DISSERTATION

INFLUENCE OF FOLIAR ABSORPTION, HERBICIDE METABOLISM, LIGHT INTENSITY, IRRIGATION, AND PORPHYRIN BIOSYNTHESIS ON CARFENTRAZONE-ETHYL SELECTIVITY

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY **W. MACK THOMPSON** ENTITLED **INFLUENCE OF FOLIAR ABSORPTION, HERBICIDE METABOLISM, LIGHT INTENSITY, AND PORPHYRIN BIOSYNTHESIS ON CARFENTRAZONE-ETHYL SELECTIVITY** BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

INFLUENCE OF FOLIAR ABSORPTION, HERBICIDE METABOLISM, LIGHT INTENSITY, IRRIGATION, AND PORPHYRIN BIOSYNTHESIS ON CARFENTRAZONE-ETHYL SELECTIVITY

Carfentrazone-ethyl is a new post emergence herbicide for broadleaf control in corn and wheat. Low use rates (0.009 kg ha^{-1}) of carfentrazone inhibit protoporphyrinogen oxidase in the chlorophyll biosynthesis pathway, which results in increased levels of protoporphyrin IX and production of singlet oxygen species in the presence of light. Plant death occurs rapidly due to membrane disruption and desiccation. Research was conducted to determine the mechanism of selectivity of carfentrazone and factors that influence crop safety. Carfentrazone absorption, translocation and metabolism were determined using radiolabelled herbicide in corn (*Zea Mays*), velvetleaf (*Abutilon theophrasti*), and soybean (*Glycine max*). Absorption and translocation were not factors contributing to carfentrazone selectivity. Metabolic half-lifes of carfentrazone correlated well with species sensitivity. Half-life of carfentrazone in corn was less than 2 h, less than 8 h in soybean, and greater than 24 h in velvetleaf. Field research utilizing shade cloth tents and furrow irrigation was conducted to determine the influence of light intensity and plant water status on carfentrazone crop [corn, soybean, and wheat (Triticum aestivum)] response. Corn was relatively insensitive to either factor. Carfentrazone caused more injury in shaded, high-moisture soybean and wheat versus unshaded, dryland treatments. The complex mechanism of action of carfentrazone introduces other means that affect a plant's ability to tolerate carfentrazone.

Research was conducted to determine the influence of water and nutrient stress on the porphyrin biosynthesis pathway. Protochlorophyllide was measured in corn and wheat placed in darkness and protoporphyrin IX was measured in light-pulsed plants following treatment with carfentrazone. Low nutrient treatments reduced protochlorophyllide production, but protochlorophyllide was not influence by water status. Protochlorophyllide levels correlated with visual crop injury. Differences in carfentrazone metabolism appear to explain selectivity among species. Environmental factors affecting flux through the porphyrin pathway can influence the carfentrazone sensitivity within a species.

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DEDICATION

In dedication of

Mack #1

Mack Peyton, Grandfather

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Chapter 1

Absorption and fate of carfentrazone-ethyl in corn (*Zea mays*), soybean (*Glycine max*), and velvetleaf (*Abutilon theophrasti*)

Abstract. Carfentrazone-ethyl absorption, translocation, and metabolism was determined in *Glycine max*, *Zea mays*, and *Abutilon theophrasti*. *G. max* absorbed greater than 90% of applied carfentrazone-ethyl within 2 hours after treatment (HAT) when nonionic surfactant (NIS) or crop oil concentrate (COC) were included in the treatment solution. The addition of 28% urea ammonium nitrate (UAN) did not improve carfentrazone-ethyl absorption in *G. max*, but in *Z. mays* and *A. theophrasti*, UAN combined with NIS or COC increased the rate of carfentrazone-ethyl absorption. Carfentrazone absorption in *A. theophrasti* 2 HAT was 70% when UAN was combined with NIS or COC compared to 40% with NIS or COC alone; however, 24 HAT, absorption with NIS and COC were similar to treatments with UAN. Carfentrazone-ethyl did not translocate from the treated leaf to other plant parts in *Z. mays* and only small amounts of radiolabeled product were detected in the rest of the shoots of *A. theophrasti* (5%) and *G. max* (12%). Herbicide metabolism in *Z. mays* and *G. max* was greater than in *A. theophrasti*. All three species converted carfentrazone-ethyl to its phytotoxic metabolite carfentrazone-chloropropionic acid; therefore, the parent molecule was considered to be the sum of the ethyl ester and its hydrolysis product. Estimated half-

lives of carfentrazone in *Z. mays*, *G. max* and *A. theophrasti* were 1, 7, and 40 h, respectively. The rate of carfentrazone metabolism corresponded to plant sensitivity (sensitivity to carfentrazone: *Z. mays*<*G. max*<<*A. theophrasti*); however, rapid absorption and translocation of carfentrazone may reduce the tolerance of *G. max*.

Nomenclature: Carfentrazone-ethyl, ethyl *a*,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoate; carfentrazone-chloropropionic acid, (2-chloro-4-[2-chloro-4-fluoro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1-*H*-1,2,4-triazol-1-yl]phenyl]propionic acid; velvetleaf, *Abutilon theophrasti* Medic. ABUTH; corn, *Zea mays* L. 'Pioneer 3655'; soybean, *Glycine max* (L.) Merr. 'Conrad'.

Key words: Metabolism; tolerance; protoporphyrin IX; protoporphyrinogen oxidase; selectivity; translocation; uptake; triazolinone.

Introduction

Carfentrazone-ethyl (formerly F8426) is a new post-emergence herbicide discovered and developed by FMC Corporation for control of several broadleaf species in corn (*Zea mays*), wheat (*Triticum aestivum*), and soybean (*Glycine max*) (Anonymous 1998). Carfentrazone-ethyl is a contact herbicide capable of producing injury symptoms (water-soaked leaves) within hours and death can occur within 24 h. Low rates of carfentrazone-ethyl (9 g ai ha^{-1}) effectively control several troublesome weeds in *Z. mays* and cereals: *Amaranthus spp.*, *Ipomoea spp.*, *Solanum spp.*, velvetleaf (*Abutilon theophrasti* Medic.), common lambsquarters (*Chenopodium album* L.), and catchweed bedstraw (*Galium*

aparine L.). It can also be effective against ALS resistant populations of kochia (*Kochia scoparia* (L.) Schrad.) and Russian thistle (*Salsola iberica* Senn. & Pau). Carfentrazone-ethyl provides producers with an additional mode of action for their herbicide-resistance management programs.

Carfentrazone-ethyl and its structurally related cousin, sulfentrazone, are aryl triazolinone herbicides (Theodoridis et al. 1992). The mode of action for both herbicides is inhibition of protoporphyrinogen oxidase (Protox) (Dayan et al. 1997a; Dayan et al. 1997b) in the chlorophyll biosynthesis pathway which results in accumulation of protoporphyrin IX (PPIX) in the cytosol (Becerril and Duke 1989; Sherman et al. 1991). PPIX is photoactive and involved in the light-dependent formation of singlet oxygen, which is responsible for plant death via membrane peroxidation (Devine et al. 1993). Other herbicides with the same mode of action include the diphenyl ethers, oxadiazon, flumiclorac, and in general, a subgroup known as phenyl heterocycles (Dayan et al. 1997a). Carfentrazone-ethyl and its hydrolysis product, carfentrazone-chloropropionic acid (CP-acid), are 50 to 100 times stronger inhibitors of Protox, based on I_{50} values, than sulfentrazone and 20 times more than acifluorfen (Dayan et al. 1997a). Protox-inhibiting herbicides, especially the diphenyl ethers, have been extensively studied (for a recent review see Dayan and Duke 1997); however, little information is available on the aryl triazolinones.

Recent studies have indicated that selectivity of aryl triazolinones (carfentrazone-ethyl and sulfentrazone) is based on rates of metabolic detoxification of the compounds (Dayan et al. 1997a; Dayan et al. 1997b; Dayan et al. 1996). All plants studied hydrolyze carfentrazone-ethyl to CP acid, but this cannot be considered a detoxifying step since the

CP acid is as phytotoxic as the parent compound (Dayan et al. 1997a) (Thompson et al., unpublished data). *G. max* further metabolizes carfentrazone compared to *A. theophrasti* and ivyleaf morningglory (*I. hederacea*) (Dayan et al. 1997a), which may explain why *G. max* is more tolerant than the weed species. Although *G. max* displayed enhanced metabolic degradation of carfentrazone, phytotoxic forms of the herbicide were present in all the species studied; therefore, the authors concluded that other factors probably are involved in protecting the plants against photodynamic damage.

Selectivity of most herbicides is due to differences in metabolic herbicide degradation between crops and weeds, although the advent of herbicide resistant crops has promoted selectivity based on resistant sites of action. The complex mechanism of action of Protox inhibitors provides additional potential sites of resistance: 1) enhanced protective antioxidant system, 2) rate of protopophyrinogen (Protogen) synthesis and enzymatic degradation, 3) conversion rate of Protogen to PPIX, and 4) rapid enzymatic degradation of PPIX (Dayan and Duke 1997).

The purpose of this study was to further evaluate the basis of selectivity of carfentrazone-ethyl in *Z. mays*, *G. max*, and *A. theophrasti* by determining absorption, translocation, and metabolism. Several adjuvant combinations were studied to aid in developing surfactant use recommendations that decrease crop response and increase weed control.

Materials and Methods

Plant Material

Z. mays (L.) ('Pioneer 3655'), *G. max* (L.) (Merr. 'Conrad'), and *A. theophrasti* were grown outdoors in 8 cm diam by 30 cm long cones[1] filled with soil [Fort Collins loam (fine loamy, mixed, mesic, Ustollic Haplargids) pH 7.7, CEC 25.1, 2.2% organic matter] amended with 10% w/w soil-less potting media[2]. Root temperature was moderated by placing plants in large plastic tubs buried in the field and cones were held in place with styrofoam lids. Plants were watered daily and fertilized weekly with the equivalent of 200 mg L^{-1} nitrogen using 20-19-18 (N-P-K) with minor nutrients. Experiments were conducted in July and August of 1995 and 1996 at Colorado State University's Agriculture Research Development and Education Center eight miles northwest of Fort Collins, CO.

Effects of Surfactants and Nitrogen on Absorption

Experiments were conducted by species using 3-leaf *Z. mays*, second to third trifoliolate *G. max*, and 5 to 10 cm *A. theophrasti*. One leaf on each plant was covered with aluminum foil and plants were oversprayed using a CO_2 backpack sprayer calibrated to deliver 187 L ha^{-1}. Carfentrazone was applied at 0.035 kg ha^{-1} to *Z. mays* and *A. theophrasti* and at 0.009 kg ha^{-1} to *G. max*. The spray solution included no surfactant, nonionic surfactant (NIS)[3], or crop oil concentrate (COC)[4], alone and in combination with urea ammonium nitrate (UAN). Adjuvants were used at the following rates: 0.25% v/v NIS, 1% v/v COC, and 2.5% v/v 28% UAN. Following herbicide application the aluminum foil was removed and the protected leaf on each plant was treated with four,

0.5 Π droplets of radiolabeled carfentrazone (2500 Bq leaf^{-1} ^{14}C-carfentrazone-ethyl, specific activity 3474 kBq mg^{-1}). Radiolabeled treatment solutions were prepared from technical-grade ^{14}C-carfentrazone, 2-fold concentrated formulation blank, distilled water, and adjuvants. Following treatment plants were immediately moved outdoors until harvest.

Plants were harvested 2, 8, and 24 h after treatment (HAT). Treated leaves were excised and vortexed for 30 s in 5 ml 10% aqueous methanol containing 0.25% NIS. Radioactivity of the leaf wash solution was determined by liquid scintillation spectroscopy (LSS) and used to estimate herbicide absorption. The remaining above ground plant material was removed at the soil surface. Both the treated leaf and the shoot material were immediately frozen in liquid N_2 and stored at –20 C for metabolite analysis and sample oxidation.

Glass cover slips were used to determine amount ^{14}C applied and the potential for carfentrazone volatilization or UV degradation. Cover slips were treated with radiolabeled carfentrazone solution as described for the treated leaves. The slides were placed near the treated plants outdoors, were collected at 0, 2, 8, and 24 HAT, and rinsed using aqueous methanol solution.

Translocation and Metabolism

Shoot material was sample oxidized[5] to determine the amount of radioactivity translocating out of the treated leaf. Fresh weights were obtained prior to oxidizing the entire shoot material. Oxidized samples were counted by LSS to determine radioactivity. Roots and soil were not analyzed due to limited translocation. The treated leaf was

analyzed for carfentrazone-ethyl, carfentrazone-chloropropionic acid, and metabolites using high-performance liquid chromatography (HPLC).

Carfentrazone and metabolites were extracted from the treated leaf by grinding samples in 10 ml water and methanol (1:9 by vol) using a tissue homogenizer[6]. Tissue samples were shaken for 1 h followed by filtration through 0.2 Πm membranes[7]. Particulate was oxidized and counted by LSS to verify ^{14}C extraction. The filtrate was reduced to 400 Πl under vacuum and 100 Πl subsamples were fractionated by C_8 reversed-phase[8] HPLC[9] coupled with inline ^{14}C detection[10]. The solvents were (A) water and methanol (1:19 by vol) and (B) acetonitrile each acidified with 0.1% (v/v) phosphoric acid. The compounds were fractionated with a binary gradient of 4% per min from 35% B to 100% B. Percent parent carfentrazone remaining was the ratio of carfentrazone-ethyl plus carfentrazone-chloropropionic acid peaks to total ^{14}C of the extract.

Data Analyses

Experiments were designed as factorials with HAT, surfactant, and UAN as factors. Treatments were replicated four times and experiments were repeated. Percentage data from absorption, translocation and metabolism experiments were arcsine transformed before statistical analyses. These transformations did not change the results of the statistical analyses; therefore, non-transformed data are presented. Bartlett's test for homogeneity of variance indicated that the variances from the replicated experiments were similar and that these data could be combined. Experiments were not analyzed across species. Mean values for absorption and translocation results were compared using Fisher's protected least significant difference (P=0.05) (Steel and Torrie 1980).

Metabolism means and standard errors are presented along with exponential decay curves calculated from raw data.

Results and Discussion

Effects of Surfactants and Nitrogen on Absorption

Carfentrazone absorption was determined by difference between amount of ^{14}C-carfentrazone applied and amount recovered in the leaf wash. Carfentrazone applied to the cover slips did not volatilize within 24 HAT and remained as intact carfentrazone-ethyl. The majority of ^{14}C-carfentrazone was recovered from the treated leaf. Overall recovery of applied ^{14}C was 85% derived from the treated leaf, shoot, and leaf wash.

The absorption and herbicidal action of carfentrazone is rapid. *A. theophrasti*, a susceptible species, began to show herbicide symptoms such as water soaked leaves 2 HAT and complete necrosis 24 HAT. *A. theophrasti* absorbed 70% of ^{14}C-carfentrazone within 2 HAT when carfentrazone was applied with NIS and UAN or COC and UAN (Figure 1). *G. max*, a moderately tolerant species, exhibited rapid absorption with greater than 90% of carfentrazone absorbed 2 HAT when surfactants were include in the treatment solution. Carfentrazone absorption 24 HAT in *A. theophrasti* was similar to values reported by Dayan and others (1997a); however, absorption in *G. max* was nearly 40% greater than that previously reported. Carfentrazone absorption in *Z. mays*, a tolerant species, was highest with COC or COC and UAN, but overall absorption in *Z. mays* was similar to absorption in *A. theophrasti*.

Urea ammonium nitrate increased the rate of carfentrazone absorption in *A. theophrasti* and *Z. mays* when applied alone and in combination with NIS or COC

(Figure 1). Addition of UAN significantly increased absorption 2 HAT, but at 24 HAT absorption was similar between treatments with surfactant and surfactant plus UAN. This indicates that UAN increases the initial rate of absorption in *Z. mays* and *A. theophrasti*, but does not significantly increase overall absorption 24 HAT. Increasing the initial rate of carfentrazone absorption with the addition of UAN could improve the rainfastness. During the first few hours after application, there appears to be a synergistic interaction between UAN and NIS. The combination of UAN and NIS increased carfentrazone absorption in *Z. mays* and *A. theophrasti* more than either adjuvant alone. Carfentrazone absorption in *G. max* was not influenced by UAN, possibly because absorption in *G. max* was very rapid and nearly complete within 2 HAT.

Carfentrazone absorption without the use of adjuvants is limited. Only 17% and 27% of carfentrazone applied without adjuvant was absorbed 2 HAT by *A. theophrasti* and *Z. mays*, respectively. *G. max* absorbed 60% or less of carfentrazone within 24 HAT, but the addition of NIS or COC increased absorption to more than 90%. In the field, carfentrazone applied without adjuvants appears to lower the herbicide efficacy due to limited absorption.

There is evidence that absorption may be a factor influencing tolerance of some species to Protox inhibitors (Komives and Gullner 1994). Reduced oxyfluorfen absorption was determined to be a factor contributing to the tolerance of rice (*Oryza sativa*) (Lee et al. 1991; Matsumoto et al. 1994); however, selectivity of carfentrazone cannot be explained by absorption. *A. theophrasti* and *Z. mays* absorbed similar amounts of carfentrazone while these two species vary widely in their susceptibility to carfentrazone. *G. max* absorbed much more carfentrazone than either *Z. mays* or *A.*

theophrasti, yet is intermediate in response to carfentrazone. Although absorption does not appear related to carfentrazone susceptibility, rapid absorption of carfentrazone in *G. max* may be a factor contributing to limited *G. max* tolerance.

Translocation and Metabolism

Carfentrazone translocation is species dependent, but does not appear to be a factor accounting for tolerance. Surfactant and UAN did not significantly influence carfentrazone translocation. Time was the only significant factor. Less than 1% and 5% of applied ^{14}C-carfentrazone was translocated out of the treated leaf 24 HAT in *Z. mays* and *A. theophrasti*, respectively (Table 1). Limited translocation in *A. theophrasti* could be due to rapid shoot necrosis following carfentrazone treatment, but lack of translocation in *Z. mays* must be due to other factors since *Z. mays* rarely exhibited injury symptoms. *G. max* translocated 12% of applied ^{14}C-carfentrazone to the shoot, and necrotic lesions were observed extending from the site of application to the leaf margin. Increased translocation in *G. max* could be related to rapid carfentrazone absorption. Although there are slight differences in carfentrazone translocation among *Z. mays*, *G. max*, and *A. theophrasti*, there is no evidence of natural tolerance to a Protox inhibiting herbicide that can be attributed to herbicide sequestration (Dayan and Duke 1997).

Carfentrazone-ethyl, and the free acid derivative, carfentrazone-chloropropionic acid (CP acid), are herbicidal with similar binding constants (258 and 285 nM), similar I_{50} values (18 and 6 nM), and induce similar levels of Proto IX accumulation in leaf disc assays (Dayan et al. 1997a). Carfentrazone-ethyl was rapidly converted to CP acid by all three species in this study. Even at 2 HAT, nearly 100% of remaining carfentrazone was in the CP acid form. Since both forms are highly active, for purposes of this study the

parent molecule is assumed to be the sum of both the ethyl ester and the CP acid of carfentrazone. Other metabolites were measured, but not identified.

Carfentrazone metabolism to non-phytotoxic metabolites appears to account for herbicide selectivity. Surfactant and UAN did not affect carfentrazone metabolism; therefore, data are averaged across surfactant and nitrogen factors (Figure 2). Eight hours after treatment, the amount of carfentrazone remaining as parent product (ethyl ester and CP acid) differed significantly among the species, where 80%, 40%, and less than 10% carfentrazone remained phytotoxic in *A. theophrasti*, *G. max*, and *Z. mays*, respectively. The rate of carfentrazone metabolism corresponded with the species sensitivity to carfentrazone. *A. theophrasti* is highly sensitive to carfentrazone compared to *G. max*, which is moderately tolerant, and *Z. mays*, which is very tolerant. Biological half lives for carfentrazone in the three species can be estimated using the fitted exponential decay equations (Figure 2). In *Z. mays* 50% of carfentrazone is metabolized to non-phytotoxic products within 1 h, *G. max* within 7 h, and *A. theophrasti* greater than 24 h.

Carfentrazone metabolism has been reported in *G. max* and *A. theophrasti* 24 HAT. Dayan and others (1997a) reported that 88% and 48% of carfentrazone remained as carfentrazone-ethyl and CP acid versus 65% and 20% in this study for *A. theophrasti* and *G. max*, respectively. Numerous differences existed between the studies, such as size of plants, growing conditions, and overspraying of plants, which could explain why results differ. *G. max* metabolism results from this study are similar to results determined for sulfentrazone, a molecule in the same family as carfentrazone, in two *G. max* varieties (Dayan et al. 1997b).

By analyzing carfentrazone absorption, translocation, and metabolism for each species, it is possible to explain how these processes influence the selectivity of carfentrazone. The most susceptible species, *A. theophrasti*, did not absorb or translocate large amounts of carfentrazone, but *A. theophrasti* was slow to metabolize carfentrazone to non-phytotoxic metabolites. Lack of metabolism appears to account for *A. theophrasti* susceptibility. *G. max*, a species of intermediate tolerance, metabolized more carfentrazone than *A. theophrasti*, but carfentrazone was rapidly and almost completely absorbed by *G. max*. Although *G. max* can metabolize carfentrazone, rapid absorption and translocation may reduce *G. max* tolerance. Rapid carfentrazone metabolism seems to explain the natural tolerance of *Z. mays* to carfentrazone and may account for lack of carfentrazone translocation. *Z. mays* may metabolize carfentrazone to polar metabolites that are sequestered or have limited phloem mobility.

This study has evaluated several possible mechanisms of tolerance to carfentrazone; however, the complex mechanism of action of carfentrazone provides several more sites which could confer natural tolerance (Dayan and Duke 1997). Additional mechanisms that should be or are being evaluated include site of action resistance (Protox) (Dayan et al. 1997a); enzymatic degradation of PPIX or Protogen (Jacobs et al. 1994; Jacobs et al. 1996; Jacobs et al. 1999); quenching of toxic oxygen species (Matsumoto et al. 1994; Sherman et al. 1991); relative carbon flow through the porphyrin biosynthesis pathway; and cytoplasmic conversion of Protogen to PPIX (Retzlaff and Böger 1996).

Sources of Materials

[1] Stuewe and Sons, Inc., Corvalis, OR 97333.

[2] Metro Mix 350, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

[3] X-77 Spreader, Loveland Industries, Inc., PO Box 1289, Greeley, CO 80632.

[4] Herbimax Oil-Surfactant Adjuvant, Loveland Industries, Inc., PO Box 1289, Greeley, CO 80632.

[5] OX-500 Biological Oxidizer, R.J. Harvey Instrument Corp., Hillsdale, NJ 07642.

[6] Tempest Homogenizer, Virtis Company, Gardiner, NY 12525.

[7] Nylaflo, Gelman Sciences, Ann Arbor, MI 48106.

[8] Zorbax SB C_8, 3 mm x 150 mm, 3.5 Γm particle size, Mac-Mod Analytical, Inc., 127 Commons Ct., Chadds Ford, PA 19317.

[9] Hitachi Instruments, Inc., San Jose, CA 95134.

[10] ERAM Detector, IN/US Systems, Inc., 5809 N. 50th St., Tampa, FL 33610-4809.

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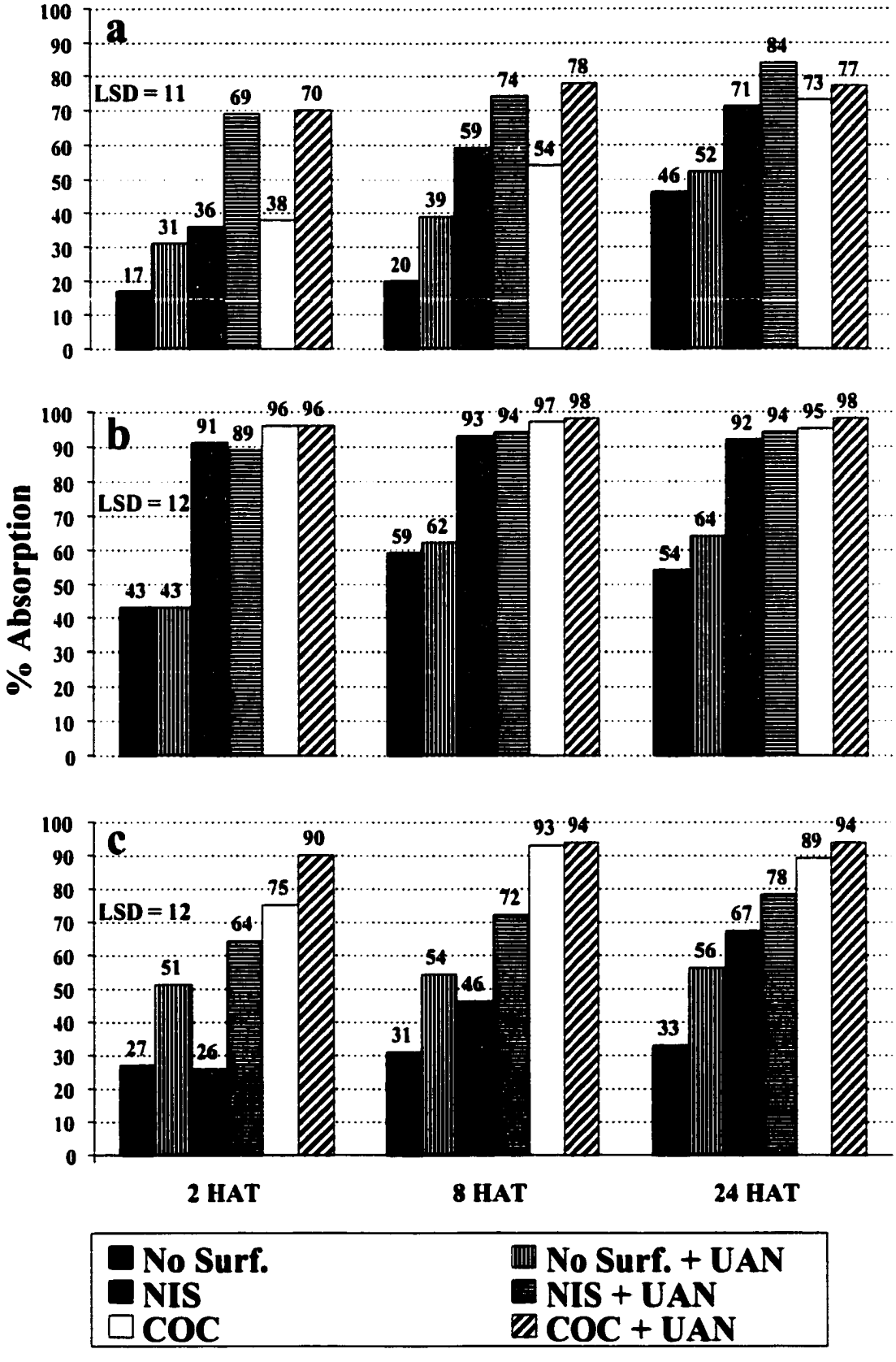
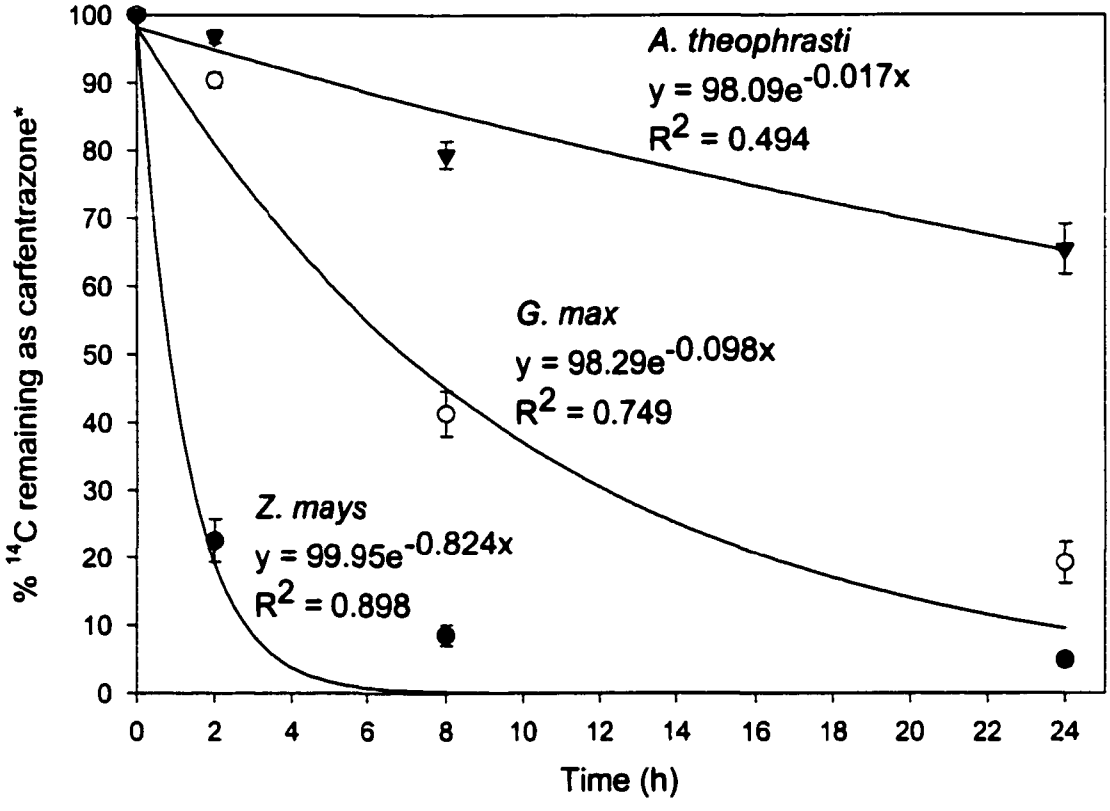
Table 1.1. Translocation of ^{14}C-carfentrazone out of the treated leaf in *A. theophrasti*, *Z. mays*, and *G. max* 2, 8 and 24 HAT. Data were averaged across factors of surfactant and nitrogen, which did not significantly affect translocation.

	Hours After Treatment			
	2	8	24	LSD
	-------- % of applied ^{14}C --------			
A. theophrasti	1	5	5	2
G. max	1	12	12	2
Z. mays	<1	<1	<1	NS

Figure 1.1. Foliar absorption of ^{14}C-carfentrazone in a) *A. theophrasti*, b) *G. max*, and c) *Z. mays* 2, 8, and 24 HAT. Carfentrazone was applied with no surfactant, urea ammonium nitrate (UAN), nonionic surfactant (NIS), NIS and UAN, crop oil concentrate (COC), and COC and UAN. NIS was used at 0.25% v/v, COC at 1.0% v/v, and 28% UAN at 2.5% v/v. Absorption was determined by difference between amount of ^{14}C applied and amount ^{14}C recovered in leaf wash.





* Includes ethyl ester and chloropropionic acid forms of carfentrazone.

Figure 1.2. Metabolism of ^{14}C-carfentrazone in the treated leaves of *A. theophrasti*, *G. max*, and *Z. mays* 2, 8, and 24 HAT. These data were averaged across surfactant and nitrogen treatments, which were not significant factors affecting carfentrazone metabolism, and were plotted as means plus or minus the standard error (n=32). The exponential decay curves were fitted to the raw data.

Chapter 2

Influence of light intensity and irrigation on crop [corn (*Zea mays*), soybean (*Glycine max*), and wheat (*Triticum aestivum*)] response to carfentrazone-ethyl.

Abstract. Environmental conditions can affect the selectivity of carfentrazone-ethyl. Crop safety becomes a concern under extreme environmental conditions. This study was initiated to determine the effect of plant moisture status and light intensity prior to herbicide treatment on crop response to carfentrazone-ethyl. The study was conducted in wheat, corn, and soybeans in 1996, a wet year, and 1997, a dry year. It was difficult to distinguish differences in visual crop injury between irrigated and dryland crops within the same year; however, injury was much higher in the wet year 1996. High moisture status plants (irrigated) appear to be slightly more sensitive than unirrigated plants. In contrast, crop injury was significantly higher in response to low light intensity prior to herbicide treatment. Soybean plants covered with 80% shade cloth for five days prior to carfentrazone application were injured 24 to 41% more than unshaded plants. Corn was relatively insensitive to either factor. Soybeans were very sensitive to carfentrazone and were highly influenced by both light intensity and irrigation. Wheat response to carfentrazone was not influenced by irrigation, but injury in 1996 was 4 times higher than 1997. Light intensity influenced wheat response to carfentrazone, where shading increased visual injury in wheat, but by less than 10%. To reduce the risk of herbicide

injury, carfentrazone-ethyl should not be applied to high moisture status plants or to crops following several cloudy days.

Nomenclature: Carfentrazone-ethyl, ethyl *a*,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoate; carfentrazone-chloropropionic acid, (2-chloro-4-[2-chloro-4-fluoro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1-*H*-1,2,4-triazol-1-yl]phenyl]propionic acid; corn, *Zea mays* L. 'Pioneer 3655'; soybean, *Glycine max* (L.) Merr. 'Conrad'; spring wheat, *T. aestivum* L. 'cv. Blanca'.

Key words: Water stress; light intensity; shading; protoporphyrin IX; protoporphyrinogen oxidase; selectivity; triazolinone.

Introduction

Carfentrazone-ethyl is a new herbicide designed for use in corn, wheat, and soybeans for selective post-emergence control of several broadleaf weeds. Low rates of carfentrazone-ethyl (9 g ai ha^{-1}) effectively control several troublesome weeds in *Z. mays* and cereals: *Amaranthus spp.*, *Ipomoea spp.*, *Solanum spp.*, velvetleaf (*Abutilon theophrasti* Medic.), common lambsquarters (*Chenopodium album* L.), and catchweed bedstraw (*Galium aparine* L.). It can also be effective against ALS resistant populations of kochia (*Kochia scoparia* (L.) Schrad.) and Russian thistle (*Salsola iberica* Senn. & Pau). Carfentrazone-ethyl provides producers with an additional mode of action to manage herbicide-resistance weeds.

Carfentrazone-ethyl and its structurally related cousin, sulfentrazone, are aryl triazolinone herbicides (Theodoridis et al. 1992). The mode of action for both herbicides is inhibition of protoporphyrinogen oxidase (Protox) (Dayan et al. 1997a; Dayan et al. 1997b) in the chlorophyll biosynthesis pathway which results in accumulation of protoporphyrin IX (PPIX) in the cytosol (Becerril and Duke 1989; Sherman et al. 1991). PPIX is photoactive and involved in the light-dependent formation of singlet oxygen, which is responsible for plant death via membrane peroxidation (Devine et al. 1993). Other herbicides with the same mode of action include the diphenyl ethers, oxadiazon, flumiclorac, and in general, a subgroup known as phenyl heterocycles (Dayan and others 1997a).

Carfentrazone-ethyl is a rapid-acting contact herbicide with little or no residual activity and is best used to control small actively growing weeds. The herbicide is quickly absorbed through plant foliage and becomes rainfast within one hour of application (Anonymous 1998). Foliage of susceptible weeds can begin to show signs of dessication within hours of treatment followed by necrosis and plant death within days. Herbicide symptoms can appear on the crop, though the crop usually recovers quickly with no yield loss. Environmental conditions and adjuvants can have a large influence on crop safety, which has been a concern for carfentrazone-ethyl. Extremes in environmental conditions such as temperature, moisture, and cultural practices may affect the activity of carfentrazone-ethyl. Crop injury has been associated with warm, moist conditions and the use of crop oil concentrates in the spray solution; however, very dry conditions can reduce herbicide efficacy and the addition of crop oil concentrate may be required to achieve adequate weed control.

Carfentrazone-ethyl selectivity appears to be due to rapid herbicide degradation by tolerant crops (Thompson and Nissen 2000); however, the complex mechanism of action of Protox inhibitors provides additional potential mechanisms of selectivity. Herbicide selectivity is often a result of differences in herbicide metabolism or sites of action. Selectivity can also be influenced by differences in herbicide absorption and translocation. Protox inhibitors are subject to these same factors, but their complex mechanism of action introduces other factors that can influence selectivity, including: 1) enhanced protective antioxidant system, 2) rate of protopophyrinogen (Protogen) synthesis and enzymatic degradation, 3) conversion rate of Protogen to PPIX, and 4) rapid enzymatic degradation of PPIX (Dayan and Duke 1997). These additional mechanisms complicate the interaction between the environmental conditions and potential crop response to carfentrazone. For example, light intensity could alter the rate of Protogen synthesis or water stress could enhance the protective antioxidant system.

The purpose of this study was to determine the influence of plant moisture status and light intensity on crop response to carfentrazone-ethyl treatments in wheat, corn, and soybean. Understanding how these environmental factors affect crop response to carfentrazone-ethyl is essential to reducing the risk of crop injury.

Materials and Methods

T. aestivum (cv. Blanca, spring wheat), *Z. Mays* (Pioneer 3655), and *G. max* (cv. Conrad) were planted May 8, 1996 and May 23, 1997 at the Colorado State University horticulture research farm 10 km northwest of Fort Collins, Colorado. Wheat was drilled in 25 cm rows with a seeding rate of 67 kg ha^{-1}, and corn and soybean were planted in 76

cm rows with seeding rates of 11 and 34 kg ha^{-1} respectively. The soil was a Nunn clay loam (fine,montmorillonitic, mesic, Aridic Argiustolls) pH 7.6, CEC 23, 1.9% organic matter.

Experiments were designed as split plots with irrigation (0 and 1.3 cm water per week) as main plots and light intensity treatments (0, 35%, and 80% shading) as sub-plots. Each main plot also included an untreated check. A-frame shade cloth tents, 3 m by 3 m, were cut and sewn from 3.7 m wide, 35% shade and 80% shade cloth[1]. Tents were placed over each sub-plot using nylon rope suspended between two steel t-posts. Tents were installed 5 days prior to carfentrazone treatment and main plots were furrow irrigated the following day (see Figure 2.1 for treatment dates).

After 5 days, shade cloth tents were removed and not replaced. Immediately following tent removal, plots were treated with carfentrazone-ethyl applied in 187 l ha^{-1} water with 0.25 % v/v non-ionic surfactant using a CO_2 backpack sprayer with a 2 m, 4 nozzle boom. Carfentrazone was applied at recommended rates 0.026 kg ha^{-1}, 0.035 kg ha^{-1}, and 0.009 kg ha^{-1} to tillering wheat, 4 –leaf corn, and 2-3 trifoliolate soybean, respectively.

Percent injury based on leaf necrosis, chlorosis, and stunting was recorded for each crop 7, 14, and 21 days after treatment (DAT). Nearby CoAgMet[2] weather station (ftc03) was used to record temperature, humidity, and precipitation during the experiments. Three additional personal weather stations[3] were used to record micro-climate temperature and humidity within the plot area. Temperature and humidity sensors were placed inside 80% shade tents in irrigated and nonirrigated plots and one station was place in the open. A quantum light meter[4] was used to measure photosynthetically active

radiation (PAR) of full sunlight and cloud cover and PAR inside 35% and 80% shade tents.

Each crop was treated separately, experiments were repeated in consecutive years, and treatments were replicated three times. Experiments were designed as split plots with irrigation treatments as main plots and light intensity treatments as subplots. Percentage data were arcsine transformed before statistical analyses, but these transformations did not change the results of the analyses; therefore, non-transformed data are presented. ANOVA was used to determine significant differences at the P δ 0.05 level. Bartlett's test for homogeneity of variance indicated that variances from the two years were not similar; therefore, data are not pooled between years. Means are presented separately for irrigation treatments averaged across light intensities (Table 2.1) and light intensity treatments averaged across irrigation treatments (Table 2.2).

Results and Discussion

Environment

Environmental conditions differed in 1996 and 1997 and affected crop response to carfentrazone-ethyl. Precipitation was greater and more frequent during the treatment period in 1996 compared to 1997 (Figure 2.1). In 1996, rainfall occurred 3 days prior to carfentrazone treatment, while no precipitation occurred during the 1997 treatment period. Consequently, plant moisture status was higher in 1996 with plants growing under field capacity and saturated conditions compared to drier conditions in 1997.

Full-sunlight photosynthetic photon flux (PPF) measured 1800 to 2000 Πmol m^{-2} s^{-1} compared to ~200 Πmol m^{-2} s^{-1} PPF on a cloudy day. PPF measured 1100 and 200 Πmol

m^{-2} s^{-1} for 35% and 80% shade cloth, respectively; therefore, 80% shade cloth simulated the amount of PPF on a cloudy day.

There was no difference in humidity or temperature between irrigated and nonirrigated plots under shade cloth tents and plots in the open. Tent material was porous and allowed enough air movement to dissipate any temperature or humidity differences; thus, the only factors that would have influenced crop response were light intensity and plant moisture status.

Plant Moisture

Wheat response to carfentrazone did not appear to be influenced by plant moisture status within the same year (Table 2.1); however, wheat was injured more than 20% in the wet year of 1996 compared to very little injury in the dry year 1997. Injury consisted mostly of necrotic patches located on leaves present at the time of treatment and specifically where the bend in the leaf was at that time. Some stunting was observed, but was not a major symptom. Wheat rapidly recovered from carfentrazone injury during the time span from 7 to 14 DAT. Wheat under irrigation appeared to recover faster than dryland wheat, but the results do not indicate any such trend. Crop injury was evaluated 21 DAT, but by that time wheat had out grown the herbicide injury and there was no difference between treated and untreated plots. Although wheat did not respond differently to irrigation treatments within an experiment, the difference in crop response between wet and dry years is considerable. Wheat appears to be more vulnerable to carfentrazone injury in wet years when the plant moisture status is very high.

Visual carfentrazone injury in corn was affected by plant moisture status. Irrigated corn exhibited slightly more visual injury in both years compared to nonirrigated corn

and corn injury appeared slightly higher in the wet year 1996 (Table 2.1). Visual injury symptoms for corn included leaf necrosis near the bend in leaf, similar to wheat, but also included leaf necrosis that would associate with where the whorl was at time of treatment. Severe injury to the whorl resulted in stunted plants and some shepherd's crooked leaves, but this was not common. High moisture status corn was injured 15 to 20% by carfentrazone, a level of injury that probably would not be commercially acceptable. Corn rapidly recovered from carfentrazone injury between 7 and 14 DAT and, based on visual symptoms, completely recovered by 21 DAT. The recommended carfentrazone rate at the time of this experiment, 0.035 kg ha^{-1}, has since been reduce to 0.009 kg ha^{-1}. The results from this study indicate that carfentrazone applied at the higher rate may cause unacceptable corn injury and high moisture status plants are more susceptible than plants under water stress.

Carfentrazone caused severe injury to soybeans (up to 75%, Table 2.2) and soybean injury appeared to respond to plant moisture status. Because 1997 was a dry year, plant moisture status varied substantially between irrigated and dryland plots and this was reflected in soybean injury which was 17% higher in irrigated plots compared to dryland plots (Table 2.1). More precipitation and timing of precipitation prior to treatment in 1996 resulted in moisture status treatments that were not well separated. Irrigated plots were injured only 6% more than dryland plots 7 DAT and were not significantly different at 14 DAT in 1996. Soybean injury in 1996 (wet year) was approximately 30% higher than 1997 (dry year) which further indicates that soybeans are more sensitive to carfentrazone in high moisture conditions. Soybean did not recover as rapidly as corn and wheat, possibly because soybean were too severely injured. Injury symptoms

consisted of severe necrotic lesions on all leaves exposed at treatment. New growth was unaffected, but some plants lost all exposed leaves. Soybeans did not completely recover by 21 DAT, but they had recovered to a point where the treatments were indistinguishable. The effect of moisture status on carfentrazone injury in soybeans is statistically significant possibly because soybeans are so sensitive to the herbicide compared to wheat and corn. Whether this indicates a trend that can be extrapolated to other crops is questionable. Corn and wheat appear to follow a similar trend, but to a much smaller extent; where as carfentrazone injury in soybean is highly influenced by plant moisture status.

Light Intensity

Light intensity prior to herbicide application affected crop response to carfentrazone for all three crops in both years with the exception of corn in 1996. Plants subjected to 80% shading were injured more by carfentrazone than plants that were not shaded (Table 2.2). Plants grown under 35% shade produced an intermediate in response. Since the measurements for these studies were visual injury ratings, it was important to distinguish between chlorosis caused by shade tents and chlorosis caused by carfentrazone. The tents covered an area 3 m wide, while only 2 m of the plot were treated with carfentrazone. Each plot had a 1 m (1 row) untreated area, subjected to shading, to use for comparison when making visual ratings.

Shading increased carfentrazone injury 4 to 8% in wheat 7 DAT (Table 2.2). Plots that had been subjected to 80% shading were easily distinguished from unshaded plots and unshaded wheat recovered faster than shaded wheat (Table 2.2). Wheat injury in 1997 (dry year) was less than 10% and would probably have been acceptable, and

possibly unnoticeable, to producers. Carfentrazone injury in 1996 would have been unacceptable to most producers with up to 25% visual injury; however, plants appeared to recover quickly and yield was probably not affected.

Carfentrazone injury in corn was not affected by light intensity in 1996, but was 9% greater for 80% shade vs. no shade in 1997. Corn rapidly recovered from injury and shading did not appear to slow recovery. Overall, corn seemed to be the least influenced by light intensity and plant moisture status.

As with plant moisture status, soybeans were affected the most by light intensity. Soybeans grown under 80% shade were 24 to 41% more injured than soybeans grown in full sunlight (Table 2.2). Soybeans grown under 35% shade were significantly more sensitive to carfentrazone compared to unshaded plants. Light intensity prior to carfentrazone application appears to influence crop response in all three species, but especially soybean.

Plant moisture status and light intensity could influence carfentrazone injury by affecting herbicide absorption, herbicide metabolism, herbicide sequestration, chlorophyll biosynthesis, Protogen to PPIX conversion, PPIX or Protogen degradation, and/or free radical detoxification (Dayan and Duke 1997). Most likely plant moisture status affects absorption, chlorophyll production, or free radical detoxification. Plants with a low moisture status could have dehydrated cuticles that would hinder rapid carfentrazone absorption resulting in less injury (Peregoy et al. 1990). Slow growing, drought stress plants may not be producing as much chlorophyll (Botha and Botha 1979); therefore, reducing the rate of porphyrin production and decreasing the potential for injury. Drought stress plants may have elevated levels of superoxide dismutase (Baisak et al.

1994; Zhang and Kirkham 1994; Zhang and Han 1997) allowing them to rapidly detoxify singlet oxygen species produced by light activated PPIX. Although air temperature was the same among plots, leaf temperature may have differed due to cooling through shading or evapotranspiration. Water stressed plants don't have the same potential to cool themselves through evaporative cooling, thus leaf temperatures may have increased. Drought stress induces compatible osmolytes which could also quench free radicals and reduce the response to carfentrazone (Hare et al. 1998). Further research is needed to discern the role of heat stress in protecting plants against Protox inhibiting herbicides.

Light intensity most likely affects the rate of chlorophyll biosynthesis. Pea (*Pisum sativum*) and barley (*Hordeum vulgare*) seedlings produced more chlorophyll under low light conditions vs. high intensity light (Mathis and Burkey 1989). Shaded plants may compensate for low light intensities by increasing chlorophyll production to produce more light harvesting complexes. Increased chlorophyll biosynthesis blocked by carfentrazone would result in more PPIX production and greater injury.

In conclusion, plant moisture status and light intensity prior to herbicide treatment influenced carfentrazone injury in wheat, corn, and soybean. The effect of plant moisture status on crop response was difficult to measure experimentally, but differences between wet and dry years indicate that moisture status does affect the level of carfentrazone injury. Plants subject to reduced light intensity using 80% shade cloth were much more sensitive to carfentrazone than plants receiving normal irradiation. Corn was the least affected by either factor while soybeans were highly influenced by both factors and were very sensitive to carfentrazone. To reduce the risk of carfentrazone injury, avoid

applying the herbicide to high moisture status crops and avoid application following several cloudy days.

Sources of Materials

[1]Centrl Bag and Burlap Co., 2715 Blake St., Denver, CO 80205-2216.

[2]CoAgMet weather station network, http://ccc.atmos.colostate.edu/~coag/, Colorado Climate Center, Atmospheric Science Department, Colorado State University, Fort Collins, CO 80523-1371.

[3]Weather Monitor II, Davis Instruments Corp., 3465 Diablo Ave., Hayward, CA 94545.

[4]LI-185b Quantum Photometric Meter, LI-COR, inc., Environmental Division, 4421 Superior St., Lincoln, NE 68504.

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Table 2.1. Effect of plant moisture status on crop tolerance to carfentrazone. Precipitation was much higher in 1996 compared to 1997. Values are averaged across light intensity treatments.

	Visual Carfentrazone Injury					
	Wheat		Corn		Soybean	
	7 DAT [a]	14 DAT	7 DAT	14 DAT	7 DAT	14 DAT
	%					
1996						
Dryland	22	12	16	5	61	38
Irrigated	24	13	20	8	67	40
	ns [b]	ns	*	*	*	ns
1997						
Dryland	2	3	12	1	20	14
Irrigated	5	3	15	5	37	31
	*	ns	ns	*	*	*

[a] DAT = days after treatment.

[b] Values followed by an asterisk are significantly different at the P δ 0.05 level.

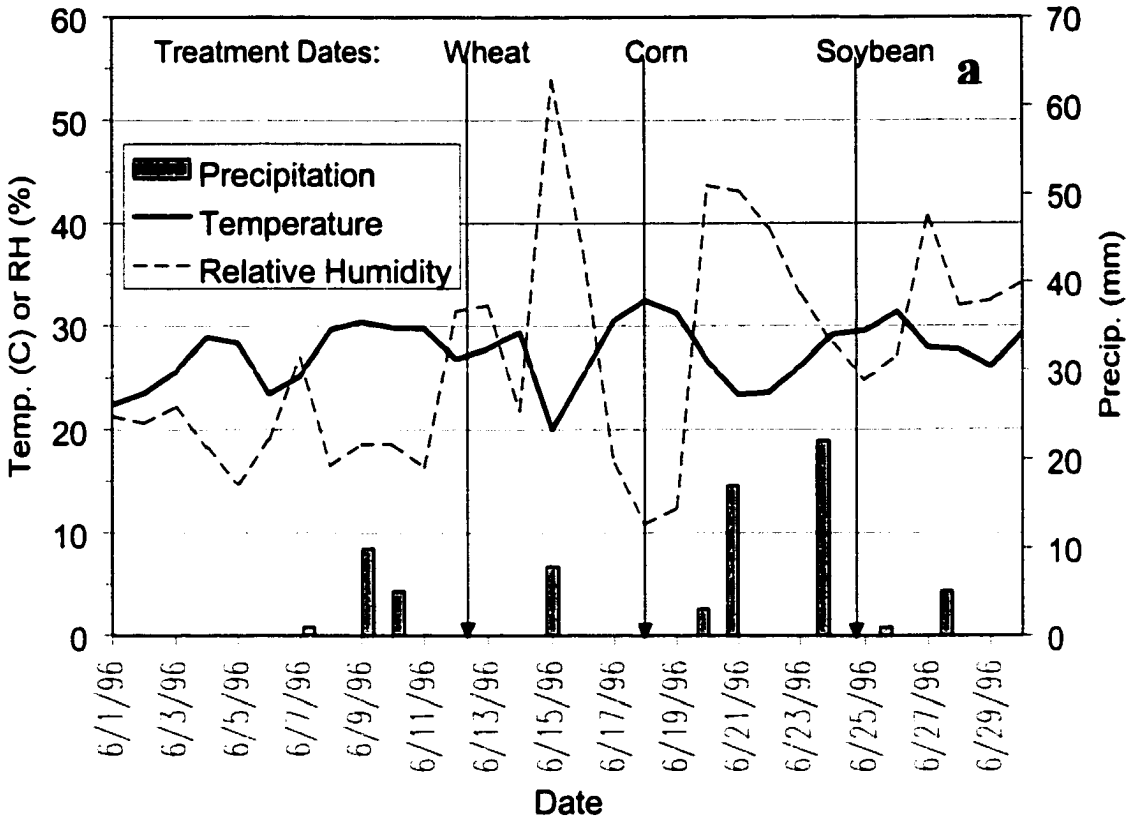
Table 2.2. Effect of light intensity on crop tolerance to carfentrazone. Precipitation was much higher in 1996 compared to 1997. Values are averaged across irrigation treatments.

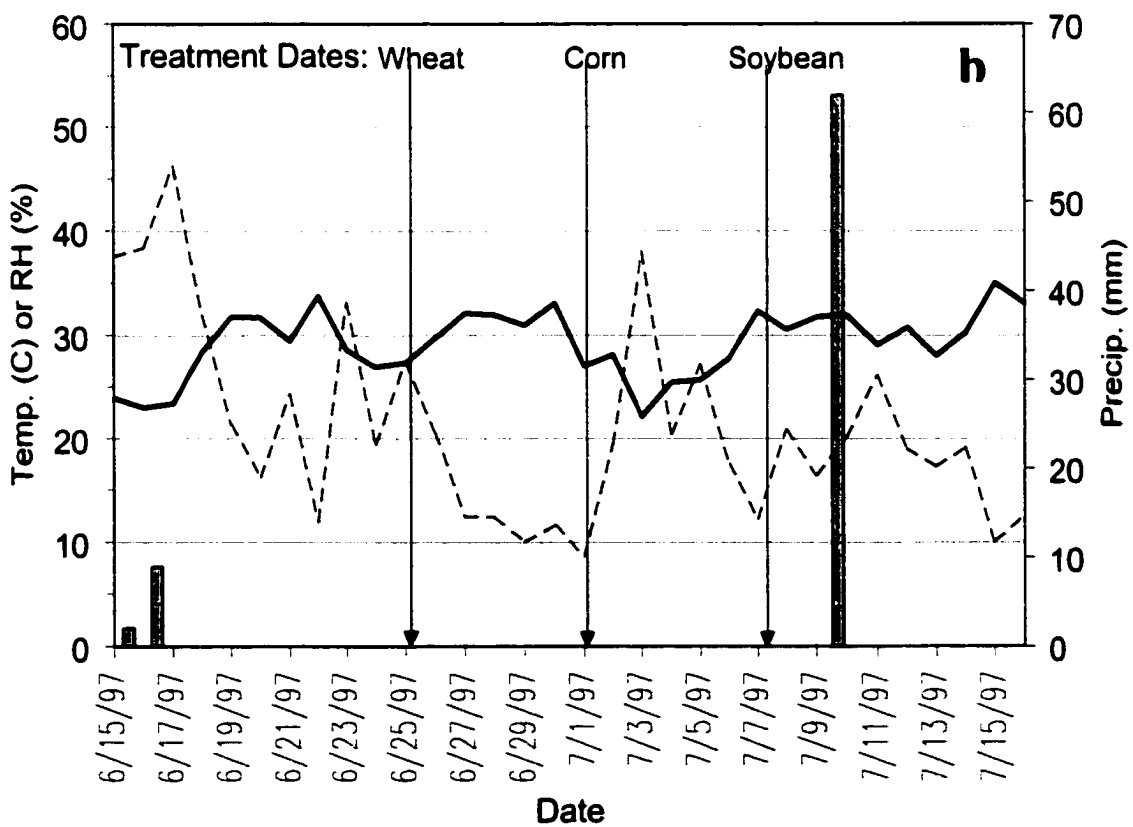
	Visual Carfentrazone Injury					
	Wheat		Corn		Soybean	
	7 DAT [a]	14 DAT	7 DAT	14 DAT	7 DAT	14 DAT
	%					
1996						
No shade	19 c[b]	7 b	17 ns	7 ns	50 c	23 c
35% shade	24 b	12 b	18 ns	6 ns	68 b	30 b
80% shade	27 a	19 a	19 ns	6 ns	74 a	33 a
1997						
No shade	2 b	0 c	10 b	1 b	17 b	16 b
35% shade	3 b	3 b	13 b	2 b	43 a	23 a
80% shade	6 a	7 a	19 a	6 a	58 a	28 a

[a] DAT = days after treatment.

[b] Values, within the same year and column, followed by different letters are significantly different at the P δ 0.05 level.

Figure 2.1. Temperature, relative humidity, and precipitation for 1996 and 1997 during the treatment period. Treatment dates for each crop are indicated by arrows. Data were retrieved from CoAgMet station ftc03.





Chapter 3

Effects of water and nutrient stress on protochlorophyllide biosynthesis and protoporphyrin accumulation in corn (*Zea mays*) and wheat (*Triticum aestivum*)

Abstract. Environmental conditions affect crop response to carfentrazone-ethyl, but the mechanisms responsible for the change in crop response have not been determined. Protochlorophyllide (Pchlide) and protoporphyrin IX (PPIX) accumulation, carfentrazone metabolism, and visual injury was measured in water and nutrient stressed corn and wheat plants to evaluate environmental conditions that could influence porphyrin biosynthesis and contribute to crop response. Pchlide levels were higher in high-nutrient plants and water stress did not affect Pchlide levels. Accumulation of Pchlide was positively correlated with herbicide injury. Flux through the porphyrin pathway appears to be affected by nutrient levels and the activity of the pathway influences herbicide sensitivity of the plant. Analysis of PPIX was inconclusive because PPIX levels did not increase within 180 h in response to 10X rate of carfentrazone-ethyl. Carfentrazone metabolism was not affected by environmental conditions; however, corn metabolized more carfentrazone than wheat 1 h after treatment. It appears that differences in herbicide sensitivity between corn and wheat are due to differences in metabolism. Herbicide metabolism, the major mechanism of carfentrazone selectivity between corn and wheat, was not affected by water or nutrient stress, but flux though the porphyrin

pathway was affected by nutrient stress resulting in differences in sensitivity to the herbicide.

Nomenclature: Carfentrazone-ethyl, ethyl *a*,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoate; carfentrazone-chloropropionic acid, (2-chloro-4-[2-chloro-4-fluoro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1-*H*-1,2,4-triazol-1-yl]phenyl]propionic acid; corn, *Zea mays* L. 'Pioneer 3655'; spring wheat, *T. aestivum* L. 'cv. Blanca'.

Key words: Metabolism; water stress; nutrient stress; tolerance; protochlorophyllide; protoporphyrin IX; protoporphyrinogen oxidase; selectivity; triazolinone.

Introduction

The aryl triazolinone herbicide carfentrazone-ethyl is a strong inhibitor of protoporphyrinogen oxidase (Protox) in the chlorophyll biosynthesis pathway. Inhibition of Protox results in accumulation protoporphyrin IX (PPIX) in the cytosol (Figure 3.1). Accumulation of PPIX is due to rapid, cytosolic, enzymatic oxidation of the Protox substrate protoporphyrinogen IX, which increases in the chloroplasts, but quickly moves to the cytosol. PPIX is photoactive and involved in light-dependent formation of singlet oxygen, which is responsible for plant death via membrane peroxidation. Herbicide induced Protox inhibition in plants is similar to the disease variegate porphyria in humans*.

* Variegate porphyria is the result of a genetic defect in Protox that makes humans very sensitive to light. The disease is suspected in helping establish vampire myths.

Recent studies have indicated that selectivity of aryl triazolinones (carfentrazone-ethyl and sulfentrazone) is based on rates of metabolic detoxification (Dayan et al. 1997a; Dayan et al. 1997b; Dayan et al. 1996). Carfentrazone-ethyl was hydrolyzed to carfentrazone-chloropropionic acid (CP acid) in all plants evaluated to date (Thompson et al unpublished data), but this cannot be considered a detoxifying step since the CP acid is as phytotoxic as the parent compound (Dayan et al. 1997a). Soybean (*Glycine max*) further metabolizes carfentrazone compared to velvetleaf (*Abutilon theophrasti*) and ivyleaf morningglory (*Ipomea hederacea*) (Dayan et al. 1997a), which may explain why soybean is more tolerant than weed species. Studies with corn (*Zea mays*), soybean, and velvetleaf also indicated selectivity of carfentrazone was due to rate of herbicide metabolism (Thompson and Nissen 2000). Although corn and soybean display enhanced metabolic degradation of carfentrazone, phytotoxic forms of the herbicide were present in all the species studied; therefore, other factors are probably involved in protecting plants against photodynamic damage.

Selectivity to many herbicides is due to differences in herbicide metabolism between species. The complex mechanism of action of Protox inhibiting herbicides introduces other means that enable plants can tolerate carfentrazone. Sensitivity to carfentrazone could be affected by protective antioxidant systems, the rate of carbon flow through the porphyrin pathway, the conversion rate of Protogen to PPIX, or the rate of PPIX and Protogen degradation (Dayan and Duke 1997). Mustard (*Brassica kaber*) Protox is very sensitive to chemical inhibitors, but PPIX does not accumulate because Protogen is rapidly degraded (Jacobs et al. 1994). Rice is tolerant to Protox inhibitors because of an

enhanced antioxidant system (Komives and Gullner 1994). Each of the above mechanisms could influence the susceptibility of species to Protox inhibitors.

Carfentrazone is a highly effective herbicide at very low rates (0.009 kg ha^{-1}). Carfentrazone-ethyl and its hydrolysis product, carfentrazone-chloropropionic acid (CP-acid), are 50 to 100 times more effective inhibitors of Protox than sulfentrazone and 20 times stronger than acifluorfen, based on I_{50} values (Dayan et al. 1997a). In the field, carfentrazone crop safety has been a concern. Environmental extremes in temperature and moisture appear to affect carfentrazone-ethyl activity. Crop injury has been associated with warm, moist conditions, while under dry conditions herbicide symptoms are lessened and delayed. Understanding resistance mechanisms responsible for carfentrazone selectivity and how they are affected by environmental conditions is key to developing guidelines to increase crop safety.

The plants protective antioxidant system and the activity of the porphyrin pathway are two likely mechanisms to be influenced by changes in the environment. There is evidence that drought stressed plants have lower rates of chlorophyll biosynthesis (Botha and Botha 1979) and elevated levels of superoxide dismutase (Baisak et al. 1994). Susceptibility of several species to acifluorfen was correlated to the amount of PPIX that accumulated in response to the herbicide and differences in PPIX accumulation appeared related to activity of the porphyrin pathway (Sherman et al. 1991). The objectives of this study were to determine the influence of water and nutrient stress on porphyrin production, herbicide metabolism, and crop sensitivity to carfentrazone.

Materials and Methods

Plant Material

Corn [*Z. mays* (L.) ('Pioneer 3655')] and wheat [*T. aestivum* (cv. Blanca, spring wheat)] were planted in washed silica sand in 4 cm diameter by 21 cm tall pots[1]. Plants were grown in the greenhouse at 27/18 C day/night temperature, 16 h day length, 50% RH, with 200 Πmol m^{-2} s^{-1} PPF supplemental lighting. Soil moisture was controlled by weighing pots daily then adding water or nutrient solution to achieve field capacity or 40% of field capacity. High-nutrient treatments were watered with one-half strength Hoagland's nutrient solution; low-nutrient treatments received tap water. Plants were harvested or treated at the four leaf stage.

Protochlorophyllide

Protochlorophyllide (Pchlide) production was determined by placing plants in the dark and harvesting two youngest leaves after 0, 30, 60, and 120 min. Harvested leaves were immediately frozen in liquid nitrogen and stored at –20 C in the dark until extraction. Samples were protected from light at all times. Leaves were homogenized[2] and extracted in 9:1 (v/v) methanol:0.1 N ammonium hydroxide (Sherman et al. 1991). Deuteroporphyrin IX 2-vinyl 4-hydroxymethyl[3] (Carlson et al. 1984) was added to the extract as an internal standard. Homogenate was centrifuged (15,000 g) for 15 min at 4^0C and the supernatant was saved. The pellet was resuspended in basic methanol, sonicated, and centrifuged (15,000 g). The supernatants were combined.

Amount of Pchlide was determined by a Hitachi HPLC[4] system composed of following modules: D-7000 interface, L-7200 autosampler, L-7100 pump with 4 way

gradient, and L-7480 fluorescence detector preceding an L-4500A diode array detector. The column was a 2.1 x 150 mm 5 Πm Zorbax SB C_{18} reversed phase column preceded by an in-line filter and Phasesep ODS guard column. Mobile phase consisted of 0.1 M ammonium phosphate pH 5.6 (solvent A) and HPLC-grade methanol (solvent B) at a flow rate of 0.3 ml min^{-1}. Solvent gradient was 70% B to 100% B over 15 min then held at 100% B for 10 min. Injection volume was 200 Πl. Commercial deuteroporphyrin and PPIX standards were used to calibrate the fluorometer. Pchlide standard was made from etiolated barley and quantified spectrophotometrically using the following equation where *E* equals extinction at the specific wavelength (nm) (Anderson and Boardman 1964).

$$\text{Pchlide} = -3.99E_{663} - 6.76E_{645} + 29.6E_{626}$$

The equation yields concentration in Πg ml^{-1}. Pchlide and PPIX were quantified using fluorescence detection with the excitation and emission wavelength settings of 400 and 620 nm for deuteroporphyrin, 440 and 630 nm for Pchlide, and 400 and 630 nm for PPIX, respectively. The photodiode array detector scanned from 300 to 700 nm to confirm peaks. Pchlide is expressed as mean nmol g^{-1} fresh weight of four replicates.

Protoporphyrin IX

PPIX accumulation in response to Protox inhibition was measured in corn and wheat plants subjected to water and nutrient stress. Plants at the 4 leaf stage were treated with 10X rate (0.26 kg ha^{-1}) of carfentrazone-ethyl. Carfentrazone-ethyl was applied with 1.0% v/v crop oil concentrate (COC)[5] using a greenhouse spray chamber calibrated to deliver 187 L ha-1 at 30 psi. Immediately following treatment plants were placed in darkness in a growth chamber and pulsed with 50 Πmol m^{-2} s^{-1} light for 1 min out of

every 10 min (Halling and Peters 1987). Two youngest leaves were harvested at 0, 30, 60, 120, and 180 min after treatment. Leaves were frozen, extracted, and analyzed using the Pchlide protocol described earlier. PPIX is expressed as nmol g^{-1} fresh weight. All PPIX treatments were triplicated.

Herbicide Metabolism

Radiolabeled ^{14}C-carfentrazone-ethyl was used to determine the effect of water and nutrient stress on carfentrazone-ethyl metabolism. Plants grown at the same time and under the same conditions as those used for Pchlide and PPIX analysis were treated at the 4 leaf stage with 3.3 kBq ^{14}C-carfentrazone-ethyl (specific activity 3474 kBq mg^{-1}). Radiolabelled ^{14}C-carfentrazone-ethyl was applied with 1.0% v/v COC to a single leaf on each plant in 5, 0.5 Π drops. Treated leaves were excised 1 hour after treatment (HAT) and vortexed for 30 s in 5 ml 10% aqueous methanol containing 0.25% nonionic surfactant (NIS)[6].

The treated leaf was analyzed for carfentrazone-ethyl, carfentrazone-chloropropionic acid, and metabolites using high-performance liquid chromatography (HPLC). Carfentrazone and metabolites were extracted from the treated leaf by grinding samples in 10 ml water and methanol (1:9 by vol) using a tissue homogenizer. Tissue samples were shaken for 1 h followed by centrifugation. Particulate was oxidized[7] and counted by liquid scintillation to verify ^{14}C extraction. The supernatant was reduced to 400 Π under vacuum and 100 Π subsamples were fractionated by C_8 reversed-phase[8] HPLC coupled with inline ^{14}C detection[9]. The mobile phase solvents were (A) water and methanol (1:19 by vol) and (B) acetonitrile each acidified with 0.1% (v/v) phosphoric acid. The compounds were fractionated with a binary gradient of 4% per min from 35%

B to 100% B. Percent parent carfentrazone remaining was the ratio of carfentrazone-ethyl plus carfentrazone-chloropropionic acid (CP-acid) peaks to total ^{14}C of the extract. Treatments were replicated 4 times.

Herbicide Injury

Water and nutrient stressed plants were treated with carfentrazone-ethyl and evaluated for visual injury. Four-leaf plants were oversprayed with a 2X rate (0.051 kg ha^{-1}) of carfentrazone-ethyl + 1.0% COC using a greenhouse sprayer as described for PPIX analysis. Visual injury ratings were taken 7 days after treatment (DAT) and each treatment was replicated five times.

Data Analysis

Experiments were designed as factorials with water and nutrient stress as factors. Treatments were replicated 3-5 times and all experiments were repeated. Percentage data from herbicide injury/metabolism experiments were arcsine transformed before statistical analyses. These transformations did not change the results of the statistical analyses; therefore, non-transformed data are presented. Bartlett's test for homogeneity of variance indicated that variances from replicated experiments were similar and that data could be combined (Snedecor and Cochran 1989). Mean values for carfentrazone metabolism were compared using Fisher's protected least significant difference (P=0.05) (Steel and Torrie 1980).

Results and Discussion

Protochlorophyllide

Protochlorophyllide oxidoreductase converts Pchlide to chlorophyllide a and is the only enzyme in the chlorophyll biosynthesis pathway that is light dependent (Reinbothe and Reinbothe 1996). Plants placed in darkness rapidly accumulate Pchlide due to inhibition of the enzyme; however, Pchlide levels quickly stabilize due to feed back inhibition. The initial rate of Pchlide production is a good indication of the flux through the porphyrin pathway and Pchlide accumulation could be representative of the overall activity of the pathway. Pchlide standards are not sold commercially because Pchlide is labile; however, Pchlide standards are easily isolated from etiolated barley and quantified using the spectrophotometric method described earlier.

Deuteroporphyrin worked well as an internal standard. It chromatographed as a symmetrical peak and eluted with baseline resolution before Pchlide and PPIX. Peak width was relatively wide, but that may have been an artifact of the large injection volume. This appears to be the first time deuteroporphyrin has been used as an internal standard for porphyrin analysis in plants.

Pchlide production in corn and wheat was not affected by water stress, but nutrient stress significantly reduced Pchlide accumulation in both species (Figure 3.2). Pchlide levels in corn rapidly increased during the first 30 min and only increased slightly over the next 90 min reaching a maximum of 3 to 6 nmol g^{-1} fresh weight. Maximum Pchlide values agree with results from earlier studies (Sherman et al. 1991). Pchlide levels in high-nutrient wheat plants were significantly higher than in low-nutrient plants at the initiation of the experiment (Figure 3.3). Wheat Pchlide levels slowly increased over the

length of the experiment. Pchlide levels were higher in corn compared to wheat, but wheat is more sensitive to carfentrazone. This suggests that the absolute amount of Pchlide accumulated is not necessarily correlated with species sensitivity to carfentrazone and that other resistance mechanisms influence species sensitivity. Relative Pchlide levels appear to be good indicators of sensitivity within a species (Figure 3.8). Wheat with the highest levels of Pchlide exhibited the most injury.

Reduced Pchlide accumulation in nutrient stressed plants is most likely due to lack of nitrogen. Nitrogen is used in the tetrapyrrole structure and in amino acids which form enzymes for the pathway. Flux through the porphyrin pathway could be reduced because of a lack of substrate (aminolevulinic acid) or lack or enzymes.

Pchlide accumulation was greatest in water-stressed high-nutrient corn and wheat 120 min after treatment; however, this may be an artifact of the plants fresh weight (Figure 3.2 and 3). Since Pchlide was determined on a per fresh weight basis, water-stressed plant tissue could weigh less, artificially inflating these values.

Protoporphyrin IX

Plants were pulsed with low intensity light every ten minutes to facilitate conversion of Pchlide to chlorophyllide (Halling and Peters 1987); otherwise, Pchlide would have accumulated and caused feed back inhibition of the porphyrin pathway. Porphyrin analysis of these plants included quantifying both PPIX and Pchlide. The low intensity light pulses were effective in avoiding accumulation of Pchlide.

After 180 min, PPIX levels had not increased above normal in corn or wheat in response to treatment with a 10X rate of carfentrazone-ethyl (Figures 3.4 and 3.5). A possible exception was high-nutrient, field capacity corn. PPIX may have just begun to

accumulate at 180 min after treatment. Previous experiments quantifying PPIX levels were conducted using leaf disks (Becerril and Duke 1989; Sherman et al. 1991). Disks were soaked in Protox inhibiting herbicide solution for up to 20 h. Although a high rate of carfentrazone-ethyl was applied to the plants, not enough time may have been allowed for the herbicide to reach the site of action. Carfentrazone-ethyl is rainfast within 1 h, but translocation to the chloroplast may take much longer. Water soaked leaves can be observed on sensitive species within a couple hours of carfentrazone treatment, indicating that 180 min should have been long enough to observe PPIX accumulation. Further research is needed to develop a method for PPIX analysis in intact plants.

Herbicide Metabolism

Both the parent molecule, carfentrazone-ethyl, and its hydrolysis product, CP-acid, are phytotoxic; therefore, metabolism results are presented as percent ^{14}C remaining as intact carfentrazone plus CP-acid. Carfentrazone metabolism did not appear to be influenced by water stress (Figure 3.6). Although not statistically significant, high-nutrient plants tended to metabolize slightly more carfentrazone than low-nutrient plants. Corn metabolized carfentrazone slightly faster than wheat, but both species had metabolized ~50% or greater of the carfentrazone in the plant to non-phytotoxic metabolites within 1 h. Overall, slight differences in metabolism did not explain differences in carfentrazone crop response to environmental factors.

Herbicide Injury

Water and nutrient stress influenced crop response to carfentrazone (Figure 3.7). In general, high-nutrient plants were injured more than low-nutrient plants. There was an interaction between the low-nutrient and water stress. Visual injury ratings in low-

nutrient water-stressed plants may have been influenced by necrosis caused by stress rather than carfentrazone, artificially inflating the injury rating. This might explain the interaction.

Drought stress did not appear to reduce crop sensitivity to carfentrazone, suggesting that the protective antioxidant system in these two crops was not enhanced by drought stress. In fact, high-nutrient water-stressed wheat was the most sensitive to carfentrazone.

There is a positive correlation for both corn and wheat between Pchlide accumulation and herbicide injury in water and nutrient stressed plants (Figure 3.8). Plants that accumulated larger quantities of Pchlide tended to be more susceptible to carfentrazone-ethyl. This would suggest that differential susceptibility to Protox inhibitors is in large part due to differences in flux through the porphyrin pathway. The relationship between Pchlide accumulation and herbicide injury is not exactly the same for both corn and wheat (regression lines are not similar and do not have similar slopes) indicating that other mechanisms affect carfentrazone selectivity. Corn appears to metabolize carfentrazone faster than wheat, which would explain why wheat is more sensitive to carfentrazone.

In conclusion, nutrient and drought stress did not appear to affect carfentrazone metabolism; thus, differences in carfentrazone crop response are not due to metabolism of carfentrazone. Pchlide levels were affected by nutrient stress. High-nutrient plants accumulated higher levels of Pchlide and there was a positive correlation between Pchlide levels and crop injury; however, Pchlide accumulation does not explain the differences between corn and wheat. More Pchlide accumulated in corn, but wheat is

more sensitive to carfentrazone. Flux of the porphyrin pathway is associated with differences in sensitivity to carfentrazone-ethyl, but crop selectivity is not based solely on carbon flow through the porphyrin pathway. Differences in carfentrazone selectivity between species are based on rates of carfentrazone metabolism, but flux through the porphyrin pathway influences sensitivity within a species.

Sources of Materials

[1] Cone-tainers, Stuewe and Sons, Inc., Corvalis, OR 97333.

[2] Tempest Homogenizer, Virtis Company, Gardiner, NY 12525.

[3] Porphyrin Products, Logan, UT 84323-0031.

[4] Hitachi Instruments, Inc., San Jose, CA 95134.

[5] Herbimax Oil-Surfactant Adjuvant, Loveland Industries, Inc., PO Box 1289, Greeley, CO 80632.

[6] X-77 Spreader, Loveland Industries, Inc., PO Box 1289, Greeley, CO 80632.

[7] OX-500 Biological Oxidizer, R.J. Harvey Instrument Corp., Hillsdale, NJ 07642.

[8] Zorbax SB C8, 3 mm x 150 mm, 3.5 Πm particle size, Mac-Mod Analytical, Inc., 127 Commons Ct., Chadds Ford, PA 19317.

%RAM Detector, IN/US Systems, Inc., 5809 N. 50th St., Tampa, FL 33610-4809.

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The author thanks FMC Corp. for financial support of this research and for supplying radiolabeled carfentrazone-ethyl and CP acid, technical grade carfentrazone and metabolite, and commercial formulation blank. Technical advice from Claude Ross and Dave Keifer from FMC Corp., Stephen Duke, and Franck Dayan was greatly appreciated.

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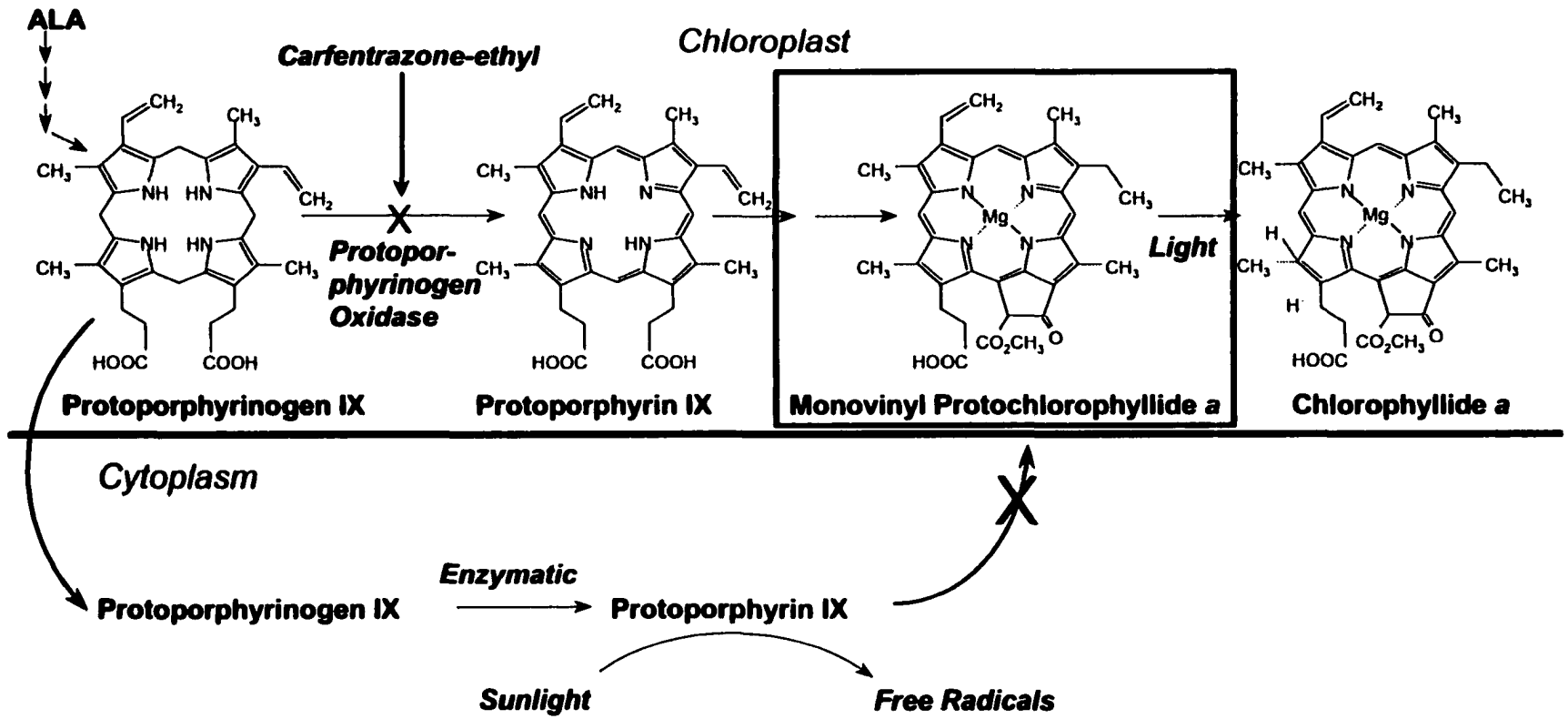

1 Figure 3.1. Simplified schematic of chlorophyll biosynthesis pathway depicting inhibition of Protox and accumulation of PPIX.

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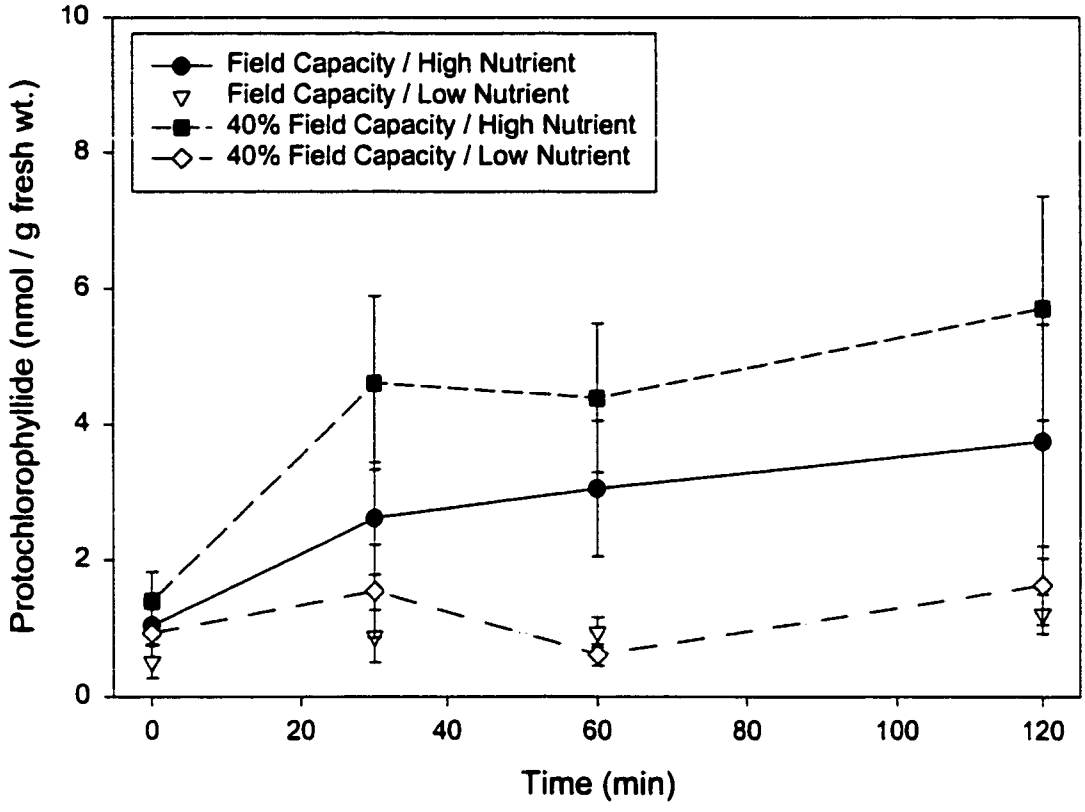


Figure 3.2. Protochlorophyllide accumulation was measured in water and nutrient stressed 4-leaf **corn** 0, 30, 60, and 120 min after being placed in darkness. Plants were grown in washed silica sand and were watered daily with one-half strength Hoagland's nutrient solution (High-nutrient) or tap water (Low-nutrient). Soil moisture status was determined by weighing cone-tainers and adjusting to field capacity or 40% of field capacity.

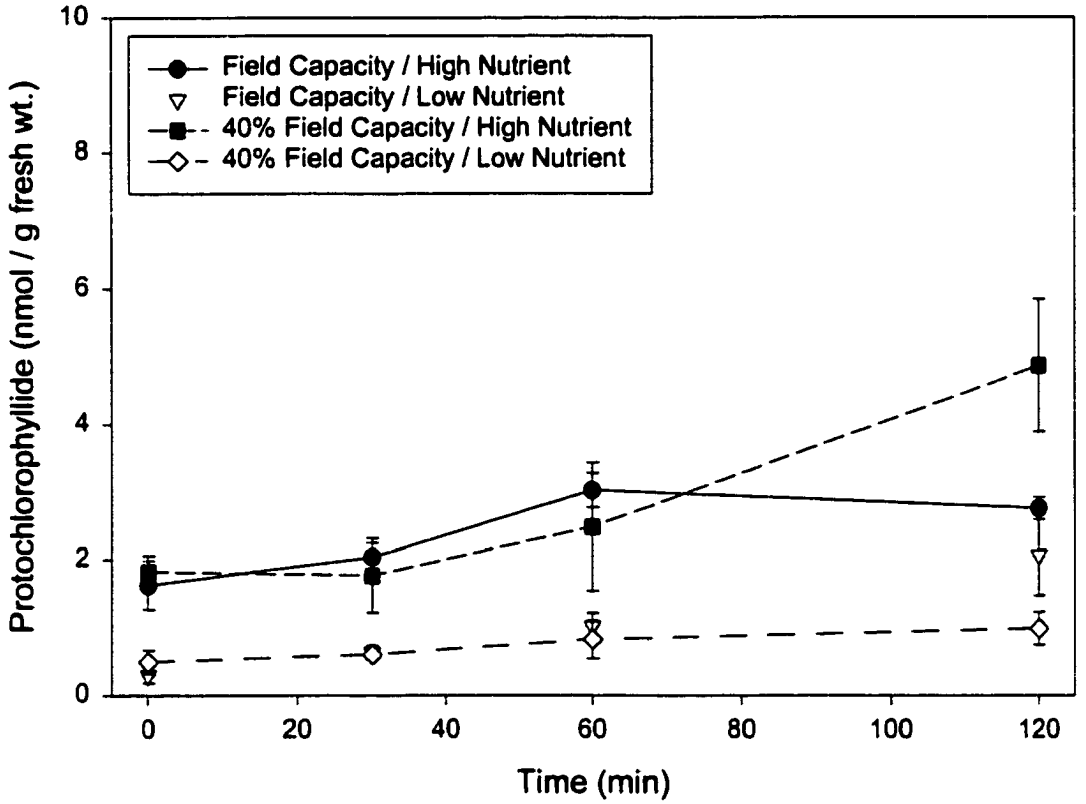


Figure 3.3. Protochlorophyllide accumulation was measured in water and nutrient stressed 4-leaf **wheat** 0, 30, 60, and 120 min after being placed in darkness. Plants were grown in washed silica sand and were watered daily with one-half strength Hoagland's nutrient solution (High-nutrient) or tap water (Low-nutrient). Soil moisture status was determined by weighing cone-tainers and adjusting to field capacity or 40% of field capacity.

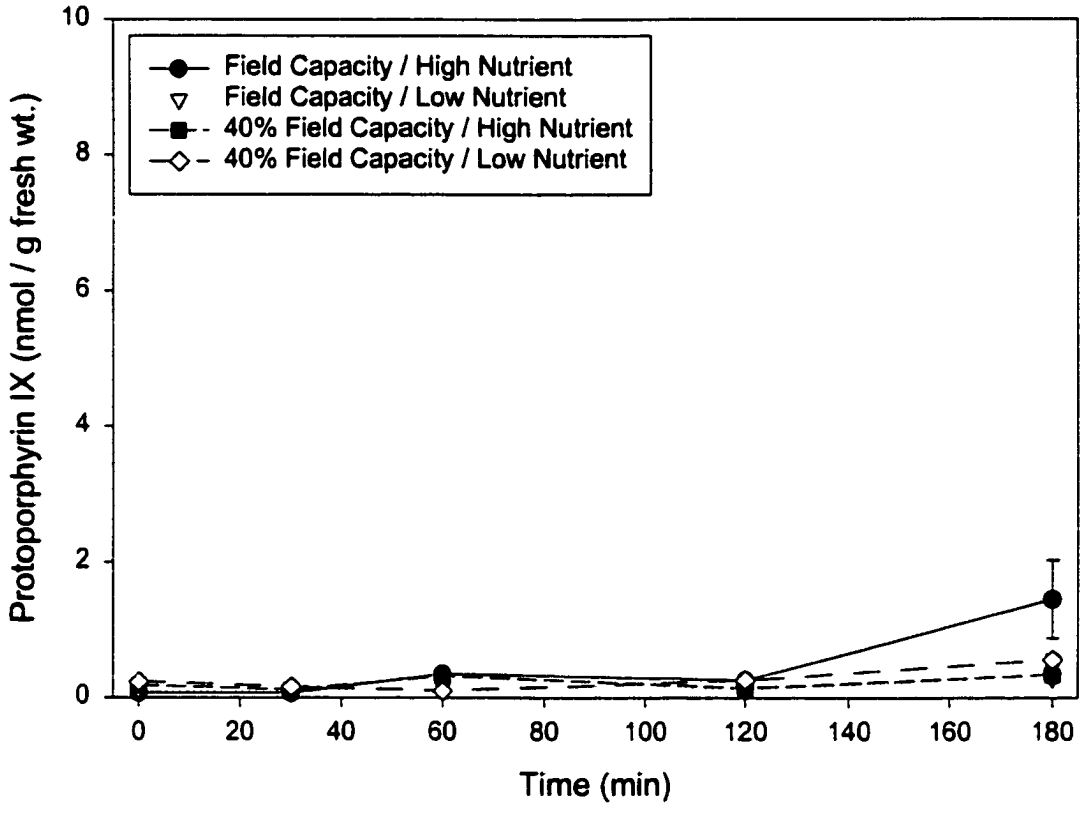


Figure 3.4. Protoporphyrin IX accumulation was measured in water and nutrient stressed 4-leaf **corn** 0, 30, 60, 120, and 180 min after being treated with 10X carfentrazone-ethyl. After herbicide treatment, plants were placed in the dark and pulse with 50 Πmol m^{-2} s^{-1} light for 1 min every 10 min. Plants were grown in washed silica sand and were watered daily with one-half strength Hoagland's nutrient solution (High-nutrient) or tap water (Low-nutrient). Soil moisture status was determined by weighing cone-tainers and adjusting to field capacity or 40% of field capacity.

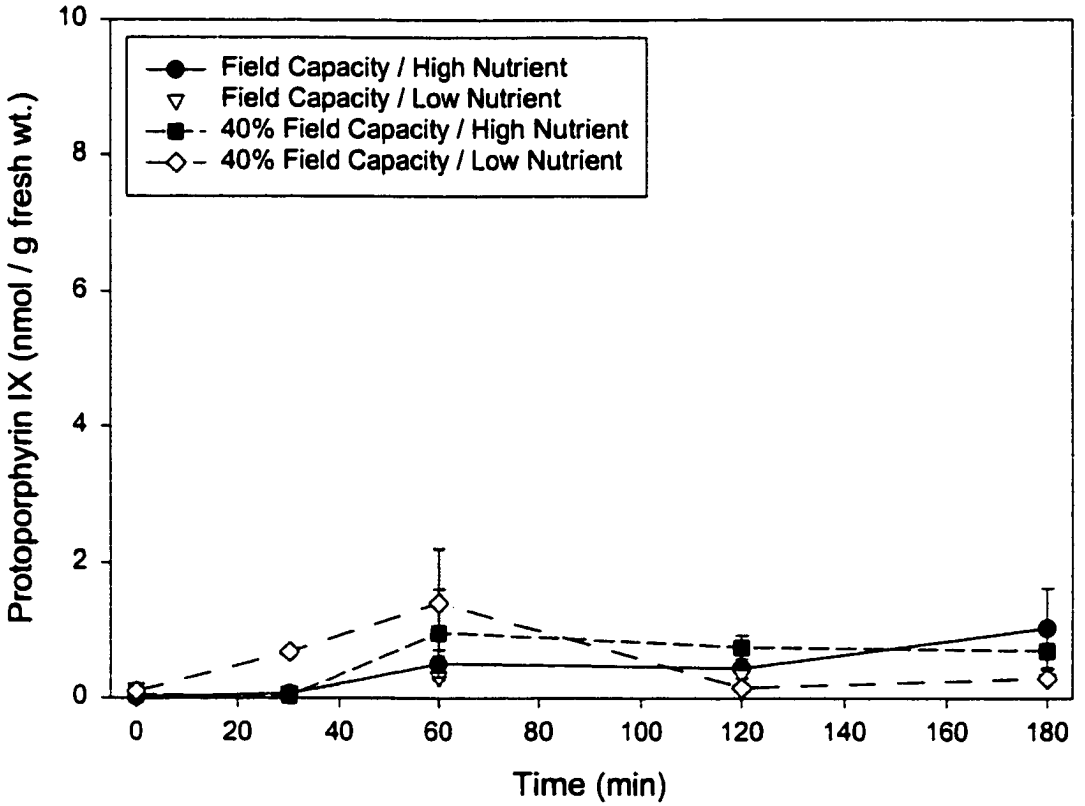


Figure 3.5. Protoporphyrin IX accumulation was measured in water and nutrient stressed 4-leaf **wheat** 0, 30, 60, 120, and 180 min after being treated with 10X carfentrazone-ethyl. After herbicide treatment, plants were placed in the dark and pulse with 50 Πmol m^{-2} s^{-1} light for 1 min every 10 min. Plants were grown in washed silica sand and were watered daily with one-half strength Hoagland's nutrient solution (High-nutrient) or tap water (Low-nutrient). Soil moisture status was determined by weighing cone-tainers and adjusting to field capacity or 40% of field capacity.

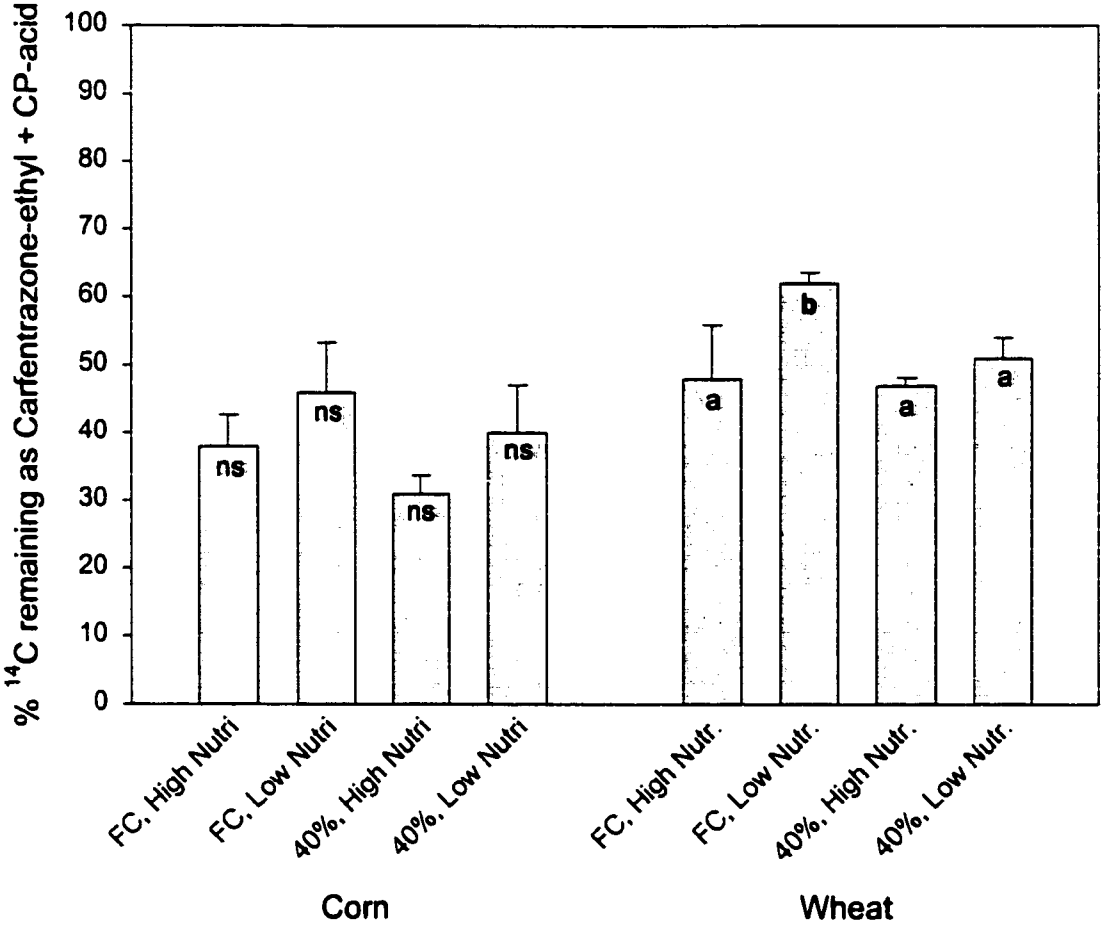


Figure 3.6. Carfentrazone-ethyl metabolism was determined 1 HAT in water and nutrient stressed corn and wheat. Results are presented as percentage of ^{14}C recovered from the treated leaf which remained as phytotoxic carfentrazone-ethyl or carfentrazone-chloropropionic acid.

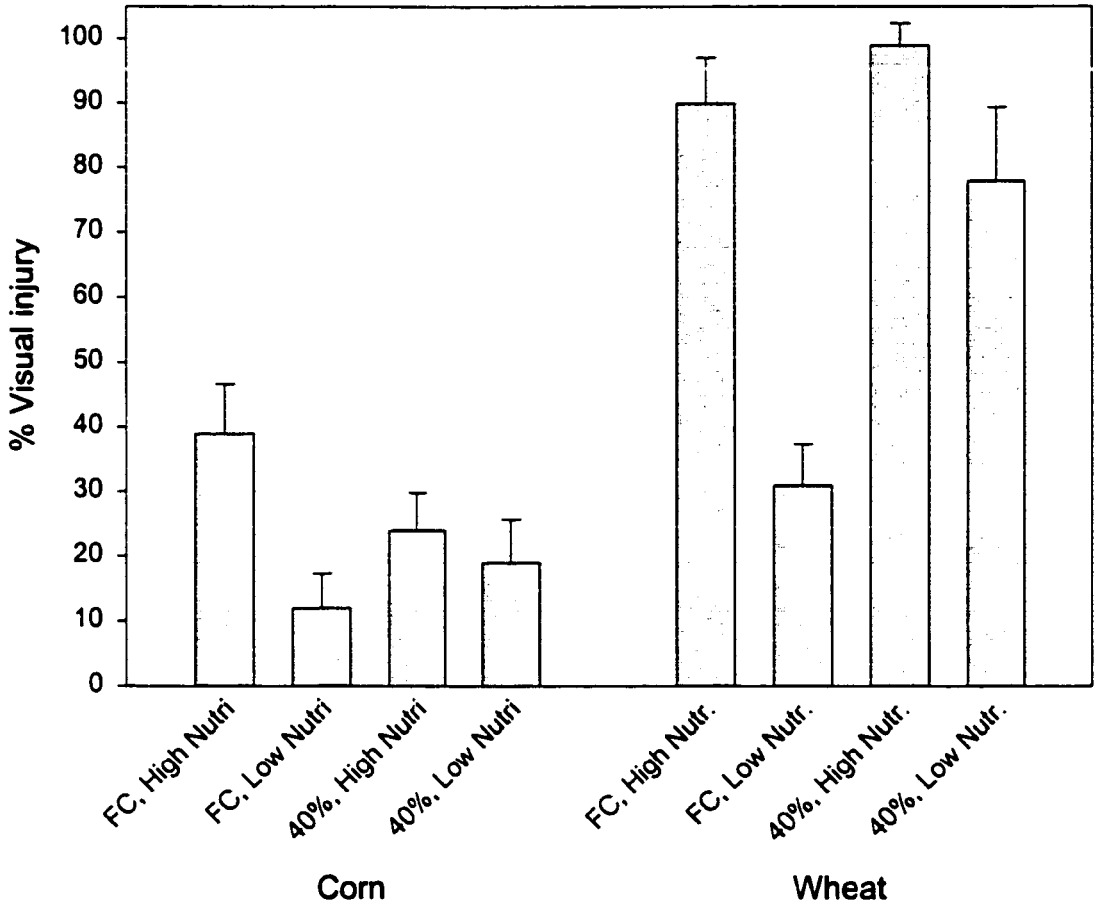


Figure 3.7. Visual injury was evaluated in water and nutrient stressed corn and wheat treated with 2X rate of carfentrazone-ethyl.

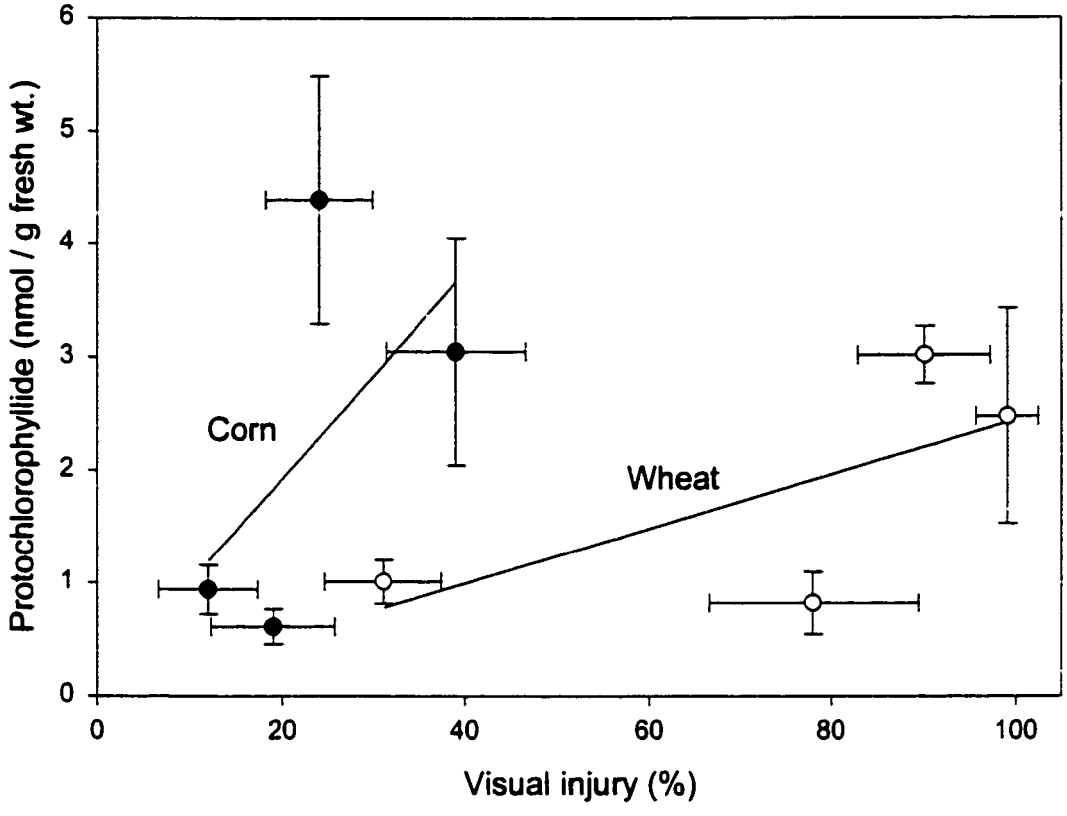
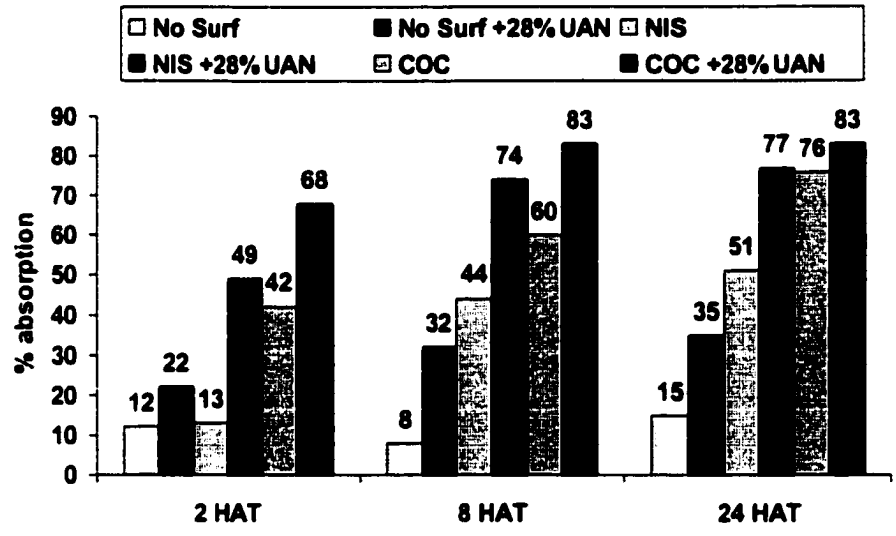


Figure 3.8. Accumulation of protochlorophyllide in water and nutrient stressed corn and wheat after 60 min of darkness *versus* visual injury caused by carfentrazone-ethyl treatment.

APPENDIX

Figure A1. F8426 Absorption in Sunflower-1996



LSD (0.05) = 18

Included in the appendix are results from experiments in which only a single trial was conducted. The experiments were conducted using the methodology in Chapter 1. Figure A1 is an extension of Figure 1.1. The sunflower data was not repeated and, therefore, not included in the first chapter. Carfentrazone absorption in sunflower appeared to be similar to corn and velvetleaf and was increased by the addition of UAN and surfactant to the treatment solution.

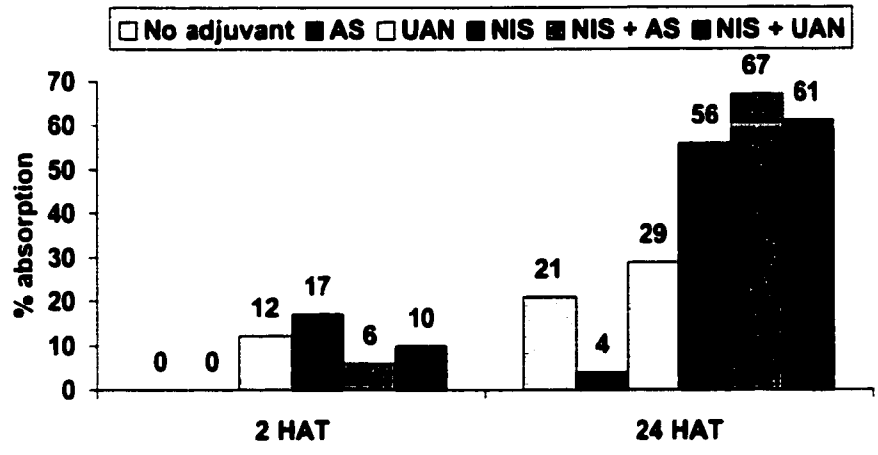
Table A1. Carfentrazone-ethyl Absorption--Cuticular Fraction

•All treatments were < 3% of applied ^{14}C

Adjuvants	Corn	Soybean	Velvetleaf
	% of absorbed ^{14}C		
NIS	10.7	1.1	4.4
NIS + UAN	6.2	1.7	2.6
COC	1.1	0.6	5.8
COC+UAN	0.6	0.5	2.1
LSD (0.05)	3.7	0.4	1.5

In 1995, the first year of the study, treated leaves were dipped 10 times in 5 ml 9:1 (v/v) hexane:acetone following the aqueous methanol rinse during harvest. This was done to remove the cuticular fraction of the leaf surface. The hexane:acetone solution was then counted by liquid scintillation to determine the amount of herbicide absorbed in the cuticle. Less than 3% of applied ^{14}C and less than 10% of absorbed ^{14}C were found in the cuticular fraction.

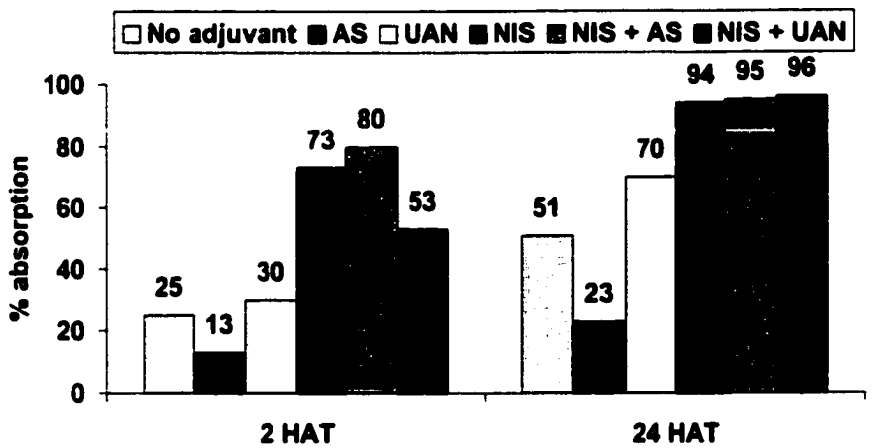
Figure A2. Effect of Nitrogen Form on Absorption of F8426 in Corn



LSD (0.05) = 11

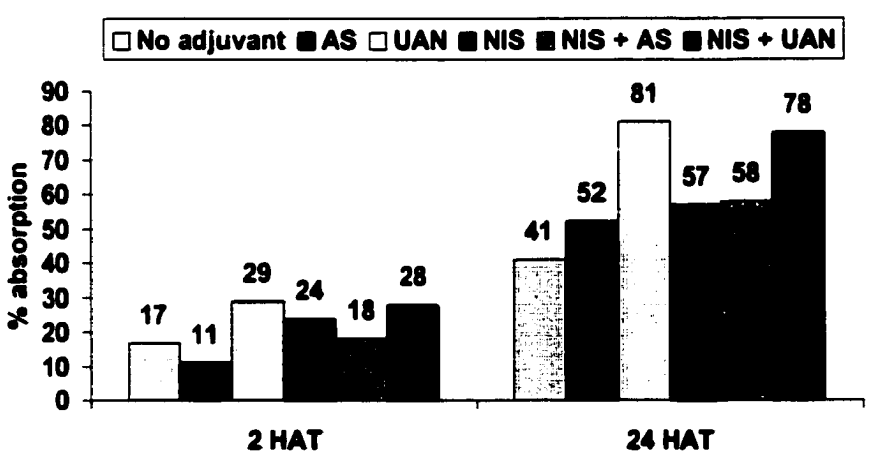
The effect of nitrogen form on carfentrazone-ethyl and CP-acid absorption was determined in corn, soybean and velvetleaf (Figures A2-A7). Plants were grown in cone-tainers in the growth chamber during the winter of 1995-96; otherwise, plants were treated as described in Chapter 1. In general, the addition of nitrogen to the spray solution increased carfentrazone absorption and was more of a factor with the CP-acid. Urea ammonium nitrate (UAN) appeared to increased absorption more than ammonium sulfate (AS) in velvetleaf, especially with CP-acid (Figure A7). Velvetleaf absorption was maximized with NIS + UAN, but this combination did not provide the most absorption in corn (NIS + AS was greater). The effect of nitrogen form appears to be species specific.

Figure A3. Effect of Nitrogen Form on Absorption of F8426 in Soybean



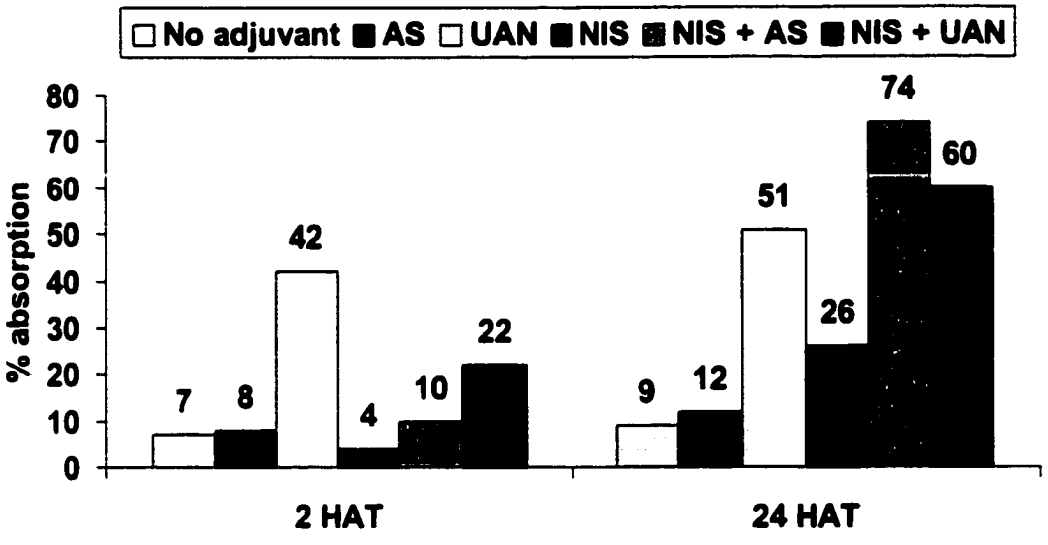
LSD (0.05) = 13

Figure A4. Effect of Nitrogen Form on Absorption of F8426 in Velvetleaf



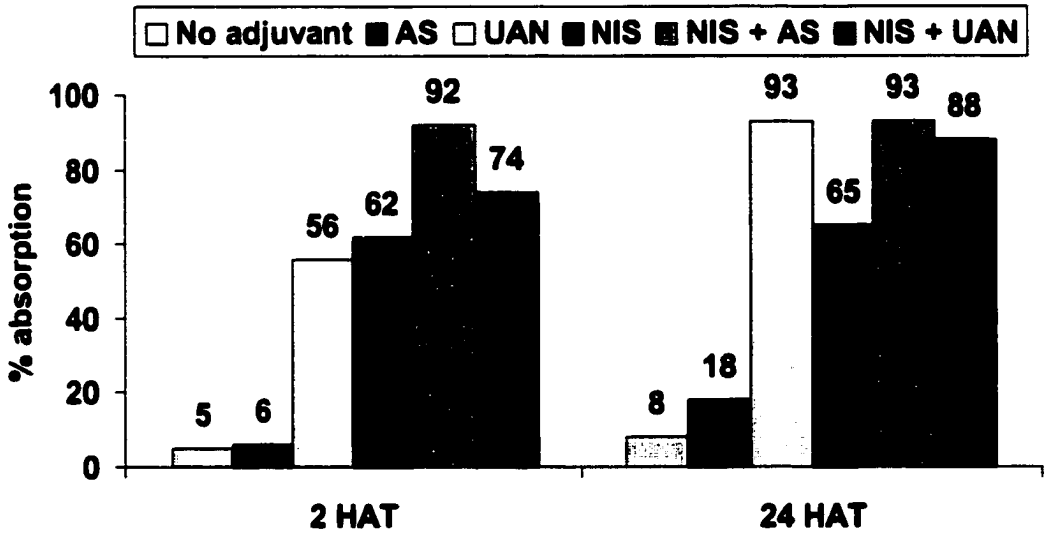
LSD (0.05) = 17

Figure A5. Effect of Nitrogen Form on Absorption of F8426 CP-acid in Corn



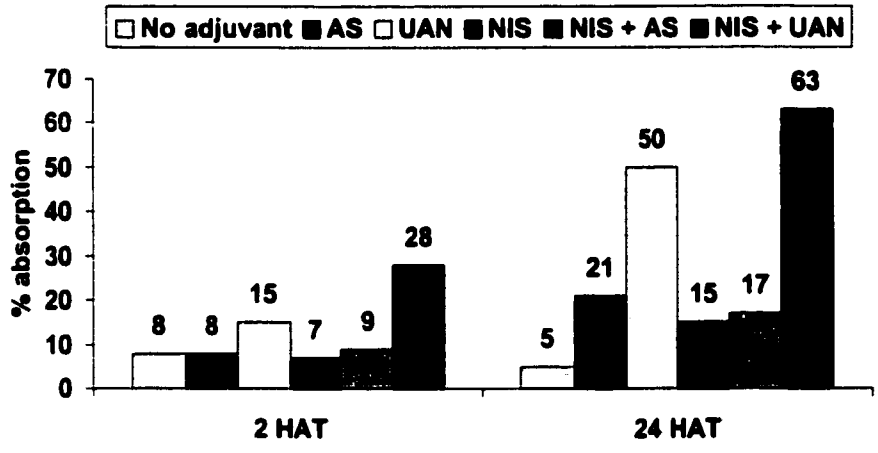
LSD (0.05) = 10

Figure A6. Effect of Nitrogen Form on Absorption of F8426 CP-acid in Soybean



LSD (0.05) = 11

Figure A7. Effect of Nitrogen Form on Absorption of F8426 CP-acid in Velvetleaf



LSD (0.05) = 9

Table A2. F8426 Absorption by Corn-1995

	2 hr.		8 hr.		24 hr.	
Surfactants	-N	+N	-N	+N	-N	+N
	---------- % ----------					
X-77	20	32	38	42	68	54
COC	77	92	91	95	95	94
Silwet L-77	14	49	52	83	53	91
Dash	83	75	97	94	98	96
No Surfactant	-	25	3	22	1	34
LSD			13			

During the first year of the study, additional surfactants were analyzed. Silwet L-77 (a silicon surfactant) and Dash were included with NIS, COC, and UAN. The silicon surfactants were developed to rapidly increase absorption via stomatal infiltration and droplet spreading. Neither surfactant performed as well as the other combinations (Tables A2-A4) and both surfactants are more expensive than NIS or COC. Because Silwet L-77 and Dash were not superior and cost prohibitive to producers they were dropped from the experiment to conserve time, labor, and laboratory resources.

Table A3. F8426 Absorption by Soybean-1995

Surfactants	2 hr.		8 hr.		24 hr.	
	-N	+N	-N	+N	-N	+N
	---------- % ----------					
X-77	88	83	91	93	86	92
COC	97	96	98	98	99	98
Silwet L-77	69	94	83	93	84	95
Dash	90	80	97	93	98	96
No Surfactant	19	19	39	48	26	52
LSD (0.05)			8			

Table A4. F8426 Absorption by Velvetleaf-1995

Surfactants	2 hr.		8 hr.		24 hr.	
	-N	+N	-N	+N	-N	+N
	---------- % ----------					
X-77	42	69	50	70	63	79
COC	31	73	43	70	62	71
Silwet L-77	26	66	36	63	59	86
Dash	44	25	63	45	63	54
No Surfactant	-	-	-	-	36	-
LSD (0.05)			13			

Table A5. Carfentrazone metabolism in Sunflower—1996.

	2 HAT	8 HAT	24 HAT	LSD (0.05)
	% carfentrazone-ethyl + CP-acid remaining			
Corn	6	1	0	2
Soybean	71	36	16	11
Velvetleaf	96	88	77	7
Sunflower	94	95	91	ns

Carfentrazone metabolism in sunflower was determined in 1996 (Table A5.) Sunflower did not metabolize carfentrazone within 24 HAT. Greater than 90% of carfentrazone remained as parent product (ethyl ester or CP-acid) in sunflower 24 HAT. Compared to velvetleaf, sunflower is more resistant to carfentrazone, yet carfentrazone was not detoxified in sunflower. Further research is needed to determine if the results are an artifact and to evaluate other mechanisms that might be contributing to the tolerance of sunflower to carfentrazone.