STUDIES ON THE MECHANISMS OF ACTION OF THE HERBICIDE SAFENER CGA-92194

by

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(ABSTRACT)

CGA-92194 {α-[1,3-dioxolan-2-yl-methoxy)imino]benzeneacetonitrile} is a herbicide safener that is used as a seed dressing agent (1.25 g ai/kg seeds) to protect grain sorghum [Sorghum bicolor (L.) Moench] against metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl-N-(2-methoxy-1-methylethyl)acetamide] injury. The potential adverse phytotoxic effects and the mechanisms of the protective action of this safener were studied in laboratory experiments.

Adverse phytotoxicity was assessed by comparing CGA-92194 and the herbicide safeners cyometrinil {(Z)-α[(cyanomethoxy)imino]benzene-acetonitrile} and flurazole [phenylmethyl 2-chloro-4-(trifluromethyl)-5-thiazolecarboxylate] for their effects on CO_2 fixation, protein, DNA, RNA and lipid syntheses of enzymatically isolated leaf cells of soybean [Clycine max (L.) Merr]. At physiological concentrations of less than 10 μM, CGA-92194, cyometrinil and flurazole were stimulatory of all metabolic processes. At 100 μM, the safeners were inhibitory of the five processes with flurazole being the most potent.

The mechanisms of the safening action of CGA-92194 were studied by examining the potential interactions of this safener with metolachlor

at the levels of uptake and macromolecular syntheses in enzymatically isolated leaf mesophyll protoplasts of grain sorghum. The influence of CGA-92194 on the *in vitro* reactivity of metolachlor with glutathione (GSH) and it metabolism by sorghum seedlings were also examined. When CGA-92194 and metolachlor were given simultaneously, CGA-92194 enhanced the uptake of ^{14}C-metolachlor into the sorghum protoplasts in a concentration-dependent pattern. Thus, interference with herbicide uptake is not involved in the protective action of this safener. Treatments with metolachlor and CGA-92194 in combination inhibited the incorporation of ^{14}C-uracil, ^{3}H-thymidine and ^{14}C-acetate into sorghum protoplast macromolecules less than metolachlor given alone, suggesting the potential involvement of a competitive antagonism in the mechanism of action of CGA-92194.

The metabolic activity and growth of sorghum seedlings grown from CGA-92194-pretreated seeds was significantly lower than that of seedlings grown from untreated seeds at 10 or 20 days after planting. The relationship of these effects of CGA-92194 to its safening action is unclear at the present time.

CGA-92194 increased the *in vitro* chemical reactivity of metolachlor for GSH in a concentration-dependent pattern. Sorghum seedlings grown from safener-pretreated seeds enhanced ^{14}C-metolachlor absorption and stimulated its metabolism *via* conjugation to GSH. This stimulation was reduced by tridiphane [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)-oxirane] a potent inhibitor of plant GSH-*S*-transferase enzymes. These results indicate that a safener-induced stimulation of the spontaneous

or enzymatic conjugation of metolachlor with GSH is most likely involved in the protective action of CGA-92194.

It is suggested that the safening mechanism of action of CGA-92194 involves a sequence of multilevel interactions which together contribute to the overall protection of grain sorghum from metolachlor injury.

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CHAPTER I

LITERATURE REVIEW AND OBJECTIVES

Some of the commercially available herbicides have limited selectivity and often fail to control weeds that are botanically related to crop plants at the species level. For example, it is difficult to control shattercane [Sorghum bicolor (L.) Moench] chemically in grain sorghum, red rice (Oryza rufipogon L.) in cultivated rice (Oryza sativa L.) itchgrass (Rottboelia exaltata L.) in corn (Zea mays L.) and wild beet (Beta vulgaris L.) in sugar beet (Beta vulgaris L.).

Over the years numerous attempts have been made to overcome the problem of limited herbicide selectivity by increasing crop tolerance to such herbicides. This has been achieved with varying degrees of success through mechanical, genetic, and chemical means. Conferring herbicide tolerance to crops mechanically is accomplished by avoiding or minimizing the contact of the herbicide with the crops. Careful timing and directed sprays of herbicide applications are often the case. The genetic approach, quite encouraging in recent years, is the most tedious (27, 51). The best illustrated example has been the transfer through conventional genetics of cytoplasmic characters from a triazine-resistant biotype of birdsrape mustard (Brassica campestris L.) to the agronomic oil crop canola or seed rape (Brassica napus L.) (4, 15). Last, but not least, crop tolerance to herbicides can be enhanced with the use of chemicals known as safeners or antidotes.

The concept that crop tolerance to herbicides can be increased with the use of other chemicals was pioneered in 1947 by Hoffman (36) who observed that 2,4,6-T (2, 4, 6-trichlorophenoxyacetic acid) reduced

the epinasty caused by 2,4-D [(2, 4-dichlorophenoxy) acetic acid] on tomato (Lycopersicon esculentum Mill.) plants. This early work was the stimulus for further research that resulted in the development of the concept of herbicide safening with the introduction, in 1962, of S-449 (4-chloro-2-hydroxyiminoacetanilide) as a safener against barban (4-chloro-2-butynyl 3-chlorophenylcarbamate) injury on wheat (Triticum aestivum L.) (38). Since then considerable attention has been given to this concept which is now recognized as an important agronomic practice (5, 28, 29, 31, 63, 65, 70).

Five chemicals that have been developed commercially are currently marketed as herbicide safeners. They are NA (napthalene-1, 8-dicarboxylic anhydride) introduced in 1969 as the first herbicide safener (37), dichlormid (2,2-dichloro-N,N-di-2-propenylacetamide), flurazole [phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate], cyometrinil {(Z)-α[(cyanomethoxy)imino]benzeneacetonitrile} and CGA-92194 {α-[(1,3-dioxolan-2-yl-methoxy)-imino]benzeneacetonitrile}. Many other chemicals with potential safening activity are in the experimental stage. The research described in this dissertation was primarily concerned with the safener CGA-92194.

Although the concept of herbicide safening has been around for more than 20 years, there are still some differences of opinion concerning the terminology used to describe these chemicals. An examination of the literature shows that these chemicals are interchangeably described as "herbicide antidotes," "herbicide safeners," "herbicide antagonists" or "crop protectants". Generally an antidote reverses or counteracts the effect of an existing intoxication. This is not the case with herbicide

antidotes which prevent rather than counteract the effects of selected herbicides. Recently, Parker (65) questioned the existence and use of the verb "to safen" in the Engligh language. A safener is defined as "a chemical used in an insecticidal or fungicidal spray to prevent damage to trees and foliage by the other ingredients in the spray".[1] Therefore a herbicide safener can be defined as a chemical agent that selectively protects crop plants from the phytotoxic effects of herbicides without protecting weeds. This protection is either the result of a specific crop/ herbicide/safener interaction or could result from the dressing of the crop seeds with the safener.

The use of safeners may allow the deliberate selective use of low cost non-selective herbicides provided that the safener itself is not expensive for the farmer. With the development of additional herbicide safeners a wider range of herbicide options could be available in minor crops for which herbicides will never be developed (65). Furthermore, the use of herbicide safeners could selectively protect the crop in case of herbicide misapplication or under poor climatic conditions. Studies with herbicide safeners could be used also to explain to some extent the mechanism of herbicide action (29).

Properties and efficacy of CGA-92194 as a herbicide safener.

CGA-92194 was synthesized in Basel, Switzerland and introduced in the U.S. in 1982 by CIBA-GEIGY Corp., to protect grain sorghum against

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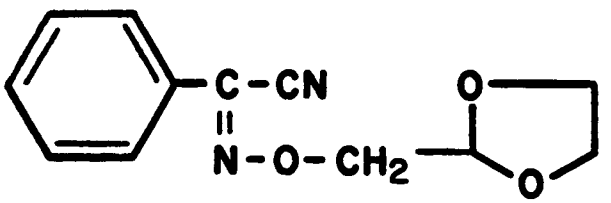
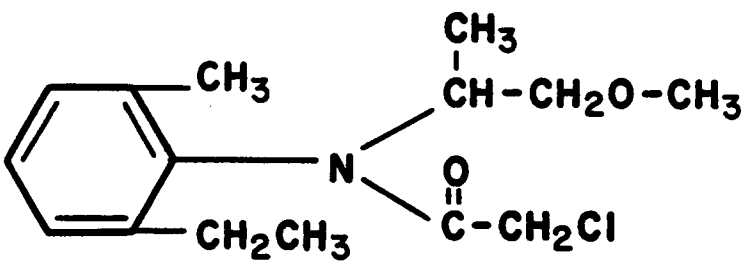
[1]Webster's Third New International Dictionary. 1981. P. B. Gove, ed. Merriam-Webster, Inc., Publishers. Boston, MA.

metolachlor injury (15, 69). This safener belongs to the class of the oxime ether derivatives whose safening properties have been reported by several investigators (8, 11, 22, 67,69). CGA-92194 was introduced as an alternative to its chemical analog cyometrinil because cyometrinil is labeled for use on high quality grain sorghum seed only at rates not exceeding 1.5 g/kg. Damage to sorghum seeds occurred when cyometrinil was used at higher rates or on other sorghum varieties (11, 69). CGA-92194 can be used on low quality sorghum seeds, yellow endosperm sorghum varieties and sudangrass [Sorghum sudanense (Piper) Stapf.] as well as on high quality sorghum seeds (69). Sorghum seeds are more tolerant to this safener, thus permitting a wider range of application rates (0.3-3.88 g/kg). Additionally, CGA-92194 offers acceptable protection to sorghum against higher rates of metolachlor (5.6 kg ai/ha) (69). The superior performance of this safener made it the leading protectant of sorghum seeds against metolachlor injury. The chemical structures of CGA-92194 and metolachlor are presented in Figure I.1 whereas the physical and chemical properties of CGA-92194 are summarized in Table I.1. CGA-92194 is a light tan crystalline solid of low volatility, practically insoluble in water. This safener remains active as a sorghum seed dressing for more than a year (15). CGA-92194 is marketed under the trade name CONCEP II® and is commonly sold coated on the sorghum seeds by Funk Seed Co.

In addition to grain sorghum, the safening ability of CGA-92194 has been tested on other crops and against injury from herbicides other than the chloroacetanilides. Early studies by Dill et al. (15) revealed that



CGA-92194



metolachlor

FIGURE I.1. Chemical structures of CGA-92194 and metolachlor.

Table I.1. Physicochemical properties of CGA-92194.[1]

Physical state:	crystalline solid
Color:	light tan
Empirical formula:	$C_{12}H_{12}N_2O_3$
M.W.	232.24
Melting point:	77.7°C
Vapor pressure (20°C)	3.9×10^{-6} mmHg
Solubility (20°C)	water: 20 ppm hexane: 0.56% methanol: 3% acetone: 25% Methylene chloride: 45%

Toxicity of the technical material

Acute oral LD_{50} (rats); >5000 mg/kg
Acute dermal LD_{50} (rats); >5000 mg/kg
Eye and skin irritation (rabbits); minimal

[1]Source: Rufener, Nyfeller and Peek. Proceedings 1982 British Crop Protection Conference-Weeds, p. 463.

the safener not only protected several hybrids of grain sorghum such as sweet sorghum, yellow endosperm sorghum and sorghum of low quality seeds but also protected grasses of the same tribe, the Andropogoneae. Itchgrass, seedling Johnsongrass [(Sorghum halepense (L.) Pers.] and sudangrass were all protected from the phytotoxic effects of metolachlor. Coats (10) also reported that CGA-92194 provided adequate protection to Sorghum vulgare L. against metolachlor. Crop protection afforded by CGA-92194 against other herbicides has been generally characterized as partial, implying the potential existence of a specific interaction between the crop, chloroacetanilide herbicides and the safener. Complete protection is an a priori condition for the ultimate development of a chemical into a commercial herbicide safener (31).

Under greenhouse conditions CGA-92194 partially protected sorghum from low rates of chlorsulfuron {2-chloro-N-[[(4-methoxy-6-methyl-1, 3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide}, fluazifop-butyl {(±)-2[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid} and sethoxydim {2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio) propyl]-3-hydroxy-2-cyclohexen-1-one} (32). Against high rates of fluazifop-butyl, sethoxydim and chlorsulfuron protection of sorghum by CGA-92194 was limited. When the same herbicides were applied to corn (Zea mays L.) the protection by the safener of the thiocarbamate-tolerant cultivar "DeKalb XL72AA" was judged satisfactory against the low rates of chlorsulfuron and sethoxydim. At higher concentrations of all herbicides the protection was only partial (33).

CGA-92194 applied alone may cause slight injury to sorghum. Reduced germination of corn seeds by CGA-92194 was reported in greenhouse studies by Henry and Hatzios (35). This safener was also toxic to the wheat cultivars "Tyler" and "McNair" (35). Laboratory studies by Ketchersid et al. (43) revealed that growth and respiration rates of grain sorghum were affected during the early stages of germination of sorghum seeds treated with the safeners.

The safening mechanism of CGA-92194

Because of the recent introduction of CGA-92194, studies on the mechanisms of its safening action are limited. Studies on the mode of action of the oxime ether safeners have been conducted primarily with cyometrinil (29). Extensive research with other safeners has shown that these compounds may protect crops against herbicide injury by one or more of the following mechanisms (29, 63, 65):

a. The safener inteferes with herbicide uptake and/or translocation in the protected plant.

b. The safener competes with the herbicide at a common site of action within the protected plant.

c. The safener stimulates herbicide degradation by the protected plant.

d. The safener protects the plant through a combination of one or more of the above mechanisms.

Information on the exact mechanisms through which cyometrinil protects grain sorghum against metolachlor is still inconclusive. This stems

partially from the fact that our knowledge of the biochemical mode of action of metolachlor or other chloroacetanilide herbicides is not complete. Metolachlor is characterized as an inhibitor of early shoot growth of germinating grass seedlings (3). Soil applications of this herbicide have revealed that the main site of entry into the plant is the emerging coleoptile rather than the root (3). Injury symptoms are mostly visible at the meristematic region of the young seedlings and include shoot deformation, leaf rolling and twisting, and the leaves sometimes fail to emerge from the coleoptile (2, 3, 24). These adverse effects of metolachlor on grain sorghum are prevented when sorghum seeds are treated with the safener CGA-92194 before planting. At a physiological level, the chloroacetanilide herbicides have multiple effects on plant metabolism. They have been reported to affect cell division and cell enlargement (12, 13, 24) and the synthesis of certain macromolecules such as proteins (12, 13, 17, 41, 51, 57, 60) and lipids (19, 21, 56, 71). Membrane integrity (20, 59), leaf nitrate reductase activity (55) and plant hormone syntheses (64, 73, 74, 75) are also affected. In addition chloroacetanilide herbicides can alkylate thiol-containing molecules (24, 54, 58).

Because the site of uptake of both metolachlor and the oxime ether safeners is mainly the young emerging coleoptile (26, 40, 67) some investigators have proposed that absorption of metolachlor is prevented in the protected plant. It has been postulated that a competitive antagonism may occur at a site of entry (23) or at a site of action (68, 72) common to the safener and the herbicide. As a result of this

competition, herbicide uptake by the plant is reduced. Experimental data supporting this hypothesis were reported by Ketchersid et al. (45) who observed that the rate of metolachlor uptake decreased considerably in excised coleoptiles of sorghum seedlings grown from seeds treated with cyometrinil. Other safeners have also been reported to reduce herbicide uptake. Dichlormid reduced the uptake of ^{14}C-EPTC (S-ethyl dipropyl carbamothioate) by corn cell suspensions (23). Similarly, NA affected metolachlor uptake and translocation in grain sorghum (1). Ebert (20) suggested that a possible mode of action for cyometrinil safening of sorghum could be the prevention of the loss of membrane integrity, influencing indirectly the uptake of metolachlor into sorghum. Strong evidence, however, supporting competitive antagonism at a site of entry or at a site of action as a potential mechanism through which oxime safeners protect sorghum from metolachlor injury is presently unavailable. Other investigators (9, 76) have suggested that a safener-induced reduction of metolachlor uptake is not the mechanism of the safening action of oxime derivatives. Christ (9) showed that cyometrinil reduced the amount of metolachlor present at a site of action of sorghum but he did not attribute this to the limited uptake of the herbicide. A stimulation of ^{14}C-EPTC uptake into corn cell suspensions in the presence of NA and cyometrinil has been reported recently by Ezra (23). Similarly, Fuerst and Gronwald (25) demonstrated a CGA-92194-induced uptake of [^{14}C]-metolachlor into excised leaves of sorghum seedlings.

The hypothesis that safeners enhance herbicide degradation in plants was originally suggested in studies examining the interactions of the herbicide EPTC and the safener dichlormid. It was proposed that dichlormid protects corn from EPTC injury by increasing the activity of glutathione-S-transferases and the amount of glutathione (GSH) which in turn detoxify the herbicide through a conjugation mechanism with the sulfoxide of EPTC (6, 7, 18, 39, 48, 49, 50, 52).

GSH is a tripeptide present in large amounts in both plant and animal systems. In plants, GSH appears to be the main long distance transport form of reduced sulfur (66). In addition, a well documented role of GSH is the detoxification of herbicides through a conjugation mechanism (33, 66). Other workers (6, 52), however, have disputed the involvement of the enzyme GSH-S-transferase in the EPTC sulfoxide conjugation of EPTC and GSH. The degradation of metolachlor in plants is poorly understood (3, 24). Dixon and Stoller (16) observed several metolachlor metabolities but no attempt was made to characterize them. In a previous study, Lamoureux et al. (47) reported three distinct metabolites of the chloroacetanilide herbicide propachlor [2-chloro-N-(1-methylethyl)-N-phenylacetamide] in the leaves of several grass crops. One of the metabolites was identified as the glutathione conjugate of propachlor. Furthermore, they found that the chloroacetanilide conjugation with GSH was not enzymatic (47). However, it has been postulated that since the substrates for GSH-S-transferases react with GSH at a finite rate in the absence of the enzyme, there is a possibility of enzyme involvement under *in vivo* conditions (34). Enzyme involvement

in the *in vitro* conjugation of the chloroacetanilide herbicide alachlor with glutathione has been recently confirmed by Mozer et al. (61), who demonstrated that chloroacetanilide degradation in plants could be mediated by two GSH-S-transferases isolated from corn. One of them was constitutively present in corn, whereas the other was induced by several herbicide safeners including dichlormid, flurazole, cyometrinil and NA (61). Recently, Fuerst and Gronwald (25) concluded that the mode of action of CGA-92194 may include a safener-induced metabolism of metolachlor *via* conjugation to GSH.

Other groups of investigators have suggested that the mechanism of action of oxime safeners could include a stimulation of a mixed-function oxidase (mfo) system that may be involved in the plant degradation of metolachlor. Mixed-function oxidases are a group of enzymes present in animals and plants, they require NADPH or NADH and molecular oxygen to function, and are inhibited by insecticide synergists (33).

Data in favor of an mfo role come from several lines of evidence. When sorghum plants were grown in nutrient solutions (53) or under conditions of excessive soil moisture (46) the safening ability of cyometrinil was strongly reduced. Ketchersid et al. (44, 46) have pointed out that oxygen was necessary for the optimum protection of sorghum by cyometrinil and that good aeration was needed for a rapid degradation of metolachlor. In a more recent study (42), these authors demonstrated that cyometrinil and CGA-92194 synergized the toxic effect of the insecticide propoxur (*O*-isoproxy phenylmethyl carbamate) on a strain of housefly (*Musca domestica* L.), by interfering with the

microsomal oxidation of this insecticide. Propoxur is metabolized by an mfo system. Finally, cyometrinil effectiveness was also reduced by the synergistic interaction of the antioxidants piperonyl butoxide {α-[(2-butoxyethyl)ethoxy)-4,5-dimethylenedioxy-2-propyltoluene} and n-propyl gallate with metolachlor on safened sorghum (30). Because sorghum coleoptiles possess the same enzymes responsible for oxidative metabolism in insects it was suggested (42) that the safening effect of CGA-92194 against metolachlor injury may involve the induction of an oxidative process mediated by an mfo system present in sorghum seedlings. Presently, however, the mfo hypothesis for the safening action of cyometrinil or CGA-92194 is speculative.

OBJECTIVES

The main objective of this research was to conduct several studies on the potential mechanism(s) of the safening action of CGA-92194 against metolachlor injury to grain sorghum. Experiments were designed to determine:

a. the potential adverse phytotoxic effects of the safeners CGA-92194, cyometrinil and flurazole.

b. the potential influence of CGA-92194 on [^{14}C]-metolachlor uptake into sorghum mesophyll protoplasts.

c. the potential interactive effects of metolachlor and CGA-92194 on the incorporation of radiolabeled precursors into macromolecules of sorghum protoplasts isolated from unsafened seedlings.

d. the effect of metolachlor on the incorporation of radiolabeled precursors into macromolecules of sorghum protoplasts isolated from seedlings unsafened or safened with CGA-92194.

e. the potential effects of CGA-92194 on the chemical and biological degradation of [^{14}C]-metolachlor.

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CHAPTER II

COMPARATIVE EFFECTS OF CGA-92194, CYOMETRINIL AND FLURAZOLE ON SELECTED METABOLIC PROCESSES OF ISOLATED SOYBEAN LEAF CELLS

ABSTRACT. The potential adverse phytotoxic effects of the herbicide safeners CGA-92194 {α-[(1,3-dioxolan-2-yl-methoxy)imino]benzene-acetonitrile}, cyometrinil [(Z)-α(cyanomethoxy)imino-benzeneacetonitrile] and flurazole [phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate] on selected metabolic processes of enzymatically isolated leaf cells of soybean [Glycine max (L.) Merr.] were compared in time-and concentration-course studies. Carbon dioxide fixation, protein synthesis, RNA synthesis, DNA synthesis, and lipid synthesis were assayed by the incorporation of $NaH^{14}CO_3$, [^{14}C]-leucine, [^{14}C]-uracil, [^{3}H]-thymidine, and [^{14}C]-acetate, respectively, into the isolated cells. CGA-92194 and cyometrinil behaved similarly and at low concentrations (0.1, 1, and 10 μM) they were stimulatory rather than inhibitory of the five metabolic processes assayed, following incubation periods of up to 2 h. At the highest concentration of 100 μM, both safeners were inhibitory of all metabolic processes of the soybean leaf cells but neither compound exhibited rapid and distinct inhibitions as might be expected in the case of inhibition of a primary target site by a potent inhibitor. At low concentrations and early incubation periods (30 and 60 min), flurazole effects of all metabolic processes were also stimulatory rather than inhibitory. However, the stimulation of CO_2 fixation by 0.1 and 1.0 μM was highly significant. At 100 μM flurazole was extremely potent on all

metabolic processes of soybean leaf cells examined. At the 2 h incubation period, flurazole was also inhibitory of all metabolic processes at concentrations lower than 100 μM. The sensitivity of the five metabolic processes to flurazole decreased in the order: photosynthesis = lipid synthesis > DNA synthesis > protein synthesis > RNA synthesis.

INTRODUCTION

The recent development and commercialization of chemicals conferring crop tolerance to nonselective herbicides is undoubtedly one of the major advances in the areas of plant growth regulation and herbicide technology. Five chemicals are presently marketed as herbicide safeners (also referred to as antidotes or protectants) and they have been the subject of several reviews (3, 2, 14, 19, 22, 23). Cyometrinil,[1] CGA-92194 and flurazole,[1] applied as seed treatments, protect grain sorghum from injury caused by chloroacetanilide herbicides such as alachlor and metolachlor (6, 7, 8, 9, 21, 25, 26).

Low potential for phytotoxicity at recommended rates and reliability under field conditions are two important considerations for the selection and development of candidate chemicals as herbicide safeners. However, since all of the currently marketed herbicide safeners contain in their molecule functional chemical groups similar to those found in many herbicides, they have the potential to cause adverse phytotoxic effects under certain circumstances. Several reports have documented the potential of the safener NA (napthalene-1,8-dicarboxylic anhydride) to interfere with the growth of corn, grain sorghum, oats, rice, and beans even at the recommended rate of 0.5% by seed weight (3, 8, 13, 15, 16, 28). Recently, Hatzios and Zama (1985) showed that changes in the chemical structure of NA could reduce the phytotoxic potential of this

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[1]Cyometrinil and flurazole are the same compounds as CGA-43089 and MON-4606, respectively in other publications.

safener without any significant loss of its antidotal activity against thiocarbamate herbicide injury to corn. Adverse effects caused by the safener dichlormid (2,2-dichloro-N,N-di-2-propenylacetamide) on the growth of corn or beans have been reported (3, 10). Reduction of the viability of sweet and yellow-endosperm sorghum seeds treated with the safeners cyometrinil and CGA-92194 has also been reported (6, 7). In addition, cyometrinil used at rates higher than 1.88 g/kg of seed caused significant phytotoxic effects on grain sorghum (6). Seed viability and growth of grain sorghum did not appear to be adversely affected by the safener flurazole (26). However, measurable effects of flurazole as well as CGA-92194 on the growth and respiration of grain sorghum seeds have been observed by Ketchersid and Merkle (20) during the early stages of germination of sorghum seeds. Under similar conditions, cyometrinil was more inhibitory than either CGA-92194 or flurazole (20).

Studies on the potential phytotoxicity of cyometrinil, CGA-92194 and flurazole on certain aspects of plant metabolism at the cellular level are not currently available. Enzymatically isolated or cultured cells of selected plant species offer an attractive and reliable system for studying selected metabolic processes (27) and screening the activity of natural or synthetic substances with phytotoxic potential (2, 11, 17).

The studies in this report were conducted to determine and compare the potential adverse effects of the safeners cyometrinil, CGA-92194, and flurazole on CO_2 fixation, and precursor incorporation into proteins, RNA, DNA and lipids in enzymatically isolated leaf cells of soybean in time-and concentration-course experiments. Soybeans were uesd in these

studies because they are a very good source of enzymatically isolated, active mesophyll cells that have been useful for screening herbicides or other growth regulators. In addition, since soybeans may follow corn or grain sorghum in crop rotation systems, they could be exposed to safener residues.

MATERIALS AND METHODS

PLANT MATERIAL. Soybean [Glycine max (L.) Merr. cv. 'Essex'] seeds were planted in plastic cups (473-ml) filled with a mixture of potting medium (Weblite Corporation, Blue Ridge, Virginia), vermiculite and sphagnum peat moss in 2:2:1 ratio. Limestone and a controlled release fertilizer (14-14-14) were added to the soil mixture to provide additional nutrients. After germination, the plants were transferred to a growth chamber with photon flux density of 50-60 W m^{-2} provided by a blend of incandescent and fluorescent lamps. A photoperiod of 16 h of light at 30°C and 8 h of dark at 20°C was maintained during the first two weeks of soybean emergence and growth. The plants were acclimated for 5 days to a 6 h photoperiod to reduce the amount of starch in the chloroplasts. A low starch content has been reported to increase the photosynthetic rates of soybean leaf cells isolated from plants exposed to a short day treatement (27).

CELL ISOLATION. Mature trifoliate leaves were detached from plants at least one hour after the initiation of the light period in the growth chamber and rinsed with distilled water. Leaf midribs were removed and

were cut into 1 mm × 1 cm strips. Two to three grams of cut leaf tissue served as a source for the enzymatic isolation of mesophyll cells through a 3-step procedure. The procedure included infiltration of the leaf tissue with the maceroenzyme (MACERASE®, Calbiochem-Behring Corp., La Jolla, California) under vacuum, maceration of the infiltrated tissue through slow magnetic stirring and repeated washings of the released cells through centrifugation. Detailed descriptions of these procedures have been given by Ashton et al. (2), Hatzios and Howe (17), and Servaites and Ogren (27). The released cells were made up to the desired volume with an incubation medium containing 0.2 M sorbitol, 2 mM $Mg(NO_3)_2$, and 1 mM $CaCl_2$. The pH of the medium was maintained at 7.8 with HEPES buffer for the CO_2 fixation assays or at 5.8 with MES buffer for the incorporation of the respective precursors into proteins, RNA, DNA, and lipids. Chlorophyll content was determined by the method of Arnon (1). The chlorophyll content of cell suspension used in this study ranged from 15 to 25 µg of chlorophyll per ml of assay medium.

TIME-COURSE AND CONCENTRATION STUDIES WITH THE THREE SAFENERS. Analytical grade samples (>95% pure) of the safeners CGA-92194 and cyometrinil, and of flurazole were provided by CIBA-GEIGY Corp., Greensboro, North Carolina and Monsanto Chemical Co., St. Louis, Missouri, respectively. The three safeners were dissolved in methanol and made up to the desired volume with distilled water so that the final methanol concentration was less than 1%. Methanol was also used in the preparation of the control (safener-free) solutions. The assay methods for the five

metabolic processes studied are available in the literature (2, 17, 27). A detailed description of these procedures is given in the Appendix. The assaying medium for all metabolic process studies contained 2 ml of the cell preparation in a 25-ml Erlenmeyer flask, 0.1 ml of radioactive substrate containing 1 μCi of radioactivity, and 0.05 ml of the safener solution making a final volume of 2.15 ml. The flasks with the assay mixtures are sealed and placed in a shaking water bath at 25°C and illuminated from above with a combination of incandescent and fluorescent lamps supplying 7.4 W m^{-2} photon flux density at the level of the flasks. Samples were removed at selected incubation period and treated as previously described (2, 17), prior to liquid scintillation counting. Carbon dioxide fixation was assayed by incubating the cells with 1 μCi of $NaH^{14}CO_3$ (sp. act. 44.4 mCi/mmol) containing 5 mM of $NaH^{12}CO_3$. Incorporation into proteins was determined by measuring the incorporation of 1 μCi of L-[U-^{14}C]-leucine (sp. act. 276 mCi/mmol) into proteins. Incorporation into RNA was assayed by measuring the incorporation of 1 μCi of [2-^{14}C]-uracil (sp. act. 55 mCi/mmol), while incorporation into DNA synthesis was assayed with [^{3}H]-thymidine (sp. act. 25 Ci/mmol). Incorporation into lipids was determined by the incorporation of 1 μCi of [1,2-^{14}C]-sodium acetate (sp. act. 56.2 mCi/mmol) into lipids. Radioactivity was determined by adding 10 ml of scintillation fluid (ACS, Amersham) and counting in a liquid scintillation spectrometer (Beckman LS-250) with a 92% counting efficiency for ^{14}C and 70% efficiency for ^{3}H. Carbon dioxide fixation was expressed as μmoles of ^{14}C fixed per mg of chlorophyll. Incorporation of precursors into proteins, RNA, and lipids

were expressed as dpm of the [^{14}C] from the respective radioactive substrate incorporated into the cells per 100 μg of chlorophyll. Incorporation into DNA was expressed as dpm of [^{3}H] incorporated into the cells per mg of chlorophyll. All of the assays were repeated 4 times, and the data were analyzed for variance in a completely randomized design. The standard errors of each mean were also calculated and used for separation of treatment means. In addition, the results were also calculated as percent inhibition caused by each concentration of the three safeners. Negative values indicate stimulation instead of inhibition.

RESULTS AND DISCUSSION

The effects of CGA-92194, cyometrinil and flurazole on CO_2 fixation, protein, RNA, DNA, and lipid syntheses of isolated soybean leaf cells are presented in Tables II.1 through II.5. Data in Table II.1 show that CGA-92194 and cyometrinil are not inhibitors of CO_2 fixation of soybean leaf cells. Appreciable inhibition of this metabolic process was observed only with the two highest concentrations (10 and 100 μM) of CGA-92194 at the 60 and 120 min incubation periods. However inhibition of this process by CGA-92194 was greatest at the 100 μM concentration. Appreciable stimulation of CO_2 fixation by the low concentrations of CGA-92194 was evident at 30 min or later incubation periods. Cyometrinil did not inhibit the fixation of $^{14}CO_2$ in soybean leaf cells at any concentration or at any incubation period examined (Table II.1). On the contrary, it appeared to stimulate this metabolic process at any incubation period examined, when used at 0.1, 1, and 10 μM concentrations. Flurazole applied at a non-

physiological concentration of 100 μM caused a rapid and distinct inhibition of the CO_2 fixation of soybean leaf cells (Table II.1). A slight inhibition of this process was also obtained with 10 μM of flurazole at the 120 min period. However, flurazole at low concentrations was stimulatory rather than inhibitory of this process. Stimulation caused by 0.1 and 1 μM of this safener at the 30 or 60 min incubation periods was highly significant (Table II.1).

A time-dependent inhibition of the incorporaion of ^{14}C-leucine into protein of isolated soybean cells was caused by 100 μM of CGA-92194. The respective inhibition percentages were 23, 33, and 47% at 30, 60 and 120 min (Table II.2). The effects of 0.1, 1 and 10 μM of CGA-92194 and of all concentrations of cyometrinil on this metabolic process were either stimulatory or slightly inhibitory at all incubation times. Comparisons of the treatment means indicate that most of these effects on protein synthesis were not significant. Flurazole at 100 μM caused a rapid and strong inhibition of the incorporation of ^{14}C-leucine into the isolated soybean leaf cells (Table II.2). Percent inhibitions at 30, 60 and 120 min were 64, 79 and 84% indicating a time-dependence of the inhibition caused by this concentration of flurazole. A slight but significant inhibition was also observed with the 10 μM concentration of flurazole. The lowest concentrations of flurazole were stimulatory rather than inhibitory of this metabolic process.

RNA synthesis of isolated soybean cells was the least sensitive process to all three safeners. Significant inhibition of the incorporation of ^{14}C-uracil into soybean cells was obtained only with

the two high concentrations (10 and 100 μM) of CGA-92194, cyometrinil and flurazole at the 2 h incubation time (Table II.3). At the highest concentration of 100 μM, flurazole was more inhibitory (52%) than either CGA-92194 (29%) or cyometrinil (32%) of this process. At 100 μM CGA-92194 and flurazole were also inhibitory of this process at the 60 min incubation period. At 30 min of incubation the three safeners at all concentrations were stimulatory.

In Table II.4, data on the effects of the three safeners on DNA synthesis of isolated soybean leaf cells are presented only for the 120 min incubation period. Incorporation of [^{3}H]-thymidine into the soybean cells was limited at 30 or 60 min of incubation and increases in incubation time did not result in linear increases in the incorporation of radioactivity into the soybean cells. Problems in studying DNA synthesis of plant cell cultures by measuring the incorporation of [^{3}H]-thymidine have been reported by other investigators. The existence of high levels of thymidine phosphorylase in most plants has been offered as a possible explanation of these problems (31). Under the conditions of this study, flurazole appeared to be a more potent inhibitor of this process than either CGA-92194 or cyometrinil (Table II.4), where percent inhibition for the 1, 10, and 100 μM concentrations of flurazole were 41, 66 and 94%, respectively. Appreciable inhibition of DNA synthesis of soybean leaf cells by CGA-92194 or cyometrinil was observed only with the highest concentration (100 μM).

CGA-92194 and cyometrinil did not adversely interfere with the incorporation of ^{14}C-acetate into isolated soybean leaf cells at any concentration of any incubation period (Table II.5). Significant stimulation of this process was observed following treatments of soybean cells with the lower concentrations of both safeners. A slight inhibition of lipid synthesis was observed only with the highest concentration of cyometrinil. A rapid and strong inhibition of the incorporation of ^{14}C-acetate into soybean cells was caused by 100 μM of flurazole. Inhibition precentage recorded at 30, 60 and 120 min were 88, 91 and 95%, respectively indicating little time-dependence of the inhibition of lipid synthesis by 100 μM of flurazole. The effects of the lower concentrations of flurazole on this process were either stimulatory or slightly inhibitory (Table II.5).

The results of this study revealed that CGA-92194 and cyometrinil had similar effects on soybean cell metabolism. At low concentrations (0.1, 1, and 10 μM) the two safeners stimulated rather than inhibited the five metabolic processes assayed following incubation periods of up to 2 h. At the highest concentration of 100 μM, both safeners were inhibitory of all metabolic processes of the soybean leaf cells but neither compound exhibited a rapid and distinct inhibition of any process as might be expected in the case of a primary target site by a potent inhibitor (2, 12). It is evident, therefore, that the potential phytotoxicity of CGA-92194 and cyometrinil at the cellular level, even at the highest non-physiological concentration of 100 μM, is very limited. However, the

potential activity of the two safeners on cellular processes other than those examined in the present study or on plant metabolism at organ or whole plant levels cannot be excluded. Ketchersid and Merkle (2) demonstrated that CGA-92194 and cyometrinil could interfere with plant metabolic processes such as respiration during the early stages of grain sorghum seed germination. A cyometrinil-induced reduction of grain sorghum seed germination has been reported also by Warmund et al. (29).

The stimulatory effects caused by the low concentrations of CGA-92194 and cyometrinil on all metabolic processes of soybean cells are not unusual. Sublethal concentrations of many herbicides or other plant growth regulators have been reported to cause stimulation of selected processes of plant metabolism (2) and the subject has been reviewed in detail by Ries (24).

The comparable activity of the two safeners on the matabolism of isolated soybean leaf cells was not unexpected. CGA-92194 is a chemical analog of cyometrinil and these oxime safeners would be expected to elicit similar biological responses on plants (5, 6, 7).

Significant stimulations of the five metabolic processes of soybean cells by the low concentrations of the safener flurazole were also evident from data generated in this study. However, at higher concentrations, and particularly at 100 μM, flurazole was extremely inhibitory of all metabolic processes of soybean cells examined. the sensitivity of the five metabolic processes to 100 μM of flurazole following 2 h of incubation period decreased in the order:

photosynthesis = lipid synthesis > DNA synthesis > protein synthesis > RNA synthesis. Interference of flurazole with the incorporation of ^{14}C-acetate into triglyceride lipids of grain sorghum seeds has been reported recently by Warmund et al. (29). A slight effect of flurazole on respiration of germinating grain sorghum seeds has been also reported (20, 29). In addition, the shikimic and phenylpropanoid acid pathways have been proposed as sites for the potential interference of flurazole with plant metabolism. Support for such a postulation comes from reports showing inhibition of anthocyanin and lignin biosynthesis in grain sorghum by 10 nM of flurazole (Dr. W. T. Molin, Monsanto Chemical Company, St. Louis, Missouri, personal communication).

Although at equimolar (100 μM) concentrations, flurazole was found to be a more potent inhibitor of all metabolic processes of soybean leaf cells examined in this study, the absence of rapid and marked inhibitions of any process, observed with concentrations of the three safeners lower than 100 μM suggests that use of CGA-92194, cyometrinil or flurazole as seed protectants of grain sorghum against chloroacetanilide herbicides is not likely to cause any significant adverse effects on plant metabolism under field conditions.

Table II.1: The effect of CGA-92194, cyometrinil and flurazole on $^{14}CO_2$ fixation of isolated soybean cells.

Incubation time (min)	Safener Concentration (μM)	CGA-92194 $^{14}CO_2$ fixation[a] (μmol $^{14}CO_2$/mg chl)	CGA-92194 Inhibition[b] (%)	Cyometrinil $^{14}CO_2$ fixation[a] (μmol $^{14}CO_2$/mg chl)	Cyometrinil Inhibition[b] (%)	Flurazole $^{14}CO_2$ fixation[a] (μmol $^{14}CO_2$/mg chl)	Flurazole Inhibition[b] (%)
30	0	12.99±1.04	0	10.14±0.24	0	16.04±0.49	0
	0.1	16.14±0.60	-24	11.43±0.51	-12	29.68±2.66	-85
	1	15.93±0.32	-22	15.70±1.73	-54	22.38±2.81	-39
	10	13.20±2.59	-1	14.55±1.79	-43	14.95±1.90	7
	100	11.45±1.55	12	10.93±0.68	-7	2.66±0.14	84
60	0	26.42±2.46	0	19.21±0.75	0	28.67±0.52	0
	0.1	28.98±1.67	-9	21.76±1.80	-13	48.77±1.13	-70
	1	25.64±0.57	-3	27.74±0.06	-44	41.61±2.13	-45
	10	22.81±2.72	14	23.84±2.89	-24	37.56±2.91	-31
	100	19.59±0.29	26	21.19±1.79	-10	2.82±0.44	91
120	0	115.21±4.18	0	85.31±4.15	0	110.70±5.30	0
	0.1	114.41±1.24	1	98.23±3.04	-15	104.46±5.36	6
	1	111.97±4.67	3	126.63±4.30	-48	96.19±2.88	14
	10	95.44±5.90	18	109.91±7.47	-28	92.83±5.60	17
	100	87.83±4.50	24	96.89±4.89	-13	3.08±0.52	98

[a]Mean values from four replications ± standard errors of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

Table II-2: The effect of CGA-92194, cyometrinil and flurazole on the incorporation of ^{14}C-leucine into isolated soybean cells.

Incubation time (min)	Safener Concentration (μM)	CGA-92194 [^{14}C]-leucine incorporated[a] (dpm x 10^{-3}/100 μg chl)	CGA-92194 Inhibition[b] (%)	Cyometrinil [^{14}C]-leucine incorporated[a] (dpm x 10^{-3}/100 μg chl)	Cyometrinil Inhibition[b] (%)	Flurazole [^{14}C]-leucine incorporated[a] (dpm x 10^{-3}/100 μg chl)	Flurazole Inhibition[b] (%)
30	0	5.02±0.95	0	4.87±0.66	0	4.17±0.37	0
	0.1	4.66±0.55	8	5.17±0.70	-6	4.72±0.24	-13
	1	5.02±0.59	0	5.84±1.16	-19	4.81±0.39	-15
	10	4.21±0.94	17	5.49±1.01	-12	3.56±0.22	15
	100	3.90±0.39	23	4.46±0.71	9	1.51±0.16	64
60	0	7.83±1.17	0	7.93±1.42	0	6.58±0.33	0
	0.1	8.45±0.77	-7	8.63±0.83	-8	7.62±1.69	-15
	1	7.15±0.92	9	9.46±0.95	-19	7.22±1.31	-9
	10	7.63±0.44	3	8.52±1.19	-7	5.37±0.58	19
	100	5.27±0.67	33	7.73±1.21	3	1.41±0.07	79
120	0	13.60±0.69	0	15.84±0.86	0	12.12±1.56	0
	0.1	14.57±1.26	-7	16.96±1.69	-7	15.03±2.10	-24
	1	14.13±2.20	-3	16.50±0.69	-4	13.33±1.26	-9
	10	13.29±1.78	3	16.92±1.20	-6	9.60±1.47	21
	100	7.21±1.48	47	12.87±1.10	19	1.97±0.16	84

[a]Mean values from four replications ± standard errors of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

Table II.3: The effect of CGA-92194, cyometrinil and flurazole on the incorporation of ^{14}C-uracil into isolated soybean cells.

Incubation time (min)	Safener Concentration (μM)	CGA-92194		Cyometrinil		Flurazole	
		[^{14}C]-uracil incorporated[a] (dpm x 10^{-3}/100 μg chl)	Inhibition[b] (%)	[^{14}C]-uracil incorporated[a] (dpm x 10^{-3}/100 μg chl)	Inhibition[b] (%)	[^{14}C]-uracil incorporated[a] (dpm x 10^{-3}/100 μg chl)	Inhibition[b] (%)
30	0	0.76±0.07	0	0.83±0.01	0	0.83±0.01	0
	0.1	0.94±0.01	-23	1.00±0.08	-9	0.94±0.01	-13
	1	0.99±0.01	-30	0.96±0.10	-15	0.89±0.01	-7
	10	0.77±0.10	-1	0.91±0.12	-9	0.80±0.01	4
	100	0.81±0.10	-6	0.91±0.04	-9	0.77±0.02	8
60	0	0.99±0.07	0	0.99±0.06	0	0.99±0.06	0
	0.1	1.11±0.11	-12	1.21±0.13	-22	0.93±0.03	7
	1	1.03±0.02	-4	1.17±0.04	-18	1.04±0.03	-5
	10	0.97±0.55	2	1.18±0.19	-19	1.01±0.06	-2
	100	0.85±0.07	14	0.94±0.06	6	0.71±0.12	29
120	0	1.54±0.61	0	1.73±0.61	0	1.73±0.61	0
	0.1	1.56±0.57	-1	1.44±0.38	17	1.39±0.10	-20
	1	1.29±0.20	17	1.35±0.04	22	1.11±0.01	36
	10	1.13±0.03	27	1.26±0.05	28	1.23±0.01	29
	100	1.10±0.06	29	1.18±0.04	32	0.72±0.01	59

[a]Mean values from four replications ± standard errors of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

Table II.4: The effect of CGA-92194, cyometrinil and flurazole on the incorporation of ^{3}H-thymidine into isolated soybean cells.

Incubation time (min)	Safener Concentration (μM)	CGA-92194 [^{3}H]-thymidine incorporated[a] (dpm x 10^{-3}/100 mg chl)	CGA-92194 Inhibition[b] (%)	Cyometrinil [^{3}H]-thymidine incorporated[a] (dpm x 10^{-3}/100 mg chl)	Cyometrinil Inhibition[b] (%)	Flurazole [^{3}H]-thymidine incorporated[a] (dpm x 10^{-3}/100 mg chl)	Flurazole Inhibition[b] (%)
120	0	3.93±0.47	0	3.93±0.47	0	4.05±0.79	0
	0.1	4.32±0.38	-9	3.72±0.02	6	4.96±0.78	-22
	1	3.91±0.55	1	3.61±0.19	9	2.41±0.73	41
	10	3.81±0.34	4	3.34±0.30	16	1.41±0.14	66
	100	3.27±0.30	17	3.07±0.26	24	0.25±0.07	94

[a]Mean values from four replications ± standard errors of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

Table II.5: The effect of CGA-92194, cyometrinil and flurazole on the incorporation of ^{14}C-acetate into isolated soybean cells.

Incubation time (min)	Safener Concentration (μM)	CGA-92194 [^{14}C]-acetate incorporated[a] (dpm x 10^{-3}/100 μg chl)	CGA-92194 Inhibition[b] (%)	Cyometrinil [^{14}C]-acetate incorporated[a] (dpm x 10^{-3}/100 μg chl)	Cyometrinil Inhibition[b] (%)	Flurazole [^{14}C]-acetate incorporated[a] (dpm x 10^{-3}/100 μg chl)	Flurazole Inhibition[b] (%)
30	0	212.34± 4.81	0	204.82± 3.24	0	220.92±0.08	0
	0.1	247.56± 0.23	-16	219.36± 4.55	-7	244.65±4.03	-10
	1	261.10± 6.40	-23	241.14± 4.76	-17	265.37±4.85	-20
	10	270.16± 7.69	-27	221.20± 5.55	-7	197.85±4.11	11
	100	182.89± 0.04	14	179.80± 5.26	13	27.21±1.65	88
60	0	278.62± 7.40	0	313.28± 7.25	0	317.96±7.95	0
	0.1	363.99± 7.90	-30	288.56± 7.82	8	369.95±8.40	-16
	1	391.20± 8.90	-40	370.16± 6.95	-18	391.52±9.30	-23
	10	418.91±10.50	-50	434.96± 3.80	-38	301.72±6.85	6
	100	293.11± 9.20	-5	287.94± 6.75	9	31.01±4.40	91
120	0	509.77± 2.34	0	494.57±10.43	0	638.70±13.55	0
	0.1	717.48±10.45	-40	507.51± 6.10	-2	731.34±16.68	-14
	1	687.42± 4.58	-34	666.83±13.29	-34	717.80±8.83	-12
	10	663.08±13.56	-30	579.64±12.10	-17	495.58±9.31	23
	100	517.77± 9.38	-1	441.38± 5.57	11	37.43±5.61	95

[a]Mean values from four replications ± standard errors of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

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CHAPTER III

INTERACTIONS BETWEEN THE HERBICIDE METOLACHLOR AND THE SAFENER CGA-92194 AT THE LEVELS OF UPTAKE AND MACROMOLECULAR SYNTHESIS IN SORGHUM

ABSTRACT. The interactions between the herbicide metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide] and the safener CGA-92194 {α-[1,3-dioxolan-2-yl-methoxy)imino]benzeneacetonitrile} at the levels of herbicide uptake and macromolecular synthesis were examined in enzymatically isolated leaf mesophyll protoplasts of grain sorghum [*Sorghum bicolor* (L.) Moench, cv G623]. When the two compounds were given simultaneously, CGA-92194 enhanced the uptake of [^{14}C]-metolachlor into sorghum protoplasts measured for 1 h, indicating that competition for uptake is not involved in the protective action of this safener. The safener-induced stimulation of metolachlor uptake was concentration-dependent and at 100 μM, CGA-92194 nearly doubled the uptake of this herbicide into sorghum protoplasts. Following 4 h of incubation, individual or combined treatments with metolachlor and CGA-92194 did not inhibit the incorporation of [^{14}C]-leucine into sorghum protoplast proteins. Treatments with metolachlor and CGA-92194 in combination, inhibited the incorporation of [^{14}C]-uracil, [^{3}H]-thymidine, and [^{14}C]-acetate into sorghum protoplast macromolecules less than metolachlor given alone, suggesting the potential involvement of a competitive antagonism in the protective action of CGA-92194. Although significant, the antagonism of metolachlor effects on nucleic acid and lipid synthesis of sorghum by CGA-92194 did not appear to be sufficient to account for the

entire mode of action of this safener. The metabolic activity of sorghum protoplasts isolated from plants grown from seeds pretreated with the safener was significantly lower than that of unsafened protoplasts. At present, it is unclear whether this safener-induced reduction in the metabolic activity of pretreated sorghum seedlings could be involved in the protective action of CGA-92194 against metolachlor injury to grain sorghum.

INTRODUCTION

In recent years, several oxime ether derivatives have been reported as effective safeners of grain sorghum against chloroacetamide herbicide injury (5, 7, 11, 17). In field tests, cyometrinil {(Z)-α[(cyanomethoxy) imino]benzeneacetonitrile} and the structurally related compound CGA-92194 were found to be effective as seed dressings at rates of 0.5-2.0 g/kg of seed against metolachlor injury to many varieties of grain, sweet, and yellow-endosperm sorghum (7, 17, 11). However, because of the inherent phytotoxicity of cyometrinil to several grain sorghum varieties, only CGA-92194 is marketed commercially as a safener for grain sorghum under the trade name CONCEP II® (11).

The mechanism(s) by which CGA-92194 protects grain sorghum against metolachlor injury is (are) not known. Extensive research on the mechanisms of the protective action of other classes of herbicide safeners such as the dichloroacetamides and NA (naphthalene-1,8-dicarboxylic anhydride) has resulted in several hypotheses suggesting that a safener could confer protection by: (1) reducing herbicide uptake and/or translocation in the protected plant, (2) counteracting herbicide phytotoxicity through a competitive inhibition at a common target site within the protected plant, (3) stimulating herbicide degradation in the protected plant, and (4) a combination of all these mechanisms (25, 26, 41, 43).

Shoot absorption through the emerging coleoptile of grasses has been shown to be more important than root uptake following soil applications of

metolachlor or other chloroacetamide herbicides (2, 40). The meristematic region of grass shoots also appears to be the site of action of these herbicides and chloroacetamides are commonly classified as inhibitors of early shoot growth of germinating grass seedlings (2, 22). Symptoms of metolachlor injury to grain sorghum seedlings include leaf or shoot deformations such as leaf twisting or rolling and at high rates leaves fail to emerge from the coleoptile of treated seedlings (42). These effects of metolachlor are counteracted by CGA-92194 on sorghum grown from seeds dressed with this safener.

The biochemical mechanism of action of chloroacetamide herbicides is poorly understood. The shoot deformations and growth inhibitions induced by these compounds could result from their reported effects on cell division and cell enlargement in coleoptile tissues of treated grass plants (8, 9, 22). At the level of cellular metabolism, physiological and biochemical processes that are affected by these herbicides include protein synthesis (8, 10, 12, 28, 35, 38), transport and membrane function (4, 37), lipid synthesis (14, 15, 36, 44), gibberellin (GA) biosynthesis (46, 47, 48), and alkylation of thiol-containing macromolecules (22, 28, 34).

Reports on the potential interactions between metolachlor and the safeners CGA-92194 and cyometrinil at a common site on sorghum are limited. In studies with a number of sorghum tissues, cyometrinil has been reported to increase (32), decrease (13, 30) or have no effect (49) on metolachlor absorption. Ebert (13) demonstrated that the cyometrinil-induced reduction of metolachlor absorption by sorghum plants was indirect resulting from the

ability of the safener to prevent the loss of cuticular integrity caused by the herbicide metolachlor on sorghum leaves. In a recent study, CGA-92194 did not antagonize metolachlor by displacing it from potential binding sites on subcellular membrane fractions (22).

Cell suspensions of maize (Zea mays L.) were very useful in studying the rapid interactions between the thiocarbamate herbicide EPTC (S-ethyl diproplycarbamothioate) and the safener dichlormid (2,2-dichloro-N,N-di-2-propenylacetamide) at the levels of uptake and site of action (18, 19). In the present study, enzymatically isolated sorghum leaf protoplasts were used to investigate the potential influence of CGA-92194 on metolachlor uptake or on its effects on protein, ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and lipid syntheses of sorghum.

MATERIALS AND METHODS

PLANT MATERIAL. Grain sorghum [Sorghum bicolor (L.) Moench cv. G623] seeds, which were either protected or unprotected with the safener CGA-92194 were used for the isolation of leaf protoplasts used in the experiments of this study. Protected seeds which had been treated with 1.25 g ai/kg of seed of CGA-92194 and along with the unprotected (control) seeds were provided by Ciba-Geigy, Corp., Greensboro, North Carolina. Sorghum seeds were grown in plastic cups (473-ml) filled with a mixture of potting medium (Weblite Corporation, Blue Ridge, Virginia), vermiculite and sphagnum peat moss in a 2:2:1 ratio. Limestone and a controlled release fertilizer (14-14-14) were added to the soil mixture to supplement nutrient levels. Emerged seedlings were watered daily and grown in a chamber with

a photosynthetic flux density of 300 $\mu E.m^{-2}.s^{-1}$, 16 h of light at 30°C and 8 h of dark at 20°C. Sorghum seedlings were maintained for 9-10 days at which time they were used for protoplast isolation.

PROTOPLAST ISOLATION. Leaf mesophyll protoplasts of grain sorghum were isolated enzymatically by adapting a slight modification of the procedures of Edwards et al. (16) and Leegood et al. (31). An appropriate number of leaves from 9-10 day old sorghum seedlings grown as described earlier were detached, washed in distilled water, dried and had their midribs removed. The leaf tissue was then cut with a sharp razor blade into transverse segments of about 0.5 mm. Cut leaf tissue (3-5 g) was then infiltrated with digestion medium containing 2% (w/v) cellulase (CELLULYSIN®, Calbiochem, La Jolla, California), 0.5% (w/v) pectinase (MACERASE®, Calbiochem), 0.5 M sorbitol, 1 mM $CaCl_2$, 0.5% (w/v) Bovine Serum Albumin (BSA) and 5 mM MES buffer (pH 5.5). The infiltrated tissue immersed into the digestion medium was then placed in a large petri dish (13-cm dia.) and was incubated for 4-5 h at 30°C under low light in a shaking water bath. A layer of parafilm was used as a cover to reduce the light at the top of the petri dish and a slow agitation (40 oscillations/min) was used to improve the digestion of the leaf tissue. At the end of the incubation period, the undigested material (e.g., epidermal tissue and vascular strands) was removed by filtration through a 500 μm nylon sieve. The filtrate containing the released protoplasts and bundle sheath strands was then filtered through an 80 μm nylon net to remove the bundle sheath strands. This filtrate was centrifuged at 160 × g for 5 min and the supernatant was

removed by suction. The pellet was then resuspended gently in 5 ml of ice-cold purification medium containing 0.5 M sorbitol, 1 mM $CaCl_2$ and 5 mM MES-KOH (pH 6.0). At the top of this medium 1 ml of ice-cold medium Dextran containing 0.5 M sucrose, 1 mM $CaCl_2$, 5 mM MES-KOH (pH 6.0), and 8% (w/v) Dextran T_{40} was added according to the procedure of Edwards et al. (16). The suspension was then centrifuged at 220 x g for 5 min and the pure protoplasts at the interface of the two purification media were collected with a Pasteur pipet in a beaker of appropriate volume. The protoplast preparation was then diluted to the desired volume with ice-cold incubation medium containing 0.4 M sorbitol, 1 mM $MgCl_2$, 1 nM $MnCl_2$, 1 mM EDTA, 2 mM KH_2PO_4 and 50 mM MES-KOH (pH 6.0). The morphological integrity and purity of the isolated protoplasts was determined by light microscopic examination. Counting of the isolated protoplasts with a corpuscule counting chamber (Hauser Scientific, Blue Bell, Pennsylvania) showed that the average number of protoplasts used in the assays of the present study was 5.6×10^5 protoplasts per ml of incubation medium. In addition, the chlorophyll content of the protoplast preparation was determined according to the method of Arnon (1).

INFLUENCE OF CGA-92194 on [^{14}C]-METOLACHLOR UPTAKE. Uptake of radiolabeled metolachlor by leaf mesophyll protoplasts isolated from unsafened sorghum was examined in the presence or absence of the safener CGA-92194 according to the procedures of Ezra et al. (19) and McDaniel (33). Aliquots of 2 ml of protoplasts suspended in the incubation medium (described in the previous section) were placed in 25 ml Erlenmeyer flasks. Metolachlor and

CGA-92194 solutions were prepared by dissolving analytical grade samples (> 95% pure) in methanol and made up to the desired volume with distilled water so that the final methanol concentration was less than 1%. Control protoplasts had methanol added in the same concentration. Aliquots of 0.1 ml of [^{14}C]-metolachlor (phenyl labeled, 75.9 µCi/mg) containing 1 µCi of radioactivity and adjusted to 10 µM with nonlabeled metolachlor were added to the protoplast suspension. CGA-92194 was used at concentrations of 0.1, 1, 10, and 100 µM and was added to the assay mixture immediately after the addition of metolachlor. The assay mixtures were incubated at 30°C for 1 h in a shaking water bath at 60 oscillations per min. Following incubation, the reaction was stopped by the addition of 4 ml of incubation medium containing 100 µM of nonlabeled metolachlor. The protoplasts were then filtered into Whatman glass-fiber (GF/C) filters and washed three times with incubation medium that contained no metolachlor. The filters were dried at room temperature and counted for radioactivity after being placed into scintillation vials with 10 ml of scintillation fluid (Scinti-Verse E®, Fisher Scientific Co., Raleigh, North Carolina). Nonspecific adsorption of [^{14}C]-metolachlor onto protoplasts surface was estimated by determining the radioactivity of protoplasts collected on GF/C filters at the beginning of the incubation period (0 h). The value obtained for nonspecific adsorption was subtracted from the total counts to give a more accurate determination of the net uptake of [^{14}C]-metolachlor by sorghum protoplasts in the presence or absence of the safener CGA-92194. The results are expressed as dpm per 100 µg of chlorophyll and these values were also calculated as percent of the control.

INTERACTION EFFECTS OF METOLACHLOR AND CGA-92194 ON THE INCORPORATION OF RADIOLABELED PRECURSORS IN SORGHUM PROPOPLASTS ISOLATED FROM UNSAFENED SEEDLINGS. The potential interactive effects of metolachlor and the safener CGA-92194 on the incorporation of radiolabeled precursors into selected macromolecules of enzymatically isolated leaf mesophyll protoplasts of grain sorghum were studied according to published procedures (3, 28). A detailed description of these procedures is given in the Appendix. Metolachlor and CGA-92194 were tested at 1, 10, and 100 μM concentrations. The assaying medium for all metabolic studies contained 2 ml of protoplasts suspended in incubation medium (same as previous) in a 25-ml Erlenmeyer flask, 0.1 ml of the appropriate radioactive precursor containing 1 μCi of radioactivity, 0.05 ml of the herbicide, and 0.05 ml of the safener solutions making a final volume of 2.2 ml. Four metabolic processes were assayed. Protein synthesis was determined by measuring the incorporation of 1 μCi of L-[U-^{14}C]-leucine (sp. act. 276 mCi/mmol) into protein. RNA synthesis was assayed by measuring the incorporation of 1 μCi of [2-^{14}C]-uracil (sp. act. 55 mCi/mmol), while DNA synthesis was assayed with [^{3}H]-thymidine (sp. act. 25 Ci/mmol). Lipid synthesis was determined by the incorporation of 1 μCi of [1,2-^{14}C]-sodium acetate (sp. act. 56.2 mCi/mmol) into lipids. Flasks with the assay mixtures were sealed and placed in a shaking water bath at 30°C and illuminated from above with a combination of incandescent and fluorescent lamps supplying 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ photon flux density at the levels of the flasks. The assay mixtures were incubated for 4 h. At the end of the incubation period, samples were collected and treated accordingly for each metabolic process as previously

described (3, 28). Radioactivity was determined by adding 10 ml of liquid scintillation fluid (ScintiVerse E,® Fisher) and counting in a Beckman LS-250 scintillation spectrometer. Protein, RNA, and lipid syntheses were expressed as dpm of the [^{14}C] from the respective radiolabeled precursor incorporated into the protoplasts per 100 µg of chlorophyll. DNA synthesis was expressed as dpm of [^{3}H] incorporated into the protoplasts per mg of chlorophyll.

STATISTICAL ANALYSIS. Data presented are the means of two experiments with two replications per experiment. The data were analyzed for variance in a four-by-four (metolachlor × safener) factorial experiment in a completely randomized design. Statistically significant interactions for each combination treatment of metolachlor and CGA-92194 were identified by the use of F tests for a two-by-two comparison of that treatment with the control and the separate levels of metolachlor and CGA-92194 involved, as had been previously described (25, 39). In addition, the expected responses for each combination treatment were calculated assuming no interactions (multiplicative model) by using the method of Colby (6) as described by Hatzios and Penner (25). For example in Table III.2, the expected response of sorghum protoplasts to the combined treatment of metolachlor at 1 µM and CGA-92194 at 1 µM was calculated as follows: (144 × 142)/132 = 154. In Table III.2 through III.5 the expected responses for each treatment combination are given in parentheses. These expected responses were then compared to the observed responses for each treatment combination (25). When an observed response is greater than its respective

expected response the interaction is antagonistic. When an observed value is less that its respective expected value, synergism is indicated. When observed and expected values are similar, the combined effect is described as additive. In the ensuing discussion, differences between observed and expected responses for each treatment combination were viewed as biologically important only when the observed response for a given treatment combination was significant by the F-test. When the observed responses for a treatment combination were not significant by the F-test the interaction was characterized as additive.

EFFECT OF METOLACHLOR ON SELECTED METABOLIC PROCESSES OF SORGHUM LEAF MESOPHYLL PROTOPLASTS ISOLATED FROM SAFENED AND UNSAFENED SEEDLINGS. In these studies, the effects of metolachlor on the incorporation of radio-labeled precursors into macromolecules were studied in sorghum leaf mesophyll protoplasts isolated from plants grown from seeds either pretreated or untreated with the safener CGA-92194. Protoplast isolation and assaying of protein, nucleic acid and lipid syntheses were conducted as previously described. Data presented are the means of two experiments with three replications per experiment. These data were analyzed for variance and the standard errors of each mean were calculated. The results are expressed as dpm per 100 µg of chlorophyll and as percent inhibition caused by each concentration of metolachlor. Metolachlor was again tested at 1, 10 and 100 µM concentrations. In addition, biomass measurements (shoot fresh weight and height) of seedlings grown from sorghum seeds safened or unsafened with the safener CGA-92194 were conducted to complement this

study. The seedlings were grown in a growth chamber as described earlier and the two parameters were measured at 10 and 20 days after planting.

RESULTS AND DISCUSSION

EFFECTS OF CGA-92194 ON UPTAKE OF METOLACHLOR. The effects of CGA-92194 on the uptake of [^{14}C]-metolachlor into sorghum leaf protoplasts are presented in Table III.1. The data show a concentration-dependent stimulation of metolachlor uptake by the safener following an 1 h incubation period. At 100 μM, CGA-92194 nearly doubled the uptake of [^{14}C]-metolachlor by the isolated leaf mesophyll protoplasts of sorghum. A time-dependent stimulation of metolachlor uptake into sorghum shoots excised from seedlings grown from seeds pretreated with the safener CGA-92194 has been reported recently by Fuerst and Gronwald (22). Cyometrinil, a structural analog of the safener CGA-92194, has also been reported to stimulate the absorption of metolachlor by sorghum seedlings (30). In addition, a cyometrinil-induced rapid stimulation of [^{14}C]-EPTC uptake into maize cells has been reported (19), although cyometrinil is ineffective as a safener of corn against thiocarbamate herbicide injury (40). These results show that a safener-induced reduction of herbicide uptake does not appear to be a mechanism involved in the protective action of CGA-92194 against metolachlor injury to grain sorghum. The physiological significance of a safener-induced stimulation of herbicide intake in protected plants is not known. It has been suggested that enhanced uptake of [^{14}C]-metolachlor in CGA-92194-treated seedlings of grain sorghum could be the result of the safener-induced stimulation of herbicide metabolism (22).

INTERACTIVE EFFECTS OF METOLACHLOR AND CGA-92194 ON THE INCORPORATION OF RADIOLABELED PRECURSORS IN SORGHUM PROTOPLASTS ISOLATED FROM UNSAFENED SEEDLINGS. Independent and combined effects of metolachlor and CGA-92194 on selected metabolic processes of sorghum leaf mesophyll protoplasts isolated from unsafened seedlings were examined to determine whether a competitive antagonism of metolachlor effects by CGA-92194 may be involved in the protective action of this safener. Toxicity was measured by the inhibition of the incorporation of radioactive precursors into cellular macromolecules such as proteins, nucleic acids and lipids (Tables III.2-III.5), since metolachlor has been reported to inhibit the biosynthesis of these macromolecules in treated plants (4, 8, 9, 10, 12, 14, 15, 28, 35, 36, 37, 38, 44). Data in Table III.2 show that metolachlor and CGA-92194 did not inhibit the incorporation of [^{14}C]-leucine into sorghum leaf mesophyll protoplasts after 4 h of incubation. Deal et al. (8) reported that at 100 µM, metolachlor and other chloroacetamide herbicides inhibited the *in vitro* incorporation of radiolabeled leucine into proteins of treated oats. However, the incubation periods (>12 h) used in their study were greater than the one used in the present study. Comparisons of the observed and expected values for each treatment combination of metolachlor and CGA-92194 and use of F-tests showed that the combined effects of metolachlor and CGA-92194 on the incorporation of [^{14}C]-leucine into protoplasts were generally additive (Table III.2). A slight synergism was observed only when 1 µM of metolachlor was combined with 1 µM of the safener. However, the magnitude of this interaction and

the rest of the data in Table III.2 indicate that within the time limits of the present study, a competitive antagonism of metolachlor effects on protein synthesis by CGA-92194 is not likely to contribute to the protective action of this safener.

The effects of metolachlor and CGA-92194 on the incorporation of radioactive precursors into nucleic acids are outlined in Tables III.3 and III.4. Comparisons between the control and metolachlor-treated or safener-treated mean observed responses show that individual treatments with metolachlor of CGA-92194 at 10 and 100 μM substantially reduced the incorporation of [^{14}C]-uracil into sorghum protoplasts following a 4 h incubation (Table III.3). At 1 μM both metolachlor and the safener were stimulatory of this process. Comparisons of the observed and expected responses for each combination treatment and use of the F-tests revealed that selected combinations of 10 and 100 μM of the safener with 10 and 100 μM of the herbicide were antagonistic (Table III.3). In all other cases, combined treatments were additive. Data in Table III.4 show that individual treatments with all concentrations of metolachlor inhibited the incorporation of [^{3}H]-thymidine into sorghum protoplasts within 4 h. Individual treatments with the safener were inhibitory of this process only at the highest concentration of 100 μM. In most cases, combined treatments with the herbicide and the safener were indicative of antagonistic interactions. However, use of the F-tests revealed that such interactions were statistically significant only when 100 μM of the safener were combined with 10 and 100 μM of the herbicide (Table III.4). The potential involvement of cell division and ell enlargement inhibitions in the

mechanism of action of α-chloroacetamide herbicides such as metolachlor has been suggested (3, 9). The inhibitory effects of these herbicides, however, on nucleic acid biosynthesis are poorly understood. In early studies on the mechanism of action of chloroacetamide herbicides, Jaworski (28) suggested an indirect effect of these herbicides on RNA synthesis and function preceding their well-documented effects on protein synthesis. He proposed that alkylation of specific mRNAs could be involved as a step in the inhibition of protein synthesis by α-chloroacetamide herbicides. Inhibition of nucleic acid synthesis by metolachlor and significant antagonism of these effects of metolachlor by the safener CGA-92194 have been reported recently by Zama and Hatzios (50) in studies with isolated soybean leaf cells. Although possible, the potential involvement of a competitive antagonism of metolachlor effects on nucleic acid synthesis in the protective action of the safener CGA-92194 is presently inconclusive and needs to be studied in more detail.

The incorporation of [^{14}C]-acetate into sorghum protoplasts was strongly inhibited by all concentrations of individually applied metolachlor or CGA-92194 within 4 h of incubation (Table III.5). A strong inhibition of lipid synthesis as measured by the incorporation of radioactive precursors into plant lipid fractions has been reported by several investigators in studies with metolachlor or other chloroacetamide herbicides (14, 15, 36, 44). It is not clear, however, whether these inhibitions might be related to the inhibitory effects of these herbicides on membrane function and protein synthesis rather than being direct effects on lipid biosynthesis (22). All combinations of metolachlor and CGA-92194

were significantly antagonistic (Table III.5). These data indicate that lipid synthesis might be a potential common site within the protected plant where a competitive antagonism between the herbicide metolachlor and the safener CGA-92194 may occur. Similar conclusions have been reported by Ebert and Ramsteiner (15) who showed that the structurally related safener cyometrinil antagonized the effects of metolachlor on epicuticular wax deposition on grain sorghum leaf surfaces.

EFFECTS OF METOLACHLOR ON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO SORGHUM PROTOPLASTS ISOLATED FROM CGA-92194 SAFENED AND UNSAFENED SEEDLINGS.

In this set of experiments, the impact of pretreating sorghum seeds with the safener CGA-92194 on the subsequent effects of metolachlor on the incorporation of radioactive precursors into cellular macromolecules of isolated leaf mesophyll protoplasts was examined. The results of these studies are outlined in Tables III.6 through III.9.

Following 4 h of incubation, metolachlor at all concentrations slightly inhibited the incorporation of [^{14}C]-leucine into protoplasts isolated from both safened or unsafened seedlings (Table III.6). However, only the inhibition induced by the highest concentration of metolachlor (100 μM) appeared to be significant as evidenced by comparisons between the mean responses of the control and the metolachlor-treated protoplasts. Thus, again, within the time limit of this study, protein synthesis did not appear to be a potential site for a competitive antagonism between the herbicide and the safener CGA-92194.

Metolachlor, at any concentration examined, did not inhibit the incorporation of [^{14}C]-uracil into sorghum protoplasts isolated from

safened or unsafened seedlings (Table III.7). A slight stimulation of this process by the lower concentrations of metolachlor was evident in both situations. Data in Table III.8 show that metolachlor inhibited the incorporation of [^{3}H]-thymidine into sorghum protoplasts only at the highest concentration of 100 μM and only in protoplasts isolated from unsafened seedlings. Metolachlor did not inhibit this process in safened protoplasts at any concentration examined (Table III.8).

A concentration-dependent effect of metolachlor on the incorporation of [^{14}C]-acetate into sorghum protoplasts isolated from unsafened seedlings is evident from data presented in Table III.9. The effect of metolachlor on the same process of protoplasts isolated from safened seedlings was less pronounced (Table III.9). Thus, data in Tables III.8 and III.9 indicate that DNA synthesis and lipid synthesis could be potential sites for a competitive antagonism between the herbicide metolachlor and the safener CGA-92194.

Comparison of the data presented in Tables III.3, III.4, and III.5 with those presented in Tables III.7, III.8, and III.9 shows that the level of incorporation of the radioactive precursors into isolated sorghum protoplasts used in the second set of experiments (Tables III.7, III.8, and III.9) was markedly reduced. Although this is difficult to explain, variations in the metabolic activity of isolated plant cells or protoplasts are not uncommon and have been observed by other investigators (23, 48). In fact, protoplast isolation is considered an "art" as well as "science" (21).

An additional observation that becomes evident from comparisons of the data presented in Tables III.6, III.7, III.8, and III.9 is that the control values for the incorporataion of the radioactive precursors into protoplasts isolated from seedlings pretreated with the safener CGA-92194 are significantly lower than those obtained with unsafened protoplasts. In most cases, the metabolic activity of protoplasts from unsafened seedlings appeared to be two-fold greater than that of the safened protoplasts (Tables III.7, III.8, and III.9). To further substantiate this observation, shoot fresh weights and heights of seedlings grown from CGA-92194-safened or unsafened seeds were determined at 10 and 20 days after planting. Data in Table III.10 show that the fresh weights and heights of seedlings grown from unsafened seeds were greater than those of the safened seedlings. A reduction of the viability of sweet and yellow endosperm sorghum seeds treated with the oxime ether safeners cyometrinil and CGA-92194 have been reported by other investigators (7, 11). In addition, cyometrinil was found to inhibit the germination of grain sorghum seeds when used at rates higher than 1.88 g/kg of seeds (7, 11). At a physiological level, CGA-92194 and cyometrinil were shown to interfere with respiration during the early stages of seed germination of grain sorghum (3). A cyometrinil-induced reduction of grain sorghum seed germination has been reported also by Warmund et al. (45). Observations on the reduction of metabolic activity of grain sorghum seedlings treated with oxime ether safeners have also been made by Dr. Ebert of Ciba-Geigy Corp., Basel Switzerland (personal communication). Whether this decrease in metabolic activity of grain sorghum seedlings induced by the safener

CGA-92194 is involved in its protective action is not known at the present time. It is conceivable that the reduced metabolic activity of safened sorghum could render these seedlings less susceptible to subsequent treatments with the herbicide metolachlor. However, future research would be needed to further substantiate this postulation and clarify its physiological significance in the protective action of the safener CGA-92194.

Table III.1: The effect of CGA-92194 on the uptake of [^{14}C]-metolachlor into sorghum leaf protoplasts after 1 h of incubation.

CGA-92194 concentration (μM)	Uptake of [^{14}C]-metolachlor (dpm x 10^{-3}/100 μg Chl)	Percent of control
0	71 ± 5	100
0.1	75 ± 1	105
1	77 ± 5	108
10	86 ± 3	121
100	141 ± 10	198

[a]Mean values from 6 replications ± standard errors of each mean.

Table III.2: Effects of metolachlor and CGA-92194 on [^{14}C]-leucine incorporation into sorghum leaf protoplasts after 4 h of incubation

Metolachlor concentration (μM)	Type of response	CGA-92194, (μM) 0	1	10	100
Metolachlor concentration	Type of response	[^{14}C]-leucine incorporated[a] (dpm x 10^{-3}/100 μg chl)			
0	observed	132±21	142±11	140±30	116±25
1	observed	144±29	130*±30	136±31	130±31
	expected		(154)	(153)	(127)
10	observed	132±31	131±20	136±15	127±19
	expected		(142)	(140)	(116)
100	observed	130±25	123±29	128±10	124±19
	expected		(140)	(138)	(115)

[a]Mean values from four replications ± standard error of each mean. Expected responses for combinations as calculated by the Colby (45) method are shown in parentheses below each observed response. Asterisks indicate significant interactions at the 5% (*) and 1% (**) levels of probability as determined by F-values for each treatment combination (see Materials and Methods).

Table III.3: Effects of metolachlor and CGA-92194 on [^{14}C]-uracil incorporation of sorghum leaf protoplasts after 4 h of incubation

		CGA-92194, (μM)			
		0	1	10	100
Metolachlor concentration (μM)	Type of response	[^{14}C]-uracil incorporated[a] (dpm x 10^{-3} μg Chl)			
0	observed	607±60	717±69	407±35	214±34
1	observed	623±44	762±71	555±12	348±41
	expected		(736)	(417)	(219)
10	observed	473±35	576±57	460*±35	265*±39
	expected		(559)	(317)	(166)
100	observed	336±13	578±55	514**±35	218**±37
	expected		(396)	(225)	(118)

[a]Mean values from four replications ± standard error of each mean. Expected responses for combinations as calculated by the Colby (45) method are shown in parentheses below each observed response. Asterisks indicate significant interactions at the 5% (*) and 1% (**) levels of probability as determined by F-values for each treatment combination (see Materials and Methods).

Table III.4: Effects of metolachlor and CGA-92194 on [^{3}H]-thymidine incorporation into sorghum leaf protoplsts after 4 h of incubation

		CGA-92194, (μM)			
		0	1	10	100
Metolachlor concentration (μM)	Type of response	[^{14}C]-thymidine incorporated[a] (dpm x 10^{-3}/mg Chl)			
0	observed	91±7	82±9	83±5	63±8
1	observed	62±6	82±7	67±5	59±4
	expected		(56)	(56)	(43)
10	observed	62±5	58±5	77±4	69*±6
	expected		(56)	(57)	(43)
100	observed	51±7	63±11	70±9	106**±26*
	expected				

[a]Mean values from four replications ± standard error of each mean. Expected responses for combinations as calculated by the Colby (45) method are shown in parentheses below each observed response. Asterisks indicate significant interactions at the 5% (*) and 1% (**) levels of probability as determined by F-values for each treatment combination (see Materials and Methods).

Table III.5: Effects of metolachlor and CGA-92194 on [^{14}]-acetate incorporation of sorghum leaf protoplasts after 4 h of incubation

		CGA-92194, (μM)			
		0	1	10	100
Metolachlor concentration (μM)	Type of response	[^{14}C]-acetate incorporated[a] (dpm x 10^{-3}/100 μg Chl)			
0	observed	136±17	29±3	49±6	30±2
1	observed	36±4	36**±5	26**±1	26**±2
	expected		(7)	(13)	(8)
10	observed	30±4	45**±6	37**±3	40**±6
	expected		(6)	(11)	(7)
100	observed	32±3	41**±6	24**±3	27**±1
	expected		(7)	(11)	(7)

[a]Mean values from four replications ± standard error of each mean. Expected responses for combinations as calculated by the Colby (45) method are shown in parentheses below each observed response. Asterisks indicate significant interactions at the 1% level of probability as determined by F-values for each treatment combination (see Materials and Methods).

Table III.6: Influence of seed pretreatment with CGA-92194 on metolachlor effects on [^{14}C]-leucine incorporation into sorghum leaf protoplasts 4 h after incubation

Metolachlor concentration (µM)	Untreated sorghum		Treated sorghum	
	[^{14}C]-leucine incorporation[a] (dpm x 10^{-3}/100 µg Chl)	Inhibition (%)	[^{14}C]-leucine incorporation[a] (dpm x 10^{-3}/100 µg Chl)	Inhibition (%)
0	123±36	0	115±27	0
1	110±28	11	91±19	21
10	104±25	16	86±15	26
100	98±21	21	76±8	34

[a]Mean values from six replications ± standard error of each mean.

Table III.7: Influence of seed pretreatment with CGA-92194 on metolachlor effects on [^{14}C]-Uracil incorporation into sorghum leaf protoplasts after 4 h of incubation

Metolachlor concentration (µM)	Untreated sorghum		Treated sorghum	
	[^{14}C]-Uracil incorporation[a] (dpm x 10^{-3}/100 µg Chl)	Inhibition[b] (%)	[^{14}C]-Uracil incorporation[a] (dpm x 10^{-3}/100 µg Chl)	Inhibition[b] (%)
0	31±2	0	12±1	0
1	39±5	-25	15±2	-25
10	33±2	-6	14±3	-16
100	32±3	-3	11±1	9

[a]Mean values from six replications ± standard error of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

Table III.8: Influence of seed pretreatment with CGA-92194 on metolachlor effects on [^{3}H]-thymidine into sorghum leaf protoplasts after 4 h of incubation

Metolachlor concentration (μM)	Untreated sorghum		Treated sorghum	
	[^{14}C]-thymidine incorporation[a] (dpm x 10^{-3}/mg Chl)	Inhibition[b] (%)	[^{14}C]-thymidine incorporation[a] (dpm x 10^{-3}/mg Chl)	Inhibition (%)
0	20±2	0	10±1	0
1	24±3	-20	10±1	1
10	22±2	-10	10±2	1
100	16±1	20	9±1	10

[a]Mean values from six replications ± standard error of each mean.

[b]A minus (-) sign preceeding a percentage value indicates stimulation instead of inhibition.

Table III.9: Influence of seed pretreatment with CGA-92194 on metolachlor effects on [^{14}C]-acetate incorporation into sorghum leaf protoplasts after 4 h of incubation

Metolachlor concentration (µM)	Untreated sorghum		Treated sorghum	
	[^{14}C]-acetate incorporation[a] (dpm x 10^{-3}/100 µg Chl)	Inhibition (%)	[^{14}C]-acetate incorporation[a] (dpm x 10^{-3}/100 µg Chl)	Inhibition (%)
0	27±3	0	16±2	0
1	23±3	15	15±2	7
10	21±1	23	12±1	25
100	12±1	56	12±2	25

[a]Mean values from six replications ± standard error of each mean.

Table III.10: Growth responses of sorghum seedlings grown from seeds pretreated with CGA-92194 or untreated[a]

Time after planting (days)	Growth parameter	Sorghum seedlings		
		Untreated (A)	CGA-92194-treated (B)	Ratio (A/B)
10	Shoot fresh wt (g)	0.34 ± 0.01	0.32 ± 0.001	1.06
	Shoot ht (cm)	12.3 ± 0.18	10.9 ± 0.25	1.13
20	Shoot fresh wt (g)	1.68 ± 0.09	1.41 ± 0.07	1.19
	Shoot ht (cm)	40.96 ± 0.07	35.95 ± 0.95	1.14

[a]Mean values from ten replicates ± standard error of each mean.

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CHAPTER IV

EFFECTS OF THE HERBICIDE SAFENER CGA-92194 ON THE CHEMICAL REACTIVITY OF METOLACHLOR WITH GLUTATHIONE AND ITS METABOLISM BY GRAIN SORGHUM SEEDLINGS

ABSTRACT. In laboratory experiments, the safener CGA-92194 {α-[(1,3-dioxolan-2-yl-methoxy)imino]benzeneacetonitrile} increased the *in vitro* chemical reactivity of the herbicide metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide] for glutathione (GSH) in a concentration-dependent pattern. Pretreatment of sorghum [*Sorghum bicolor* (L.) Moench, cv. G623] seeds with the safener CGA-92194 (1.25 g ai/kg of seeds) enhanced significantly the amount of [^{14}C]-metolachlor absorbed by the roots and translocated to the shoots and leaves of grain sorghum seedlings grown hydroponically in a nutrient solution. Thin layer chromatographic (TLC) analysis of methanol-soluble extracts from roots and shoots or leaves of sorghum seedlings treated with [^{14}C]-metolachlor revealed the detection of 8 to 10 metabolites of this herbicide. One of the major metabolites detected was tentatively identified as the conjugate of metolachlor with GSH. A safener-induced stimulation of metolachlor metabolism *via* conjugation to GSH was observed in sorghum seedlings grown from seeds pretreated with CGA-92194. Addition of tridiphane [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane], a potent inhibitor of plant GSH-*S*-transferase enzymes, to the nutrient solution of hydroponically grown sorghum seedlings reduced the stimulatory effect of the safener CGA-92194 on the metabolism of metolachlor *via* conjugation to GSH. It is suggested that the safener CGA-92194 may protect grain sorghum against injury from the herbicide metolachlor by stimulating the spontaneous and enzymatic conjugation of this herbicide with GSH.

INTRODUCTION

The selectivity of chloroacetanilide herbicides metolachlor and alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide] on some crops such as grain sorghum is marginal. Crop damage is commonly observed under field conditions and injury symptoms include shoot deformations and leaf rolling or twisting (1, 15, 31). The tolerance of grain sorghum to the chloroacetanilide herbicides can be enhanced chemically with the use of herbicide safeners (7, 8, 10, 33, 34). Pretreatment of grain sorghum seeds with the safeners flurazole [phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate] and CGA-92194 is known to increase the tolerance of this crop to high rates of the herbicides alachlor and metolachlor (3, 33, 34).

The exact mechanisms of the protective action of herbicide safeners are not known (16). One of the most popular hypotheses proposed suggests that a safener may protect grass crops against herbicide injury by stimulating the degradation of a given herbicide in the protected plant (16, 32).

The degradation of chloroacetanilide herbicides in higher plants is poorly understood at the present time. A number of *in vivo* and *in vitro* studies have demonstrated the importance of glutathione (GSH) conjugation in the deactivation of these herbicides (2, 11, 17, 24, 25, 26, 27, 30). Glutathione-S-transferase enzymes mediating the conjugation of chloroacetanilide herbicides to GSH are constitutively present in tolerant plants and GSH conjugation is considered the major factor contributing to the selectivity of these herbicides (30). Other biotransformations that

could possibly be involved in the metabolism of the chloroacetanilide herbicides by higher plants include aryl hydroxylation, catabolism of the GSH conjugate to its cysteine derivative or direct conjugation of the parent molecule to cysteine, and conjugation of the catabolized GSH conjugate to glucuronide (11, 17, 20, 24).

The protective mechanism of several herbicide safeners including flurazole and cyometrinil against alachlor injury to corn has been attributed, at least partially, to their ability to stimulate the activity or induce the de novo synthesis of GSH-S-transferase enzymes that conjugate alachlor to GSH (30). Recently, Fuerst and Gronwald (13) showed that the safening action of CGA-92194 and of other safeners against metolachlor injury to grain sorghum may be the result of a safener-induced metabolism of this herbicide via GSH conjugation. In another study (13), pretreatment of corn seedlings with the chloroacetamide herbicide CDAA (2-chloro-N,N-di-2-propenylacetamide) increased the GSH content and GSH-S-transferase activity of corn roots conferring a degree of tolerance to corn against subsequent applications of the herbicide. The proposal, however, that oxime ether safeners such as CGA-92194 and cyometrinil protect corn or grain sorghum against metolachlor injury by an enhancement of herbicide metabolism has been questioned by some investigators who failed to observe such effects in their studies with both safeners (22, 28, 35).

Tridiphane is a new herbicide being developed as a synergist of atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine] for the postemergence control of grass weeds such as giant foxtail (Setaria

faberi Herrm.) and large crabgrass [Digitaria sanguinalis (L.) Scop.] (12). Tridiphane has been shown to be a potent inhibitor of the enzymatic conjugation of atrazine with GSH (23, 36, 37, 38). Recently, Ezra et al. (12) examined the potential of tridiphane in synergizing the herbicidal activity of other herbicides known to be detoxified via enzymatic conjugation to GSH. They showed that tridiphane was an effective synergist of alachlor and improved significantly the control of proso millet (Panicum miliaceum L.) in corn by this herbicide.

The objectives of this research were to: (a) examine the potential effect of CGA-92194 on the chemical reactivity of [^{14}C]-metolachlor with [^{3}H]-glutathione under in vitro conditions; (b) study the degradation of [^{14}C]-metolachlor in sorghum seedlings grown from seeds safened or unsafened with CGA-92194; and (c) evaluate the potential effects of tridiphane on the conjugation of metolachlor with glutathione in sorghum seedlings safened or unsafened with CGA-92194.

MATERIALS AND METHODS

CHEMICALS. L-(glycine-2-^{3}H)gluthathione (sp. act. 1300 mCi/mol) was purchased from New England Nuclear. Nonlabeled GSH was purchased from Sigma Chemical Co. Analytical grade and uniformly ^{14}C-ring-labeled metolachlor (sp. act. 21.5 mCi/mmol) as well as analytical grade CGA-92194 were provided by CIBA-GEIGY Corp. The initial [^{14}C]-metolachlor stock solution was purified by TLC to remove contaminants and the non-herbicidal isomer of metolachlor. α-Chloroacetamide compounds such as metolachlor are known to occur in two isomeric/rotameric forms, due to steric hindrance of the ortho-substituent on the phenyl ring (15). The plates used were silica gel 60 F precoated plates developed in butanol/acetic acid/water, 12:3:5, and visualized with autoradiography (Kodak No-Screen X-ray film). All other chemicals used were reagent grade. Buffers were made in sterilized, deaerated, distilled water to an ionic strength of 0.1 M by the method of Cherry (5).

POTENTIAL EFFECT OF CGA-92194 ON THE CHEMICAL REACTIVITY OF METOLACHLOR. The effect of CGA-92194 on the chemical degradation of metolachlor was studied by adapting the procedures described by Lamoureux (26) and by leavitt and Penner (27). CGA-92194, [^{14}C]-metolachlor and [^{3}H]-GSH were reacted by the addition (in sequence) to 10 ml test tubes of: (a) 1 ml phosphate buffer solution (0.1 M) pH 7.0 deaerated with N_2; (b) 100 µl of CGA-92194 at concentrations of 1, 50 and 100 µM; (c) 0.2 µM of phenyl labeled [^{14}C]-metolachlor (sp. act. 21.5 mCi/mmol) diluted with nonlabeled herbicide in 100 µl of methanol; (d) 2 µM of reduced GSH in 0.2 ml of

deaerated phosphate buffer; (e) and 0.65 nm of [^{3}H]-GSH (sp. act. 1300 mCi/mmol) in 100 µl of 0.05 N acetic acid. The test tubes containing the reaction mixtures were sealed under a N_2 atmosphere and incubated for 3 h at 30°C in a water bath without shaking. At the end of the incubation period, the reaction was stopped immediately by freezing the mixtures in a dry-ice acetone bath and then lyophylizing them. The dried residue was extracted with 500 µl of methanol, centrifuged (160 x g) and 100 µl of the supernatant were subjected to TLC on 60 F silica gel precoated plates (E. Merck). The plates were developed in butanol/acetic acid/water mixture (12:3:5 v/v/v). Both [^{14}C]-metolachlor and [^{3}H]-GSH standards were cochromatographed to facilitate the identification of the possible reaction products. The developed plates were analyzed qualitatively by autoradiography and quantitatively by liquid scintillation spectrometry. The TLC plates were exposed to X-ray film for 12-15 days and the film was developed to locate the radioactive spots. Areas of the TLC plates corresponding to radiolabeled spots were scraped, dissolved in 10 ml of scintillation fluid (Scinti Verse E®, Fisher) and counted in a liquid scintillation spectrometer (Beckman LS-250) with a 92% counting efficiency for ^{14}C and 70% efficiency for ^{3}H.

METABOLISM [^{14}C]-METOLACHLOR BY SORGHUM SEEDLINGS.

Untreated and CGA-92194-treated (1.25 g ai/kg) sorghum seeds were planted in a mixture of potting medium of Weblite (Weblite Corp., Blue Ridge, Virginia), vermiculite and sphagnum peat moss in 2:2:1 ratio. The seeds were germinated in the dark for 2 days at 30°C to promote primary

root elongation (6). The sorghum seedlings were transferred to 100 ml of half-strength Hoagland nutrient solution (pH 5.6) (18) in foil-wrapped 150 ml glass containers, one plant per container. The nutrient solution was aerated continuously and replaced every 2 days. The plants were allowed to grow on 0.1 μM metolachlor in a growth chamber with a photon flux density of 50-60 W m^{-2} provided by a blend of incandescent and fluorescent lamps. A photoperiodic cycle of 16 h of light at 30°C and 8 h of dark at 20°C was maintained throughout the experiment. Exposure of the hydroponically grown sorghum seedlings to a sublethal dose of metolachlor (0.1 μM) was used because several recent reports have demonstrated a stimulation of the biochemical mechanisms involved in the degradation of selected herbicides following such pretreatments (13, 19). When the plants were 9-10 days old, [^{14}C]-metolachlor (0.22 μCi) adjusted to 10 μM with unlabeled metolachlor was added to the nutrient solution. In a second experiment, 12.5 μM of technical grade tridiphane, the GSH-S-transferase inhibitor, were added to the nutrient solution at the same time as [^{14}C]-metolachlor. Plants were harvested at 6, 24, and 48 h after treatment and the roots were washed in distilled water to remove any radioactivity from their surface. The plants were then separated into shoots plus leaves and root parts. The parts were weighed and then homogenized in 80% methanol. [^{14}C]-metolachlor and potential metabolites were extracted following the procedures described in Figure IV.1. Methanol extracts that contained more than 1500 dpm were analyzed by TLC. The amounts of radioactivity in the parent [^{14}C]-metolachlor and in the

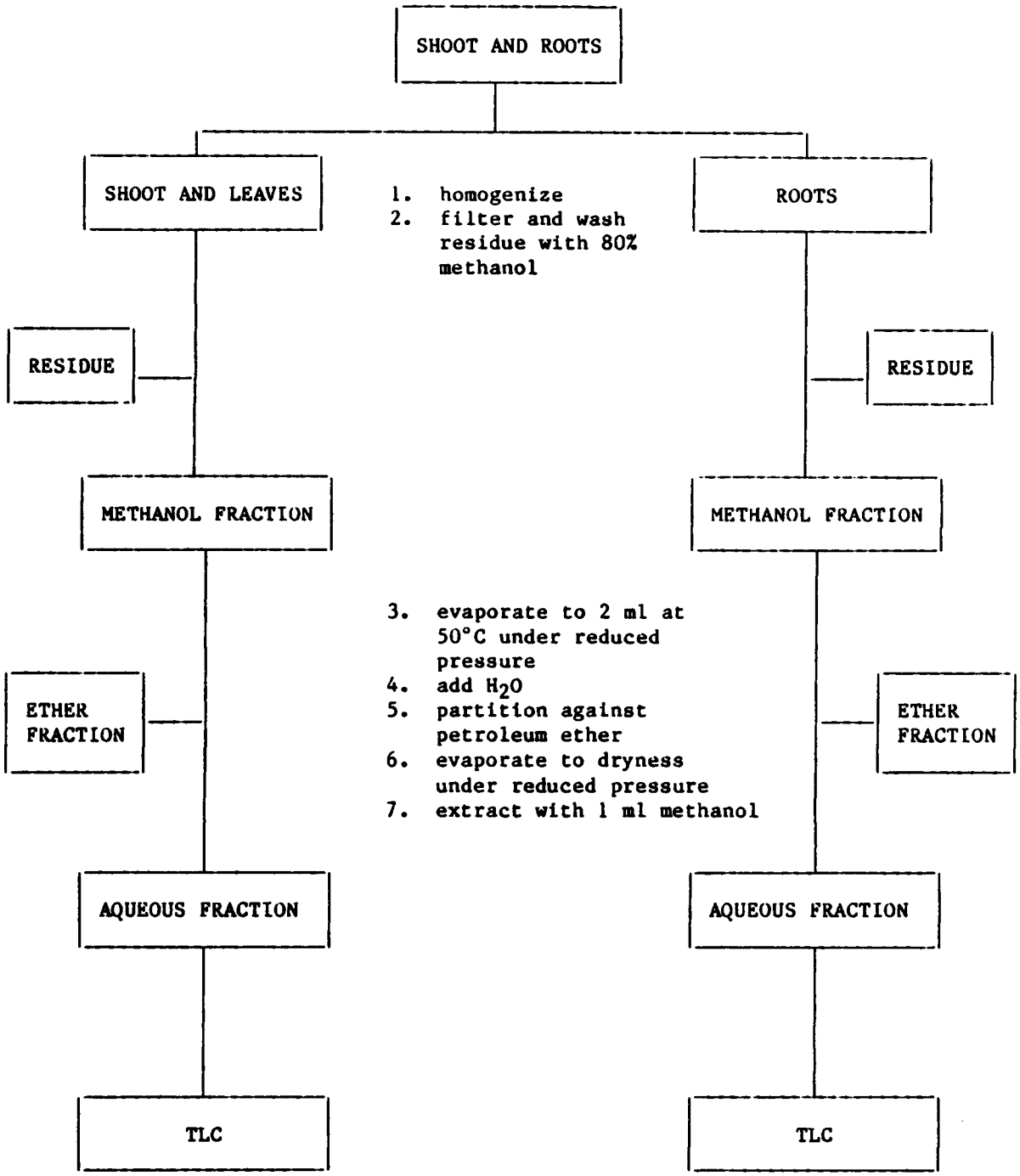

FIGURE IV.1: Extraction procedure for [^{14}C]-metolachlor and its metabolites from grain sorghum seedlings.

metabolites were analyzed qualitatively and quantitatively as in the previous study.

The quantitation results are expressed as percent of the total radioactivity recovered in the metabolites since the major interest in this study was to examine the extent of degradation of the parent compound. The experiment was repeated twice and the data were analyzed for variance in a completely randomized design. Duncan's multiple range test was used for separation of the treatment means. Comparisons between the metabolism of [^{14}C]-metolachlor in CGA-92194-safened or unsafened sorghum seedlings were made with the help of the ratios of the data obtained for each of these treatments (safened vs. unsafened). Similarly, ratios of the data obtained for the tridiphane-treated vs. the non-treated sorghum seedlings were used to assess the effects of this herbicide on the metabolism of [^{14}C]-metolachlor by sorghum and its influence by the safener CGA-92194.

RESULTS AND DISCUSSION

THE EFFECT OF CGA-92194 ON THE CHEMICAL DEGRADATION OF [^{14}C]-METOLACHLOR. The results of this study are presented in Table IV.1. Autoradiography revealed 2 main products resulting from the reaction of [^{14}C]-metolachlor with [^{3}H]-glutathione. Thin layer chromatographic analysis showed that these reaction products chromatographed at Rf values of 0.51 and 0.61, respectively (Table IV.1). The relative amount of radioactivity (dpm) associated with these products increased significantly in the presence of the safener CGA-92194 following a concentration-dependent pattern. However, at safener concentrations higher than 50 μM the radioactivity associated with the dual labeling of these products (^{14}C and ^{3}H) remained relatively constant. The reaction product chromatographing at Rf 0.51 appears to be the glutathione conjugate of metolachlor (GS-metolachlor) on the basis of previous studies by Leavitt and Penner (27). The chemical identity of the reaction product corresponding to Rf values of 0.61 (unknown in Table IV.1) was not assessed. It may very well be a catabolite of the GS-metolachlor resulting from the hydrolytic cleavage of the glutamate moiety of GSH. However, additional analysis is needed to positively characterize this product. The results of this study support earlier reports by other investigators (27, 29) that chloroacetanilide herbicides react spontaneously with thiol compounds such as GSH under *in vitro* conditions. In addition, they show that the safener CGA-92194 increases somewhat the chemical reactivity of metolachlor for its conjugation with GSH. The exact mechanism by which

this is accomplished is not known. The potential formation of chemical complexes of chloroacetanilide herbicides with oxime ether safeners has been postulated by Chang (4). However, experimental evidence for the involvement of such complexes in the safening action of these compounds against chloroacetanilide herbicide injury to sorghum is presently not available.

ABSORPTION AND DISTRIBUTION OF [^{14}C]-METOLACHLOR IN SORGHUM SEEDLINGS SAFENED OR UNSAFENED WITH CGA-92194. The distribution of ^{14}C recovered in various fractions (plant parts, nutrient solution, root wash) is shown in Table IV.2. The average recovery of the total ^{14}C applied was 86.3%. Data in Table IV.2 show that the absorption of [^{14}C]-metolachlor by sorghum roots and its translocation to shoots and leaves increased significantly with time. Radioactivity recovered in the methanol-insoluble (bound) residues of sorghum roots or shoots and leaves was very little (data not shown). Overall, root fractions contained 2-3 times more radioactivity than shoots and leaves within the time limits of this study. These results are in agreement with those of Dixon and Stoller (9), who reported that following treatment with [^{14}C]-metolachlor most of the recovered ^{14}C was present in the roots of corn at 48 h after treatment.

Pretreatment of sorghum seeds with the safener CGA-92194 increased significantly the amount of recovered ^{14}C absorbed by the roots of sorghum seedlings at 6 and 24 h after treatment. At 48 h, pretreatment with CGA-92194 did not increase the amount of ^{14}C recovered in the roots

of sorghum seedlings treated with [^{14}C]-metolachlor. In fact, a slight but significant (P = 0.01) inhibition of [^{14}C]-metolachlor absorption was evident in the presence of CGA-92194.

The amount of ^{14}C recovered in the leaves and shoots of sorghum seedlings treated with [^{14}C]-metolachlor at 24 and 48 h was also significantly higher in seedlings grown from seeds safened with CGA-92194 (Table IV.2). These results are in agreement with those of Fuerst and Gronwald (14) and those reported in Chapter III of this dissertation. In both cases, it was shown that the safener CGA-92194 stimulated the uptake of [^{14}C]-metolachlor into sorghum leaves or isolated leaf mesophyll protoplasts in a concentration-dependent fashion.

Individual treatments with tridiphane reduced the distribution of ^{14}C recovered in the roots or leaves and shoots of sorghum seedlings only at the 48 h period (Table IV.2). Application of tridiphane in combination with CGA-92194 minimized the stimulatory effect of the safener on metolachlor uptake by sorghum roots and in most cases these results appeared to be synergistic.

METABOLISM OF [^{14}C]-METOLACHLOR IN SORGHUM SEEDLINGS SAFENED OR UNSAFENED WITH CGA-92194 AS INFLUENCED BY TRIDIPHANE. Analysis of the extracts of sorghum plant parts according to the procedures of Figure IV.1 showed that most of the recovered radioactivity was associated with the methanol-extractable fractions of sorghum roots or shoots and leaves. The ether-soluble fractions of both roots and shoots plus leaves had low amounts of radioactivity which was associated primarily with the parent

[^{14}C]-metolachlor. These results are in agreement with those of Dixon and Stoller (9) who also reported that most of the recovered radioactivity extracted from corn roots and shoots or leaves was associated with the methanol-soluble fraction. Therefore, only the methanol-soluble fractions of sorghum roots and shoots plus leaves were used for further analysis through TLC procedures for the separation and characterization of [^{14}C]-metolachlor and its metabolites in sorghum. These results are presented in Tables IV.3, IV.4, and IV.5. [^{14}C]-metolachlor was metabolized extensively in both roots and shoots plus leaves of treated sorghum seedlings. Autoradiography and quantitation analysis of the TLC plates revealed the detection of 8 major spots from the methanol-soluble extracts of sorghum roots while 10 spots were found in the methanol-soluble extracts of sorghum shoots and leaves at 6, 24, and 48 h after treatment with [^{14}C]-metolachlor (Tables IV.3, IV.4, IV.5).

The major metabolites of [^{14}C]-metolachlor in root extracts of sorghum, designated as unknowns 1, 2, 3, and 4, had Rf values of 0.24, 0.33, 0.41, and 0.51, respectively. In shoots and leaves the major metabolites are designated as unknowns 5, 6, 3, 7, and 8 with Rf values of 0.19, 0.29, 0.41, 0.48, and 0.53, respectively.

Unknown 3 with Rf value of 0.41 was present in both roots and leaves of sorghum seedlings and was tentatively identified as the conjugate of metolachlor with GSH on the basis of cochromatography with the GS-metolachlor conjugate obtained in the previously described study of the chemical reactivity of metolachlor with GSH under *in vitro* conditions. The parent [^{14}C]-metolachlor chromatographed at Rf values

of 0.75 to 0.77 in the root and leaf or shoot extracts of sorghum. Metabolites with higher or lower polarity than the GS-metolachlor conjugate were present in both root and leaf extracts of sorghum plants. However, the characterization of these metabolites was not assessed in the present study. Whether any of these metabolites represent secondary conjugates of the GS-metolachlor conjugate, catabolites of the GS-metolachlor conjugate, or oxidized (hydroxylated) products of the parent herbicide remains to be determined. Acylation of the GSH-conjugates of several herbicides with malonic acid is known to occur and the subject was reviewed recently (24). In addition, the GSH-conjugates of several herbicides are known to be catabolized to their cysteine derivatives (25). The catabolism of the GS-metolachlor conjugate to its mercaptan followed by conjugation to glucuronide has been reported in metabolic studies of metolachlor in corn and soybean (11). Finally, alkyl hydroxylated metabolites have been reported as major metabolic products of the herbicide metolachlor during its degradation by a soil actinomycete (21).

Pretreatment of sorghum seeds with the safener CGA-92194 appeared to stimulate the metabolism of metolachlor via conjugation to GSH. The percent radioactivity corresponding to the GS-metolachlor was generally higher in the roots and leaves of safened sorghum seedlings than in unsafened seedlings as can be seen from the ratios of the data generated in these treatments (Tables IV.3, IV.4, and IV.5). This observation was particularly evident for the 6 and 48 h treatment periods since the ratios of the percent radioactivity corresponding to the GS-metolachlor

in control and safened seedlings favored the treated seedlings (Tables IV.3 and IV.5). At 24 h, the ratio of the data for safened and unsafened seedlings with regard to the GS-metolachlor conjugate was close to 1.0 indicating the absence of a safener effect on the metabolism of metolachlor at this period (Table IV.4).

Addition of the GSH-S-transferase inhibitor, tridiphane, to the nutrient solutions of safened or unsafened sorghum seedlings appeared to minimize the stimulatory effect of CGA-92194 on the metabolism of [^{14}C]-metolachlor *via* conjugation to GSH. With a few exceptions, the ratios of percent radioactivity corresponding to the GS-metolachlor in control and CGA-92194 treated sorghum seedlings in the presence of tridiphane was close to 1.0 indicating the absence of a safener-induced stimulation of metolachlor metabolism by sorghum roots or shoots and leaves (Tables IV.3, IV.4, and IV.5). This is further evidenced from the ratios of the percent data corresponding to the GS-metolachlor in control (unsafened) and CGA-92194 safened sorghum seedlings in the presence or absence of tridiphane (last columns of Tables IV.3, IV.4, and IV.5). The effects of tridiphane were more pronounced in the safened sorghum seedlings than in the control seedlings. These results agree with those of Ezra et al. (12) who recently reported that tridiphane synergized the activity of the chloroacetanilide herbicide alachlor on proso millet under field conditions. These effects were attributed to the inhibition by tridiphane of the proso millet GSH-S-transferase that conjugates alachlor to GSH.

Overall, the results of the present study indicate that the safener CGA-92194 may protect grain sorghum against injury from the herbicide metolachlor by stimulating the spontaneous or enzymatic conjugation of this herbicide with glutathione. In addition, they provide the framework for further studies that could be done to elucidate more precisely the mechanism of the safening action of this or other herbicide safeners.

Table IV.1. Reaction of ^{3}H-GSH with ^{14}C-metolachlor.

Reactants and Reaction Products	TLC[a] peaks R_f	CGA-92194, µM							
		0		1		50		100	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
		dpm							
^{3}H-GSH	0.15	0	122760	0	102590	0	127360	0	121550
GS-metolachlor	0.51	310	905	640	1225	2370	1850	1820	1990
Unknown	0.61	160	200	545	500	10120	5196	12320	5025
^{14}C-metolachlor	0.84	46635	0	50530	0	43245	0	42815	0

[a]Silica gel 60F, Merk plates developed in butanol/acetic acid/H_2O, 12:3:5.

Table IV.2. Distribution of ^{14}C recovered following application of ^{14}C-metolachlor to 10-day old sorghum seedlings[a].

Absorption time	Treatment	Fraction			
		Roots	Leaves and Shoots	Nutrient Solution	Root Wash
(h)		% of ^{14}C recovered			
6	Control	3.6 b	1.2 a	92.0 ab	0.2 a
	CGA-92194	4.2 a	1.1 a	84.8 c	0.1 a
	Tridiphane	3.5 b	1.0 a	89.2 b	0.4 a
	CGA-92194 + Tridiphane	2.4 c	0.9 a	95.4 a	0.3 a
24	Control	6.1 b	3.3 b	83.8 a	0.4 a
	CGA-92194	8.0 a	4.2 a	64.0 c	0.3 a
	Tridiphane	6.2 b	3.1 a	80.0 a	0.4 a
	CGA-92194 + Tridiphane	6.6 b	2.1 b	72.7 b	0.3 a
48	Control	20.3 a	5.2 b	54.0 b	0.5 a
	CGA-92194	18.2 b	6.7 a	45.0 c	0.3 a
	Tridiphane	11.9 d	4.1 c	82.4 a	0.3 a
	CGA-92194 + Tridiphane	14.3 c	4.7 d	49.4 b	0.5 a

[a]Numbers within the same column for any given absorption time followed by similar letters are not significantly different at the 0.01 level by Duncan's Multiple Range Test.

Table IV.3. TLC analysis of methanol-soluble metabolites extracted from roots and shoots plus leaves of sorghum seedlings at 6 h after treatment with ^{14}C-metolachlor[a].

		Tridiphane, µM							
		0			12.5				
		CGA-92194, g/kg			CGA-92194, g/kg				
		0	1.25	Ratio of	0	1.25	Ratio of	Ratio of	Ratio of
Metabolite	R_F[b]	(A)	(B)	A/B	(C)	(D)	C/D	A/C	B/D
		(% of radioactivity chromatographed)							
ROOTS									
	0.00	2 e	8 de		5 e	6 c			
	0.15	2 e	4 e		5 e	5 c			
Unknown 1	0.24	19 b	9 cd		23 a	10 b			
Unknown 2	0.33	15 b	12 bcd		10 cd	17 a			
Unknown 3	0.41	14 bc	23 a	0.60	13 bc	20 a	0.65	1.07	1.15
Unknown 4	0.51	33 a	15 b		15 bc	12 b			
	0.64	4 de	11 bcd		7 de	8 bc			
	0.75	9 cd	14 bc		17 b	20 a			
SHOOTS AND LEAVES									
	0.00	3 f	3 e		9 bcd	7 cd			
	0.10	5 ef	4 e		9 bcd	5 d			
Unknown 5	0.19	13 b	12 bc		13 ab	14 ab			
Unknown 6	0.29	8 cde	10 cd		10 bcd	12 abc			
Unknown 3	0.41	10 bcd	18 a	0.55	11 abc	10 bcd	1.11	0.90	1.8
Unknown 7	0.48	12 bc	12 bc		7 cd	8 cd			
Unknown 8	0.53	10 bcd	11 bcd		11 abc	7 cd			
	0.65	6 def	7 de		6 d	7 cd			
	0.71	9 bcde	6 de		8 d	10 bcd			
	0.77	19 a	15 ab		14 a	16 a			

[a]Means within columns for any given plant part followed by similar letters are not significantly different at the 0.01 by Duncan's multiple range test.

[b]^{14}C-metolachlor and metolachlor chromatographed at R_f 0.75 (roots) and 0.77 (Leaves). The GSH conjugate of metolachlor chromatographed at R_f 0.41.

Table IV.4. TLC analysis of methanol-soluble metabolites extracted from roots and shoots plus leaves of sorghum seedling at 24 h after treatment with ^{14}C-metolachlor[a].

		Tridiphane, µM							
		0			12.5				
		CGA-92194, g/kg			CGA-92194, g/kg				
Metabolite	R_F[b]	0 (A)	1.25 (B)	Ratio of A/B	0 (C)	1.25 (D)	Ratio of C/D	Ratio of A/C	Ratio of B/D
		(% of radioactivity chromatographed)							
ROOTS									
	0.00	1 d	5 e		7 de	6 e			
	0.15	3 d	4 e		4 e	7 e			
Unknown 1	0.24	18 b	21 b		21 b	12 cd			
Unknown 2	0.33	22 b	10 cd		10 d	17 b			
Unknown 3	0.41	30 a	35 a	0.85	29 a	27 a	1.07	1.11	1.29
Unknown 4	0.51	11 c	6 de		7 de	8 de			
	0.64	4 d	4 e		5 e	5 e			
	0.75	6 d	11 c		15 c	14 bc			
SHOOTS AND LEAVES									
	0.00	2 e	2 d		5 de	4 c			
	0.10	3 e	6 cd		6 cd	5 c			
Unknown 5	0.19	11 c	12 b		15 a	15 ab			
Unknown 6	0.29	26 a	22 a		13 ab	14 b			
Unknown 3	0.41	24 a	24 a	1.00	15 a	18 a	0.83	1.60	1.33
Unknown 7	0.48	17 b	11 bc		9 bcd	7 c			
Unknown 8	0.53	8 cd	6 cd		10 bc	7 c			
	0.65	2 e	4 d		5 de	6 c			
	0.71	1 e	3 d		6 cd	6 c			
	0.77	4 de	6 cd		10 bc	15 ab			

[a]Means within columns for any given plant part followed by similar letters are not significantly different at the 0.01 by Duncan's multiple range test.

[b]^{14}C-metolachlor and metolachlor chromatographed at R_f 0.75 (roots) and 0.77 (Leaves). The GSH conjugate of metolachlor chromatographed at R_f 0.41.

Table IV.5. TLC analysis of methanol-coluble metabolites extracted from roots and shoots plus leaves of sorghum seelings at 48 h after treatment with ^{14}C-metolachlor[a].

		Tridiphane, µM							
		0			12.5				
		CGA-92194, g/kg			CGA-92194, g/kg				
Metabolite	R_F[b]	0 (A)	1.25 (B)	Ratio of A/B	0 (C)	1.25 (D)	Ratio of C/D	Ratio of A/C	Ratio of B/D
		(% of radioactivity chromatographed)							
ROOTS									
	0.00	3 c	2 d		4 d	2 d			
	0.15	3 c	3 d		6 d	5 cd			
Unknown 1	0.24	26 a	11 c		25 a	23 a			
Unknown 2	0.33	21 b	22 b		17 bc	23 a			
Unknown 3	0.41	27 a	39 a	0.69	18 b	20 a	0.90	1.5	1.85
Unknown 4	0.51	4 c	6 d		7 d	7 bc			
	0.64	3 c	4 d		5 d	4 cd			
	0.75	7 c	6 d		13 c	10 b			
SHOOTS AND LEAVES									
	0.00	2 f	2 e		3 d	2 d			
	0.10	3 ef	2 e		3 d	2 d			
Unknown 5	0.19	12 bc	15 b		20 b	11 bc			
Unknown 6	0.29	16 b	14 b		17 b	15 b			
Unknown 3	0.41	24 a	35 a	0.68	25 a	30 a	0.83	0.96	1.16
Unknown 7	0.48	15 b	11 bc		10 c	10 bc			
Unknown 8	0.53	7 cde	4 de		6 d	6 cd			
	0.65	5 def	3 de		6 d	6 cd			
	0.71	5 def	3 de		3 d	7 cd			
	0.77	8 cd	7 cd		6 d	4 d			

[a]Means within columns for any given plant part followed by similar letters are not significantly different at the 0.01 by Duncan's multiple range test.

[b]^{14}C-metolachlor and metolachlor chromatographed at R_f 0.75 (roots) and 0.77 (Leaves). The GSH conjugate of metolachlor chromatographed at R_f 0.41.

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CHAPTER V

SUMMARY AND CONCLUSIONS

This research was conducted to study the potential mechanisms of the safening action of the herbicide safener CGA-92194 against metolachlor injury to grain sorghum. Previous studies have suggested that herbicide safeners may protect crop plants by interferring with herbicide uptake and/or translocation in the protected plant, by competing with the herbicide at some common site in the protected plant and by stimulating the herbicide degradation in the protected plant. The present research examined these hypotheses as they relate to the mechanism of the safening action of CGA-92194.

Experiments were designed specifically to determine:

a. the potential adverse phytotoxic effects of the safeners CGA-92194, cyometrinil and flurazole.

b. the potential influence of CGA-92194 on [^{14}C]-metolachlor uptake into sorghum leaf mesophyll protoplasts.

c. the potential interactive effects of metolachlor and CGA-92194 on the incorporation of radiolabeled precursors into macromolecules of sorghum protoplasts isolated from unsafened seedlings.

d. the effect of metolachlor on the incorporation of radiolabeled precursors into macromolecules of sorghum protoplasts isolated from seedlings unsafened or safened with CGA-92194.

e. the effects of CGA-92194 on the chemical and biological degradation of [^{14}C]-metolachlor.

The results of the phytotoxicity study revealed the absence of a rapid and marked inhibition of the metabolic processes investigated at safener concentrations of 10 µM or lower. These findings suggest that CGA-92194, cyometrinil and flurazole are not phytotoxic at a physiological concentrations of 10 µM. The field recommended rate of CGA-92194 (1.25 g ai/kg) seed) corresponds to a concentration of about 10 µM. When used at recommended rates the three safeners are not likely to cause significant adverse effects on plant metabolism under field conditions.

A safener-induced reduction of herbicide uptake and/or translocation into the protected plant cannot be counted as a mechanism of the safening action of CGA-92194 as metolachlor uptake by sorghum protoplasts doubled in the presence of CGA-92194.

Interactive studies between metolachlor and CGA-92194 demonstrated that the metolachlor-induced reduction of the incorporation of radio-labeled precursors into nucleic acid and lipids of sorghum protoplasts was antagonized by the safener CGA-92194. These results indicate that a competitive antagonism at the sites of nucleic acid and lipid syntheses may be involved in the mechanism of action of this safener.

The metabolic activity and growth of sorghum seedlings grown from safened seeds was lower than that of unsafened seeds. The reasons for this decrease in metabolic activity are not known. Although it is conceivable that this safener-induced reduction of the metabolic activity of safened sorghum could render these seedlings less susceptible to subsequent treatments with the herbicide metolachlor, further research is needed to substantiate this postulation.

The data on the chemical degradation of metolachlor showed that CGA-92194 increased the *in vitro* chemical reactivity of metolachlor for its conjugation with glutathione (GSH). Additionally, CGA-92194 enhanced metolachlor absorption and translocation into sorghum plants and stimulated the metabolism of this herbicide *via* conjugation to GSH. This stimulation was reduced in the presence of tridiphane, an inhibitor of plant GSH-*S*-transferases. These data suggest that CGA-92194 may protect grain sorghum by inducing the stimulation of metolachlor metabolism *via* enzymatic conjugation to GSH.

APPENDIX

ASSAYS OF HERBICIDE ACTIVITY WITH ISOLATED PLANT CELLS AND PROTOPLASTS

Assays of Herbicide Activity with Isolated Plant Cells and Protoplasts[1]

Reagents, Apparatus, and Materials

Erlenmeyer flasks (25-ml)

Rubber stoppers for 25-ml Erlenmeyer flasks

Fixed-volume or adjustable micropipetters (volumes needed include 50, 100, 500 and 1000 µl)

Disposable tips for micropipetters

Shaking water bath illuminated from above with a combination of incandescent and fluroscent lamps; a Gilson differential respirometer illuminated from below can be also used for this purpose.

Test tubes (20-ml)

Conical centrifuge tubes (15-ml)

Benchtop clinical centrifuge

Vacuum pump or water aspirator

$NaH^{14}CO_3$ (sp. act. 44.4 mCi/mmol)

L-[U-^{14}C]-leucine (sp. act. 276 mCi/mmol)

[2-^{14}C]-uracil (sp. act. 55 mCi/mmol) (labeled uridine could be also used)

[methyl-^{3}H]-thymidine (25-50 Ci/mmol)

[1,2-^{14}C]-acetic acid, sodium salt (sp. act. 56.2 mCi/mmol)

Scintillation pads (2.3-cm Whatman #3 paper filter discs)

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[1]Adapted from Hatzios, K. K. 1985. Use of isolated plant cells and protoplasts in herbicide research. Pages 000-000 in N.D. Camper, ed., Research Methods in Weed Science, 3rd ed., Southern Weed Science Society, Champaign, IL. (in press).

2.1-cm glass fiber filter discs

90% formic acid or 20% Trichloroacetic acid (TCA)

5% and 10% w/v ice-cold TCA

80% Ethanol

Acetone

Diethyl ether

0.35 M H_2SO_4 + 0.05 M CH_3COOH

Chloroform:Methanol (2:1, v/v)

20-ml scintillation vials (glass or plastic)

Scintillation fluid (e.g., ACS, Aquasol, Scintiverse E, etc.)

Liquid Scintillation Counter

Herbicidal solutions

GENERAL PROCEDURE

1. 2 ml of the cell or protoplast preparation suspended in the appropriate incubation/assay medium are placed into a 25-ml Erlenmeyer flask. The exact number of flasks and ml of cell or protoplast preparations needed will be dependent upon the number of treatments and replications to be included in a particular study.

2. To each flask 0.1 ml of the appropriate radioactive substrate containing 1 μCi of radioactivity is added.

3. 0.05 ml of the appropriate herbicide solution containing the desired concentrations to be tested is then added to the appropriate flask(s). Most commonly herbicide concentrations tested range from 10^{-9} to 10^{-3} M plus a control.

4. The flasks are stoppered with rubber stoppers and placed in a shaking water bath illuminated from above or below with approximately 7 W/m^2 radiant energy and they are incubated for the desired length of time (e.g., 15, 30, 60, 120, 240 min, etc.) at 20 ± 5°C for C_3 or at 30 ± 5°C for C_4 plant cellular protoplast preparations.

5. At the end of each incubation period, appropriate volumes of samples (100 or 500 µl) are collected with a micropipetter and analyzed further according to specific procedures outlined in the following sections.

SPECIFIC PROCEDURES

Photosynthesis

Assay medium for leaf mesophyll cells of C_3 plants (e.g., soybean)

0.2 M Sorbitol (the concentration of osmoticum will vary with plant species examined)

5mM KNO_3

2 mM $Mg(NO_3)_2$

1 mM $CaCl_2$

50 mM HEPES buffer, pH 7.8 (TRIS or Tricine buffers can also be used)

1 µCi $NaH^{14}CO_3$ (concentration as desired, usually 5 mM)

Cell preparation (8 to 20 µg Chl per 2 ml of the assay assay medium used)

Assay medium for leaf mesophyll protoplasts of C_4 plants (e.g., grain sorghum)

0.4 M Sorbitol (Mannitol can be also used)

1 mM $MgCl_2$

1 mM $MnCl_2$

1 mM EDTA

2 mM KH_2PO_4

50 mM Tricine buffer, pH 7.8 (HEPES or TRIS buffers can also be used)

1 µCi of $NaH^{14}CO_3$(used at 5 or 6 mM)

5 mM Pyruvate, potassium salt

Protoplast preparation (20 to 30 µg Chl per assay)

Steps 1 through 4 of the general procedures are carefully followed. At the end of each selected incubation period, a 100-µl sample is removed from every flask with a micropipetter and placed on a 2.3-cm Whatman 3 MM filter paper disc (scintillation pad) suspended on straight pins stuck into a mounting board. The discs are then acidified with 0.1 ml of 90% formic acid or 20% TCA. In studies with propoplasts of C_4 plants, the samples are treated also with 0.1 ml of 10 mM phenylhydrazine. Acidification with formic or TCA acids releases any unfixed $^{14}CO_2$ while the phenylhydrazine stabilizes any ^{14}C labeled oxaloacetate by forming the phenylhydrozone.[2] Oxaloacetate is labile

[2]Leegood, R. C., G. E. Edwards, and D. A. Walker. 1982. Chloroplasts and protoplasts. Pages 94-111 in J. Coombs and D. O. Hall, eds. Techniques in Bioproductivity and Photosynthesis. Pergamon Press, Oxford.

and in the presence of divalent cations or cellular components is decarboxylated to pyruvate and CO_2. The discs containing the collected samples are then dried in a hood and the radioactivity is determined by placing the discs into scintillation vials, adding 10 ml of an appropriate scintillation fluid and counting the vials in a liquid scintillation counter. The counts per minute (cpm) recorded for each sample are then converted to disintegrations per minute (dpm) by taking into account the counting efficiency of the scintillation counter. The results can be presented either as dpm/mg Chl or as μM $^{14}CO_2$ fixed per mg of chlorophyll per hour of incubation time by using the following formula:

$$\mu M\ CO_2/mgChl/h = \frac{\text{dpm/mg Chl} \times [\mu M] \text{ of } NaH^{14}CO_3 \text{ used}}{\mu Ci \text{ of radioactivity used} \times \text{incubation time (h)}}$$

If 5 mM of $NaH^{14}CO_3$ and 1 μCi of radioactivity are used, then the photosynthetic rate for a 60-min incubation period would be given by the formula:

$$\mu M\ CO_2/mgChl/h = \frac{\text{dpm/mg Chl} \times 5000\ \mu M \text{ of } NaH^{14}CO_3 \text{ used}}{2.2 \times 10^6 \text{ dpm} \times 1 \text{ h}}$$

Macromolecular Syntheses (Protein, RNA, and DNA) in Isolated Cells and Protoplasts

Assay medium for cells of C_3 plants (e.g., soybean)

0.2 M. Sorbitol

5 mM KNO_3

2 mM $Mg(NO_3)_2$

1 mM $CaCl_2$

50 MES, pH 5.8

1 μCi of the respective radioactive substrate, e.g.;

L-[U-^{14}C]-leucine for protein synthesis

[1-^{14}C]-uracil for RNA synthesis

[methyl-^{3}H]-thymidine for DNA synthesis

Cells (8 to 20 μg Chl per assay)

Assay medium for protoplasts of C_4 plants (e.g., grain sorghum)

0.4 M Sorbitol

1 $MgCl_2$

1 $MnCl_2$

1 mM EDTA

50 mM MES, pH 5.8

1 μCi of the respective radioactive substrate (as given above)

Protoplasts (20 to 30 μg Chl per assay)

Steps 1 to 4 of the general procedures are followed carefully. At the end of each incubation time, a 500-μl sample is collected from each flask with a micropipetter and placed in a 20-ml test tube. To each tube 2 ml of ice-cold trichloroacetic acid (10% w/v for protein synthesis and 5% w/v for nucleic acid synthesis) are added and the tubes are stoppered with parafilm or rubber stoppers and left overnight at 4°C in a refrigerator. The TCA-insoluble protein or nucleic acid

precipitates are then collected by filtering each sample through a 2.1-cm glass fiber filter disc. The discs are then washed successively with 6 ml (3 × 2 ml) of ice-cold TCA (5% w/v for nucleic acid synthesis and 10% w/v for protein synthesis), 6 ml (3 × 2 ml) of 80% ethanol, 2 ml of acetone, and 4 ml (2 × 2 ml) of diethyl ether. The discs are then put into scintillation vials, dried in an oven (40-50°C for 30 min), acclimated to room temperature and prepared for radioactivity determination as described in the previous assays. Most commonly, the results of macromolecular syntheses in isolated plant cells or protoplasts are expressed as dpm of radioactivity incorporated into a 100 µg of chlorophyll (dpm/100 µg Chl).

Lipid Synthesis

Assay medium for cells of C_3 plants (e.g., soybean)

0.2 M. Sorbitol

5 mM KNO_3

2 mM $Mg(NO_3)_2$

1 mM $CaCl_2$

50 MES, pH 5.8

1 µCi of [1,2-^{14}C]-acetic acid

Cells (8 to 20 µg Chl per assay)

Assay medium for protoplasts of C_4 plants (e.g., grain sorghum)

0.4 M Sorbitol

1 $MgCl_2$

1 $MnCl_2$

1 mM EDTA

2 mM KH_2PO_4

50 mM MES, pH 5.8

1 μCi [1,2-^{14}C]-acetic acid

Protoplasts (20 to 30 μg Chl per assay)

Steps 1 to 4 of the general procedures are followed carefully. The procedure for sample analysis to determine the incorporation of labeled acetate into lipids has been described by Ashton et al.[3] At the end of each incubation period, a 500-μl sample is pipetted from each flask and placed in a 15-ml conical centrifuge tube. Then, 2 ml of 0.35 M H_2SO_4 + 0.05 M CH_3COOH mixture is added to the sample and the tubes are allowed to sit in the acids for at least 15 min at room temperature. Then they are centrifuged for 10 min at 160 × g at room temperature. The supernatant is removed by suction and discarded. Four ml of chloroform/methanol mixture (2:1, v/v) are then added to each tube and mixed. The tubes are stoppered and left overnight at room temperature. Two ml of distilled water are added to each tube and the mixture is centrifuged at 160 × g for 50 min. The top layer is removed by suction and the procedure is repeated three times. The chloroform solution is then filtered through a 2.1-cm glass fiber filter disc into scintillation vials, and the discs are washed with 4 ml (2 × 2 ml) of chloroform/methanol (2:1, v/v) solution. The filtered solution in each vial is

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[3]Ashton, F. M., O. T. DeVilliers, R. K. Glenn, and W. B. Duke. 1977. Localization of metabolic sites of action of herbicides. Pestic. Biochem. Physiol. 7:122-141.

then dried under a hood or under a current of air. The vials with the dried samples are prepared for radioactivity determination with the addition of 10 ml of scintillation fluid. Lipid synthesis results are expressed as dpm of acetate incorporated into lipids per 100 μg of chlorophyll (dpm/100 μg Chl).

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