DIFFERENTIAL TOLERANCE OF CORN HYBRIDS TO METOLACHLOR AND ITS REGULATION BY THE SAFENER BENOXACOR

by

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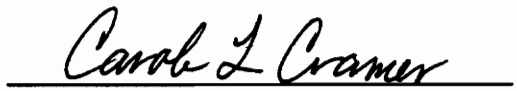
Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
in
Plant Physiology

APPROVED:

K. K. Hatzios, Chairman



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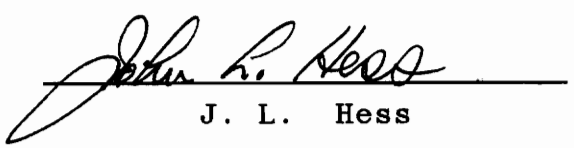

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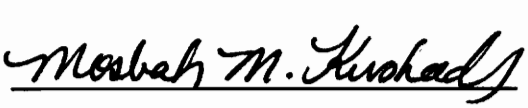

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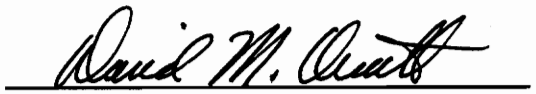

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JUNE, 1991

Blacksburg, Virginia

DIFFERENTIAL TOLERANCE OF CORN HYBRIDS TO METOLACHLOR AND ITS REGULATION BY THE SAFENER BENOCACOR

by

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(ABSTRACT)

Determining the basis of intraspecific herbicide tolerance was expected to be a useful way of revealing factors which are regulated by safeners in providing their protective effect. Differential tolerance to the chloroacetanilde herbicide metolachlor and the thiocarbamate herbicide EPTC was examined in 11 corn hybrids. Tolerance to one of these herbicides does not imply similar tolerance to the other. Detoxication of these herbicides in plants is mediated via conjugation with glutathione (GSH). GSH levels from 1.8 to 2.4 µmol/g fresh weight were determined for the eleven corn hybrids tested. There was no correlation between GSH content and herbicide tolerance. The monooxygenase inhibitor piperonyl butoxide (PBO) acted synergistically with EPTC on 8 of the tested corn hybrids. A similar antagonism by the oxygen evolving compound calcium peroxide provided additional evidence for the importance of oxidative processes in EPTC tolerance which were not important in determining metolachlor tolerance. The more rapid

absorption and greater accumulation of ^{14}C-metolachlor by 'Northrup-King 9283' corn relative to 'Cargill 7567' corn at least partially explains the increased susceptibility of the former hybrid to metolachlor. The *in vitro* metabolism of ^{14}C-metolachlor was similar for both hybrids. A lag in the expression of glutathione *S*-transferase (GST) activity during early seedling development of 'Northrup-King 9283' corn may be of additional significance in its limited tolerance to metolachlor. The safener benoxacor was effective in protecting 'Northrup-King 9283' and other susceptible corn hybrids from metolachlor injury. Benoxacor had no effect on metolachlor uptake or the rate of non-enzymatic conjugation of metolachlor. Seedlings of 'Cargill 7567' and 'Northrup-King 9283' treated with 1 µM benoxacor metabolized metolachlor to the GS-conjugate at a rate 1.7 times that of untreated seedlings. GST activity was stimulated by 35% by similar treatment. GST isozymes with metolachlor conjugating activity (GST-metolachlor activity) were found in the cytosol and microsomal fractions of corn extracts. At least two GST-metolachlor isozymes were separated by DEAE-Sepharose chromatography. The activity of both isozymes was increased by benoxacor treatment. It appears that benoxacor regulates metolachlor tolerance by inducing GST isozymes that consequently increase the rate of metolachlor detoxication.

ACKNOWLEDGEMENTS

I would like to express my thanks to Dr. Kriton Hatzios for his guidance and support; and the remainder of my committee, Dr. Carole Cramer, Dr. John Hess, Dr. Mosbah Kushad, and Dr. Dave Orcutt for their assistance and review of this dissertation. Financial support of this research was provided by a research grant from Ciba-Geigy Corporation, Greensboro, NC. Partial support was also provided by the Herman Frasch Foundation of the American Chemical Society, Washington, DC. I am especially grateful to Dr. Larry Moore, Chairman; Department of Plant Pathology, Physiology, and Weed Science; Virginia Polytechnic Institute and State University, Blacksburg, VA; for providing departmental support of my graduate education.

I will always be grateful to my wife and partner in life's adventures, Becky, for her constant love and understanding; and to my step-children, Chris and Katie, who have done much to enrich my life during the last few years. I am deeply indebted to Sue Meredith for all her help in the greenhouse and in the lab. Thanks go also to my many friends at the Glade Road Research Facility.

I am forever grateful to my family for providing me with a happy childhood, a stimulating intellectual environment, and an upbringing steeped in Southern culture. This dissertation is dedicated to the memory of my grandmothers,

Mrs. Lucille "Mama" Martin and Mrs. Flora "Granny" Cottingham. These extraordinary women were my first instructors in Botany, Horticulture, and Weed Science. Surely their love of gardening and the beauty of nature helped to determine my destiny as a plant scientist.

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LIST OF ABBREVIATIONS

alachlor, *N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetanide

atrazine, 6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine

benoxacor, [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4 benzoxazine]

CDNB, 1-chloro-2,4-dinitrobenzene

CGA-154281, benoxacor

CGA-180937, metolachlor + benoxacor (30:1, w/w)

dichlormid, 2,2-dichlor-*N*,*N*-di-2-propenylacetamide

DUAL, formulated metolachlor herbicide

EPTAM, formulated EPTC herbicide

EPTC, S-ethyl dipropylcarbamothioate

flurazole, phenylmethyl-2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate

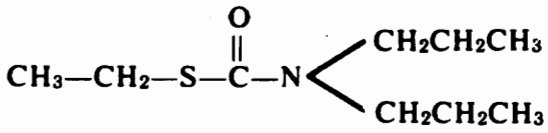
GSH, reduced glutathione

GST, glutathione *S*-transferase [EC 2.5.1.18]

metolachlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide

PBO, piperonyl butoxide

STRUCTURAL FORMULAS OF EPTC, METOLACHLOR, AND BENOXACOR



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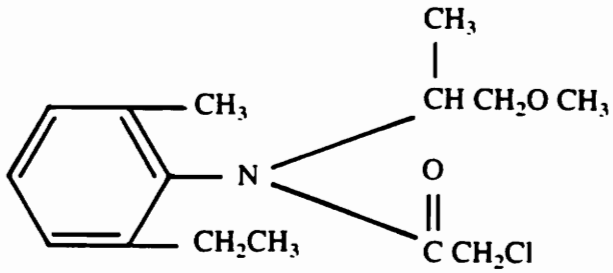
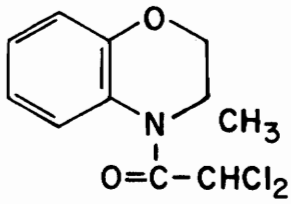


Metolachlor



Benoxacor

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I. INTRODUCTION

The success of modern chemical weed control is primarily due to the selectivity of herbicide action. The basis of this concept is that weeds can be controlled without injury to the crop plant (1). A certain measure of selectivity can be afforded by cultural practices which minimize the exposure of the crop plant to phytotoxic chemicals (1). This concept has been expanded and capitalized on to its fullest by exploiting the inherent tolerance of crop plants to certain herbicides. Thus, a herbicide can be applied to control noxious weeds which are sensitive to its phytotoxic action without any injury to the crop plant. This approach is not without its limitations. It seems that botanically and/or physiologically similar weeds will always be present which can tolerate particular herbicides or herbicide mixtures as well as the crop (2). Crop tolerance is not always absolute and under certain conditions injury may occur. Of additional concern is the development of weed tolerance, or resistance, following continued usage of certain herbicides (3).

Herbicide tolerance may be determined by a number of factors (4). These include plant modifications which limit the uptake/and or translocation of the herbicide, the ability of a tolerant plant to detoxify the herbicide,

modification of a target site so that it is not sensitive to the herbicide, increased synthesis of a target enzyme in response to the herbicide, or the synthesis of substrates which can reverse the herbicide effect. Understanding these factors is important to weed scientists in designing new herbicides, to plant breeders in developing tolerant crops, and to plant physiologists using herbicides as tools in understanding fundamental processes in plants.

The development of herbicide safeners in the last two decades has done much to extend the concept of herbicide selectivity. The most successful commercialized safeners are dichloroacetamide derivatives which are very effective in protecting corn against injury from thiocarbamate and chloroacetanilide herbicides (5). Other safeners include oxime ethers and 2,4-disubstituted thiazoles which are commercially useful in protecting sorghum from chloroacetanilide herbicide injury (5). In general, the safeners presently available are most effective in protecting grass crops against injury from thiocarbamate, chloroacetanilide, and aryloxyphenoxypropionate herbicides (5). Some degree of inherent tolerance appears to be necessary for safeners to achieve their protective function. They essentially confer enhanced tolerance to the protected crop plant.

The exact mechanisms by which safeners exert their

protection is unknown. Hatzios (5) has summarized four general mechanism which may be involved in herbicide safener action. These include: (a) a safener-induced reduction in herbicide uptake and/or translocation; (b) competition of the herbicide and safener at a common site of action within the protected plant; (c) a safener-induced stimulation of herbicide detoxication within the protected plant; (d) a combination of the above processes. Note that these mechanisms are directly analogous to the determinants of herbicide tolerance given above.

The view that herbicide safeners act primarily by the enhancement of metabolic detoxication of selected herbicides has gained wide acceptance. In this role safeners may induce the formation of specific co-factors such as reduced glutathione (GSH) or enzymes involved in herbicide biotransformation such as mixed function oxidases (monooxygenases), glutathione *S*-transferases (GSTs), and glucosyl transferases (6,7,8,9,10).

The metabolism of both thiocarbamate and chloroacetanilide herbicides involves conjugation with glutathione in a process enzymatically catalyzed by glutathione *S*-transferase (10, 11). Moreover, the GS-conjugation of thiocarbamate herbicides is obligately preceded by a sulfoxidation believed to be mediated by a microsomal monooxygenase (12,13). Similarly, monooxygenase-

mediated reactions may be occurring in the final steps of chloroacetanilide metabolism (11). Certain safeners have been shown to increase GSH levels (6,7,14,15). Gronwald et al. (15) found the degree of protection conferred by safeners to grass crops against chloroacetanilide herbicides to be strongly correlated with the ability of safeners to stimulate GST activity.

In addition to the above similarities in metabolism and ability to be protected by safeners the chloroacetanilide and thiocarbamate herbicides share other common properties including similar utility, and proposed mechanism(s) of action (16). These herbicides have been shown to inhibit a variety of physiological processes (16,17,18) and it is perhaps this lack of specificity in their mode of action that has allowed their protracted use without the development of weed resistance (3). This lack of a complete understanding of their mode of action warrants continued research on these herbicides.

Differential tolerance to thiocarbamate and chloroacetanilide herbicides and differential response to safeners have been observed for a number of corn lines (19,20,21,22,23,24). These differences may be useful in further understanding the basis of both endogenous and safener-conferred herbicide tolerance. These studies however have not determined if tolerance or susceptibility to one

class of herbicide is paralleled by similar tolerance to the other.

Recently Ciba-Geigy synthesized a new dichloroacetamide safener, benoxacor (also known by the code name CGA-154281), for use with the cloroacetanilide herbicide metolachlor in corn (25). This is the first safener designed for this specific application. The chemical structures of the herbicides EPTC and metolachlor, and of the safener benoxacor are given on page ix. Benoxacor has been shown to stimulate the metabolism of metolachlor by certain corn lines (24,26,27). This increase in metabolism appears to be due to the induction of GST activity without a corresponding increase in GSH content (27). This safener has also been reported to stimulate the metabolism of the sulfonylurea herbicide primisulfuron presumably by the induction of a cytochrome P450-dependent monooxygenase (28). A number of questions remain to be answered concerning the mechanism of action of this safener, particularly in terms of its effect on herbicide uptake, glutathione-mediated metabolism, and involvement in oxidative processes.

GOALS AND OBJECTIVES OF THE DISSERTATION

The overall goal of this dissertation research was to elucidate the mechanism(s) which determine the tolerance of corn to the chloroacetanilide herbicide metolachlor, both naturally occurring and that induced by the herbicide safener benoxacor. Specifically, the objectives of these studies were:

a) to compare the growth responses of eleven corn hybrids to treatment with EPTC and metolachlor and ascertain the importance of glutathione levels, glutathione *S*-transferase activity and oxidative processes in these responses;

b) to determine the basis of the differential response of the metolachlor-tolerant corn hybrid, 'Cargill 7567', and the metolachlor-susceptible corn hybrid, 'Northrup-King 9283' in terms of herbicide uptake and translocation, glutathione *S*-transferase activity, and metabolism of metolachlor;

c) to characterize the safener benoxacor by examining its efficacy, uptake and translocation;

d) to study the mechanism of action of benoxacor in terms of its effect on metolachlor uptake, non-enzymatic conjugation of metolachlor with glutathione, and involvement of oxidative processes;

e) to determine the effects of benoxacor on metolachlor

metabolism, glutathione *S*-transferase activity, and glutathione content in seedlings of two corn hybrids known to respond differentially to metolachlor to determine if predisposition to herbicide injury is a factor in their response to the safener;

f) to describe the glutathione *S*-transferase isozymes from the metolachlor-tolerant corn hybrid, 'Cargill 7567', and the metolachlor-susceptible corn hybrid, 'Northrup-King 9283';

g) to develop a molecular probe for a corn glutathione *S*-transferase gene using polymerase chain reaction (PCR) technology.

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II. COMPARATIVE RESPONSES OF SELECTED CORN (*Zea mays*) HYBRIDS TO EPTC AND METOLACHLOR

INTRODUCTION

The thiocarbamate herbicide EPTC and the chloroacetanilide herbicide metolachlor are widely used to control grass weeds in corn (*Zea mays* L.). Corn is marginally tolerant to these and other thiocarbamate and chloroactanilide herbicides. These are growth retardant type herbicides with incompletely characterized mechanisms of action. The inhibition of multiple physiological processes including the synthesis of proteins, nucleic acids, lipids, flavonoids, and isoprenoids including gibberellins is a characteristic shared by both classes of herbicides (1). The injury symptoms displayed by susceptible plants, severe stunting and twisting as a result of abnormal leaf emergence, is nearly indistinguishable for the two types of herbicides. Differential tolerance to thiocarbamate herbicides has been reported by a number of investigators (2, 3, 4). Similarly, differential tolerance to chloroacetanilide herbicides has also been observed (5, 6). Herbicide tolerance is believed to be largely a function of the ability of a plant to detoxify the herbicide in question to non-phytotoxic metabolites. Thiocarbamate and chloroacetanilide herbicides are primarily detoxified in

tolerant plants by conjugation with reduced glutathione (GSH) (7). This reaction is enzymatically catalyzed by glutathione-*S*-transferase (GST) (EC 2.5.1.18). GST activity has been described from corn with the sulfoxide of the thiocarbamate EPTC as substrate (8), with the chloroacetanilides alachlor and metolachlor (9, 10), and from sorghum with metolachlor (11).

Thiocarbamate herbicides must first be oxidized to their respective sulfoxide before conjugation to glutathione occurs (12). This reaction is believed to be catalyzed by a microsomal monooxygenase (EC 1.14.14.1) (12, 13) or a peroxygenase (14). Microsomal monooxygenase preparations from grain sorghum capable of metabolizing metolachlor *in vitro* have been reported (15), but the significance of such reactions in chloroacetanilide herbicide metabolism *in vivo* remains unclear. Piperonyl butoxide (PBO) is a potent inhibitor of microsomal monooxygenase activity (16) and is commonly used as an insecticide synergist. Hatzios found synergistic interactions between EPTC and PBO in corn (17) and between metolachlor and PBO in sorghum (18). Molecular oxygen is an obligatory substrate for monooxygenase activity (19).

Metolachlor injury on corn or grain sorghum has been reported to be particularly severe under conditions of excessive soil moisture (20). Hatzios (18) suggested that

the increased metolachlor-induced injury, observed under these conditions, might be related to decreased oxygen availability influencing monooxygenase activity in a manner similar to inhibition by PBO. Since most plant seeds require the presence of oxygen during the early stages of germination, recent research has explored the practical advantages of crop seed coating with peroxide-containing dressings, which will liberate oxygen at a controlled rate. The topic has been reviewed by Leaver and Roberts (21). Based on the aforementioned discussion, it can then be hypothesized that the presence of excess oxygen generated by peroxide-containing seed dressings may enhance the oxidative metabolism of soil-applied herbicides such as EPTC and metolachlor and improve the tolerance of corn to these herbicides.

The objectives of the present study were to: (a) compare the growth responses of eleven corn hybrids to EPTC and metolachlor treatments, (b) determine and compare the glutathione levels and glutathione *S*-transferase activities of these eleven hybrids of corn to ascertain whether these factors contribute to herbicide tolerance of corn, and (c) characterize the involvement of oxidative processes in the metabolism of EPTC and metolachlor in corn by studying the interactions of EPTC and/or metolachlor with the antioxidant piperonyl butoxide and the oxygen-evolving compound calcium

peroxide.

MATERIALS AND METHODS

Chemicals. EPTC (formulated EPTAM herbicide) and metolachlor (formulated DUAL herbicide) were provided by Stauffer Chemical Co., Westport CT; and Ciba-Geigy Corp., Greensboro, NC, respectively. Piperonyl butoxide (PBO) was a gift of Endura Company, Bologna, Italy. Calcium peroxide was purchased from Alpha Products, Danvers, MA. All other chemicals were from Sigma, St. Louis, MO.

Plant Material and Chemical Application. The following eleven corn lines were utilized in the present study: 'Cargill 7567'; 'Northrup-King 9283'; 'Great Lakes 584'; 'DeKalb' hybrids XL67 and XL72AA; and 'Pioneer' hybrids 3147, 3179, 3318, 3377, 3475 and 3744. The known history of the growth responses of some of these hybrids to selected thiocarbamate and chloroacetanilide herbicides has been summarized in Table 1. As can be seen, not all of these hybrids have been tested for their response to EPTC or metolachlor.

Plants were grown from seed in a 1:1:1 (v/v/v) mixture of potting medium (Weblite, Weblite Corp., Blue Ridge, VA), vermiculite, and peat, three plants per 236-ml styrofoam cup, under greenhouse conditions. Cups were half filled with soil mixture, seeds placed on this surface and were then covered with a 3 cm deep layer of the same soil treated

Table 1. Known history of the response of the eleven corn hybrids to thiocarbamate or chloroacetanilide herbicide treatment.

Number	Hybrid code name	Response[a] to EPTC	Response[a] to Metolachlor	Reference
1	Cargill 7567	NT[b]	Tolerant	27
2	Great Lakes 584	NT	Tolerant	27
3	DeKalb XL72AA	Tolerant	NT	3, 4
4	Pioneer 3377	Tolerant	NT	30
5	Pioneer 3179	Susceptible	NT	29, 30
6	Pioneer 3475	Tolerant	NT	30
7	Pioneer 3744	NT	Susceptible	27
8	Pioneer 3147	Tolerant	NT	29, 30
9	Northrup King 9283	NT	Susceptible	27
10	Pioneer 3318	Susceptible	NT	30
11	DeKalb XL67	Susceptible	NT	3, 4

[a] Growth response of corn hybrids treated with EPTC or metolachlor applied at rates as high as 6.7 kg/ha.

[b] NT = not tested

with the appropriate chemical; 6.7 kg/ha EPTC, 6.7 kg/ha metolachlor, 6.7 kg/ha EPTC plus 6.7 kg/ha PBO, or 6.7 kg/ha metolachlor plus 6.7 kg/ha PBO. Untreated controls were also included. The herbicide treatments used in this study were twice the maximum recommended use rates for both herbicides (22). Previous studies (17,18) have shown an equivalent PBO treatment as that used in this investigation to effect herbicide synergism without influencing plant growth when applied alone. Greenhouse conditions used in all experiments included temperatures of 25 ± 5 °C and a 14-hr photoperiod with a photosynthetic photon flux density (PPFD) of 400 $\mu E/m^2/s$.

Calcium Peroxide Treatment. For these experiments the only corn hybrid evaluated was 'Northrup-King 9283', which is susceptible to both EPTC and metolachlor. Planting procedures were as above except that granular calcium peroxide ($CaO_2 \cdot 8H_2O$, 60%) was applied at 0.2 and 0.4g per cup to the untreated soil surface before planting the seeds and covering with 3 cm of either untreated soil or soil treated with either EPTC or metolachlor at 6.7 kg/ha.

Evaluation of Growth Response. Ten days after planting corn seedling shoot heights and dry weights were determined. Data presented are the means and standard errors of two experiments with three replications per treatment. In some cases Colby's method (23) was utilized to characterize

treatment interactions.

Total Glutathione Analysis. Leaf tissue of untreated ten-day old corn seedlings was collected and frozen immediately in liquid nitrogen. After grinding of the frozen leaf material to a fine powder in a mortar and pestle, 0.5 g sub-samples were extracted with 5 ml of 5% trichloroacetic acid (TCA). The resulting extract was then clarified by centrifugation at 20,000 x *g* for 15 min. The supernatant was diluted 25-fold with 0.35 M Na-K phosphate buffer, pH 7.5, and the total glutathione content was determined by the method of Tietz (24). Data presented are the means and standard errors of five independent observations from two growth harvests.

GST Assays. Leaf tissue of untreated ten-day old corn seedlings was collected and frozen immediately in liquid nitrogen. One g of leaf tissue was homogenized with a mortar and pestle in 5 ml of ice-cold 0.1 M potassium phosphate buffer (pH 6.9) and 0.2 g of polyvinyl-polypyrrolidone (PVPP). The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged for 20 min at 20,000 x *g*. GST enzyme activity was determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (GST-CDNB activity) and with ^{14}C-metolachlor as substrate (GST-metolachlor activity). GST-CDNB activity was determined according to

Mannervik and Guthenberg (25). The reaction mixture contained 2.0 ml 100 mM potassium phosphate buffer (pH 6.9), 0.9 ml 3.3 mM GSH, 100 µl 30 mM CDNB, and 10 µl enzyme extract. The change in absorbance due to formation of the GS-CDNB conjugate was measured at 340 nm in a Hitachi Model 100-10 spectrophotometer (Hitachi Ltd., Tokyo) at 25 °C. Correction for the non-enzymatic formation of the GS-CDNB conjugate was made by measuring and subtracting the rate in the absence of enzyme. A molar extinction coefficient of 10 mM/cm (25) was used to calculate enzyme activity.

GST-metolachlor activity was determined essentially as described by Mozer et al. (9) with slight modifications. The reaction mixture contained 30 µl potassium phosphate buffer (pH 6.9), 10 µl 60 mM GSH, 10 µl metolachlor (13 nCi carbonyl-labeled ^{14}C-metolachlor, specific activity 59.5 µCi/µmol), and 10 µl enzyme extract. After incubating for 60 min at 30 °C the reaction was terminated by the addition of 60 µl 5% trichloroacetic acid (TCA) and 1 ml of methylene chloride. The reaction tunes were mixed by vortexing for 10 sec and the organic and aqueous phases separated by a 2 min microcentrifugation. The radioactivity in a 60 µl aliquot of the aqueous phase containing the GS-metolachlor conjugate was determined by liquid scintillation spectrometry (LSC). Blanks containing all components except plant extract were included to correct for non-enzymatic conjugation.

Enzyme activities for both substrates are expressed on a per mg protein basis. Protein content was determined by the dye binding method of Bradford (26) with bovine serum albumin as standard. Data presented are the means and standard errors of six independent determinations from three growth harvests.

RESULTS

Influence of EPTC and Metolachlor on Corn Seedling Growth. Differential tolerance to both EPTC and metolachlor was observed for the eleven corn hybrids examined in this study (Fig. 1). Five of the eleven corn hybrids; 'Cargill 7567', 'Great Lakes 584', 'DeKalb XL72AA', and 'Pioneer' 3377 and 3179 were exceptionally tolerant to EPTC at the 6.7 kg/ha rate utilized. For this group of five corn hybrids growth inhibition by EPTC ranged from 0 to 9% and there was no visible herbicide damage. A second group of four corn hybrids; 'Pioneer' 3475, 3744, 3147, and 'Northrup-King 9283' were intermediate in their growth response to EPTC with growth inhibition of from 22 to 40%. The 'Pioneer 3318' and 'DeKalb XL67' hybrids of corn were very susceptible to EPTC with growth inhibitions greater than 70% (Fig. 1). In general, the growth responses of some of the tested corn hybrids to EPTC treatment were similar to those reported previously (Table 1). The only discrepancy

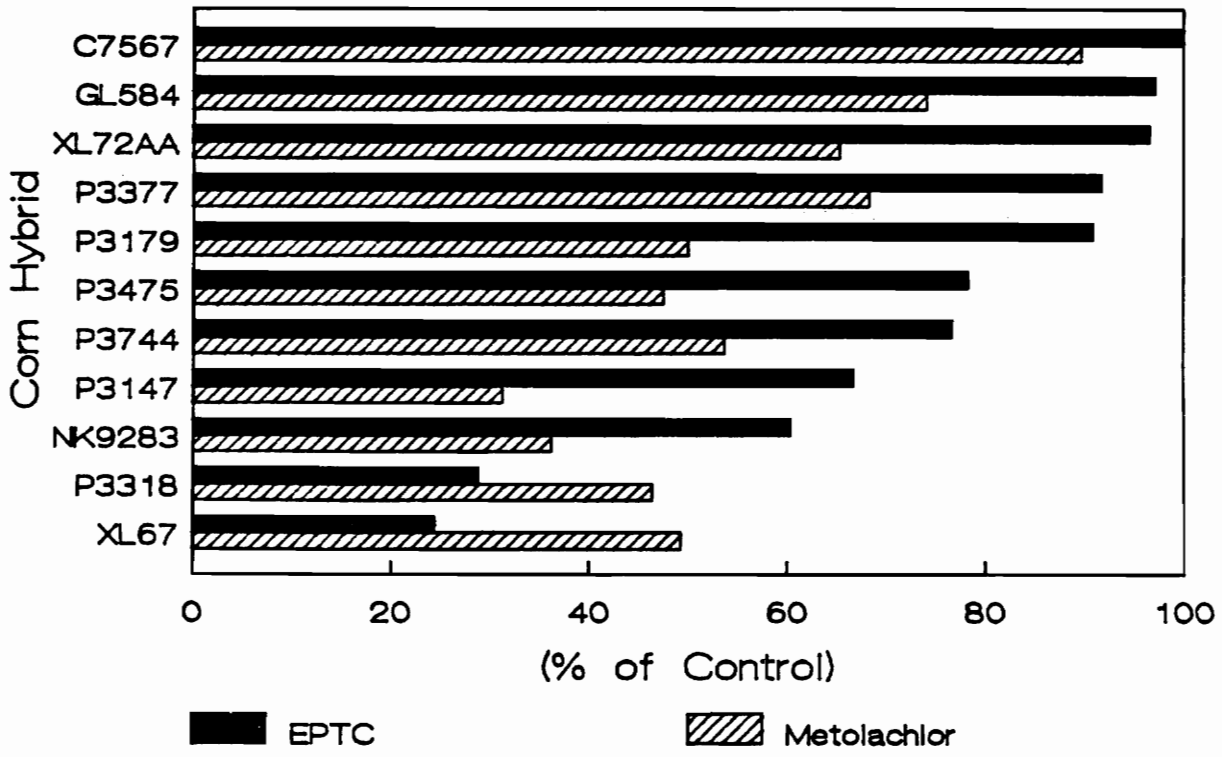

Figure 1. Growth response (shoot height) of 10-day old corn hybrids to pre-plant incorporated treatments with 6.7 kg/ha of EPTC and metolachlor.

observed was related to the 'Pioneer 3179' hybrid of corn which has been previously reported to be susceptible to EPTC (24), but was found to be tolerant in our studies.

Data in Figure 1 illustrate that in this study seedling growth was more severely inhibited by metolachlor than by EPTC for nine of the eleven hybrids. The two hybrids that were most susceptible to EPTC phytotoxicity, 'Pioneer 3318' and 'DeKalb XL67' were effected to a much lesser extent by metolachlor. Growth inhibition for these two hybrids was about 70% for EPTC as compared to 50% for metolachlor. Only 'Cargill 7567', with a growth reduction of 10% could be described as metolachlor tolerant. Growth inhibition by metolachlor at the 6.7 kg/ha rate ranged from 30 to 70% for the other hybrids. These results are in agreement with those of Rowe and Penner (27) characterizing the 'Cargill 7567' and 'Great Lakes 584' corn hybrids as tolerant and the 'Pioneer 3744' and 'Northrup-King 9283' hybrids as susceptible to similar metolachlor treatment.

Relationship of Glutathione Content to Herbicide Tolerance. Total glutathione contents and relative tolerance to EPTC and metolachlor of the eleven corn hybrids examined in this study are presented in Table 2. Values range from 1.8 to 3.0 µmol GSH per g fresh weight of shoot tissue. There is no apparent relationship between glutathione content and herbicide tolerance. In fact; the

Table 2. Glutathione content and tolerance ratings of 10-day old seedlings of selected corn hybrids to EPTC and metolachlor treatments.

Hybrid	Glutathione Content (µmol GSH/g FW)	Tolerance Rating[a]	
		EPTC	Metolachlor
Cargill 7567	2.28 ± 0.37	+++	+++
G. Lakes 584	2.22 ± 0.50	+++	++
DeKalb XL72AA	2.37 ± 0.50	+++	+
Pioneer 3377	2.03 ± 0.34	+++	+
Pioneer 3179	1.99 ± 0.47	+++	+
Pioneer 3475	1.81 ± 0.37	++	-
Pioneer 3744	2.12 ± 0.41	++	+
Pioneer 3147	2.34 ± 0.56	+	-
N-K 9283	1.97 ± 0.28	+	-
Pioneer 3318	2.99 ± 0.56	-	-
DeKalb XL67	2.40 ± 0.50	-	-

[a] Relative tolerance rating of corn hybrids to treatments with 6.7 kg/ha of EPTC or metolachlor are as follows:

+++ = ≤ 10% inhibition of shoot height

++ = 11-25% inhibition of shoot height

+ = 26-50% inhibition of shoot height

- = > 50% inhibition of shoot height

corn hybrid with the highest observed glutathione content, 'Pioneer 3318', was very susceptible to injury by both EPTC and metolachlor. The lack of a definite positive correlation between GSH content and tolerance of corn hybrids or inbreds to thiocarbamate herbicides has been reported previously by Lay and Niland (2).

Relationship of GST Activity to Herbicide Tolerance. GST activities and relative tolerance to metolachlor of the eleven corn hybrids examined in this study are presented in Table 3. In general higher GST activities were associated with greater metolachlor tolerance. Hybrids which were inhibited 50% or less by 6.7 kg/ha metolachlor treatment had GST-metolachlor activities on average about 75% higher and GST-CDNB activities about 30% higher than the more susceptible hybrids. There were, however, susceptible hybrids with GST activities equivalent to some of the more tolerant hybrids.

Interactive Effects of EPTC or Metolachlor with PBO. A synergistic effect was observed on the response of eight of the eleven corn lines examined in this study when the antioxidant PBO was applied in combination with EPTC (Fig. 2). This synergism was more pronounced on corn lines that were intermediate in their response to EPTC. The most tolerant corn hybrid, 'Cargill 7567'; and the two most EPTC-susceptible hybrids, 'Pioneer 3318' and 'DeKalb XL67'

Table 3. GST activity and metolachlor tolerance of 10-day old seedlings of selected corn hybrids.

Hybrid	GST-Metolachlor (pmol/min/mg)	GST-CDNB (µmol/min/mg)	Tolerance[a]
Cargill 7567	12.1 ± 1.0	8.3 ± 1.4	+++
G. Lakes 584	8.9 ± 1.8	11.5 ± 1.0	++
DeKalb XL72AA	7.2 ± 2.4	10.2 ± 1.3	+
Pioneer 3377	9.9 ± 1.2	10.5 ± 1.7	+
Pioneer 3179	10.1 ± 0.6	6.4 ± 1.3	+
Pioneer 3475	8.0 ± 1.3	4.0 ± 1.4	-
Pioneer 3744	10.9 ± 1.5	11.2 ± 2.4	+
Pioneer 3147	5.6 ± 1.8	15.7 ± 3.4	-
N-K 9283	5.2 ± 0.6	4.7 ± 0.9	-
Pioneer 3318	2.5 ± 0.6	6.0 ± 1.0	-
DeKalb XL67	6.8 ± 1.0	6.6 ± 0.8	-

[a] Relative tolerance rating of corn hybrids to treatments with 6.7 kg/ha of metolachlor are as follows:

+++ = ≤ 10% inhibition of shoot height

++ = 11-25% inhibition of shoot height

+ = 26-50% inhibition of shoot height

- = > 50% inhibition of shoot height

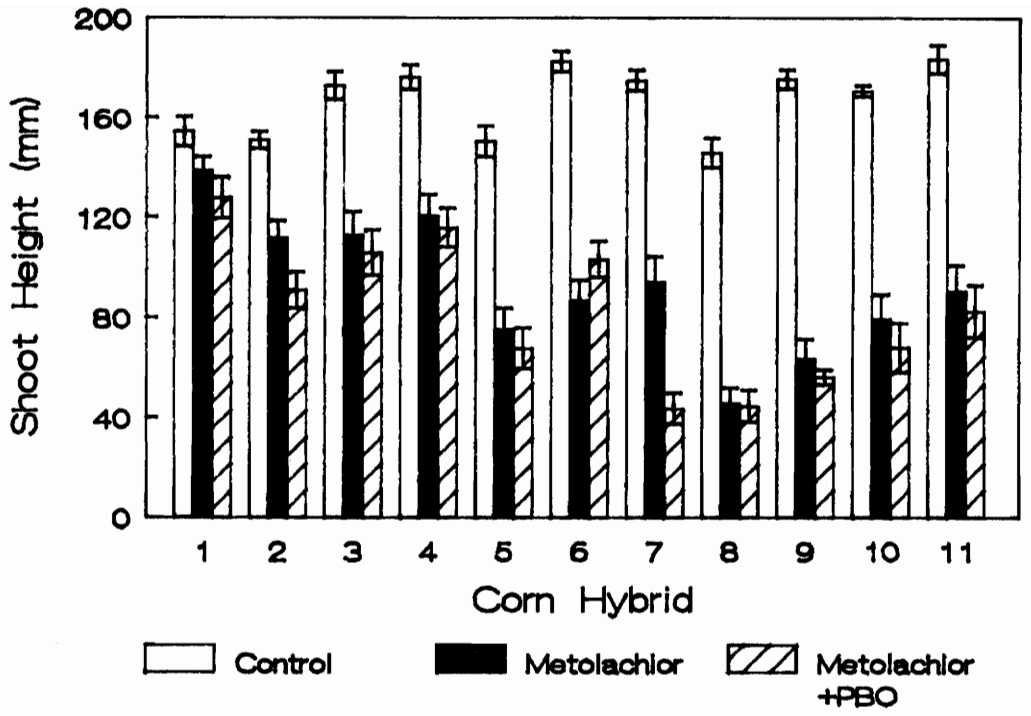

Figure 2. Combined effects of EPTC and PBO on shoot height of 10-day old seedlings of selected corn hybrids. Both EPTC and PBO were applied at 6.7 kg/ha. For numbering system of corn hybrids used see Table 1.

did not appear to be influenced by the PBO treatment. PBO acted synergistically in combination with metolachlor mainly on the corn hybrid, 'Pioneer 3744', and to a lesser extent on the 'Great Lakes 584' hybrid (Figure 3). The combined effects of PBO and metolachlor on all other corn hybrids appeared to be additive.

Influence of Calcium Peroxide Soil Treatment on Growth Response of 'Northrup-King 9283' Corn to EPTC or Metolachlor. The effects of EPTC or metolachlor on shoot height and dry matter accumulation for the herbicide-sensitive corn hybrid 'Northrup-King 9283' grown in the presence of two different calcium peroxide treatments are shown in Tables 4 and 5. In the absence of calcium peroxide, shoot growth was inhibited about 50% and dry weight was reduced about 30% by either herbicide. The 0.2 g calcium peroxide treatment appeared to have an antagonistic effect on EPTC (observed value < expected value). In contrast, the same treatment had a synergistic effect on metolachlor for this corn hybrid (observed value > expected value). The higher calcium peroxide treatment (0.4 g) did not result in further synergism of metolachlor nor antagonism of EPTC.

DISCUSSION

Based on the similarities of thiocarbamate and

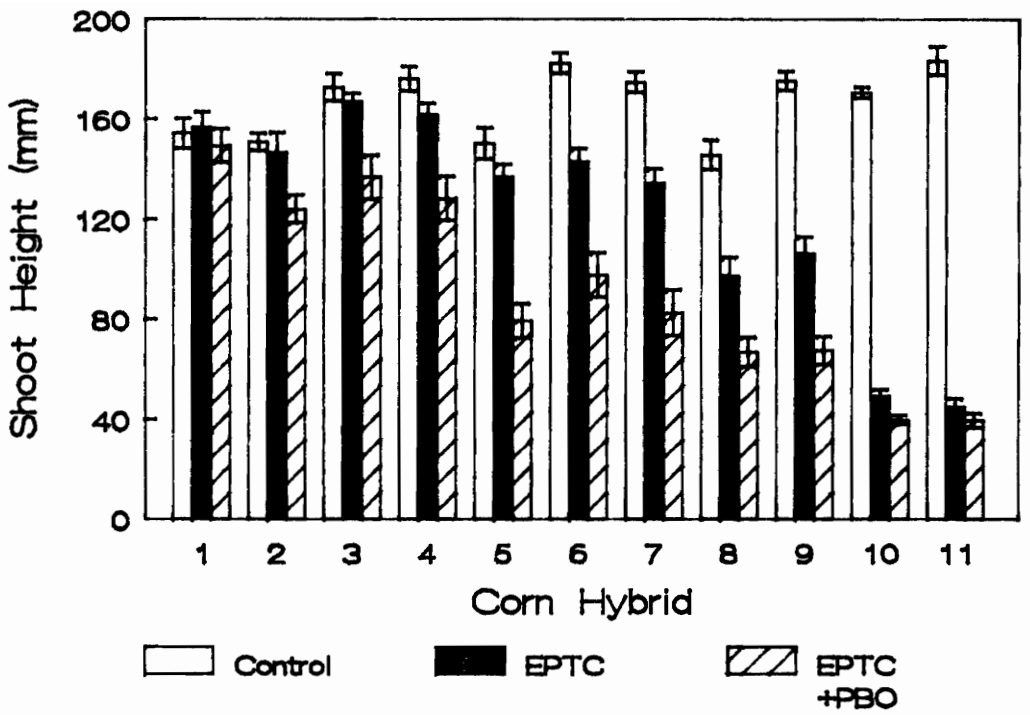

Figure 3. Combined effects of metolachlor and PBO on shoot height of 10-day old seedlings of selected corn hybrids. Both metolachlor and PBO were applied at 6.7 kg/ha. For numbering system of corn hybrids used see Table 1.

Table 4. Interactions of EPTC or metolachlor with calcium peroxide on 10-day old seedlings of 'Northrup King 9283' corn.

CaO_2 (g)	Herbicide treatment (6.7 kg/ha)		
	Control	EPTC	Metolachlor
	---------- Shoot height (mm) ----------		
0.0	133 ± 10	71 ± 6	79 ± 12
0.2	133 ± 13	98 ± 5	52 ± 6
		(71)	(79)
0.4	142 ± 13	79 ± 7	68 ± 10
		(76)	(84)

Values in parentheses represent expected responses that were calculated according to Colby's method (23).

Table 5. Interactions of EPTC or metolachlor with calcium peroxide on 10-day old seedlings of 'Northrup King 9283' corn.

CaO_2 (g)	Herbicide treatment (6.7 kg/ha)		
	Control	EPTC	Metolachlor
	---------- Shoot dry weight (mg) ----------		
0.0	76 ± 12	50 ± 16	55 ± 9
0.2	85 ± 18	62 ± 13	40 ± 7
		(56)	(62)
0.4	87 ± 20	57 ± 10	47 ± 8
		(57)	(63)

Values in parentheses represent expected responses that were calculated according to Colby's method (23).

chloroacetanilide herbicides it was expected that the degree of tolerance or susceptibility of a particular corn hybrid to EPTC would be paralleled by a similar degree of tolerance or susceptibility to metolachlor. The results of the present study confirm previously published reports demonstrating intraspecific differential responses of several crop cultivars or genotypes to thiocarbamate and chloroacetanilide herbicides (2-5, 27-30). As a group the eleven corn hybrids examined in this study exhibited differential responses to both herbicides (Fig. 1). Some hybrids were quite tolerant while others were severely injured by EPTC and metolachlor at the 6.7 kg/ha rate used in these experiments. Most of the tested corn hybrids were more susceptible to treatment with metolachlor than EPTC. The only exceptions were the two hybrids most susceptible to EPTC, 'Pioneer 3318' and 'DeKalb XL67', which were more sensitive to the phytotoxic effect of EPTC than that of metolachlor.

It is apparent from these results that the degree of tolerance observed for one of these herbicides is not necessarily matched by a similar degree of tolerance to the other. There may be several reasons for this disparity; including differences in uptake and translocation of the herbicides, differences in the metabolism of the herbicides, and unexplained differences in the mechanisms of action of

the two herbicides. The tolerance of the 'DeKalb XL72AA' hybrid of corn to EPTC has been shown to be controlled by a single recessive gene (4). The biochemical nature of the product(s) expressed by this gene is not known at the present time. Peptides such as glutathione (GSH) and enzymes such as glutathione-*S*-transferases (GSTs) and mixed-function oxidases play a key role in the biotransformations of thiocarbamate and chloroacetanilide herbicides in corn and other grass species (7-15). The genetic basis of the tolerance or susceptibility of the remaining corn hybrids to EPTC and/or metolachlor is not presently available. The control of the EPTC or metolachlor tolerance in corn genotypes by single or multiple gene systems and the exact products coded by such gene systems remain to be elucidated.

There has been considerable interest in the role of glutathione in the metabolic detoxication of both thiocarbamate and chloroacetanilide herbicides. Increased glutathione levels have been observed in response to certain herbicide safeners by a number of researchers (8, 11, 31, 32) who have suggested that this increase confers enhanced herbicide tolerance. This would imply that plants with higher endogenous glutathione levels would tend to be more tolerant to EPTC and metolachlor. The findings presented here for 10-day old seedlings (Table 2) do not support such

a hypothesis. Perhaps the importance of glutathione content in determining differential herbicide tolerance is more apparent during early seedling growth in herbicide treated soils.

A relationship between GST activity and metolachlor tolerance was suggested by this study (Table 3). Although there were exceptions, higher values of GST activity were generally characteristic of the more tolerant corn hybrids. Multiple GST isozymes with varying substrate specificities are known to exist in corn (9,10). In this study both a general substrate (CDNB) and a specific substrate (^{14}C-metolachlor) were used to measure GST activity. The latter substrate is perhaps the best measure of GST activity directly concerned with metolachlor detoxication. Measurements of GST activity with CDNB as substrate must be interpreted with caution as this method may detect GST activity not associated with metolachlor detoxication. Similarly, crude extracts may contain isozymes for which the GST-metolachlor assay has not been optimized. This may explain why corn hybrids with marked differences in metolachlor tolerance, such as the DeKalb hybrids 'XL67' and 'XL72AA', have similar GST-metolachlor activities. Examples of an uncertain correlation between corn tolerance to EPTC and GST activity have been reported by Lay and Niland (2) with selected corn hybrids and inbred lines. In this study,

corn GST activity catalyzing the conjugation of EPTC to GSH was not determined due to the unavailability of the specific substrate (^{14}C-EPTC-sulfoxide) required for such assays.

In this study PBO was utilized as an indirect method of assessing the contribution of oxidative reactions in the metabolism of EPTC and metolachlor. The sulfoxidation of EPTC is enzymatically catalyzed by a microsomal monooxygenase and is a requisite step to subsequent glutathione conjugation (12). An inhibition of this oxidative reaction should indirectly result in a reduction in the rate of EPTC detoxication. The data presented here (Fig. 2) support this assumption. In general PBO had no influence on the effect of metolachlor (Fig. 3) indicating a lack of involvement in this pathway of metolachlor metabolism.

Calcium peroxide treatments have been used to increase the oxygen content available to seeds during seed germination (21). This oxygen liberating compound could conceivably stimulate the activity of oxygen-dependent monooxygenase and thus stimulate metabolism. The objective of these experiments were to determine the influence of enhanced oxygen content on the growth response of a corn hybrid susceptible to both EPTC and metolachlor. Treatment effects were most pronounced for the 0.2 g calcium peroxide treatment. The antagonistic effect on EPTC is presumably

due to an increase in EPTC sulfoxidation and subsequent GSH conjugation. The reason for the observed synergistic effect on metolachlor is unclear. Perhaps uptake of the herbicide is more rapid or some as yet unidentified process is rendered more sensitive to metolachlor at higher oxygen concentrations.

The identification and characterization of corn lines with differential tolerance to thiocarbamate and chloroacetanilide herbicides will no doubt be useful to the further understanding of the mechanism of action of these herbicides and the safeners that are used to chemically regulate the tolerance of corn to these herbicides. These experiments suggest that oxidative reactions are important in determining tolerance to EPTC but are not involved in determining tolerance to metolachlor. They also suggest a relationship between metolachlor-tolerance and GST activity. The role of glutathione as a limiting factor in determining tolerance to either herbicide appears unlikely. Differential herbicide tolerance is probably controlled by multiple factors including herbicide absorption and metabolism characteristics. These factors may be further modified by developmental and environmental conditions. Further studies are needed to address these phenomena.

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III. BASIS OF DIFFERENTIAL METOLACHLOR TOLERANCE BY 'CARGILL 7567' AND 'NORTHRUP-KING 9283' CORN

INTRODUCTION

Differences in absorption and metabolism have been shown to be the basis of interspecific differences in tolerance to certain chloroacetanilide herbicides. Uptake and translocation of alachlor was greater for wheat, a susceptible species, than for soybean a resistant species (1). Yellow nutsedge, a weed which can be controlled by metolachlor, was shown to absorb metolachlor more rapidly and to a greater extent than corn (2). Enhanced metabolism of chloroacetanilides has been attributed as a major factor in determining chloroacetanilide selectivity (2,3,4). The enhanced metabolism observed in tolerant species is thought to be due to the ability of these species to maintain high levels of reduced glutathione (GSH) or the expression of glutathione *S*-transferase (GST) isozymes with greater herbicide specificity (5,6).

While corn is generally tolerant to the chloroacetanilide herbicide metolachlor, differential tolerance has been observed for a number of inbred and hybrid corn lines (7,8,9). The objectives of this study were: (a) to further document the differential effect of metolachlor on the growth of the metolachlor-tolerant corn hybrid, 'Cargill

7567' and the metolachlor-susceptible corn hybrid, 'Northrup-King 9283' in a greenhouse study; (b) to determine the relative rates of metolachlor uptake and translocation for these two corn hybrids; (c) to quantify the expression of GST activity of both hybrids on a developmental scale; and (d) to determine the relative rates of metolachlor metabolism of these hybrids.

MATERIALS AND METHODS

Seeds of 'Cargill 7567' and 'Northrup-King 9283' corn were kindly provided by Cargill Co., Minneapolis, MN and Northrup-King Co., Minneapolis, MN. ^{14}C-metolachlor was provided by Ciba-Geigy Corp., Greensboro, NC. HPLC grade solvents and additional chemicals were purchased from commercial vendors.

Greenhouse Study. Seeds of 'Cargill 7567' and 'Northrup-King 9283' corn were planted in 236-ml styrofoam cups, 3 seeds/cup, in a 1:1:1 (v/v/v) mixture composed of potting medium (Weblite, Weblite Corp., Blue Ridge, VA), vermiculite, and peat. The soil mixture was treated pre-plant incorporated with metolachlor at 6.7 kg/ha. Three cups were planted per treatment and the experiment was repeated twice in time. The greenhouse temperature was maintained at 25 ± 5 °C. Natural sunlight provided an approximate 14-hr photoperiod with a photosynthetic photon

flux density (PPFD) of 400 µE/m²/s. Shoot heights were determined 6 and 14 days after planting and herbicide injury was evaluated by comparing metolachlor treated plants to untreated controls.

Uptake Study. The plant material for these experiments was obtained by germinating seeds of 'Cargill 7567' and 'Northrup-King 9283' corn on two layers of moistened filter paper (Whatman #1) in Petri dishes in a dark growth chamber at 30° C. After 72 hr the emerging coleoptiles were about 25 mm in length. At this time, a 2 µl drop of ^{14}C-metolachlor (3.3 nCi ø-labeled ^{14}C-metolachlor, specific activity 21.6 µCi/µmol) was applied to the coleoptile. The seedlings were then placed in a sealed plastic box containing water saturated paper towels to maintain a high relative humidity and reduce evaporation. The humidity chambers were then returned to the growth chamber. Seedlings were removed at 0, 0.5, 1, 2, 4, and 8 hr after treatment. Shoot tissue was cut from the rest of the seed and rinsed in 1.0 ml of 80% methanol. After rinsing the shoots were combusted in a biological sample oxidizer (Packard Tri-Carb model 306, Packard Instrument Co., Downers Grove, IL) and the amount of radioactivity absorbed determined by liquid scintillation spectrometry (LSC) (Beckman LS5000TA, Beckman Instruments, Inc., Fullerton,CA). The remaining tissue, root plus seed, were

similarly oxidized and the radioactivity was quantified by LSC. Three seedlings were utilized per treatment time and the experiment was repeated three times.

GST Analysis. A liquid culture method as described by Dean et al. (10) was used for this experiment. Seeds of both corn hybrids were germinated for 48 hr on H_2O saturated filter paper before being transferred to incubation medium consisting of 10 mM HEPES (pH 7.5), plus 1 mM $CaCl_2$. Fifteen seedlings were placed in each of six flasks containing 100 ml of incubation medium and were maintained at 30 °C in a dark growth chamber. The liquid cultures were aerated vigorously throughout incubation for 12, 24, 36, 48, and 72 hr. Every 24 hr the incubation medium was drained, the seedlings were rinsed with distilled water, and fresh medium was added to the culture flasks.

At each sampling interval two seedlings were removed from each of the six flasks. After discarding the seeds, the shoot and root tissue of six seedlings were combined and ground to a powder with a mortar and pestle under liquid nitrogen. To this powder 0.1 g of polyvinyl-polypyrrolidone (PVPP) was added followed by 3 ml of 0.1 M potassium phosphate buffer, pH 6.9. The resulting slurry was homogenized with mortar and pestle, centrifuged at 20,000 x *g* for 20 min, and the supernatant utilized as the source of enzyme. Protein was determined by the dye binding method of

Bradford (11).

GST activity was determined essentially as described by Mozer et al. (12) with slight modifications. The enzyme assay was performed as follows: in a 1.5 ml microfuge tube 10 µl of extract was mixed with 30 µl of 100 mM potassium phosphate buffer (pH 6.9), 10 µl of 60 mM GSH, and 10 µl of 60 µM metolachlor (containing 13 nCi carbonyl-labelled ^{14}C-metolachlor, specific activity 59.5 µCi/µmol), giving final concentrations of 10 mM GSH and 10 µM metolachlor. After 60 min incubation at 30 °C the reactions were stopped by the addition of 60 µl of 5% trichloroacetic acid (TCA). Parent herbicide and GS-metolachlor were fractionated by the addition of 1 ml of methylene chloride to the reaction. The reactions were then vortexed for 10 sec and microcentrifuged for 2 min. Sixty µl of the aqueous phase containing the GS-metolachlor conjugate was counted using LSC. Blanks containing all components except plant extract were included to correct for non-enzymatic conjugation.

Metabolism Study. Seeds were germinated on H_2O saturated filter paper for 3-days 30 °C in a dark growth chamber. Six excised apical sections (20 mm) were placed in vials containing 10 nCi ø-labelled ^{14}C-metolachlor in 400 µl of incubation medium (10 mM HEPES, 1 mM $CaCl_2$, pH 7.5) for 1, 2, 4, and 8 hr at 27 °C. After incubation the apices were removed, rinsed with 80% methanol, and the

absorbed ^{14}C-metolachlor extracted by grinding the apices in 1 ml of 80% methanol. The extract was clarified by centrifuging for 5 min in a microcentrifuge and the radioactivity contained in the extract was determined by counting a 60 µl aliquot of the supernatant. At least 90% of the applied radioactivity was extracted by this method. To determine the amount of ^{14}C-metolachlor conjugated with metolachlor an additional 60 µl aliquot was removed, mixed with 60 µl of H_2O, and fractionated with 1 ml of methylene chloride. The experiment was performed twice with at least three replicates per treatment.

RESULTS

Effect of Metolachlor on Growth of 'Cargill 7567' and 'Northrup-King 9283' Corn Seedlings. The results of the greenhouse study reported here clearly demonstrate the differential response of these two corn hybrids to metolachlor (Figure 1). Shoot height of both corn hybrids was reduced when grown in soil treated with 6.7 kg/ha metolachlor. Shoot height of six-day old 'Cargill 7567' was reduced by 52% and 'Northrup-King 9283' was reduced by 68% . At this age the seedlings of both varieties showed typical chloroacetanilide herbicide injury symptoms; stunted growth, rolling and twisting of primary leaves. These symptoms were more pronounced for the 'Northrup-King' hybrid. After 14

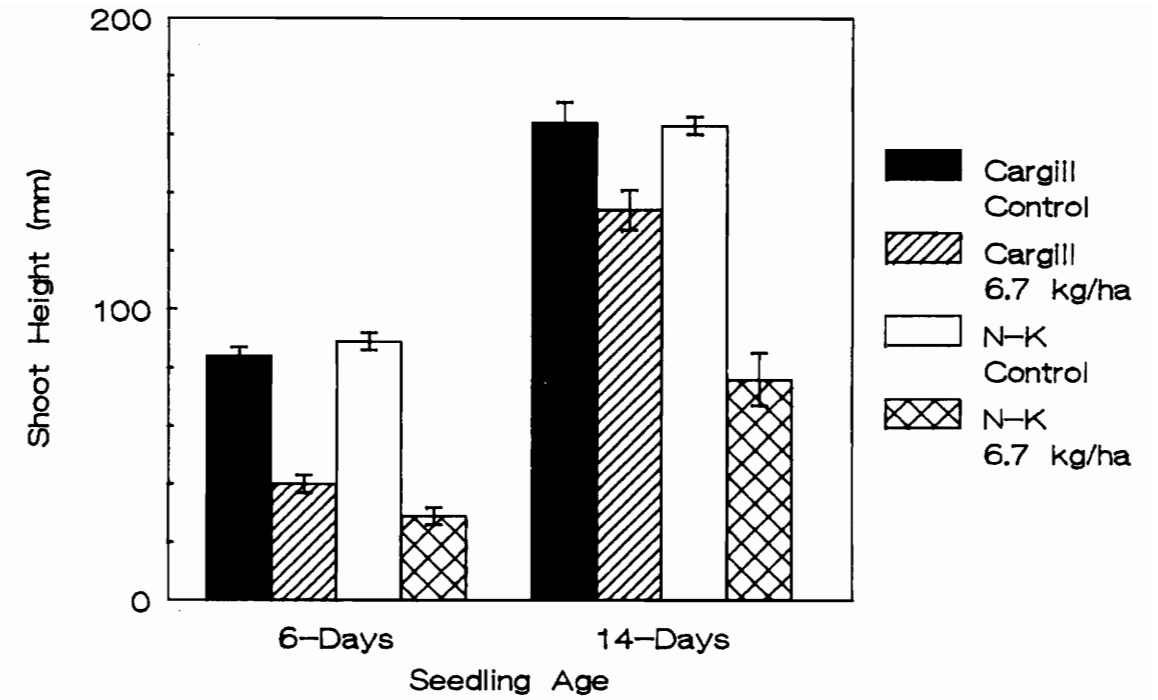

Figure 1. Growth response of 'Cargill 7567' and 'Northrup-King 9283' corn seedlings to 6.7 kg/ha metolachlor treatment. Shoot heights were determined 6 and 14 days after planting.

days shoot height of 'Cargill 7567' and 'Northrup-King 9283' treated with 6.7 kg/ha metolachlor were reduced by 18% and 53%, respectively. At this time herbicide injury was no longer apparent for the 'Cargill' plants although they were somewhat shorter than the untreated controls. In contrast, plants of the 'Northrup-King' variety were unable to recover from the effects of herbicide toxicity and after 14 days still showed twisted leaves and stunted growth.

Uptake and Translocation of Metolachlor. Uptake of ^{14}C-metolachlor by shoots of 3 day-old 'Cargill 7567' and 'Northrup-King 9283' corn is shown in Figure 2. Uptake of ^{14}C-metolachlor is more rapid during the first hour of exposure by the metolachlor-susceptible 'Northrup-King' corn hybrid than for the metolachlor-tolerant 'Cargill' variety. Eight hours after application 80% of the applied radioactivity was found in the shoots of 'Northrup-King 9283' as compared to 63% in shoots of 'Cargill 7567'. Even after 8 hr exposure to the shoot applied ^{14}C-metolachlor only slight amounts of radioactivity were found in the root and seed tissue of either corn hybrid (Table 1).

Developmental Progression of GST Activity. Glutathione *S*-transferase activity was measured at six time intervals during seedling development of 'Cargill 7567' and 'Northrup-King 9283' corn (Fig. 3). GST activity was similar for both corn hybrids 48 and 60 hr after imbibition

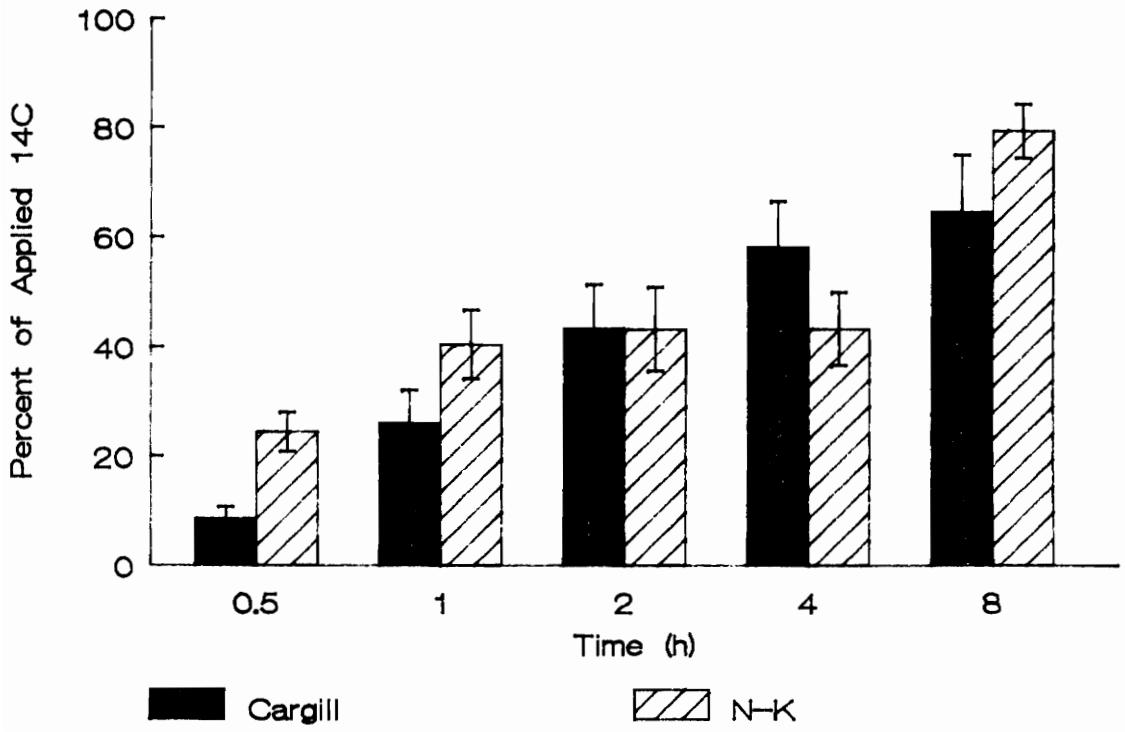

Figure 2. Absorption of ^{14}C-metolachlor by shoots of 3-day old 'Cargill 7567' and 'Northrup-King 9283' corn seedlings.

Table 1. Translocation of ^{14}C into seed and root tissue after application of ^{14}C-metolachlor to the coleoptiles of 3-day old corn seedlings.

Time (hr)	Cargill 7567 --dpm[a]--	Cargill 7567 % of applied	Northrup-King 9283 --dpm--	Northrup-King 9283 % of applied
0.5	N.D.[b]	N.D.	19 ± 4	.2
1	25 ± 12	.3	19 ± 14	.2
2	39 ± 7	.5	31 ± 9	.4
4	116 ± 24	1.6	78 ± 27	1.1
8	174 ± 22	2.4	93 ± 31	1.3

[a] Data, expressed as dpm per plant, are the mean and standard error of nine observations from three experiments.

[b] N.D. = not detectable.

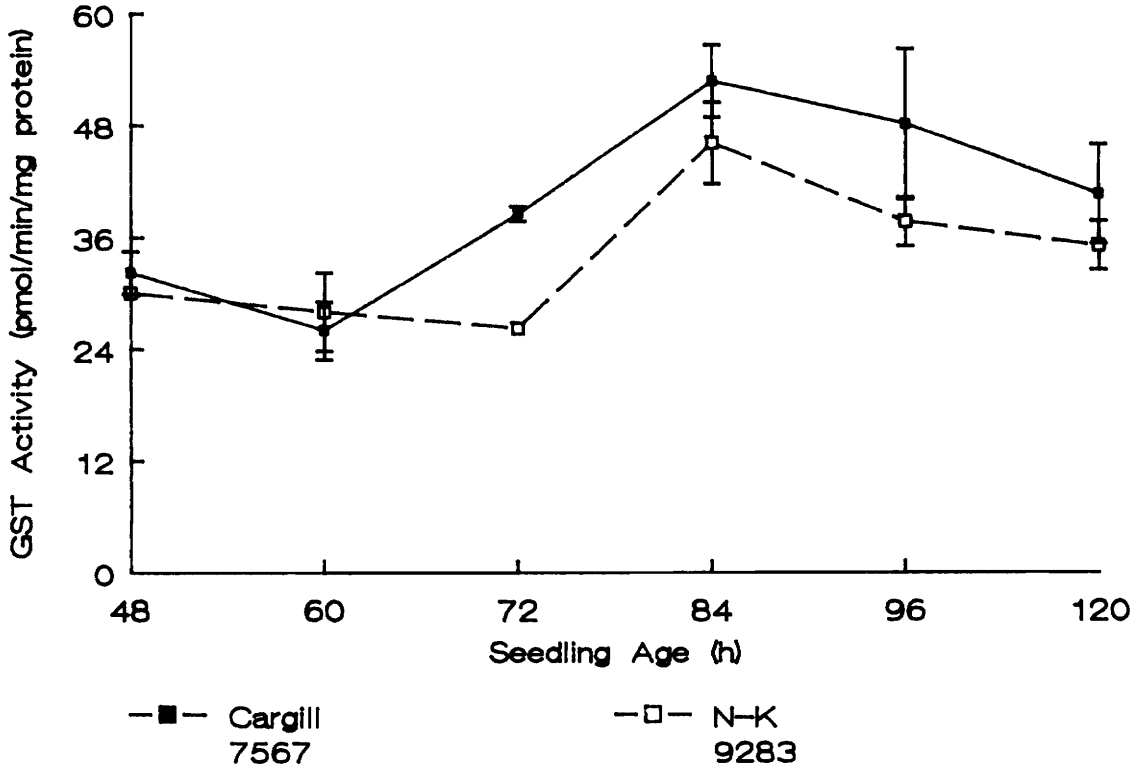

Figure 3. GST-metolachlor activity of 'Cargill 7567' and 'Northrup-King 9283' corn as a function of seedling age.

began. At 72 hr GST of the metolachlor-tolerant corn hybrid, 'Cargill 7567', showed a marked increase in activity which was not observed for the less tolerant 'Northrup-King' corn hybrid until the 84 hr sampling time. By 84 hr the GST activity of both hybrids was at its maximum level after which time it began to decline. From 72 to 120 hr the activity of 'Cargill 7567' GST tended to be greater than that of 'Northrup-King 9283'.

Metolachlor Metabolism. Metabolism of ^{14}C-metolachlor to the GSH-conjugate by excised apical sections of 'Cargill 7567' and 'Northrup-King 9283' corn is shown in Figure 4. The rate of metabolic conversion of ^{14}C-metolachlor to this product was similar for both hybrids. After 1 hr of incubation approximately 25% of the extractable radioactivity was found in the aqueous phase corresponding to the GS-conjugate of metolachlor. The rate of metolachlor metabolism was greatest during the following hour of incubation. After 2 hr of incubation about 75% of the radioactive substrate was metabolized by both hybrids. Further incubation resulted in some additional metabolism but at a much reduced rate.

DISCUSSION

The results of the greenhouse study reported here clearly demonstrate the differential response of these two corn

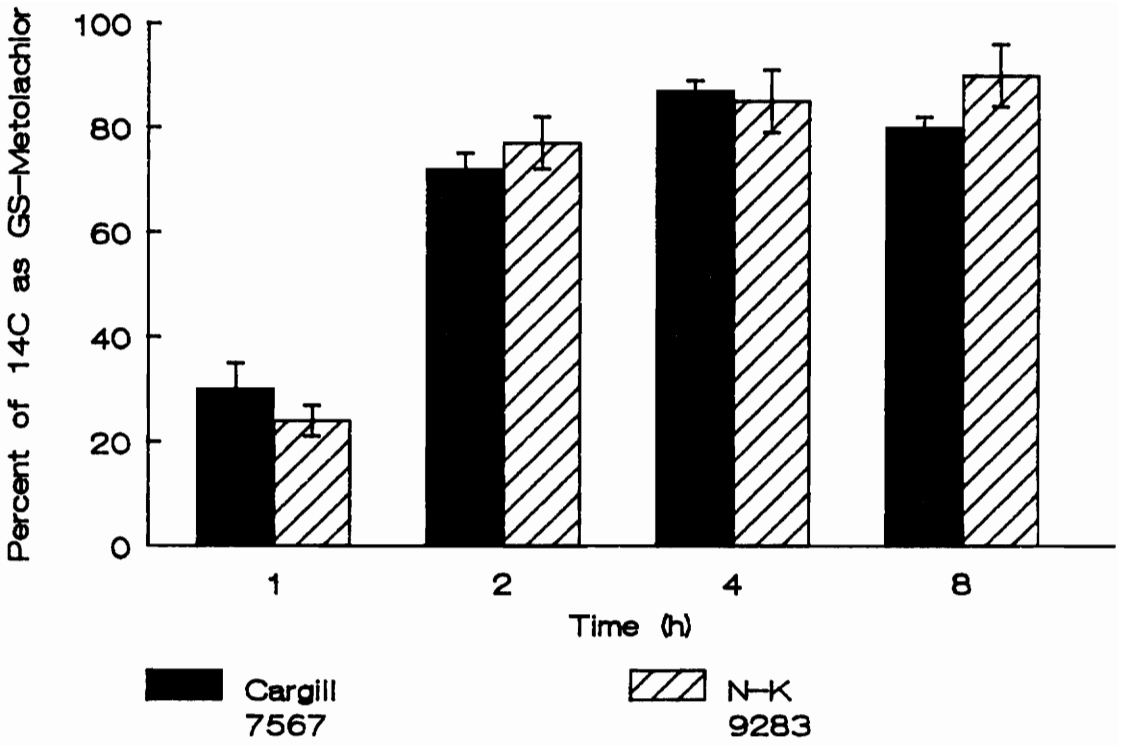

Figure 4. Metabolism of ^{14}C-metolachlor by excised apical sections of 3-day old 'Cargill 7567' and 'Northrup-King 9283' corn seedlings. Data presented as the percent of extractable radioactivity found as the GS-metolachlor conjugate.

hybrids to metolachlor. These results are consistent with those reported in Chapter II, as well as previous reports of 'Cargill 7567' corn's tolerance and 'Northrup-King 9283' corn's susceptibility to metolachlor (8,9).

In this study ^{14}C-metolachlor applied to the coleoptiles of 3-day old seedlings was more rapidly absorbed during the early stages of exposure and accumulated to a somewhat greater extent in the susceptible hybrid, 'Northrup-King 9283' (Figure 2). Only limited basipetal movement of ^{14}C-metolachlor was observed for either corn hybrid (Table 1). Dixon and Stoller (2) reported a similar low level of translocation for corn even after much longer exposures to shoot applied ^{14}C-metolachlor. These results suggest that the more rapid uptake of metolachlor by 'Northrup-King 9283' corn could be a determining factor in the increased sensitivity of this hybrid and that translocation is of little importance in determining either tolerance or susceptibility.

Measurement of GST activity as a function of seedling age revealed an interesting developmental progression (Figure 3). Seventy-two hours after beginning germination the GST activity of 'Cargill 7567' exceeded that of 'Northrup-King 9283' by 30%. It may be possible that a delay in GST expression during a critical stage of development could result in herbicide injury. At this age

the emerging shoots were about 25 mm in length. In a soil situation this would place the growing shoot well within the zone of herbicide treatment. The apparent lag in GST activity observed for 'Northrup-King 9283' could in part explain the relative sensitivity of this corn hybrid to metolachlor.

It was expected that the differential GST activity discussed above would be paralleled by a similar difference in the metabolism of metolachlor by 3-day old corn shoots. However, the metabolism studies presented here do not support this hypothesis (Figure 4). Under the conditions of this study metolachlor was metabolized equally by the two corn hybrids. Perhaps when uptake kinetics and other factors influencing the effective concentration of a soil applied herbicide are considered this discrepancy may be resolved.

It should be noted that the injury to the metolachlor-susceptible hybrid utilized in this study was not so extensive as to lead to morbidity. In general corn is quite tolerant to metolachlor. The situation described here is a case of limited intraspecific differential tolerance and as such the factors conferring the observed differences between these two corn hybrids were expected to be more subtle than might be observed if comparing either corn hybrid with a very susceptible species. It seems clear

that the basis of differential metolachlor tolerance for these two corn hybrids is in part due to differences in uptake of metolachlor. It also appears that an enhanced expression of GST activity during a critical stage of exposure to metolachlor could provide a margin of tolerance to a developing seedling. The developmental control of herbicide tolerance warrants further investigation.

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IV. STUDIES ON THE MODE OF ACTION OF THE METOLACHLOR SAFENER BENOXACOR FOR CORN (*Zea mays* L.)

INTRODUCTION

Herbicide safeners are chemicals which can extend the use of certain herbicides by somehow protecting the crop plant from the phytotoxic effect of the herbicide. This protection is limited to a few crop species; primarily large seeded grasses such as corn, rice, and sorghum; and mainly with herbicides of the thiocarbamate and chloroacetanilide classes (1). The chloroacetanilide herbicide metolachlor is one of the most widely used herbicides for the control of grass weeds in corn production (2). Corn is generally tolerant to metolachlor, but under certain conditions severe injury can occur (3). Conditions favoring metolachlor injury to corn include high application rates, high soil moisture, low temperature, as well as the intrinsic sensitivity of certain corn lines (3,4). This tendency towards injury necessitates the use of a safener for use with metolachlor in corn. However, most of the herbicide safeners developed for use with corn are specifically formulated for use with thiocarbamate herbicides, particularly EPTC (1). Recently Ciba-Geigy Corporation synthesized a new safener, benoxacor (also referred to as CGA-154281), for formulation with metolachlor

for use in corn (5).

Although herbicide safeners have been known and used for about twenty years their exact mechanisms of action have not been elucidated. Hatzios (1) has summarized four general mechanisms of action for herbicide safeners. First, the safener may interfere with herbicide uptake and/or translocation; second, the safener may enhance the detoxication of the herbicide within the protected plant; third, the safener and herbicide may compete for a common target site; and fourth, action of the safener may involve a combination of these processes. Competition for a common site (competitive antagonism) is an unlikely mechanism for the benoxacor/metolachlor interaction due to the proposed multiple sites of action of metolachlor (2,6). It seems most likely that benoxacor acts by regulating the amount of metolachlor that reaches its site(s) of action. This mechanism has been described as the "biochemical antagonism hypothesis" of safener action (1). The central element of this hypothesis is that the safener reduces or eliminates completely the amount of herbicide that would otherwise reach its site of action. This could result from either a safener-induced reduction in the rate of herbicide penetration and/or translocation or a safener-induced enhancement of the detoxication of the herbicide.

Reports of safener effects on herbicide uptake and/or

distribution are variable and contradictory. For example, a significant reduction of metolachlor uptake was observed for cyometrinil-treated grain sorghum (7) while a significant stimulation of metolachlor uptake was observed for grain sorghum treated with the closely related safener oxabetrinil (8,9). Still others have found no effects of safeners on herbicide uptake and/or translocation (10,11).

The influence of herbicide safeners on the metabolism of thiocarbamate and chloroacetanilide herbicides has been reviewed by Gronwald (12). Effects include increases in the cofactor glutathione (GSH) and stimulation of glutathione *S*-transferase (GST) activity including the induction of novel isozymes of GST with increased specificity for herbicide substrates. Benoxacor has been shown to stimulate the metabolism of metolachlor by certain corn lines (4,13,14). This increase in metabolism appears to be due to the induction of GST activity without a corresponding increase in GSH content (13).

Safeners have also been shown to influence the rate of non-enzymatic conjugation of chloroacetanilide herbicides with GSH (15,16). The rate of this process, although quantitatively small in relation to the corresponding enzymatic process, must be considered when characterizing the mechanism of action of safeners.

Researchers are also interested in the effects of

herbicide safeners on the metabolism of herbicides of other classes. In a recent report, Fonné-Pfister et al. (17) observed a 2-fold increase in the metabolism of the sulfonylurea herbicide primisulfuron by benoxacor-treated corn seedlings. This enhanced metabolism was believed to be due to the induction of novel cytochrome P450 monooxygenase isozymes.

In the present study, experiments were carried out to characterize the mechanism of action by which the safener benoxacor protects corn against metolachlor injury. The specific objectives were as follows: (a) to determine the efficacy of benoxacor when used with metolachlor; (b) to determine the rate of benoxacor uptake and translocation in comparison to that of metolachlor, (c) to determine the effect of benoxacor treatment on metolachlor uptake; (d) to determine the involvement of any oxidative processes in the action of benoxacor by studying the interactions of the safener with the antioxidant piperonyl butoxide (PBO); (e) to determine the effect of benoxacor on the non-enzymatic glutathione-mediated detoxication of metolachlor *in vitro*.

MATERIAL AND METHODS

Chemicals. Metolachlor (formulated DUAL herbicide), CGA-180937 (formulated DUAL + benoxacor, 30:1 w/w), benoxacor, ^{14}C-metolachlor, and ^{14}C-benoxacor were provided

by Ciba-Geigy Corporation, Greensboro, NC. Formulated piperonyl butoxide (PBO) was a gift of Endura Company, Bologna, Italy. Other chemicals were purchased from commercial vendors.

Plant Material and Chemical Application. For greenhouse studies seeds of 'Cargill 7567', 'Northrup-King 9283', and Pioneer hybrids '3744' and '3147' were planted in a 1:1:1 (v/v/v) mixture of potting medium (Weblite, Weblite Corp., Blue Ridge, VA), vermiculite, and peat in 236-ml styrofoam cups. The cups were half filled with soil mixture, seeds were placed on this surface and were then covered with a 3 cm deep layer of the same soil treated with the appropriate chemical. The greenhouse temperature was maintained at 25 ± 5 °C. Natural sunlight provided an approximate 14-hr photoperiod with a photosynthetic photon flux density (PPFD) of 400 $\mu E/m^2/s$ during the course of these experiments.

Safener Efficacy Study. Seeds were planted as described above with the following soil treatments: metolachlor (6.7 kg/ha), CGA-180937 (6.7 kg/ha metolachlor + 0.22 kg/ha benoxacor), or untreated. Three seeds were planted per cup, three cups were prepared per treatment and the experiment was repeated twice in time. Ten days after planting, shoot heights and dry weights were determined.

Benoxacor Uptake. Seeds of 'Cargill 7567' and 'Northrup-King 9283' corn were germinated on two layers of

water saturated filter paper (Whatman #1) in Petri dishes in a dark growth chamber at 30° C. After 72 hr the emerging coleoptiles were about 25 mm in length. At this time, a 2 µl drop of ^{14}C-benoxacor (3.3 nCi ø-labeled ^{14}C-benoxacor, specific activity 5.9 µCi/µmol) was applied to the coleoptile. The seedlings were then placed in a sealed plastic enclosure containing water saturated paper towels to maintain a high relative humidity and reduce evaporation. The humidity chambers were then returned to the growth chamber. Seedlings were removed at 0, 0.5, 1, 2, 4, and 8 hr after treatment. The shoot axes were excised, rinsed with 80% methanol, combusted in a biological sample oxidizer (Packard Tri-Carb model 306, Packard Instrument Co., Downers Grove, IL) and the amount of radioactivity absorbed determined by liquid scintillation spectrometry (LSC) (Beckman LS5000TA, Beckman Instuments, Inc., Fullerton, CA). The remaining plant tissue, root plus seed, were oxidized and the radioactivity in these tissues was quantified by LSC. Three seedlings were utilized per treatment time. Data presented are the means and standard errors of three experiments with three replications per treatment.

Effect of Benoxacor and Metolachlor Treatment on ^{14}C-Metolachlor Uptake. Seeds of 'Northrup-King 9283' corn were grown in a dark growth chamber at 30 °C for 3 days on 3 layers of paper towels saturated with either benoxacor

(1µM), metolachlor (30 µM), or the two in combination (1 µM benoxacor + 30 µM metolachlor). Uptake of shoot applied ^{14}C-metolachlor (ø-labeled ^{14}C-metolachlor, specific activity 21.6 µCi/µmol) was performed as previously described.

Interactive Effect of PBO with CGA-180937. Seeds of 'Cargill 7567', 'Northrup-King 9283', and Pioneer hybrids '3744' and '3147' were grown as described above. Chemical treatments included 6.7 kg/ha metolachlor, 6.7 kg/ha CGA-180937, 6.7 kg/ha CGA-180937 + 6.7 kg/ha PBO, 6.7 kg/ha PBO, and untreated controls. Three cups were planted per treatment and the experiment was repeated twice in time. The greenhouse temperature was maintained at 25 ± 5 °C with a 14-hr photoperiod. Ten days after planting shoot heights and dry weights were determined.

Effect of Benoxacor on Metolachlor Conjugation with GSH. Non-enzymatic conjugation of metolachlor with reduced glutathione (GSH) was determined in a 60 µl reaction mixture containing 50 mM potassium phosphate buffer, pH 7.0; 1 mM GSH; 50 µM metolachlor (containing 13 nCi carbonyl-labeled ^{14}C-metolachlor, specific activity 59.5 µCi/µmol); and benoxacor to give final concentrations of 1, 10, 50, 100 µM. The reaction vials were incubated at 30 °C for 1 and 4 hr at which time 60 µl of 5% trichloroacetic acid (TCA) and 1.0 ml of dichloromethane were added. After vortexing and

centrifugation in a microcentrifuge a 60 µl sample of the GS-metolachlor containing aqueous phase was removed and quantified by LSC.

RESULTS

Efficacy of Benoxacor. Treatment with 6.7 kg/ha metolachlor resulted in an inhibition of shoot height and a reduction in above ground biomass of the four corn hybrids tested which was relieved by addition of benoxacor to the herbicide formulation (Figures 1 and 2). The protective action of benoxacor was especially apparent for the metolachlor-susceptible 'Northrup-King 9283' and 'Pioneer 3744' and '3147' corn hybrids. These hybrids were severely stunted and displayed classic chloroacetanilide injury symptoms as a result of the treatment with metolachlor but appeared healthy and normal when benoxacor was present. The Pioneer hybrids were more severely injured by metolachlor treatment and appeared to be less protected by the safener than the other hybrids tested.

Uptake and Translocation of Benoxacor. ^{14}C-Benoxacor is rapidly absorbed through the coleoptiles of 'Cargill 7567' and 'Northrup-King 9283' corn. Absorption and translocation of the labeled compound was similar for both hybrids (Table 1). At least 30% of the radioactive benoxacor was absorbed in the first 30 min after

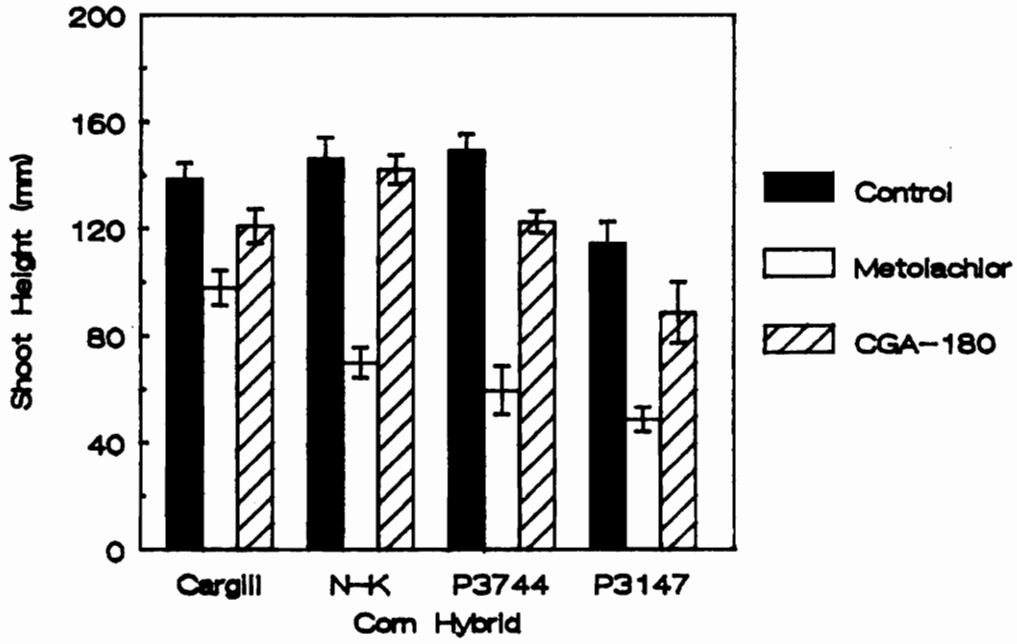

Figure 1. Effect of 6.7 kg/ha metolachlor and 6.7 kg/ha CGA-180937 (metolachlor + benoxacor, 30:1, w/w) treatment on shoot height of 10-day old seedlings of selected corn hybrids.

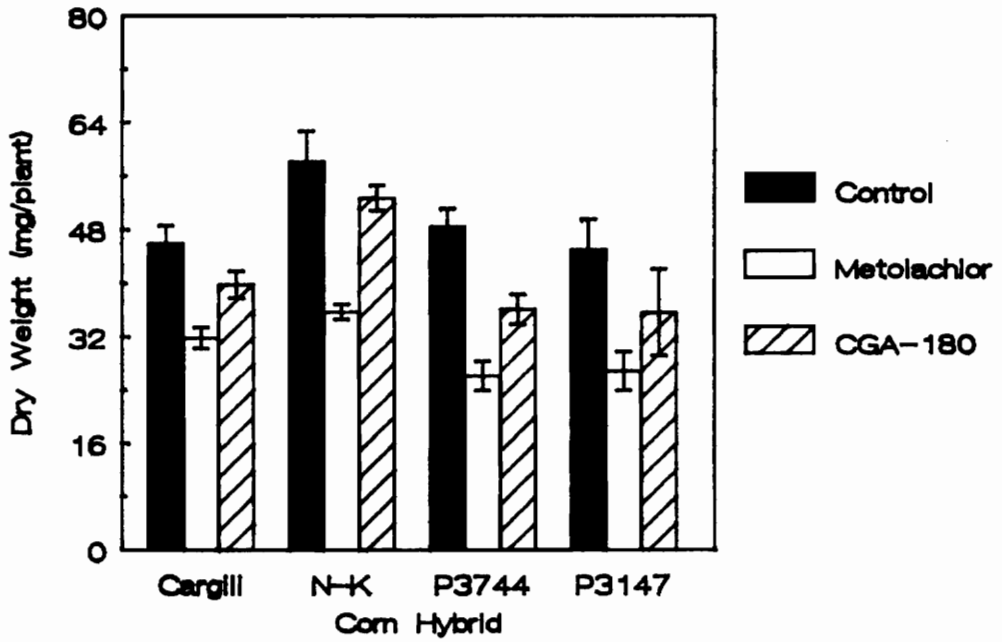

Figure 2. Effect of 6.7 kg/ha metolachlor and 6.7 kg/ha CGA-180937 (metolachlor + benoxacor, 30:1, w/w) treatment on shoot dry weight of 10-day old seedlings of selected corn hybrids.

Table 1. Uptake and distribution of ^{14}C-benoxacor in 3-day old 'Cargill 7567' and 'Northrup-King 9283' corn seedlings.

Treatment (hr)	% of applied ^{14}C-benoxacor			
	'Cargill 7567'		'Northrup-King9283'	
	Shoot	Root[a]	Shoot	Root
0.5	35.0 ± 6.7	0.4 ± 0.1	30.2 ± 3.9	0.8 ± 0.1
1	33.0 ± 5.2	0.7 ± 0.2	49.0 ± 7.0	1.0 ± 0.2
2	76.9 ± 7.7	2.0 ± 0.3	68.5 ± 8.6	2.0 ± 0.3
4	75.6 ± 9.7	4.1 ± 0.7	78.3 ± 7.7	3.0 ± 0.4
8	91.2 ± 8.9	4.8 ± 0.9	91.7 ± 6.6	5.9 ± 0.7

[a] Includes remaining seed tissue.

application of ^{14}C-benoxacor to coleoptiles of the two corn hybrids. More than 70% of the radioactive safener was absorbed in 2 hr and by the end of the experiment 90% of the applied radioactivity had been absorbed. Relatively small amounts of labeled benoxacor were translocated from the site of application to the root and seed tissue of these seedlings. After 8 hr about 5% of the applied radioactivity was found in these tissues.

Effect of Pretreatment on Metolachlor Uptake. The uptake of ^{14}C-metolachlor by shoots of 'Northrup-King 9283' corn grown in the presence of benoxacor, metolachlor, or CGA-180937 is shown in Figure 3. None of these treatments appeared to influence metolachlor uptake relative to the untreated controls.

Interactive Effect of PBO with CGA-180937. The addition of the antioxidant PBO had no effect on the protective ability of benoxacor (Figures 4 and 5). Interestingly, treatment with PBO alone appeared to have a slight negative effect on the growth of the two Pioneer corn hybrids. This effect was similar to the depression of growth associated with CGA-180937 treatment. No growth effects were observed with PBO alone for either 'Cargill 7567' or 'Northrup-King 9283'.

Effect of Benoxacor on Non-enzymatic Conjugation. Non-enzymatic conjugation of ^{14}C-metolachlor to GSH was not

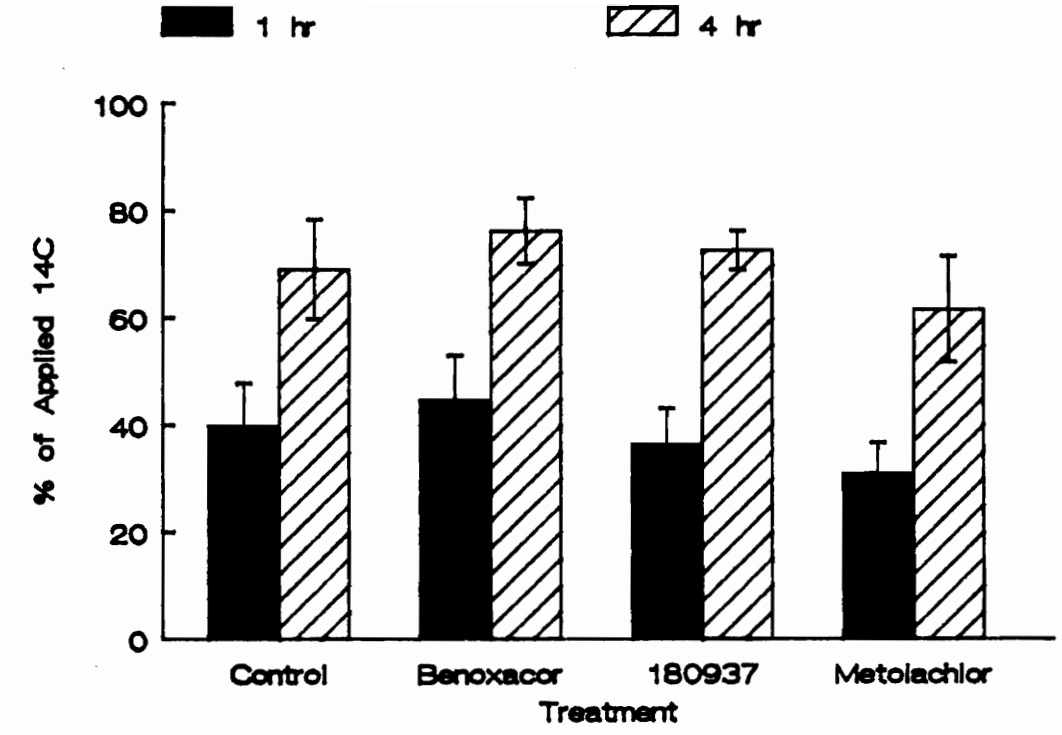

Figure 3. Effect of growing 'Northrup-King 9283' corn for 3-days in the presence of 1 µM benoxacor, 30 µM metolachlor, or 30 µM CGA-180937 (metolachlor + benoxacor, 30:1, w/w) on subsequent uptake of ^{14}C-metolachlor. Uptake was measured 1 and 4 hr after application ^{14}C-metolachlor.

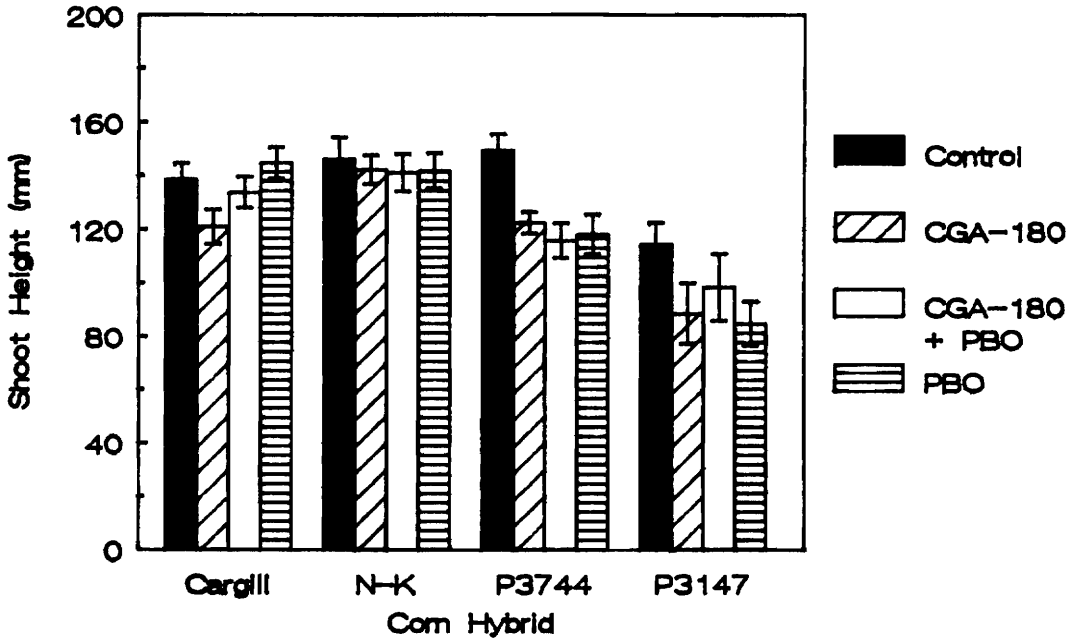

Figure 4. Interactive effect of PBO with CGA-180937. Effect on shoot height of 10-day old seedlings of selected corn hybrids. Chemicals were applied pre-plant incorporated at 6.7 kg/ha.

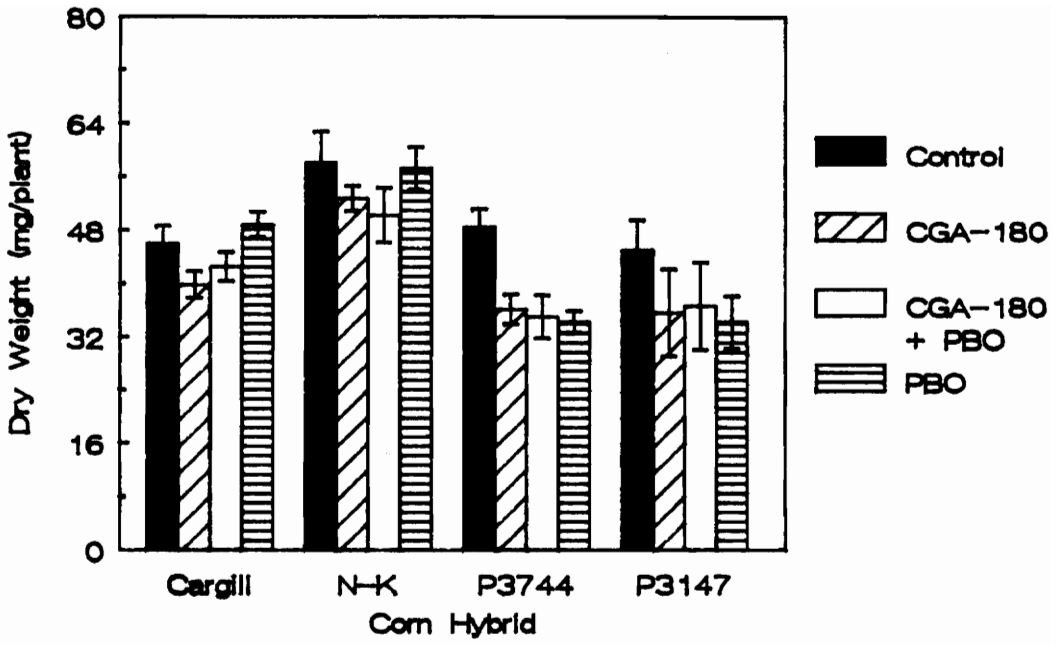

Figure 5. Interactive effect of PBO with CGA-180937. Effect on shoot dry weight of 10-day old seedlings of selected corn hybrids. Chemicals were applied pre-plant incorporated at 6.7 kg/ha.

affected by benoxacor concentrations of up to 100 µM (Figure 6). After 1 hr about 900 dpm equivalent to 40 pmol of ^{14}C-metolachlor was conjugated with GSH. Additional incubation resulted in only a modest increase in the amount of ^{14}C-metolachlor forming the GS-metolachlor conjugate.

DISCUSSION

Benoxacor was shown to be effective in protecting corn from metolachlor injury (Figures 1 and 2). This safening effect was most apparent for the metolachlor-susceptible 'Northrup-King 9283' hybrid. The lack of complete recovery by the Pioneer hybrids and their apparent sensitivity to PBO treatment in the absence of any other chemical may be related to the basis of their susceptibility to metolachlor. In a recent report, Rowe et al. (14) observed a similar response to benoxacor treatment for 'Cargill 7567', 'Northrup-King 9283', and 'Pioneer 3744' corn in a greenhouse study of the same duration reported here. In a 21 day field test carried out by these same investigators (14) growth of benoxacor-treated 'Pioneer 3744' was equal to that of similarly treated metolachlor-tolerant corn hybrids. This suggests that the growth inhibition observed in short term greenhouse experiments is transitory.

Benoxacor uptake was rapid and proceeded at a similar rate for both 'Cargill 7567' and 'Northrup-King 9283' corn.

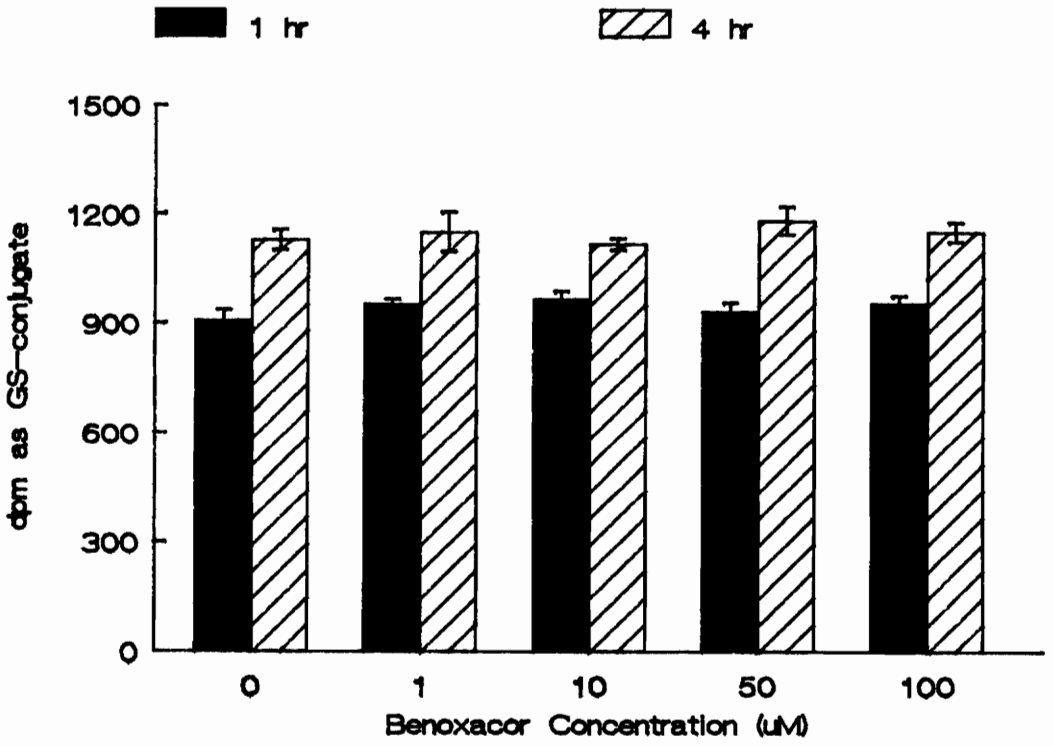

Figure 6. Effect of benoxacor concentration on the non-enzymatic conjugation of metolachlor with glutathione *in vitro*. The amount of ^{14}C-metolachlor forming the glutathione conjugate was determined after 1 and 4 hr.

Figure 7 is presented to compare benoxacor uptake as determined in this study (Table 1) to metolachlor uptake as previously determined for 'Northrup-King 9283' corn (Chapter III, Figure 2). Similarly Figure 8 is presented to compare the translocation of benoxacor observed in this study (Table 1) with that of metolachlor (Chapter III, Table 1) in this corn hybrid. After 2 h benoxacor is about 70% absorbed compared to 45% aborption of metolachlor. In addition to being more rapidly absorbed, benoxacor was also shown to be more mobile than metolachlor when translocation is similarly compared. This greater rate of uptake of the safener is of interest considering that these compounds are normally applied together. About 5% of the applied radioactive benoxacor was translocated to the root and seed tissue in this study. In contrast only about 2% of an equivalent amount of radioactive metolachlor was translocated in the same time. The importance of this is unclear but may relate to the same property that allows the safener to enter the plant more rapidly than the herbicide. The potential for competition for the same active site is suggested by these results. This however is unlikely considering the ratio of herbicide to safener in typical formulations (30:1, w/w) and the proposed multiple sites of action of metolachlor (2,6). The more rapid uptake of benoxacor relative to metolachlor could allow the induction of processes involved in

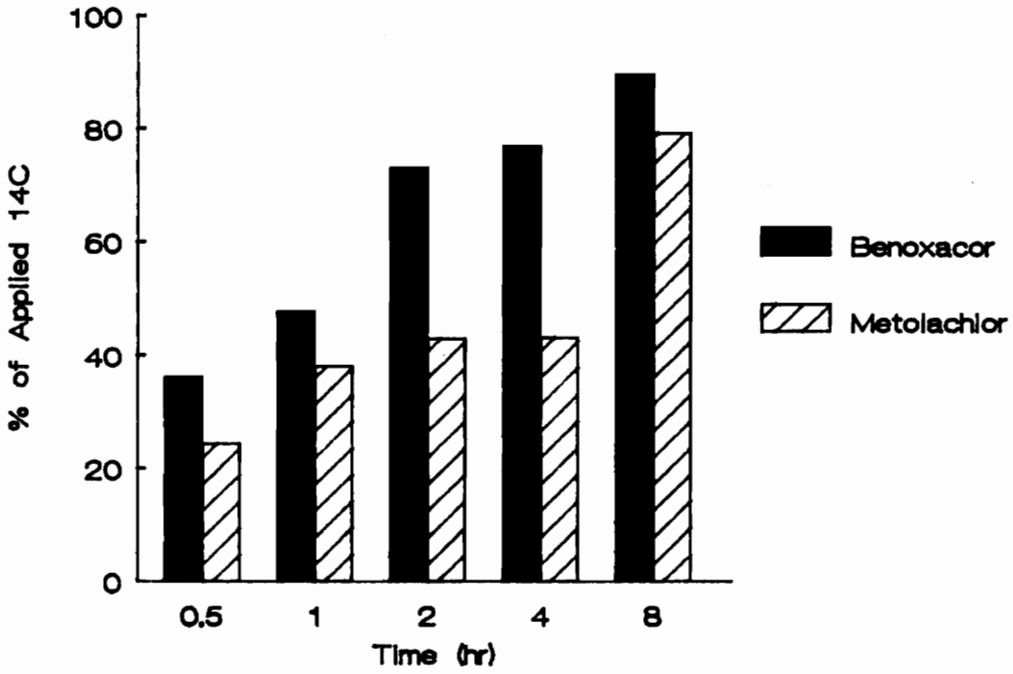

Figure 7. Comparison of benoxacor and metolachlor uptake by shoots of 3-day old 'Northrup-King 9283' corn.

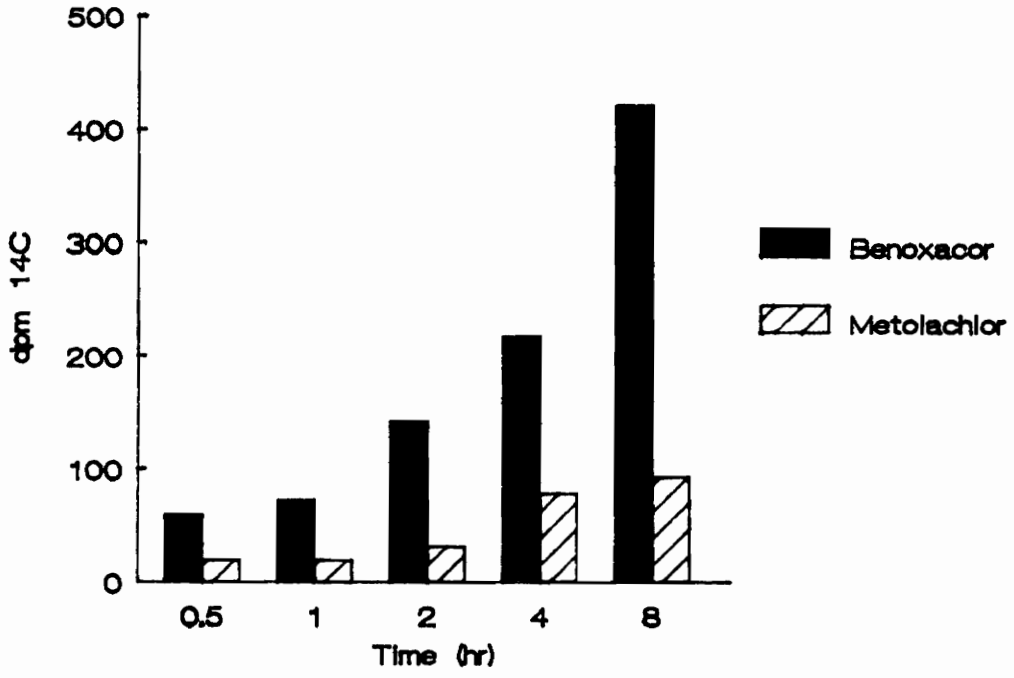

Figure 8. Accumulation of benoxacor and metolachlor in root and seed tissue of 3-day old 'Northrup-King 9283' corn after shoot application of the radioactive chemicals.

metolachlor detoxication prior to the arrival of the herbicide at its target site(s).

When metolachlor uptake was examined using seedlings that were grown in the presence of benoxacor, metolachlor, or the two combined no differences were observed relative to untreated controls. Therefore a reduction in herbicide uptake does not appear to be involved in the protective mechanism of benoxacor.

Inclusion of the antioxidant compound piperonyl butoxide (PBO) had no effect on the safening ability of benoxacor. This indirect evidence suggests that oxidative processes are of little importance in the mechanism of action of this safener when used with metolachlor. The induction of monooxygenase activity described previously when benoxacor was used with primisulfuron (17) is nevertheless of interest to the overall mechanism of action of this safener. It is possible that benoxacor regulates a family of enzymes involved in herbicide detoxication including monooxygenases and GSTs. The relative importance of these induced enzymes to the protective effect depends on the particular herbicide being safened. Oxidative reactions do not appear to be important in the primary metabolism of metolachlor by plants (18). Consequently, the inhibition of monooxygenase activity would have little effect on the subsequent detoxication of metolachlor.

The contribution of non-enzymatic conjugation with GSH to the metabolism of chloroacetanilide herbicides relative to that catalyzed by GST isozymes has been a matter of controversy (12). The degree of non-enzymatic conjugation reported here is minimal compared to the observed rates of GST-catalyzed conjugation reported earlier (see Chapter 2). The failure of this safener to influence non-enzymatic conjugation eliminates the involvement of this process in its mechanism of action.

From the results presented here it appears that the mechanism of action of benoxacor must be based primarily on the observed ability of this safener to stimulate the metabolism of metolachlor in protected plants (4,13,14). The enhancement of metolachlor metabolism by benoxacor was the subject of subsequent studies discussed in the next chapter.

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V. INFLUENCE OF THE SAFENER BENOXACOR ON THE METABOLISM OF METOLACHLOR IN CORN

INTRODUCTION

Benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4 benzoxazine] is a recently developed safener synthesized by Ciba-Geigy Corporation for use in corn as a formulated mixture with the herbicide metolachlor (1). The mechanism by which this safener confers its protective effect is believed to be by enhancing the detoxication of metolachlor in treated plants. The chloroacetanilide class of herbicides, to which metolachlor belongs, are detoxified in corn (2,3) and sorghum (4,5) by conjugation with the tripeptide glutathione (GSH). This conjugation has been shown to occur both as an enzymatic process (3,6,7) catalyzed by glutathione-S-transferase isozymes (GSTs) and nonenzymatically (8). The relative contributions of these two processes is currently a matter of controversy (9). There is a growing body of evidence suggesting that herbicide safeners confer their protection primarily by inducing GST isozymes specific for chloroacetanilide herbicides (4,7,10,11,12).

The objectives of this study were to determine the effects of benoxacor on metabolism of metolachlor, GST induction, and glutathione content in seedlings of two

hybrid corn lines. These hybrids, known to respond differentially to metolachlor (13), were utilized in order to determine if predisposition to herbicide injury (susceptibility or tolerance) is a factor in their response to the safener.

MATERIALS AND METHODS

Chemicals. Analytical grade (>95% purity) metolachlor, benoxacor, and radiolabeled metolachlor (carbonyl ^{14}C labeled, sp. act. 59.5 µCi/mmol) were provided by Ciba-Geigy Corp., Greensboro, NC. All other chemicals were obtained from commercial sources.

Metolachlor Metabolism Study. For metabolism experiments seeds of both corn hybrids were germinated on filter paper saturated either with distilled H_2O or 1 µM benoxacor at 30°C in a dark growth chamber. After 72 h, apical sections (20 mm) of the seedlings were excised and placed, six per vial, in 400 µl of incubation medium (1 mM $CaCl_2$, 10 mM HEPES, pH 7.5) and 10 nCi ^{14}C-metolachlor for 1, 2, 4, and 8 h at 27°C. After incubation the apices were removed, rinsed with 80% methanol, and the absorbed ^{14}C-metolachlor extracted by grinding in 1 ml of 80% methanol. The extract was clarified by centrifuging for 5 min in a microcentrifuge and the radioactivity contained in

the extract was determined by liquid scintillation spectrometry. The amount of ^{14}C-metolachlor metabolized to the glutathione conjugate was determined by mixing a 60 µl aliquot of the extract with 60 µl of H_2O, and fractionating with 1 ml of methylene chloride.

Metolachlor metabolism by unsafened seedlings was also determined in the presence of varying concentrations of benoxacor. These experiments were conducted as above except for the inclusion of 0, 1, 10, and 50 µM benoxacor to the metabolism reaction mix.

Influence of Benoxacor on GST Activity. Seeds of the metolachlor-tolerant 'Cargill 7567' and the metolachlor-susceptible 'Northrup-King 9283' corn hybrids were germinated for 48 h on H_2O saturated filter paper at 30°C in a dark growth chamber before being transferred to continuously aerated incubation medium containing 0, 0.2, 2, 5, 10, or 20 mg/l benoxacor. After 24 h the seedlings were removed from the liquid medium, rinsed, and frozen immediately in liquid nitrogen for GST analysis.

GST and GSH Assays. For GST activity determinations 6 seedlings were pulverized in liquid nitrogen and homogenized with a mortar and pestle in 3 ml of 0.1 M K-phosphate buffer, pH 6.9, and 0.1 g polyvinyl-polypyrrolidone (PVPP). The resulting homogenate was centrifuged at 20,000 x g for 20 min and the supernatant was used as enzyme source. GST

activity was determined with ^{14}C-metolachlor as substrate. The enzyme reaction contained 30 µl of 0.1 M K-phosphate buffer, pH 6.9; 10 µl of 60 mM GSH; 10 µl of 60 µM metolachlor (1.3 nCi/µmol); and 10 µl of plant extract. Enzyme reactions were incubated for 1 h, at 30°C. Assays were terminated by the addition of 60 µl of 5% trichloroacetic acid (TCA). Metolachlor conjugated to GSH was determined by fractionating with 1 ml of methylene chloride and determining the radioactivity remaining in the aqueous phase by liquid scintillation spectrometry.

The total glutathione content of 72 h old seedlings was determined by the method of Tietz (14) as described by Gronwald et al.(7).

Protein content was determined spectrophotometrically by the Coomassie blue G-250 dye-binding assay (15) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Apical sections from seedlings grown in the presence of 1 µM benoxacor were found to metabolize metolachlor to a greater extent than control sections in a 1 h incubation (Table 1). A similar enhancement of metolachlor metabolism (65-70%) was observed for both corn varieties. This increase in herbicide detoxication is consistent with the findings of other investigators for a number of chemicals

Table 1. Formation of the GS-metolachlor conjugate in unsafened and benoxacor-treated corn seedlings*

Corn Hybrid	Unsafened	Benoxacor**	Ratio
	% of extracted ^{14}C as GS-metolachlor		
Cargill 7567	29.6 ± 5.3	48.8 ± 6.4	1.65
Northrup-King 9283	23.9 ± 2.8	41.4 ± 5.6	1.73

* Data presented represent the average and standard errors of two experiments with 3 replications.

** 1 µM benoxacor was included in the imbibing solution of treated seeds.

safening chloroacetanilide herbicides. (9,16).

Various concentrations of benoxacor were tested for their ability to directly influence the metabolism of metolachlor by excised apical sections of 'Northrup-King 9283' corn which had not previously been treated with the safener (Table 2). No effect was observed with any of the concentrations tested. This suggests that the safener must be present for some time before its action occurs, and that there is no direct interaction between the herbicide and the safener. This finding fits well with the concept of safener action occurring at the molecular level with enzyme induction being a prerequisite for enhanced herbicide metabolism (10,17).

The effect of benoxacor concentration on GST activity is shown in Figure 1. The lowest safener concentration tested (0.2 mg/l) resulted in the greatest stimulation of GST activity. This approximately 35% increase in GST activity was observed for both of the corn varieties tested. The level of induction reported here is substantially less than the four to five-fold increase reported by other investigators using different corn lines (18,19). There is however no reason to believe that GST activity need be elevated to such extremes to prevent the phytotoxic effects of metolachlor. When untreated 72 h old seedlings were compared the GST activity of the metolachlor-tolerant

Table 2. Effect of benoxacor on GS-metolachlor conjugation by unsafened 'Northrup-King 9283' shoots when benoxacor is added to the reaction mixture*

Benoxacor (μM)	% of ^{14}C-metolachlor
0	36.0 ± 2.6
1	34.2 ± 3.8
10	38.2 ± 3.8
50	35.0 ± 5.7

* Data presented as percent ^{14}C-metolachlor partitioning into the aqueous phase after fractionation with methylene chloride. Data presented represent the average and standard errors of two experiments with 3 replications.

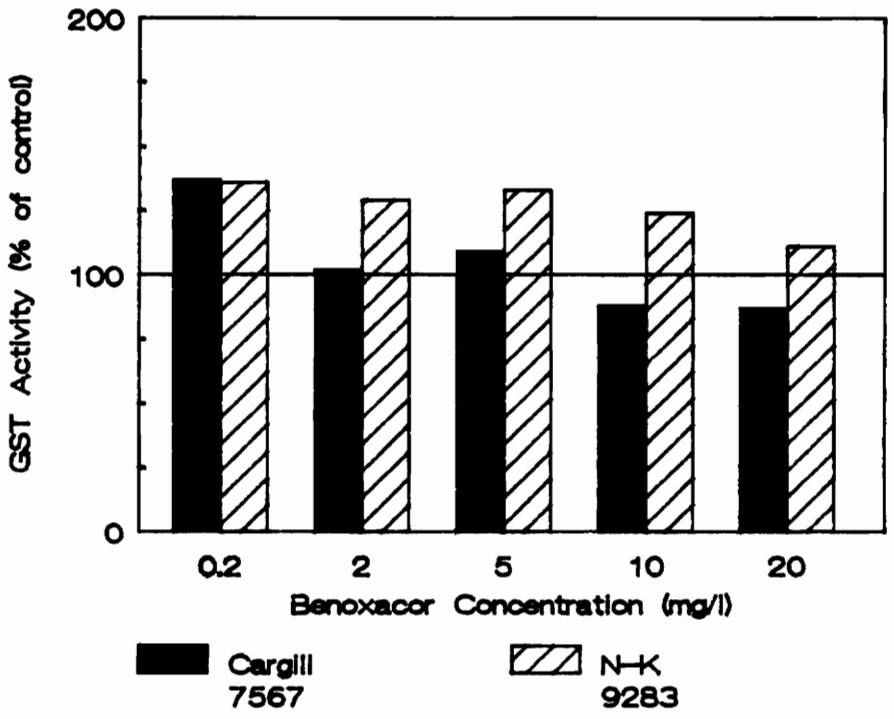

Figure 1. Effect of benoxacor concentration on GST-metolachlor activity as percent of untreated controls. Control values were 41.2 and 28.0 pmol/min/mg protein for 'Cargill 7567' and 'Northrup-King 9283', respectively.

'Cargill-7567' corn was found to be 35% greater than that of the susceptible 'Northrup-King 9283' corn (13). Therefore, the increase in GST shown here for the susceptible variety would be sufficient to raise its GST activity to an equivalent level of the naturally tolerant corn variety. This is supported by our results on the significant enhancement of metolachlor metabolism by benoxacor on both corn lines shown in Table 1. It has been shown that GST isozymes with greater affinity for chloroacetanilide herbicides can be induced by herbicide safeners (4,7,10,11,12). It is expected that the induction of GST activity shown here involves such a mechanism.

Interestingly, the two corn hybrids respond differently to higher concentrations of the safener (Figure 1). GST activity of the tolerant corn variety, 'Cargill-7567', was only induced slightly by 2 mg/l and 5 mg/l benoxacor (2% and 9% respectively) and was inhibited by higher concentrations of the chemical. GST activity of the susceptible corn hybrid, 'Northrup-King 9283', was induced to the same level at safener concentrations up to 5 mg/l, was only reduced slightly by higher concentrations of the safener, and was not inhibited by any of the concentrations tested. The reason for this differential response to the safener is unknown, but is believed to be related to the biochemical and physiological differences underlying the differential

metolachlor tolerance observed for these two corn hybrids.

The effect of benoxacor on the total glutathione content of 72 h old corn shoots is shown in Table 3. An increase in the glutathione content of the metolachlor-tolerant corn hybrid, 'Cargill 7567', was observed but no change in glutathione content was observed for the susceptible hybrid. Previous studies have shown different herbicide safeners to have variable effects on glutathione content of protected crops. Lay and Cassida (20) found an increase in glutathione in the roots of corn treated with dichlormid, Gronwald et al. (7) found dichlormid and flurazole treatment increased glutathione levels in sorghum shoots but did not find increases in glutathione content with naphthallic anhydride or oxabetrinil treatments. Viger et al. (19) found benoxacor to have no effect on the glutathione content of 'Pioneer 3906' corn. In our study the glutathione content of untreated shoots (about 1.2 µmol/g fresh weight) was similar for both varieties and is two-times the level of glutathione reported for sorghum shoots (7). These findings suggest that glutathione level is not the limiting factor in determining the metolachlor tolerance of these two corn varieties, and also suggest that regulation of glutathione content is not essential to the mechanism of action of benoxacor.

Table 3. Effect of benoxacor on glutathione content of 72-hr old corn shoots*

Corn Hybrid	Control	1µM benoxacor
	µmol/g fresh weight	
Cargill 7567	1.25 ± 0.05	1.50 ± 0.04
Northrup-King 9283	1.20 ± 0.07	1.30 ± 0.09

* Data presented are the average and standard error of at least 4 determinations.

CONCLUSION

The dichloroacetamide safener benoxacor is capable of stimulating GST activity with an associated increase in the metabolism of metolachlor to the glutathione conjugate. The increase in GST activity observed in this study is believed to be sufficient to account for the safening effect of this compound. The failure of benoxacor to elevate glutathione levels in the metolachlor-susceptible corn variety and the relatively high endogenous glutathione content of both varieties tested suggest that regulation of glutathione content is not a primary mechanism of action of this safener. Further characterization of the GST isozymes from these corn varieties is underway to determine if the increase in GST activity reported here involves the stimulation of constitutive enzyme activity or the induction of newly synthesized GST isozymes. The variable effect of benoxacor on the two corn varieties tested in regard to the regulation of GST and glutathione content suggests fundamental differences in their physiology which may be useful in explaining the basis of differential herbicide tolerance.

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VI. STUDIES ON THE GLUTATHIONE *S*-TRANSFERASE ISOZYMES OF CORN

INTRODUCTION

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of enzymes catalyzing any reaction in which the glutathione thiolate ion (GS^-) is a participant (1). As such they catalyze the conjugation of a variety of naturally occurring and xenobiotic compounds, including pesticides, with glutathione to form polar, less toxic metabolites. GSTs appear to be ubiquitous enzymes having been described from mammals (2), insects (3), higher plants (4), fungi (5), and bacteria (6). They are dimeric, multifunctional proteins usually found as cytosolic isozymes. At least 10 GST isozymes have been found in the cytosolic fraction of rat liver (2). Membrane bound GSTs have also been described from rat liver (7) and plants (8,9).

The first report of an atrazine-specific GST activity in tolerant plants in 1970 (10) demonstrated the potential importance of GST in herbicide selectivity. Subsequent studies with an atrazine-susceptible corn mutant verified that the atrazine-specific GST activity was the basis of atrazine selectivity (11). In the following two decades research on GSTs in crop plants has continued unabated. Much of this research has focused on the ability of

herbicide safeners to induce GST activity (12-16). Gronwald (17) has proposed that the degree of protection provided to grain sorghum and corn against injury from chloroacetanilide herbicides correlates strongly with the ability of safeners to enhance GST activity.

In corn, three GST isozymes have been well characterized in terms of their physical properties and specificities towards chloroacetanilide herbicides (13, 18-20). These corn GSTs have been summarized in a review by Timmerman (4).

GST I is a constitutive isozyme with activity against both 1-chloro-2,4-dinitrobenzene (CDNB) and the chloroacetanilide herbicide alachlor (13). GST I activity can also be induced by safener treatment (14). From the N-terminal amino acid sequence of the GST I subunit designated GST A, oligonucleotide probes were synthesized to isolate cDNA clones of the GST I gene (14). One of these cDNA clones, designated pMON9000 was used as a probe to demonstrate a 4-fold increase in mRNA encoding a subunit of GST I in response to flurazole treatment. Subsequently, pMON9000 was used as a probe to isolate the first plant GST genomic clone (21).

GST II was identified only from corn seedlings treated with the safeners flurazole or dichlormid (13). This inducible GST also has activity towards CDNB and alachlor.

GST III is a constitutive enzyme with a greater

specificity towards alachlor than GST I (18). This isozyme was shown to be responsible for 80% of the alachlor or metolachlor conjugating capacity of 'Pioneer 3320' corn (20). GST III cDNA clones have been described by independent researchers (18,22). Moore et al. (18) determined that the gene designated GST IIIA was a low or single copy gene. They also successfully expressed GST IIIA in *E. coli*. Later, Grove et al. (22) isolated and sequenced a GST III cDNA clone believed to be identical to GST IIIA but with eight reading frame shifts believed to be due to errors in the original sequence. The corrected sequence revealed partial amino acid similarity with rat GSTs and about 45% amino acid similarity to GST I (4).

Recent reports, utilizing fast protein liquid chromatography (FPLC) to separate GST isozymes, describe the induction of additional GST isozymes in response to safener treatments (16,23). It seems likely that, in addition to the three well characterized corn GSTs, other isozymes including the atrazine-specific GSTs (4) and the microsomal GSTs associated with the conjugation of cinnamic acid (glutathione *S*-cinnamoyl transferases) (8,9) exist which remain to be characterized.

The purpose of this study was to investigate further the GSTs responsible for metolachlor detoxication in corn and to develop techniques for investigating the regulation of GST

activity by the metolachlor safener benoxacor. The specific objectives were to: (a) compare the sub-cellular distribution of GST activity in the metolachlor-tolerant corn hybrid, 'Cargill 7567' and the metoalchlor-susceptible corn hybrid 'Northrup-King 9283'; (b) determine the effect of metolachlor, benoxacor, or the two chemicals in combination (CGA-180937) on the activity and sub-cellular distribution of GST activity in 'Northrup-King 9283'; (c) to separate and characterize the GST isozymes from both untreated and benoxacor-treated corn seedlings; and (d) to construct a molecular probe for corn GST III using polymerase-catalyzed chain reaction (PCR) technology.

MATERIALS AND METHODS

Plant Material and Chemical Treatments. Seeds of 'Cargill 7567' and 'Northrup-King 9283' corn were rinsed for 5 min with tepid tap water, blotted dry, and placed on three layers of paper towels in 20 x 30 cm plastic containers with tight fitting lids. The paper towels were saturated with distilled water (150 ml), the boxes placed in a dark growth chamber at 30 °C, and allowed to germinate for 72 hr. To determine the influence of benoxacor, metolachlor, or the two chemicals in combination (CGA-180937) on GST activity seeds of 'Northrup-King 9283' were prepared as above with the inclusion of 1 µM benoxacor, 30 µM metolachlor, or 30 µM

CGA-180937 (metolachlor:benoxacor 30:1, v/v) in the imbibing solution.

Preparation of Sub-cellular Membrane Fractions. Microsomes from 3-day old etiolated corn seedling shoots were prepared according to McFadden et al. (24) with slight modification. Two grams of excised shoots were ground to a powder with liquid nitrogen and homogenized at 4 °C with a mortar and pestle in 6 ml of buffer containing 0.2 M Tris-HCl (pH 7.8), 25 mM mercaptoethanol, 1 mM ethylene-diamine tetraacetic acid (EDTA) and 10% glycerol (w/v) at pH 7.8. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA), centrifuged for 30 min at 4,000 x *g* and the supernatant centrifuged for 90 min at 100,000 x *g*. The 100,000 x *g* supernatant (soluble fraction) was used directly for determination of protein and GST activity. The 100,000 x *g* pellet (microsomal fraction) was resuspended in 1 ml of extraction buffer for assay.

Enzyme Assays. GST enzyme activity was determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (GST-CDNB activity) and with ^{14}C-metolachlor as substrate (GST-metolachlor activity). GST-CDNB activity was determined according to Mannervik and Guthenberg (25). The reaction mixture contained 2.0 ml 100 mM potassium phosphate buffer (pH 6.9), 0.9 ml 3.3 mM GSH, 100 µl 30 mM CDNB, and 10 µl enzyme extract. The change in

absorbance due to formation of the GS-CDNB conjugate was measured at 340 nm in a Hitachi Model 100-10 spectrophotometer (Hitachi Ltd., Tokyo) at 25 °C. Correction for the non-enzymatic formation of the GS-CDNB conjugate was made by measuring and subtracting the rate in the absence of enzyme. A molar extinction coefficient of 10 mM/cm (25) was used to calculate enzyme activity.

GST-metolachlor activity was determined essentially as described by Mozer et al. (13) with slight modifications. The reaction mixture contained 30 µl potassium phosphate buffer (pH 6.9), 10 µl 60 mM GSH, 10 µl metolachlor (13 nCi carbonyl-labeled ^{14}C-metolachlor, specific activity 59.5 µCi/µmol), and 10 µl enzyme extract. After incubating for 60 min at 30 °C the reaction was terminated by the addition of 60 µl 5% trichloroacetic acid (TCA) and 1 ml of methylene chloride. After vortexing for 10 sec and microcentrifugation for 2 min the radioactivity in a 60 µl aliquot of the aqueous phase containing the GS-metolachlor conjugate was determined by liquid scintillation spectrometry (LSC). Blanks containing all components except plant extract were included to correct for non-enzymatic conjugation.

Enzyme activities for both substrates are expressed on a per mg protein basis. Protein content was determined by the dye binding method of Bradford (26) with bovine serum

albumin as standard.

Partial Purification of Corn GSTs. The following procedures follow the method described by Mozer et al. (13) for the purification of corn GSTs. Approximately 100 grams of 'Northrup-King 9283' seeds were grown as above for 4 days. Safener treatment was made by adding 30 µM CGA-180937 to the imbibing solution. Forty grams of excised seedling shoot tissue was frozen with liquid nitrogen and ground to a powder in a Waring blender. The powder was the suspended in ice-cold 0.2 M Tris-HCl (pH 7.8), containing 1 mM EDTA and 10% (w/v) polyvinylpolypyrrolidone, filtered through 4 layers of cheesecloth and centrifuged 10 min at 12,000 x *g*. This crude extract was then treated with 0.1 volume of 1.4% protamine sulfate and centrifuged for 30 min at 20,000 x *g* to remove nucleic acids.

A 30-70% $(NH_4)_2SO_4$ precipitate was obtained from the resulting supernatant by the addition of solid $(NH_4)_2SO_4$ and centrifugation for 30 min at 20,000 x *g*. The resulting protein pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.3), and desalted on a column of Sephadex G-25M (Pharmacia PD10, Pharmacia Fine Chemicals AB, Uppsala, Sweden) equilibrated with the same buffer. This preparation was applied to a 1.5 x 25 cm column of DEAE-Sepharose CL-6B (Sigma Chemical Co., St. Louis) equilibrated with 10 mM potassium phosphate buffer (pH 7.3). The column was

developed by elution with 100 ml of equilibration buffer, followed by a 400 ml linear gradient of 10-200 mM potassium phosphate buffer (pH 7.3). The chromatography was performed at 4 °C at a flow rate of 0.6 ml/min. Six ml fractions were collected and protein determined by absorbance at 280 nm and GST activity with CDNB. Selected fractions were pooled, concentrated by $(NH_4)_2SO_4$ precipitation and GST-activity determined with both CDNB and ^{14}C-metolachlor as substrates.

RNA Isolation. RNA was extracted according to Lawton et al. (27) with modifications. Four-day old CGA-180937 treated corn shoots (1 g fresh weight) were ground to a fine powder with liquid nitrogen in a mortar and pestle. The powdered plant material was transferred directly to a 15 ml centrifuge tube containing 1 ml 0.1 M Tris-HCl (pH 9.0) and 1 ml Tris-saturated phenol and homogenized with 3-30 sec pulses with a tissue homogenizer (Tissumizer, Tekmar Co., Cincinnati). After centrifugation for 5 min at setting 6 in a clinical centrifuge the aqueous phase was transferred to a clean centrifuge tube. The organic phase from the initial extraction was re-extracted with 1 ml 0.1M Tris (pH 9.0), and the resulting aqueous phase combined with the first. The combined aqueous phases were extracted three times with 2 ml of Tris-saturated phenol, followed by one extraction with 2 ml of chloroform. At this point the RNA-containing solution (ca. 2 ml) was divided and transferred to two 1.5

ml microcentrifuge tubes and 0.3 ml of 8M $LiCl_2$ was added to each and incubated overnight at 4 °C. Following microcentrifugation for 15 min the supernatant was discarded and the resulting pellets containing RNA were combined and resuspended in 100 µl distilled water, transferred to a fresh microcentrifuge tube, mixed with 1 ml of 3M Na-acetate, and microcentrifuged again for 15 min. The resulting pellet was resuspended in 200 µl distilled water, 20 µl 3M Na-acetate, 400 µl ice cold 100% ethanol, and placed at -70 °C for at least 30 min before centrifuging for 15 min. After microcentrifugation the supernatant was discarded, the pellet rinsed with 200 µl ice cold 70% ethanol, microcentrifuged again for 5 min, and the pellet dried under vacuum. Before use the RNA was resuspended in 100 µl distilled water and its concentration determined spectrophotometrically at 260 nm. The yield of RNA was greater than 100 µg/g fresh weight with an A_{260}/A_{280} ratio of approximately 2.0. Poly A^+ mRNA was prepared from total RNA with an oligo (dT)-cellulose spun column (5 Prime-3 Prime Inc., West Chester, PA).

Single Strand cDNA Synthesis. Poly A^+ mRNA (1.2 µg) was heated at 65 °C for 3 min and then placed on ice. The cDNA synthesis reaction contained 1.2 µg mRNA, 4 µl 5X reverse transcriptase buffer, 4 µl 10 µM dNTP mix, 2 µl oligo(dT)n, 0.5 µl RNasin ribonuclease inhibitor (3,500

units/ml; Promega Corp., Madison, WI), 6.5 µl H_2O, and 1 µl reverse transcriptase (200 units/µl; BRL, Life Technologies, Inc., Bethesda, MD). The reaction was incubated at 42 °C for 2 hr followed by 3 min at 95 °C and used directly in the polymerase-catalyzed chain reaction (PCR).

PCR-mediated Amplification. The published sequences of cDNA clones designated GST IIIA (18) and GST III (22) were utilized in designing oligonucleotide primers for use in amplifying mRNA from 'Northrup-King 9283' corn encoding GST III using PCR as described by Mullis and Faloona (28). These sequences are nearly identical with the exception of one nucleotide in GST IIIA which is absent in GST III, the addition of 7 extra nucleotides in GST III, and six base substitutions. Both sequences terminate at the same codon. The oligonucleotide primer 5'-AAGCTTTACGGGATGCCGCTG-3' was synthesized with one base substitution (G to T) to provide a Hind III site for subcloning. Similarly, the downstream oligonucleotide primer 5'-AAGCTTGGCGAGCTGCTCCGCGT-3' contains two base substitutions (G to C, G to T) for the same purpose. These primers span a 420 nucleotide region of corn mRNA coding for GST III.

PCR was conducted in an Ericomp twin block thermocycler (Ericomp Co., San Diego, CA). The PCR reaction contained 20 µl cDNA (prepared from 1.2 µg poly A^+ mRNA), 40 pmol of each primer, 16 µl dNTPs, 10 µl 10X PCR buffer, 30 µl 5 mM $MgCl_2$,

and water to a final volume of 100 µl. Conditions of the first PCR cycle were as follows: denaturation at 95 °C for 5 min, primer hybridization at 72 °C for 5 min during which time 0.5 µl Taq DNA polymerase (5 units/µl; Perkin Elmer Cetus, Norwalk, CT) were added, and polymerase reaction at 68 °C for 2 min. The reactants were then overlayed with mineral oil. PCR was continued for 35 cycles with the following conditions: denaturation temperature 95 °C, 1 min; primer hybridization 68 °C, 3 min; polymerase reaction 72 °C, 2 min. The PCR reaction was ended with an additional 15 min cycle at 72 °C. Amplified products were analyzed by electrophoresis on 1.2% agarose gels (29) either directly from the PCR reaction mixture or after phenol/ether precipitation.

RESULTS AND DISCUSSION

Soluble and Microsomal GST Activity of Corn. In this study, soluble and microsomal GST-metolachlor activity was similar for both 'Cargill 7567' and 'Northrup-King 9283' corn hybrids (Table 1). The sub-cellular fractionation revealed a substantial microsomal GST-metolachlor activity. Microsomal GST activity was first described from cell cultures of *Phaseolus vulgaris* and leaves of *Pisum sativum* (8). These preparations were quite active in catalyzing the conjugation of cinnamic acid with GSH but did not catalyze

Table 1. Sub-cellular distribution of GST-metolachlor activity in shoots of 3-day old corn seedlings.

	GST-Metolachlor Activity			
	Soluble		Microsomal	
Corn Hybrid	(pmol/min/mg)	Total[a]	(pmol/min/mg)	Total
Cargill 7567	34.0	474	21.3	69
Northrup-King 9283	33.3	464	23.2	74

[a]Total units of GST activity calculated by multiplying enzyme activity, expressed as pmol GS-metolachlor conjugate formed per min per mg protein, times total protein in respective fraction.

metolachlor conjugation. Recently microsomal GST from Black Mexican Sweetcorn cell cultures was also reported to have glutathione *S*-cinnamoyl transferase activity but were not tested for GST-metolachlor activity (9). Although the microsomal GST-metolachlor activity demonstrated here represents only a fraction of the total GST-metolachlor activity (ca. 13%) it may contribute considerably to the native tolerance of corn to metolachlor. It would be interesting to determine if susceptible weed species possess a similar microsomal GST-metolachlor. Such a study might provide further insight on the basis of metolachlor selectivity.

Effect of Metolachlor and Benoxacor Treatment on Corn GST Activity. The influence of metolachlor, benoxacor, and the metolachlor/benoxacor mixture (CGA-180937) on GST activity in 'Northrup-King 9283' corn is shown in Table 2. As reported previously (4) GST-CDNB activity was much higher (10^6 times) than that of GST-metolachlor activity. Both soluble GST-metolachlor and soluble GST-CDNB activities were stimulated by treatment with 1 µM benoxacor, or 30 µM CGA-180937 (metolachlor:benoxacor, 30:1 w/w). There was no effect of metolachlor treatment on soluble GST-metolachlor activity but GST-CDNB activity was inhibited by metolachlor. This is in contrast to a recent report (16) that metolachlor treatment stimulated sorghum GST-metolachlor activity at

Table 2. Soluble and membrane-bound GST activities of 3-day old 'Northrup-King 9283' corn shoots treated with benoxacor, metolachlor, or the two combined.[a]

	GST-Metolachlor (pmol/min/mg)		GST-CDNB (µmol/min/mg)	
Treatment	Soluble	Microsomal	Soluble	Microsomal
Control	29.7	23.8	39.2	6.4
Benoxacor	42.0	24.8	46.2	9.1
CGA-180937	40.7	21.5	53.8	9.6
Metolachlor	31.8	25.2	28.8	8.5

[a] Seeds were imbibed with either 1µM benoxacor, 30µM metolachlor, or 30µM CGA-180937 (metolachlor:benoxacor, 30:1 w/w).

concentrations up to 160 µM but had no effect on GST-CDNB activity. This discrepancy could be explained by the presence of two cytosolic GST isozymes in corn; one with activity towards both substrates which is inhibited by metolachlor, and another inducible by metolachlor with GST-metolachlor activity but little GST-CDNB activity. This would result in an inhibition of GST-CDNB activity without a net loss in GST-metolachlor activity relative to the untreated plants.

Microsomal GST-metolachlor activity was not influenced by any of the herbicide/safener treatments (Table 2) indicating that this fraction contains a distinct GST species that has not been substantially contaminated by cytosolic GST. In contrast microsomal GST-CDNB activity was enhanced by 30-50% by all of the treatments utilized. This suggests that these microsomal preparations contain an inducible GST isozyme which does not possess GST-metolachlor but which can be measured with CDNB as substrate. Based on the recent paper by Edwards and Dixon (9) it would appear likely that this isozyme will prove to be a glutathione *S*-cinnamoyl transferase upon further examination.

Partial Purification of Corn GSTs. When a protein extract from 4-day old untreated 'Northrup-King 9283' shoots was chromatographed on DEAE-Sepharose (Figure 1) one peak of GST-CDNB activity was eluted at a buffer

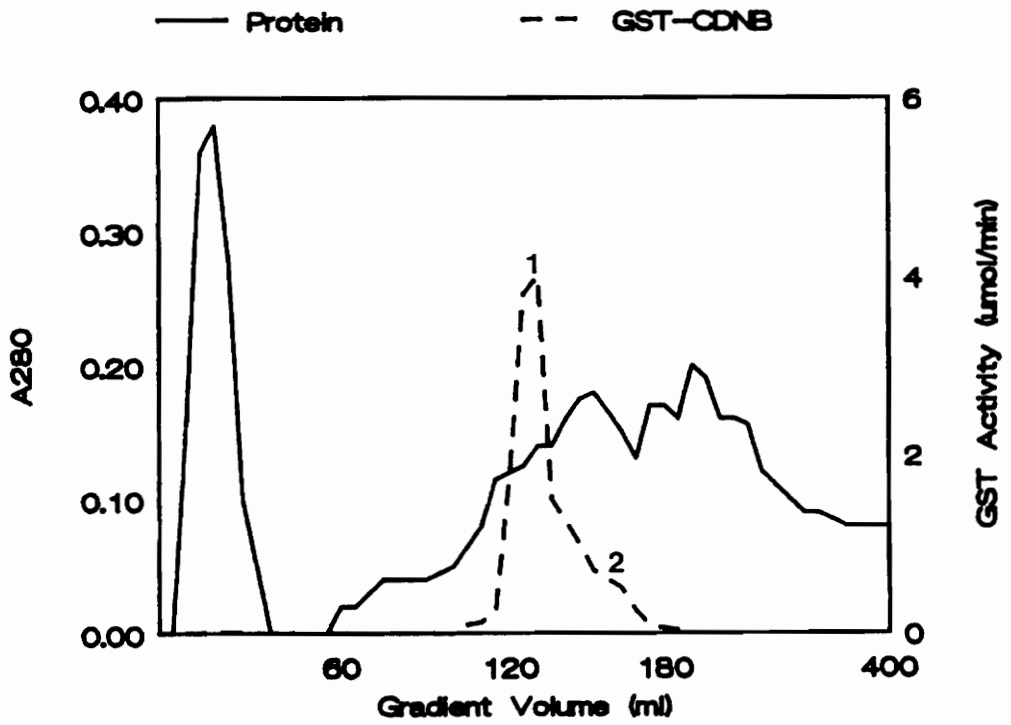

Figure 1. DEAE-Sepharose chromatography of GST activity extracted from shoots of untreated 4-day old 'Northrup-King 9283' corn.

concentration of approximately 50 mM corresponding to GST I as described by Mozer et al. (13). Fractions within this peak (designated fraction 1) were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, and assayed for GST-metolachlor activity with ^{14}C-metolachlor as substrate. Additional fractions within the tailing region of the major GST-CDNB peak (designated fraction 2) were also pooled, concentrated, and assayed. Chromatography of an extract obtained from CGA-180937 treated shoots resulted in a similar elution profile except that a second peak of GST-CDNB activity became apparent (Figure 2). Fractions from this chromatogram were pooled, concentrated, and assayed as above.

After analysis of the pooled and concentrated fractions from either untreated or CGA-180937 treated corn it was apparent that at least two distinct GST isozymes were separated by the DEAE-Sepharose chromatography. (Figure 3). This differs from the previously reported study using this same method (13). Upon examination of the elution profiles presented by these researchers, it appears that they did not consider that the obvious shoulder of GST-CDNB activity eluting immediately after GST I on chromatograms of untreated corn extracts might represent a unique GST species. Assay of this shoulder with both CDNB and ^{14}C-metolachlor revealed a definite difference in its

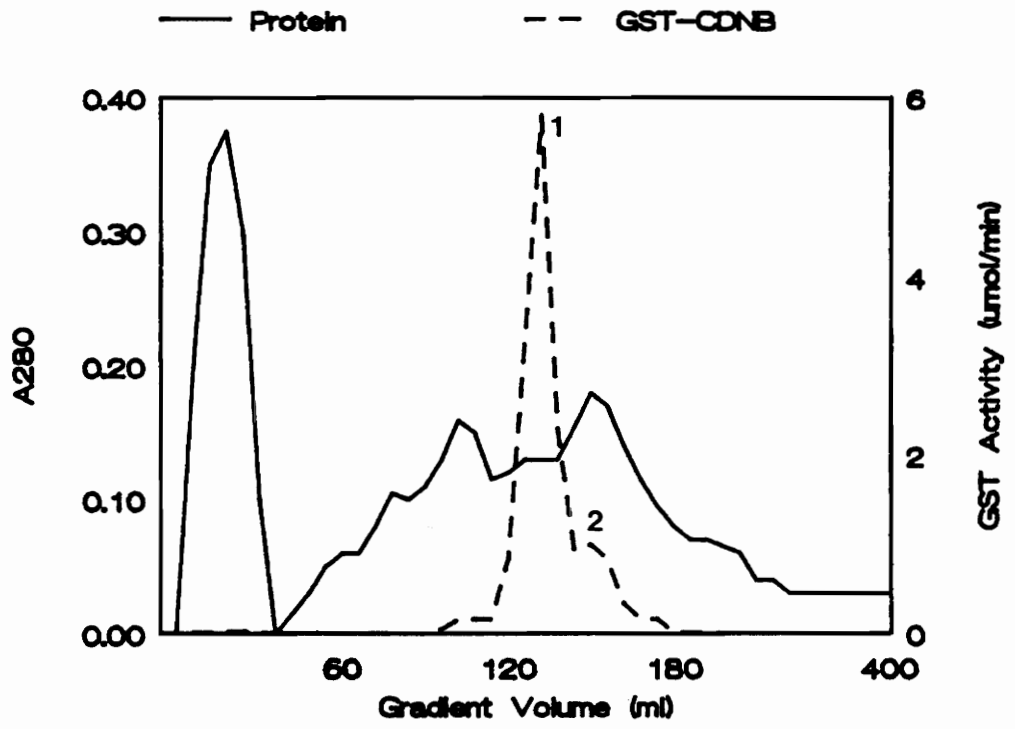

Figure 2. DEAE-Sepharose chromatography of GST activity extracted from shoots of 4-day old 'Northrup-King 9283' corn grown in the presence of CGA-180937.

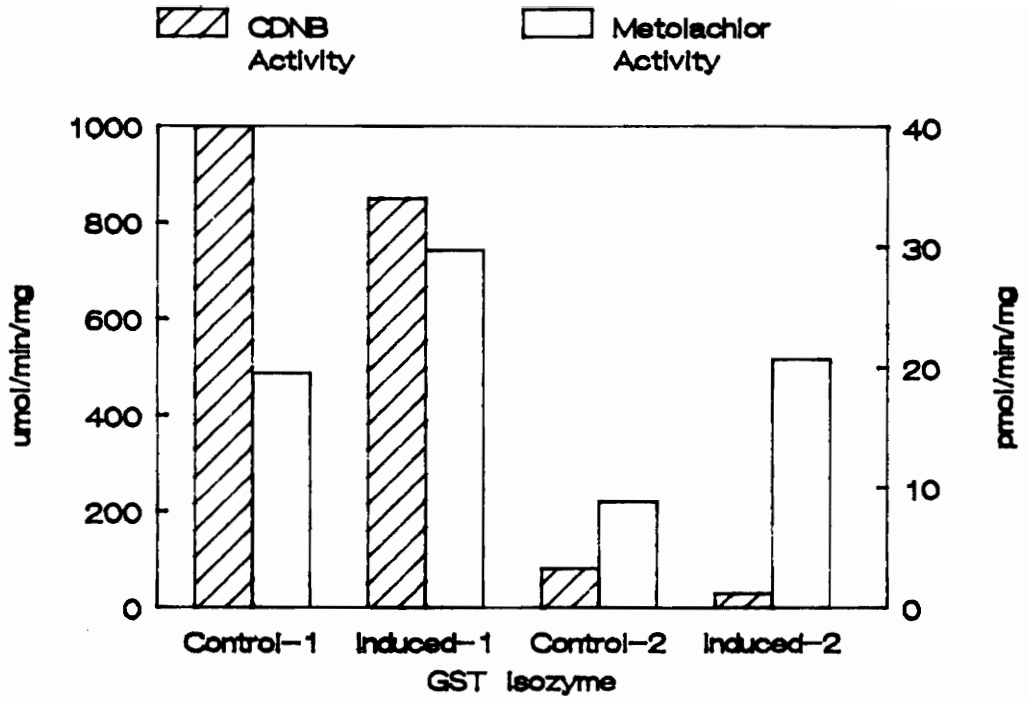

Figure 3. Comparison of GST isozymes from 'Northrup-King 9283' corn separated by DEAE-Sepharose chromatography.

relative affinity towards CDNB and metolachlor as compared to the Fraction 1-GST peak (Figure 3). Safener treatment increases the activity of the Fraction 2 isozyme thus making it more easily resolved on the chromatogram of proteins from safener-treated corn.

The GST isozymes from 'Northrup-King 9283' are compared in Figure 3. Fraction 1-GST has a high level of CDNB-GST activity in both untreated and CGA-180937 induced corn shoots. CGA-180937 treatment increases the GST-metolachlor activity of this isozyme. Based on its elution from DEAE-Sepharose and substrate specificity Fraction 1-GST is probably GST I as described by Mozer et al. (13). Fraction 2-GST is distinguished by its relatively low affinity for CDNB. The specific activity of Fraction 2-GST was doubled by CGA-180937 induction (Figure 3).

It remains to be determined if Fraction 2-GST is the same as GST II previously described (13) or is in actuality the same isozyme as GST III (18,22). Recently Dean et al. (23) examined the GST activity of untreated and benoxacor-treated corn shoots by FPLC. Multiple peaks of GST with activities towards a variety of substrates were detected. From untreated shoots two peaks of GST-metolachlor activity were detected. A third peak of GST-metolachlor activity was detected in extracts from benoxacor-treated corn shoots. This is additional evidence for the existence of three

chloroacetanilide specific GSTs in corn; GST I and GST III which are constitutive, and GST II which is only present in safener treated corn (4). It is possible that GST II was not detected in the chromatograms presented (Figures 1 and 2) here due to its low expression relative to the constitutive isozymes. The benoxacor-induced isozyme revealed by FPLC analysis (23) in fact had a much lower GST-metolachlor activity than did the two constitutive GST-metolachlor isozymes. This may be indicative of a fundamental difference in the mechanism of action of benoxacor as compared to other safeners. The safeners flurazole and dichlormid seem to act primarily by inducing a novel isozyme, GST II (13). Benoxacor may act primarily by regulating the activities of GST I and GST III while inducing GST II to a lesser extent.

Construction of GST Molecular Probe. The herbicide safener flurazole has previously been shown to cause a 4-fold increase in mRNA coding for a subunit of corn GST I (14). This increase was believed to be due to either transcriptional activation or an increase in the half-life of the mRNA. More recently Dean et al. (16) suggested that *de novo* protein synthesis was required for the action of certain safeners and that gene activation was necessary for their action. To extend this line of research on the molecular mechanisms of safener action to benoxacor a gene

specific probe for corn GST was needed. Using synthetic oligonucleotide primers and cDNA prepared from CGA-180937-treated 'Northrup-King 9283' corn mRNA, PCR (28) was utilized to obtaining an amplified DNA sequence homologous to a published GST III cDNA clone (18,22). The DNA fragment so obtained was to be sub-cloned and used as a probe to study the regulation of GST at the transcriptional level.

The products of a PCR reaction are shown in Figure 4. One band approximately 600-bp in length is apparent. The two oligonucleotide primers span a region of the GST III clone 420-bp in length. The size discrepancy observed on this gel was somewhat disturbing. It is possible that the amplified fragment is not homologous to GST III. This information will have to be obtained by sequence analysis.

The GST III gene was selected for initial experiments not only because sequence information was available, but because of the specificity of the GST III isozyme for chloroacetanilide herbicide substrates (20). Fully induced corn (ie. treated with CGA-180937) was utilized to maximally enrich the population of GST mRNAs available for conversion to cDNA and subsequent amplification by PCR. It is believed that due to sequence homology and the efficiency of PCR, this method could potentially yield a family of molecular probes for future investigations on the regulation of GSTs at the molecular level.

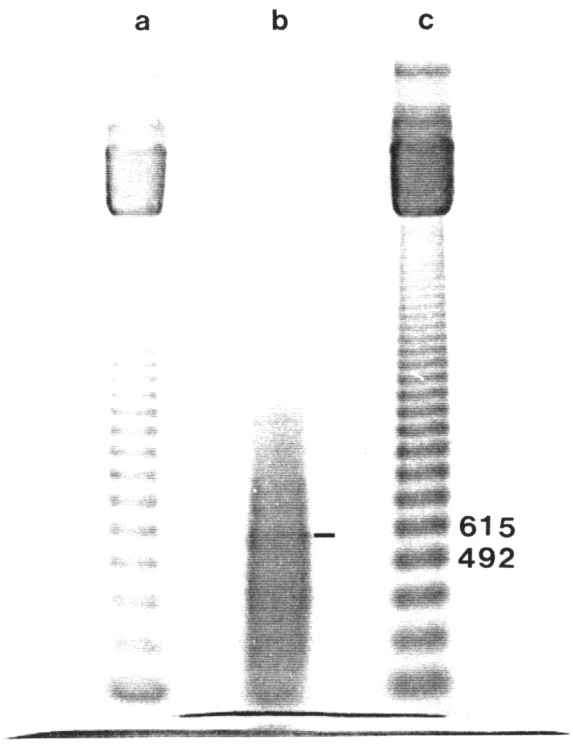

Figure 4. Agarose gel analysis of DNA amplified by PCR from 'Northrup-King 9283' cDNA (Lane b). 123-bp DNA ladder used as standards (Lanes a and c).

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VII. SUMMARY AND CONCLUSIONS

The overall objectives of the experiments reported in this dissertation were to determine the basis of differential metolachlor tolerance in corn and how this tolerance was enhanced by a new corn safener, benoxacor. Eleven corn hybrids were selected for initial screening. Some background information on the relative tolerance of these hybrids to either thiocarbamate or chloroacetanilide herbicides was available (see Chapter 2, Table 1) but none had been tested for their tolerance to herbicides of both classes. It was thought, due to similarities in use, proposed mechanisms of action, and injury symptoms produced by these herbicides (1), that the corn hybrids would respond similarly to both the thiocarbamate, EPTC, and the chloroacetanilide, metolachlor. This assumption proved to be incorrect. In general most of the tested hybrids were less sensitive to EPTC than to metolachlor. However some hybrids were quite tolerant to EPTC at the relatively high rate utilized (6.7 kg/ha) but were seriously injured by a comparable application of metolachlor. Perhaps most interesting were the two corn hybrids which, although quite susceptible to both herbicides, were somewhat more tolerant to metolachlor.

The selectivity of both EPTC and metolachlor is believed

to be primarily due to the rapid metabolism of these chemicals to non-phytotoxic GS-conjugates within tolerant plants (2). This implies that more tolerant corn hybrids might have higher endogenous levels of GSH. For the eleven corn hybrids tested glutathione levels do not appear to be a limiting factor in either EPTC or metolachlor tolerance in corn, and no correlation between GSH and tolerance was observed.

The importance of oxidative reactions in determining EPTC tolerance was demonstrated by the addition of the antioxidant piperonyl butoxide (PBO) to soil treatments of EPTC. This ability of moderately tolerant corn hybrids to resist injury was significantly reduced; presumably due to a decrease in the metabolism of EPTC to EPTC-sulfoxide which is a prerequisite to GSH conjugation (3). Additional support for the role of oxidative metabolism in determining EPTC tolerance was provided by increasing the oxygen available to germinating seeds with the oxygen-evolving compound calcium peroxide. In this experiment, the observed reduction in EPTC injury was believed to be due to enhanced sulfoxidation and subsequent GS-conjugation. Oxidative reactions were not of primary importance in determining metolachlor tolerance.

From the initial herbicide screening experiments two corn hybrids, 'Cargill 7567' and'Northrup-King 9283', which

were metolachlor-tolerant and susceptible respectively, were chosen for further examination of the basis of differential metolachlor tolerance. Specifically; metolachlor uptake, GST activity, and *in vitro* metabolism rates of the two hybrids were compared. These factors determine the rate and amount of metolachlor reaching its active site(s) and consequently the plants tolerance to the herbicide (2).

Shoot absorption of ^{14}C-metolachlor was more rapid for 'Northrup-King 9283' corn. This factor could contribute substantially to the limited metolachlor-tolerance observed for this hybrid. No differences in the rate of GS-metolachlor conjugation were observed between the two hybrids. Perhaps the most notable observation in this set of experiments was the differential expression of GST-metolachlor activity during seedling development. This activity increases markedly between 60 and 84 hr after imbibition of 'Cargill 7567' corn. A similar increase is observed for 'Northrup-King 9283' GST-metolachlor activity but there is a 12 hr lag in its initiation. The hybrids express their maximum GST-metolachlor activity 84 hr after imbibition implying that their potential to detoxify metolachlor is the same.

Metolachlor is a soil-applied herbicide. Consequently in a field situation the coleoptile of a germinating corn seedling must pass through a region of herbicide-treated

soil as it emerges. It is likely that the coleoptile is within this potentially phytotoxic region 3 to 4 days after the beginning of germination. Therefore, the temporal expression of GST activity may be a crucial factor in determining the relative tolerance of a corn hybrid to metolachlor. The limited tolerance of the 'Northrup-King' corn hybrid may arise as a result of a its more rapid uptake of metolachlor coupled with its delayed expression of GST-metolachlor activity early in development. The developmental regulation of GST activity has heretofore received little attention in the literature on plant GSTs. Edwards and Owen (4) observed differential expression of GST isozymes in 7-day and 14-day suspension-cultured corn cells. Three GST isozymes were present in 7-day old cell cultures. After 14 days one of those isozymes had disappeared. This area of research certainly merits further examination.

Herbicide safeners act essentially by enhancing the tolerance of crop plants to certain herbicides while not influencing the herbicides weed control ability. Benoxacor (also known by the code name CGA-154281) was shown to be effective in protecting corn hybrids with varying metolachlor tolerance from injury by the herbicide. A number of experiments were performed to elucidate the probable mechanism of action of benoxacor. This investigation was based primarily on the assumption that the

mechanism involved a biochemical antagonism between the safener and the herbicide (5). The basic premise of such a mechanism is that the safener reduces or eliminates completely the amount of herbicide that would otherwise reach its site of action. This decrease could result from either a safener-induced reduction in the rate of herbicide penetration and/or translocation or a safener-induced enhancement of the detoxication of the herbicide. No evidence of a benoxacor-mediated reduction in metolachlor uptake was observed. It seems likely that the mechanism of benoxacor action primarily involves the stimulation of metolachlor detoxication.

Seedlings of 'Cargill 7567' and 'Northrup-King 9283' corn, when treated with 1 µm benoxacor, metabolized metolachlor to the GS-conjugate at a rate approximately 1.7 times that of untreated seedlings. GST-metolachlor activity of both corn hybrids was stimulated 35% by low concentrations (0.2 mg/l) of benoxacor. Higher concentrations resulted in no further stimulation of GST activity for 'Northrup-King 9283' corn but were ineffective in inducing 'Cargill 7567' GST activity or were inhibitory. Another difference between the two corn hybrids was noted when glutathione levels of nontreated and benoxacor treated shoots were analyzed. Glutathione levels of the metolachlor-susceptible corn hybrid were not affected by

benoxacor treatment, but an increase was noted for shoots of the tolerant corn hybrid. Benoxacor had no effect on the non-enzymatic conjugation of metolachlor with GSH. These results suggest that benoxacor confers its safening effect by stimulating the enzymatically catalyzed conjugation of metolachlor with GSH. GSH is not believed to be a limiting factor in this process nor is its regulation a major component of the mechanism of action of benoxacor.

A comparison of benoxacor and metolachlor uptake and translocation found the safener to be more rapidly absorbed and more mobile than metolachlor. This suggest the possibility of competition for the same active site (competitive antagonism) (5) . This, however, is an unlikely mechanism considering the ratio of herbicide to safener in typical formulations is 30:1 (w/w) and that metolachlor probably has multiple sites of action (1,6). The more rapid uptake of benoxacor relative to metolachlor could allow the induction of processes involved in metolachlor detoxication (ie. GSTs) prior to the arrival of the herbicide at its target site(s). Some length of time is apparently necessary for benoxacor to exert its influence as addition of benoxacor directly to the *in vitro* assay failed to stimulate metolachlor metabolism by previously untreated corn shoots.

A recent report, describing induction of a novel

cytochrome P450 monooxygenase isozyme by benoxacor (6), prompted an experiment to determine the interactive effects of PBO with the safener. Inclusion of this monooxygenase inhibitor had no effect on the ability of benoxacor to provide protection against metolachlor injury. The induction of monooxygenase activity involved in the metabolism of the sufonylurea herbicide primisulfuron (6) is nevertheless of interest to the overall mechanism of action of benoxacor. It is possible that this safener regulates a family of enzymes involved in herbicide detoxication including monooxygenases and GSTs. The relative importance of these induced enzymes to the protective action of the safener depends on the particular herbicide/safener combination being used. Oxidative reactions do not appear to be involved in the primary metabolism of metolachlor by plants (7). Consequently, the inhibition of monooxygenase activity would have little effect on the subsequent detoxication of metolachlor.

The GST isozymes of corn involved in metolachlor detoxication were partially characterized by a combination of sub-cellular fractionation, anion exchange chromatography, and assay with two distinct substrates. The sub-cellular fractionation yielded a cytosolic fraction with both GST-CDNB and GST-metolachlor activity which was stimulated at least 30% by benoxacor treatment. The

GST-CDNB activity was inhibited by metolachlor treatment suggesting the presence of two cytosolic GST isozymes. A substantial microsomal GST-metolachlor activity was also detected. Although this activity represented only about 13% of the total GST-metolachlor activity it may contribute to the native tolerance of corn to metolachlor. Microsomal GST-metolachlor activity was not affected by benoxacor treatment. A microsomal GST with limited GST-CDNB activity was also observed. This isozyme, induced by benoxacor and metolachlor may be a glutathione *S*-cinnamoyl transferase (8,9).

DEAE-Sepharose chromatography of untreated and safener-induced corn extracts revealed the presence of at least two constitutive GST-metolachlor isozymes which were further induced by benoxacor treatment. It is unclear how these isozymes exactly relate to the previously reported corn GST-metolachlor isozymes (10). One of these isozymes is believed to GST I based on its elution from the DEAE-Sepharose column. The identity of the second isozyme (Fraction 2-GST) remains to be determined. It has very little activity towards CDNB but is quite active in catalyzing metolachlor conjugation to GSH. This fraction eluted differently from GST II as previously described (11). It may have been ignored by these researchers because of its low affinity for CDNB. It is possible that benoxacor may

primarily regulate the activities of GST I and GST III while inducing GST II to a lesser extent. This result may partially explain why benoxacor does not stimulate GST activity to the same extent as some other safeners which are very effective inducers of GST II activity (11).

Initial efforts to construct a molecular probe for the corn GST III gene using PCR technology were begun during the course of this dissertation work. To date a cDNA fragment of about 600-bp has been amplified, which is somewhat larger than the anticipated fragment size of 420-bp. The reason for this size discrepancy is unknown but could be due to amplification of something other than GST III. This can only be resolved by sequence analysis.

Overall the results of these studies indicate that tolerance to metolachlor is determined by a complex interaction of factors which vary even within closely related members of the same species. The most important factor in determining tolerance is the ability to detoxify metolachlor. Absorption characteristics are also important factors as there appears to be a correlation between uptake and susceptibility. In general corn is tolerant to metolachlor by virtue of GST isozymes with activity towards metolachlor and adequate levels of GSH for conjugation. GST activity is apparently under developmental control which may be further modified as a result of environmental factors.

The increased chance of metolachlor injury in cool, wet soils (12) can be explained as follows: metolachlor availability is increased by high soil moisture (12), less than optimal temperature may interfere with the normal developmental progression of GST activity, consequently the seedling is exposed to higher than normal metolachlor concentrations without the necessary detoxication system in place.

The corn safener benoxacor can effectively prevent metolachlor injury by stimulating the enzymatic detoxication of metolachlor. The safener may possibly override developmental controls allowing the expression of constitutive GSTs in greater abundance and at earlier times or induce novel GST isozymes. The effect of this safener on GST activity is somewhat less dramatic than that of some other safeners (11,13). It is however sufficient to raise the GST activity of a susceptible hybrid to the level of an inherently tolerant hybrid.

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