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# ABSTRACT

The role of the maize secondary metabolites, hydroxamic acids, in the interactions of maize, *Zea mays* L., and western corn rootworm, *Diabrotica virgifera virgifera* LeConte, was investigated under greenhouse and laboratory conditions.

An accurate high-performance liquid chromatography (HPLC) method was developed for the separation and quantification of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones in maize root extracts. Four compounds, found in maize roots, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-(4H)-one(DIMBOA),2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3(4H)-one ($DIM_2BOA$), 2-hydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one (HMBOA), and 6-methoxybenzoxazolinone(MBOA), were separated and identified within 30 min on a C18 reversed-phase column using a gradient of methanol and phosphoric acid. This approach represents the first gradient HPLC method to separate a variety of hydroxamic acids and derivatives in maize root extracts.

The concentrations of hydroxamic acids and related compounds, DIMBOA, $DIM_2BOA$, HMBOA, and MBOA, in roots of 1-5 week old maize plants were determined by HPLC. The highest concentrations of $DIM_2BOA$, HMBOA and total related compounds were found in maize root extracts when maize roots were 2 weeks old and the maize plant was approximately 15 cm in height. The highest concentrations of DIMBOA equivalents were found in 4 week old maize root extracts, which overlapped with the time of development of western corn rootworm larvae. The distribution of individual compounds in different tissues (cortex, stele and complete organ) of various root parts (first set of nodal roots, secondary roots, primary root, mesocotyl and adventitious roots from

mesocotyl) was also determined. Hydroxamic acids and related compounds are concentrated in the cortex of maize roots, which is the feeding site of western corn rootworm larvae. The total concentration of related compounds and the concentration of each individual compound except HMBOA in the complete organ of nodal roots was significantly higher than in any other part of maize roots. The high concentrations of these substances in the cortex of maize root may be relevant to the resistance of maize varieties to subterranean pest insects.

Thirty three inbred maize lines from different latitudinal geographical regions were surveyed for concentrations of hydroxamic acids. All were found to contain four major hydroxamates: DIMBOA, $DIM_2BOA$, HMBOA, and MBOA. There was a trend showing that differences in concentrations of hydroxamic acids characterized the different geographical groups, and that the intermediate temperate region (ITR) group had higher levels of all individual compounds, especially DIMBOA equivalents and the total amount of hydroxamic acids. In addition, several maize lines commonly used in corn rootworm resistance studies were analyzed for their hydroxamic acid levels. A positive correlation between concentration of DIMBOA in maize roots and in maize leaves was found.

DIMBOA, the major hydroxamic acid present in maize roots, and MBOA, the degradation product of DIMBOA, were studied for their toxic and deterrent effects on western corn rootworm larvae. Exogenously applied DIMBOA and MBOA separately caused mortality in western corn rootworm larvae feeding on fresh corn roots. The $LC_{50}$ (lethal concentration for 50% mortality) value of DIMBOA was 153 ppm (fiducial limits 108-209) and the $LC_{90}$ value was 917 ppm (560-2297). The $LC_{50}$ value of MBOA was 718 ppm (529-1033) and the $LC_{90}$ value was 2457 ppm (1524-7139). The deleterious effects of DIMBOA and MBOA on western corn rootworm larvae are possibly due to both

feeding deterrence and toxicity. In a replicated pot trial during two growing seasons, two maize lines developed by Agriculture Canada from International Centre for Maize and Wheat Improvement (CIMMYT) collections, ITR 3872 with a high DIMBOA content in roots, and NTR-2 Ger. 4042 with a low DIMBOA content in roots, were evaluated for resistance to western corn rootworm larvae. The results indicated that the high DIMBOA line (but not the low DIMBOA line) stressed western corn rootworm larvae to produce inferior adults based on the measurement of adult emergence number, adult weight, and adult head-capsule width. The effect of western corn rootworm on both corn lines with different DIMBOA levels was measured based on plant growth parameters including plant height, stem thickness, plant fresh weight, root fresh weight, plant dry weight, and root dry weight. ITR 3872 (high DIMBOA) showed significantly less damage than NTR-2 Ger. 4042 (low DIMBOA) in almost all plant parameters measured. Under controlled environment conditions, seven maize lines with various hydroxamic acid levels were grown with infestations by western corn rootworm larvae. The results revealed that all larval development parameters measured were significantly correlated to the hydroxamic acid levels in maize roots. The results suggest that hydroxamic acids may in some instances contribute to the resistance of maize to western corn rootworm larvae.

The behavioral responses of western corn rootworm larvae to naturally occurring and synthetic hydroxamic acids were investigated. When maize roots were treated with different hydroxamic acids which act as feeding deterrents in other insects, neonate larvae of western corn rootworm responded by significantly reducing the number of turns, while area searched and locomotory rate significantly increased. These responses were dependent on the concentrations of the related compounds and clearly suggested the behaviour modifying effect of these substances.

# RESUME

Nous avons étudié dans des conditions de serre et de laboratoire le rôle des métabolites secondaires du maïs, les acides hydroxamiques, sur les interactions entre le maïs *Zea mays* L. et la chrysomèle du maïs *Diabrotica virgifera virgifera* LeConte.

Nous avons mis au point une méthode précise de chromatographie en phase liquide à haute performance (HPLC) pour séparer et quantifier, dans des extraits de racines de maïs, les 1,4-benzoxazines-3-ones et les benzoxazolines-2-ones. Quatre composés trouvés dans les racines de maïs, le 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-(4H)-one (DIMBOA), 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3(4H)-one ($DIM_2BOA$), 2-hydropxy-7-methoxy-1,4-benzoxazin-3(4H)-one(HMBOA), et le 6-methoxybenzoxazolinone (MBOA), ont pu être séparés et identifiés en utilisant une colonne C-18 à phase renversée avec un gradient de méthanol et d'acide phosphorique. Ceci représente la première méthode HPLC à gradient proposée pour séparer divers acides hydroxamiques et leurs dérivés dans les extraits de racine de maïs.

En utilisant le HPLC sur les racines de maïs agées de 1 à 5 semaines, nous avons déterminé les concentrations des acides hydroxamiques et de leurs dérivés DIMBOA, $DIM_2BOA$, HMBOA et MBOA. Les concentrations les plus élevées de $DIM_2BOA$, HMBOA et de l'ensemble de leurs dérivés se trouvent dans les extraits de racines de plants de 2 semaines d'approximativement 15 cm de hauteur. Les concentrations les plus élevées d'équivalents DIMBOA se trouvent dans les extraits de racines de 4 semaines, période qui chevauche celle du développement des larves de chrysomèles. La distribution relative de

ces composés a également été étudiée dans différents tissus (cortex, stèle, toute la racine) de différentes parties de la racine (première racine nodulaire, racines secondaires, racine primaire, mésocotyle et racines adventives du mésocotyle). Les acides hydroxamiques et leurs dérivés sont concentrés dans le cortex des racines de maïs, qui est le lieu où les larves de chrysomèles se nourissent. Dans l'ensemble du nodule radiculaire, la concentration totale des dérivés et de chacun des composés, excepté HMBOA, s'est avérée plus haute que dans toutes les autres parties de la racine. Il est possible que la forte concentration de ces substances dans le cortex de la racine du maïs soit importante dans la résistance du maïs aux insectes nuisibles souterrains.

Nous avons examiné les concentrations en acides hydroxamiques de 33 lignées de maïs croisées, provenant de latitudes différentes. Toutes contiennent les 4 principaux hydroxamates: DIMBOA, $DIM_2BOA$, HMBOA et MBOA. Les différentes concentrations de ces acides hydroxamiques apparaissent faiblement caractéristiques des groupes géographiques, et le groupe ITR contient le plus fort taux de chacun des composés, particulièrement d'équivalents DIMBOA, et d'acides hydroxamiques dans leur ensemble. De plus, nous avons étudié les niveaux d'acides hydroxamiques de plusieurs lignées de maïs utilisées fréquemment dans les études de résistance contre la chrysomèle. Nous avons trouvé une corrélation entre la concentration de DIMBOA dans les racines et les feuilles de maïs.

Les effets toxiques et de déterrence, sur la chrysomèle, du DIMBOA, l'acide hydroxamique principal de la racine du maïs, et du MBOA, le produit de dégradation du DIMBOA, ont été étudiés. En application externe, le DIMBOA et le MBOA séparément provoquent la mort des larves de chrysomèles nourries sur des racines fraîches de maïs.

Avec le DIMBOA la valeur du $LC_{50}$ (concentration léthale pour 50% de la population) est de 153 ppm (limite de fiducie 108-209), celle du $LC_{90}$ de 917 ppm (560-2297). Avec le MBOA le $LC_{50}$ est de 718 ppm (529-1033), et le $LC_{90}$ de 2457 ppm (1524-7139). L'effect nocif du DIMBOA et du MBOA sur les larves de chrysomèles est probablement dû à un double effet d'anti-appétance et de toxicité.

Nous avons examiné, sur des larves de chrysomèles, la résistance de 2 cultivars mis au point par Agriculture Canada à partir de collections provenant du CIMMYT, ITR 3872, à fort contenu de DIMBOA dans les racines, et NTR-2 Ger. 4042, à faible contenu de DIMBOA dans les racines. Le stress sur les larves provoqué par la lignée de maïs à fort contenu de DIMBOA (et non par la lignée à faible contenu de DIMBOA) engendre des adultes inférieurs si l'on se base sur le nombre d'émergences, le poids et la largeur de la capsule cervicale des adultes.

Nous avons ausi examiné l'effet de la chrysomèle sur ces 2 lignées de maïs en mesurant les paramètres de croissance tels que la hauteur de la plante, l'épaisseur de la tige, le poids de la plante fraîche et séchée, le poids des racines fraîches et séchées. Pour presque tous les paramètres mesurés, la différence est significative entre les moindres dégats subis par ITR (fort en DIMBOA), et ceux plus importants subis par NTR (faible en DIMBOA). L'infestation par les larves de chrysomèles a été ensuite faite en environnement contrôlé sur 7 lignées de maïs à niveaux variables d'acides hydroxamiques. Les résultats montrent une corrélation significative entre tous les paramètres de développement larvaire et les niveaux d'acides hydroxamiques dans les racines du maïs. Ceci suggère la posibilité d'une contribution des acides hydroxamiques à la résistance du

maïs aux larves de chrysomèles.

Nous avons enfin étudié le comportement des larves de chrysomèles face à des acides hydroxamiques naturels et synthétiques. Lorsque les racines du maïs sont traitées avec différents acides hydroxamiques qui agissent comme déterrents chez d'autres insectes, la réponse des larves nouveau-nées est une réduction significative du nombre de tours, et une augmentation significative de l'aire de recherche et de la locomotion. L'intensité de ces réponses varie avec la concentration de la substance étudiée, ce qui démontre clairement le rôle modificateur de ces dernières sur le comportement de l'insecte.

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# TABLE OF CONTENTS

# FIGURES

# TABLES

# CHAPTER I.

# GENERAL INTRODUCTION

## 1.1. The Importance of Plant Resistance to Insects in Integrated Pest Management (IPM) System

Since the 1960's, integrated pest management (IPM) has become the predominant philosophy for pest insect control. The basic strategy of IPM is to utilize all suitable techniques and methods in as compatible a manner as possible and prevent insect populations from attaining their economic injury level, while avoiding unfavourable ecological, economic, and sociological consequences. Plant resistance to insects is one of the most important tactics of integrated pest management.

Humans have manipulated plant phenology to avoid pest insect damage to their crops since the dawn of history. The characteristics of insect-tolerant Chinese millet cultivars were first recognized by Chinese farmers over 1400 years ago, when 13 of 86 millet cultivars were found to be tolerant to pest insect attack (Jia 528). The study of plant resistance to insects may date back to the earliest days of applied entomology. About 200 years ago, it was reported that a wheat variety "Underhill" was resistant to the Hessian fly (Havens 1792). This report is generally believed to be one of the earliest examples of documentation of plant resistance to insects. Another classic example of plant resistance to pest insects and pathogens was reported in 1890. At that time, the French wine industry was seriously damaged by grape phylloxera, a root aphid, *Daktalosphaira*

*vitifoliae* (Fitch), and faced possible destruction. It was protected by the successful grafting of European grape vines onto resistant rootstock from North America.

The theory, study and use of plant resistance to insects has greatly matured since the publication of a classical text on the subject "*Insect Resistance in Crop Plants*" written by R.H. Painter (1951). In his treatment of the subject, Painter (1951) defined plant resistance to insects as the relative amount of heritable qualities possessed by the plant which influence the ultimate degree of damage done by the insect. This broad concept reflects the complexity of the phenomenon and involves three points of view: (1) plant resistance is heritable and controlled by genetic factors; (2) plant resistance is relative and can be measured only by comparison with other genotypes of the same species; and (3) plant resistance is variable, and can be modified by physical, chemical and biological factors.

As well, Painter (1951) proposed three general mechanisms to account for plant resistance to insects: (1) non-preference [term "antixenosis" proposed by Kogan and Ortman (1978) to replace non-preference; intended to parallel antibiosis], which is the insect response to plants that are unattractive or unsuitable to serve as hosts, resulting from negative reactions to searching for food, oviposition sites, or shelter; (2) antibiosis, which adversely affects insect biology, causing mortality, reduced growth, reproduction and survival, prolonged periods of development in immature stages; and (3) tolerance, which enables a host plant to withstand infestation and/or recover from damage caused by an insect population that would severely damage susceptible plants of the same species. These three mechanisms have been accepted and used as a framework by most researchers in this

field.

For many cases of identifiable plant resistance to insects, the mechanisms of resistance are unknown or poorly understood. However, several kinds of mechanism have been recognized. Mechanisms of plant resistance to insects are generally considered under two major groupings: (1) morphological and (2) biochemical bases (Norris and Kogan 1980). Morphological resistance factors, such as colour, shape, hairiness, waxiness, solidness, and trichomes, can physically interfere with insect host-selection, feeding, ingestion, absorption, mating, and oviposition behaviour. Biochemical resistance factors mainly refer to plant secondary metabolites, which are believed to be a major component of the plant's total defense armament against insects. They contribute to antibiosis by adversely affecting insect biology.

Plant resistance to insects has been discovered in many crops in the world. With the advent of integrated pest management, the use of insect resistant plant varieties, in combination with other control tactics, to reduce crop loss caused by insects is perhaps the most effective, convenient, economical and environmentally accepted method of pest insect control (Adkisson and Dyck, 1980).

Plant resistance to insects in some cases can be used as a principle method of control. In fact, a few pest insects have been controlled for many years by use of resistant varieties alone. Insects for which this has been true most often have been those with a high host specificity, such as Hessian fly (*Mayetiola destructor*) (Havens 1792; Painter 1958; Maxwell 1972), grape phylloxera (*Daktalosphaira vitifoliae*) (Painter 1951; Martin 1973), spotted alfalfa aphid (*Therioaphis maculata*) (Howe and Smith 1957).

However, high levels of plant resistance are present in only a few crop varieties. In most cases, the resistance is moderate or low. Even those with low and/or moderate levels of resistance, can offer a number of advantages when they are integrated with other control methods, such as cultural, chemical, and biological control.

Variations in cultural practices, such as adjusting planting dates and crop stubble burning, were first used by Chinese rice farmers to reduce pest insect population over 2000 years ago (Flint and vandenBosch 1981). There are a few documented examples to show the integration of plant resistance to insects with cultural control practices. The incorporation of plant resistance to insects into early maturing varieties would avoid the peak pest insect population and could greatly reduce pest insect damage (e.g. rice and brown planthopper, Heinrichs et al. 1986; cotton and pink bollworm, Walker and Niles 1971).

There are also positive interactions between plant resistance and chemical control methods. The major advantage of using plant resistance integrated with insecticides is to reduce the numbers of pest insects in each generation and slow the pest population growth. This can result in reductions of the concentration and amount of the insecticides needed and increase the efficiency of insecticides applied (Adkisson and Dyck 1980).

Generally, resistant varieties are compatible with biological control and the activities of insect parasites and predators are not adversely affected by plant resistance (Bergman and Tingey 1979). Resistant varieties may reduce the populations of pests, improve the pest/natural enemies ratio, and enable the natural enemies to be more effective (Starks et al. 1972; Kartohardjono and Heinrichs 1984). However, several recent studies have

documented the adverse effects of plant secondary compounds involved in plant resistance to insects (antibiosis) on beneficial insects (Boethel and Eikenbary 1986).

Clearly, plant resistance to insects, either as a principle method or as integrated with other control practices, can offer a great number of advantages, including specificity to one or several pests, cumulative effectiveness, persistence for a long period time, economic (no extra cost to farmer) and compatibility with other control methods.

## 1.2. Literature Review

### 1.2.1. Maize: The third major cereal crop in the world

#### 1.2.1.1. Crop value

Maize (*Zea mays* L.) is the third largest cereal crop after wheat and rice and generates 8.9% of the total world food production (FAO 1974). Maize is the most widely distributed cereal throughout temperature, subtropical and tropical zones. More than 110 million hectares of land in 134 countries were planted to produce about 300 million metric tons of maize grain in 1975 (Ortega et al. 1980). Maize is the major food for more than 100 million people in the world (Chiang 1978).

In North America, three types of maize are grown: sweet, silage, and grain (Hudon and Ogilvie 1984). Among them, 90% of the total tonnage is grain maize (Chiang 1978). In Canada, approximately 7 million metric tons of grain maize were produced in 1986

(Anonymous 1987). In developing countries, maize is grown primarily for human consumption. Maize also has many other uses. It yields more industrial products than any other grain (Jugenheimer 1976). More than 300 commercial items are derived from the maize grain alone (Hartman et al. 1981).

### 1.2.1.2. The maize plant

Maize is a member of the grass family, Gramineae (Poaceae). As an annual crop, maize is grown and matured in one season. The plant characteristics include: strong erect stalks, conspicuous nodes on the stem, long narrow leaves spaced alternately on the stem, a fibrous root system, and separate male and female flowers on the same plant.

The origin and evolution of maize are still matters of some controversy and speculation. One theory suggests that maize originated in Mexico and/or South America about 7,000 years ago (Mangelsdorf 1974).

Maize is considered to be a warm-season crop, requiring temperatures higher than 20 °C during the day and higher than 14 °C during the night (Hartman et al. 1981). Maize requires abundant sunlight for optimum yield, and day length obviously affects the rate of maturity (Hartman et al. 1981). Maize is classified as a short day plant, longer days tend to increase the duration of the vegetative stage and the size and number of leaves in a plant (Bockholt 1979), and make the plant fail to mature before frost (Hartman et al. 1981).

### 1.2.1.3. The pest insect complex of maize

Maize in North America is attacked by a complex of pest insects from the time it is planted to the time it is stored. This complex includes root feeders (corn rootworms, *Diabrotica* spp.), leaf feeders (armyworms, *Spodoptera* spp.; corn leaf aphid, *Rhopalosiphum maidis*), stem borers (European corn borer, *Ostrinia nubilalis*; south western and sugar cane borer, *Diatraea* spp.), ear feeders (corn earworm, *Heliothis zea*), and stored grain insects (Angoumois grain moth, *Sitotroga cerealella*; maize weevil, *Sitophilus zeamais*) (Ortega et al. 1980). Other equivalent pest insects exist in Asia and Africa.

Pest insects and diseases are the major factors limiting the increase of maize yields. The annual maize grain losses from the pest complex (insects, diseases, and weeds) have been estimated to be about 34% in the world, of which 12% is solely due to insect damage (Crammer 1967, cited by Ortega et al. 1980).

Of the many pest insects of maize, corn rootworms (*Diabrotica* spp.) are the most economically important in North American maize production. It has been estimated that the annual cost due to corn rootworm, combining insecticides to control rootworm and crop losses caused by rootworm damage, exceeds $1 billion in the United States (Metcalf 1986). Comparable figures are not available for Canada.

### 1.2.2. Corn rootworm: The most economically important pest insect in North America maize production

#### 1.2.2.1. History and host relationships of *Diabrotica* spp.

*Diabrotica* is a large genus of galerucine Chrysomelids. In North America, the most economically important taxa in this genus are three corn rootworms: the western corn rootworm (*Diabrotica virgifera virgifera* LeConte), the northern corn rootworm (*D. longicornis barberi* Smith and Lawrence), and the southern corn rootworm (*D. undecimpunctata howardi* Barber) (Metcalf 1986). *D. virgifera* was first described by LeConte in 1868 from specimens collected from the flowers of *Cucurbita foetidissima* near Wallace, Kansas (Smith and Lawrence 1966), and was first found attacking maize in 1909 near Fort Collins, Colorado (Gillete 1912). *D. longicornis* was first described by Say in 1824 as *Galleruca longicornis*, and later, Smith and Lawrence (1966) designated a neotype for *D. longicornis* due to the loss of original specimens, and recognized two subspecies: *D. longicornis longicornis* for Say's species and *D. longicornis barberi* for a new subspecies.

Based on voltinism and host specificity, Wilcox (1972) divided the species of *Diabrotica* found in the United States into two taxonomic groups: the *fucata* group and the *virgifera* group. The species classified in the *fucata* group (e.g. southern corn rootworm) are multivoltine polyphagous and overwinter as adults. The species classified in the *virgifera* group (e.g. northern and western corn rootworm) are univoltine, oliphagous

(or monophagous) and overwinter in the soil as eggs. It is believed that the difference in voltinism between the *fucata* group and the *virgifera* group is related directly to host range and indirectly to climate (Branson and Krysan 1981). In fact, the larvae of the *fucata* group were found to feed on plants of eight families (Isley 1929; Pitre and Kantack 1962), but the larval host range of the *virgifera* group was limited only to certain species of the family Gramineae, and maize is the most favourable host for larval development (Branson and Ortman 1970).

The host relationships within the *virgifera* group are also different. For example, the adult feeding and oviposition habits for western and northern corn rootworm are not identical: western corn rootworm adults feed on leaves as well as on silks, pollen and young kernels of maize, and deposit eggs in the same maize field as they developed; northern corn rootworm adults feed on silks, pollen and young kernels of maize, but not on leaves (Ludwig and Hill 1975), and tend to lay eggs in the field that did not bear maize in the previous season (Anonymous 1976). This suggests that western corn rootworm has a closer relationship to maize than northern corn rootworm.

An understanding of the host relationships of corn rootworm is useful for designing management of the pest. Since northern corn rootworm adults do not feed on corn leaves, they tend to move to other fields with fresh silks, pollen and young kernels for feeding and oviposition when corn plants become mature. Therefore, crop rotation, which has been recommended as a control approach, fails frequently with northern corn rootworm in some places at some times due to the adult feeding-oviposition habits.

### 1.2.2.2. Life cycle and bionomics of *D. v. virgifera*

Of the three economically damaging corn rootworms, the western corn rootworm is most important. The range of distribution of western corn rootworm has extended from Central America to North America and eastward Ontario in Canada (Krysan 1986). Our research group has monitored the increase in populations of western corn rootworm from 1986 - 1990 at the Central Experimental Farm, Ottawa, and it is considered an increasingly serious threat to the economy in Ontario, Canada.

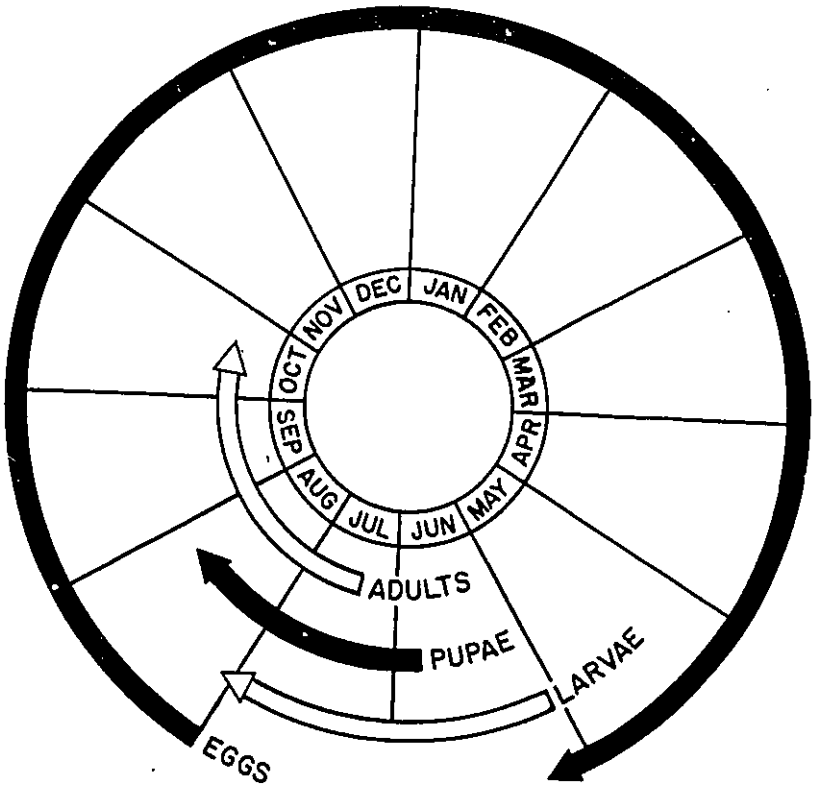
Western corn rootworm is well known to be univoltine, to have an egg diapause, and to overwinter in soil as diapausing eggs (Fig. 1.1). Under laboratory conditions, it is necessary to chill eggs for a minimum 5 months at 5 °C to break egg diapause (Wilde 1971; Branson et al. 1988). The threshold temperature for hatching and the thermal constant for the first hatching were estimated at 12.8 °C and 380 degree-days respectively (Wilde 1971). In the field, western corn rootworm adult females usually deposit most of their eggs in the top 15 cm of soil and between the rows (Pruess et al. 1968). The horizontal distribution of eggs varies with the tillage and irrigation methods, and the vertical distribution of eggs varies with soil moisture (Pruess et al. 1968; Gustin 1979).

The overwintered eggs begin hatching in the late spring. Larvae feed on the roots of maize for several weeks, passing through three instars. Temperature affects larval development. Under laboratory conditions, it has been found that the temperature threshold for larval development is 11.7 °C, and the thermal constant from hatching to adult emergence is 975 degree-days (Kuhlman et al. 1970). As well, moisture is critical for

**Fig. 1.1.**

Life cycle of western corn rootworm

(Adapted from Dominique and Yule, 1984).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larval survival (Jackson 1986). A subterranean larval movement in the soil is likely important for locating the host. In field studies, western corn rootworm larvae were found to move as far as 50 cm below the soil surface (Suttle et al. 1967). The larvae are the most destructive of all stages causing yield reduction through direct damage to the root system and indirect damage by lodging plants. The economic threshold has been estimated at 10 larvae per plant (Peters 1963).

Adult emergence begins in midsummer. After emergence, adults feed on leaves, pollen, silks, and young kernels of maize. Adults can also cause some economic damage by burrowing into the immature ear and by causing poor kernel set as they feed on silks. The effects of temperature on the adult stage have received little attention. However, light has been found to affect adult longevity and fecundity. A twelve hour photophase appears to be optimal for adult oviposition (Ball 1971).

Under field conditions, western corn rootworm mating generally happens shortly after emergence of adults (Branson et al. 1977). Egg laying begins a few weeks after adult emergence and continues until the adults are killed by frost. In the laboratory, the western corn rootworm has a mean pre-oviposition period of 14.3 days, a mean longevity of 94.8 days, and a mean fecundity of 1023 eggs per female (Branson and Johnson 1973).

### 1.2.2.3. Control of *Diabrotica* and host-plant resistance in maize

Chemical control is a major strategy to suppress corn rootworm populations and 50-60% of total maize acreage is treated with soil insecticides annually in the United States

(Metcalf 1986). The amount of insecticide used against *Diabrotica* spp. is greater than any other pest of maize in the United States (Suguiyama and Carlson 1985). As early as in 1948, the insecticide benzene hexachloride (BHC) was used to control corn rootworm and was shown to be effective in reducing corn rootworm larval damage (Hill et al. 1948). Subsequently, different insecticides, formulations, application rates and zones were evaluated for corn rootworm control (e.g. Muma et al. 1949; Cox and Lilly 1953; Apple 1957, 1960, 1961). Since large-scale applications were made to control corn rootworm, insect populations resistant to insecticides have appeared and spread very quickly. Corn rootworm resistance to insecticides is generally considered as an important factor which greatly decreases the efficiency of insecticides. Moreover, enhanced biodegradation of insecticides is another major cause for inconsistency in root protection by insecticides. Therefore, alternation of insecticides against corn rootworm has been strongly recommended. This strategy may cope with enhanced biodegradation of insecticides and can avoid or delay the development of insect resistance to insecticides (Felsot 1989). However, corn rootworm resistance and biodegradation of insecticides have frequently resulted in the withdrawal of insecticides from the market. For example, the following insecticides have been removed from the market for corn rootworm control between 1950 and 1983: benzene hexachloride, aldrin, dieldrin, heptachlor, chlordane, parathion, diazinon, disulfoton, fensulfothion, isofenphos, carbaryl, metalkamate, landrin, and carbofuran (Metcalf 1986).

The recommendations of chemical control for corn rootworms include choices of insecticides, formulations, application time, and application zones. In Ontario, 91.8% of growers practised chemical application in 15-cm bands at planting time (Ellis 1982); it is

currently recommended that the granular insecticides dyfonate, thimet, and furandan should be applied at the application rate of 55, 75, and 110 g per 100 meters of row respectively (Anonymous 1990).

Chemical control decisions are generally based on estimates of adult populations in the previous summer because larval damage is difficult to predict. The economic threshold for applying a soil insecticide at planting time is 0.75 beetles per plant in continuous cornfields and 0.5 beetles per plant in first-year cornfields during the oviposition period (Levine and Oloumi-Sadeghi 1990). However, it has been reported that one western equivalent (WE), where 1 WE = 1 beetle of western corn rootworm or 2 beetles of northern corn rootworm per plant, did not cause damage in corn fields in Ontario (Ellis et al. 1989).

It has long been understood that crop rotation, which can interrupt the insect's life cycle, is a practical control method. This strategy was designed based on the insect's biology, such as univoltine and oligophagous (or monophagous) characteristics, and has been successfully used as a method to control corn rootworms in Nebraska (Hill et al. 1948). Generally, two facts should be considered when crop rotation is used. First, a number of crops in the family of Gramineae other than maize may serve as the larval host and hence the selection of crops in the rotation should be carefully managed. Second, western corn rootworm has a closer relationship to maize than northern corn rootworm, and hence crop rotation for western corn rootworm may be more effective than for northern corn rootworm. On the other hand, the northern corn rootworm has been found to remain in diapause for more than one year, but extended diapause was not found in

western corn rootworm (Krysan et al. 1984, 1986). Extended diapause found in northern corn rootworm may be the best explanation for the ineffectiveness of rotation in controlling northern corn rootworm.

Cultural control is another tactic for suppressing corn rootworm population. The tillage system may affect western corn rootworm emergence. Compared to conventional tillage, conservation tillage can delay western corn rootworm emergence (Gray and Tollefson 1988). It has been found that root damage, numbers of corn rootworm larvae and adults are significantly reduced, and larval, pupal and adult development are significantly delayed by late planting (Bergman and Turpin 1984).

Corn rootworms have a few natural enemies, such as parasitoids and pathogens. *Celatoria broticae*, a tachinid parasitoid, can be reared from northern corn rootworm in Kansas (Chiang 1973). *Steinernema feltiae*, an entomogenous nematode, affects susceptibility of western corn rootworm in the laboratory (Jackson and Brooks 1989), and *Beauveria bassiana*, a fungus, can cause natural epizootics in corn rootworm populations in the laboratory (Maddox and Kinney 1989). However, the efficacy of these biological agents in controlling corn rootworms in the field is not clear; and the role of these biological agents in corn rootworm management is not yet developed.

Plant resistance to insects has been successfully used as a major control method in many crops in the world (see section 1.1.). Since the 1960's, scientists have been searching for maize resistance to the larvae of corn rootworm. As described in section 1.1, there are three mechanisms to account for plant resistance to insects, antixenosis (i.e. non-preference), antibiosis, and tolerance. With respect to maize and corn rootworm, the terms

antixenosis and antibiosis were considered as synonymous and referred to as antibiosis, because larval mobility is limited, and any effects of antixenosis may cause some effects of antibiosis (Branson 1986).

It is very difficult to evaluate maize tolerance to corn rootworm under natural field conditions. This is not only because corn rootworm larvae are found only underground, but also because the larval populations vary greatly, possibly from none to more than 100 larvae per plant in the same field or from one row to another (Branson 1986). Even so, the tolerance of maize to corn rootworm larvae has been extensively studied. One of the outstanding tolerant maize lines is SD-10, which has a superior capacity to regenerate a root system after rootworm damage (Shank et al. 1965). Generally, tolerance may be evaluated in a controlled infestation condition based on the measurement of a root damage rating (1-6 and 1-9 rating system) (Hill and Peters 1971; Welch 1977), root lodging (Rogers et al. 1977), root pulling (Ortman et al. 1968), and root volume (Zuber et al. 1971).

The antibiosis of maize to corn rootworm larvae has received little attention. Only one study reported the results of resistance to corn rootworm larvae in three experimental maize hybrids, which is due to antibiosis, rather than tolerance (Branson et al. 1983).

A few studies have concentrated on the resistance of maize to leaf and silk feeding of adult corn rootworm (Sifuentes and Painter 1964; Hagen and Anderson 1967; Reissig and Wilde 1971). The resistance of maize to corn rootworm adult feeding was independent of larval feeding on roots (Fitzgerald and Ortman 1964).

### 1.2.3. Hydroxamic acids: The defence chemicals in the Gramineae

#### 1.2.3.1. Hydroxamic acids in Gramineae

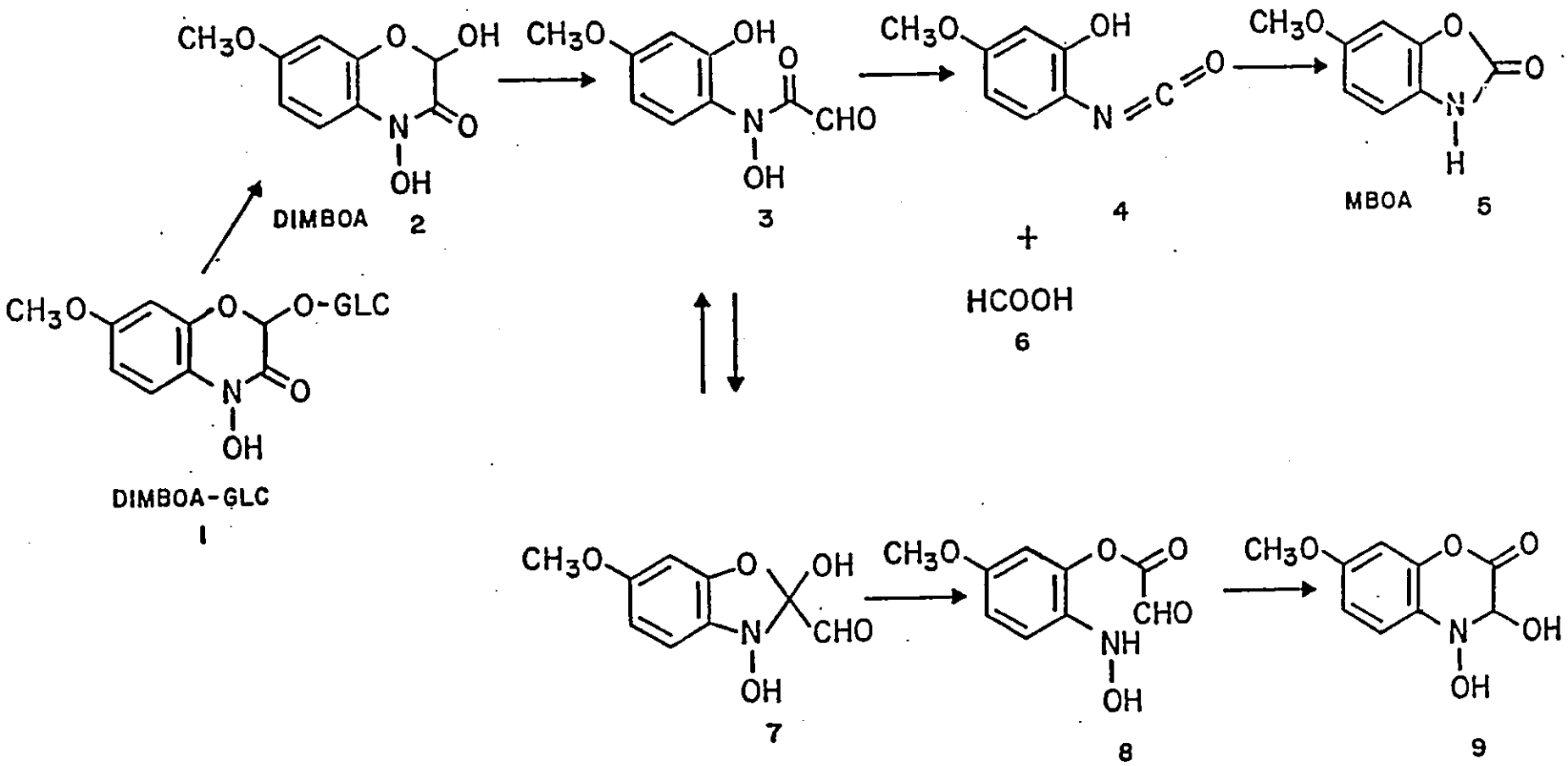
Hydroxamic acids are naturally present as the 2-D-ß-D-glucosides in intact tissue in several species of Gramineae, such as maize, wheat (Wahlroos and Virtanen 1959), and rye (Honkanen and Virtanen 1960). When plant tissues are injured, as would occur by insect feeding, the glucoside form undergoes enzymatic conversion to the aglucone by the hydrolytic enzyme ß-glucosidase. The major aglucone produced by maize is 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one(DIMBOA) (Fig. 1.2, 2). DIMBOA decomposes in a reaction which is pH and temperature dependent (Woodward et al. 1978; Perez and Niemeyer 1986), to yield 6-methoxybenzoxazolinone (MBOA) (Fig. 1.2, 5), with the liberation of formic acid (Fig. 1.2, 6). As the pH of the reacting medium becomes low, the yield of MBOA from DIMBOA decreases and results instead in the formation of 3,4-dihydroxy-7-methoxy-1,4-benzoxazin-2-one(an isomer of DIMBOA) (Fig. 1.2, 9) (Bravo and Niemeyer 1986).

Hydroxamic acids have been found in several members of the Gramineae excluding maize, such as *Triticum aesestum*, *Chusquea cumgii*, *Secale cereale*, and *Coix lachryma-jobi* (Argandoña et al. 1981; Zùñiga et al. 1983; Nagao et al. 1985; Grambow and Luckge 1986). Within the members of Gramineae, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) is the major hydroxamic acid present in rye, DIMBOA is the main one in maize and wheat, 2-hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one(HMBOA), the lactam of DIMBOA,

**Fig. 1.2.**

Chemical degradation of DIMBOA-Glc.

(Adapted from Bravo and Niemeyer, 1986).


DIMBOA-GLC
1
DIMBOA
2
3
4
+
HCOOH
6
MBOA
5
7
8
9

is also found in maize (Woodward et al. 1979a), and 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one ($DIM_2BOA$) is found only in maize. As well, MBOA does not occur *in vivo* (Hofman and Hofmanova 1971).

Hydroxamic acids are biosynthesized and accumulated throughout the growth of the plant (Klun and Robinson 1969). They are not present in seeds of cereals (Argandoña et al. 1980), and appear upon germination in maize (Klun and Robinson 1969), wheat and rye (Argandoña et al. 1980). Generally, the concentrations of hydroxamic acids are highest in the root, and then in decreasing order of concentration in the stalk, whorl and leaf (Klun and Robinson 1969). Also, the amount of hydroxamic acids vary with plant age. Younger parts of the plant contain higher hydroxamic acid levels than older ones (Argandoña et al. 1981; Guthrie et al. 1986). The total amount of hydroxamic acids and their glucosides may be more than 1% of the dry weight of young plants (Woodward et al. 1978).

Environmental conditions such as temperature and photoperiod may affect the levels of hydroxamic acids in plants. Thompson et al. (1970) reported that maize roots grown in low temperature contained low levels of hydroxamic acids, but the opposite was found in aerial parts of wheat seedlings, i.e. lower growth temperature increased hydroxamic acid levels (Epstein et al. 1986). Longer photophase leads to lower hydroxamic acid levels in wheat seedlings (Epstein et al. 1986). In maize, longer photoperiod did not affect the concentrations of either DIMBOA or its glucoside (Brandes and Heitefuss 1971).

The biosynthetic pathway of hydroxamic acids has not been fully elucidated, but the

aromatic ring of DIMBOA is derived from the products of the shikimic acid pathway (Reimann and Byerrum 1964; Tipton et al. 1973).

### 1.2.3.2. Biological activity of hydroxamic acids

There is an abundant literature on the biological activity of hydroxamic acids. A review article on this aspect has recently been published (Niemeyer 1988). Here, only a brief survey is offered.

The role of DIMBOA and MBOA in the resistance of maize to European corn borer has been extensively studied. It has been shown that high DIMBOA maize lines are highly resistance to the first generation of European corn borer (Klun and Brindley 1966; Reid 1988). When DIMBOA was added to artificial diets on which larvae feed, it had antibiotic effects, as reflected in higher larval mortality, slower larval development, lower larval and pupal weight, poorer matings, and fewer offspring (Reed et al. 1972; Robinson et al. 1982; Campos 1989). The modes of action of DIMBOA and MBOA are different (Campos 1989). Dietary tests with European corn borer indicate that DIMBOA resulted in a decrease in the approximate digestibility (AD), while MBOA resulted in a decrease in the efficiency of conversion of digested food (ECD). This suggests that DIMBOA has an effect on digestion, while MBOA has an effect on the internal metabolism of the insect. Enzyme assays *in vitro* also showed that the decrease in the utilization of food resulted from an inhibition of digestive proteases (Campos 1989, Houseman et al. 1991).

There is an inverse relationship between hydroxamic acid levels in the maize plant and numbers of the corn leaf aphid, *Rhopalosiphum maidis*, infesting the plant (Long et al. 1977). Similar relationships were obtained between hydroxamic acid levels in wheat plants and infestations of wheat aphids, such as the rose-grain aphid, *Metopolophium dirhodum* (Argandoña et al. 1980), the greenbug, *Schizaphis graminum* (Argandoña et al. 1981), and the English grain aphid, *Sitobion avenae* (Bohidar et al. 1986). When DIMBOA was incorporated into holidic diets, it has exhibited both antibiotic (Long et al. 1977; Argandoña et al. 1981; Zùñiga et al. 1983) and antifeedant (Argandoña et al. 1983; Corcuera et al. 1985) effects on cereal aphids.

Varietal correlations exist between hydroxamic acid levels in maize plant and resistance of maize to pathogens, such as fungi (northern corn leaf blight, *Helminthosporium turcicum*) (Long et al. 1975) and bacteria (stalk rot, *Erwinia* spp.) (Corcuera et al. 1978). Germination of spores of *H. turcicum* was inhibited in DIMBOA solutions (Couture et al. 1971), and the lag phase of bacterial growth was prolonged (Corcuera et al. 1978).

Excluding the biological activities of hydroxamic acids in the resistance of cereals to insects, fungi, and bacteria, hydroxamic acids were also associated with triggering the reproduction of grass-feeding mammals, with allelopathic effects of cereals, and with the detoxification of herbicides and pesticides (Niemeyer 1988).

## 1.3. Hypothesis and Objectives

### 1.3.1. Hypothesis

A review of the literature reveals that plant resistance to insects has been successfully used as a major strategy against pest insects in many crops in the world. Unfortunately, the control of *Diabrotica* spp., the most economically important pest insects in maize in North America, is still mainly dependent on insecticides. The study of maize resistance to corn rootworms has concentrated only on the tolerance in some maize cultivars to larval feeding (Chiang and French 1980), and very little information is known about maize resistance (antibiosis) to corn rootworm (Branson et al. 1983). In fact, nothing is known about the mechanisms of maize resistance to corn rootworm, nor has any study been completed on how maize secondary metabolites affect this insect, and how corn rootworms respond to the naturally occurring secondary compounds present in maize. The fact that hydroxamic acids have been associated with resistance of cereals to many kinds of pest insects leads logically to a hypothesis that these compounds may provide an important role in the resistance of maize to corn rootworm.

### 1.3.2. Objectives

The overall objectives of this study are to elucidate the potential of hydroxamic acids in the resistance of maize to the western corn rootworm, and to identify the

biological activity of these secondary compounds to western corn rootworm larvae. The specific objectives of this study are:

(1). To separate, identify, and quantify hydroxamic acids in maize roots;

(2). To determine the distribution of hydroxamic acids in maize root tissues and the relation to the location of western corn rootworm larvae in maize roots;

(3). To survey the concentrations of hydroxamic acids in maize germplasm and their relation to geographical origin;

(4). To examine the resistance of maize varieties to western corn rootworm (plant responses);

(5). To examine the effects of hydroxamic acids on the development of western corn rootworm (insect responses);

(6). To determine the behavioral responses of western corn rootworm larvae to naturally occurring and synthetic hydroxamic acids.

# CHAPTER II.

# SEPARATION, IDENTIFICATION, AND QUANTIFICATION OF 1,4-BENZOXAZIN-3-ONES AND RELATED COMPOUNDS IN MAIZE ROOTS

## 2.1. Introduction

It is well known that 1,4-benzoxazin-3-ones are present in several species of the Gramineae. Some of these compounds and their decomposition products play an important role in plant defence against pest insects and diseases. Due to their biological activities, 1,4-benzoxazin-3-ones (e.g. DIMBOA) and related compounds (e.g. MBOA) have attracted attention. Interest in these compounds has resulted in the development of various methods to quantify them.

During the past three decades, several methods have been developed to separate and to quantify 1,4-benzoxazin-3-ones and related compounds. The simplest method available is to measure total hydroxamic acid content by measuring the absorbance of the blue complex formed between hydroxamic acids and ferric chloride ($FeCl_3$) (Hamilton 1964; Long et al. 1974; Argandoña et al. 1981). This method can only estimate total cyclic hydroxamic acids because $FeCl_3$ does not react with benzoxazolinones. This approach is not highly specific to hydroxamic acids and is limited by the fact that benzoxazolinones can not be quantified.

Another method is based on the assumption that 1 mole hydroxamic acids

(DIMBOA) produces 1 mole of benzoxazolinone (MBOA) after degradation, and measures hydroxamic acid content as benzoxazolinones. The benzoxazolinones were quantified by isotopic dilution (Klun and Brindley 1966), infrared spectrophotometry (Scism et al. 1974), fluorometry (Bowman et al. 1968), gas-liquid chromatography (GLC) (Tang et al. 1975), and by high-performance liquid chromatography (HPLC) (Pessi and Scalorbi 1979). However, it has been found that the amount of MBOA formed from DIMBOA varies with temperature, pH, and composition of the reaction medium and always yields less than 75% of MBOA from DIMBOA (Woodward et al. 1978). Therefore, it is clear that these methods lead to a certain amount of error in estimates of DIMBOA content in plant extracts.

More recently, GLC and HPLC methods have been developed which can separate and quantify individual cyclic hydroxamic acids and related compounds (Tang et al. 1975; Woodward et al. 1979b; Gutierrez et al. 1982; Niemeyer et al. 1989). All of these methods measure 1,4-benzoxazin-3-one and related compounds indirectly as aglucones because enzymatic hydrolysis of the parent glucosides normally occurs during extraction. Lyons et al. (1988) presented an HPLC method to measure 1,4-benzoxazin-3-one directly as parent glucosides in situations where the enzymatic hydrolysis of the glucosides is prevented during extraction.

Most of the authors cited above used plant leaves (maize or wheat) as experimental materials to quantify the compounds concerned although Klun and Robinson (1969) have reported that maize root, compared with maize stalk, whorl and leaf, contains the highest concentration of 1,4-benzoxazinones. Maize roots have been used as experimental materials

to quantify total cyclic hydroxamic acids (Thompson et al. 1970; Argandoña and Corcuera 1985), but neither research groups quantified the individual hydroxamic acids and related compounds. To understand the chemical ecology of maize root-subterranean pest interactions, it is necessary to develop a method for the quantitative determination of the content of individual hydroxamic acids and related compounds in maize root.

In this chapter, an accurate HPLC method for separation and quantitation of 1,4-benzoxazin-3-ones and related compounds in maize root extracts was developed. The major related compounds present in maize root extracts were identified as two hydroxamic acids, 2,4-dihydrox-7-methoxy-1,4-benzoxazin-3-(4H)-one (DIMBOA), 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3(4H)-one ($DIM_2BOA$), one lactam, 2-hydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one (HMBOA), and one benzoxazolinone, 6-methoxybenzoxazolinone (MBOA) (Fig. 2.1). This approach represents the first gradient HPLC method to separate a variety of hydroxamates and derivatives in maize root extracts.

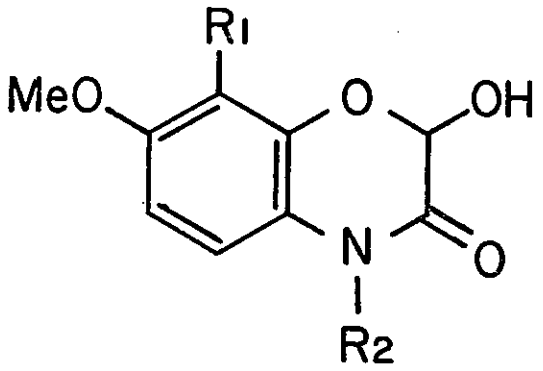
## 2.2. Materials and Methods

### 2.2.1. Extraction of 1,4-benzoxazin-3-ones and related compounds from maize root

**Extract sample preparation.** Seeds of maize (*Zea mays* L.) (hybrid 359GC3-6 x 46-43) were soaked in tap water for 24 h and planted in a plastic pot (15 cm x 15 cm) with a growth medium of one part perlite and one part vermiculite. Greenhouse temperature was maintained at approximately 22/20 °C day/night and the photoperiod was

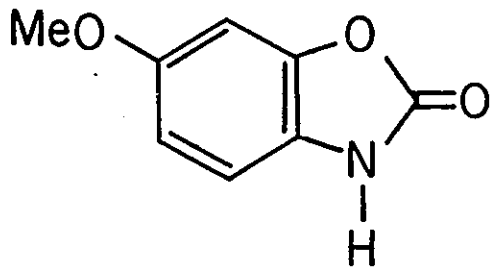
**Fig. 2.1.**

Chemical structures of 1,4-benzoxazin-3-ones and related compounds found in maize root extracts




1,4-benzoxazin-3-ones

| R1 | R2 | Abbreviation |
|---|---|---|
| H | OH | DIMBOA |
| MeO | OH | $DIM_2BOA$ |
| H | H | HMBOA |




benzoxazolin-2-one
(MBOA)

12L : 12D (12 h light provided by natural sunlight supplemented with 3 h artificial light (incandescent) at the beginning and at the end of the 12 h photophase). After two weeks of growth under these conditions, maize roots were taken out and washed with tap water followed by distilled water.

**Extraction procedure.** The extraction procedure was a modification of the method described by Gutierrez et al.(1982) and a flow chart of the procedure is shown in Fig. 2.2. One gram of the fresh root material was cut into small pieces and homogenized with 3 x 5 mL distilled water using a mortar and pestle and placed in 25 mL Erlenmeyer flask. The slurry was incubated at room temperature (25 °C) overnight in order to facilitate enzymatic hydrolysis of the DIMBOA glucoside. Afterwards, the slurry was sonicated in a cleaning bath (Branson B-220) for 3 minutes then filtered through four layers of bleached cheesecloth into a 125 mL Erlenmeyer flask. The filtrate (pH approximately 5.8) was adjusted to pH 2 with 2N HCl, heated to 65 °C for 1 minute, and cooled in an ice bath for 10 minutes to coagulate the proteins. The resulting filtrate was filtered through Whatman No. 42 filter paper in a Buchner funnel to remove the proteins. Then, the resulting filtrate was extracted with 2 x 10 mL ethyl acetate. The aqueous phase was discarded, and the pooled organic phases were evaporated to dryness under vacuum at 40 °C. Final complete dryness was carried out under a stream of nitrogen. Next, the residue was resuspended in 1 mL of HPLC grade ethyl acetate and stored in a freezer for later analysis by HPLC. Before analysis, the extracts were evaporated under vacuum and completely dried under a stream of nitrogen, then redissolved in 1 mL of HPLC grade of methanol. All extracts were filtered through 0.5 micron nylon millipore filters with a 2.5 mL Luer lock syringe and a millipore filter holder before injection.

**Fig. 2.2.**

Flow chart of hydroxamic acid extraction procedure

for two-week-old maize roots

One gram fresh root

↓

Homogenized in 3 x 5 mL $dH_2O$

↓

Incubated at 25 °C, over night

↓

Filtered through cheesecloth

↓ (Discard residue)

Adjust filtrate to pH 2 with 2N HCl

↓

Heat to 65 °C, 1 min; cooled in ice 10 min.

↓

Filter through filter paper (Whatman No. 42)

↓

Extract with 2 x 10 mL ethyl acetate

↓ (Discard aqueous phase)

Pool organic phase

↓

Evaporate under vacuum at 40 °C

↓

Dry under stream of nitrogen

↓

Resuspend in 1 mL ethyl acetate for storage

↓

Completely dry under nitrogen

↓

Resuspend in 1 mL methanol; filtered

↓

Analysis by HPLC

### 2.2.2. Separation, identification, and quantification of extracts

**Apparatus and HPLC procedures.** The root extract in methanol was analyzed using a Perkin-Elmer HPLC system equipped with a Model 250 Binary Pump, and a Model LC-480 Auto Scan Diode Array Detector. Twenty microliters of the extract sample in methanol was injected into the liquid chromatography system. The sample was run under the following conditions: Ultrasphere ODS 5 $\mu$, 4.6 mm x 25 cm reversed-phase C18 column (Beckman); flow rate of 1 mL/min; detection wavelength of 265 nm; an online UV scan of 190-400 nm; and a two-solvent system, solvent A 100% methanol and solvent B 20 mM $H_3PO_4$ (pH 2.3), was used as a mobile phase. A gradient program was processed as follows: Solvent A at the initiation was 10%; then when the sample was injected, 10% A was linearly altered to 56% A in 25 min, and then to 100% A in 2 min. This ratio was held for 4 min to clean the column, after which the mobile phase was returned to the starting concentration (10% A) in 2 min. This ratio was maintained for an additional 2 min to equilibrate the column.

**Identification of extracts.** Identification of 1,4-benzoxazin-3-ones and related compounds in maize root extracts was carried out by comparison of retention times, UV spectra and by peak enrichment of known standard compounds. All standard compounds (DIMBOA, $DIM_2BOA$, HMBOA, and MBOA) were synthesized by Atkinson (1989).

**Quantification of extracts.** Quantifications of related compounds were derived from standard curves created by a dilution series (0 $\mu$g - 20 $\mu$g) of different compounds. Three replicates were prepared for each concentrations. Absorbance units of standards were

correlated to concentration of related compounds injected. Thus, concentrations of related compounds in extracts were determined from the formulas for the regression lines.

### 2.2.3. Recovery of 1,4-benzoxazin-3-ones and related compounds from extracts

Different concentrations of DIMBOA, $DIM_2BOA$, HMBOA, MBOA were separately added to an Erlenmeyer flask (125 mL) containing 1 g of homogenated maize root tissue and the extraction procedure processed as described above. Triplicate treated root samples were prepared for individual extraction and analysis. In order to detect how much of the reference compounds were lost by binding to the tissue, a triplicate of different concentrations of the reference compounds were added to an Erlemeyer flask containing solvent (ethyl acetate) alone and the same extraction procedure carried out.

## 2.3. Results and Discussion

### 2.3.1. Separation, identification, and quantification of extracts

The purity of DIMBOA, $DIM_2BOA$, HMBOA, MBOA standards was checked by HPLC. Under the HPLC procedures described above, each of the standards showed one peak in the chromatogram. When a standard mixture of the $DIM_2BOA$, HMBOA, DIMBOA, and MBOA was injected into the HPLC system, four peaks were resolved within 25 min (Fig. 2.3.). The retention times of the mixture of four standards (Table 2.1) were virtually identical to retention times when they were injected individually.

**Table 2.1.** Retention time of the mixture of 4 standard compounds in the chromatogram

| Standard compound | Retention time (min) |
|---|---|
| $DIM_2BOA$ | 18.95 |
| HMBOA | 20.10 |
| DIMBOA | 20.83 |
| MBOA | 24.32 |

A typical chromatogram of a maize root sample extracted for 1,4-benzoxazin-3-ones and related compounds is shown in Fig. 2.4. $DIM_2BOA$, HMBOA, DIMBOA and MBOA were identified as the major compounds in corn root extracts based on comparison of retention times and spectra. Other minor hydroxamates appear to be present in the extract sample shown in Fig. 2.4, but we have, so far, no available data for their identification and quantification.

Figure 2.5 shows typical UV spectra of 1,4-benzoxazin-3-ones and related compounds in maize root extracts which are useful in confirmation of the identification of the compounds concerned. $DIM_2BOA$, HMBOA, and DIMBOA have similar but distinct UV spectra with absorbance maxima at wavelengths of 264 nm, 261 nm (shoulder 289 nm), and 264 nm (shoulder 292 nm) respectively. MBOA has two absorbance peaks with maxima of 230 nm and 292 nm.

**Fig. 2.3.**

HPLC chromatogram of mixtures of standards of 1,4-benzoxazin-3-ones and related compounds separated by the methods described in the text: (1) $DIM_2BOA$ (5 $\mu$g/mL), (2) HMBOA (5 $\mu$g/mL), (3) DIMBOA (20 $\mu$g/mL), (4) MBOA (25 $\mu$g/mL).

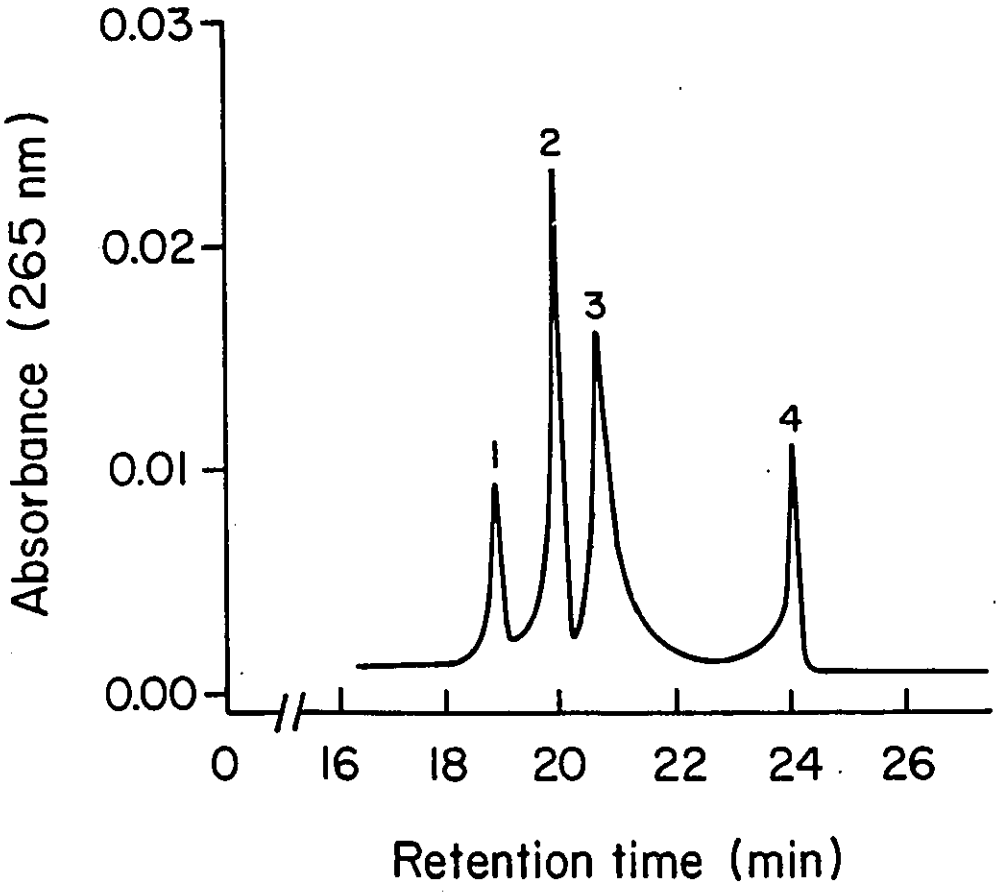

0.03
0.02
0.01
0.00
Absorbance (265 nm)
1
2
3
4
0
16
18
20
22
24
26
Retention time (min)

**Fig. 2.4.**

HPLC chromatogram of maize root extracts separated by the conditions as described in the text: (1) $DIM_2BOA$, (2) HMBOA, (3) DIMBOA, (4) MBOA.

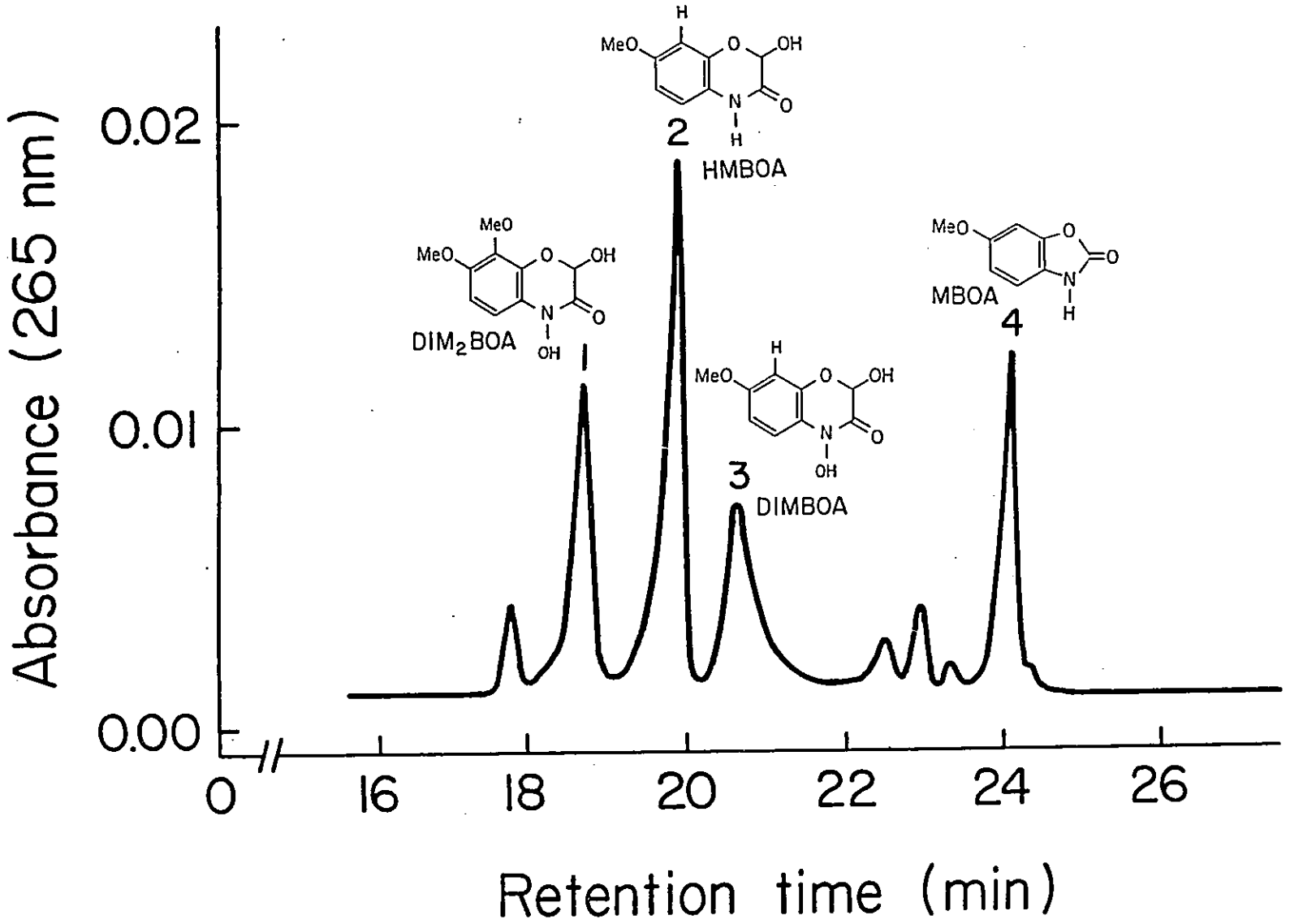
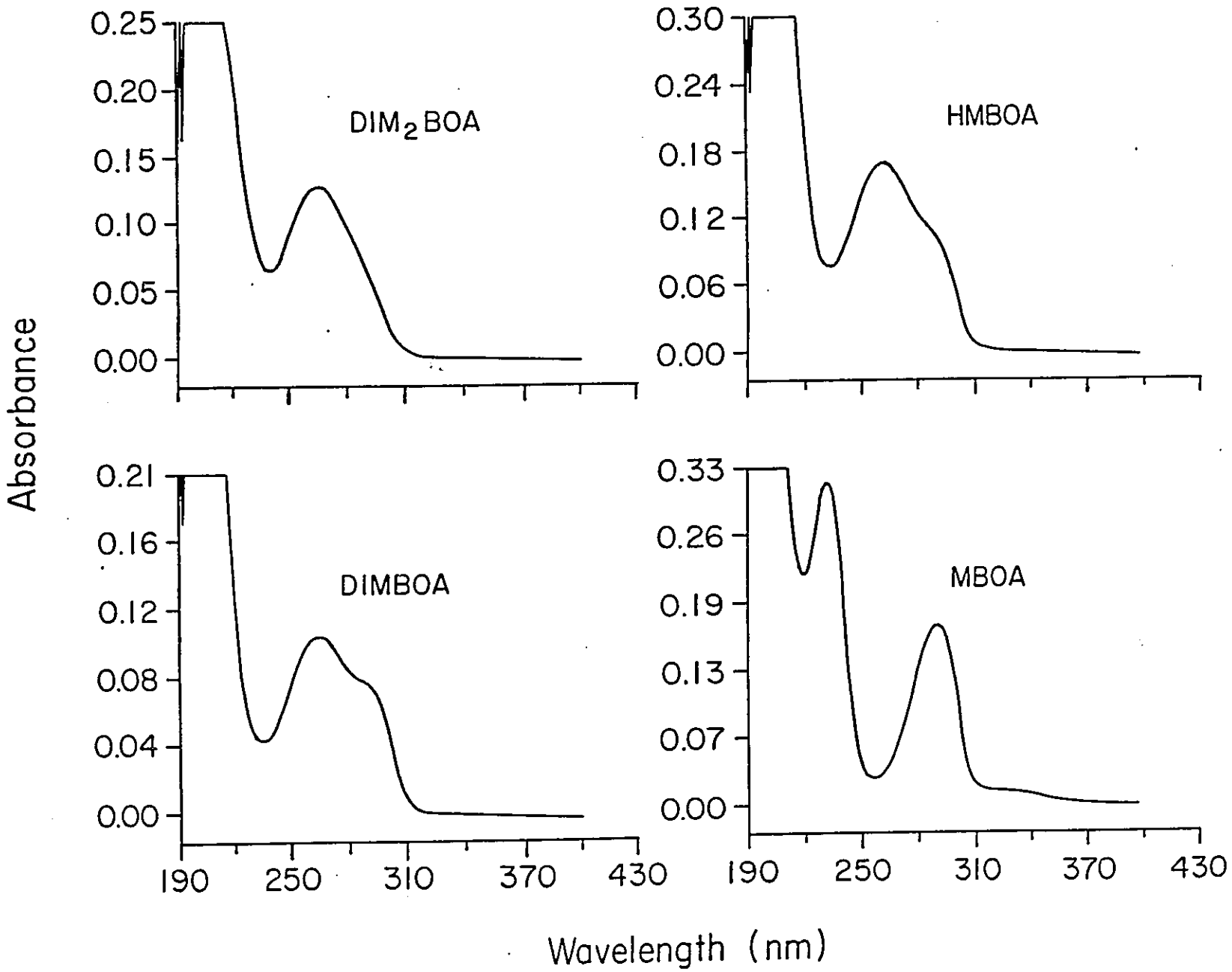

Absorbance (265 nm)
0.02
0.01
0.00
0
16
18
20
22
24
26
Retention time (min)
MeO
OH
O
N
H
2
HMBOA
DIM2BOA
1
3
DIMBOA
MBOA
4

**Fig. 2.5.**

UV spectra of 1,4-benzoxazin-3-ones and related compounds in a maize root extract. (1) $DIM_2BOA$, (2) HMBOA, (3) DIMBOA, (4) MBOA.


Absorbance
0.25
0.20
0.15
0.10
0.05
0.00
DIM2BOA
0.30
0.24
0.18
0.12
0.06
0.00
HMBOA
0.21
0.16
0.12
0.08
0.04
0.00
DIMBOA
0.33
0.26
0.19
0.13
0.07
0.00
MBOA
190
250
310
370
430
Wavelength (nm)

Quantitative estimates of the identified compounds were derived from standard curves for $DIM_2BOA$, HMBOA, DIMBOA, and MBOA (Fig. 2.6). Regression formulae for the quantification of different compounds in practical samples are shown in Table 2.2.

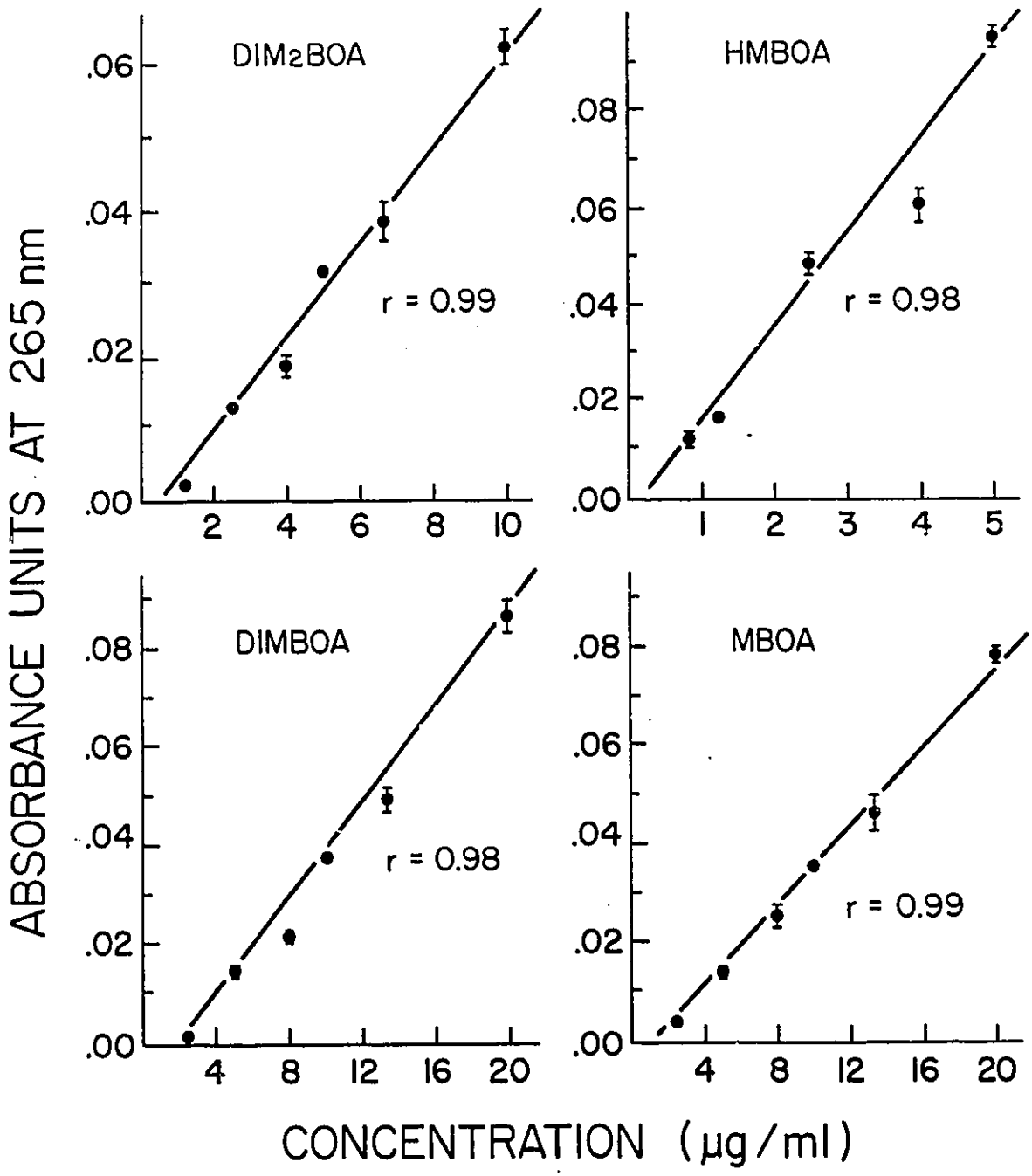
**Table 2.2.** Regression formulae for the quantification of related compounds in practical samples

| Compound | Regression formula* | $r^2$ value | P | Formula No. |
|---|---|---|---|---|
| $DIM_2BOA$ | Y = 31.99 + 7548.50 X | 0.98 | 0.0013 | [2.1] |
| HMBOA | Y = 11.42 + 2627.00 X | 0.96 | 0.0091 | [2.2] |
| DIMBOA | Y = 96.29 + 10898.50 X | 0.98 | 0.0001 | [2.3] |
| MBOA | Y = 63.79 + 12360.50 X | 0.98 | 0.0001 | [2.4] |

* Y represents the dependant variable ($\mu$g/mL sample), X represents the independent variable (absorbance units).

**Fig. 2.6.**

Calibration curves of $DIM_2BOA$, HMBOA, DIMBOA and MBOA for quantitation of related compounds in maize root extracts. Error bars indicate standard deviation (those less than 1% not shown).


DIM2BOA
r = 0.99
HMBOA
r = 0.98
DIMBOA
r = 0.98
MBOA
r = 0.99
ABSORBANCE UNITS AT 265 nm
CONCENTRATION (µg/ml)

Based on the fact that MBOA is the decomposition product of DIMBOA, and that the decomposition of DIMBOA to MBOA is not stoichiometric (Woodward, et al. 1978), DIMBOA equivalents were estimated from the recovery of DIMBOA from samples with and without added plant tissue, and from the recovery of MBOA when known amounts of DIMBOA were added to plant tissue. A correction factor of 0.946 for the MBOA produced was obtained which could be used to infer the original concentration of DIMBOA in the tissue:

$$\text{DIMBOA equivalents} = \text{HPLC-DIMBOA} + 0.946 \times \text{HPLC-MBOA} \qquad [2.5]$$

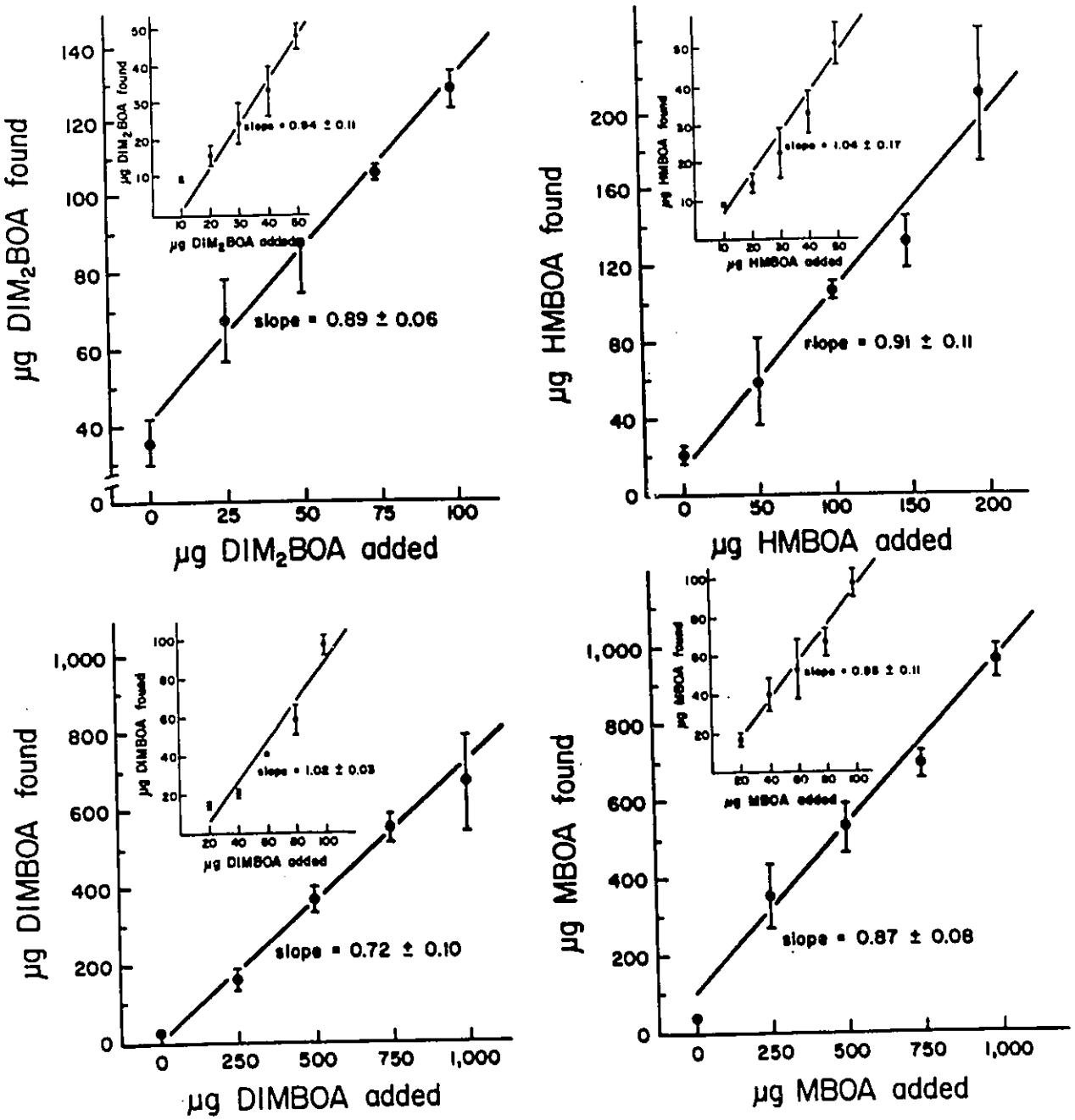
The limits of detection of the present method were determined from a dilution series. It appears that this method can detect 1 $\mu$g/mL for $DIM_2BOA$, DIMBOA and MBOA, and 0.5 $\mu$g/mL for HMBOA, in the injected solution.

### 2.3.2. Recovery of 1,4-benzoxazin-3-ones and related compounds from extracts

The accuracy of the present method was evaluated by a recovery test. Triplicated known amounts of each compound were individually added to an Erlenmeyer flask containing 1 g of homogenated root sample and processed according to the extraction procedure. The results are shown in Fig. 2.7. The intercept values in these plots represent the original amounts of each known compound found in the root sample without adding any known compounds. The recoveries are represented by the slopes of the regressions. The recoveries of $DIM_2BOA$, HMBOA, DIMBOA and MBOA were 89 $\pm$ 4%, 91 $\pm$ 11%, 72 $\pm$ 10%, and 87 $\pm$ 8% (mean $\pm$ S.D.). The recovery of DIMBOA was relatively low,

**Fig. 2.7.**

Recovery of $DIM_2BOA$, HMBOA, DIMBOA, MBOA added to maize root samples prior to extraction and HPLC analysis. In each plot, the intercept values with the y axes represent the original amount of the compounds present in the samples, the slope of each regression is equal to the fraction of the compounds recovered. The figures in the corners show the recovery of the compounds added to solvent (ethyl acetate) alone prior to extraction and HPLC analysis. Bars indicate standard deviation.


µg DIM2BOA found
µg DIM2BOA added
slope = 0.89 ± 0.06
µg HMBOA found
µg HMBOA added
slope = 0.91 ± 0.11
µg DIMBOA found
µg DIMBOA added
slope = 0.72 ± 0.10
µg MBOA found
µg MBOA added
slope = 0.87 ± 0.08

due to decomposition of DIMBOA to MBOA. When the extraction was carried out with solvent (ethyl acetate) alone(no added plant tissue), the recoveries of $DIM_2BOA$, HMBOA, DIMBOA, and MBOA were obviously increased (Fig. 2.7). This may be the result of cyclic hydroxamic acids reacting with plant tissue. Cyclic hydroxamic acids react with protein thiol groups, which is one possible cause of yield loss and one of the unavoidable problems in quantification of cyclic hydroxamic acids (Niemeyer, et al. 1982; Pèrez and Niemeyer, 1985).

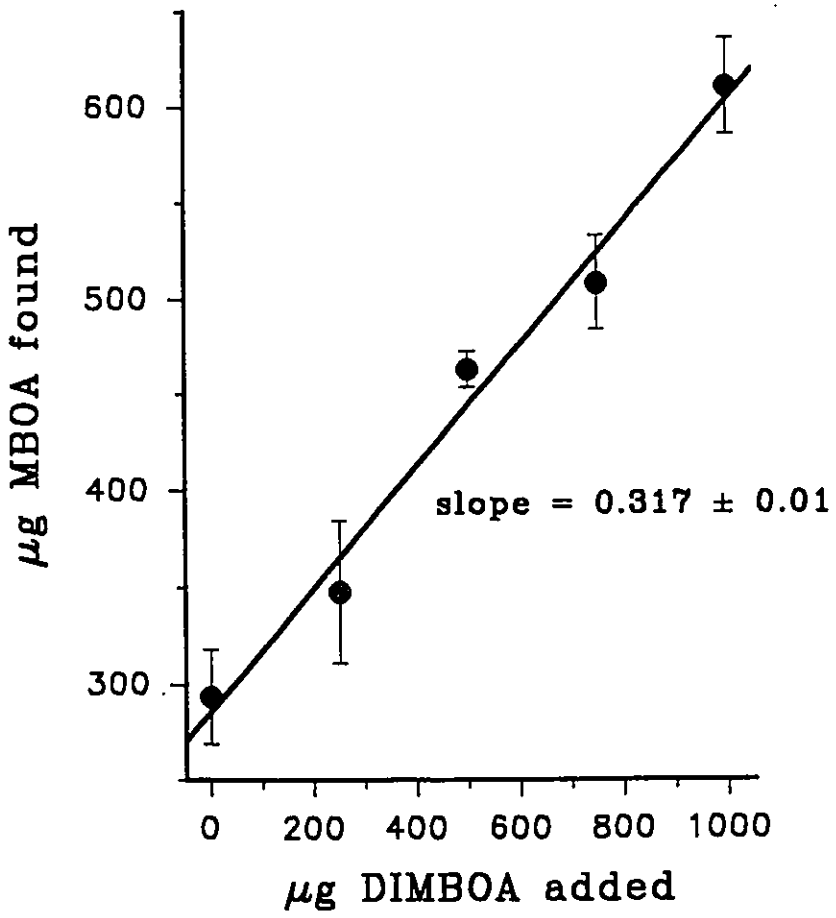
The standard deviations in Fig. 2.7 are large for most data points, in comparison with the standard deviation in the standard curves (Fig. 2.6), indicating that the main source of error appeared in the extraction procedure rather than in the separation method.

Fig. 2.8 showed the recovery of MBOA when known amounts of DIMBOA were added to an Erlenmeyer flask containing 1 g of homogenated root sample and processed by the extraction procedure. As before, the recovery is represented by the slope of the regression. The recovery of MBOA (when DIMBOA was added) was 31.7 $\pm$ 1% (mean $\pm$ S.D.). This value can be used for the calculation of DIMBOA equivalents (see section 2.3.1).

The HPLC procedure reported here is a rapid and accurate method for separation and quantification of individual 1,4-benzoxazin-3-ones and related compounds in maize root extracts. Compared with the GC method, the present HPLC method eliminates the derivatization step and can separate and quantify at least four of 1,4-benzoxazin-3-ones and

**Fig. 2.8.**

Recovery of MBOA when DIMBOA was added to maize root samples prior to extraction and HPLC analysis. Explanation as Fig. 2.7.


600
500
400
300
μg MBOA found
slope = 0.317 ± 0.01
0
200
400
600
800
1000
μg DIMBOA added

related compounds within 30 min. Therefore, the method could be helpful for researchers studying the biosynthesis of 1,4-benzoxazin-3-ones and related compounds in plant root systems. However, it is especially useful for those who investigate the potential role of 1,4-benzoxazin-3-one and related compounds in the resistance of maize to subterranean pests, such as corn rootworm, *Diabrotica* spp.

# CHAPTER III.

# DISTRIBUTION AND VARIATION OF HYDROXAMIC ACIDS IN THE MAIZE ROOT SYSTEMS

## 3.1. Introduction

Although it has been known for over three decades that several hydroxamic acids are present in maize with the highest concentration in roots, and in decreasing order of concentration in stalk, whorl, and leaf (Klun and Robinson 1969), the distribution of these compounds in specific tissues of these organs is unclear. Hydroxamic acids in maize leaf and root are preferentially concentrated around vascular tissues based on detection by the ferric chloride ($FeCl_3$) method (Argandoña and Corcuera 1985), but the individual compounds in the tissues of these organs were neither identified nor quantified.

Concentrations of hydroxamic acids in maize varieties vary with the age of the plant. The levels of DIMBOA and DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) detected by the technique of isotopic dilution were highest in the embryonic plant and declined thereafter (Klun and Robinson 1969). Similarly, the total hydroxamic acids in roots of 4-8 day old maize seedlings when measured with $FeCl_3$ reagent decreased with age (Argandoña and Corcuera 1985). However, the variation of individual hydroxamic acids in maize with root system development is unknown.

The first instar of western corn rootworm concentrates in the adventitious roots within the maize root systems (Strnad and Bergman 1987), and the cortex of the maize

root is the feeding site of western corn rootworm larvae (Chiang 1973). Based on these facts, several questions should be considered: Is the location of western corn rootworm larvae in the maize root system regulated by the hydroxamic acids content? Is there a relation between the distribution of hydroxamic acids in different root tissues (cortex and stele) and the feeding site of western corn rootworm larvae? Does the development period of western corn rootworm overlap the time period for highest concentrations of hydroxamic acid? To answer these questions, as well as to understand the chemical ecology of maize roots and their subterranean pest interactions, it is necessary to know the location and the distribution of the hydroxamates in the different tissues of the various parts of maize root system, and the variation of these compounds during maize root growth. The purpose of this chapter is to determine the distribution of individual compounds in the maize root system, and the variation of these compounds within a five-week period of root development.

## 3.2. Materials and Methods

### 3.2.1. Sample preparation

**Plant growth conditions.** Maize plants (*Zea mays* L.) (hybrid 359GC3-6 X 46-43) were grown in plastic pots (15 by 15 cm) with a growth medium of perlite/vermiculite (1:1) in a greenhouse. The greenhouse temperature was maintained at approximately 22/20°C day/night, and the photoperiod was 12 hrs provided by natural sunlight plus 3 hrs artificial light at the beginning and at the end of the photoperiod.

**Sample preparation for age variation test.** After 1-5 weeks of growth under the above conditions, maize roots were harvested and washed with tap water and then with distilled water. One gram of this fresh root material was cut in small pieces and homogenized with 2 x 5 mL distilled water using a mortar and pestle. The slurry was extracted by a modification of the method described by Gutierrez et al. (1982) (see Chapter II). Four replicates were prepared for every sample.

**Sample preparation for distribution test.** Two-week old maize root samples were used for the distribution studies. The location of root parts sampled are shown in Fig. 3.1. The whole root system was divided into five parts: first set of nodal roots, secondary roots, primary root, mesocotyl, and adventitious roots from the mesocotyl (Fig. 3.1.). One gram of the fresh material from each part of the root system was homogenized and extracted as described above. Meanwhile, each part of the root system (except the secondary roots) was divided into cortex and stele (vascular cylinder). Cortex and stele were separated by twisting and pulling out the stele. Five hundred milligrams of the fresh materials were homogenized and extracted as described before. Triplicates were prepared for every sample.

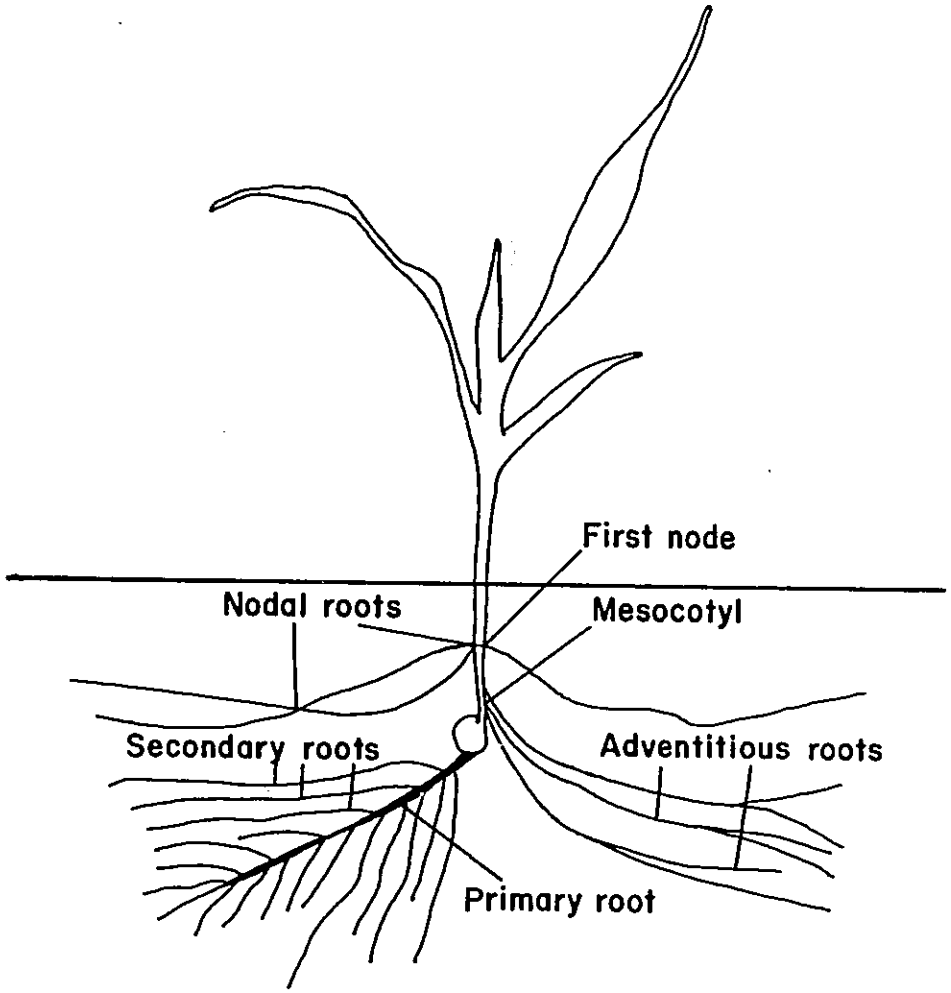
### 3.2.2. Distribution of hydroxamic acids in the maize root system detected by HPLC

All extract samples were analyzed by an HPLC procedure as described previously (see Chapter II). All HPLC data were subjected to analysis of variance (ANOVA) (SAS Institute 1982). The concentrations of all individual compounds were analyzed as the

**Fig. 3.1.**

Samples of maize root parts taken when plants were two weeks old

(Adapted from Esau 1977).


First node
Nodal roots
Mesocotyl
Secondary roots
Adventitious roots
Primary root

dependant variables, and maize root ages (1-5 weeks) and maize root tissues (cortex, stele, and complete organ) of different maize root parts (first set of nodal roots, secondary roots, primary root, mesocotyl and adventitious roots from the mesocotyl) were analyzed as the independent variables. When significant differences were found by ANOVA, Duncan's multiple range test and t test were applied for comparisons among the means (Steel and Torrie 1980; SAS Institute 1982).

## 3.3. Results and Discussion

### 3.3.1. Variation of hydroxamic acids during a five-week period of maize root development

$DIM_2BOA$, HMBOA, DIMBOA equivalents and total hydroxamic acids found in maize root extracts varied significantly ($P < 0.05$) with root age (Table 3.1). A multiple range test (Table 3.2) indicated that mean $DIM_2BOA$ and HMBOA concentrations reached a maximum at 2 weeks and then declined. By contrast, DIMBOA equivalents accumulated more slowly with peaks at 4 weeks. In all cases, the maximum concentrations were significantly higher than week 1 concentrations. These trends of accumulation of hydroxamic acids or their derivatives have also been observed in leaf tissue. For example, MBOA derived from leaves is generally highest at 15 cm height and declines afterwards through growth dilution (Klun and Robinson 1969; Guthrie et al. 1986). The high concentrations of DIMBOA equivalents found at 4 weeks may be important in biochemical defense against western corn rootworm, because western corn rootworm larvae are in the first instar at that time.

**Table 3.1.** ANOVA of all individual compounds found in maize root extracts at different maize root ages

| Source of | | $DIM_2BOA$ | | HMBOA | | DIMBOA equiv.* | | Total | |
|---|---|---|---|---|---|---|---|---|---|
| variation | df | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P > F |
| Root age | 4 | 4.86 | 0.0114 | 10.68 | 0.0004 | 4.12 | 0.0207 | 5.21 | 0.0088 |

* DIMBOA equiv. = DIMBOA equivalents. These were estimated from HPLC determination of DIMBOA and its decomposition product MBOA (see Chapter II).

**Table 3.2.** Mean content of hydroxamic acids at different maize root ages ($\mu$g/g fresh tissue)

| Maize root age (weeks) | $DIM_2BOA$ | HMBOA | DIMBOA equiv.[a] | Total |
|---|---|---|---|---|
| 1 | 20.52 b[b] | 50.30 b | 42.35 c | 115.17 b |
| 2 | 53.99 a | 82.41 a | 58.00 cb | 254.45 a |
| 3 | 5.54 b | 12.50 c | 72.26 abc | 93.76 b |
| 4 | 6.70 b | 18.46 c | 102.32 a | 132.55 b |
| 5 | 14.07 b | 18.54 c | 81.40 ab | 117.28 b |

a. DIMBOA equiv. were as in Table 3.1.

b. Means in the same column followed by the same letter are not significantly different ($P > 0.05$, Duncan's multiple range test).

### 3.3.2. Distribution of hydroxamic acids in the maize root system as detected by HPLC

When the concentrations of all individual compounds and total compounds in the complete organ of the different parts of the root were analyzed by Duncan's multiple range test, they were almost always higher in nodal roots than in other parts of root (Fig. 3.2). This result is consistent with the observation that the level of hydroxamic acids within a plant varies with the age of the plant part concerned, and at any given plant age, hydroxamic acid contents are always higher in younger plant parts (Argandoña et al. 1981). The nodal root system is the youngest part in the maize root system. It is initiated at about 7 days after planting and the first set of nodal roots starts elongation at about 10 days after planting (Ritchie et al. 1986). Clearly, when root samples were taken at 2 weeks old, the first set of nodal roots were actually only 5 days old. Hence, it is not surprising that nodal roots had a higher hydroxamic acids content than other parts of maize root. As well, there was an obvious trend showing that the concentrations of HMBOA, DIMBOA equivalents and total compounds detected in the adventitious roots were less than in the other parts of the roots (Fig. 3.2). In all tissues, DIMBOA equivalents were significantly higher than HMBOA and $DIM_2BOA$ (Fig. 3.2).

We found that the concentrations of total hydroxamic acids and all individual compounds except HMBOA in the cortex of nodal roots were significantly higher ($P < 0.05$) than that in the stele of nodal roots (Fig. 3.3, A). As well, the concentrations of DIMBOA equivalents and total hydroxamic acids in the cortex of adventitious roots, and

Fig. 3.2.

Concentrations of hydroxamic acids in different root parts when maize roots were 2 weeks old. Same letters in the top of columns within the same compounds indicate there are not significantly different ($P > 0.05$, Duncan's multiple range test) among the tissues. DIMBOA equiv. were as in Table 3.1.

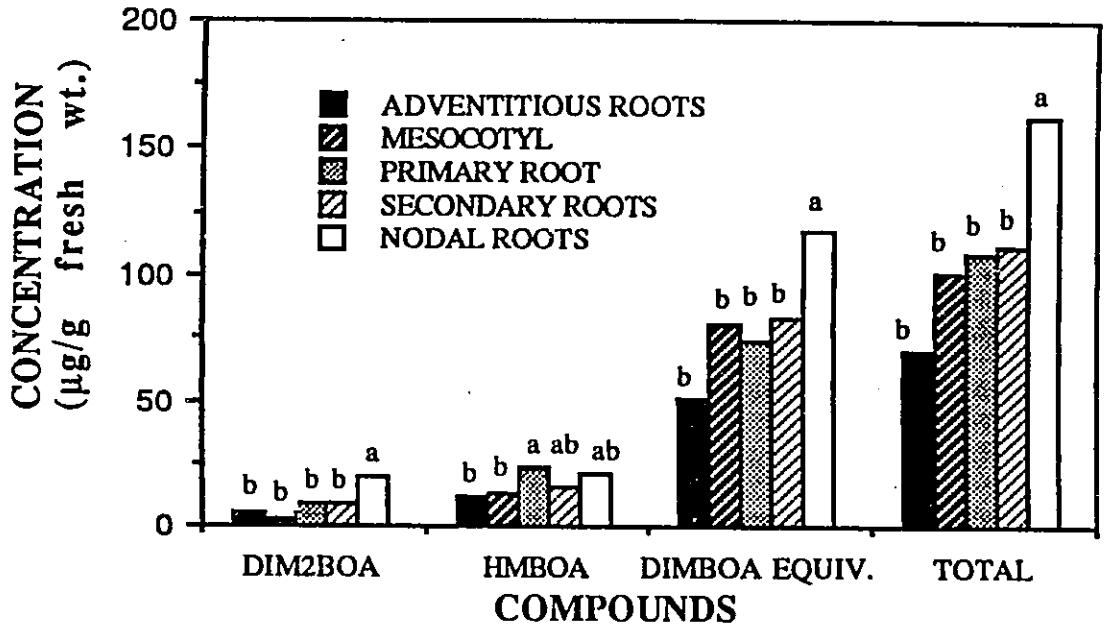
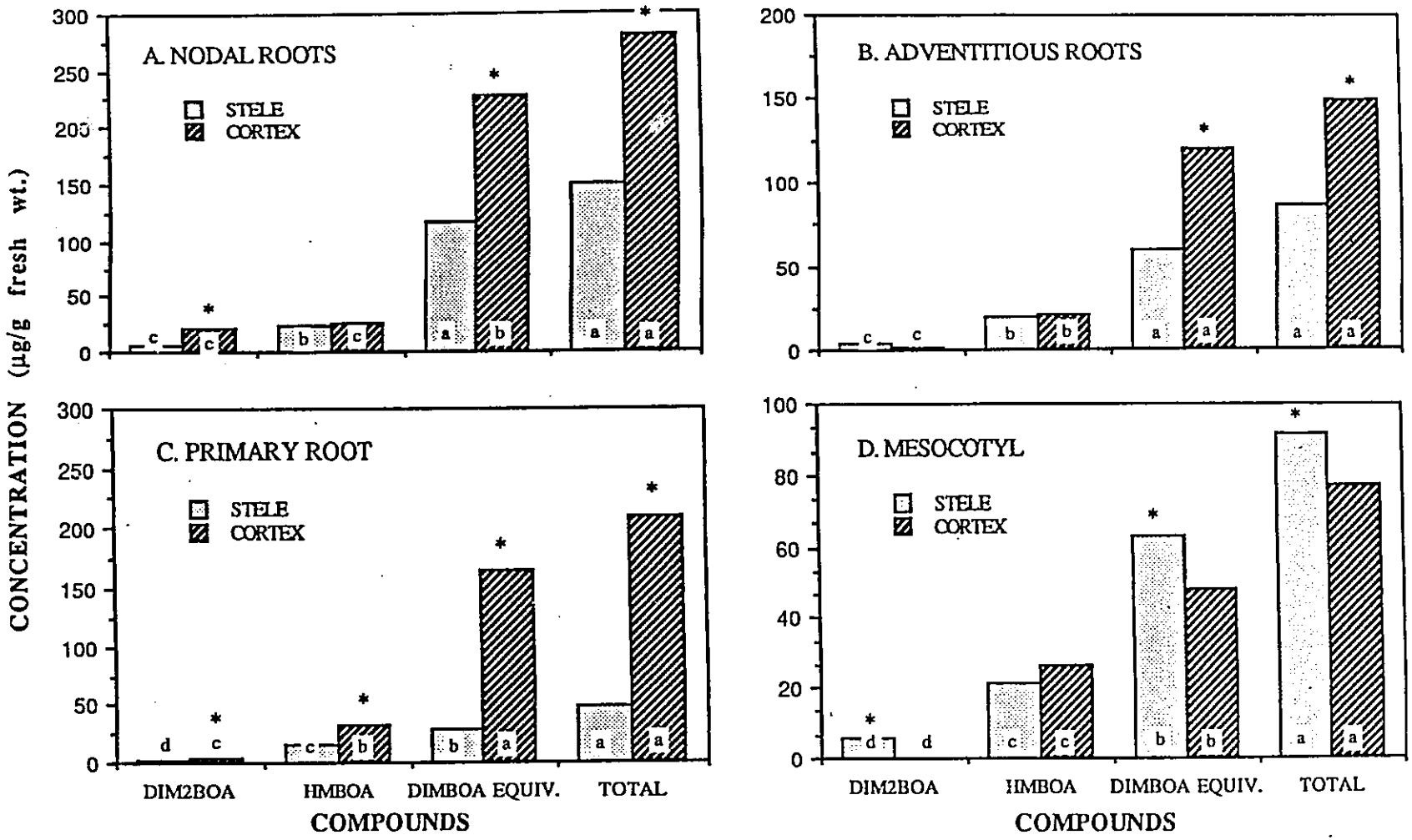

200
150
100
50
0
CONCENTRATION
(µg/g fresh wt.)
ADVENTITIOUS ROOTS
MESOCOTYL
PRIMARY ROOT
SECONDARY ROOTS
NODAL ROOTS
b b b b a
b b a ab ab
b b b b a
b b b b a
DIM2BOA
HMBOA
DIMBOA EQUIV.
TOTAL
COMPOUNDS

**Fig. 3.3.**

Concentrations of hydroxamic acids in different tissues of (A) nodal roots, (B) adventitious roots from the mesocotyl, (C) primary root and (D) mesocotyl when maize roots were 2 weeks old. An asterisk indicates there was significant difference ($P < 0.05$, t-test) between tissues. Same letters in the base of columns within the same compounds indicate amounts were not significantly different ($P > 0.05$, Duncan's multiple range test) among the tissues. DIMBOA equiv. were as in Table 3.1.


A. NODAL ROOTS
B. ADVENTITIOUS ROOTS
C. PRIMARY ROOT
D. MESOCOTYL
STELE
CORTEX
CONCENTRATION (µg/g fresh wt.)
DIM2BOA
HMBOA
DIMBOA EQUIV.
TOTAL
COMPOUNDS

all individual compounds and total related compounds in the cortex of primary root were significantly higher ($P < 0.05$) than that in the stele of each respective root part (Fig. 3.3, B and C). However, in the mesocotyl, a significantly higher ($P < 0.05$) concentration $DIM_2BOA$, DIMBOA equivalents and total hydroxamic acids were found in the stele (Fig. 3.3, D).

DIMBOA was the major hydroxamic acid in all tissues of various parts of maize roots. It was significantly more concentrated than $DIM_2BOA$ and HMBOA in all samples determined (Fig. 3.3), and it accounted for a major proportion of total related compounds in the stele of nodal roots, cortex of primary root, and all tissues of adventitious roots (Fig. 3.3, A, B and C). In addition, the concentrations of HMBOA in all tissues of different root parts (except in the cortex of nodal roots) were significantly higher ($P < 0.05$) than $DIM_2BOA$ (Fig. 3.3). In general, we conclude that hydroxamic acids are concentrated in the cortex of maize roots, and that DIMBOA is the major hydroxamic acid present in different tissues of various maize root parts.

The high concentration of hydroxamic acids observed in the cortex tissue of maize root, which is the feeding site of western corn rootworm larvae, may be important in the observed resistance of high hydroxamic acid maize varieties to this insect (see Chapter V). The distribution of hydroxamic acids in different root parts may also explain corn rootworm preference for certain root parts. In another plant-insect system, the hydroxamic acid content in wheat leaf tissue regulates the distribution of the aphid *Schizaphis*

*graminum* on the leaves of wheat (Argandoña et al. 1981). The low concentration of HMBOA, DIMBOA equivalents, and total of these compounds observed in maize adventitious roots in our study may partly explain why western corn rootworm larvae (first instar) prefer these root structures (Strnad and Bergman 1987). These compounds are already known to play an important role in the protection of maize against insects feeding on the aerial part of the plant (Campos et al. 1988, 1989). Their significance as chemical barriers towards root feeding insects should not be underestimated.

# CHAPTER IV.

# VARIATION OF HYDROXAMIC ACIDS IN MAIZE ROOTS IN RELATION TO GEOGRAPHIC ORIGIN OF MAIZE GERMPLASM

## 4.1. Introduction

In most maize growing environments, maize insect pests and diseases are considered a major threat to maize production. It is well understood that breeding maize for improved resistance to insect pests and diseases and the use of resistant plants to reduce crop loss caused by these pests are an effective, convenient, economical, and environmentally acceptable method of insect pests and diseases control (Adkisson and Dyck 1980). Both resistant germplasm sources and significant physiological biochemical traits are required to assist progress in plant breeding programs.

Although a single species, maize germplasm possesses a great deal of genetic diversity with great variation for most important economic traits, which may supply the raw materials for maize breeding programs. There are a number of germplasm banks throughout the world to conserve potential sources of genetic diversity of maize (William 1984), including an important collection maintained at the International Centre for Maize and Wheat Improvement, El Batan, Mexico (CIMMYT) (Harlan and Starks 1980). CIMMYT has also been instrumental in the development of elite germplasm pools for

different latitudinal regions of the world.

Plant secondary metabolites have been demonstrated to be protective agents in the defence of plants against insect pests and diseases (Mckey 1979). Hydroxamic acids have been shown to play an important role in the defense of plants against several pest insects, bacteria, and fungi (see Chapter I). Development of inbred maize lines with high levels of DIMBOA, a major hydroxamic acid present in maize, has been one of the principal means for control of the European corn borer, *Ostrinia nubilalis* (Lynch 1980). As well, it has been reported that resistance of maize germplasm to European corn borer is related to geographical origin, and the geographical origin is correlated with DIMBOA content, and with resistance to corn borer damage (Reid et al. 1990).

In addition to its function of absorbing water and mineral substances from soil to supply plant growth, another major function of the maize root system is mechanically to support the above ground plant. Undoubtedly a large maize root system is important in plant tolerance to unfavourable growth conditions (such as insects, diseases, etc.). Hence, a large maize root system has been considered to be a major contributor to tolerance of maize to western corn rootworm, *Diabrotica virgifera virgifera* (Ortman et al. 1974).

The purpose of this chapter is to survey the concentrations of hydroxamic acids in maize germplasm, to demonstrate if the concentrations of these compounds found in maize root or if maize root size are related to geographical origin of maize germplasm. In addition, the relation between the concentration of DIMBOA in maize roots and that in maize leaves was investigated.

## 4.2. Materials and Methods

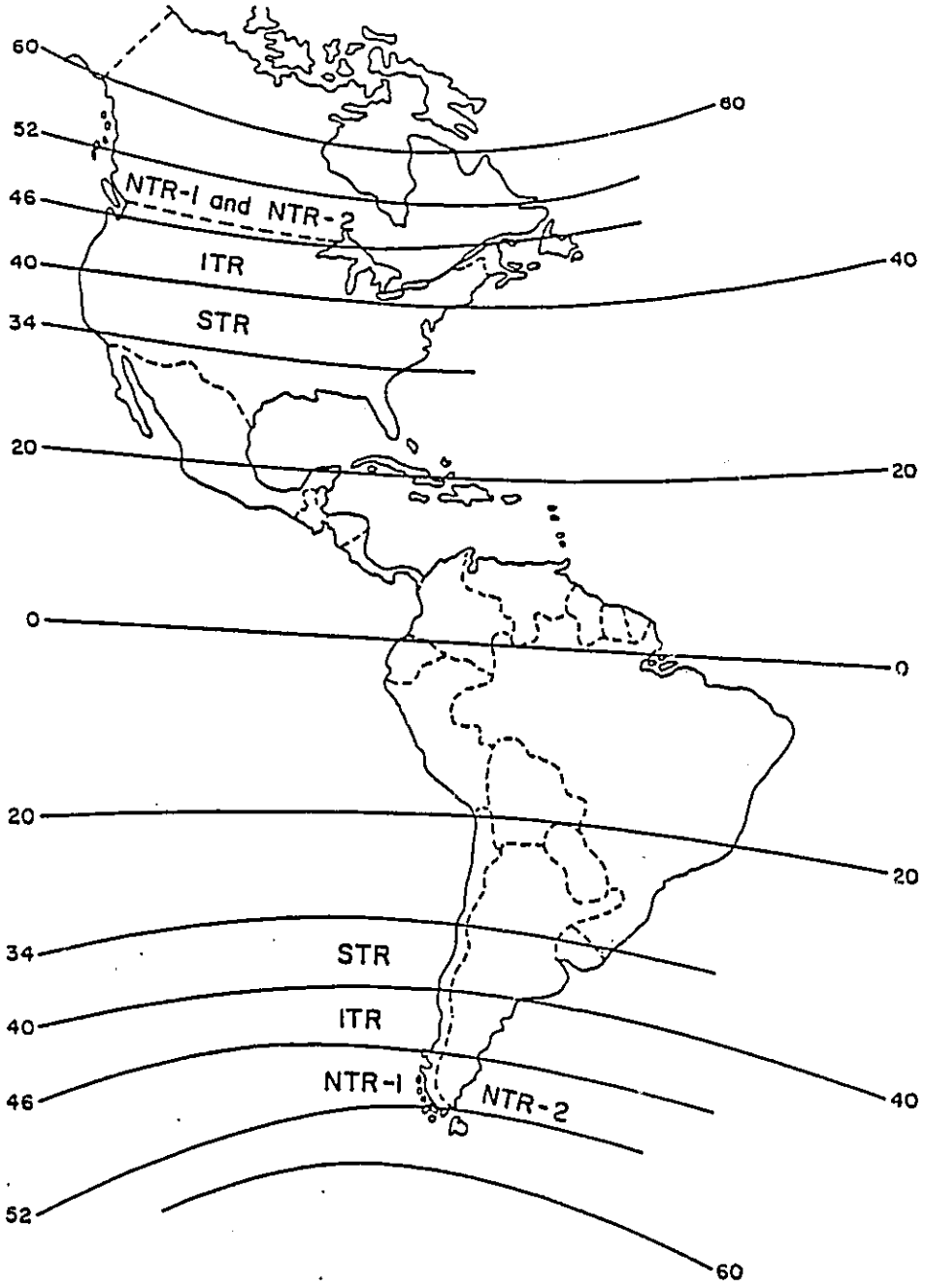
### 4.2.1. Plant materials

Thirty three inbred maize lines selected from 6 germplasm groups were used in this study. Of these, 27 lines were from latitudinal pools developed by CIMMYT and cooperating international centres: the southern temperate region pool (STR), 34-40° N-S of the equator; the intermediate temperate region pool (ITR), 40-46° N-S of the equator; the northern temperate region pool (NTR-1), 46-52° N-S of the equator; and the northern temperate region (NTR-2), 46-52° N-S of the equator (this group was from five CIMMYT cooperator countries: Canada, Poland, Germany, Switzerland, and Holland). Another 6 inbred lines were from Argentine landraces (Cateto E and Cateto C) and Mexican landraces (Mexico) respectively. The origin of all maize lines used in this study is mapped in Fig. 4.1. All of these inbred lines were selected for adaptation and grown for seed increase at the Plant Research Centre, Agriculture Canada, Ottawa (46° N latitude).

In addition, 6 other maize lines, which are usually used as materials for studying maize resistance to corn rootworm, were also analyzed for their chemical levels.

**Fig. 4.1.**

Map of latitudinal groups of maize inbred lines and races used in this study (Adapted from Reid et al. 1988).


60
52
46
40
34
20
0
20
34
40
46
52
60
NTR-I and NTR-2
ITR
STR
STR
ITR
NTR-I
NTR-2
60
40
20
0
20
40
60

### 4.2.2. Plant growth conditions and measurement of root-system size

Seeds of all different maize inbred lines were soaked in tap water for 24 hrs and planted in plastic pots (15 cm by 15 cm), five plants per pots, with a growth medium consisting of one part of perlite and one part of vermiculite in a greenhouse. The greenhouse temperature was maintained at approximately 22/20°C day/night, with a photoperiod of 12 : 12 (L : D). After 2 weeks of growth under these conditions, ten maize plants for each lines were randomly selected, and the roots were harvested and washed with tap water, then with distilled water. The maize roots were blotted dry with a piece of tissue paper, and then, weighed individually.

### 4.2.3. Quantification of hydroxamic acids by HPLC

One gram of dry root, blotted dry with paper towel, material was cut in small pieces and the extraction procedures were performed by a modification of the method described by Gutierrez et al. (1982) (see Chapter II). Four replicates were prepared for each maize line. All extract samples for different maize germplasms were analyzed by a Perkin-Elmer HPLC system equipped with a Model 250 Binary Pump, and a Model LC-480 Auto Scan Diode Array Detector. The method used for the separation and quantification of the compounds was previously described in Chapter II.

### 4.2.4. Statistical analysis.

A normality test and a homogeneity test were performed to determine if the data were parametric. Based on results of these tests, parametric analysis of variance (ANOVA) (SAS Institute 1982) or nonparametric analysis of variance (Kruskal-Wallis test) (Anonymous 1991), as required, were performed for all HPLC data to determine the differences of concentration of individual compound content by genotype and geographical group. When significant differences were found by analysis of variance, Duncan's multiple range test was applied for comparisons among the means (Steel and Torrie 1980). As well, a SAS program was performed to determine the correlation between the concentrations of DIMBOA in maize roots and that in maize leaves. DIMBOA concentrations in maize leaves were analyzed by Reid (1988).

## 4.3. Results and Discussion

### 4.3.1. Survey of concentrations of hydroxamic acids in root extracts of maize germplasm of different geographical origins

The ANOVA of hydoxamic acids in different maize lines (Table 4.1) indicated that the concentrations of all individual compounds determined were significantly different in various maize lines ($P < 0.01$). The concentrations of hydroxamic acids in the roots of different maize lines is shown in Table 4.2.

**Table 4.1.** ANOVA of all individual compounds found in maize root extracts of maize gemplasms from various geographical origins

| Source of variation | df | $DIM_2BOA$ | | HMBOA | | DIMBOA equiv.* | | Total amount | | Root wt. | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P >F |
| Corn line | 32 | 21.21 | 0.0001 | 41.22 | 0.0001 | 10.79 | 0.0001 | 11.88 | 0.0001 | 30.06 | 0.0001 |

* DIMBOA equiv. = DIMBOA equivalents.

**Table 4.2.** Concentrations of related compounds in the roots of maize germplasm of various geographic origins ($\mu$g/g fresh wt.)

| Maize line | Total | DIMBOA equiv.* | HMBOA | $DIM_2BOA$ |
|---|---|---|---|---|
| ITR 3872 | 1140.5 A | 921.1 A | 86.9 A | 120.9 A |
| NTR-1 3983 | 444.3 B | 327.1 B | 68.5 B | 35.1 CDE |
| ITR 3865 | 392.8 BC | 296.4 BC | 30.1 EF | 61.7 B |
| NTR-1 3946 | 359.0 BCD | 248.7 BCD | 30.1 EF | 70.1 B |
| NTR-1 3962 | 296.0 CDE | 186.5 DEF | 32.6 DE | 69.5 B |
| NTR-2 4071 | 281.0 CDEF | 215.0 CDE | 22.3 FGH | 38.8 CD |
| ARGEN 2030 | 248.7 DEFG | 213.0 CDE | 13.5 HIJKL | 15.5 FGHI |
| ARGEN 2032 | 218.5 EFGH | 177.9 DEFG | 21.4 GH | 11.7 FGHI |
| NTR-2 4065 | 202.3 EFGHI | 140.4 DEFG | 15.2 HIJK | 42.7 C |
| STR 3794 | 191.2 EFGHI | 120.0 EFGH | 39.7 CD | 26.5 DEFG |
| NTR-2 4072 | 184.8 EFGHI | 138.6 EFGH | 18.9 HIJ | 22.9 DEFGH |
| STR 3815 | 184.3 EFGHI | 115.2 EFGH | 41.5 C | 22.5 DEFGH |
| STR 3805 | 163.2 EFGHI | 103.2 EFGH | 31.4 E | 22.9 DEFGH |
| ITR 3862 | 143.0 FGHI | 99.8 EFGH | 28.7 EFG | 10.2 GHI |
| MEXICO 5 | 135.6 GHI | 100.5 EFGH | 19.1 HI | 12.3 FGHI |
| ARGEN 2051 | 132.9 GHI | 89.3 FGH | 10.6 IJKL | 28.6 CDEF |
| NTR-2 4081 | 132.6 GHI | 106.0 EFGH | 9.3 KL | 13.4 FGHI |
| MEXICO 55 | 123.4 GHI | 87.0 FGH | 14.2 HIJK | 19.0 EFGHI |
| ITR 3878 | 118.7 GHI | 88.1 FGH | 12.4 IJKL | 14.5 FGHI |
| NTR-2 4042 | 118.3 GHI | 102.1 EFGH | 9.8 JKL | 2.7 I |
| NTR-2 4036 | 117.2 GHI | 100.9 EFGH | 6.8 KL | 6.3 HI |
| MEXICO 212 | 111.5 GHI | 86.4 FGH | 14.2 HIJK | 7.8 HI |
| NTR-2 4050 | 94.9 HI | 64.0 GH | 10.1 IJKL | 18.3 FGHI |
| STR 3802 | 92.4 HI | 65.9 GH | 11.2 IJKL | 11.8 FGHI |
| NTR-2 4022 | 82.5 HI | 62.7 GH | 7.5 KL | 10.3 GHI |
| NTR-2 4064 | 82.4 HI | 57.7 H | 7.3 KL | 15.6 FGHI |
| ITR 3853 | 81.9 HI | 60.0 GH | 10.1 IJKL | 8.5 HI |
| STR 3790 | 77.1 HI | 54.7 H | 12.4 IJKL | 7.0 HI |
| NTR-1 3971 | 73.0 HI | 49.9 H | 10.6 IJKL | 10.1 GHI |
| NTR-1 3945 | 72.2 HI | 64.1 GH | 1.7 L | 2.7 I |
| NTR-2 4018 | 68.1 I | 51.2 H | 4.9 L | 10.2 GHI |
| NTR-2 4034 | 60.1 I | 42.5 H | 4.8 L | 11.1 GHI |
| NTR-2 4021 | 56.8 I | 44.9 H | 6.1 KL | 4.0 I |

* DIMBOA equiv. = DIMBOA equivalents.

Maize line ITR 3872 had highest concentrations of $DIM_2BOA$,HMBOA, DIMBOA equivalents, and total hydroxamic acids. The levels of total hydroxamic acids, DIMBOA equivalents, HMBOA, and $DIM_2BOA$ in ITR 3872 were 20, 20, 14, and 30 times more than that in NTR-2 4021, the line with the lowest levels of these substances. The dramatic differences in the concentrations of hydroxamic acids present in various maize lines provides the variation required to select maize lines with high levels of hydroxan. acids in breeding program. High hydroxamic acid maize lines would be of agronomic value because hydroxamic acids have been shown to be a resistant factor to many pest insects and pathogens of maize (see Chapter I), including corn rootworm (see Chapter V).

### 4.3.2. Variation of hydroxamic acids in maize roots in relation to geographical origin of maize

All HPLC data were pooled by different geographical groups and analyzed by Kruskal-Wallis test. No significant difference in concentrations of related compounds was found between various groups (Table 4.3). However, there is a trend showing that the ITR group had higher levels of all individual compounds, especially DIMBOA equivalents and the total amount of hydroxamic acids (Fig. 4.2). Groups STR, NTR-2, Argentine, and Mexican landraces contained a lower concentrations of $DIM_2BOA$, HMBOA (except STR group), DIMBOA equivalents, and the total amount of these compounds (Fig. 4.2).

Maize root-system size measured as root fresh wt. was significantly different in various maize lines ($F = 30.06$, $P = 0.0001$), and in different groups ($F = 2.91$, $P =$

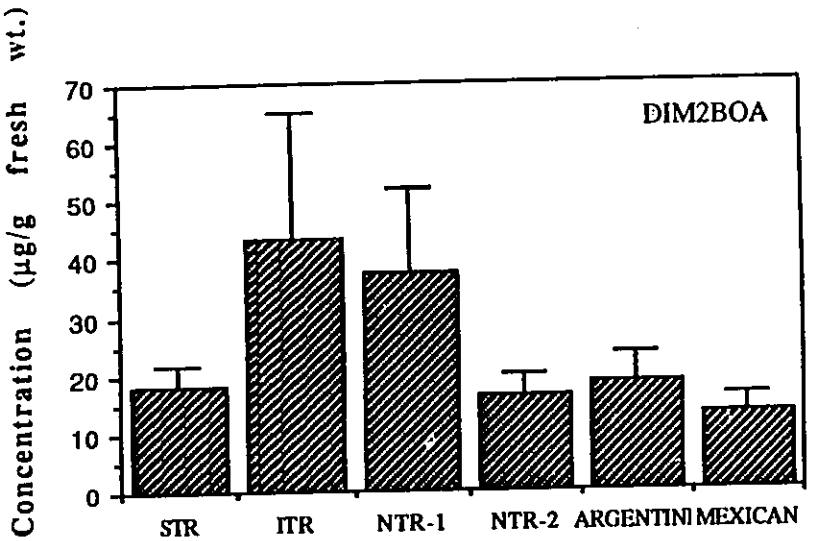
**Table 4.3.** ANOVA of all individual compounds found in maize root extracts at different maize geographical groups

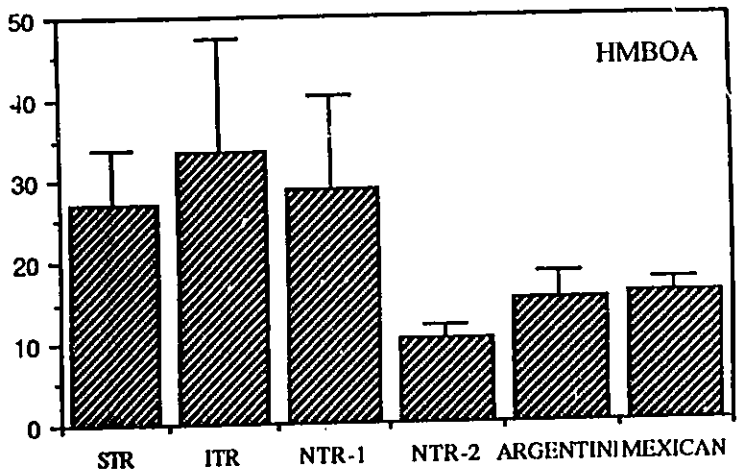
| Source of variation | df | $DIM_2BOA$ | | HMBOA | | DIMBOA equiv.* | | Total amount | | Root wt. | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P >F |
| Group | 9 | 1.19 | 0.3411 | 2.17 | 0.0886 | 1.39 | 0.2588 | 0.60 | 0.7027 | 2.91 | 0.0322 |

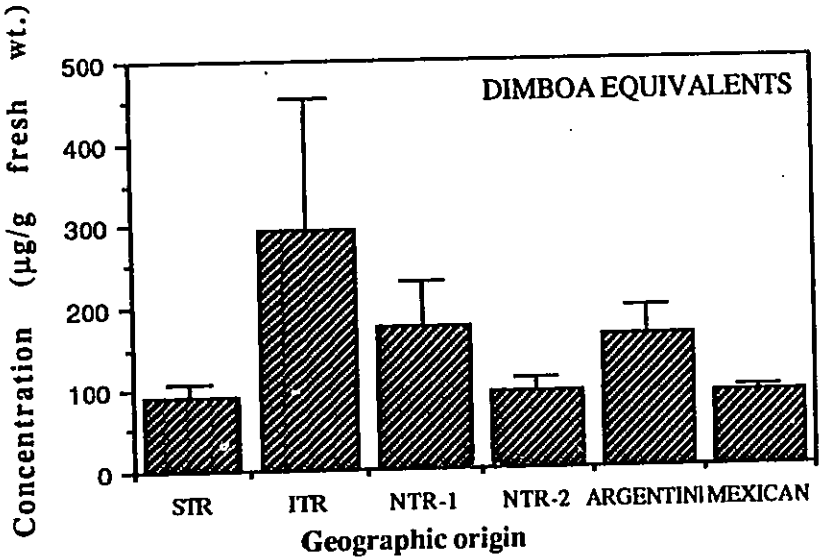
* DIMBOA equiv. = DIMBOA equivalents.

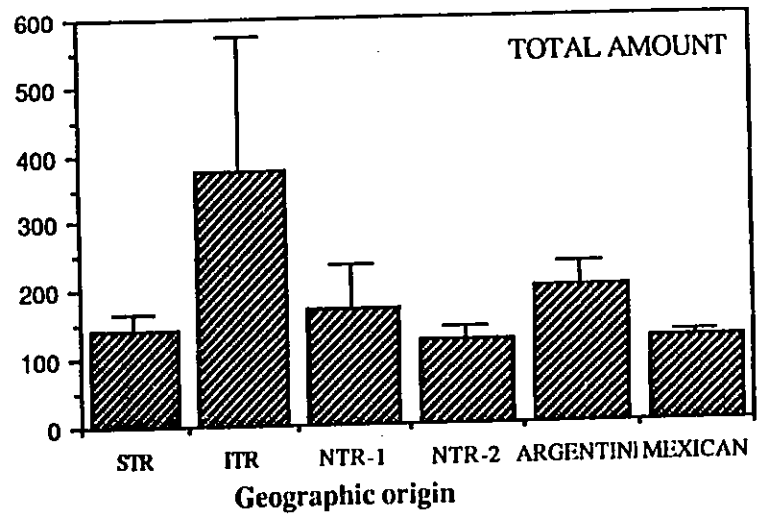
**Fig. 4.2.**

Concentrations of $DIM_2BOA$, HMBOA, DIMBOA equivalents, and the total amount of the compounds in different maize inbred lines in relation to geographical groups. Bars indicate standard error of mean.


DIM2BOA
Concentration (µg/g fresh wt.)
70
60
50
40
30
20
10
0
STR
ITR
NTR-1
NTR-2
ARGENTINI
MEXICAN


HMBOA
50
40
30
20
10
0
STR
ITR
NTR-1
NTR-2
ARGENTINI
MEXICAN


DIMBOA EQUIVALENTS
Concentration (µg/g fresh wt.)
500
400
300
200
100
0
STR
ITR
NTR-1
NTR-2
ARGENTINI
MEXICAN
Geographic origin


TOTAL AMOUNT
600
500
400
300
200
100
0
STR
ITR
NTR-1
NTR-2
ARGENTINI
MEXICAN
Geographic origin

0.0322). Maize root fresh wt. in STR and Mexican groups was significantly higher than that in ITR and Argentine groups (Fig. 4.3). The groups which have higher hydroxamic acids levels have smaller root system. The lower phytochemical levels in large roots may be a result of growth dilution. However the opposite result was obtained in some other individual maize lines such as SD-10, CH807-44, and CH663-8 (see section 4.3.4).

None of the maize germplasm from different geographical groups had been deliberately selected for either hydroxamic acids levels or corn rootworm resistance. Thus, the trend showing that the ITR group had higher levels of hydroxamic acids is only attributed to the individual gemplasm included in the population. In fact, a large variability in concentrations of related compounds was found in each groups, especially the ITR group, which may explain why a significant correlation of the levels of related compounds to geographical origin was not found even though the differences between groups appear to be great (Fig. 4.3).

### 4.3.3. Correlation between concentration of DIMBOA in maize roots and that in maize leaves

A positive significant correlation ($r = 0.40$, $P = 0.0280$, $n = 30$) was found between the concentration of DIMBOA in maize roots and that in maize leaves (Fig. 4.4). Because it has been shown that DIMBOA is a resistance factor to the European corn borer (see Chapter I) and to western corn rootworm (see Chapter V), the correlation of DIMBOA levels in maize roots to those in maize leaves, may be important in developing

**Fig. 4.3.**

Maize root fresh wt. at two weeks age in relation to geographical groups. Same letters at the top of columns indicate means are not significantly different ($P > 0.05$, Duncan's multiple range test).

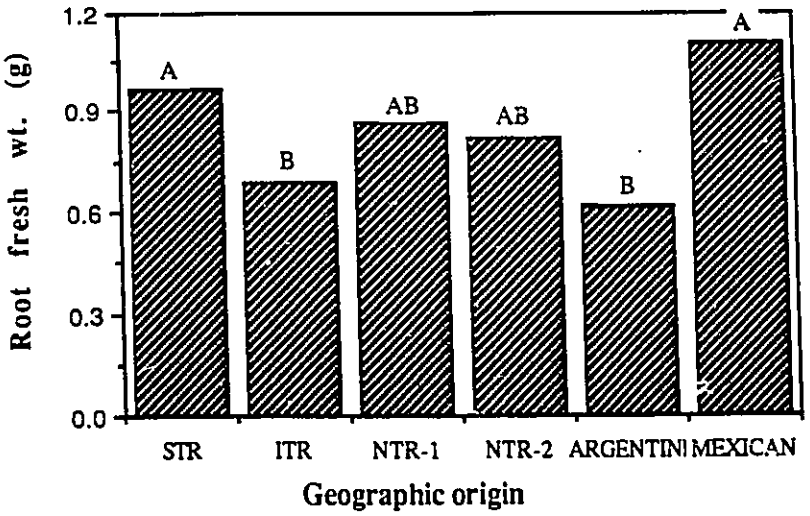
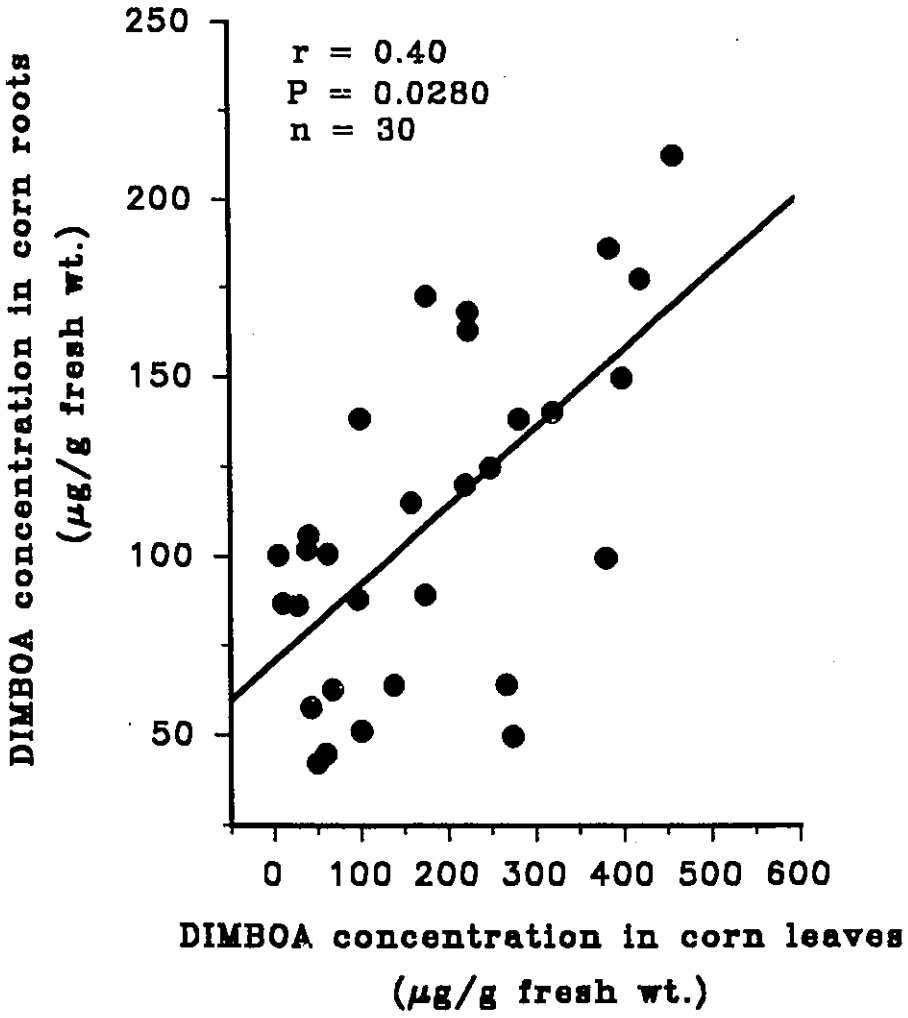

Root fresh wt. (g)
1.2
0.9
0.6
0.3
0.0
A
B
AB
AB
B
A
STR
ITR
NTR-1
NTR-2
ARGENTINI
MEXICAN
Geographic origin

**Fig. 4.4.**

Correlation between the concentration of DIMBOA in maize roots and that in maize leaves.


r = 0.40
P = 0.0280
n = 30
250
200
150
100
50
0
100
200
300
400
500
600
DIMBOA concentration in corn roots
(μg/g fresh wt.)
DIMBOA concentration in corn leaves
(μg/g fresh wt.)

maize breeding strategies. Development of inbred maize lines with high levels of DIMBOA in leaves or in roots may possibly be resistant to both European corn borer and corn rootworm.

### 4.3.4. Variation of related compounds present in the maize lines usually used in corn rootworm resistance studies

In study of maize resistance to corn rootworm, SD-10, a well known tolerant maize line to corn rootworm since the 1960's (Shank et al. 1965), is usually used as a resistant check, and A632 is used as a susceptible check. As well, in another project for screening maize inbred resistance to western corn rootworm, maize inbreds CH807-44 and CH663-8 were resistant to western corn rootworm, and that inbreds CK29 and CM7 were susceptible to western corn rootworm (Olechowski, unpublished data). All of these maize lines were analyzed for the levels of hydroxamic acids, and the results of analysis were pooled in Fig. 4.5. The concentrations of $DIM_2BOA$, HMBOA, and the total amount of hydroxamic acids in the known tolerant line SD-10 were significantly ($P < 0.05$) higher than that in A632, the line used as a susceptible check (Fig. 4.5). As well, levels of all individual compounds and the total amount of hydroxamic acids present in corn rootworm resistant maize lines CH663-8 and CH807-44 were significantly ($P < 0.05$) more than that in corn rootworm susceptible lines CK29 and CM7 (Fig. 4.5).

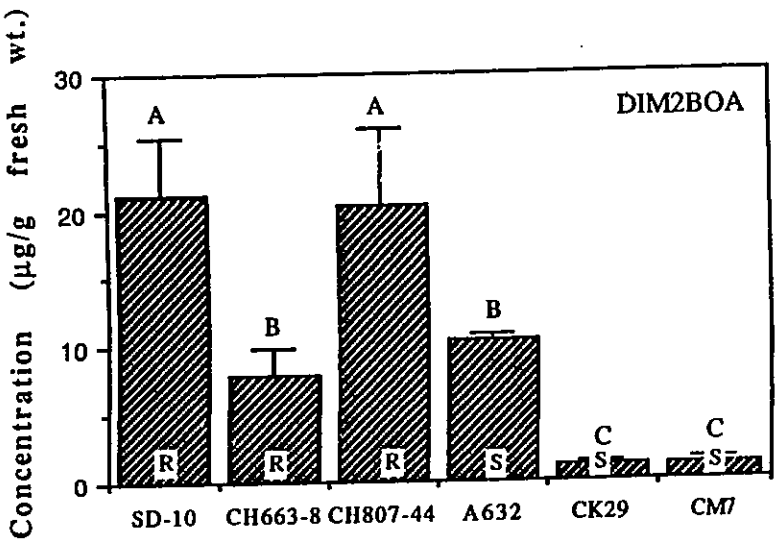
Fig. 4.6 presents root system size of these 6 maize lines. Clearly, SD-10, CH807-44, and CH663-8 have a significantly larger root system (root fresh wt.) than A632,

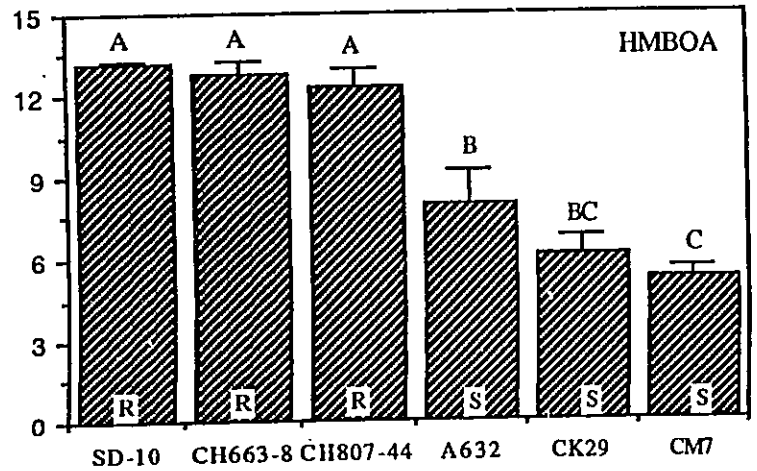
CK29, and CM7 (Fig. 4.6). A large root system found in maize lines SD-10, CH807-44, and CH663-8 undoubtedly contribute to these lines tolerance or resistance to corn rootworm. On the other hand, high levels of hydroxamic acids in these lines (Fig. 4.5) may also explain their observed resistance to corn rootworm.

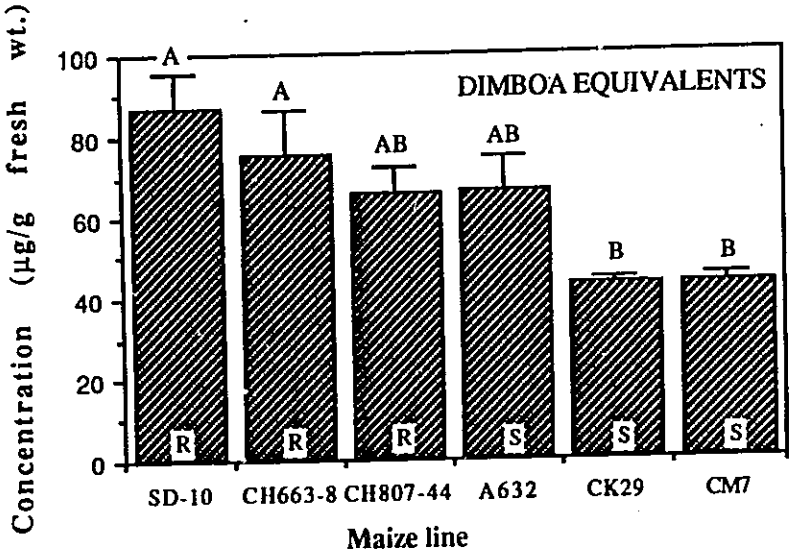
It has been shown that the larval growth parameters, such as mean number of larvae developed, mean larvae wt., and larval head-capsule width, are negatively correlated to the concentration of hydroxamic acids in maize roots (see Chapter V). It will be of interest to relate insect parameters with the related compounds levels in these maize lines.

**Fig. 4.5.**

Concentrations of $DIM_2BOA$, HMBOA, DIMBOA equivalents, and the total amount of the compounds in the maize lines usually used as materials for corn rootworm resistance study. Same letters at the top of columns within the same compounds indicate means are not significantly different ($P > 0.05$, Duncan's multiple range test). R = resistant, S = susceptible.


Concentration (µg/g fresh wt.)
DIM2BOA
30
20
10
0
A
B
A
B
C
C
R
R
R
S
S
S
SD-10
CH663-8
CH807-44
A632
CK29
CM7


HMBOA
15
12
9
6
3
0
A
A
A
B
BC
C
R
R
R
S
S
S
SD-10
CH663-8
CH807-44
A632
CK29
CM7


Concentration (µg/g fresh wt.)
DIMBOA EQUIVALENTS
100
80
60
40
20
0
A
A
AB
AB
B
B
R
R
R
S
S
S
SD-10
CH663-8
CH807-44
A632
CK29
CM7
Maize line

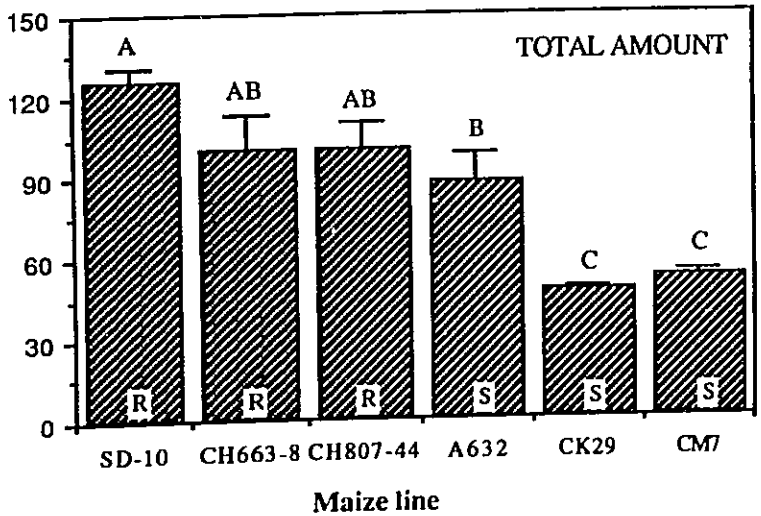
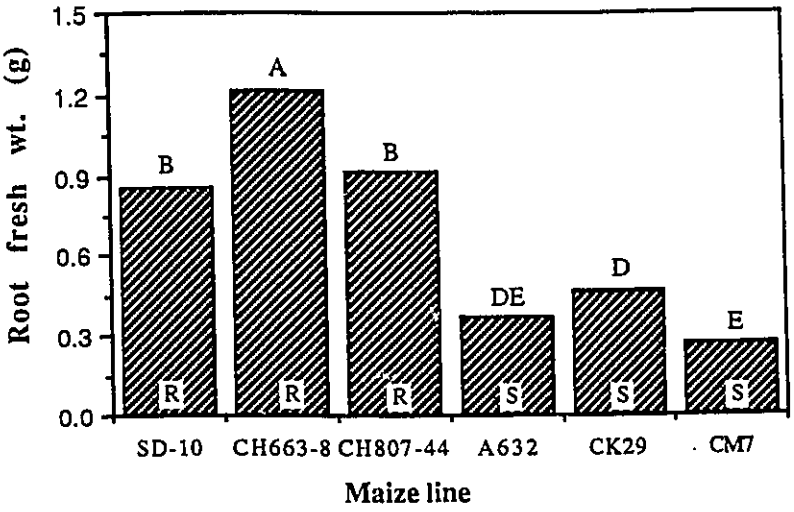

TOTAL AMOUNT
150
120
90
60
30
0
A
AB
AB
B
C
C
R
R
R
S
S
S
SD-10
CH663-8
CH807-44
A632
CK29
CM7
Maize line

**Fig. 4.6.**

Root fresh wt. at two weeks of age in the maize lines usually used as materials for corn rootworm resistance studies. Same letters at the top of columns indicate means are not significantly different ($P > 0.05$, Duncan's multiple range test).


Root fresh wt. (g)
1.5
1.2
0.9
0.6
0.3
0.0
A
B
B
DE
D
E
R
R
R
S
S
S
SD-10
CH663-8
CH807-44
A632
CK29
CM7
Maize line

# CHAPTER V.

# ROLE OF HYDROXAMIC ACIDS AND OTHER PLANT METABOLITES IN THE INTERACTIONS OF MAIZE AND WESTERN CORN ROOTWORM

## 5.1. Introduction

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) is considered an increasing threat to corn production in North America. It is well known that the use of resistant plants to reduce crop loss caused by insects, especially for those with a site of damage in roots, which are more difficult to control than leaf feeding insects, is an effective, convenient, economical, and environmentally acceptable method of insect control (Adkisson and Dyck 1980). Since the 1960's, scientists have been searching for corn varieties with resistance to corn rootworms. Most research has reported tolerance to rootworm feeding in the form of a large root system or the capability to regenerate a root system after damage, rather than antibiosis (Shank et al. 1965; Ortman et al. 1974; Branson et al. 1982, 1983). Branson et al. (1983) first reported resistance to western corn rootworm in three experimental maize hybrids, which was due to antibiosis or antixenosis, rather than tolerance. However, nothing is known about the mechanisms of maize resistance to corn rootworm, nor has any work been done on how maize secondary metabolites such as hydroxamic acids play a role in the defence of maize

against this insect, and how corn rootworms respond to the naturally occurring secondary compounds present in maize.

Because plant resistance to insects is complex, it is logical to hypothesize that factors, other than hydroxamic acids, may also be involved in the relationship between corn and corn rootworm.

The primary metabolites in plant, such as sugar and nitrogen, play a role in the nutritional relationship between plants and phytophagous insects. There is much evidence in the literature indicating the importance of these substances as they occur as phagostimulants (Mattson 1980; Schonhoven 1982).

Sugar content in plants can be important to phytophagous insects, usually as a feeding stimulant or a nutrient improving growth and survival. For example, low levels of soluble sugar content in the host plant of *Brevicryne brassicae* limits reproduction and development of winged forms in the aphid (Evans 1938). As well, the content of total sugar in corn silks was related to the damage by corn earworm, *Heliothis zea* (Boddie), and that the susceptible corn lines had higher total sugar than resistant ones (Knapp et al. 1966).

Plant nitrogen, which can be measured as total nitrogen, soluble nitrogen, amino acids, or protein nitrogen, is a component of plant tissue which is critical for phytophagous insects (Strong et al. 1984). An increase in nitrogen content in plants often improves growth efficiencies, growth rates, reproduction of individual insects, and increases the average population size (Onuf 1978; Prestidge 1982). Resistance of pea plants, *Pisum sutivum*, to the pea aphid, *Acychosiphon pisum*, was due to low amino acid content

(Auclair et al. 1957). Similarly, the silks of certain corn lines resistant to corn earworm had lower concentrations of amino acids than those of susceptible ones (Knapp et al. 1966); the average content of free amino acids in the winter wheat varieties resistant to the English grain aphid, *Sitobion avenae*, was 40.0% less than in two susceptible ones (Xie et al. 1987). However, the relationship between sugar and nitrogen in corn roots and western corn rootworm development is unknown.

The purpose of this chapter is to examine the role of DIMBOA in the resistance of selected corn lines to western corn rootworm larvae, and to investigate the possible role of sugar and nitrogen in the interaction of corn and western corn rootworm.

## 5.2. Materials and Methods

### 5.2.1. Absorbance of DIMBOA by corn roots

Since there is no artificial diet available for western corn rootworm larvae, it is necessary to develop a new method to determine the effect of plant secondary compounds on western corn rootworm larvae. The method designed was to put seed with freshly germinated 2-3 cm long roots in Petri dishes lined with filter paper on the bottom, and then water them with a solution of the compound of interest. The idea was to allow the roots to absorb the compound, and then infest the surface of roots with larvae.

To determine if and how much of the compound was absorbed by corn roots, appropriate amounts of a 400 ppm DIMBOA solution were added to the Petri dishes as

described above. Treated roots were kept in 25°C for time intervals of 0, 1, 2, 3 days. One gram of fresh roots (3 replicates were prepared) was extracted for DIMBOA analysis (see Chapter II) at each time interval. In addition, samples treated with distilled water were used as control.

### 5.2.2. Toxicity of DIMBOA and MBOA to western corn rootworm neonates

Germinated hybrid corn seeds with fresh roots (2-3 cm long) were placed in Petri dishes with one filter paper (9.0 cm) on the bottom of each dish, and watered with 1.7 mL of a solution of known concentration of DIMBOA (0, 50, 100, 200, 400, 800 $\mu$g per mL) and MBOA (0, 200, 400, 800, 1600 $\mu$g per mL), respectively. Western corn rootworm neonates were then carefully placed on the corn roots. Ten larvae (five seeds in each dish and two larvae per seed) were used for each treatment, and three replicates were prepared at each concentration. The dishes were kept in the incubator (25°C, 10L : 14D). The whole experiment was repeated three times. After a 24 or 36-h period, the filter paper was moistened with 0.5 ml of the appropriate solutions. Seventy-two hours after treatment, the mortality of the larvae at the different concentrations was recorded. Abbott's formula (Abbott 1925) was used to correct for control mortality (less than 15%). If the average control mortality exceeded 15%, the data were not used. A probit analysis was generated by SAS procedure (SAS Institute 1982) for calculation of $LC_{50}$ value.

### 5.2.3. Effect of western corn rootworm on plant growth parameters: Plant responses

**DIMBOA analysis.** Twenty four different corn lines developed by R. I. Hamilton, Plant Research Centre, Agriculture Canada, Ottawa from synthetic varieties obtained from the International Centre for Maize and Wheat Improvement (CIMMYT) were used as the materials for analysis. Fresh root samples (1 g from a 20-day old plant) were extracted by the method described by Gutierrez et al. (1982). The root extracts were analyzed by a Beckman HPLC equipped with a Model 165 Variable Wavelength Detector, a Model 110A Solvent Metering Pump System, and a Model 420 System Controller Programmer. The samples were run under the following conditions: Ultrasphere ODS reverse-phase C18 column; isocratic elution with 40% methanol and 58% water : 2% acetic acid (pH 2.5); flow rate of 1 mL per min; detection wavelength of 265 nm; and an online UV scan of 240-400nm.

**Effect of western corn rootworm on plant growth parameters of two different corn lines.** Based on the results of DIMBOA analyses of 24 four corn lines, two corn lines from different latitudinal regions (ITR 3872 with high DIMBOA content and NTR-2 Ger. 4042 with low DIMBOA content), were selected as the test varieties. The western corn rootworm eggs were provided by T.F. Branson, U.S.D.A., Brookings, SD, for the 1988 test and by A.W. Schaafsma, Ridgetown College, Ridgetown, Ont. for the 1989 test. After 4 months at 5°C, eggs were screened from the soil, suspended in a 0.125% agar solution (Palmer et al. 1977), and adjusted to 200 eggs per ml. Random samples of eggs from the agar solution were maintained in the incubator at 25°C to determine their

viability. Mean hatches were 32.0% in 1988 and 38.3% in 1989. The potting medium, composed of one part top soil, one part perlite and one part peat moss, was pasteurized (180°C, 30 min.) before use. The selected corn line seeds were planted in plastic pots (20 by 20 cm), and the pots were artificially infested at the rate of 0, 100, 200, 400 eggs per pot in 1988, and 0, 200, 400, 800 eggs per pot in 1989. Ten plants (one plant per pot) were planted for each treatment. The plant pots were kept in the greenhouse at 20°C, with a photophase of 14 h, and watered once a day. At 7 weeks after infestation, plant height was measured vertically from the soil surface to the tip of plant, stem thickness was measured at the base of stem, and the fresh weight of the above ground plant, the fresh weight of the root, the dry weight of the above ground plant, the dry weight of the root were recorded. All plant parameter data were analyzed by analysis of variance (ANOVA) program (SAS Institute 1982). The significance ($P < 0.05$) of the effect of each factor and all possible interactions between these factors were considered. When the differences found by ANOVA were significant, Duncan's multiple range test was applied for comparisons between different means (Steel and Torrie 1980).

### 5.2.4. Effect of different maize lines on western corn rootworm: Insect responses

**Effect of two different corn lines on western corn rootworm adults.** Two corn lines as described above (ITR 3872 with high DIMBOA content, NTR-2 Ger. 4042 with low DIMBOA content) were used as the test varieties. The procedures for the pot trial test in greenhouse were similar as described above. The egg samples from agar solution

maintained in the incubator had mean hatches of 38.3% in 1989 and 44.5% in 1990. Three replicates were prepared: one was performed in 1989, and the others in 1990. The infestation rate was 200 eggs per pot for all of three replicates. Before adult emergence (ca. 7 weeks after infestation), all pots were caged with nylon mesh. Adults were collected every other day until emergence was completed. All adults were weighed immediately after collection and the number of emerged adults was recorded. Subsequently, adults were killed at -20°C, air dried at room temperature, and the head-capsule widths of 20 randomly-selected adults were measured. All data were analyzed by a t-test program to determine if the means from the two different corn lines were significantly different ($P < 0.05$) (SAS Institute 1982).

**Effect of seven different corn lines on western corn rootworm larvae.** In a previous study (see Chapter IV), 40 inbred maize lines from different latitudinal regions were used as the materials for the phytochemical analysis of hydroxamic acids present in the roots. Based on the results of that study, 7 inbred maize lines (ITR 3872, NTR-1 3983, ITR 3865, NTR-1 3946, NTR-2 Canada 4072, NTR-2 Germany 4042, and NTR-2 Switzerland 4034) with different levels of related compounds were selected as the materials in this study.

Western corn rootworm eggs, after 4 months at 5°C, were screened from the soil, suspended in a 0.125% agar solution (Palmer et al. 1977), and adjusted to 100 eggs per ml. The plastic container (ca. 30 ml volume) with 5 ml egg/agar suspension (ca. 500 eggs) were incubated at 25 °C for 2 weeks. On day 11 after incubation, seeds of 7 maize

lines (as mentioned above) were soaked in tap water for 24 h. Forty germinated seeds for each maize lines were put in the bottom of a plastic container (11 by 13 cm), and the soil with eggs from one egg container was evenly spread over the seeds. Afterward, 50 ml distilled water and 200 g of soil were added, and the containers were incubated at 25°C for 2 weeks. After 2 weeks, the larvae in each containers were extracted by hand. All larvae in different maize lines were collected, larvae were weighed individually, and head-capsule width of 30 randomly-selected larvae was measured under a binocular microscope.

### 5.2.5. Determination of sugar and nitrogen

**Plant materials.** Of seven corn lines which were used for studying the effects on western corn rootworm larvae (section 5.2.3), five corn lines (ITR 3872, NTR-1 3946, NTR-2 Canada 4072, NTR-2 Ger. 4042, NTR-2 Switz. 4034) were used as materials for determining the contents of sugar and nitrogen.

**Quantification of total sugar.** The total sugar was determined by the method of Dubois et al. (1956). One gram of fresh corn root was cut into small pieces and extracted in 25 mL 50% ethanol under dark condition over night. The residues were separated by filtration through a millipore filter funnel. One mL of this filtrate was pipetted into a test tube and mixed with 1 mL 5% phenol solution, and then, 5 mL concentrated sulfuric acid ($H_2SO_4$) was rapidly added to the mixture. The tubes were slightly shaken in order to obtain good mixing. The samples were maintained at room temperature for 30 min, and

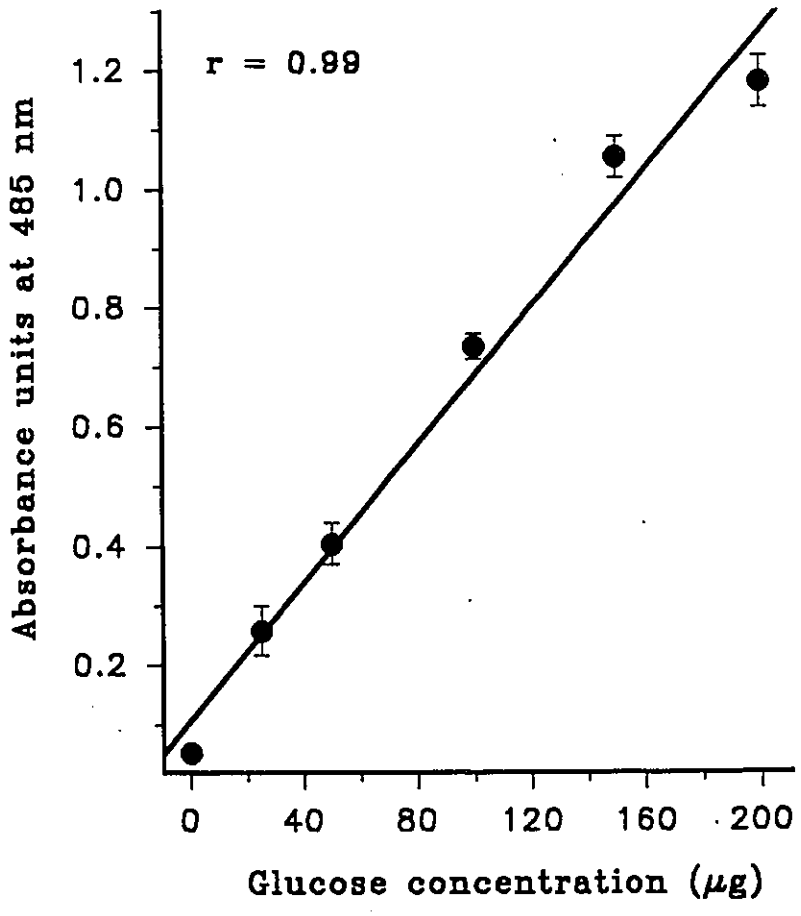
the absorbance was detected at 485 nm by a Varian CARY 2200 Spectrophotometer. Three replicates were prepared for each corn line. Quantification of total sugar was derived from a standard curve created by a dilution series (0 $\mu$g - 200 $\mu$g) of glucose. Quantitative estimates of total sugar were derived from a standard curve for glucose (Fig. 5.1). The regression formula for the quantification of total sugar was:

$$[\text{Glucose}]\ (\mu\text{g/g fresh wt.}) = 4212.50\ A - 398.25 \qquad [5.1]$$

**Quantification of total nitrogen.** A half gram sample of dried corn roots were used to determine total nitrogen. Three replicates were prepared for each corn line. These determinations were performed by Janet Gale at the Central Experimental Farm, Agriculture Canada, Ottawa, Ontario, by Kjeldahl nitrogen analysis.

**Fig. 5.1.**

Calibration curve of glucose for quantification of total sugar in corn root. Error bars indicate standard deviation.


r = 0.99
Absorbance units at 485 nm
1.2
1.0
0.8
0.6
0.4
0.2
0
40
80
120
160
200
Glucose concentration (μg)

## 5.3. Results and Discussion

### 5.3.1. Absorbance of DIMBOA by corn roots

HPLC analysis showed that when roots were incubated in a 400 ppm DIMBOA solution, they absorbed the compound (Fig. 5.2). Compared with controls, significantly more DIMBOA was found in the samples treated with DIMBOA at day 1, 2, and 3 (Fig. 5.2). The result suggested that the method could be used to test the effect of the compound on corn rootworm larvae.

### 5.3.2. Toxicity of DIMBOA and MBOA to western corn rootworm neonates

The data from the toxicity studies showed that there was a high positive correlation between the percent mortality of the western corn rootworm in probit scale and the log DIMBOA concentration ($r = 0.94$) (Fig. 5.3). As well, a significant correlation between the percent mortality of the western corn rootworm in probit scale and the log MBOA concentration was found ($r = 0.98$) (Fig. 5.4). Using these data, $LC_{50}$ and $LC_{90}$ values and their 95% fiducial limits were determined for both DIMBOA and MBOA, and shown in Table 5.1.

We found that almost all larvae placed on corn roots in the different treatments bored into the roots within 24 h. However, 24 or 36 h after application of DIMBOA and MBOA solution around the roots, most larvae in the treatments at higher concentrations

**Fig. 5.2.**

Concentration of DIMBOA found in corn roots treated with DIMBOA solution (400 ppm) and with distilled water (control).

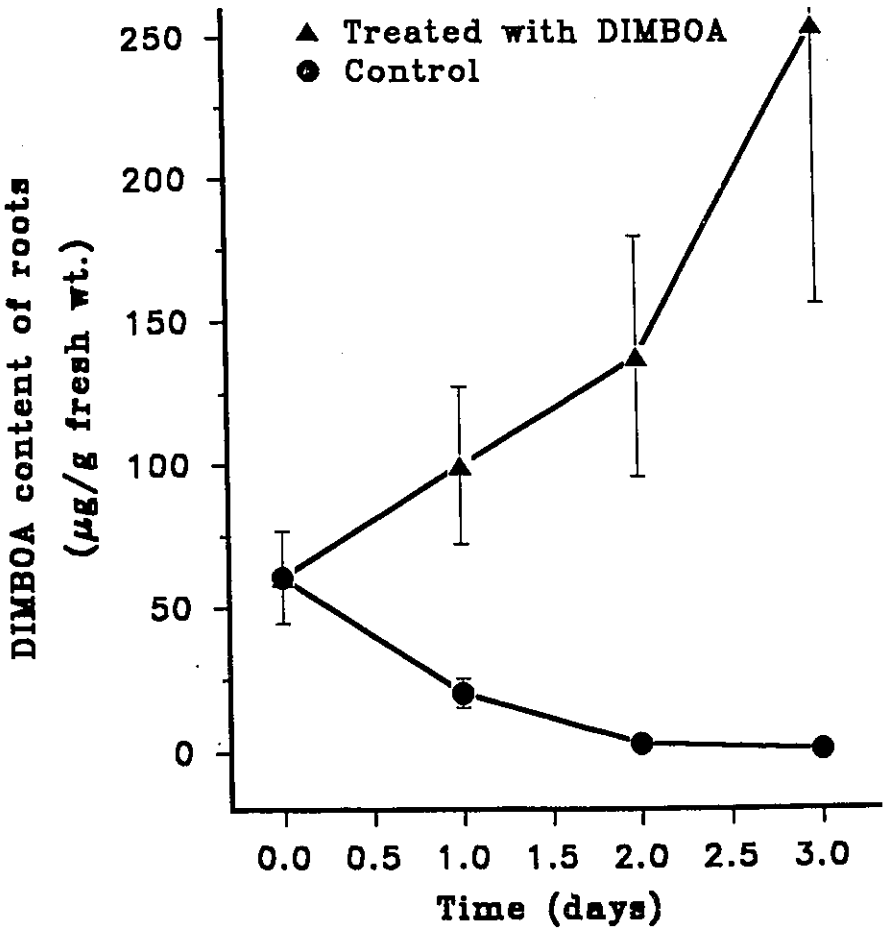

▲ Treated with DIMBOA
● Control
DIMBOA content of roots
(μg/g fresh wt.)
250
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (days)

**Fig. 5.3.**

The probit analysis of rootworm mortality

at different DIMBOA concentrations.

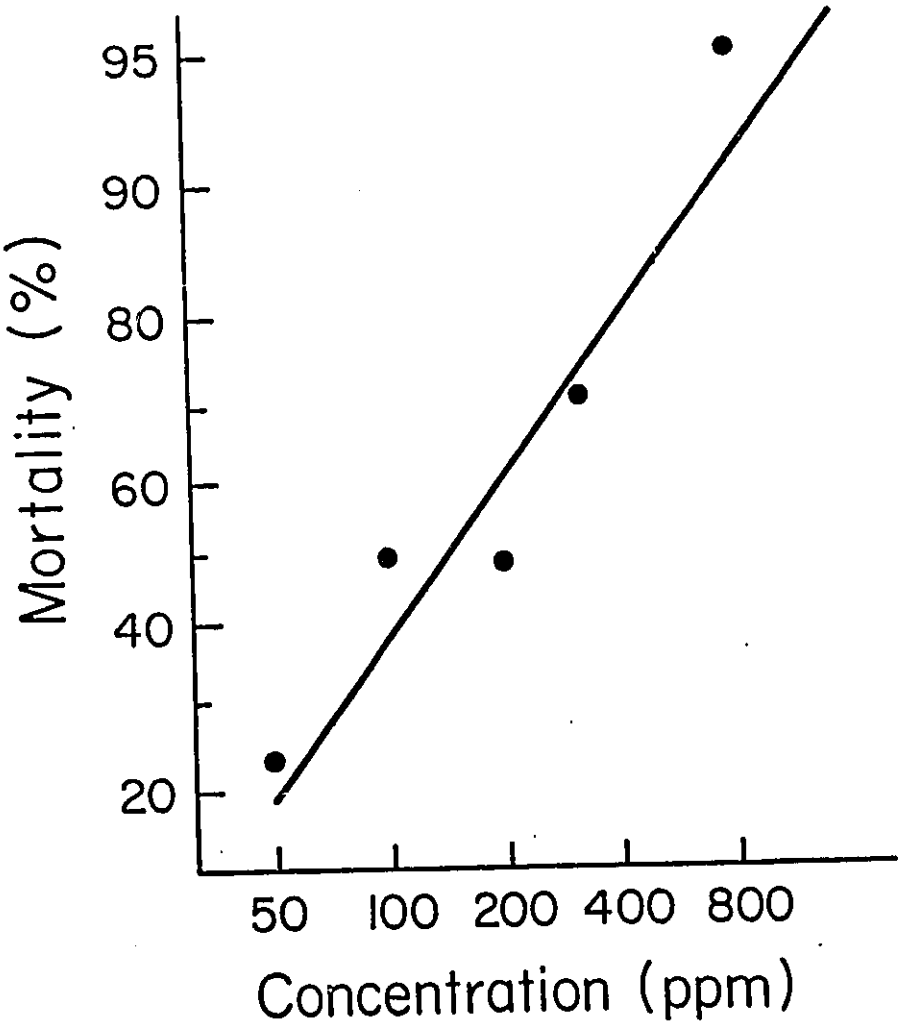

95
90
80
60
40
20
Mortality (%)
50
100
200
400
800
Concentration (ppm)

**Fig. 5.4.**

The probit analysis of rootworm mortality

at different MBOA concentrations.

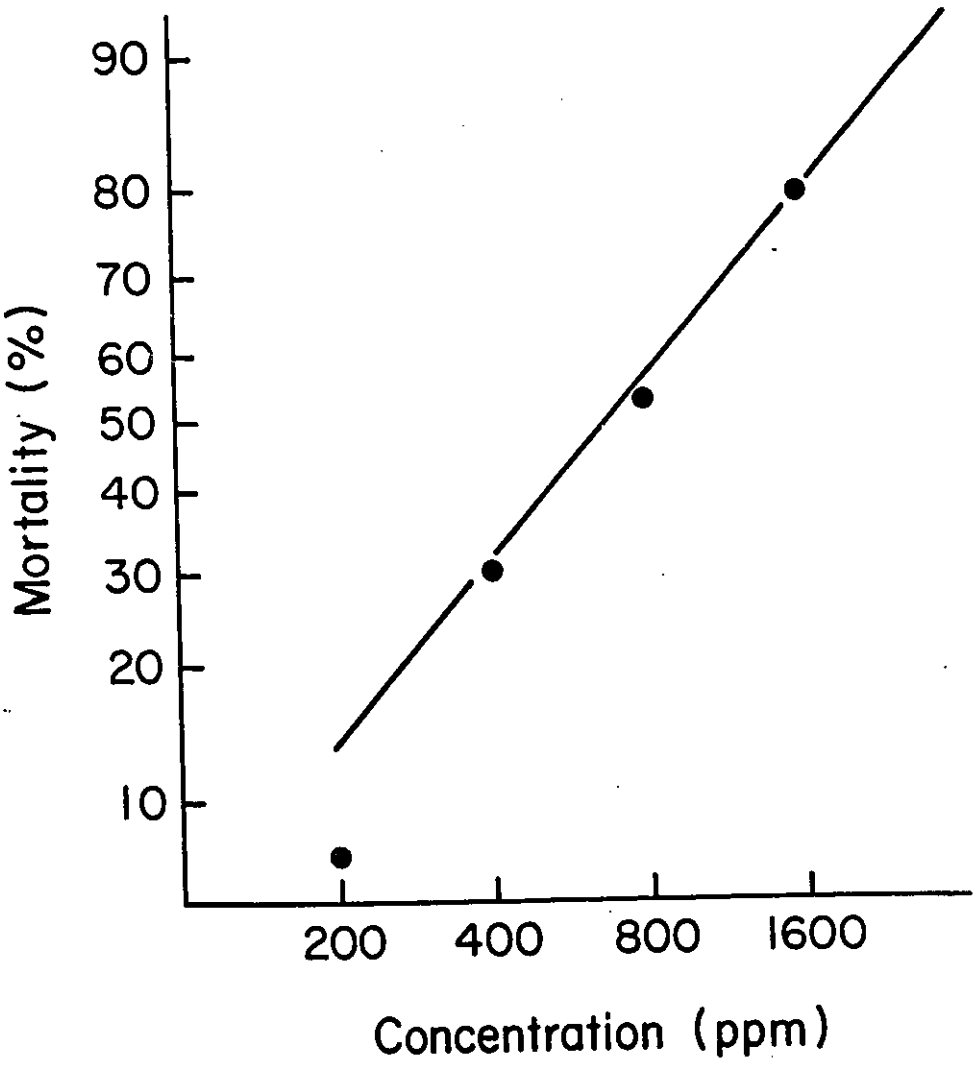

90
80
70
60
50
40
30
20
10
Mortality (%)
200
400
800
1600
Concentration (ppm)

**Table 5.1.** $LC_{50}$ and $LC_{90}$ values with 95% fiducial limits for DIMBOA and MBOA against western corn rootworm larvae

| Compound | Total no. of larvae | $LC_{50}$ (ppm) | $LC_{50}$ fiducial limits | $LC_{90}$ (ppm) | $LC_{90}$ fiducial limits |
|---|---|---|---|---|---|
| DIMBOA | 450 | 153 | 108 - 209 | 917 | 560 - 2297 |
| MBOA | 450 | 718 | 529 - 1033 | 2457 | 1524 - 7139 |

came out of the roots and died on the filter paper. We conclude that DIMBOA and MBOA act as a dose-dependent feeding deterrent, and starvation may be a contributing cause of death. In addition, we found that some larvae died in treated corn roots, which suggests possible toxicity of DIMBOA and MBOA to western corn rootworm larvae. Thus, the deleterious effects of DIMBOA and MBOA on western corn rootworm larvae are similar to those observed with other insects such as the European corn borer (Campos et al. 1989). In the present study, the result is consistent with that obtained by Campos et al. (1988, 1989), showing that DIMBOA is more toxic to insects than MBOA. Studies of nutritional indices should clarify the mode of action of DIMBOA on rootworms.

### 5.3.3. Effect of western corn rootworm on plant growth parameters: Plant responses

The pot trial results obtained in 1988 and 1989 indicated that the high DIMBOA corn line (ITR 3872) was significantly less damaged than the low DIMBOA corn line (NTR-2 Ger. 4042) when infested by western corn rootworm. An analysis of variance revealed that all plant growth parameters measured except plant height showed significant reductions ($P < 0.05$) in the low DIMBOA line (Table 5.2). Different infestation rates also were significantly different ($P < 0.05$) in the reduction of corn growth based on the measurement of plant growth parameters (Table 5.2). The interaction between corn line and year was not significant ($P > 0.05$) in any of the plant growth parameters measured (Table 5.2). However, the interaction between corn line and infestation rate was significant ($P < 0.05$) in all plant growth parameters measured except stem thickness, which suggests that the effect of corn lines on western corn rootworm depends on the level of infestation.

**Table 5.2.** ANOVA of plant height, stem thickness, plant fresh weight, root fresh weight, plant dry weight, root dry weight in 1988 and 1989 pot trials

| Source of | df | Plant height | | Stem thickness | | Plant fresh wt. | | Root fresh wt. | | Plant dry wt. | | Root dry wt. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| variation | | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P > F | F-value | P > F |
| Model | 22 | 26.68 | 0.0001 | 18.75 | 0.0001 | 15.55 | 0.0001 | 18.84 | 0.0001 | 15.09 | 0.0001 | 18.91 | 0.0001 |
| Rep. | 9 | 3.52 | 0.0008 | 3.20 | 0.0019 | 1.08 | 0.3887 | 0.70 | 0.7068 | 0.92 | 0.5081 | 0.87 | 0.5514 |
| Year | 1 | 0.51 | 0.4773 | 23.38 | 0.0001 | 18.03 | 0.0001 | 5.50 | 0.0614 | 3.04 | 0.0845 | 2.54 | 0.1146 |
| CL | 1 | 1.47 | 0.2284 | 37.00 | 0.0001 | 14.83 | 0.0002 | 10.47 | 0.0018 | 18.18 | 0.0001 | 13.76 | 0.0004 |
| IR | 3 | 177.57 | 0.0001 | 101.35 | 0.0001 | 83.13 | 0.0001 | 118.37 | 0.0001 | 92.41 | 0.0001 | 118.94 | 0.0001 |
| CL*Year | 1 | 2.10 | 0.1506 | 1.29 | 0.2593 | 1.03 | 0.3133 | 3.46 | 0.0666 | 0.42 | 0.5194 | 4.88 | 0.0560 |
| IR*Year | 2 | 0.98 | 0.3780 | 1.82 | 0.1668 | 12.19 | 0.0001 | 1.90 | 0.1566 | 5.44 | 0.0059 | 0.59 | 0.5589 |
| CL*IR | 3 | 4.56 | 0.0049 | 1.14 | 0.3379 | 3.12 | 0.0300 | 3.37 | 0.0225 | 2.53 | 0.0260 | 2.87 | 0.0415 |
| CL*IR*Year | 2 | 0.19 | 0.8259 | 9.74 | 0.0001 | 0.09 | 0.9099 | 6.16 | 0.0032 | 0.19 | 0.8245 | 4.87 | 0.0101 |

Rep. = replicates; CL = corn line; IR = infestation rate.

The relationship of all plant growth parameters measured to infestation rate of western corn rootworm for 1988 is given in Fig. 5.5. These graphs show that as the infestation rate increased, the response of the low DIMBOA line (NTR-2 Ger. 4042) plants was greater than that of the high DIMBOA line (ITR 3872) in all plant growth parameters measured except plant height. When these two corn lines were compared by a t-test at different infestation rates (100 or 200 eggs per pot), the high DIMBOA line showed significantly ($P < 0.05$) less damage than the low DIMBOA line. Differences were observed in all plant growth parameters measured except plant height. When these two corn lines were infested with 400 eggs per pot, both corn lines showed similar damage, and none of the plant growth parameters measured was significantly different ($P > 0.05$) (Fig. 5.5). The data indicate that corn resistance to these insects is a relative characteristic, and ITR 3872, even with a higher level of resistance, is seriously damaged under the feeding pressure of such a high insect population.

The data obtained in 1989 (Fig. 5.6) were consistent with those obtained in 1988. None of the plant growth parameters measured for the two corn lines were significantly different ($P > 0.05$) in the control (not infested with eggs) except for a higher root biomass in the low DIMBOA line (Fig. 5.6). A lower root biomass found in the high DIMBOA line is, perhaps, a cost for maintaining higher DIMBOA levels, but further investigation is required to confirm it. As well, Fig. 5.6 shows the relationship of all plant growth parameters to infestation rate of western corn rootworm. It indicates that as the infestation rate increased, the low DIMBOA corn line had a greater response than the high DIMBOA line in all plant parameters measured. When these two corn lines were

**Fig. 5.5.**

Relation of plant growth parameters (plant height, stem thickness, plant fresh weight, plant dry weight, root fresh weight, and root dry weight) with infestation rate for western corn rootworm comparing a high DIMBOA (ITR 3872) and low DIMBOA (NTR-2 Ger. 4042) corn line in 1988 pot trial. Asterisks indicate the plant parameter is significantly different between two corn lines at that infestation rate ($P < 0.05$; Duncan's multiple range test). Bars indicate standard error of mean.

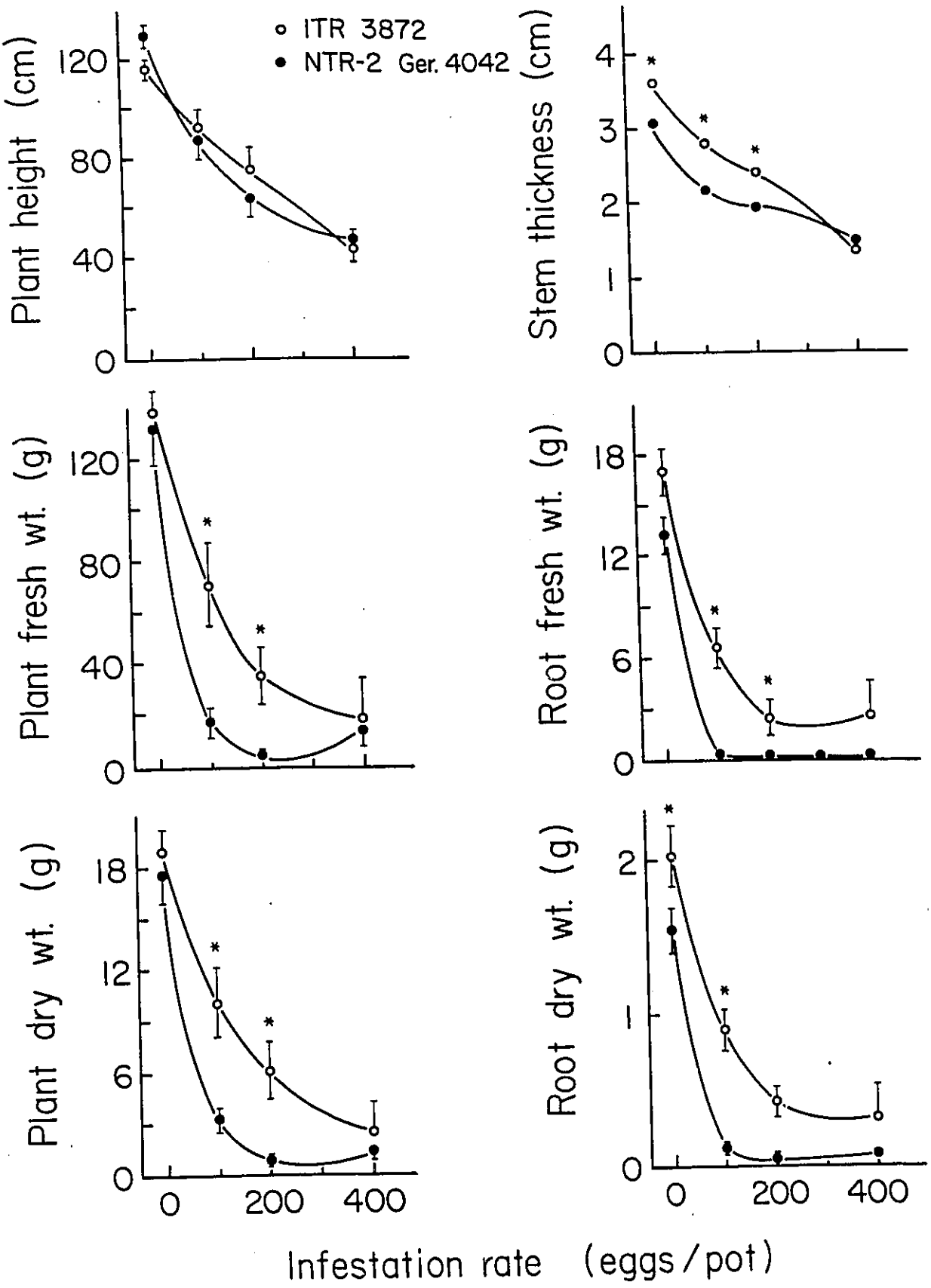
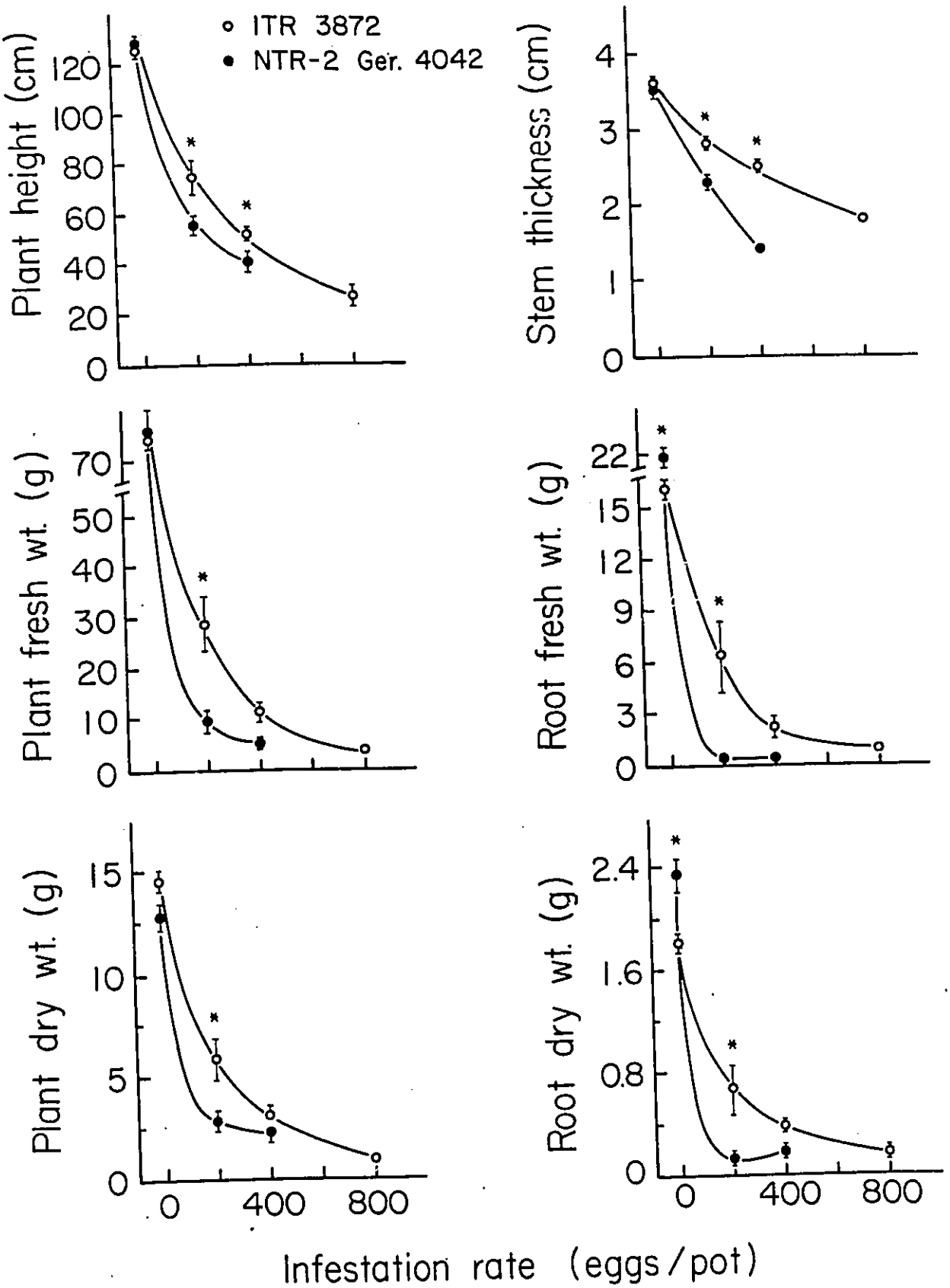

ITR 3872
NTR-2 Ger. 4042
Plant height (cm)
120
80
40
0
Stem thickness (cm)
4
3
2
1
0
Plant fresh wt. (g)
120
80
40
0
Root fresh wt. (g)
18
12
6
0
Plant dry wt. (g)
18
12
6
0
Root dry wt. (g)
2
1
0
0
200
400
Infestation rate (eggs/pot)

**Fig. 5.6.**

As in Fig. 5.5 for 1989 pot trial. All plants of low DIMBOA line (NTR-2 Ger.4042) in the infestation rate of 800 eggs per pot died before assessment.


ITR 3872
NTR-2 Ger. 4042
Plant height (cm)
Stem thickness (cm)
Plant fresh wt. (g)
Root fresh wt. (g)
Plant dry wt. (g)
Root dry wt. (g)
Infestation rate (eggs/pot)

compared at the infestation rate of 200 eggs per pot, the high DIMBOA line showed significantly ($P < 0.05$) less damage than low DIMBOA line in all plant growth parameters (Fig. 5.6). When both corn lines were infested with 400 eggs per pot, the results were the same as that obtained in 1988.

In the treatment of 800 eggs per pot (Fig. 5.6), all plants of the low DIMBOA line died and 40% of the high DIMBOA corn line survived. Thus, the high DIMBOA line, even with a higher level of resistance, suffered substantial mortality when highly infested. These data also agree with the results of 1988.

### 5.3.4. Effect of different maize lines on western corn rootworm: Insect responses

**Effect of two different corn lines on western corn rootworm adults.** The results obtained in both years were consistent in showing that the adult emergence number, adult weight, and adult size (adult head-capsule width) were all reduced in the high DIMBOA corn line. In the 1989 test, there was an obvious trend showing a lower mean number of adults and lower mean weight of adults emerged from the high DIMBOA corn line as compared to the low one, even though they were not statistically significant ($P > 0.05$) (Table 5.3). In the 1990 test, the results indicated that the mean number of adults emerged from the high DIMBOA line was significantly ($P < 0.05$) less than that from the low one, and that adults emerged from the high DIMBOA line were significantly ($P < 0.05$) smaller than those from the low DIMBOA line based on measurement of adult mean weight (replicate II) and adult head-capsule width (replicate II, III) (Table 5.3).

**Table 5.3.** Effect of different corn lines with different DIMBOA content on adult emergence, adult wt., and adult head-capsule width of western corn rootworm

| Replicates (year) | Corn line | Mean number | Mean wt. (mg) | Head-capsule width (mm) |
|---|---|---|---|---|
| I (1989) | ITR 3872 | 1.30 a[1] (0.62) | 8.72 a (0.60) | N.D.[2] |
| | NTR-2 Ger.4042 | 2.30 a (1.02) | 9.66 a (0.31) | N.D. |
| II (1990) | ITR 3872 | 2.70 a (0.73) | 6.81 a (0.20) | 1.17 a (0.01) |
| | NTR-2 Ger.4042 | 5.44 b (0.58) | 8.23 b (0.19) | 1.22 b (0.01) |
| III (1990) | ITR 3872 | 2.90 a (0.57) | 8.08 a (0.30) | 1.16 a (0.01) |
| | NTR-2 Ger.4042 | 5.50 b (0.38) | 8.28 a (0.18) | 1.23 b (0.01) |

1. Means in the same column within the same replicate followed by the same letter are not significantly different (P = 0.05; t-test). Standard error of mean in parentheses.
2. N.D. = not determined.

Population density of western corn rootworm has been reported as a factor that influences size, longevity, and fecundity of adult western corn rootworm (Branson and Sutter 1985; Weiss et al. 1985). We believe that DIMBOA is a phytochemical factor which stresses larvae to produce inferior adults, but further investigation is required to elucidate its mode of action.

**Effect of seven different corn lines on western corn rootworm larvae.** Significant differences ($P < 0.05$) in the concentration of DIMBOA were found in different corn lines selected by ANOVA. The correlation analysis revealed a significant inverse relationship ($r = -0.81$, $P = 0.0265$) between the concentration of DIMBOA in roots and the total number of larvae developed. In high DIMBOA corn lines, the total number of larvae developed was significantly less than that developed in low DIMBOA corn lines (Fig. 5.7).

As well, mean wt. of western corn rootworm larvae was negatively related to the content of DIMBOA in corn roots (Fig. 5.8). A significant correlation ($r = -0.95$, $P = 0.0013$) between DIMBOA content and mean wt. of western corn rootworm larvae was found by correlation analysis.

The body size of western corn rootworm larvae developed in high DIMBOA varieties was stressed as measured by larval head-capsule width. The larval head-capsule width in high DIMBOA corn varieties was significantly smaller than that in low DIMBOA varieties (Fig. 5.9). An inverse relationship between DIMBOA content and larval head-capsule width was also significant ($r = -0.94$, $P = 0.0016$).

**Fig. 5.7.**

Relationship between DIMBOA content and total number of western corn rootworm larvae. Total number of larvae plotted versus DIMBOA content in roots (Log scale).

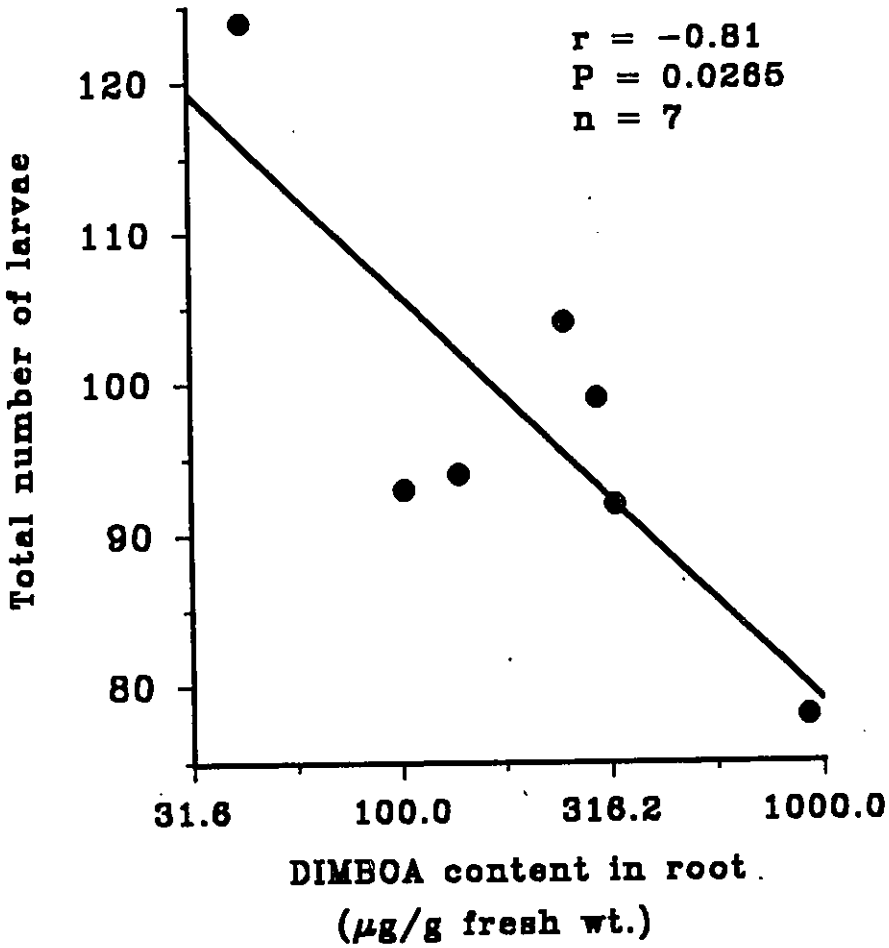

r = −0.81
P = 0.0265
n = 7
120
110
100
90
80
Total number of larvae
31.6
100.0
316.2
1000.0
DIMBOA content in root
(μg/g fresh wt.)

**Fig. 5.8.**

Relationship between DIMBOA content and mean wt. of western corn rootworm larvae. Mean wt. of larvae plotted versus DIMBOA content in roots (Log scale).

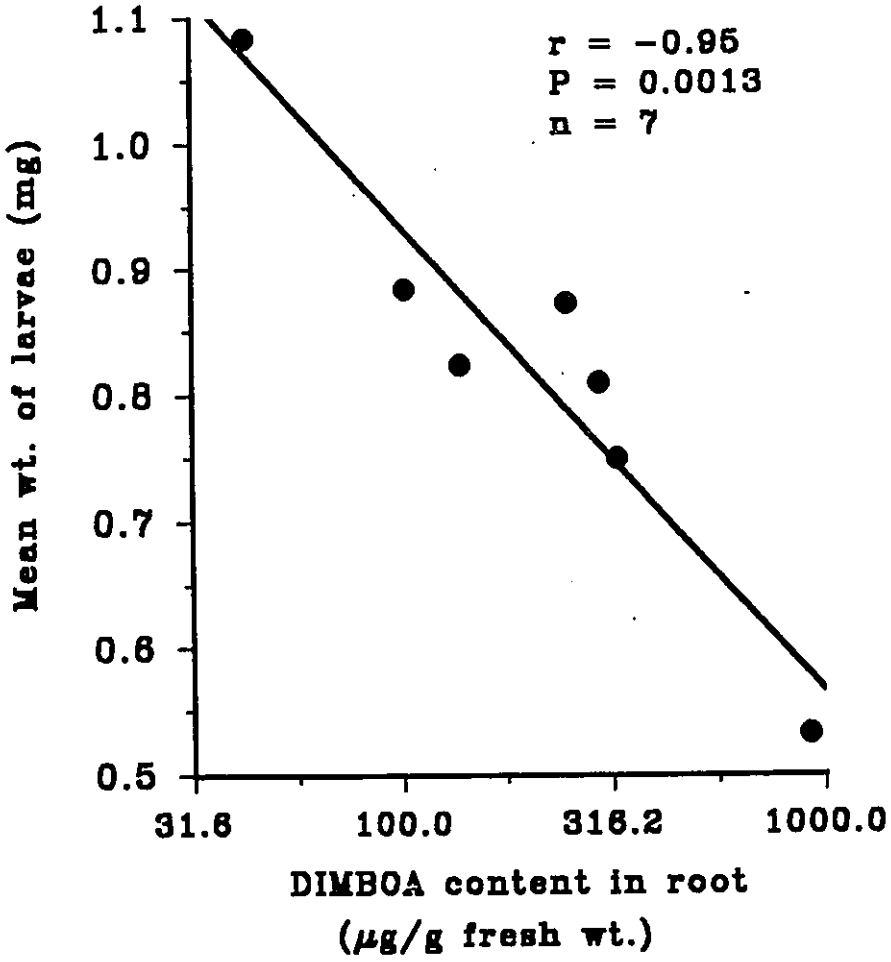
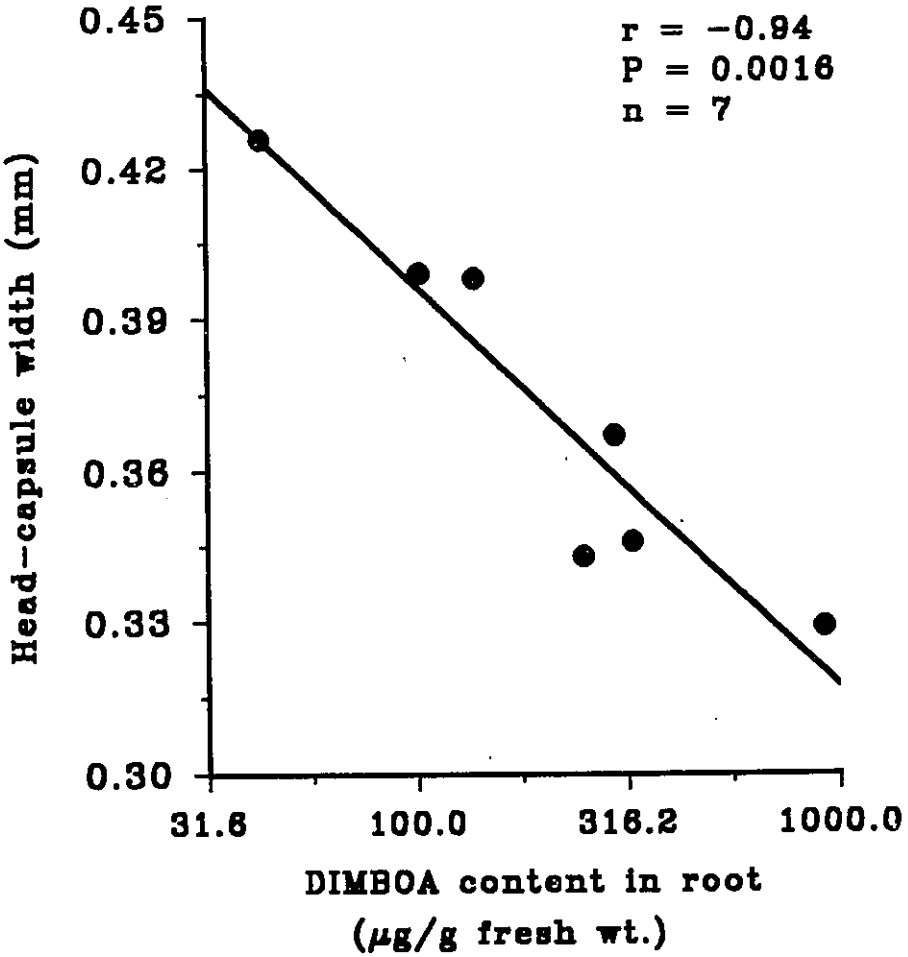

r = −0.95
P = 0.0013
n = 7
Mean wt. of larvae (mg)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
31.6
100.0
316.2
1000.0
DIMBOA content in root
(μg/g fresh wt.)

**Fig. 5.9.**

Relationship between DIMBOA content and head-capsule width of western corn rootworm larvae. Mean width of larval head capsule plotted versus DIMBOA content in roots (Log scale).


r = −0.94
P = 0.0016
n = 7
0.45
0.42
0.39
0.36
0.33
0.30
Head-capsule width (mm)
31.6
100.0
316.2
1000.0
DIMBOA content in root
(μg/g fresh wt.)

Clearly, the western corn rootworm larvae developed in the high DIMBOA line were stressed. We believe that the stress observed in the insects developed from the high DIMBOA lines is related to the *in vivo* levels of DIMBOA.

Painter (1951) proposed three mechanisms of plant resistance to insects: nonpreference and antibiosis, which are characterized by an insect response to a host plant, and tolerance, which is characterized by a host plant response to an insect attack. In the case of the corn rootworm and corn, Ortman et al. (1974) defined tolerance as a capability to regenerate a root system after damage. The data in this study suggest that the resistance shown by ITR 3872 was not tolerance according to this definition since the parameters in root mass were significantly different between control and infested treatments. The data presented here are consistent with an antibiosis mechanism based on the effect of the phytochemical DIMBOA on western corn rootworm. The phytochemical analysis of these two corn lines showed that DIMBOA content in roots of the resistant line ITR 3872 was 1304 $\mu$g per g fresh root, 3-fold more than that in the susceptible line NTR-2 Ger. 4042 (411 $\mu$g per g fresh root). We have recently determined that hydroxamic acids detected by HPLC are concentrated in the cortex of corn root (see Chapter III) which is the feeding site of western corn rootworm larvae. Based on the observations described above, we believe that DIMBOA is a major factor which contributes to ITR 3872 resistance to western corn rootworm.

### 5.3.5. Relationship between levels of sugar and nitrogen and larval growth parameters of western corn rootworm

**Determination of sugar.** The total sugar (Table 5.4) present in corn roots was significantly different in the five corn lines ($F = 4.82$, $P < 0.05$). It ranged from 3.22 $\mu$g/g fresh root to 4.08 $\mu$g/g fresh root. The corn lines NTR-1 3946 and ITR 3872 showed the highest levels of sugar, and NTR-2 4034 had the lowest levels of sugar.

**Determination of total nitrogen.** Table 5.4 also showed the results of the determination of total nitrogen in different corn lines. Significant differences in the content of total nitrogen were found in various corn lines ($F = 40.28$, $P < 0.01$). The corn line ITR 3872 showed the highest percentage of nitrogen in roots, and the line NTR-2 4042 had the lowest percentage of nitrogen in roots, while intermediate levels were found in the other corn lines (Table 5.4).

**Relationship between levels of sugar and nitrogen and larval growth parameters of western corn rootworm.** The results indicated that a significant negative correlation existed between sugar content and larval head-capsule width ($r = -0.895$, $P < 0.05$) (Fig. 5.10), and all other larval growth parameters measured were also negatively related to sugar content in corn roots (Appendix 2).

Sugar is generally believed to be a feeding stimulant to many phytophagous insects (Schoonhoven 1982). However, sugar was found to be a resistance factor in the interactions of peas and pea aphid (Auclair et al. 1957), and winter wheat and the English grain aphid (Xie et al. 1987). Aphids on high sugar plants have to suck much more plant sap in order

**Table 5.4.** Concentrations of total sugar and nitrogen in maize roots

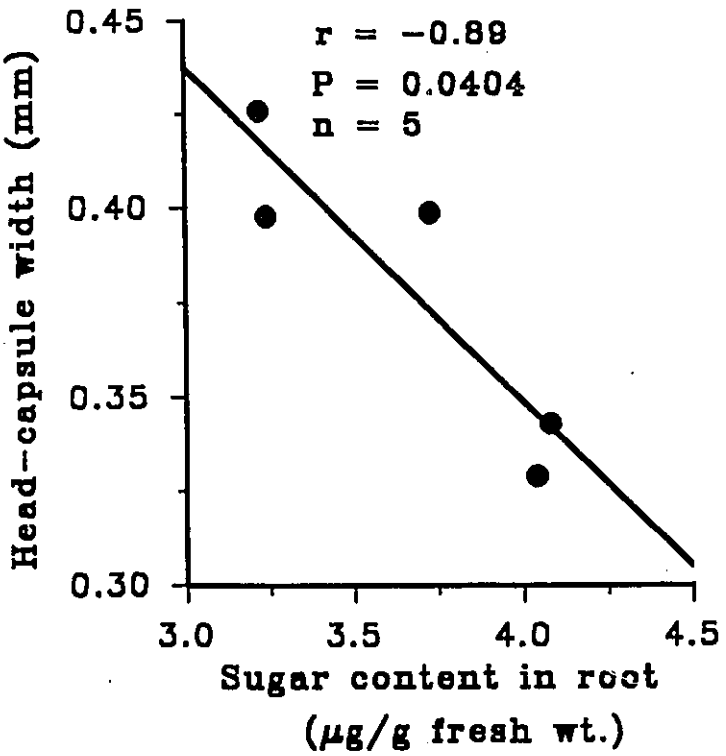
| Corn lines | Total sugar ($\mu$g/g fresh wt.) | Total nitrogen (%) |
|---|---|---|
| ITR 3872 | 4.04 a* | 2.36 a |
| | (0.28) | (0.05) |
| NTR-1 3946 | 4.08 a | 2.10 b |
| | (0.14) | (0.03) |
| NTR-2 Canada 4072 | 3.24 b | 2.01 b |
| | (0.06) | (0.05) |
| NTR-2 Switz. 4034 | 3.22 b | 2.04 b |
| | (0.25) | (0.02) |
| NTR-2 Ger. 4042 | 3.73 ab | 1.73 c |
| | (0.14) | (0.01) |

* Means in the same column followed by the same letter are not significantly different ($P > 0.05$; Duncan's multiple range test). Standard error of mean in parentheses.

**Fig. 5.10.**

Relationship between sugar content and

head-capsule width of western corn rootworm larvae.


r = −0.89
P = 0.0404
n = 5
0.45
0.40
0.35
0.30
Head-capsule width (mm)
3.0
3.5
4.0
4.5
Sugar content in root
(μg/g fresh wt.)

to obtain sufficient nitrogen component (Schaefer 1938).

As well, negative trends between all larval growth parameters measured and total nitrogen in corn roots were found although these correlations were not significant ($P > 0.05$) (Appendix 3). Generally, plant nitrogen is positively related to phytophagous insect growth (Mattson 1980). The trends observed here indicated that total nitrogen contents in corn roots may reflect the amounts of hydroxamic acids. In fact, a significantly positive correlation between total nitrogen contents and DIMBOA concentrations in corn roots was obtained ($r = 0.806$, $P < 0.05$). In another plant-insect system, no correlation was found between total nitrogen in tomato leaves and number of the tomato fruitmoth larvae per leaf in the field, nor with the consumption of tomato leaf disks by tomato fruitmoth in the laboratory (Estay 1987).

In conclusion, the role of plant primary metabolites, such as sugar and nitrogen, in the interaction of plants and insects is very complex, and may vary greatly depending upon the species. In the present study, the fact that the sugar and nitrogen contents were negatively correlated to the larval growth of western corn rootworm suggests that sugar may be linked to some other biologically active compound, and the role of nitrogen in this interaction is believed to be linked to hydroxamic acids.

# CHAPTER VI.

# BEHAVIORAL RESPONSES OF WESTERN CORN ROOTWORM NEONATES TO NATURALLY OCCURRING AND SYNTHETIC HYDROXAMIC ACIDS

## 6.1. Introduction

Behavioral bioassays for western corn rootworm larvae have been developed to determine the role of carbon dioxide ($CO_2$) released by roots of corn plant and volatile semiochemicals from corn seedlings in host location by western corn rootworm larvae (Strnad et al. 1986; Strnad and Bergman 1987; Hibbard and Bjostad 1988). The results revealed that both a plant primary metabolite ($CO_2$) and secondary metabolites (volatile semiochemicals) are involved as the attractive agents in the orientation of western corn rootworm larvae. As well, volatile chemicals from corn silks have been demonstrated to be attractants to adults of western and northern corn rootworm (Prystupa et al. 1988). However, the behavioral responses of western corn rootworm larvae to plant secondary metabolites, which are known as protective agents in the defence of plant against insect pests and diseases, are unknown. Although volatile chemicals are well known to be involved in the orientation of insect to plant, nonvolatile chemicals usually serve as the factors which lead to final acceptance or rejection (Beck and Schoonhoven 1980).

The search orientation in animals is recognized to take place in two phases: ranging and local search (Jander 1975). The former is characterized by relatively straight locomotion in which the animal has limited resource information, and the latter is

characterized by increased turning rate and decreased locomotory rate when animals receive information of suitable resources (Bell 1985). In a study of host searching behaviour with corn rootworm, Strnad and Dunn (1990) demonstrated that a decreased search area and locomotory rate and a increased turning rate and path crossings occurred after western corn rootworm larvae contacted host roots as compared with non-host roots.

Although some hydroxamic acids have been shown as resistance factor of corn to corn insect pests and pathogens (e.g. Klun and Brindly 1966; Long et al. 1977; Campos et al. 1988, 1989), including rootworm (see Chapter V), it is not clear what behavioral responses western corn rootworm larvae have after contact with naturally occurring or synthetic hydroxamic acids, and it is unknown if the host searching behaviour is affected by these secondary metabolites. The objective of this chapter is to characterize searching behaviour of western corn rootworm larvae after contact with maize roots treated with various hydroxamates, and to determine the choice behaviour shown by western corn rootworm larvae on maize roots treated with different compounds.

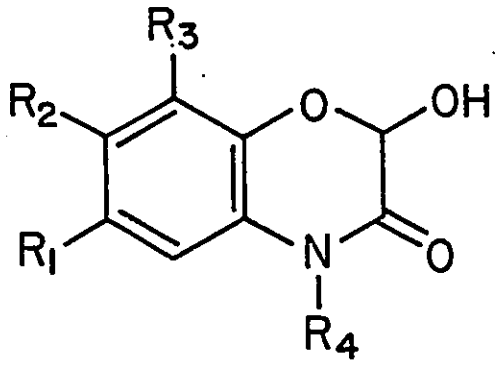
## 6.2. Materials and Methods

### 6.2.1. Sample preparation

**Source of compounds.** The compounds used in this study include those naturally occurring in maize roots and those synthesized. All these compounds were synthesized in our laboratory and represent a good range of known chemical trends, either unimolecular decomposition rates, or reduction by thiol (Atkinson 1989). Complete synthetic details are described by Atkinson (1989). The structures of these chemicals are shown in Fig. 6.1.

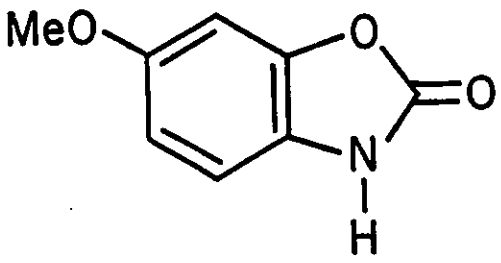
**Fig. 6.1.**

Chemical structures of naturally occurring and synthetic 1,4-benzoxazin-3-ones and benzoxazolin-2-one used in this study.




1,4-benzoxazin-3-ones

| $R_1$ | $R_2$ | $R_3$ | $R_4$ | Abbreviation |
|---|---|---|---|---|
| H | MeO | H | OH | DIMBOA |
| H | MeO | MeO | OH | $DIM_2BOA$ |
| MeO | MeO | H | OH | 6,7-diMeO |
| H | t-Bu | H | OH | 7-t-Bu |
| H | Me | H | OH | 7-Me |
| H | H | H | OH | DIBOA |
| H | Cl | H | OH | 7-Cl |
| H | F | H | OH | 7-F |
| H | Meo | H | H | HMBOA |




benzoxazolin-2-one (MBOA)

**Maize root materials.** Dried maize kernels (unspecified hybrid) were washed in detergent solution for 3 min, rinsed thoroughly, and soaked in distilled water for 24 h. Soaked seeds were rinsed again with distilled water, placed on moistened paper, covered with another moistened paper towel, kept in a closed plastic container, and incubated at room temperature (25 °C). Three to four days later, fresh maize roots (2-3 cm long) were removed for bioassays.

### 6.2.2. Choice study

Fresh maize roots were dipped for 30 seconds in ethanolic solution of various hydroxamates (500 ppm) or distilled water with the same amount of solvent (0.1% EtOH) (solvent control). One treated and one control root were placed 1 cm apart on moistened filter paper in a plastic petri dish (9 cm). One neonate western corn rootworm was placed between the roots. The dish was covered, sealed with parafilm, and kept in a incubator (25 °C, dark condition). The larval position (in root, on root, or out of root) was examined for each root (treated and control) at 24 h after infestation. Thirty replicates (30 larvae) were prepared for each compound, and the whole experiment was repeated four times.

### 6.2.3. Host searching behaviour

**Effects of hydroxamic acids on localized search.** The behavioral responses of western corn rootworm neonate to maize root treated with hydroxamic acids were recorded

by a modification of the method described by Strnad and Dunn (1990). Fresh maize roots were dipped in a solution of compound (500 ppm) for 30 seconds. Distilled water alone was served as a true control and distilled water with the same amount of solvent (0.1% EtOH) served as a solvent control. Individual neonates (<24 h old) of western corn rootworm were placed on the surface of treated roots. After a 5-min period of contact, the larva was transferred to the centre of a moistened filter paper (Whatman No. 1, 24 cm) on a square glass plate (30 by 30 cm) with 4 supports (3 mm high) in each corner, and then covered with a second glass plate. An acetate sheet was placed on the top of second glass plate. During a 5-min period, the path of larval travel was traced on the acetate sheet by following the larval travel with a felt pen. Meanwhile, the number of turns (defined as any change of the larval body in axis of travel) was recorded using a hand counter. Thirty larvae were used for each treatment (compounds and controls), and a fresh area of filter paper was used for each larva. Finally, acetate sheets were copied to grid paper (1 square per mm). The area searched ($mm^2$) was determined by counting the number of squares intersected by the path. Path lengths were measured with a map wheel and converted to locomotory rate (mm/min).

**Concentration-dependence of localized search.** To determine if the effects of hydroxamic acids on the localized search of western corn rootworm neonates depends on the concentration of the compounds, different concentrations of the selected compounds were used for testing the search behaviour of western corn rootworm larvae. Two compounds, DIMBOA and MBOA, were selected, and three concentrations, 250, 500, and 1000 ppm, for each were used in this study. Number of turns, area searched, and locomotory rate were determined as described above.

### 6.2.4. Statistical analysis

The data from choice tests were calculated as percent control. Since there were no larvae found on the root or out of the root in some treatments, the number of larvae on root and out of root was transformed with square root (X+1) (Snedecor and Cochran 1980) for analysis. All data were tested by a normality test. If they were normal, an analysis of variance (ANOVA) program (SAS Institute 1982) was performed. When the differences found by ANOVA were significant ($P < 0.05$), Duncan's multiple range test was applied for comparisons between different means (Steel and Torrie 1980).

## 6.3. Results and Discussion

### 6.3.1. Choice study

The normality test showed that the behavioral data were normal, and consequently parametric analysis was performed. All hydroxamates used in this study were shown to be effective deterrents in the choice test design (Table 6.1). Of the three naturally occurring hydroxamic acids (1, 2, 9) in maize roots, two (1, 9) strongly affected rootworm feeding, which was only 58.40% and 55.05% of control. Compound 3 showed the least effect possibly due to its previously observed rapid decomposition which was the highest of all compounds tested (Atkinson 1989). There were no significant differences ($P > 0.05$) between the results obtained with the 7-substituted series, except for 7-Cl, which showed the highest behavioral effect (Table 6.1).

**Table 6.1.** Western corn rootworm larval position (% control) in a choice test with fresh maize roots treated with different hydroxamates

| Compounds | | Larval position | | |
|---|---|---|---|---|
| No. | Substituent | In root (%) | On root (%) | Out of root (%) |
| 3 | 6,7-diMeO | 79.7 a* | 174.8 a | 141.4 a |
| | | (13.2) | (19.3) | (0.0) |
| 2 | 7,8-diMeO | 77.8 a | 138.4 a | 126.3 abc |
| | ($DIM_2BOA$) | (7.7) | (26.5) | (9.8) |
| 6 | 7-H | 73.2 ab | 125.7 a | 149.1 a |
| | (DIBOA) | (8.5) | (12.3) | (25.5) |
| 4 | 7-t-Bu | 68.5 ab | 147.2 a | 139.0 a |
| | | (4.6) | (23.6) | (15.0) |
| 8 | 7-F | 67.9 ab | 141.3 a | 92.7 bc |
| | | (6.9) | (29.1) | (7.3) |
| 5 | 7-Me | 64.3 ab | 121.5 a | 134.2 a |
| | | (1.6) | (3.9) | (15.5) |
| 10 | MBOA | 62.5 ab | 121.3 a | 80.2 c |
| | | (8.6) | (22.6) | (12.2) |
| 1 | 7-MeO | 58.4 ab | 152.8 a | 115.9 abc |
| | (DIMBOA) | (8.6) | (20.3) | (10.0) |
| 9 | HMBOA | 55.1 ab | 122.2 a | 153.7 a |
| | | (7.2) | (17.4) | (21.5) |
| 7 | 7-Cl | 48.1 b | 167.2 a | 120.7 abc |
| | | (2.3) | (16.2) | (11.9) |

* Means in the same column followed by the same letter are not significantly different ($P > 0.05$; Duncan's multiple range test). Standard error of mean in parentheses.

Generally, choice designs are useful in detecting small differences in food acceptability (Schoonhoven 1982). It is clear that when fresh maize roots were treated with various hydroxamates, all compounds tested reduced the food acceptability, showing that the percentage of rootworm found into roots decreased and the percentage of rootworm stayed on the surface of roots increased (Table 6.1). The number of rootworm larvae which stayed on the surface of the roots in all treatments was approximately 20-75% more than in the control, and there were no significant differences ($P > 0.05$) between various compounds because of the high standard errors.

### 6.3.2. Host searching behaviour

**Effects of various compounds in localized search.** Two controls were designed in order to see if the control with solvent (0.1% EtOH) affected behavioral responses of western corn rootworm larvae. The result showed that there were no significant differences ($P > 0.05$) between the solvent treated control and the true control (distilled water alone) in all behavioral parameters studied (Table 6.2). Fig. 6.2 showed two distinct path patterns resulted from 5 min contact with corn roots treated with related compounds and control. Compared to controls, the searching behaviour of neonate western corn rootworm larvae was obviously affected by treating maize roots with hydroxamate solution (Fig 6.2, Table 6.2). Neonate western rootworm larvae exposed to maize roots treated with various hydroxamates significantly ($P < 0.05$) decreased the number of turns, but increased their search area and locomotory rate significantly ($P < 0.05$) (Table 6.2). The naturally

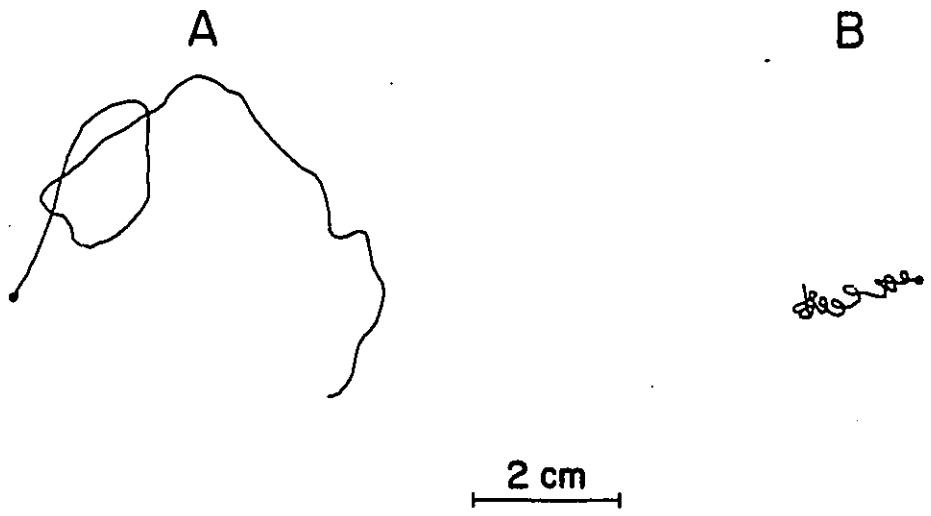
**Table 6.2.** Number of turns, area searched, and locomotory rate of western corn rootworm neonates during a 5-min host-searching period after removal from maize root treated with hydroaxamates solution

| Compounds No. | Substituent | Number of turns | | Area searched ($mm^2$) | | Locomotory rate (mm/min) | |
|---|---|---|---|---|---|---|---|
| | Control | 61.3 | a* | 91.7 | f | 16.8 | b |
| | ($H_2O$ only) | (4.9) | | (8.1) | | (1.4) | |
| | Control | 58.5 | a | 116.9 | ef | 20.1 | b |
| | (with solvent) | (5.7) | | (9.2) | | (1.5) | |
| 9 | HMBOA | 36.2 | bc | 158.9 | cde | 27.5 | a |
| | | (2.8) | | (13.8) | | (2.3) | |
| 1 | 7-MeO | 30.4 | bc | 165.7 | bcd | 31.9 | a |
| | (DIMBOA) | (3.2) | | (11.8) | | (1.9) | |
| 2 | 7,8-diMeO | 26.9 | cd | 157.2 | cd | 28.1 | a |
| | ($DIM_2BOA$) | (3.7) | | (18.5) | | (2.2) | |
| 10 | MBOA | 25.4 | cd | 203.8 | ab | 22.5 | a |
| | | (2.5) | | (15.8) | | (2.1) | |
| 4 | 7-t-Bu | 24.2 | cd | 171.4 | abc | 28.8 | a |
| | | (1.7) | | (12.9) | | (1.7) | |
| 5 | 7-Me | 22.0 | cd | 132.9 | cdef | 21.6 | b |
| | | (2.5) | | (17.1) | | (2.3) | |
| 8 | 7-F | 20.1 | d | 210.5 | a | 33.3 | a |
| | | (1.9) | | (15.0) | | (2.0) | |
| 7 | 7-Cl | 18.3 | d | 176.4 | abc | 27.7 | a |
| | | (1.7) | | (14.1) | | (1.9) | |
| 6 | 7-H | 17.6 | d | 124.8 | def | 20.8 | b |
| | (DIBOA) | (2.1) | | (13.7) | | (1.9) | |
| 3 | 6,7-diMeO | 16.9 | d | 176.8 | abc | 28.3 | a |
| | | (1.5) | | (13.3) | | (1.7) | |

* Means in the same column followed by the same letter are not significantly different ($P > 0.05$; Duncan's multiple range test). Standard error of mean in parentheses.

**Fig. 6.2.**

Pathways of neonate western corn rootworm larvae during 5 min localized search resulting from 5 min contact with maize roots treated with hydroxamate (6,7-diMeO) (A), and with distilled water (control) (B). Dot indicates beginning point in each path.


A
B
2 cm

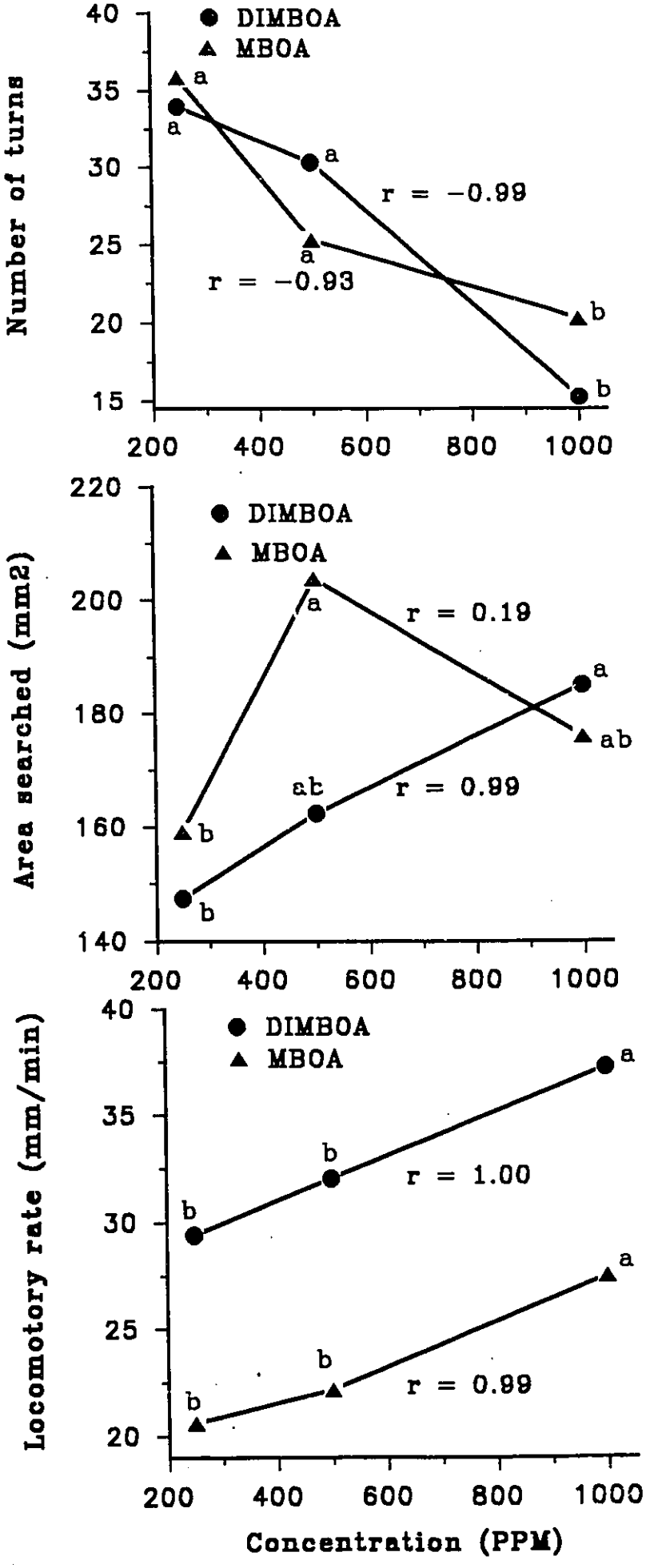
occurring compounds DIMBOA, as well as its amide HMBOA and its decomposition product MBOA, and $DIM_2BOA$ showed similar effects on number of turns, area searched (except MBOA), and locomotory rate. In general, the compounds 3, 4, 5, and 7 of 7-substituted series showed higher activities in this respect (Table 6.2).

All trials in this study appear to show activities for all compounds tested, but no correlation of these activities to known chemical trends, such as unimolecular decomposition rates, reduction by thiols, nor case of hemithoacetal formation, was found.

**Concentration-dependence of localized search.** Localized search orientation of insects can be modified by sensory information from internal and external sources (Bell 1985). For example, the search path of starved fruit fly, *Drosophila melanogaster*, on and around a 3.5 cm patch of sucrose is dependent on sucrose concentration (Bell 1985). The area searched by *D. melanogaster* on a patch of 0.5 M sucrose is significantly larger than that of a patch of 0.125 M sucrose (Bell 1985). The present results showed that the effects of hydroxamic acids on the localized search of western corn rootworm larvae were also dependent on the concentrations of the hydroxamic acids (Fig. 6.3). As the concentrations of DIMBOA and MBOA increased, larvae exposed to treated maize roots significantly decreased the number of turns, but increased their searched area (except for MBOA) and locomotory rate. At 250 and 500 ppm of DIMBOA and MBOA, the trends were evident but not significant ($P > 0.05$). However, the parameters were significantly different ($P < 0.05$) when insects were exposed to maize roots treated with 1000 ppm of DIMBOA and MBOA (Fig. 6.3). Considering that DIMBOA and MBOA were feeding deterrents for western corn rootworm (see Chapter V), it is not surprising to find that the number of

**Fig. 6.3.**

Number of turns, area searched, and locomotory rate of neonate western corn rootworm larvae during a 5 min host-searching period resulting from 5 min contact with maize roots treated with various concentrations (250, 500, and 1000 ppm) of DIMBOA and MBOA. Each figure is the mean of 30 larvae which individually tested. Means followed by the same letter (within the data for the same compound) are not significantly different ($P > 0.05$; Duncan's multiple range test).


DIMBOA
MBOA
Number of turns
40
35
30
25
20
15
200
400
600
800
1000
a
a
a
a
b
b
r = −0.99
r = −0.93
DIMBOA
MBOA
Area searched (mm2)
220
200
180
160
140
200
400
600
800
1000
a
a
ab
ab
b
b
r = 0.19
r = 0.99
DIMBOA
MBOA
Locomotory rate (mm/min)
40
35
30
25
20
200
400
600
800
1000
Concentration (PPM)
a
b
b
a
b
b
r = 1.00
r = 0.99

turns decreased and area searched and locomotory rate increased when larvae exposed to maize roots treated with higher concentrations of the selected compounds.

Generally, an insect's search orientation is controlled by information either stored genetically or learned from the sensory system (Bell 1985). After neonate larvae of western corn rootworm made contact with maize roots, they switched from ranging to a localized search. Our assay involves contact of neonate larvae with maize roots, and hence chemoreception of nonvolatiles is possible. After encountering food resources of the controls in which feeding stimuli are present, neonate rootworm larvae shift their locomotion from straight to convoluted patterns. Our results are consistent with the concept of local search (Bell 1985; Visser 1988). When maize roots were treated with different hydroxamates which act as feeding deterrents for other insects (Campos et al. 1988, 1989), neonate larvae of western corn rootworm responded by significantly reducing the number of turns, while area searched and locomotory rate significantly increased. The path patterns recorded after larvae contacted maize roots treated with compounds in our study are similar to those recorded with non-host plants or possible host plants which contain feeding deterrents (Strnad and Dunn 1990).

# CHAPTER VII.

# GENERAL CONCLUSIONS AND DISCUSSION

## 7.1. Main conclusions

This study has investigated the interactions of maize and western corn rootworm, and has led me to the following conclusions.

(1). A gradient HPLC method developed for the analysis of hydroxamic acids revealed that $DIM_2BOA$, HMBOA, DIMBOA, and MBOA were the major related compounds in maize roots. Of these, DIMBOA is the major hydroxamic acid present in maize roots (Chapter II, Xie et al. 1991a).

(2). The maximum concentrations of hydroxamic acids during the development of roots occurs at the same time as the development of western corn rootworm larvae. The hydroxamic acids are concentrated in the cortex of corn roots, which is the feeding site of western corn rootworm. These results support the hypothesis that DIMBOA content is important in resistant of maize to western corn rootworm (Chapter III, Xie et al. 1991b).

(3). DIMBOA and MBOA are toxic to western corn rootworm. High DIMBOA maize lines are significantly less susceptible to western corn rootworm than low DIMBOA lines based on the measurement of plant growth parameters and insect development

parameters. Significant negative correlations exist among the levels of hydroxamic acids in maize lines and insect development parameters. (Chapter V, Xie et al. 1990; Xie et al. 1991c).

(4). Significant differences in hydroxamic acid levels exist among the various maize germplasm types. It appears that the concentrations of hydroxamic acids in maize roots are also related to geographic origin of maize germplasm. The ITR group has higher levels of all individual compounds, especially DIMBOA equivalents and total amount of hydroxamic acids (Chapter IV, Xie et al. 1991c).

(5). A significant correlation between the concentration of DIMBOA in maize roots and in maize leaves was found, which implies that breeding maize varieties with high DIMBOA levels in maize roots or in maize leaves may give multi-resistance to both corn rootworm and the European corn borer (Chapter IV).

(6). It was revealed that naturally occurring and synthetic hydroxamic acids reduced the food acceptability and affected the host searching behaviour of western corn rootworm larvae, which suggested that hydroxamic acids were acting as behaviour-modifying chemicals (Chapter VI, Xie et al. 1991d).

(7). This research represented the first evidence showing that phytochemical factors, such as hydroxamic acids, were involved in the resistance of maize to western corn rootworm.

## 7.2. Discussion

### 7.2.1. Evolution of maize and corn rootworm

**Coevolution of plants and insects.** Insects have been eating plants for over 300 million years (Melcalf 1979). The persistence of plants shows their effective defense against herbivorous insects, and the abundance of insects demonstrates their ability to overcome the defense of plants. Ehrlich and Raven (1964) proposed stepwise coevolution as a distinct evolutionary process to explain the evolution of chemical defense in plant. In their model, plant-insect coevolution is a five-step sequence: (1). Plants produce novel secondary phytochemicals by mutation and recombination, and these new secondary substances reduce the suitability of the plant as food for insects; (2). Insect feeding is reduced due to deterrent properties of new secondary chemicals, and plants, protected from the attack of herbivorous insects, undergo evolutionary radiation in a new adaptive zone; (3). Insects, by mutation and/or recombination, evolve mechanisms to resist or overcome the secondary substances produced by plants; (4). Adapted insects undergo their own evolutionary radiation and enter a new adaptive zone; (5). The cycle may be repeated. As a result, more complex plant secondary substances and further specialized insects are produced.

**Evolution of maize and corn rootworm.** As for maize (*Zea mays*) and corn rootworm (*Diabrotica v. virgifera*), there is no direct evidence to show that they have coevolved. However, there is ample evidence to show that maize and *D. v. virgifera* evolved in the same general region, the tropics or subtropics (Smith 1966, Galinat 1977).

As described in Section 1.2, the cultivation of maize started in Mexico and South America about 7000 years ago (Mangelsdorf 1974). Although it is difficult to determine when *D. virgifera* became a significant pest of maize, based on the evidence that the farmers of Mesoamerica traditionally hill their corn to prevent maize from lodging, Melhus et al. (1954) believed that corn rootworm has probably been a significant problem on maize for 5000 years. It is believed that *D. virgifera* "followed" the cultural diffusion of maize cultivation into the temperature United States and Canada (Branson and Krysan 1981). The evolution and survival of maize is directly dependent on human practices, and thus an abundance of *D. virgifera* and its specialization on maize is indirectly dependent on human activities (Mangelsdorf 1974, Branson and Krysan 1981).

It is well established that most plant secondary compounds are involved with external regulation, mediating interactions between plants and herbivores, or between plants and other plants (Hsiao 1969). Although the production of secondary compounds is under genetic control, human practices, such as selection and hybridization, as well as biotic stress, such as insect pressure, affect both the presence and amount of specific compounds (McKey 1979). In the case of maize, human selection and hybridization of maize has been directed to yield and performance, but has often ignored chemical and other plant defenses, which has enabled herbivorous insects, including *D. virgifera*, to more successfully exploit maize as a host. In developing countries, the replacement of traditional varieties of maize with high yield varieties is a well documented cause of increased pest problems (Fortier et al. 1982). On the other hand, so many herbivorous insects have been associated with maize (Chiang 1978; Ortega et al. 1980), that these insects have most probably applied sufficient selection pressure upon maize to elaborate effective chemical defenses in at least

some genotypes. Therefore, it is not surprising to find maize to have evolved a broad biochemical resistance against herbivorous insects including *D. virgifera*.

The role of plant secondary compounds in the interactions of plant and herbivores may be either positive (attractive) or negative (defensive) (Fig. 7.1). The present study revealed that hydroxamic acids acted as allomones, but not kairomones. In this study, hydroxamic acids acted as toxicants and/or antifeedants, which prevented or interrupted feeding activity and caused mortality (Chapter V); as repellents, which oriented locomotion away from the source of stimulus (host) (Chapter VI); and as growth regulators, which regulated rootworm growth and stressed larvae to produce inferior adults (Chapter V).

In addition to corn rootworm, there is abundant evidence showing that hydroxamic acids are deleterious to several other cereal pests. So far, at least five herbivorous insect species have been found to be affected by hydroxamic acids (Table 7.1).

The present study of the role of hydroxamic acids in the interactions of maize and western corn rootworm is consistent with those obtained in other insect systems in several aspects:

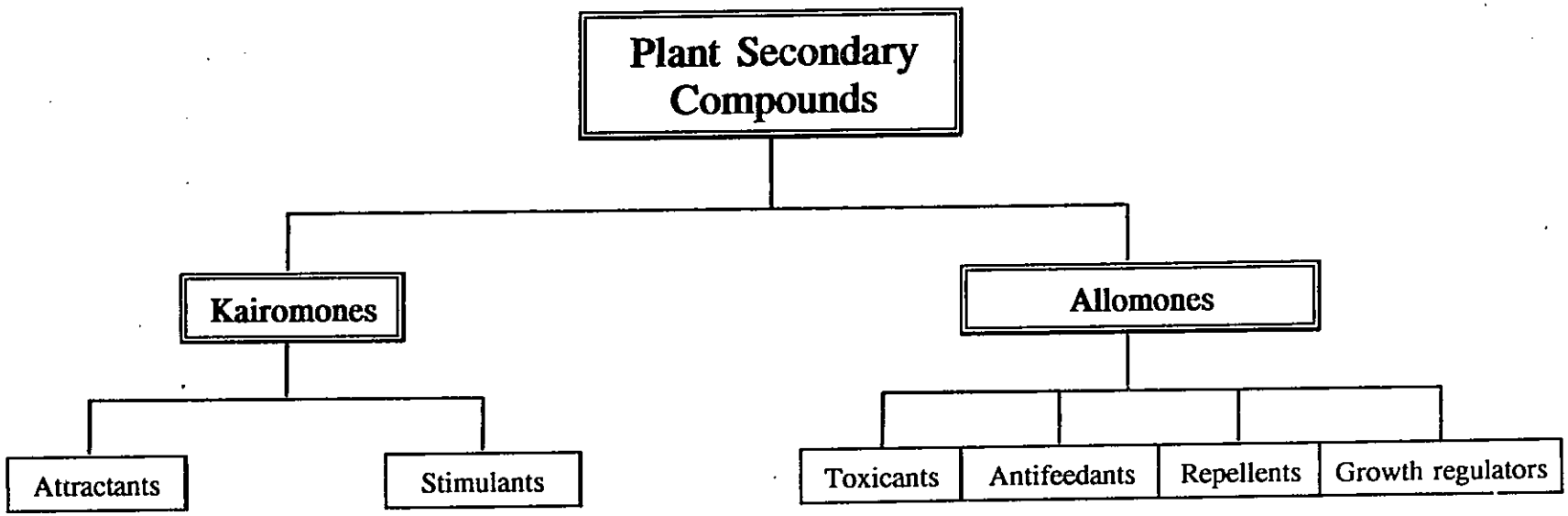
(1). Hydroxamic acids acts as a dose-dependent feeding deterrent to western corn rootworm (Xie et al. 1990). However, as indicated by Robinson et al. (1982) working with European corn borer, it was difficult to discriminate between behaviour and physiology (starvation vs. toxicity). In our study, starvation, in addition to feeding deterrence, may also be involved in larval death.

(2). Significant correlations between DIMBOA concentrations in maize roots and larval development parameters of western corn rootworm were found in this study. Similar

**Fig. 7.1.**

Potential of plant secondary compounds

in the interactions of plant and other organisms.


Plant Secondary Compounds
Kairomones
Allomones
Attractants
Stimulants
Toxicants
Antifeedants
Repellents
Growth regulators

relationships have been found in other insect systems as indicated in Table 7.1. The range of DIMBOA concentrations in roots of different maize lines used in this study was 43-921 μg/g fresh tissue. It is in the same ranges as found by other researchers in leaves, such as Klun and Brindley (1966) (20-220 μg/g fresh tissue), Reed et al. (1972) (500-3500 μg/g fresh tissue), Long et al. (1977) (80-1480 μg/g fresh tissue), and Reid (1988) (15-480 μg/g fresh tissue).

(3). Argandoña et al. (1981) have demonstrated that hydroxamic acid content in wheat leaf tissue can regulate the distribution of the aphid *Schizaphis graminum* on the leaves of wheat. In our study, the low concentrations of HMBOA, DIMBOA equivalents, and the total of these compounds were observed in maize adventitious roots (Xie et al. 1991b) which is the preferable part of western corn rootworm larvae (first instar) (Strnad and Bergman 1987). This result suggested that the distribution of western corn rootworm larvae may be regulated by the presence of hydroxamic acids.

(4). A trend showing that hydroxamic acid levels in different maize lines were related to geographical origin was obtained in the chemical survey (Xie et al. 1991c), which is consistent with that observed by Reid et al. (1989).

(5). When a DIMBOA solution (1 mg DIMBOA/mL $H_2O$) was sprayed on maize plants susceptible to the European corn borer, the number of larvae moving off the plants obviously increased (Robinson et al. 1982). Similarly, when maize roots were treated with DIMBOA solution (500 ppm), larvae of western corn rootworm showed nonpreference, and host searching behaviours were modified, which suggested that hydroxamic acids were acting as behaviour-modifying chemicals (Xie et al. 1991d).

**Table 7.1.** Interactions between hydroxamic acids and herbivorous insects

| Plant | Insect | Compound | Activity | Varietal correlation | References |
|---|---|---|---|---|---|
| Maize | *Ostrinia nubilalis* (European corn borer, ECB) | MBOA | Inhibiting rate of borer pupation | A correlation exists between MBOA concentrations in leaves and resistance rating (1-9 scale) (n = 11) | Klun and Brindley (1966) |
| Maize | ECB | MBOA | Increasing larval mortality, decreasing sex ratio and fecundity, prolonging the time to pupation and adult emergence | --- | Campos et al. (1988) |
| Maize | ECB | DIMBOA | Prolonging the time to pupation, increasing larval mortality | --- | Klun et al. (1967) |
| Maize | ECB | DIMBOA | Increasing larval mortality, slowing larval development, decreasing larval wt. and adult fecundity | A correlation exists between DIMBOA concentrations in leaves and larval mortality (n = 6) | Reed et al. (1972) |
| Maize | ECB | DIMBOA | Nonpreference | Leaf DIMBOA concentration was significantly related to the wt. of larvae (n = 6, r = -0.83), and the number of larvae established (n = 6, r = -0.92) | Robinson et al. (1978) |
| Maize | ECB | DIMBOA | Prolonging the time to pupation, decreasing larval wt. Nonpreference | --- | Robinson et al. (1982) |

Table 7.1. Continued

| Plant | Insect | Compound | Activity | Varietal correlation | References |
|---|---|---|---|---|---|
| Maize | ECB | DIMBOA | Reducing larval feeding rate | Leaf DIMBOA concentration was related to borer leaf-feeding rate in both laboratory (n = 48, r = -0.57) and field (n = 102, r = -0.46) | Reid (1988) |
| Maize | ECB | DIMBOA | Increasing larval and pupal mortality, decreasing pupal and adult wt. and fecundity, prolonging the time to pupation and adult emergence | --- | Campos et al. (1989) |
| Maize | *Rhopalosiphum maidis* (Corn leaf aphid) | DIMBOA | Increasing aphid mortality | A correlation is obtained between DIMBOA concentration in leaves and aphid infestation in inbred maize lines (n = 12, r = -0.72) | Long et al. (1977) |
| Maize | *Diabrotica v. virgifera* (Western corn rootworm) | DIMBOA, MBOA and analogues | Increasing larval mortality, inhibiting larval development and adult emergence, reducing body wt. and size of larvae and adults. Modifying behaviour | Highly negative correlations were found between DIMBOA concentrations in roots and the number of larvae developed (n = 7, r = -0.81), larval wt. (n = 7, r = -0.95), and larval head-capsule width (n = 7, r = -0.94) | Xie et al. (1990), (1991d) |

Table 7.1. Continued

| Plant | Insect | Compound | Activity | Varietal correlation | References |
|---|---|---|---|---|---|
| Wheat | *Metopolophium dirhodum* (Rose-grain aphid) | DIMBOA | Decreasing survival and growth rate of aphid | A correlation exists between DIMBOA concentration in leaves and aphid survival, aphid growth rate ($n = 20$, $r = -0.94$) | Argandoña et al. (1980) |
| Wheat | *Schizaphis graminum* (Greenbug) | DIMBOA | Decreasing survival and field population of aphid | A correlation exists between DIMBOA concentration in leaves and aphid survival, aphid field population | Argandoña et al. (1981) |
| Wheat | *Sitobion avenae* (English grain aphid) | Total hydroxamic acids | Reducing the rate of reproduction | Total amount of hydroxamic acids in the leaves of 6 wheat cultivars is related to the intrinsic rate of natural increase of *S. avenae*. | Bohidar et al. (1986) |

* Not indicated.

### 7.2.2. Implications of this study to corn rootworm management

It is well established that IPM is the best available pest management system which utilises all suitable techniques and/or tactics in a compatible manner to reduce pest populations and maintain them below economic injury levels (Smith and Reynolds 1966). Among the components of the IPM system, host-plant resistance could be an important tactic of corn rootworm management. The present study has demonstrated that resistance (antibiosis) is present in corn varieties to corn rootworm, and that hydroxamic acids are contributing to this resistance. What implications can be drawn from this study for corn rootworm management?

(1). Phytochemical indicators of resistance may accelerate the process of screening. The basic procedure for plant resistance breeding program is screening of collected germplasm materials. The normal screening procedures involve artificially infesting with insects and evaluating plant reaction to insect damage. It is a long term process requiring considerable financial resources and labour. Since there is a clear inverse relationship between levels of hydroxamic acids in corn roots and performance of western corn rootworm as demonstrated in this study, the levels of hydroxamic acids could be used as phytochemical indicators of resistance. However, some caution is needed, since entirely relying on a single factor such as hydroxamic acids may lead to problems, as other underlying factors other than hydroxamic acids may also be involved in the interaction of maize and corn rootworm. Final verification always requires examination of field performance.

(2). Searching for corn resistance to corn rootworm among tropical or subtropical corn germplasm may be more successful than from temperat germplasm. From an evolutionary point of view, these races of corn from tropical or subtropical reigns have had the longest association with corn rootworm, and would be the most likely to have evolved an effective chemical defense system. The fact that there are higher concentrations of hydroxamic acids in the genotypes of corn from tropical or subtropical regions suggests that it may be more successful to select corn rootworm-resistant genotypes from those originating in tropical or subtropical areas. On the other hand, synchrony between *Diabrotica* spp and their host is a crucial factor in resistance (Melhus et al. 1954), and there are always problems of adaptation of exotic material.

(3). Breeding corn varieties with high hydroxamic acid levels may lead to multi-resistance to both corn rootworm and the European corn borer. It has been demonstrated that hydroxamic acids contribute resistance to both the European corn borer (Klun et al. 1970) and western corn rootworm (see Chapter V). The fact that there is a significant positive correlation between levels of DIMBOA in corn roots and that in corn leaves implies that it is possible to breed corn varieties with multi-resistance to both the European corn borer and corn rootworm.

### 7.2.3. Future work

(1). $DIM_2BOA$, and HMBOA are also present in corn root extracts of different corn lines. The role of these compounds, if any, in the resistance of corn to western corn

rootworm should be elucidated.

(2). It is important to determine the mode of action of DIMBOA and MBOA, especially their effect on corn rootworm food consumption and utilization. Since there is no artificial diet available for western and northern corn rootworm at the moment, it would be most practical to use southern corn rootworm as the test species.

(3). It has been reported that DIMBOA inhibited the digestive processes of the European corn borer and reduced the tryptic and chymotrypic activity of the European corn borer (Houseman et al. 1991). It is important to determine if digestive enzyme systems and other enzyme systems in corn rootworms are inhibited by DIMBOA and MBOA.

(4). It has been reported that the metabolism, penetration, and partitioning of [$^{14}$C]aldrin in western corn rootworm are different from that in the closely related and sympatric northern corn rootworm (Siegfried and Mullin 1990). It is important to determine the differences between the toxicity of DIMBOA and MBOA to western corn rootworm and to northern corn rootworm.

(5). Determination of the distribution of the labelled DIMBOA and MBOA in western and northern corn rootworm, in order to detect the differences of excretion and metabolism of hydroxamic acids in both species, is an essential next step in understanding the insect's responses to plant defense.

(6). With the use of DIMBOA analogues, it would be interesting to determine the structure-activity relationship of these compounds on corn rootworms.

(7). It is important to determine the potential of western corn rootworm to develop resistance to high DIMBOA content corn lines. This could be investigated by recurrent

selection of insects cultured on high DIMBOA lines.

(8). It is important to carry out field trials to determine responses of high DIMBOA corn lines on corn rootworm damage under natural conditions. This is the ultimate demonstration of the role of hydroxamic acids in the resistance of maize to corn rootworm.

# CHAPTER VIII.

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# Appendix 1.

## Distribution of hydroxamic acids in maize root as detected by $FeCl_3$ reagent

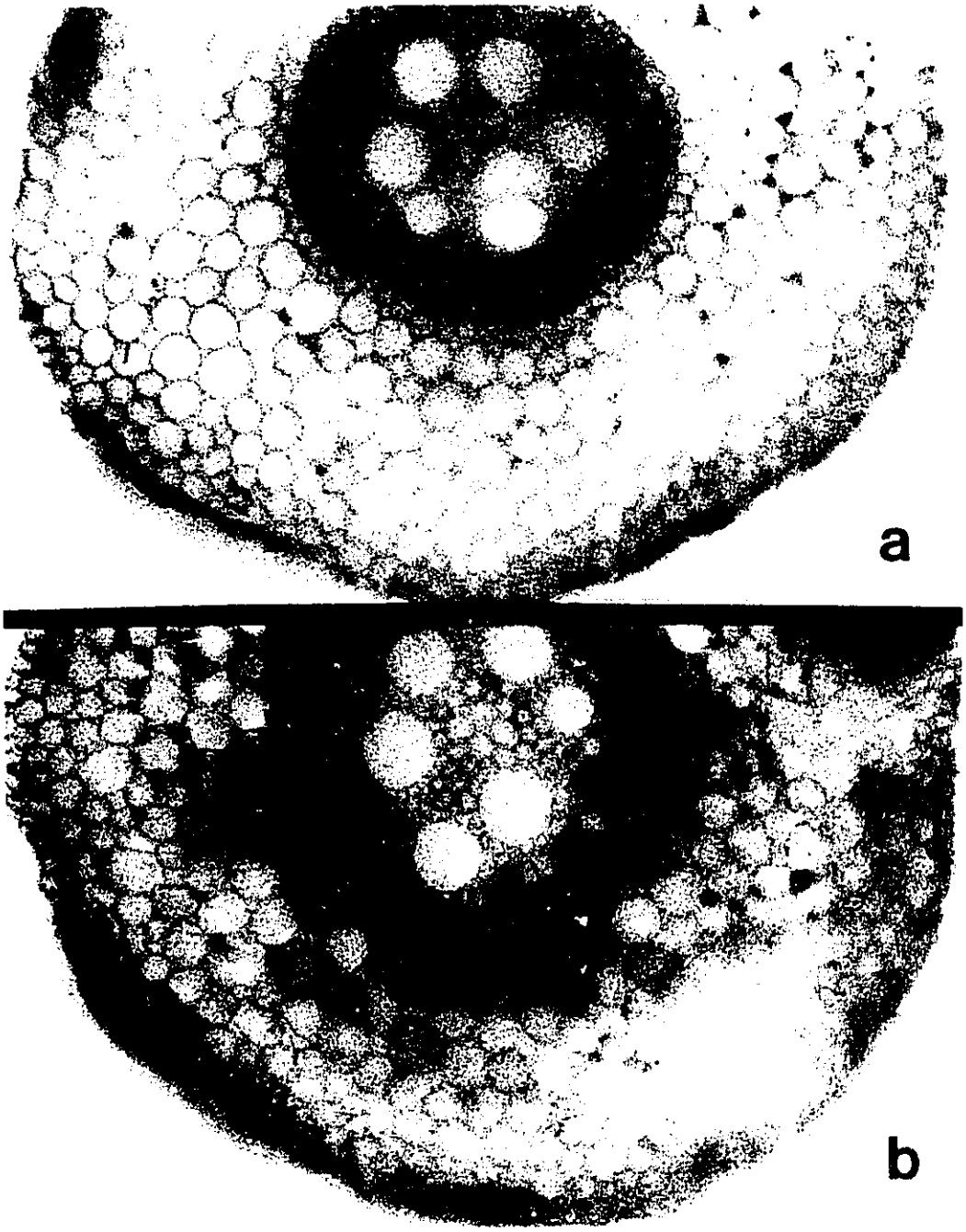
In addition to the HPLC method for detecting the distribution of the related compounds in maize root, ferric chloride ($FeCl_3$) was used as a reagent to determine the localization of total hydroxamic acids in maize roots. Based on the results of DIMBOA analysis for 24 different maize lines from various latitudinal regions, two maize lines, ITR 3872 with high DIMBOA content in root and NTR-2 Ger. 4042 with low DIMBOA content in root, were used as the test varieties. The plants were grown in the same greenhouse conditions as described above. After 10 days of growth under those conditions, maize roots were taken out and washed with tap water, then with distilled water. The primary root was carefully sectioned by hand and then mixed with a drop of ferric chloride reagent (2.5 g $FeCl_3 \cdot 6H_2O$, 25 ml 95% ethanol, and 0.25 ml 6N HCl, Woodward et al. 1978). The colour in hand-sectioned samples was examined with an Olympus Vanox microscope equipped with bright field optics.

When ferric chloride reagent was added to the cross section of fresh maize primary root, a light blue colour appeared in approximately three layers of cortex cells adjacent to the endodermis (Fig. Appendix 1, b), and the colour disappeared within 1 min. Under the microscope, the difference in colour between control (no reagent added, Fig. Appendix 1, a) and treatment with reagent (Fig. Appendix 1, b) was very evident. Although ferric chloride reagent is not a highly specific one (it can give a blue complex with some

phenolics), we believe that the colour reaction was mainly due to hydroxamic acids in the cortex for two reasons. First, maize phenolics are more stable than hydroxamates which are known to degrade rapidly after tissue damage (Honakanen and Virtanen 1960; Woodward et al. 1978), and second, when we compared the colour which appeared in maize lines of known DIMBOA content, the blue colour appearing in the cortex of the high DIMBOA line was obviously darker than that of low DIMBOA line. The result is highly consistent with the results obtained by HPLC as described in Chapter III.

**Fig. Appendix 1.**

Cross section of fresh maize root at 10 days. **a.** Control. x130. **b.** With ferric chloride reagent, showing some cortex cells adjacent to endodermis stained dark (blue in original preparation). x130.


a
b

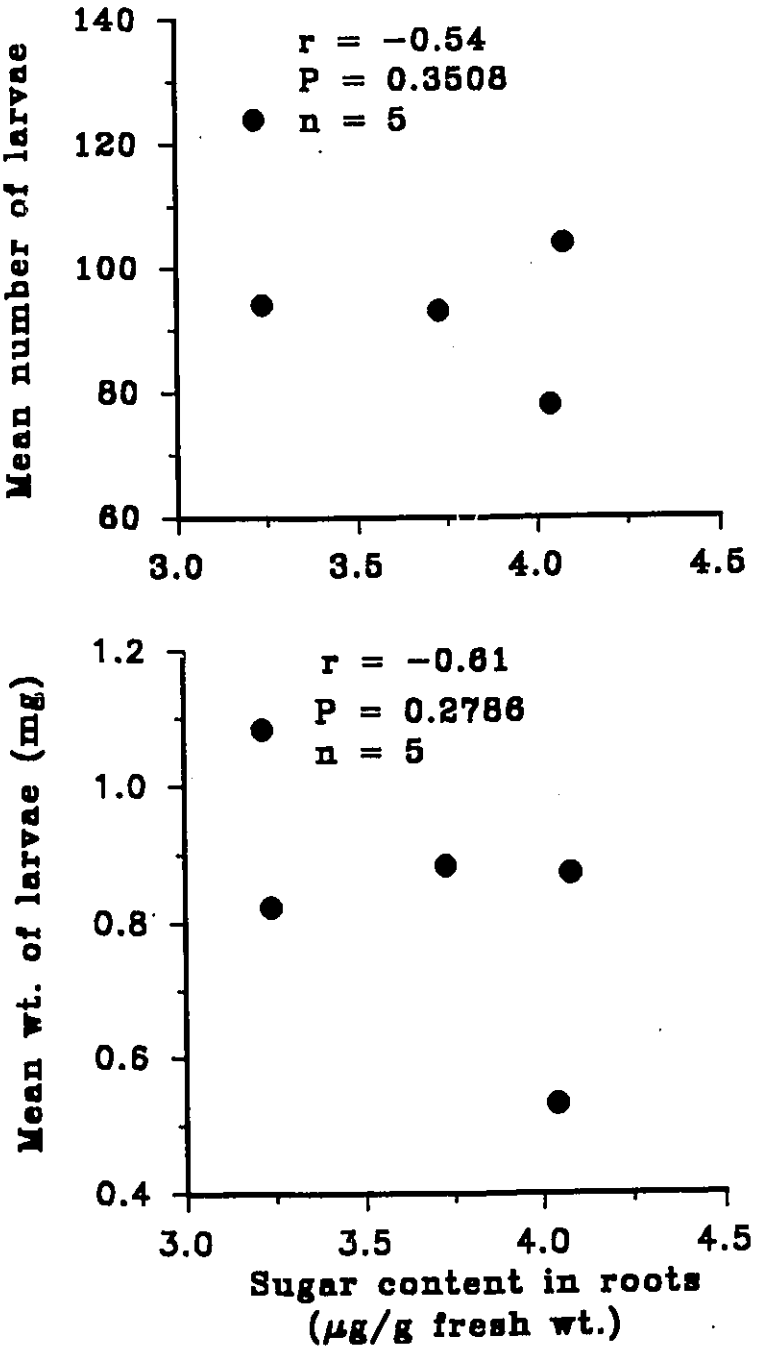
## Appendix 2.

## Relationship between western corn rootworm larval growth parameters and total sugar content in corn roots at two weeks of age

Negative correlations were found between total sugar content in corn roots and mean number of western corn rootworm larvae developed ($r = -0.54$, $P = 0.3508$, $n = 5$), and mean wt. of western corn rootworm larvae ($r = -0.61$, $P = 0.2786$, $n = 5$) (Fig. Appendix 2).

**Fig. Appendix 2.**

Relationship between western corn rootworm larval growth parameters (mean number of larvae developed, and mean wt. of larvae) and total sugar content in corn roots at two weeks age.


r = −0.54
P = 0.3508
n = 5
Mean number of larvae
140
120
100
80
60
3.0
3.5
4.0
4.5
r = −0.61
P = 0.2786
n = 5
Mean wt. of larvae (mg)
1.2
1.0
0.8
0.6
0.4
3.0
3.5
4.0
4.5
Sugar content in roots
(μg/g fresh wt.)

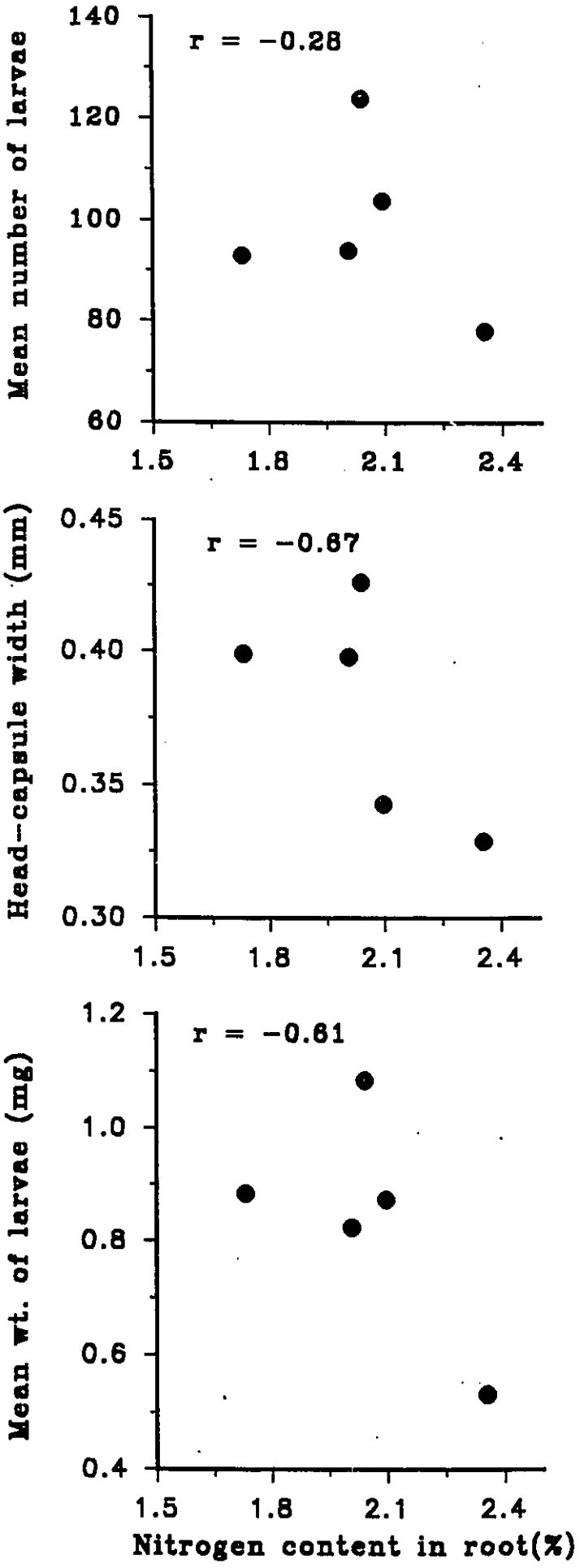
## Appendix 3.

## Relationship between western corn rootworm larval growth parameters and total nitrogen content in corn roots at two weeks of age

Negative correlations were found between total nitrogen content in corn roots and mean number of western corn rootworm larvae developed ($r = -0.28$, $P = 0.5179$, $n = 5$), head-capsule width of western corn rootworm larvae ($r = -0.67$, $P = 0.2176$, $n = 5$), and mean wt. of western corn rootworm larvae ($r = -0.61$, $P = 0.2754$, $n = 5$) (Fig. Appendix 3).

**Fig. Appendix 3.**

Relationship between western corn rootworm larval growth parameters (mean number of larvae developed, larval head-capsule width, and mean wt. of larvae) and total nitrogen content in corn roots at two weeks age.


r = −0.28
Mean number of larvae
140
120
100
80
60
1.5
1.8
2.1
2.4
r = −0.67
Head-capsule width (mm)
0.45
0.40
0.35
0.30
r = −0.61
Mean wt. of larvae (mg)
1.2
1.0
0.8
0.6
0.4
Nitrogen content in root(%)

## Appendix 4.

## Paper chromatography to determine sugar

Paper chromatography to determine sugar was performed on Whatman No. 1 filter paper. The developing solvents used were benzene : n-butanol : pyridine : water (1 : 5 : 3 : 3). Paper was run over night. Sugars were detected by spraying phthalate and comparing with standard mixture (Mukherjee and Srivastava 1952).

The main sugar present on chromatography of corn root was identified as glucose.

## Appendix 5.

## Effects of Azadirachtin on the Western Corn Rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae)

Corn rootworms (*Diabrotica* spp., Coleoptera: Chrysomelidae) are serious pest insect of corn production. It is estimated that farmers in the United States have losses of over $1 billion each year as a result of crop damage and treatment costs for this pest (Metcalf 1986). Chemical control is the main method of suppressing corn rootworm populations and the amount of insecticide used against *Diabrotica* spp. is greater than for any other pests of corn in the United States (Suguiyama and Carlson 1985). The development of nontoxic and biodegradable alternatives to chemical insecticides is highly desirable.

Azadirachtin, a natural substance from the neem tree, *Azadirachta indica* A. Juss (Meliaceae), has biological effects on more than 200 species of insect and can act as an antifeedant, a repellent, a toxicant, and a growth disruptant, or a growth regulator, or both (Warthen 1979, 1989; Saxena 1989). This compound is nontoxic to mammals, rapidly degraded in the environment, nonpersistent in sunlight (Stocke and Redfern 1982), and may be one of the more promising botanical substances for insect control. The antifeedant effects of azadirachtin on adult *Diabrotica* have been demonstrated (Reed et al. 1982), and Landis and Gould (1989) have shown feeding deterrence of neem extracts on the economically-important larval stage of southern corn rootworm, *Diabrotica undecimpunctata*

*howardi* Barber. The present report examines the effects of pure azadirachtin on the western corn rootworm, *Diabrotica virgifera virgifera* LeConte.

Pure azadirachtin, extracted from neem seeds by the method of Schroeder and Nakanishi (1987), was used in this study. Identification of azadirachtin was confirmed by fast atom bombardment (FAB), mass spectrometry, $^1$H-NMR and high-performance liquid chromatography (HPLC). Purity was better than 95%. Preparation of 22, 23 dihydro ($H_2$-aza) or ditritio azadirachtin ($T_2$-aza) was achieved by reduction of the 22, 23 double bond with $H_2$ or tritium gas respectively, using methods reported previously (Turner et al. 1987). Completion of the reduction was confirmed by FAB. Radio-purity of $T_2$-aza was assessed by HPLC of the reduction product. HPLC fractions were collected and counted; over 90% of counts appeared in the fraction with a similar retention time to $H_2$-aza.

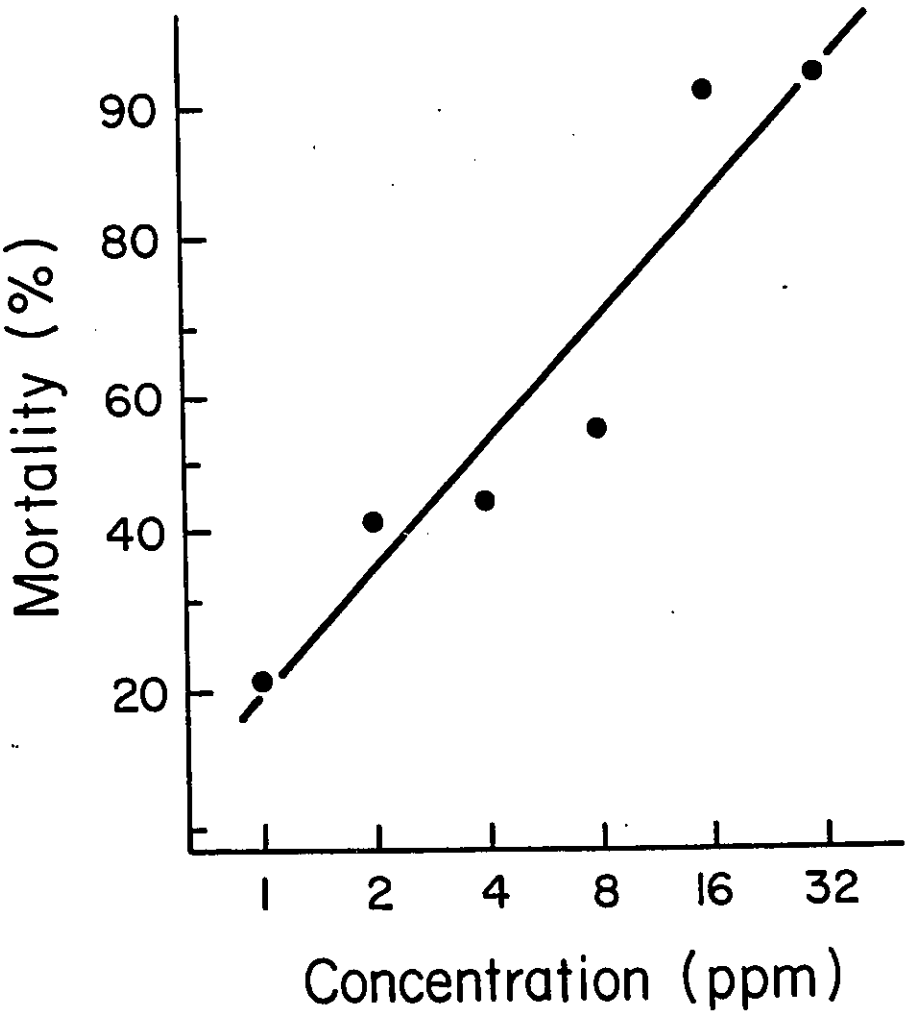
For determination of the toxicity of azadirachtin to rootworm, germinated hybrid corn seeds with fresh roots (2-3 cm long) were placed in Petri dishes lined with one piece of filter paper (9.0 cm), and then moistened with 1.7 ml of an aqueous azadirachtin solution. Concentrations of azadirachtin used were 1, 2, 4, 8, 16, and 32 $\mu$g per ml with distilled $H_2O$ as the control. Newly-hatched larvae were placed on the corn roots. Ten larvae were used for each treatment, five replicates were prepared at each concentration, and the whole experiment was repeated twice. Seventy two hours after treatment, mortality was recorded. Abbott's formula (Abbott 1925) was used to correct for control mortality (less than 20%). The probit analysis generated by the SAS (SAS Institute 1982) program was used for calculation of the lethal concentration for 50% and 90% mortality ($LC_{50}$ and $LC_{90}$).

The results for the two trials were similar and therefore were combined. The probit plot obtained demonstrated an excellent correlation between the mortality of the western corn rootworm larvae and the logarithm of azadirachtin concentration ($r^2$ = 0.94) (Fig. Appendix 5). Western corn rootworm larvae were found to be very sensitive to pure azadirachtin with $LC_{50}$ (95% fiducial limits) of 3.9 (2.5 - 5.9) ppm, and an $LC_{90}$ (95% fiducial limits) of 29.7 (16.5 - 92.4) ppm.

In a subsequent pot trial, four treatments were designed: (1) uninfested control (no eggs, no azadirachtin); (2) with eggs, 10 ppm azadirachtin solution applied to the soil; (3) with eggs, azadirachtin solution (100 ppm dissolved in 0.1% citowet) sprayed on the leaves; and (4) with eggs, no azadirachtin (infested control). Corn seeds were planted in plastic pots (20 cm by 20 cm), and the pots were artificially infested at the time of planting for treatments 2, 3, and 4 (200 eggs per pot, hatchability: 55.9%). There were 10 pots (one plant per pot) for each treatment. Two weeks after infestation, 10 ppm azadirachtin solution (200 ml per pot, once a day) was applied to the soil for 3 days to ensure that azadirachtin was present at the time of hatching for treatment 2; for the other treatments, the same amount of water was applied. For treatment 3, a 100 ppm azadirachtin solution (dissolved in 0.1% aqueous citowet) was sprayed on leaves, until runoff; applications were made daily for 3 days. For the other treatments, the same amount of water with 0.1% citowet was sprayed. Just prior to the azadirachtin treatment, plant height for all four treatments was measured. Seven weeks after infestation, plant height and stem thickness were measured, and fresh weight of plant, fresh weight of root, dry weight of plant, and dry weight of root were recorded.

**Fig. Appendix 5.**

The probit analysis of western corn rootworm mortality at different azadirachtin concentrations.


Mortality (%)
90
80
60
40
20
1
2
4
8
16
32
Concentration (ppm)

The results demonstrated that plant height prior to application of azadirachtin (2 weeks after infestation) did not differ significantly ($P > 0.05$) among the treatments (Table 1). Seven weeks after infestation, plants in the infested control group (treatment 4) showed heavy damage as measured in all parameters when compared with uninfested controls, whereas application of 10 ppm azadirachtin solutions applied to the soil (treatment 2) offered complete protection. None of the plant parameters in treatment 2 showed significant differences from the uninfested control (treatment 1), but were significantly different ($P < 0.05$) from the infested control (treatment 4) (Table 1). Application of 100 ppm azadirachtin solutions to corn leaves (treatment 3) significantly ($P < 0.05$) reduced damage as measured by plant height, but not other parameters (Table 1).

Our data confirm that azadirachtin is toxic to corn rootworm larvae and establishes that it protects corn from rootworm damage when applied to the soil as a drench. The systemic movement of azadirachtin from leaves to roots is suggested by the results of the foliar applications, which provided partial protection of plants. To further investigate systemic movement, 50 $\mu$g of $H_2$-aza + $T_2$-aza (specific activity, 4.38 Ci/mole) dissolved in 1 ml of 25% MeOH containing 0.1% citowet was applied by Hamilton syringe on leaves of 4-week old corn seedlings held hydroponically in Hoagland's medium. In all seven replicates radioactive label was detected after 24 h in washed roots and root medium, corresponding to a mean (standard error) of 62.6 (21.0) ng equivalents and 429.0 (177.0) ng equivalents, respectively. This result confirms systemic movement of label from the leaf to root. Although the magnitude of transport is not large, it suggests that sufficient azadirachtin or its metabolites may move to roots after it is applied to the leaves, and that it acts directly on rootworms.

**Table 1.** Corn growth parameters in pot trials with various azadirachtin and western corn rootworm (WCR) treatments

| Treatment | WCR infestation (eggs/pot) | Azadirachtin treatment | Plant height$_1$[a] (cm) | Plant height$_2$ (cm) | Plant thickness (cm) | Plant fresh wt. (g) | Root fresh wt. (g) | Plant dry wt. (g) | Root dry wt. (g) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | none | 41.00 a[b] | 137.00 a | 3.47 a | 73.34 a | 20.23 a | 14.04 a | 2.38 a |
| | | | (5.01) | (8.56) | (0.49) | (16.73) | (4.27) | (4.16) | (0.59) |
| 2 | 200 | 10 ppm | 41.60 a | 135.10 a | 3.39 ab | 68.40 a | 22.95 a | 13.53 ab | 2.84 a |
| | | in soil | (3.47) | (7.46) | (0.40) | (16.12) | (7.27) | (4.99) | (0.81) |
| 3 | 200 | 100 ppm | 37.70 a | 104.50 b | 2.93 bc | 45.18 b | 4.10 b | 8.64 bc | 0.72 b |
| | | on leaves | (2.91) | (19.21) | (0.36) | (23.08) | (1.72) | (4.53) | (0.27) |
| 4 | 200 | none | 39.30 a | 74.00 c | 2.73 c | 25.33 b | 2.16 b | 5.19 c | 0.43 b |
| | | | (2.98) | (17.45) | (0.38) | (14.67) | (0.97) | (2.71) | (0.15) |

a. Plant height$_1$ was measured at the time before azadirachtin treatment (2 weeks after infestation), other parameters were measured around the time of adult emergence (7 weeks after infestation).

b. Means in the same column followed by the same letter are not significantly different ($P > 0.05$; Tukey's test) (SD in parentheses).

The overall results of this study are encouraging enough to suggest that field trials of pure azadirachtin or other neem products containing this substance for the control of corn rootworms may be warranted.