the recommended rate for field application (0.06 g granules per tub, 10 mg terbufos per kg soil). For each treatment the experiment was repeated at least 9 times.

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Fisher's LSD test was used for all *a posteriori* mean comparisons ($\alpha = 0.05$).

Soil Bioassays with Insecticide and Corn Extract-Coated Granules.

Concentrated Corn Extract (FS). Feeding stimulants (FS) for WCR larvae were extracted in solution from germinating corn seeds. To remove fungal spores, untreated dried corn seeds (*Zea mays*, cv 58-DL, Novartis, Basel, Switzerland) were soaked for 24 h in soapy water (1 drop of Ivory Dishwashing Liquid, Procter & Gamble, Cincinnati, Ohio, per liter of water), and rinsed thoroughly with water. The washed seeds were germinated 3 d on blotter paper (Steel Blue, Anchor Paper Company, St. Paul, Minnesota) in a closed polyethylene tub (30 x 15 x 8 cm), where the plants typically reached a shoot length of 1 cm and a root length of 6 cm. The 3-d-old corn seeds (70 g wet wt) were soaked in a round-bottom flask filled with 300 ml acetone. After 4 days the liquid was filtered through filter paper (Whatman No. 2, Cat No. 1004-090, Springfield Mill, Maidstone, Kent, England) and most of the acetone was removed using a rotary evaporator, leaving an extract that was concentrated 8-fold by volume.

Granule Preparation. Granules were prepared from dried spent brewers grain (obtained at a local brewery), processed corn cob granules (2 mm diameter, Mt. Pulaski Co., Mt. Pulaski, Illinois) and clay granules (Special Kitty® cat litter, Bentonville, Arkansas, sieved to obtain granules 2 mm in diameter). Insecticide was added to the feeding stimulant solution (FS, concentrated corn extract described previously) and the resulting solution (30 ml) was shaken in a closed container with 30 g of spent grain or processed cob granules until the dry material was thoroughly coated. For clay granules, 15 ml of solution was used to treat 30 g of clay granules. For negative

controls, granules were prepared with the insecticide solution in water (no corn extract).

Soil Bioassay Procedure. Soil tub bioassays were conducted as described previously using 0.3 g of each of the following granules: spent grain plus FS (0.03% or 1% thiamethoxam), spent grain without FS (0%, 0.03% or 1% thiamethoxam), processed cob plus FS (0.03% thiamethoxam), processed cob without FS (0% or 0.03% thiamethoxam), clay granules plus FS (1% thiamethoxam), clay granules without FS (1% thiamethoxam). The tests were repeated using 1 g of each granule type. Control tubs contained untreated soil and positive controls were conducted using Counter 20CR insecticide granules (American Cyanamid, Mount Olive, New Jersey, 0.3 g, 60 mg terbufos per kg soil). Counter 20CR granules (20% terbufos) are applied at a conventional rate of 103 mg terbufos per linear meter of corn row. Field observations indicated that the Counter CR granules are dispersed throughout a cross-section of soil that is 10 cm wide and 10 cm deep, indicating a conventional application rate of 60 mg terbufos per kg of soil. For each treatment the experiment was repeated at 10 times.

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Fisher's LSD test was used for all *a posteriori* mean comparisons ($\alpha = 0.05$).

1998 Field Trials. In 1998, tests were conducted at a cornfield located at the Agricultural Research Demonstration and Education Center (ARDEC, Colorado State University, Fort Collins, Colorado). The site had been planted with continuous corn for at least 5 years. Treatments were applied by hand immediately following planting of the corn seed. One set of treatments included yeast granules containing 4.4% terbufos prepared by American Cyanamid (Mount Olive, New Jersey) using the method described above. The treated yeast granules were tested at rates of 1, 3.28 or 10 g per m of corn row. Control yeast granules (with no insecticide) were tested at 3.28 g per m

of corn row. The granules were placed in a trench dug 2 inches deep and 4 inches to either side of the corn seed. A second set of treatments included clean cracked corn (poultry feed purchased at a local Country General Farm and Ranch store) and spent grain that were coated with insecticide solution as described previously. Treatments included spent grain (0.2% carbaryl), cracked corn (0.02% carbaryl), spent grain (0.02% fipronil), cracked corn (0.02% fipronil), Counter 20CR and untreated controls. Counter 20CR was applied at 504 or 168 g per m of corn row and the spent grain, processed corn cob and clay granules were applied at a rate of 3.28 g per m of corn row. All treatments were applied in a banded pattern 3 inches wide directly over the corn row and were covered with a thin layer of soil. The plots (4 of each treatment) were 15.2 m in length and were arranged in a randomized complete block pattern.

Root ratings were conducted in late July. The feeding damage for each plant was evaluated using the Iowa 1-6 scale (Hills and Peters 1971), in which a rating of 1 indicates no visible rootworm feeding scars and a rating of 6 indicates at least three nodes of roots destroyed.

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Dunnett's test was used for *a posteriori* mean comparisons with a control ($\alpha = 0.05$) (Winer 1971).

1999 Field Trials. Field tests were conducted in 1999 at 2 locations: The Agricultural Research, Demonstration and Education Center (ARDEC, Fort Collins, Colorado) and a commercial farm near Yuma, Colorado. All treatments were applied at planting time in a standard T-band pattern over the corn row using modified Winter-Stieger meters (Read Brothers, Inc., Henry, Illinois). Plots (6 of each treatment at ARDEC and 8 of each treatment at the Yuma site) were 12.1 m in length and were arranged in a randomized complete block pattern.

Treatments were comprised of spent grain, processed cob and clay granules coated with insecticide and FS. Treatments included spent grain plus FS (2% thiamethoxam), spent grain without FS (2% thiamethoxam), processed cob plus FS (2% thiamethoxam), processed cob without FS (2% thiamethoxam), clay granules plus FS (2% thiamethoxam), and clay granules without FS (2% thiamethoxam). Additional treatments included processed cob plus FS (5% tefluthrin), processed cob plus FS (3.3% terbufos), untreated processed cob, untreated spent grain, Force 3G and untreated controls. Force 3G was applied at the rate of 420 mg per m of corn row and all other granules were applied at a rate of 840 mg per m of corn row.

Two varieties of corn seed were used: Pioneer seed (cv. 3055) and Novartis seed (cv. 58-DL, Novartis, now Syngenta, Basel, Switzerland). Counter 20CR and untreated controls were applied to both types of corn. Treatments applied only to the Novartis seed included Force 3G, processed cob without FS (2% thiamethoxam), and processed cob plus FS (2% thiamethoxam). The remaining treatments were applied only to the Pioneer corn seed. Corn roots were evaluated in late July using the Iowa 1-6 scale (described previously).

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Dunnett's test was used for *a posteriori* mean comparisons with a control ($\alpha = 0.05$) (Winer 1971).

2000 Field Trials. Field tests were conducted in 2000 at 2 commercial farms near Haxtun, Colorado. All treatments were applied in a standard T-band pattern directly over the corn row using modified Winter-Stieger meters. Plots (8 of each treatment) were 12.1 m in length and were arranged in a randomized complete block pattern. Treatments applied at planting included: Force 3G (420 mg per m of corn row), processed cob plus FS (2% thiamethoxam) at 420 and 840 mg per m of corn row, clay granules without FS (2% thiamethoxam) at 840 mg per m of corn row, and clay gran-

ules plus FS (2% thiamethoxam) at 840 mg per m of corn row. Three treatments were applied at cultivation: processed cob plus FS (2% thiamethoxam) at 420 mg per m of corn row, clay granules plus FS (2% thiamethoxam) at 840 mg per m of corn row, and clay granules without FS (2% thiamethoxam) at 840 mg per m of corn row. Corn roots were evaluated in mid July using the Iowa 1-6 scale (described previously)

Statistical Analysis. Analysis of variance was conducted with Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts). Dunnett's test was used for *a posteriori* mean comparisons with a control ($\alpha = 0.05$) (Winer 1971).

Results

Bioassays with Terbufos-Treated Yeast Granules In soil tub tests with terbufos-treated yeast granules, significantly fewer larvae were recovered for all treatments than from the untreated control tubs (14.66 ± 1.31) ($F=18.21$, $df=93$, $p < 0.05$) (Fig. 1A). Yeast granule treatments with significantly fewer larvae recovered than the Counter 15G insecticide (10 mg terbufos) (6.88 ± 1.53) included: 1 g granules with 1% (10 mg) terbufos (1.22 ± 0.43), 1 g granules with 5% (50 mg) terbufos (2.0 ± 0.92), 3 g granules with 1% (30 mg terbufos) (0.60 ± 0.26), and 3 g granules with 5% (150 mg) terbufos (0.80 ± 0.32). The fewest number of larvae were recovered from tubs with 3 g yeast granules containing 5% terbufos, and this treatment was not significantly different from 1 g yeast granules with 1% terbufos or 3 g yeast granules with 5% terbufos. There was no significant difference among the 1 g granules with 0 % terbufos and 3 g granules with 0 % terbufos and the insecticide. All of the yeast granule treatments produced significantly more CO_2 (Fig. 1B) than the untreated controls ($F=4.94$, $df=47$, $p < 0.05$). The highest CO_2 concentration was in the tub containing 3 g granules with 5% (150 mg) terbufos (6.34 ± 0.92 mmol/mol). The CO_2 con-

centration in the control tubs (2.00 ± 0.12 mmol/mol) was not significantly different from the concentration in tubs with Counter 15G insecticide (2.81 ± 0.17 mmol/mol).

Soil Bioassays with Insecticide and Corn Extract-Coated Granules. In soil tub bioassays using 0.3 g of spent grain granules, significantly fewer larvae were recovered for every granule treatment than for the controls ($F=20.18$, $df=69$, $p < 0.05$) (Fig. 2A). Significantly fewer larvae were recovered for the 0.03% thiamethoxam plus FS than for 0.03% thiamethoxam alone. For the 0.3 g rate of processed cob granules, significantly fewer larvae were recovered for the 0.03% thiamethoxam and 0.03% thiamethoxam plus FS than for the controls ($F=30.71$, $df=49$, $p < 0.05$). The 0% thiamethoxam was not significantly different from the controls (Fig. 2B). For the 0.3 g rate of clay granules, significantly fewer larvae were recovered for the 1% thiamethoxam plus FS and for the Counter 20CR insecticide than from the controls or the 1% thiamethoxam ($F=22.79$, $df=39$, $p < 0.05$) (Fig. 5.2C).

For the 1 g rate of spent grain granules, significantly fewer larvae were recovered from tubs with every treatment than from the controls ($F=46.72$, $df=69$, $p < 0.05$) (Fig. 5.2A). For the 1 g rate of processed cob granules, significantly fewer larvae were recovered for the 0.03% thiamethoxam and the 0.03% thiamethoxam plus FS treatments than for the controls ($F=29.47$, $df=49$, $p < 0.05$). The 0% thiamethoxam was not significantly different from the controls (Fig. 5.2B). For the 1 g rate of clay granules, 2 treatments, the 1% thiamethoxam plus FS and the Counter 20CR, had significantly fewer larvae recovered than untreated controls or the 1% thiamethoxam alone ($F=23.67$, $df=39$, $p < 0.05$) (Fig. 5.2C).

1998 Field Trials. In 1998, treatments that produced root ratings significantly lower than the untreated control (3.30 ± 0.19) included Counter 20CR insecticide at 504 mg per m of corn row (2.29 ± 0.21), Counter 20CR insecticide at 168 mg per m of

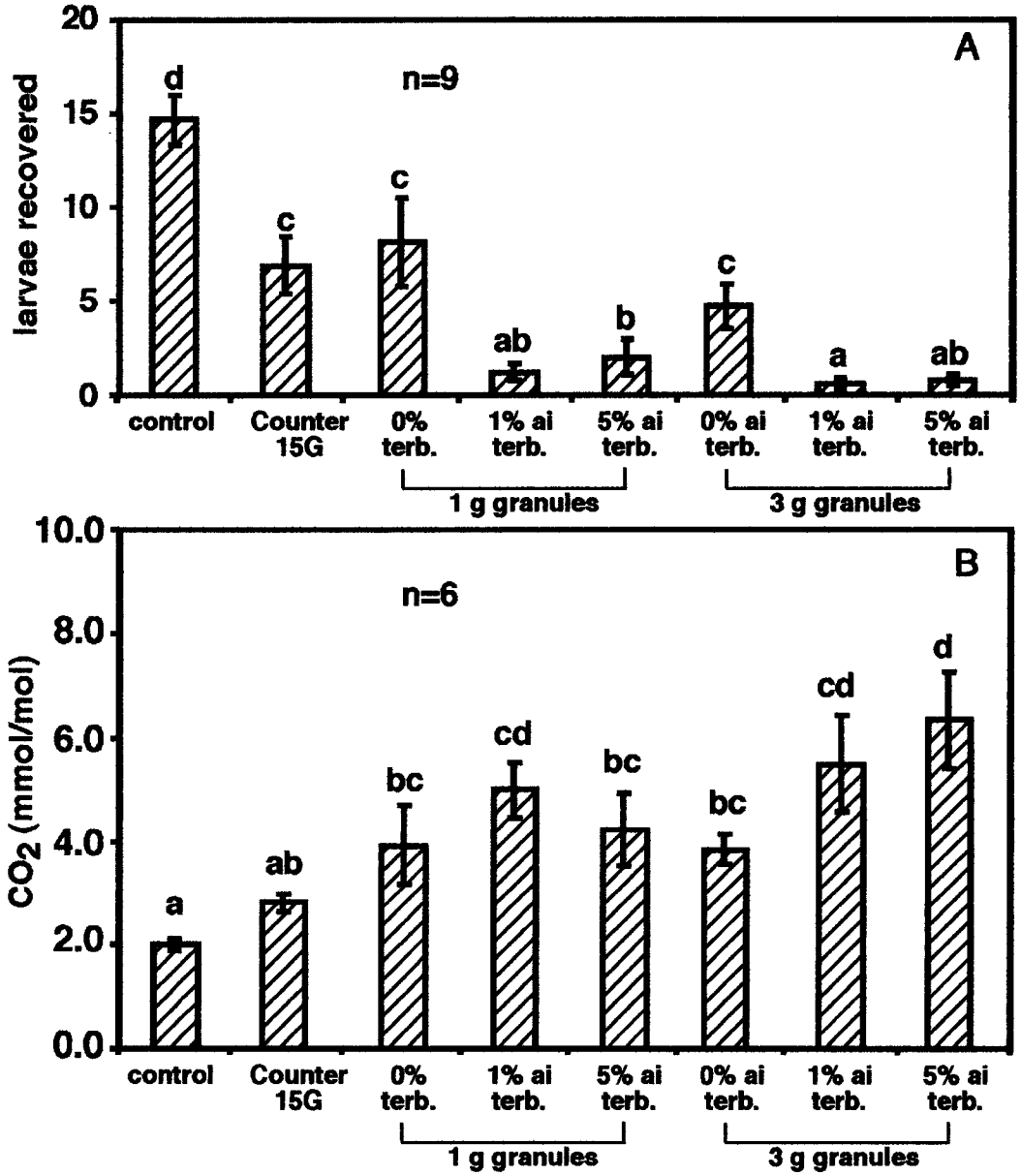

Fig. 5.1. (A) Neonate larvae recovered from corn in soil tubs containing terbufos-treated yeast granules. (B) Carbon dioxide concentrations from soil tubs containing terbufos-treated yeast granules. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors.

corn row (2.50 ± 0.16), and spent grain with 0.02% fipronil (2.46 ± 0.16), ($F=1.76$, $df=223$, $p < 0.05$).

1999 Field Trials. In 1999 at the ARDEC site, 8 treatments produced root ratings significantly lower than the untreated control (3.71 ± 0.30, Novartis seed; 3.71 ± 0.49, Pioneer seed) ($F=6.08$, $df=145$, $p < 0.05$) (Fig. 5.3). These included Counter 20CR insecticide (2.13 ± 0.21, Novartis seed; 2.38 ± 0.22, Pioneer seed), Force 3G insecticide (2.79 ± 0.25), clay granules without FS (2% thiamethoxam) (1.90 ± 0.16), clay granules plus FS (2% thiamethoxam) (1.94 ± 0.11), spent grain without FS (2% thiamethoxam) (2.29 ± 0.16), spent grain plus FS (2% thiamethoxam) (1.96 ± 0.33).

In 1999 at the Haxtun site, 7 treatments produced root ratings significantly lower than the untreated control (4.39 ± 0.17, Novartis seed; 4.58 ± 0.17, Pioneer seed) ($F=18.64$, $df=206$, $p < 0.05$) (Fig. 5.4). These included: Counter 20CR insecticide (3.36 ± 0.24, Novartis seed; 2.94 ± 0.11, Pioneer seed), Force 3G insecticide (2.93 ± 0.20), spent grain without FS (2% thiamethoxam) (2.91 ± 0.12), spent grain plus FS (2% thiamethoxam) (2.69 ± 0.13), processed cob without FS (2% thiamethoxam) (3.03 ± 0.20), and processed cob plus FS (2% thiamethoxam) (3.63 ± 0.20).

2000 Field Trials. In 2000 at field #1, 7 treatments produced root ratings significantly lower than the untreated control (3.86 ± 0.16) ($F=4.84$, $df=239$, $p < 0.05$) (Fig. 5A). These included Force 3G insecticide (2.70 ± 0.14), clay granules without FS (2% thiamethoxam) (2.84 ± 0.17), clay granules plus FS (2% thiamethoxam) (2.70 ± 0.17), processed cob plus FS (2% thiamethoxam) at 840 mg per m of corn row (3.26 ± 0.21), processed cob plus FS (2% thiamethoxam) at 420 mg per m of corn row (3.11 ± 0.14), clay granules without FS (2% thiamethoxam, applied at cultivation time) (3.03 ± 0.15), and clay granules plus FS (2% thiamethoxam, applied at cultivation time) (3.04 ± 0.23).

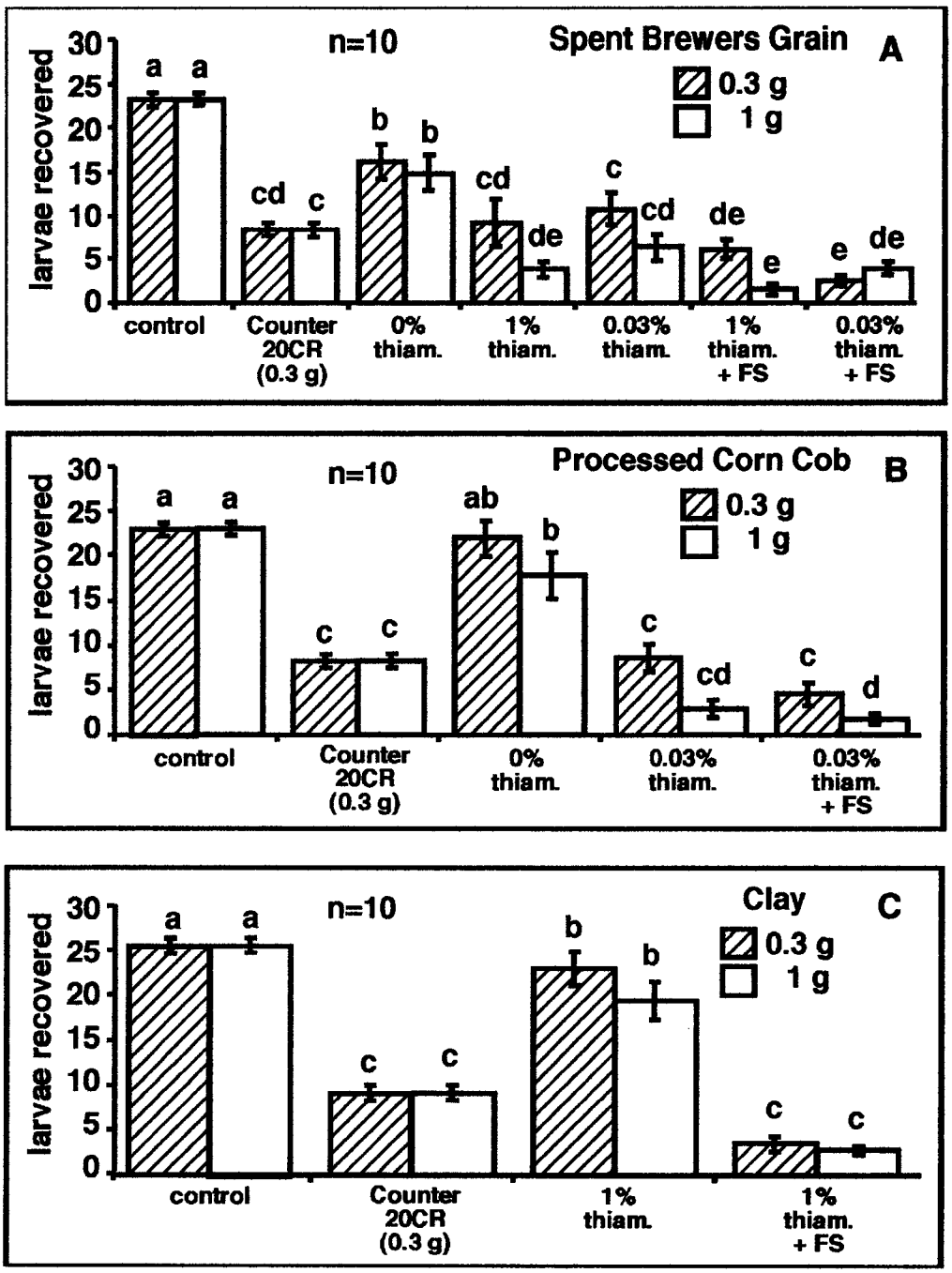

Fig. 5.2. (A) Neonate larvae recovered from corn in soil tubs containing 0.3 g (hatched bars) and 1 g (white bars) spent grain granules treated with thiamethoxam. (B) Neonate larvae recovered from corn in soil tubs containing 0.3 g (hatched bars) and 1 g (white bars) processed cob granules treated with thiamethoxam. (C) Neonate larvae recovered from corn in soil tubs containing 0.3 g (hatched bars) and 1 g (white bars) clay granules treated with thiamethoxam. Significant differences ($p < 0.05$) are indicated by different lower case letters. Comparisons are made among treatments with the same fill pattern. Bars represent standard errors.

In 2000 at field #2, 6 treatments produced root ratings significantly lower than the untreated control (4.12 ± 0.21) ($F=7.89$, $df=239$, $p < 0.05$) (Fig. 5B). These included Force 3G insecticide (2.50 ± 0.18), clay granules without FS (2% thiamethoxam) (2.28 ± 0.18), clay granules plus FS (2% thiamethoxam) (3.03 ± 0.23), processed cob with plus FS (2% thiamethoxam) at 0.2 lb per acre (2.82 ± 0.29), clay granules plus FS (2% thiamethoxam, applied at cultivation time) (2.79 ± 0.24), and processed cob plus FS (2% thiamethoxam, applied at cultivation time) (3.09 ± 0.25).

Discussion

CO_2-generating granules containing insecticide prevented larvae from locating growing corn roots in soil bioassays. The best results with insecticide-treated yeast granules were obtained using 3 g of granules containing a total of 30 mg terbufos, but yeast granules containing no insecticide provided control comparable to that of Counter 15G insecticide granules. The CO_2 concentration in soil tubs treated with 3 g yeast granules (with no insecticide) was approximately 4 mmol per mol, and this was similar to the CO_2 concentration produced by corn growing in soil (4.36 ± 0.31 mmol/mol) (Bernklau and Bjostad 1998b). In an earlier study that employed the same soil bioassay (Chapter 3), 6 grams of untreated yeast granules (mixed throughout the soil) produced CO_2 concentrations between 7 and 8 mmol/mol. Although the CO_2 concentrations were slightly higher, the 6 g granule treatment used in the previous study was not as effective (10.54 ± 1.72 larvae recovered) as the 3 g granule treatment in the current study (4.70 ± 1.17 larvae recovered). The two experiments were conducted using the same methodologies, but at different times of the year and with different soil sources. This suggests that the activity of the CO_2 formulations may be influenced by

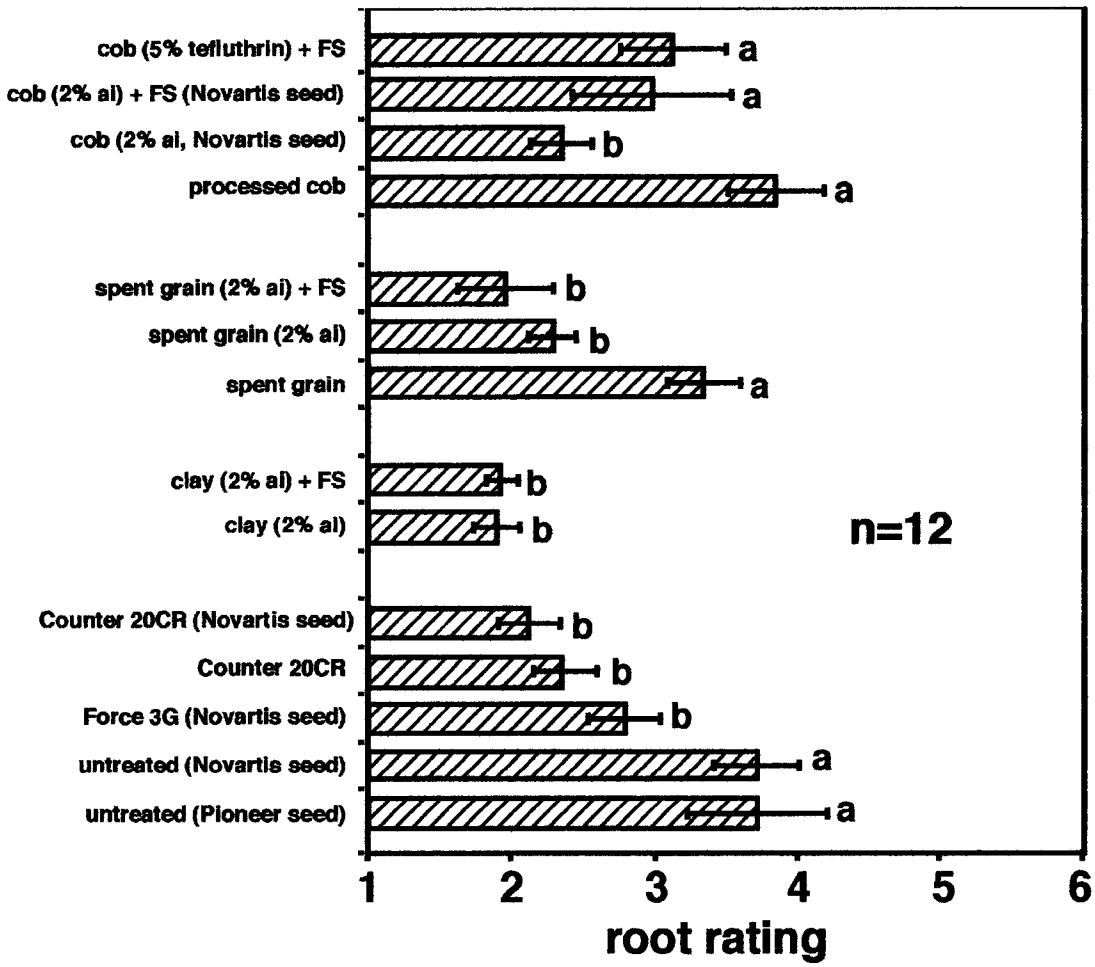

Fig. 5.3. Root ratings for 1999 field treatments at the ARDEC site. Significant differences (Dunnett's test, $p < 0.05$) between treatments and the untreated controls are indicated by different lower case letters.Bars represent standard errors.

environmental factors such as temperature and moisture and by specific characteristics of a soil.

In the current study, spent brewers grain coated with thiamethoxam and FS was an effective treatment at both rates tested. Untreated spent grain (1 g) prevented 50% of the larvae from locating the corn and the addition of feeding stimulants plus 0.03% thiamethoxam reduced larval recovery to less than 16%. Processed cob granules treated with FS and 0.03% thiamethoxam provided better control than Counter 20CR insecticide. When soil was treated with clay granules coated with FS and 1% thiamethoxam, fewer than 16% of larvae were recovered from the corn.

The spent brewers grain and processed corn cob used in the current study are organic materials that release CO_2 into the soil as they decompose. In preliminary laboratory experiments, the CO_2 concentration in soil containing processed cob (1 g per 300 g soil) was greater than 6 mmol per mol after 3 days, and spent grain produced concentrations in excess of 20 mmol/mol. For the yeast granules, CO_2 is produced by the micro-organisms (*Saccharomyces cerevisiae*) that are sustained by nutrients incorporated into the granules. The highest concentration of CO_2 measured in soil bioassays with 1 g yeast granules was 5.01 ± 0.53 mmol/mol, which is comparable to the CO_2 production of the processed cob. Clay granules in soil did not generate CO_2, except when they were coated with the feeding stimulant extract, which can be metabolized by soil microbes.

In field trials, treatments of CO_2-generating materials coated with feeding stimulants and insecticide were effective in preventing damage to corn roots. The most consistent results were obtained with clay granules containing thiamethoxam and feeding stimulants, and these granules provided control comparable to that of the Force and Counter 20CR insecticide. A rate of thiamethoxam 2-fold less than the prescribed rate provided adequate control of WCR when used in conjunction with feeding stimu-

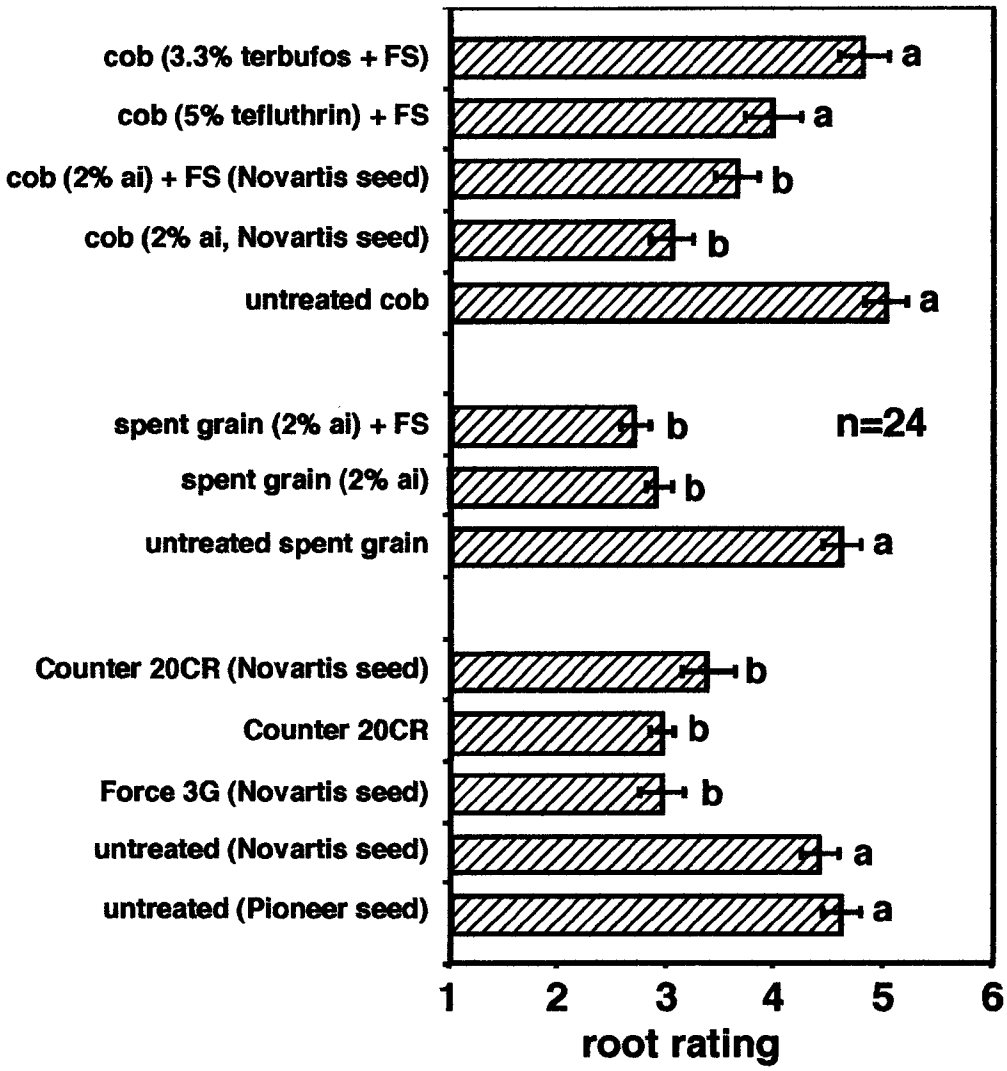

Fig. 5.4. Root ratings for 1999 field treatments at the Haxtun site. Significant differences (Dunnett's test, $p < 0.05$) between treatments and the untreated control are indicated by different lower case letters. Bars represent standard errors.

lants and CO_2. At least one formulation of each granule type (spent grain, processed cob and clay) with thiamethoxam reduced damage to corn roots by rootworm larvae. The uniform size, availability and ease of handling make cob and clay granules the best candidates for future field treatments.

We have determined from soil scouting that WCR larval hatch in eastern Colorado typically begins during the first week of June and continues until the end of July, with a peak in late June. CO_2-generating formulations applied at planting in late April to mid May must produce CO_2 for 8 to 9 weeks to provide complete control of WCR larvae. Corn growers sometimes apply soil insecticides during cultivation, rather than at planting. If CO_2-generating granules were applied at cultivation in mid-June, the formulations would still be producing sufficient amounts of CO_2 at the time of peak larval hatch.

The activity of the CO_2-generating materials may have been enhanced by the clay-loam soil texture at the test fields in Colorado. Rootworm larvae move readily through loam and silty clay soils (MacDonald and Ellis 1990) and have been shown to survive best in soils that are uncompacted and have greater pore continuity (Ellsbury et al. 1994). Sandy to silty soils allow faster diffusion of CO_2, but clay soils have greater water-holding capacity, which could extend the time of CO_2 generation by the moisture-dependant granules. In contrast, CO_2-generation might be abbreviated in soils with higher organic content where the granules would tend to decompose more quickly.

CO_2 as a soil treatment has been shown to increase the health and production of certain crops (Mauney and Hendrix 1988, Novero et al. 1991, Ariezo et al. 1993) and the effects of CO_2 on soil microflora may be partially responsible for this benefit. CO_2 production in the soil, and the subsequent acidification of the soil, improves conditions for beneficial rhizosphere bacteria (Glass and Siddiqi 1981, Bashan and

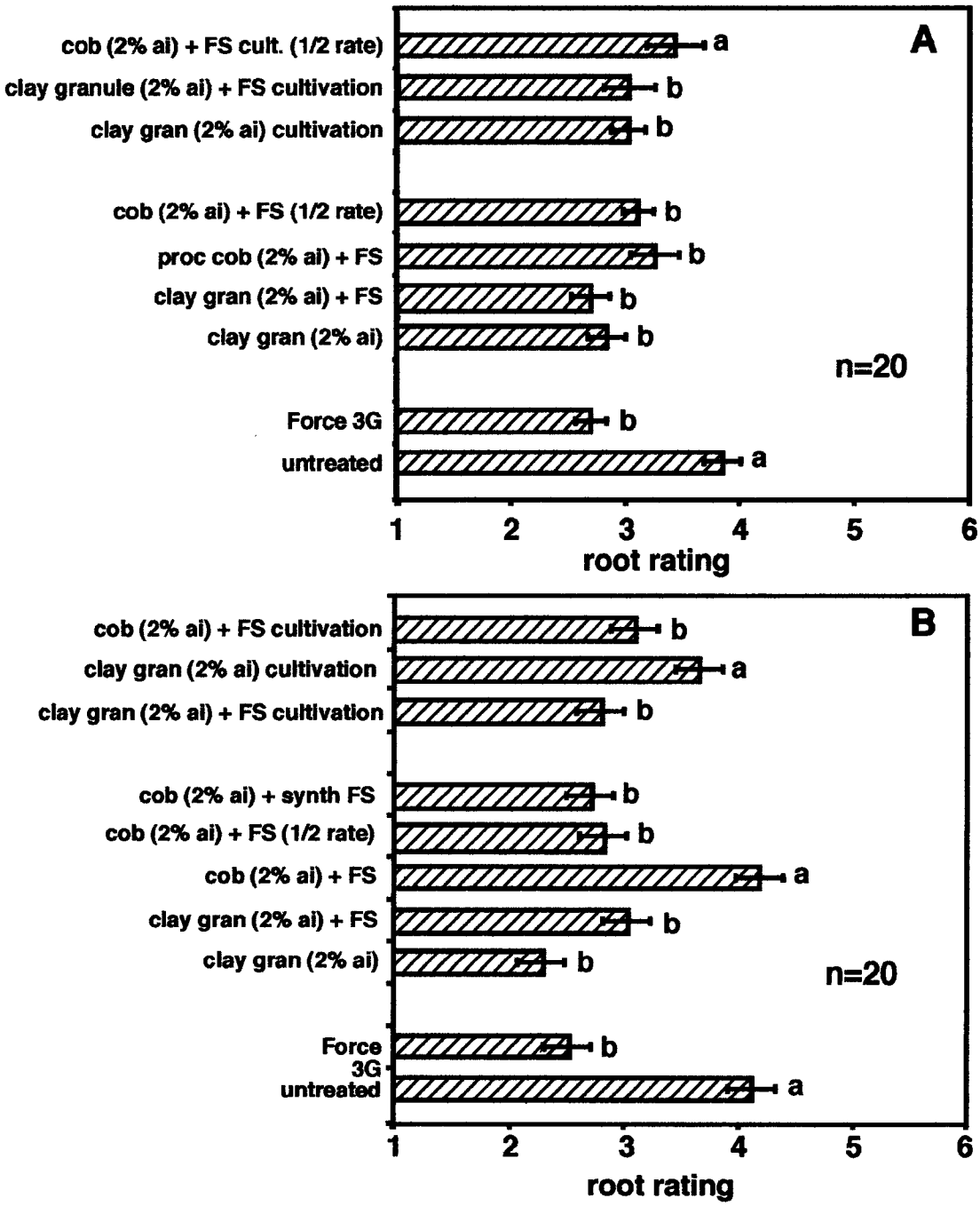

Fig. 5.5. (A) Root ratings for 2000 field treatments at field #1. (B) Root ratings for 2000 field treatments at field #2. Significant differences (Dunnett's test, $p < 0.05$) between treatments and the untreated control are indicated by different lower case letters. Bars represent standard errors.

Levanony 1989). It is unlikely that CO_2-generating treatments present in the soil for 9-12 weeks would significantly effect the microflora, because short-term fluctuations in soil CO_2 levels occur naturally as a result of seasonal changes and other normal environmental factors (Brady 1990).

The insecticide thiamethoxam used in this study is a second generation neonicotinoid insecticide developed by Syngenta Crop Protection as a replacement for the organophosphate soil insecticides that are gradually being withdrawn from the market (Schroeder and Dumbleton 2001, Steffey 2002). Thiamethoxam is sold under the trade name Cruiser® (Syngenta Crop Protection, Basel, Switzerland) as a seed treatment on corn, rape, wheat, sorghum, potatoes, and canola for the control of a variety of soil insects (Schroeder and Dumbleton 2001, Steffey 2002). In the current field tests, granules containing organophosphate insecticides (terbufos and tefluthrin) were not as effective against WCR larvae as granules containing thiomethoxam, even with the addition of feeding stimulants and CO_2. Some insecticides are repellent to rootworm larvae (Hibbard and Bjostad 1989) and the efficacy of these chemical may be reduced by larval avoidance. Terbufos has been shown to be less effective in providing plant protection against rootworm larvae at sub-lethal levels when compared to tefluthrin (Michaelides et al. 1997). In the current study, CO_2-generating granules containing tefluthrin and terbufos did not provide adequate control of WCR larvae, indicating that the attraction of larvae to CO_2 may not be strong enough to overcome the repellent effect of some organophosphate insecticides.

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CHAPTER 6

ATTRACTION OF TERMITES TO CARBON DIOXIDE

CO_2 is an attractant for a number of soil-dwelling organisms, including numerous insect larvae *(1-12)*, insect adults *(11, 13)*, mites *(11, 12)*, chilopods *(12)*, nematodes *(4, 14-19)*, and bacteria *(20)*. Although subterranean termites are among the most abundant and widely distributed soil insects, we were intrigued to find that they have apparently never been tested for attraction to CO_2. Termite nest environments normally contain concentrations of CO_2 between 0.3 and 5% *(21, 22)* and levels as high as 15% have been reported *(21)*. The concentration of CO_2 in ambient soil atmosphere outside the nest is much lower (0.04%) *(22)*. Damp wood that is used as a food source by termites emits CO_2 in concentrations as high as 15% *(23)*. We considered the possibility that termites might associate higher CO_2 levels with nest vicinity or food, and that they may follow a CO_2 gradient to locate the source.

We first investigated the attraction of subterranean termites to CO_2 using a behavioral bioassay in the laboratory. The experiments were conducted with field-collected termite workers *(24)* using a modified glass T-tube apparatus that allowed termites to choose between ambient air and a defined concentration of CO_2. We prepared various concentrations of CO_2 in 35-ml polyethylene syringes and mounted the syringes on a syringe pump for delivery into the bioassay apparatus (1 ml/min on each side), and we used GC-MS-SIM (m/z 44) to measure the CO_2 concentrations *(23)*. We tested CO_2 concentrations of 1, 2, 5, 10, 20, 50 and 500 mmol CO_2 per mol air against ambient air, which had a CO_2 concentration of 1 mmol/mol. Three subterranean termite species, *Reticulitermes flavipes* (Kollar), *R. virginicus* Banks and *R. tibialis* Banks, were attracted to a range of CO_2 concentrations between 5 and 50 mmol/mol (Fig. 6.1).

We next tested a variety of CO_2-generating materials in a behavioral choice test in soil-filled containers in the laboratory. For each bioassay, 200 termite workers were introduced into a center chamber from which they had access to two foraging

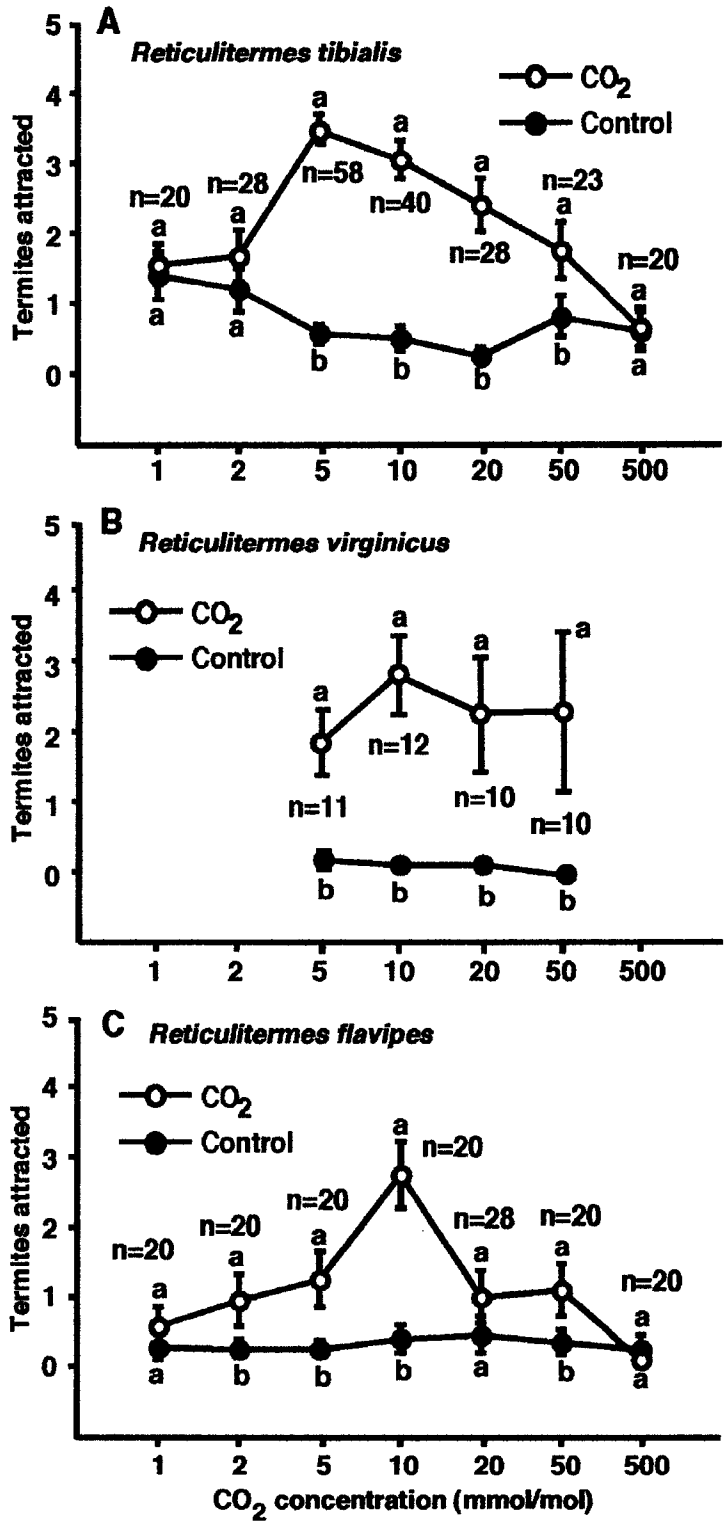

Fig. 6.1. Dose response of termite workers to CO_2 vs. ambient air control. Number of termites attracted to CO_2 for (A) *Reticulitermes tibialis*, (B) *Reticulitermes virginicus*, and (C) *Reticulitermes flavipes*. For all graphs, open circles represent the CO_2 and closed circles represent the ambient air control. Significant differences ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

chambers. Both chambers contained a food source (wafer of yellow pine wood) and one chamber also contained a CO_2-generating material. We selected materials that produced CO_2 in excess of 8 mmol/mol after 24 hours when placed in soil containing 15% moisture by weight, as determined by analysis of soil atmosphere samples with GC-MS-SIM. We attempted to choose non-exotic materials that could be used inexpensively and on a large scale. The test materials included processed corn cob granules, dried spent brewers grain (a waste material from the brewing of beer), whole malted barley, baking powder and effervescent tablets of sodium bicarbonate and citric acid *(25)*.

Significantly more termites ($p < 0.05$) foraged in the CO_2-generating chamber than in the control chamber after 14 days for the baking powder, effervescent tablet, processed corn cob and activated charcoal treatments (Fig. 6.2A). The termites consumed more wood in the CO_2-baited chamber than in the untreated chamber for 3 treatments: effervescent tablets (140 mg vs. 40 mg), activated charcoal (190 mg vs. 50 mg) and malted barley (210 mg vs. 150 mg). The spent brewers grain treatment produced the highest and most consistent levels of CO_2, sustaining a CO_2 concentration of more than 8 mmol/mol for 14 days (Fig. 6.2B).

We evaluated three of the CO_2 baits in field tests in eastern Colorado. Our experimental sites were cattle ranches bordered with old wooden fenceposts, many of which were infested with termites (*R. tibialis*). The CO_2-generating baits were mixed into moist soil and we used the treated soil to fill termite bait stations constructed of polyethylene jars (500 ml) that were perforated with 3 mm holes to allow termites to enter and leave freely. A wafer of yellow pine wood was placed on top of the soil in each station as a food source, and the stations were placed in pairs near infested fenceposts. Two stations were buried 1 meter away from the post and 1.5 meters apart, with both stations on the same side of the fenceline *(24)*. For each test, one of

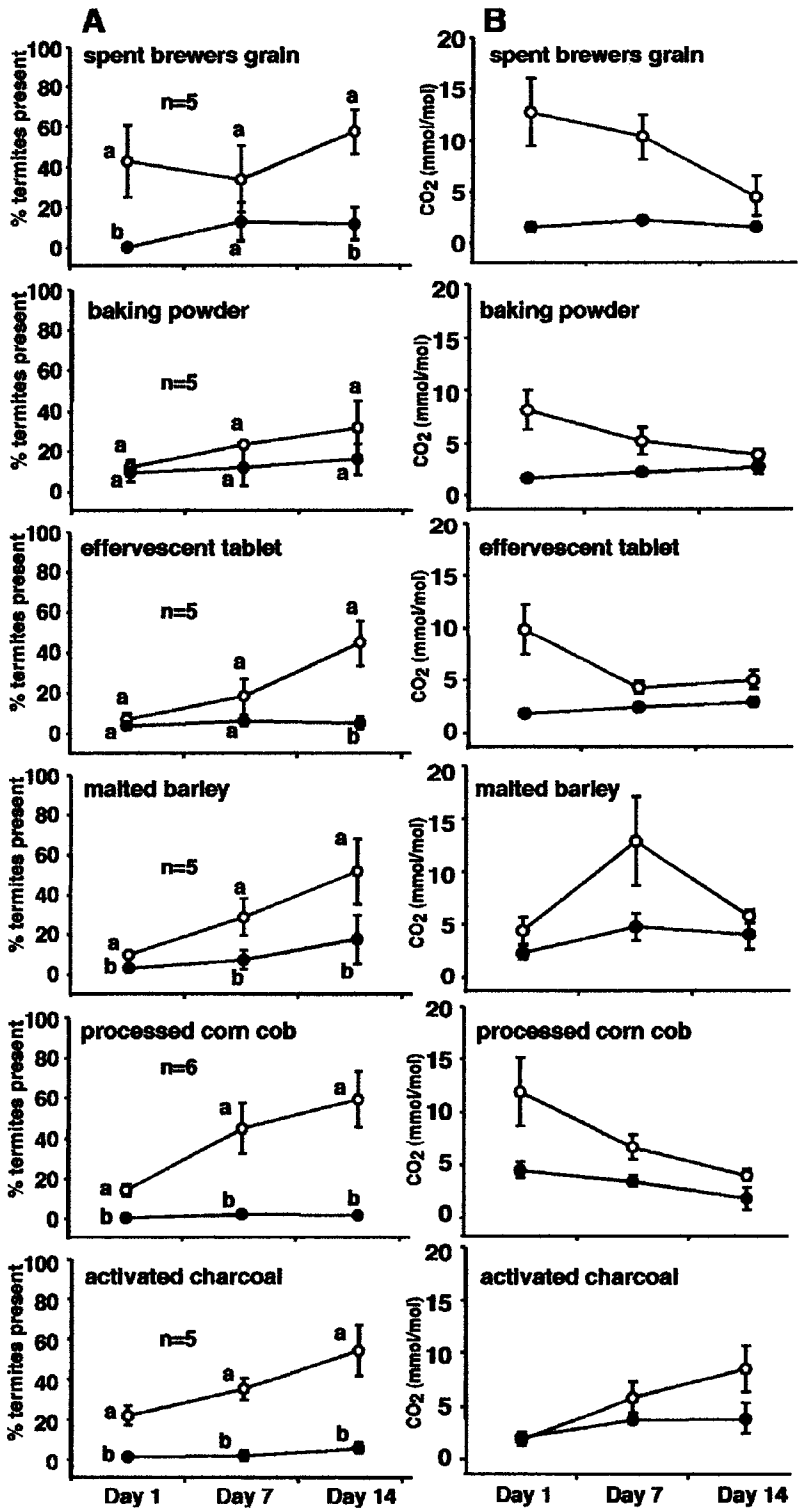

Fig. 6.2. Behavioral bioassays with termites in soil. (A) Percent termites foraging in sources of CO_2-generating baits vs. controls. (B) CO_2 concentrations in sources of CO_2-generating baits and in controls as measured by GC-MS-SIM. For all graphs, open circles represent the source containing the CO_2 bait and closed circles represent the controls. Significant differences in termite numbers ($p < 0.05$) are indicated by different lower case letters. Bars represent standard errors (some standard error bars are too small to be seen).

the two stations contained a CO_2-generating bait and the other contained only moist soil. For controls, two stations were placed in the same manner, but neither station contained a CO_2-generating bait. The stations were monitored weekly over a 6 to 8-week period for the presence of foraging termites and evidence of termite feeding. The number of stations located by foraging termites steadily increased throughout the season for all of the CO_2-generating baits. In contrast, very little activity occurred in the control stations (Fig. 6.3). The CO_2 baits also reduced the amount of time required for termites to locate the point source. Termites discovered only one of the control stations, and did so after 5 weeks (Fig. 6.3).

Baiting strategies for termite control have recently gained popularity due to the withdrawal of chlordane, chlorpyrifos and other termiticides from the market. The development of insect growth regulators and slow-acting toxicants, and the subsequent incorporation of these materials into termite baits, has led to the introduction of commercial products such as the Sentricon Colony Elimination System® (Dow Agrosciences), Outpost™ (Bayer), Exterra™ (Ensystex), Firstline® (FMC), Terminate™ (Spectracide) and Subterfuge® (BASF). Researchers have mixed opinions about the success of baiting techniques and current efforts are focused on improving specific aspects of these systems, including the addition of attractants and/or bait enhancers *(25)*. In a baiting system, the active ingredient is typically introduced into a station only after termites are detected in that station, and, depending on the species, weeks may pass before termites locate a station and begin to forage. In our field tests, *R. tibialis* workers were always found in CO_2-baited stations in the first week, and more than 50% of the total stations in which termites foraged during the season contained feeding termites by the third week. These results indicate that CO_2 can be used as an attractant to quickly bring termites directly to a point source. An attractant

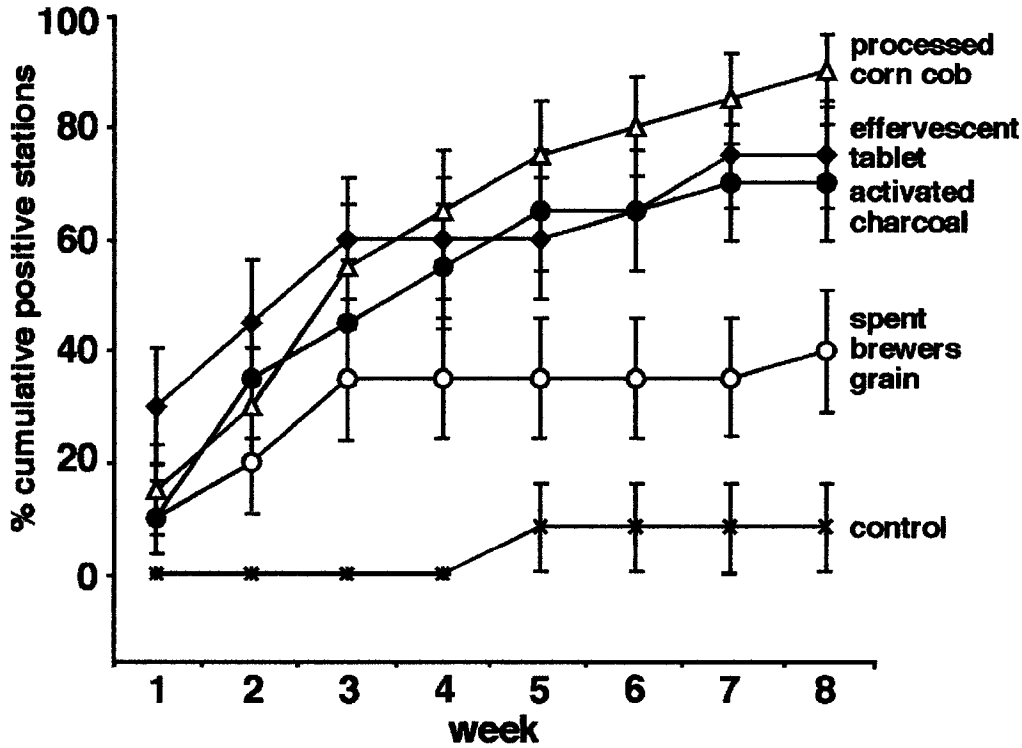

Fig. 6.3. Termite foraging in stations containing CO_2 baits placed near termite-infested fenceposts on cattle ranches in 2000. Graph shows the cumulative number of stations visited at least once by termites up to the time shown. Bars represent standard errors for binomial distribution (spent brewers grain, n=20: processed corn con, n=20; activated charcoal, n=20; effervescent tablet, n=20; control, n=12).

such as CO_2 has the potential to reduce the time interval between station placement and introduction of the active ingredient and thereby improve the effectiveness of the baiting system.

The bioassay response of subterranean termites to CO_2 is consistent with the known attraction for other soil-dwelling arthropods. CO_2 is a small molecule that can readily diffuse through micropores in the soil, creating a concentration gradient for soil organisms to follow. Termites may use CO_2 to orient towards a source of decaying wood or to locate the nest when returning from foraging. CO_2 concentrations measured inside termite nests (0.3 to 5%) *(21)* are consistent with the attractive range demonstrated in our behavioral bioassays.

The economic impact of termites in the United States each year may exceed $11 billion *(26)* and the majority of damage to homes and other structures is caused by subterranean termites, the group that includes *Reticulitermes. flavipes, R. virginicus* and *R. tibialis (27).* Environmental concerns have prompted the withdrawal of foam insulation products that are manufactured using chlorofluorocarbons (CFCs), methylene chloride, or 1.1.1 trichloroethane. Replacements for the CFC products include polyurethane foams that are manufactured using liquid carbon dioxide as an expanding agent *(28).* Foam products manufactured with this process will contain carbon dioxide gas that may be released from the foam insulation and into the soil over time. Our research suggests that structures constructed with CO_2-containing foams may be more susceptible to termite attack.

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APPENDIX A

SUPPLEMENTAL MATERIAL FOR

CHAPTER 6

Methods and Materials

Insects. Experimental termites were collected in the field using wood frame traps consisting of a square wood frame (6 by 6 in), with several pieces of double-corrugated cardboard cut to fit the center of the frame and held in place by a 0.25 in wire mesh. Traps were completely buried in a hole (30 cm deep) dug at the base of a wooden fencepost. The traps were checked after two weeks and termites found in the traps were placed in covered plastic tubs. *Reticulitermes flavipes* were collected from Michigan, *Reticulitermes tibialis* were obtained from rangeland sites in eastern Colorado and *Reticulitermes virginicus* were obtained from northern Virginia. Termites were kept in sealed plastic tubs containing soil and moist cardboard.

CO_2 Dose Response Bioassays. The choice-test bioassay apparatus was constructed from a glass T-tube (5 mm inside diameter, 5 mm stem, with each branch 4.5 cm long). Each branch of the 'T' was bent downward (2.5 cm from the junction of the 'T') at a 45° angle to form a pitfall trap. A 5 mm NMR cap (cat. no. 100-0050, Drummond Scientific, Broomall, Pennsylvania) with a 1 mm pinhole in it was firmly pushed over the end of each bent branch and the cap was connected with a 25 cm length of Teflon tubing (0.8 mm inside diameter) to a 35 ml polyethylene syringe (cat. no. 106-0490, Sherwood Medical, St. Louis, Missouri). The two 35-ml syringes were clamped onto to a syringe pump (Sage Model 355, Fisher Scientific, Pittsburgh, Pennsylvania) adjusted to provide an airflow of 1 ml per min into each choice arm of the bioassay apparatus. Prior to bioassays, 5 termite workers were placed into a small plastic holding container and held for 20 minutes. The syringe pump was turned on, and after 3 minutes of pumping, the holding container was gently connected to the end of the central arm of the T-tube, allowing termites to crawl out and enter the apparatus. Bioassays were conducted for 15 min, after which the number of termites in each

pitfall was recorded. After each test, all glass parts of the apparatus were washed with soap and water, rinsed with water and heated at 80°C in an oven for 30 min.

CO_2 concentrations were measured using a Hewlett-Packard Series II 5890 gas chromatograph interfaced with a Hewlett-Packard 5971 mass selective detector was operated in selected ion monitoring mode (SIM) for m/e 44 with a methyl silicone capillary column (30 m long,x 0.32 mm inside diameter, RSL-150, Alltech, Deerfield, Illinois). A 10-mmol/mol mixture of CO_2 (a 300-ml glass bottle into which 3 ml of CO_2 was injected) was used as a standard to calculate the CO_2 concentrations of the unknown samples. To prepare treatments, a 35-ml syringe was rinsed with distilled water and partially filled (5 ml) with ambient air. An amount of 100% CO_2 was obtained with a smaller glass syringe from a tank and injected into the 35-ml syringe. Ambient air was then drawn into the 35-ml syringe to fill it and mix the gases by turbulence as the syringe was loaded. A 2nd 35-ml polyethylene syringe was filled with ambient air for a control. Bioassays were conducted with *R. tibialis* and *R. flavipes* for 1, 2, 5, 10, 50 and 500 mmol per mol concentrations of CO_2 and with *R. virginicus* for 2, 5, 10 and 50 mmol per mol concentrations of CO_2.

Statistical analysis of choice-test data were T-tests conducted to compare the number of termites attracted to the CO_2 vs. air for each concentration of CO_2 using Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts) with $\alpha = 0.05$.

Soil Bioassays. For choice-test bioassays in soil, the bioassay apparatus was constructed from 3 4-ounce polyethylene jars with screw cap lids. The jars were connected with 0.5 cm diameter Tygon tubing inserted into holes drilled midway between the top and bottom and each jar was filled 2/3 with clean soil mixed to contain 15% moisture. The middle jar (harborage) and one of the outer foraging jars con-

tained no treatment. The second foraging jar contained a CO_2-generating formulation. A wafer of yellow pine wood (6 cm x 6 cm x 0.5 cm thick) was placed on top of the soil in each of the foraging chambers. A total of 200 termite workers (*R. tibialis*, with a few soldiers) were introduced into the middle jar on day 0. On days 1, 7 and 14 the number of termites in the treatment and control foraging chambers were recorded. At the end of the bioassay, the wood was removed, oven dried (40 degrees C for 2 days) and weighed. The CO_2-generating materials included: 2 g dried spent grain (obtained from a local brewery), 2 g processed corn cob (2 mm diameter, Mt. Pulaski Co., Mt Pulaski, Illinois), 2 g malted barley (Beer Beer & More Beer, Concord, California), and effervescing tablets (2 g Fizzi brand tablets, Premiere Innovations, Pacific Palisades, California). CO_2 concentrations were measured in the treated soil at day 1, day 7 and day 14 using GS-MS-SIM. Day 1 was counted as 24 hours after introduction of the termites. A jig to measure CO_2 was constructed from a piece of glass tubing (5 cm long x 1 mm ID) inserted through a hole drilled into the side of the jar (halfway up). The needle of a 10-µl Hamilton syringe was inserted into the glass tube and a 5-µl sample of soil headspace was removed for analysis. Statistical analysis of soil bioassay data were T-tests conducted to compare the number of termites foraging in the CO_2 chamber vs. the untreated chamber for each treatment using Minitab (Addison-Wesley Publishing Co. Inc., Reading, Massachusetts) with $p=0.05$. Figure S1 shows the amount of wood (grams) consumed by termites during the 2-week bioassay.

Field Tests. Field tests were conducted in 1999 and 2000 at 4 sites: the Central Plains Experimental Range located north of Nunn, Colorado, ranch properties north of Fort Collins, Colorado and another one east of Wellington, Colorado and a ranch property in the foothills near Bellvue, Colorado. These sites all contain long lines of old fenceposts, many of which are infested with termites (*R. tibialis*). Stations

were 8-ounce polyethylene jars (with screwcap lids) with 36 holes (3 mm) drilled around the circumference and filled to within 3 cm of the top with soil containing 15% moisture (300 g soil). Treatments were mixed into the soil prior to filling the jars. The jars were placed as pairs with one control (unbaited) and one CO_2-baited jar buried (in holes 30 cm deep) 1 meter away from an infested fencepost and 1 meter apart with both stations on the same side of the fenceline. For controls, 2 stations (both unbaited) were placed in the same manner.

NOTE: Fig. 6.3. shows the percent cumulative stations visited at least once by termites up to that week of the study for each treatment. Both baited and unbaited stations wearere included in the total number. The objective of the study was to determine if CO_2 attracts termites to an area and if it results in increased foraging activity within that area. To make this determination, only one station at each fencepost required baited. For Figure 3, the error bars represent the standard errors for binomial distribution.

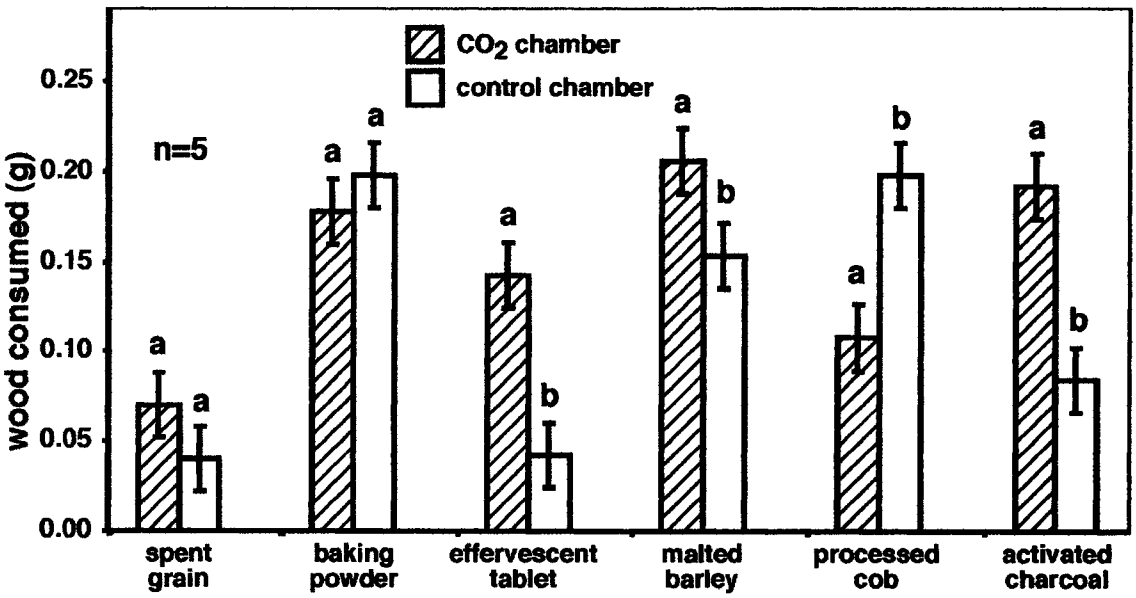

Fig. S1. Wood (grams) consumed by termites after 2 weeks in treated (CO_2-generating material) and control (untreated) soil jars.

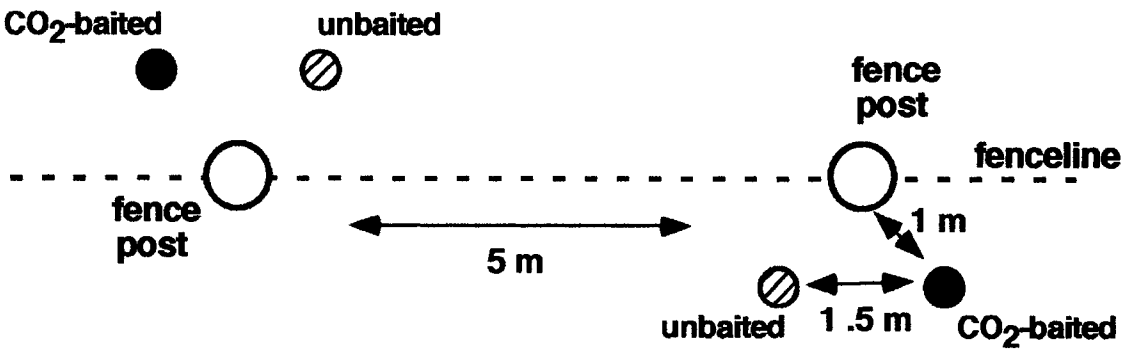

Fig. S2. Placement of bait stations around fenceposts.