Clomazone: Toxicity, Biotransformation, Resistance and Interaction with P450 Inhibitors in Rice (*Oryza sativa*) and Watergrasses (*Echinochloa* spp.)

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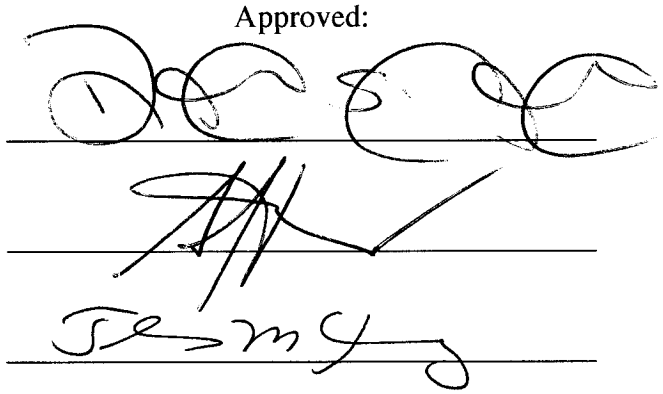

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ABSTRACT

Rice (*Oryza sativa*), early watergrass (*Echinochloa oryzoides*; EWG; biotypes resistant (R) and susceptible (S) to thiocarbamates and aryloxyphenoxy propionate herbicides), and late watergrass (*Echinochloa phyllopogon*; LWG; R and S biotypes) were exposed to clomazone for 7 d. Whole-plant change in fresh weight and ß-carotene concentrations were measured. For growth, the No Observed Effect Concentrations (NOECs) were 7.9, 0.21, 0.21, 0.46, and 0.46 μM clomazone for rice, EWG (R), EWG (S), LWG (R), and LWG (S), respectively, while the concentrations causing 25% inhibition in response (IC25s) were 5.6, 0.46, 0.42, 0.92 and 0.79 μM clomazone, respectively. For rice, EWG (R), EWG (S), LWG (R), and LWG (S), the ß-carotene NOECs were 0.21, < 0.08, < 0.08, 0.08 and 0.46 μM clomazone, respectively and IC25s were 0.42, 0.08, 0.08, 0.33 and 0.54 μM, respectively. At a field-relevant clomazone application rate resistance to clomazone was observed in EWG (R) and LWG (R) as measured by growth and ß-carotene, respectively. Due to similar sensitivity between rice and late watergrass, use of clomazone in rice culture may require the use of a safening technique.

Rice and EWG (S) were exposed to ^{14}C-labeled clomazone to determine accumulation, biotransformation and mass balance. In terms of extractable ^{14}C, rice contained more total residues than EWG ($p < 0.05$), but the concentration in EWG was significantly higher ($p < 0.01$). More metabolized residue was measured in EWG compared to rice ($p < 0.01$). Both species produced hydroxylated forms of clomazone, β-D-glucoside conjugates, and unidentified polar metabolites. The suspected active

metabolite, 5-ketoclomazone, was found at a significantly higher concentration in EWG vs. rice (21 ± 2 vs. 5.7 ± 0.5 pmol g^{-1}, respectively; $p < 0.01$). Qualitatively and quantitatively, more clomazone metabolism was observed in the relatively sensitive EWG compared to rice.

R and S biotypes of EWG and LWG were exposed to clomazone alone and in combination with P450 inhibitors. Growth reduction (GR_{25}, GR_{50}, and GR_{75}) values were lower for S biotypes than for R biotypes ($p < 0.05$) with exposure to clomazone alone indicating resistance to clomazone in the R biotypes. In combination with clomazone disulfoton increased 20-d growth in LWG (R) by as much as 5-fold, compared to application of clomazone alone. Oxydemeton methyl increased 20-d growth by as much as 6-fold. Disulfoton and oxydemeton methyl reduced β-carotene levels compared to treatment with clomazone alone. For EWG (S), disulfoton increased 4-d growth by 46% and increased β-carotene levels by 52%. Demeton-S increased β-carotene levels by 48%. At 6800 g ai ha^{-1} PBO increased growth and β-carotene levels by 28 and 48%, respectively. These results indicate that P450 enzymes serve both to activate and detoxify clomazone.

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Section I. Introduction

Clomazone chemistry

Clomazone, 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone, is a bleaching herbicide produced by the FMC Corporation under the commercial names Command® and Cerano®. The structure of clomazone is shown in Fig. 1 and physical properties are shown in Table 1.

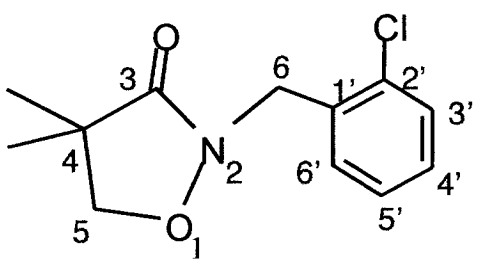

Figure 1. Structure of clomazone

Table 1. Clomazone chemical/physical properties from Herbicide Handbook (1994) unless noted otherwise

Property	Value
Molecular Formula	$C_{12}H_{14}ClNO_2$
Molecular Weight	239.7
Density	1.192 g/mL @ 20°C
Boiling Point	275.4°C @ 760 mm Hg
Vapor Pressure	1.44 X 10^{-4} mm Hg @ 25°C
Water Solubility	1100 mg/L @ 25°C
Henry's constant (K_H)[1]	1.68 X 10^{-6}
K_{ow}	350
K_{oc}	300 mL/g
K_d[2]	0.47-5.30

1. Calculated: K_H = P/SRT, where P = vapor pressure = 1.89 X 10^{-7} atm (at 25°C), S = water solubility = 1100 mg/L = 4.59 X 10^{-3} mol/L, R = ideal gas constant 0.082057 L atm $mol^{-1}K^{-1}$, T = temperature = 25°C.
2. From Loux *et al.* (1989b).

Clomazone Use

Effective against grasses and broadleaf weeds, clomazone is used on a number of crops, including soybeans, cotton, tobacco and dry-seeded rice (FMC Corporation, 2001). Cerano® is a newer clomazone formulation, designed specifically for aerial application to flooded fields. This formulation consists of 5% microencapsulated active ingredient (ai) in extruded clay granules. Efficacy studies have shown this formulation to work well for weed control, but severe phytotoxicity to rice plants has occurred (Cheetham, pers. comm. 2001). Nonetheless, California rice growers need new herbicides for control of watergrasses, which have developed resistance to much-used herbicides, such as thiobencarb, molinate, bensulfuron-methyl and bispyribac-sodium (Fischer *et al.*, 2000).

Environmental Fate

The environmental fate of clomazone has been studied in a number of systems and a variety of soils. Reported half-lives of clomazone range from 5-117 days. Differences in observed half-lives are due to a number of factors, including soil texture, temperature, soil moisture, application methods, and herbicide formulation (Loux *et al.*, 1989a, Mills *et al.*, 1989, Curran *et al.*, 1992, Kirksey *et al.*, 1996, Mervosh *et al.*, 1995a, 1995b, Locke *et al.*, 1996, and Thelen *et al.*, 1988).

Clomazone does not bind tightly to soils, as demonstrated by Freundlich constants ranging from 0.47-5.30 (Loux *et al.*, 1989b) and 3.29 (Mervosh *et al.*, 1995c). Although it was found to bind to clay, clomazone binding correlated only with soil organic matter content in real soil samples (Loux *et al.*, 1989b). Temperature, moisture and incubation

time all affect the soil binding characteristics of clomazone (Mervosh *et al.*, 1995c, Locke *et al.*, 1996).

Clomazone water solubility is 1100 mg/L and its vapor pressure is 1.44×10^{-4} mm Hg (Herbicide Handbook, 1994). The resulting dimensionless Henry's constant of 1.6×10^{-6} (Table 1) indicates that clomazone volatilization from water will be insignificant. However, studies have shown significant volatilization of clomazone from soil, which can be minimized by incorporation (Thelen *et al.*, 1988). Locke *et al.* (1996) found that soil moisture decreased the volatilization rate of clomazone from soil, while Mervosh *et al.* (1995a, 1995b) observed no moisture effect. As expected, volatilization has been found to increase with temperature (Locke *et al.*, 1996, and Mervosh *et al.*, 1995a, 1995b).

Degradation of clomazone was found to be dependent on soil microbial activity, with the overall degradation rate (including mineralization rate) dependent on temperature and moisture (Mervosh *et al.* 1995a). Soil texture is also expected to have an effect on microbial degradation rates due to its effect on availability of clomazone to microbes. Bioavailability of clomazone to corn was greater in a silt loam soil with 1.3% organic matter than in a silty clay loam with 5.8% organic matter (Loux *et al.*, 1989a). In contrast, Schmelzer *et al.* (2005) have shown that microbial transformation of thiobencarb to deschlorothiobencarb is greatly enhanced by a 2% rice-straw soil amendment. One explanation for this enhancement is that the straw serves as a carbon source for the soil microbes, providing ample nutrients for rapid growth. Microbial degradation of clomazone will likely be similarly affected by addition of rice straw.

Abiotic degradation of clomazone is reported to be insignificant (Herbicide Handbook, 1994). Specifically, clomazone degradation by sunlight in aqueous solutions is reportedly slow and hydrolysis in acidic, basic and neutral aqueous solutions reportedly does not occur. However, these conclusions are apparently from unpublished studies, as no reference is given in the Herbicide Handbook. An exhaustive literature search has found no studies of clomazone photolysis or hydrolysis. Section III of this dissertation presents data that indicate possible photo-enhancement of clomazone mineralization.

The effect of herbicide formulation on clomazone movement in the environment has been studied. Off-site movement of clomazone impregnated onto dry fertilizer was less than for clomazone applied in water carriers (Halstead and Harvey 1988). Starch encapsulation did not affect mobility in soil, but did reduce volatilization (Mervosh *et al.*, 1995b).

Phytotoxicity

The most readily observable symptom of clomazone toxicity is bleaching. Duke *et al*, (1985) reported that clomazone caused ultrastructural damage to chloroplasts, reduced levels of carotenoids and phytols, inhibited starch mobilization, and blocked both diterpene and tetraterpene synthesis in morningglory. Duke *et al.*, (1991) found inhibition of terpenoid synthesis, sesquiterpenoid accumulation, and damage to etioplasts in cotton exposed to clomazone.

Mueller *et al.*, (2000) found that a clomazone metabolite, 5-ketoclomazone, inhibits the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS). This enzyme catalyses an early step in the non-mevalonate isoprenoid biosynthetic pathway (Mueller *et al.*, 2000). Among the compounds produced by this pathway are the growth-regulating

gibberellins and the carotenoid pigments (Sponsel 2002). Ordinarily, plants are protected from oxidative damage by the anti-oxidant capabilities of the carotenoids, particularly β-carotene (Taiz and Zeiger 1998). Reduction of β-carotene levels due to exposure to clomazone leads to the bleaching effect. Ketoclomazone was found to be the most active among 3,5-isoxazolidinones during early studies of this class of herbicides (Chang *et al.*, 1987). However, it was found to be relatively unstable in field conditions, and further research led to the more stable, but still very active, clomazone.

Clomazone injury in rice

Clomazone-induced injury has been observed in water- and drill-seeded rice with chlorosis observed at application rates as low as 0.28 kg ha^{-1} and reduced grain yield with application as low as 0.84 kg ha^{-1} (Bollich *et al.* 2000). In experiments on dry-seeded rice, fourteen cultivars suffered chlorosis due to clomazone exposure as low as 0.56 kg ai ha^{-1}, but all went on to produce normal grain yields with clomazone application as high as 1.1 kg ai ha^{-1} (Scherder *et al.* 2004). Bleaching and reduction in rice grain yield were observed in a medium-grain rice cultivar with application of 1.12 kg ai ha^{-1} clomazone (Zhang *et al.* 2004). The most commonly grown rice in California is a medium-grain variety, M202 (California Cooperative Rice Research Foundation 2005). Further, it has been found that rice injury by clomazone is more severe on silt loam soils than on silty clay soils (Jordan *et al.* 1998). Many rice fields in California lie on the eastern side of the Sacramento Valley where the soils are classified as loam to sandy loam (TenBrook *et al.* 2004), which could potentially enhance rice damage by clomazone.

Biotransformation

Plants are capable of extensive biotransformation of xenobiotics through three types of reactions. Phase I reactions include oxidation, reduction and hydrolysis. Phase I reactions usually detoxify compounds, increase water solubility and create substrates for Phase II reactions. In Phase II reactions glutathione, glucosyl or malonyl conjugates are formed, and in Phase III reactions Phase II metabolites are further conjugated or are converted to insoluble residues which are stored in vacuoles or are incorporated into the plant structure (Hatzios, 1997). Cytochrome P450 enzymes and glutathione S-transferases both provide very important detoxification routes for plants, and differential activity of these two enzyme systems is key to herbicide selectivity (Barrett *et al.*, 1997; Cole *et al.*, 1997). In the case of clomazone, which apparently requires oxidation to an active form, high P450 activity would be expected to increase toxicity.

Weimer *et al.* (1992) studied clomazone metabolism in cell cultures of soybean (clomazone-tolerant) and velvetleaf (clomazone-sensitive). No qualitative differences in metabolites were observed between soybean and velvetleaf. Oxidative cleavage of clomazone to produce 2-chlorobenzyl alcohol, followed by conjugation to glucose was the major metabolic reaction observed in both species.

The study by Meuller *et al.*, (2000), mentioned previously, raises the interesting possibility that 5-ketoclomazone, rather than the parent compound, is the herbicidally active compound via the inhibition of the enzyme DXS. 5-Ketoclomazone was identified by Liu *et al.* (1996) as a metabolite formed by a number of bacterial species. Formation

of ketones is also a common metabolic pathway in plants (The Royal Society of Chemistry, 1998). ElNaggar *et al.* (1992) reported formation of 5-ketoclomazone by soybean, a species tolerant to clomazone. Weimer *et al.* (1992) found that velvetleaf produced clomazone metabolites at a faster rate than soybean, which could explain the relative insensitivity of soybean. Similarly, sensitive tomato shoots were found to metabolize clomazone to a greater extent than tolerant bell pepper shoots, while pepper roots metabolized a larger percentage of clomazone than tomato roots (Weston and Barrett, 1989). This agrees with the findings of Mueller *et al.* (2000) that a clomazone metabolite, 5-ketoclomazone, is the herbicidally active compound and that it targets the plastidic, non-mevalonate isoprenoid synthetic pathway, which functions in the shoots.

Liu *et al*, (1996) identified 13 microbial metabolites of clomazone in studies of metabolism by pure bacterial cultures. The most commonly formed metabolites were 5-hydroxy clomazone, 5-ketoclomazone, hydroxymethyl clomazone, 6-ketoclomazone and 3'-hydroxymethyl clomazone. Mervosh *et al.* (1995) found one unidentified metabolite in their study of microbial degradation. A study by FMC Corporation (Froelich *et al.*, 1984) found one anaerobic microbial metabolite, N-[(2'-chlorophenyl)methyl]-3-hydroxy-2,2-dimethyl propanamide. This metabolite was also identified by Liu *et al*, (1996), but in an aerobic system.

Herbicide Resistance in Watergrasses

Echinochloa spp. have become difficult to control in California rice culture due to the development of herbicide-resistant biotypes (Fischer *et al.* 2000a). This resistance to thiocarbamates and aryloxyphenoxy propionate herbicides has been linked to cytochrome

P450 activity (Fischer *et al.* 2000b). Higher levels of P450 enzymes and activity were found in resistant vs. susceptible biotypes of *E. phyllopogon* and enzyme induction was found to be substrate-specific, indicating involvement of P450 isozymes (Yun *et al.* in press). Recently, clomazone-resistant biotypes of *Echinochloa* spp. have been observed in the field (Fischer and Cheetham, in press).

Research Objectives

Rice growers in California need new herbicides to control watergrasses (*Echinochloa* spp.), which have developed resistance to currently-used herbicides. The biochemistry of clomazone toxicity and detoxification in rice and watergrasses has not been previously studied. To be able to use clomazone for weed control without harming rice, the relative sensitivity of rice vs. watergrass needs to be known. The objective of Section II was to determine relative sensitivity to clomazone of rice (*Oryza sativa*) and early and late watergrasses (*Echinochloa oryzoides* and *Echinochloa phyllopogon*, respectively). This was accomplished by measuring effects of clomazone on growth and β-carotene levels. Growth is usually considered a general, integrative physiological endpoint, but it is possible that clomazone affects growth directly by interfering with gibberellin synthesis via the non-mevalonate isoprenoid pathway. Measurement of β-carotene levels provides a direct measure of inhibition of the same pathway.

If selectivity mechanisms are understood, it may be possible to develop crop safening schemes that exploit differences between weed and crop species, such that weeds can be controlled without crop damage. The objective of Section III was to determine differences in accumulation and biotransformation of clomazone between rice and early watergrass (a biotype susceptible to thiocarbamates, aryloxyphenoxy-

propionates and bispyribac-sodium). To further elucidate clomazone selectivity and resistance mechanisms, and to investigate possible clomazone safeners, the objectives of Section IV were to determine if watergrasses resistant to other herbicides are also resistant to clomazone, and to study the effect of P450 inhibitors on clomazone toxicity in early and late watergrasses.

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Section II: Comparative actions of clomazone on ß-carotene levels and growth in rice (*Oryza sativa*) and watergrasses (*Echinochloa* spp.)

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1 INTRODUCTION

Clomazone is an herbicide produced by the FMC Corporation under the trade names Command® and Cerano 5 MEG®. Effective against grasses and broadleaf weeds, clomazone is used on a number of crops, including soybeans, cotton, tobacco and dry-seeded rice.[1]

While clomazone has shown promise for weed control in rice culture, excessive crop injury has been observed in early field tests in California (Albert Fischer, pers. comm.). Nonetheless, California rice growers need new herbicides for control of watergrasses, which have developed resistance to herbicides such as thiobencarb, molinate, bispyribac-sodium and bensulfuron-methyl.[2-4] Safe and effective use of clomazone on rice in California may be possible, but better understanding of differential clomazone sensitivity between rice and weeds is needed.

By inhibiting production of pigments, particularly carotenoids, clomazone exposure leads to bleaching, as plants are left defenseless against oxidative damage caused by absorption of ultraviolet light.[5] The role of 5-ketoclomazone, the active metabolite of clomazone, in blocking the ß-carotene biosynthetic pathway is shown schematically in Fig. 2.[6-8]

This investigation was designed to provide information regarding the comparative toxic actions of clomazone on ß-carotene levels and growth in rice (*Oryza sativa* L.),

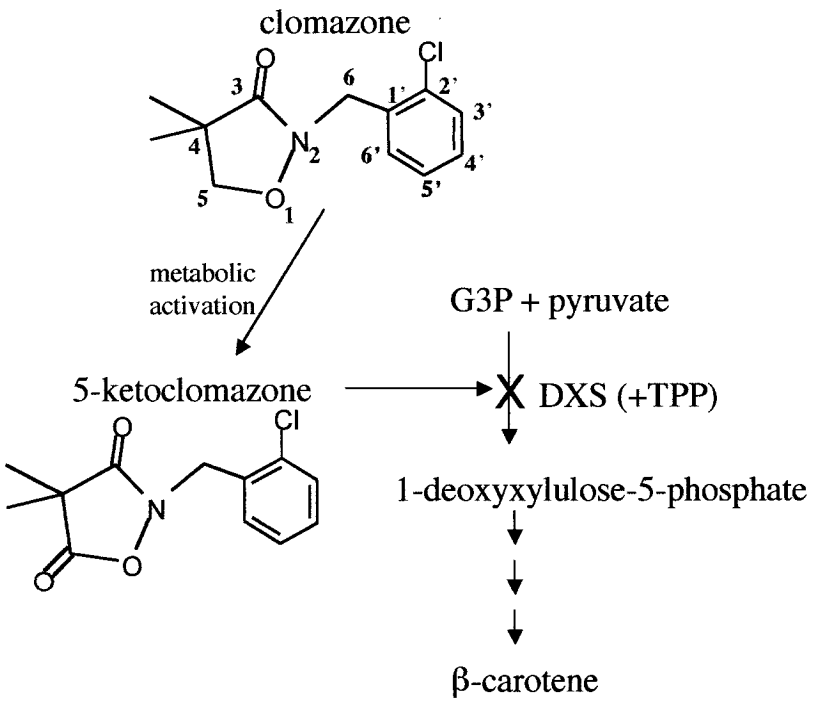

Figure 2. Schematic of clomazone action on the plastidic ß-carotene synthetic pathway; the clomazone metabolite, 5-ketoclomazone, blocks DXS; G3P = glyceraldehyde-3-phosphate; DXS = 1-deoxyxylulose-5-phosphate synthase; TPP = thiamine pyrophosphate cofactor.

early watergrass (*Echinochloa oryzoides* (Ard.) Fritsch), and late watergrass (*Echinochloa phyllopogon* (Stapf) Koss). The mechanism for thiobencarb resistance to watergrasses is unknown.[3] However, if it is a very general mechanism, such as glutathione or glucose conjugation, it should impart resistance to clomazone as well. To determine if this might be the case, biotypes that have shown resistance to thiobencarb were studied along with biotypes that are susceptible to thiobencarb. Growth is a general physiological endpoint that integrates numerous biochemical effects, while ß-carotene

content provides a direct measure of the functioning of the non-mevalonate isoprenoid synthetic pathway.

2 MATERIALS AND METHODS

2.1 Experimental design

Seeds of rice (*O. sativa* L.; variety M202) and thiobencarb-resistant (R) and thiobencarb-susceptible (S) biotypes of early and late watergrasses (*E. oryzoides* and *E. phyllopogon*, respectively) were obtained from the California Rice Experiment Station (Biggs, CA). Seeds were germinated for 7-10 d in deionized water at room temperature. Rice seeds were pre-treated with 3% bleach for 5 min, then rinsed three times in deionized water prior to germination. Seedlings were blotted dry, weighed and placed in 250 x 50 mm test tubes (4 seedlings per tube = replicate) containing 36 mL 0.5X Hoagland's solution (pH 6.2 ± 0.2; Sigma Aldrich Corp., St. Louis, MO) plus technical grade clomazone (FMC Corporation, Philadelphia, PA) at concentrations ranging from 0.08-7.9 μM. Tubes were capped with polyurethane foam plugs and placed in a controlled growth chamber (30°C day:16°C night; 14 h day:10 h night; rel. humidity: 30% day:80% night; maximum photosynthetically active radiation: 800 μmol $m^{-2}s^{-1}$). Exposure tubes were randomized in the growth chamber and were not moved during the test. After a 7-d exposure period, plants were rinsed, blotted dry, weighed and frozen at –80°C prior to ß-carotene analysis. Plants (10-20 replicates) were analyzed at each exposure concentration for changes in growth. The non-destructive fresh-weight growth measurement was used so that the same plants could be used for subsequent β-carotene measurements. Three replicates were analyzed for ß-carotene levels.

2.2 Confirmation of test solution concentrations

Clomazone test solutions were extracted on C18 SPE columns (Alltech Associates, Deerfield, IL) and analyzed by HPLC (HP1090 with diode array detector, Agilent Technologies, Palo Alto, CA) to confirm initial concentrations. SPE columns were prepared with at least two bed volumes of methanol, followed by two bed volumes of 0.5X Hoagland's solution. After drying under vacuum, columns were eluted with 3 X 0.5 mL methanol and extract was volume reduced to 1.0 mL under N_2 gas; SPE extraction efficiency was 99.1 $\pm$ 2.6%. HPLC analysis was accomplished using an Alltech Partisil, ODS-3, 5 μm, C18 column (gradient elution; 0.01% acetic acid:acetonitrile; 0-10 min, 10:90; 10-15 min, to 20:80; 15-20 min, 20:80; 20-25 min, to 35:65; 25-35 min, 35:65; 35-45 min, to 78:22; 45-47 min to 10:90; 47-52 min, 10:90; 1.0 mL/min). Clomazone was detected by ultraviolet absorbance (240 nm) using a diode array detector and had a retention time of 43.6 min.

2.3 β-carotene analysis

Whole plants were extracted and pigment analysis was accomplished according to previously described methods with some modification.[9,10] Plants (4 per replicate) were homogenized (Tekmar tissue homogenizer, model TP 18/10 S1, Cincinnati, OH) in 12.5 mL 7:2 acetone:methanol solution. The tissue probe was rinsed twice with 5-10 mL 7:2 acetone:methanol. Rinsate was added to homogenate, which was then centrifuged for 5 min at 1500 rpm (495 g). Supernatant was transferred to a 100-mL round bottom flask. Solids were resuspended in extraction solvent, vortexed for 30 s, centrifuged again and supernatant was added to the round bottom flask. Ethanol (2-3 mL) was added to aid in

water removal, and then the extract was evaporated to dryness at 40°C under vacuum and redissolved in 3 mL hexane. Pigment extracts were loaded onto 200-mg silica SPE columns (Alltech Associates, Inc.) and ß-carotene was eluted with 2 x 3 mL of hexane. Extracts were volume-reduced under N_2 gas to a final volume of 4.5 mL. Acetone (0.5 mL) was added to bring the final solvent ratio to 9:1 (hexane:acetone). Absorbance at 436 nm was recorded and the extinction coefficient of 0.196 mL μg^{-1} [10] was used to calculate ß-carotene concentrations; extraction efficiency was 100 $\pm$ 6.2%.

2.4 Statistics

Linear interpolation was used to determine concentrations of clomazone producing 25 and 50% reductions in response compared to controls (IC25 and IC50, respectively; ICp v. 2.0, USEPA, Environmental Effects Research Laboratory, Duluth MN; ToxCalc v. 5.0.20, Tidepool Scientific Software, McKinleyville, CA). The IC25 and IC50 were both determined because, while the 50% effect level is commonly reported, some of the treatments in this study did not cause a 50% reduction in response. Thus the IC25 values were calculated to facilitate comparisons between groups. ANOVA with Dunnett's post-test was used to determine no observed effect concentration (NOEC) values (ToxCalc v. 5.0.20). Comparisons between IC values were made using ANOVA with Tukey-Kramer multiple comparison tests (Instat v. 3.05, GraphPad Software, San Diego).

3 RESULTS AND DISCUSSION

Table 2 shows IC25, IC50 and NOEC values calculated for both growth and ß-carotene endpoints. To facilitate sensitivity comparisons between thiobencarb-resistant

Table 2. Clomazone toxicity as measured by β-carotene levels and growth in rice and watergrasses (value ± sd).

Endpoint	Rice	EWGR[a]	EWGS[b]	LWGR[c]	LWGS[d]
ß-carotene					
IC25 (μM)	0.42 ± 0.26	0.08 ± 0.02	0.08 ± 0.02	0.33 ± 0.09	0.54 ± 0.15
IC50 (μM)	1.9 ± 0.3	0.33 ± 0.20	0.33 ± 0.04	> 3.9	0.88 ± 0.15
NOEC (μM)	0.21	< 0.08	< 0.08	0.08	0.46
Growth					
IC25 (μM)	5.6 ± 1.6	0.46 ± 0.06	0.42 ± 0.08	0.92 ± 0.451	0.79 ± 0.08
IC50 (μM)	> 7.9	2.1 ± 0.8	1.2 ± 0.2	> 3.9	3.7 ± 0.2
NOEC (μM)	7.9	0.21	0.21	0.46	0.46

[a] EWGR: Early Watergrass R (thiobencarb (TB) –resistant)
[b] EWGS: Early Watergrass S (TB-susceptible)
[c] LWGR: Late Watergrass R (TB-resistant)
[d] LWGS: Late Watergrass S (TB-susceptible)

and -sensitive biotypes of watergrasses, and between rice and watergrasses, ratios of IC25 and IC50 values were calculated and are shown in Table 3.

Figs. 3 and 4 show dose-response curves for the ß-carotene and growth endpoints, respectively. At lower clomazone concentrations, the early watergrass R and S curves are similar for both endpoints, but they diverge slightly at higher clomazone concentrations for the growth endpoint. This is supported by the finding of a significant difference between R and S early watergrass for the growth IC50s, but not for the IC25s (Table 3).

The R and S dose-response curves for late watergrass diverge for both endpoints (Figs. 3 and 4), with the R biotype responding less to higher clomazone concentrations (IC50 R/S ratios > 1.0; Table 3). While there was no significant difference in sensitivity for growth at lower clomazone concentrations (IC25 ratio ~1.0), for ß-carotene levels the S biotype was significantly less sensitive to clomazone than the R biotype (IC25 R/S ratio < 1.0; Table 3).

Table 3. Ratios of IC25 and IC50 values for thiobencarb-resistant vs. -susceptible watergrasses and for rice vs. watergrasses; ratios of ~ 1.0 indicate no difference in sensitivity; Resistant/Susceptible ratios > 1.0 indicates that the resistant biotype is less sensitive to clomazone than the susceptible biotype; Rice/Watergrass ratios > 1.0 indicate that rice is less sensitive to clomazone than the watergrass.

	IC25		**IC50**	
Resistant/Susceptible	**ß-carotene**	**Growth**	**ß-carotene**	**Growth**
EWG[a]	1.0	1.1	1.0	1.8[c]
LWG[b]	0.6[c]	1.2	> 4.5	> 1.1
Rice/Watergrass				
Resistant EWG	5.0[c]	12.3[c]	5.8[c]	> 3.8
Susceptible EWG	5.0[c]	13.5[c]	5.8[c]	> 6.6
Resistant LWG	1.3[c]	6.1[c]	< 0.48	~ 2[d]
Susceptible LWG	0.8[c]	7.1[c]	2.4[c]	> 2.1

[a]EWG: Early Watergrass
[b]LWG: Late Watergrass
[c] significant difference between numerator and denominator ($p < 0.01$)
[d] indeterminate values in numerator and denominator

For rice no significant difference was observed for the growth endpoint at any of the concentrations tested (NOEC = 7.9 μM), but the ß-carotene levels showed considerable sensitivity (NOEC = 0.21 μM). The IC25 calculated for growth is 5.6 μM, which is lower than the NOEC (7.9 μM). This is an artifact of the statistical methods used to derive these values. The NOEC calculation simply compared each treatment group to the control group, while the IC25 calculation involved a curve-smoothing technique to create a monotonically decreasing dose-response curve, effectively discounting the growth stimulation observed at the lowest clomazone concentrations. Both the NOEC and

the IC25 are well above levels of clomazone that would be applied to a rice field[1], thus the statistical artifact does not affect conclusions drawn from the data.

The ratios in Table 3 indicate that rice is considerably, and in many cases statistically significantly ($p < 0.01$), less sensitive to clomazone than either of the watergrasses for the growth endpoint. Differences in ß-carotene levels indicates that rice is less sensitive than early watergrass (R and S), but the results for late watergrass vary with clomazone concentration. At low clomazone concentrations, rice is more sensitive than the S biotype of late watergrass, but less sensitive than the R biotype (IC25 ratios; Table 3). Conversely, at higher clomazone concentrations, rice is less sensitive than the S biotype of late watergrass, but more sensitive than the R biotype (IC50 ratios; Table 3). This result is likely due to the fact that clomazone toxicity is dependent on the balance between two different enzyme systems: those that activate the herbicide (likely P450 enzymes)[11] and those that detoxify it. Differing rates of these reactions in different species could lead to the observed differences in sensitivity at higher versus lower concentrations.

Clomazone reduced ß-carotene levels in rice and watergrasses at very low concentrations. Effect on growth was less severe, and limited to watergrasses. The recommended application rate[1] of 0.67 kg ha^{-1} would leave 2.3 μM clomazone in a paddy with a 10 cm flood, assuming all of the clomazone was in the water column. Reported K_d values range from 0.47-5.30,[12,13] thus with partitioning into sediment, and assuming equilibrium conditions between water and a mixed sediment surface layer, there would be

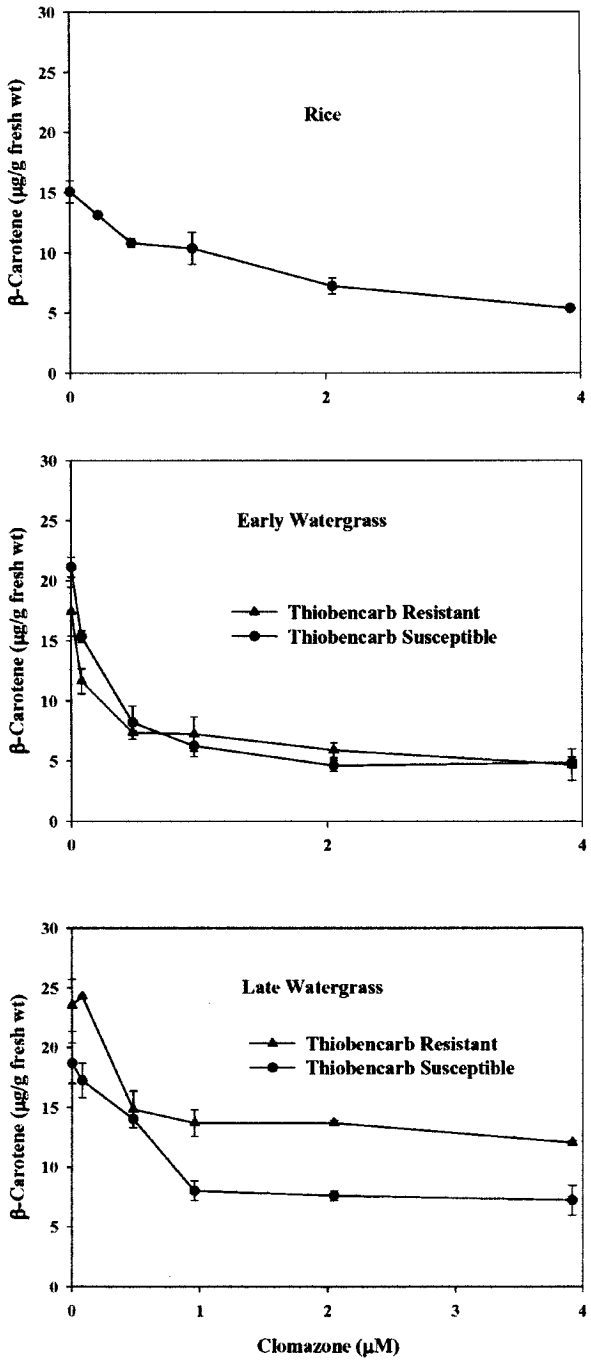

Figure 3. Clomazone dose-response curves for ß-carotene levels (μg g^{-1} fresh wt; mean ± se).

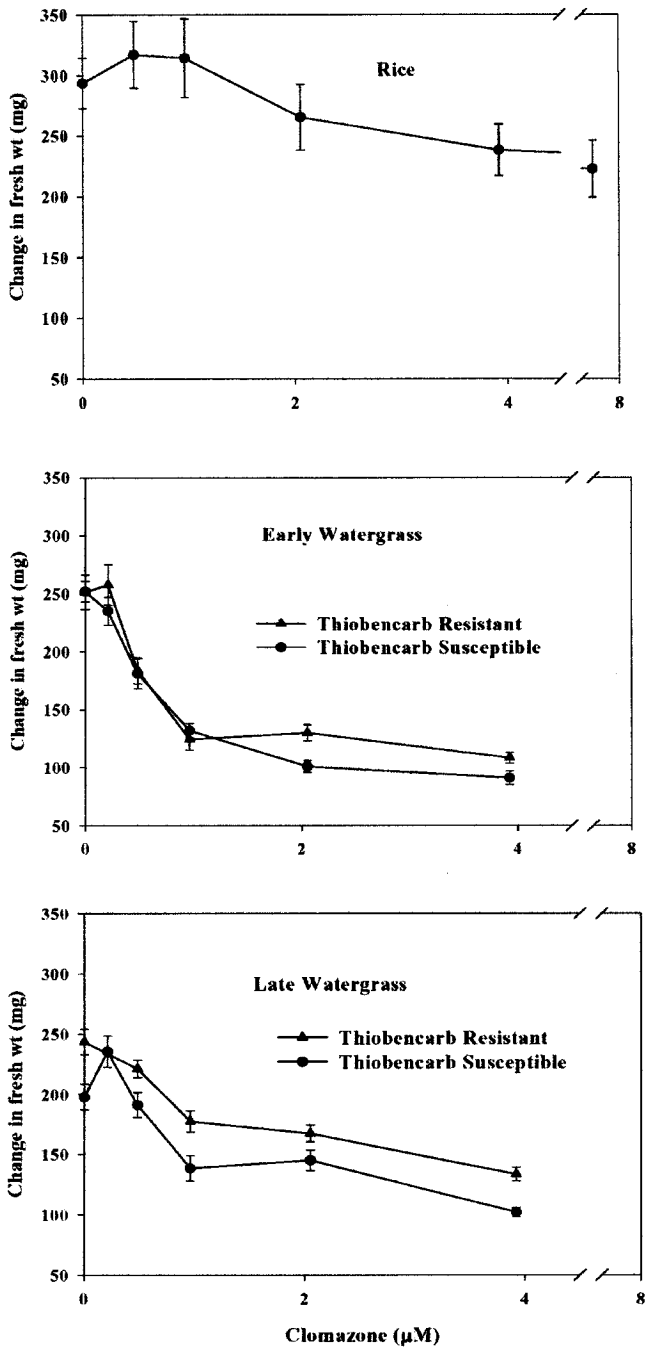

Figure 4. Clomazone dose-response curves for growth as measured by change in fresh weight (mg; mean ± se).

0.36-1.6 μM clomazone in the water. These concentrations are above or near all of the IC25 and NOEC values determined in this study for ß-carotene, and for watergrass growth. However, rice growth would not be expected to be affected at those levels. That rice growth was not affected by clomazone in this study could be due to the supply of nutrients and energy contained in its relatively large seeds. Clomazone disrupts photosynthetic processes, but, with ample energy in its seeds, the rice plants were likely not dependent on photosynthetic energy for growth during the 7-d exposure period. Conversely, the watergrasses, with relatively small seeds, would likely need energy from photosynthesis to maintain growth over the same exposure period. Whether plants that have grown well, but have suffered clomazone bleaching, could recover and produce normal yields is an unexplored question.

No clear pattern of resistance to clomazone emerges for the watergrasses from the current study (Table 3). Thus it is not likely that the resistance mechanism present in the thiobencarb-resistant biotypes of watergrasses will be effective against clomazone. However, differences were observed between rice and watergrasses. Differential sensitivity to clomazone may be due to differential uptake, translocation, activation or detoxication. No differences in uptake, translocation or detoxication of clomazone were found in a study of soybean and velvetleaf.[14] Clomazone detoxication through metabolism, particularly by conjugation with glucose and glutathione[15,16] may also play a role in differential clomazone sensitivity. Several studies have shown that various plants are able to metabolize clomazone to different degrees.[14,17-20]

The current study has shown that, with respect to production of ß-carotene, rice and late watergrass are quite similar in sensitivity to clomazone. Thus to be able to use clomazone for control of late watergrass without injuring rice, a safening technique should be developed based on physiological and biochemical mechanisms of clomazone activity in plants.

Two promising avenues for further research are the mechanisms of clomazone transformation to the active 5-ketoclomazone, and of the interaction of 5-ketoclomazone with the enzyme 1-deoxyxylulose-5-phosphate synthase (DXS). First, activation of clomazone by a P450 enzyme is suggested by a study in which the organophosphates phorate and disulfoton (known P450 substrates) protected cotton from clomazone toxicity, but aldicarb (not a P450 substrate) did not.[11] Second, the enzyme DXS requires thiamine pyrophosphate (TPP) as a cofactor.[8] Structural similarities between 5-ketoclomazone and thiamine pyrophospahte suggest the possibility of competition at the DXS-TPP binding site. These mechanisms, if fully understood, may be exploited in development of chemical safeners for clomazone.

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Section III. Biotransformation of clomazone in rice (*Oryza sativa*) and early watergrass (*Echinochloa oryzoides*)

1. Introduction

Watergrasses are difficult to control in California rice culture because they have developed resistance to herbicides such as thiobencarb, molinate, bispyribac-sodium, fenoxaprop-ethyl, and bensulfuron-methyl [1-3]. Clomazone, an herbicide produced by the FMC Corporation under the trade names Command® and Cerano 5 MEG®, has shown promise for weed control, however rice injury has occurred in early field tests (Albert Fischer, pers. comm. 2005). Clomazone toxicity is thought to depend on a plant's ability to oxidize the parent compound to the active metabolite, 5-ketoclomazone [4], then subsequently detoxify it. Differences in herbicide metabolic capabilities between rice and watergrasses could be exploited to develop herbicide safeners.

Clomazone biotransformation has been previously investigated in soybean, corn, smooth pigweed, velvetleaf, cotton, soil, and microbial culture [5-15]. Most studies have found no qualitative or quantitative differences in metabolism between tolerant and sensitive plant species, although many involved analytical methods that may not have been sufficient to fully separate polar metabolites [5, 6, 8]. In cell cultures, Norman *et al.* [7] found that tolerant soybean, compared to cotton, produced a qualitatively different metabolite profile, converting more clomazone to polar metabolites at 6 and 24 h after treatment. However, after 48 h, differences in total metabolite levels were no longer evident. Weimer *et al.* [9, 11] found that sensitive velvetleaf metabolized clomazone

more rapidly and/or to a greater extent than the tolerant soybean. The HPLC methods utilized by Weimer *et al.* [9, 11], which included fairly extensive aqueous gradient steps, provided better metabolite resolution than did previous studies, but still no qualitative differences were observed between tolerant and sensitive species.

This investigation provides a comparison of clomazone accumulation, uptake and biotransformation by rice and early watergrass, which have been shown to possess different tolerances to clomazone [16]. HPLC methods were optimized for separation of polar metabolites, while quantitation by analysis of ^{14}C residues allowed for detection of metabolites at pmol levels.

2. Materials and Methods

2.1 Chemicals

^{14}C aromatic-ring-labeled clomazone (17.9 mCi/mmol dissolved in ethyl acetate; 95.5% purity confirmed by HPLC), technical grade clomazone (I), 4', 5-dihydroxy clomazone (II), clomazone ring-open derivative (IV), 3'-hydroxyclomazone (V), 5-hydroxyclomazone (VI), 5'-hydroxyclomazone (VII), and 5-ketoclomazone (VIII) were obtained from FMC Corporation (Philadelphia, PA). 2-Chlorobenzyl alcohol (III) and 0.5N KOH were purchased from Fisher Scientific (Hampton, NH). β-D-glucosidase (from almonds; 3.7 units mg^{-1}) was purchased from Sigma Aldrich Corp. (St. Louis, MO). Hoagland's No. 2 basal salt mixture was obtained from Sigma Aldrich and was used to prepare 0.5X solution at pH 6.2 $\pm$ 0.2. Ultima Gold™ scintillation cocktail was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

2.2 Plants

Seeds of rice (*Oryza sativa* L; variety M202) and early watergrass (*Echinochloa oryzoides* (Ard) Fritsch; EWG) were obtained from the California Rice Research Station (Biggs, CA). After a 3-d period of germination on moist filter paper in the dark, seeds were treated with 3% bleach for 5 min, thoroughly rinsed with deionized water, and then transferred to individual 7-mL vials of deionized water. Seedling development continued under ambient laboratory conditions for another 7 d prior to beginning exposures. Seedlings of uniform size (0.5 leaf stage) were selected for use in the experiments.

2.3 Laboratory exposures and wet weight analysis

Test solutions were prepared by adding 5 uL ^{14}C-labeled clomazone in ethyl acetate to 500 mL of 0.5X Hoagland's solution for a final clomazone concentration of 0.29 μM. Initial concentration of clomazone was confirmed by sampling 2 x 200 μL aliquots of test solution for analysis by liquid scintillation counting (LSC). Experiments were carried out in Gledhill flasks to allow for mass balance determination. Briefly, each flask included a reservoir of 0.5 N KOH to trap evolved $^{14}CO_2$ and a polyurethane foam (PUF) plug to collect volatilized compounds. Plants (25 per replicate) were weighed and placed into flasks which were then sealed with rubber septa and placed in a controlled-environment growth chamber (30°C day:16°C night; 14 h day:10 h night; rel. humidity: 30% day:80% night; maximum irradiance: 800 μmol $m^{-2}s^{-1}$ PPFD). After 7 d plants were removed the from flasks, rinsed 3 times with deionized water, blotted dry, weighed and frozen at –80°C prior to extraction. Two separate experiments were run, each with two replicates of each plant species.

2.4 Metabolite extraction

A two-step extraction procedure was developed to maximize recovery of clomazone and metabolite standards. Quantitation of metabolites was by radiotracer analysis, thus cleanup steps were omitted to minimize analyte loss. To extract non-polar compounds, whole plants were homogenized for 1 min in 5 mL methanol plus 10 mL hexane using a Tekmar model TP 18/10 S1 tissue homogenizer (P.O. Box 429576 Cincinnati, OH 45242-9576). The hexane fraction was transferred to 2 X 40-mL centrifuge tubes and the homogenization procedure was repeated with 2 X 10 mL hexane. Remaining plant tissue was further homogenized for 1 min in 12.5 mL 90:10 acetone:water to extract polar compounds. After transferring material to a 40-mL centrifuge tube the extraction tube and homogenizer probe were rinsed twice with 5 mL 90:10 acetone:water and rinsate was added to the centrifuge tube. All extracts were centrifuged for 5 min at 2000 rpm (880 g) and supernatants were transferred to 250-mL round bottom flasks. Pellets in the acetone:water extracts were resuspended in the same solvent mixture, vortexed for 1 min, and centrifuged. Supernatants were added to the appropriate round bottom flasks. Extracts were reduced under vacuum at 40°C. Hexane fractions were reduced to approx. 1 mL while acetone:water extracts were reduced to 3-6 mL, then were frozen for at least 1 h to precipitate solids, and were centrifuged again. Final extracts were stored at –20°C in amber vials until analyzed. Table 4 shows extraction recoveries for clomazone and each metabolite standard as analyzed by C_{18} HPLC.

Table 4. Extraction recovery and HPLC retention times (RT) of clomazone and metabolite standards; the C_{22} method did not resolve 3'-hydroxyclomazone and the ring-open derivative.

No.	Compound	Rec (%)	se	n	C_{18} RT (min)	C_{22} RT (min)
I	clomazone	83	2	6	40.3	41.2
II	4',5-dihydroxyclomazone	123	17	6	26.6	13.8
III	2-chlorobenzyl alcohol	45	10	6	31.4	21.2
IV	clomazone ring-open derivative	99	7	6	32.8	23.2
V	3'-hydroxyclomazone	134	14	6	34.5	23.2
VI	5-hydroxyclomazone	77	5	6	35.6	27.8
VII	5'-hydroxyclomazone	122	10	6	36.4	30.8
VIII	5-ketoclomazone	60	10	4	41.6	38.7

2.5 Mass Balance Determination

At the end of the 7-d exposure period, ^{14}C residues in PUF plugs, KOH solution (2 x 1 mL aliquots) and test solution (2 x 200 μL aliquots) were quantified by LSC using a Packard Tri-Carb model 2000CA liquid scintillation analyzer (PerkinElmer; Boston, MA). Automatic quench correction was via an internal standard, and each sample was counted for 5 min. PUF plugs were placed into 15-mL scintillation vials, and after addition of scintillation cocktail, plugs were squeezed several times to soak. Blanks of PUF, KOH and Hoagland's solution were all counted and final sample counts were blank-corrected.

2.6 β-D-glucosidase treatment

Polar extracts were treated with β-D-glucosidase to identify clomazone conjugates. Each extract (0.5 mL) was evaporated to dryness, resuspended in 0.5 mL 0.2 M sodium acetate buffer at pH 5.0, and then added to 1.5-mL Eppendorf tubes containing

approx. 18 U (5.0-5.2 mg) of β-D-glucosidase. Samples were incubated for 4 h at 37°C and then transferred to amber vials for storage at –20°C until analyzed. A positive control of *p*-nitropheny-β-D-glucoside was included to monitor the hydrolytic reaction. Recovery of residues through this procedure was 73.7 ± 6.2% for rice and 57.5 ± 4.7% for EWG. Reported results were corrected accordingly.

2.7 HPLC methods

Metabolite extracts were initially analyzed by two different HPLC methods using a Hewlett-Packard model 1090 integrated HPLC (Palo Alto, CA) with a diode-array detector (220 nm) and a Gilson model 203 fraction collector (Middleton, WI). First, a quantitative method utilizing an Intersil ODS 3 C_{18} column (250 X 4.6 mm, 5 μm; Alltech Associates, Inc., Deerfield, IL) was used with a gradient mobile phase of 0.01% acetic acid:acetonitrile (85:15 v/v for 10 min, changed to 65:35 over 20 min, changed to 40:60 over 5 min and held for 5 min, changed to 30:70 over 1 min and returned to 15:85 over 2 min; 1.0 mL min^{-1}). Second, a qualitative method utilizing a Chromegabond reverse-phase C_{22} column (250 X 4.6 mm, 5 μm; ES Industries, West Berlin, NJ) was used with a gradient mobile phase of 0.01% acetic acid:methanol (60:40 v/v for 15 min, changed to 45:55 over 10 min and held for 10 min, changed to 30:70 over 5 min and returned to 60:40 over 2 min; 1 mL min^{-1}). Compounds tentatively identified by the C_{18} method were confirmed by the C_{22} method. Retention times for clomazone and metabolite standards are shown in Table 4.

A third, qualitative HPLC method was developed for analysis of polar extracts to try to separate the most polar metabolites using the same C_{22} column as above, but with a

different gradient mobile phase of 0.01% acetic acid:methanol (75:25 v/v for 40 min, changed to 0:100 over 20 min, held for 2 min and returned to 75:25 over 5 min). For this aqueous C_{22} method, the 4 replicates from each plant were combined and injected as one sample. By this method, all metabolite standards eluted between 48 and 58 min. Reverse phase C_{18} guard columns (7.5 X 4.6 mm, 5 μm) were used for all the methods above.

2.8 Microbial controls

To control for possible microbial and/or photochemical degradation of clomazone, Gledhill flasks containing test solutions, but no plants, were prepared and incubated for 7 d. Mass balance was determined as described above. Five replicates were incubated in light and another five in darkness.

2.9 LC/MS

To attempt to identify unknown polar metabolites plants were exposed to unlabeled clomazone and extracted, as described above (n = 3 for each species). Extracts were fractionated by HPLC according to retention times of ^{14}C-residue peaks. Fractions from replicates were combined and volume reduced prior to direct injection into a Finnegan LCQ mass spectrometer operating in negative mode with an electrospray ionization source.

2.10 Statistics

Metabolite levels before and after β-D-glucosidase treatment and percent metabolites in rice vs. EWG were compared using t-tests (Excel v. X).

3. Results and Discussion

3.1 Mass balance

Table 5 shows the mass balance for rice and EWG test systems. Over the 7-d exposure period, the plants accumulated a small, but measurable, percentage of the applied radiotracer. Given that 101 ± 1% of applied ^{14}C label was recovered in controls without plants, it may be assumed that ^{14}C not accounted for in solution or in the volatile or CO_2 fractions must have been absorbed by or adsorbed to the plants. This fraction includes unbound and bound residues as well as those lost during metabolite extraction. Rice accumulated more total ^{14}C residues than EWG (16.8 ± 1.0 nmol vs. 7.8 ± 1.9 nmol, respectively; $p < 0.05$), but on a nmol g^{-1} basis, there was no difference between the two species (rice = 4.0 ± 0.5 nmol g^{-1}; EWG = 6.8 ± 1.8 nmol g^{-1}; $p > 0.05$). More ^{14}C residues were extracted from rice (7.7 ± 0.5) compared to EWG (4.8 ± 0.5; $p < 0.05$), but residue concentration in EWG was significantly higher (rice = 1.8 nmol g^{-1}; EWG = 4.2 nmol g^{-1}; $p < 0.01$). The greater clomazone sensitivity in EWG compared to rice cannot be explained by differential uptake of clomazone, but may be due, in part, to the higher concentrations of extractable (i.e. bioavailable) clomazone and metabolites in EWG. Controls incubated in light without plants lost 6.2 ± 1.3% of applied ^{14}C to mineralization and 0.2 ± 0.1% to evaporation; 95 ± 1% remained in solution. Controls kept in darkness lost 2.2 ± 0.5% to mineralization and 0.2 ± 0.04% to evaporation; 98 ± 0.4% remained in solution. The difference in mineralization between light and dark controls was significant ($p < 0.05$) indicating possible photo-enhancement of microbial degradation.

Table 5. ^{14}C mass balance for rice and watergrass exposures. Portion bound or lost in extraction was determined by subtraction; all other values were measured.

Rice	uCi	se	n	% of total
Remaining in Solution	2.19	0.03	3	86.1
Volatilized	0.005	0.000	4	0.2
CO_2	0.04	0.01	4	1.5
Plant (total)	0.31	0.04	4	12.2
Extracted	0.14	0.01	4	(5.4)
Bound/Lost in extraction	0.17	0.04		(6.8)
Total applied	2.54	0.03		100.0
Watergrass				
Remaining in Solution	2.41	0.04	4	92.5
Volatilized	0.006	0.001	4	0.2
CO_2	0.05	0.00	4	1.9
Plant (total)	0.14	0.05	4	5.4
Extracted	0.09	0.01	4	(3.3)
Bound/Lost in extraction	0.05	0.05		(2.1)
Total applied	2.61	0.01		100.0

The microbial activity indicated by production of $^{14}CO_2$ leads to the conclusion that the test solutions may have contained small quantities of clomazone degradation products in various stages of decomposition. Production of $^{14}CO_2$ did not differ between rice and watergrass systems (1.5 and 1.9%, respectively; $p > 0.05$), but was lower in test systems than in the controls ($p < 0.05$), probably due to $^{14}CO_2$ uptake by the plants.

3.2 Metabolite profiles

Fig. 5 shows metabolite profiles for rice and EWG and identifies peaks that co-eluted with analytical standards I-VIII by C_{18} HPLC. Compounds present in at least 3 of

the 4 replicates are included. The identities of all compounds, except IV and V in rice, were confirmed by C_{22} HPLC (compounds IV and V were not resolved by this HPLC method). Compounds found in rice included I, III, IV, V, VI, VII, and VIII, while EWG produced I, II, III, V, VI, VII, and VIII. Both species produced several unidentified metabolites.

The basal microbial activity observed in some controls means that at least some of the metabolites extracted from the plants may not have been produced by them. However, plants were rinsed and blotted dry prior to freezing, so it is highly unlikely that detectable residues were transferred to the extraction process via test solutions. Uptake and transport of relatively small water-soluble molecules may proceed via the apoplast [17]. Thus, microbial transformations that increase xenobiotic polarity would not necessarily completely eliminate residue uptake. Results of this investigation represent uptake and metabolism of clomazone, as well as possibly small quantities of one or more microbial metabolic products, which reflects a realistic field scenario.

Table 6 provides a quantitative metabolite profile for polar and non-polar plant extracts. Some compounds, such as II and IV, that appear in Fig. 5 do not appear in the Table 6. This is because when considering the polar and non-polar fractions independently, these compounds were not found in at least 3 of 4 replicates. Table 7 compares percent total metabolites between rice and EWG. Peaks that eluted after VIII are included for mass balance, but are not discussed as they represent compounds that eluted during a 100% organic cleaning phase of the HPLC gradient.

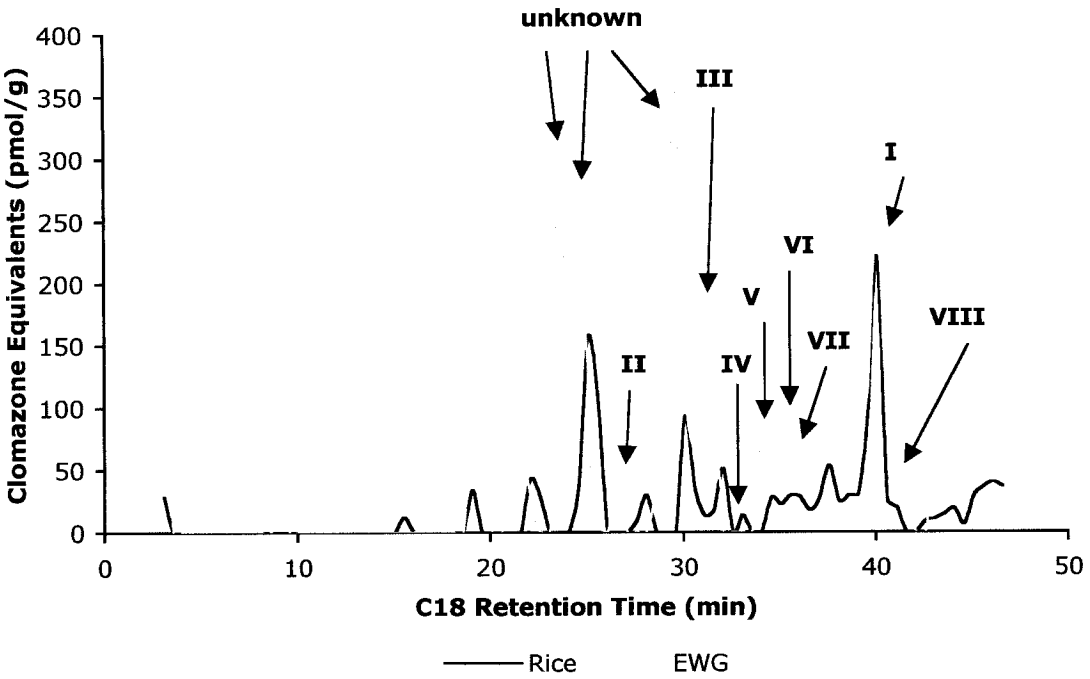

Fig. 5. Clomazone metabolites from rice and early watergrass (EWG) resolved by C_{18} HPLC.

Expressed as a percentage of total ^{14}C recovered, more clomazone metabolites were found in EWG compared to rice (84.1% vs. 67.9%; $p < 0.01$). EWG produced two large, unidentified peaks that rice did not produce (RT 23.6-24.1 and 29.1-30.6). Both rice and EWG produced large, unidentified peaks at RT 24.6-25.6, representing 22.7 and 12.1% of metabolites for rice and EWG, respectively (difference in percent of total not significant; $p > 0.05$); these peaks were subsequently identified as β-D-glucoside conjugates (see below). The unidentified peaks that appeared for both rice and watergrass between 37.1 and 37.6 min coincide with the largest impurity peak found in a purity analysis of the ^{14}C-labeled clomazone.

Table 6. Metabolite profiles for rice and early watergrass (EWG); polar metabolites are shown before and after treatment with β-D-glucosidase; *p < 0.05; **p < 0.01 for changes with treatment; results are from C_{18} HPLC method (mean ± se; • = not detected).

		Polar				**Non-Polar**	
Retention Time (min)	Elutes with	Rice (pmol/g) Before	After	EWG (pmol/g) Before	After	Rice (pmol/g)	EWG (pmol/g)
3.1		29 ± 2	49 ± 3**	24 ± 15	300 ± 31**	•	•
15.6		•	41 ± 7	•	•	•	•
19.1-19.6		34 ± 13	•	180 ± 39	•	•	•
21.1		•	•	•	•	•	12 ± 3
21.6		•	32 ± 4	•	•	•	•
22.1-22.6		42 ± 7	•	•	233 ± 22	•	10 ± 1
23.6-24.1		•	•	424 ± 98	94 ± 5*	•	10 ± 1
24.6-25.6		264 ± 66	•	376 ± 92	•	•	22 ± 3
27.6-28.1		29 ± 5	80 ± 12**	•	•	•	•
29.1-30.6		•	•	609 ± 162	•	•	•
31.1-31.6	III	•	33 ± 1	241 ± 17	•	12 ± 1	59 ± 9
32.1		•	32 ± 2	•	•	•	•
32.6-33.1	IV	•	51 ± 8	•	105 ± 9	•	•
33.6		•	61 ± 20	•	118 ± 2	•	•
34.1-35.1	V	53 ± 5	200 ± 32**	195 ± 39	550 ± 47**	•	•
35.6	VI	29 ± 2	31 ± 5	103 ± 18	210 ± 51	•	•
36.1-36.6	VII	26 ± 3	42 ± 7	125 ± 12	348 ± 60*	3.9 ± 0.4	13 ± 1
37.1-37.6		61 ± 4	88 ± 8*	88 ± 10	•	8.4 ± 1.6	32 ± 4
38.1		24 ± 2	•	66 ± 5	•	•	•
38.6		29± 5	42 ± 9	62 ± 3	•	•	•
39.1-40.6	I	121 ± 10	80 ± 8*	399 ± 17	267 ± 36*	241 ± 23	126 ± 7
41.1-41.6	VIII	•	•	•	•	5.7 ± 0.5	21 ± 2
42.6-46.6		91 ± 7	•	•	•	58 ± 2	105 ± 4
Total		832 ± 70	862 ± 45	2892 ± 221	2225 ± 106	329 ± 23	410 ± 14

Metabolite VIII, which is an active metabolite of I [4], was found in very small amounts in the non-polar extracts of both rice and EWG. Comprising < 1% of total metabolites in both species, the concentration of VIII was significantly higher in EWG vs. rice (21 ± 2 vs. 5.7 ± 0.5 pmol g^{-1}, respectively; $p < 0.05$). ElNaggar *et al.* [10] found that VIII comprised < 5% of the total metabolites produced, and it was present in the β-D-glucoside-conjugated fraction, indicating that it existed as a hydrolyzed product in the plant. The chemistry of VIII is very complicated in that it can hydrolyze into a polar, water-soluble product, but in the keto form, it is very non-polar and insoluble in water. In the current study, no VIII was recovered after treatment of the polar extract with β-D-glucosidase indicating either that it had not been hydrolyzed and conjugated, or that it

Table 7. Comparison of percent metabolites produced between rice and early watergrass (EWG); only metabolites found at 5% or greater and detected metabolite standards are included; asterisks indicate significant difference between rice and EWG (mean ± se; * p = 0.05; ** p = 0.01; • = not detected).

RT (min)	Elutes with	Rice % of total	EWG % of total
19.1-19.6		2.9 ± 1.1	5.5 ± 1.2
23.6-24.1		•	13.1 ± 3.1
24.6-25.6		22.7 ± 5.9	12.1 ± 2.9
29.1-30.6		•	18.4 ± 5.1
31.1-31.6	III	•	9.1 ± 0.8
34.1-35.1	V	4.6 ± 0.5	5.9 ± 1.2
35.6	VI	2.5 + 0.2	3.1 ± 0.6
36.1-36.6	VII	2.6 ± 0.3	4.2 ± 0.5*
37.1-37.6		6.0 ± 0.5	3.6 ± 0.4**
39.1-40.6	I	32.1 ± 2.9	15.9 ± 1.2**
41.1-41.6	VIII	0.5 ± 0.1	0.6 ± 0.1
42.6-46.6		5.0 ± 0.4	3.2 ± 0.2**

was lost to volatilization or container adsorption upon cleavage of the β-D-glucoside. Another possible explanation for low levels of VIII is that it is a transitory product that does not accumulate, but reaches a steady state. This is supported by the work of ElNaggar *et al.* [10] in which the percentage of VIII did not change appreciably between plants harvested after 30 d compared to those harvested after 60 d (4.1 vs. 3.7%), while levels of unidentified, polar metabolites increased from 6.4 to 11.1% and the level of I decreased form 7.9 to 2.9% during the same time interval.

3.3 LC/MS

Results of LC/MS analyses were inconclusive as none of the spectral peaks matched molecular weights of metabolites identified by HPLC or other potential metabolites, such as glutathione conjugates. The extraction method used for both analyses involved minimal clean-up steps as it had been optimized for quantitation by LSC. This, combined with the very low concentrations of metabolites, resulted in MS signals that were generally poor and noisy.

3.4 β-D-glucosidase treatment

Results of treatment of the polar extracts with β-D-glucosidase are shown in Fig. 6, and are quantified in Table 6. For both rice and EWG, some peaks were either lost or significantly reduced, indicating cleavage of β-D-glucoside conjugates. Most notably, a large peak at RT 24.6-25.6 disappeared for both rice and EWG, and peaks at RT 23.6-42.1 and RT 29.1-30.6 for EWG significantly reduced and disappeared, respectively. A small peak at RT 19.1-19.6 also completely disappeared in both species. Concomitant with peak loss was the appearance of new peaks, or significant increases in peak size. In

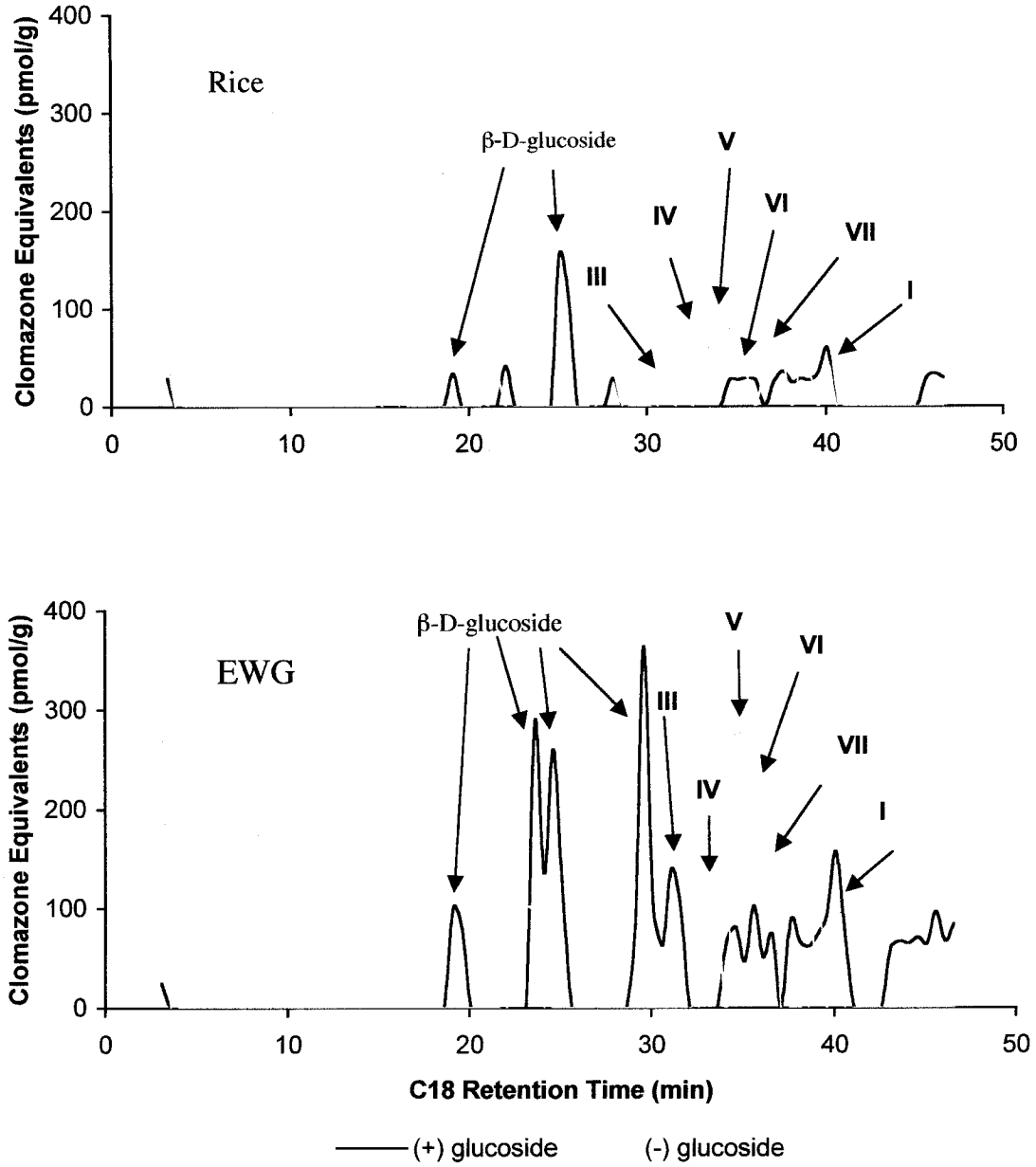

Fig. 6. Polar metabolites from rice and early watergrass (EWG) before and after treatment with β-D-glucosidase.

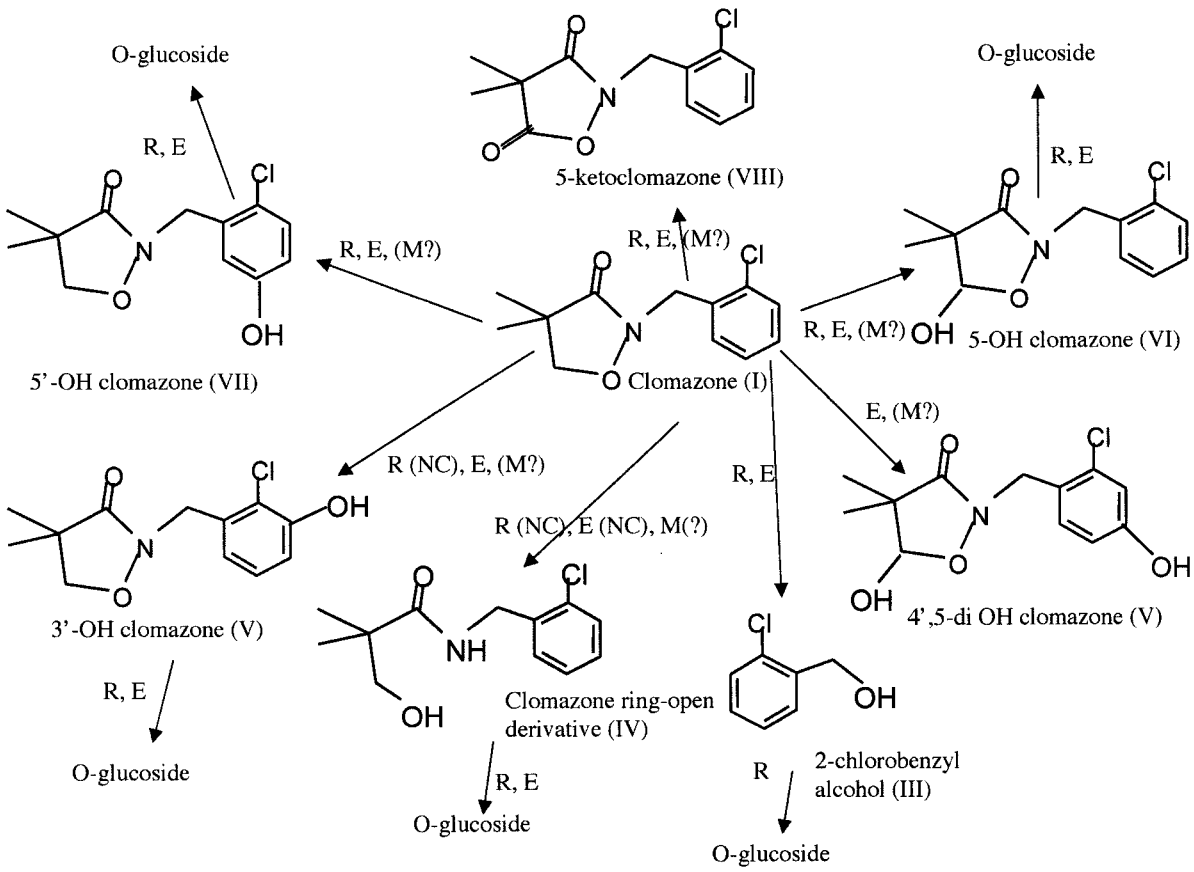

Fig. 7. Proposed biotransformation pathway for clomazone in rice (R) and early watergrass (EWG) based on results in Fig. 5 and Table 6; M (?) = possible microbial transformation; NC = not confirmed.

rice, new peaks appeared at RT 15.6, 21.6, 31.3-31.6 (elutes with III), 32.1, 33.6 and 32.6-33.1 min (elutes with IV), while peak sizes increased for RT 3.1, 27.6-28.1, 34.1-35.1 (elutes with V) and 37.1-37.6 min ($p < 0.01$, 0.01, 0.01, 0.05, respectively). For EWG new peaks appeared at RT 22.1-22.6, 32.6-33.1 (elutes with IV) and 33.6 min, while peak sizes increased for RT 3.1, 23.6-24.1, 34.1-35.1 (elutes with V) and 36.1-36.6 min (elutes with VII; $p < 0.01$, 0,05, 0.01, 0.05, respectively). The increased peak size at RT 35.6 min for EWG (elutes with VI) was not significant ($p > 0.05$). These results indicate that both rice and EWG either produced or absorbed phase I metabolites in the form of hydroxylated clomazone, which were subsequently conjugated with glucose. While it is not possible to discern whether phase I reactions were accomplished by microbes or plants, the glucoside conjugates were undoubtedly plant products because microbes do not produce glucosides to any appreciable extent [18]. Based on this study, a proposed biotransformation pathway for clomazone in rice and EWG is shown in Fig. 7.

The appearance of IV in the β-D-glucosidase-treated extracts of both rice and EWG was unexpected because this product has only been reported in studies of microbial degradation of clomazone [13, 14]. This suggests that this compound may have been formed by microbial action prior to uptake by the plants, and was subsequently conjugated to glucose.

Analysis of β-D-glucosidase-treated extracts by the C_{22} HPLC method revealed that the largest polar peaks, which eluted in the first 10 min had not changed. To try to resolve these peaks, the extracts were re-analyzed by a C_{22} method with a prolonged aqueous phase gradient. Three large peaks were successfully resolved into 4-5 peaks, but

no differences were evident between treated and untreated extracts, indicating that no β-D-glucosides were present.

A somewhat surprising result was the appearance or increase in size of polar peaks after treatment with β-D-glucosidase, particularly the more than 10-fold increase in the size of the peak at 3.1 min for EWG. While the change in this peak was significant ($p < 0.01$) for both rice and EWG, it was particularly dramatic for EWG. This peak is composed of compounds that elute with the solvent front and thus would likely be small, charged molecules. The HPLC method used in this study included an acidic aqueous phase, which would result in charged amine groups. Glutathione conjugation is a common xenobiotic detoxification mechanism in plants [19]. Vencill *et al.* [6] found that clomazone formed conjugates with glutathione non-enzymatically. Plants generally further process glutathione conjugates into glutamylcysteine, cysteine, sulfhydryl, thiolactyl, malonyl or other products [20, 21]. Several of these contain amine groups that could be positively charged under acidic conditions. It is possible that the peak at 3.1 min represents a compound, such as III, which was originally conjugated with β-D-glucose and with glutathione. Upon cleavage of the β-D-glucosidase moiety, the remaining, smaller, charged molecule would be expected to elute with the solvent front.

Conclusion

TenBrook and Tjeerdema [16] found that EWG was more sensitive to clomazone than rice as measured by reduction in growth and reduction in β-carotene levels. It would be logical to expect more metabolic activity in rice, which is consistent with findings of Norman *et al.* [7] that tolerant soybean plants metabolized a larger percentage of

clomazone than did susceptible cotton. However, results of the current study are similar to those of Weimer *et al.* [9, 11] in which the more sensitive species metabolized clomazone either more rapidly or to a greater extent than the more tolerant species. Similarly, sensitive tomato shoots were found to metabolize clomazone to a greater extent than tolerant bell pepper shoots, while pepper roots metabolized a larger percentage of clomazone than tomato roots [5]. The shoots contain the plastidic, non-mevalonate isoprenoid synthetic pathway, which clomazone targets directly [4]. Herbicide sensitivity associated with high metabolic activity is indicative of a proherbicide and supports the finding of Mueller *et al.* [4] that a clomazone metabolite, 5-ketoclomazone, is an active herbicide, or at least a precursor to an ultimately active form. Activation of clomazone to 5-ketoclomazone requires oxidation of the parent compound. Oxygenases that would ordinarily detoxify a xenobiotic substrate would convert clomazone to its toxic metabolite, thus causing harm rather than conferring protection. It may be possible to utilize oxygenase inhibitors as safeners to block the activation of clomazone in crop species, thereby protecting them from clomazone damage. Indeed, Culpepper *et al.* [15] found that organophosphate insecticides, which are known cytochrome P450 inhibitors, protected cotton against clomazone injury. Additionally, disulfoton applied as a seed treatment protected rice (*Oryza sativa;* cultivar "Cypress") from clomazone injury [22], indicating that there is potential for using P450-inhibiting insecticides as safeners in rice culture.

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Section IV. Clomazone resistance and effects of P450 inhibitors on clomazone toxicity in *Echinochloa oryzoides* and *Echinochloa phyllopogon*

INTRODUCTION

Clomazone is a bleaching herbicide used to control annual broadleaf weeds and grasses (Weed Science Society 1994). In California, the Cerano® formulation has been developed specifically for control of barnyard grass (*Echinochloa crus-galli* (L.) Beauv. var. crus-galli), sprangletop (*Leptochloa fascicularis* (Lam.) A. Gray) and watergrasses (*Echinochloa* spp.) in water-seeded rice culture (California Department of Pesticide Regulation 2003). Resistance to the herbicides molinate, thiobencarb, fenoxaprop-ethyl and bispyribac-sodium has been reported in *Echinochloa oryzoides* (Ard.) Fritsch and *Echinochloa phyllopogon* (Stapf) Koss (Fischer *et al.* 2000a). Clomazone resistance in watergrasses has also been observed in California rice fields (Fischer and Cheetham, in press). Resistance of *E. phyllopogon* to bispyribac-sodium has been related to cytochrome P450 activity (Fischer *et al.* 2000b). Higher levels of substrate-specific induction of cytochrome P450 enzymes have been observed in resistant (R) *E. phyllopogon* exposed to herbicides compared to susceptible (S) biotypes, indicating the involvement of multiple P450 isozymes (Yun *et al.*, in press).

Rice (*Oryza sativa* L.) tolerance to clomazone is higher than that of *Echinochloa* spp. with respect to growth, but a biotype of *E. phyllopogon* cross-resistant to thiocarbamates, aryloxyphenoxy-propionates, and bispyribac-sodium was found to have the same tolerance as rice with respect to the bleaching effect as measured by β-

carotene production (TenBrook and Tjeerdema 2005). Bleaching and growth reduction of rice is of concern when clomazone is used. To safely control grasses in rice, it may be necessary to utilize herbicide safeners to protect rice plants. Safener use with clomazone presents a challenge due to the likelihood that it is a proherbicide that requires metabolic activation, an idea first proposed by Norman and Leibl (1989). The metabolite 5-ketoclomazone, but not clomazone itself, inhibits the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which is the first enzyme in the plastidic non-mevalonate isoprenoid biosynthesis pathway (Mueller *et al.*, 2000). Further, Chang *et al.* (1987) had originally intended to develop the herbicidally very active 5-ketoclomazone as a new herbicide, but found it to be unstable in field trials, thus leading to the development of clomazone. The hydrolyzed, hydroxamic acid form of 5-ketoclomazone is also herbicidally active (Chang *et al.*, 1987). The form of clomazone that is ultimately toxic is not known, but it seems certain that the oxidation to 5-ketoclomazone is a required step in the activation process.

The non-mevalonate isoprenoid synthetic pathway produces carotenoids as well as gibberellins (Sponsel 2002), which play a major role in plant growth regulation (Ross *et al.* 1997). Thus, the inhibition of DXS by the active metabolite of clomazone can have direct effects on both growth and β-carotene levels.

While the mechanism of clomazone activation to 5-ketoclomazone is not known, it is likely the due to the action of cytochrome P450 enzymes which are abundant in plants and play a major role in biotransformation of xenobiotics (McGonigle *et al.* 1997).

Thus, the same class of enzymes that may activate clomazone also likely play a role in its detoxification. According to McGonigle *et al.* (1997), the other major detoxifying enzyme system in plants consists of the glutathione S-transferases (GSTs). Clomazone will react non-enzymatically with glutathione, indicating that this conjugation reaction is another probable pathway for clomazone detoxification (Vencill *et al.* 1990).

The organophosphate insecticide disulfoton, a known P450 inhibitor, protected rice against clomazone injury (Jordan *et al.* 1998). Also, disulfoton and phorate (another P450 inhibitor) both protected cotton against clomazone injury (Culpepper *et al.* 2001). In cotton treated with clomazone, phorate prevented loss of chlorophyll and carotenoids, decreased overall metabolism of clomazone, and inhibited an NADPH-dependent P450 reaction in corn shoot microsomes (Ferhatoglu *et al.*, 2004). These studies support the proposition that a P450-activated metabolite is the active form of clomazone.

Inhibition of P450 enzymes ought to reduce the effects of clomazone, as the transformation to the active herbicide should be inhibited. This hypothesis was tested by exposing R late watergrass and S early watergrass to clomazone alone and to clomazone in combination with P450 inhibitors piperonyl butoxide (PBO), disulfoton, oxydemeton methyl or demeton-S in both greenhouse and laboratory experiments. The R late watergrass biotype was chosen for the inhibitor studies because it had exhibited resistance to clomazone in the field (Fischer and Cheetham, in press) and the S early watergrass was chosen due to its extreme susceptibility to clomazone (TenBrook & Tjeerdema 2005). Fresh weight and β-carotene analyses were used to assess responses to

clomazone. Additionally, the P450-based herbicide resistance mechanism reported by Fischer *et al.*, (2000a), ought to render watergrasses more susceptible to the proherbicide Clomazone because higher P450 activity should produce more of the active metabolite. To test this hypothesis R and S biotypes of late watergrass were exposed to clomazone in greenhouse studies and effects on growth were measured.

MATERIALS AND METHODS

Chemicals

Technical grade clomazone {2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone}, Command® (micro-encapsulated liquid) and Cerano® (extruded clay granule) formulations were obtained from FMC Corporation (Philadelphia, PA). Technical grade disulfoton {O,O-diethyl S-(2-ethylthioethyl)phosphorodithioate} and demeton-S {O,O-diethyl S-(2-ethylthioethyl)phosphorothioate}were purchased from ChemService (West Chester, PA), and piperonyl butoxide (PBO; {5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole}) was purchased from Sigma-Aldrich (St. Louis, MO). The Disyston® formulation of disulfoton {O.O-diethyl S-[2-(ethylthio)ethyl] phosphorodithioate} was purchased from Bayer CropScience (Research Triangle Park, NC) and the MSR® formulation of oxydemeton methyl {S-[2-(ethylsulfinyl)ethyl]O,O-dimethyl phosphorothioate} was obtained from Gowan Company (Yuma, AZ). Certified analytical standards were used to build external calibration curves for each herbicide or inhibitor used in the laboratory study. Certified standards of clomazone, disulfoton and PBO (98, 98 and 90% pure, respectively) were purchased from Absolute Standards, Inc. (Hamden, CT); demeton-S (99% pure) was

purchased from Protocol Analytical, LLC (Middlesex, NJ). Hoagland's No. 2 basal salt mixture (Sigma-Aldrich, St. Louis, MO) was reconstituted in deionized water and adjusted to pH 6.2 $\pm$ 0.2.

Plants

Seeds of early and late watergrasses, which had been collected from California rice farms, were obtained from the California Rice Experiment Station (Biggs, CA). Two S and three R biotypes of *E. phyllopogon* (EPS1, EPS2, EPR1, EPR2, EPR3) were used in the resistance experiments; EPR1 and one S biotype of *E. oryzoides* (EOS1) were used in the inhibitor experiments. These biotypes have been designated R or S based on tolerance to molinate, thiobencarb, fenoxaprop-ethyl and bispyribac sodium (Fischer *et al.* 2000a).

Effects of P450 inhibitors--greenhouse

In 2005 experiments were conducted to determine effects of P450 inhibitors on clomazone action in the late watergrass biotype EPR1. Germinated seeds were planted in pots filled with Yolo clay loam soil (fine-silty, mixed, non acid, thermic Typic Xerorthents, 1.7% organic matter; 10 plants per pot). Plants were treated at the 0.5 to 1 leaf-stage of growth. On the day prior to clomazone application the plants were sprayed with either DiSyston® (ai disulfoton; 0, 106, 212, 424, 848 or 1696 g ai ha^{-1}) or MSR® (ai oxydemeton methyl; 0, 106, 212, 424, 848 or 1696 g ai ha^{-1}). Approx. 18 h after pre-treatment, plants were sprayed with Command® (ai clomazone; 0, 673 g ai ha^{-1}). A cabinet sprayer equipped with an 8001E-VS (Spraying Systems, Wheaton, IL) flat-fan even-spray nozzle to deliver 380 L ha^{-1} at 275 kPa pressure was used for applications. Pots were placed under a flood (10 cm). Average greenhouse temperature was 24° C with

a 16-h day; 8-h night photoperiod. Natural light was supplemented with 900 μmol m^{-2} s^{-1} PPFD from metal halide lamps. Above-ground portions of 5 plants from each replicate were harvested after 4 d and were weighed and frozen at –80°C for later β-carotene analysis; above-ground portions of the remaining 5 plants in each replicate were harvested 21 days after treatment (DAT) and were weighed. Treatments were a factorial combination of clomazone and inhibitor rates arranged in a completely randomized design (n=3), and the experiment was repeated.

Effects of P450 inhibitors--laboratory

Seeds of S early watergrass biotype EOS1 were germinated on moist towels in the dark for 3 d and then were treated for 5 min in 3% bleach and thoroughly rinsed prior to transfer to 7-mL vials containing deionized water. After 7 d, seedlings at the 0.5 leaf-stage were selected for uniformity and used in the experiments. To aid in dissolution of clomazone and P450 inhibitors, methanol dosing solutions were individually prepared. Technical grade chemicals were added to methanol to a final volume of 0.5 mL and dosing solutions were then added to 100 mL 0.5X Hoagland's solution in 500-mL biochemical oxygen demand bottles. Final concentrations of clomazone were 0 and 2.8 μM. Assuming a 10 cm flood, these concentrations are equivalent to applications of 0 and 660 g ai ha^{-1}. Each clomazone concentration was tested in combination with PBO (5.6 or 20.1 μM; equivalent to 1900 or 6800 g ai ha^{-1}), disulfoton (0.22 or 1.5 μM; equivalent to 60 or 420 g ai ha^{-1}) or demeton-S (1.2 μM; equivalent to 320 g ai ha^{-1}) with 4-6 replicates for each combination of herbicide plus inhibitor. Controls of methanol alone and of each P450 inhibitor alone were included.

Ten seedlings were blotted dry, weighed and placed in each test bottle. Bottles were then sealed with a ground glass stopper and silicone grease and placed randomly in a growth chamber (30°C day: 16°C night; 14 h day:10 h night; relative humidity: 30% day:80% night; maximum irradiance: 800 μmol m^{-2} s^{-1} PPFD). After 4 d plants were removed from test solutions, rinsed with deionized water, blotted dry, weighed and frozen at –80°C prior to β-carotene analysis. Growth was recorded as the 4-d change in wet weight.

Resistance experiments

Greenhouse experiments were conducted in 2003 and 2005 to determine clomazone resistance in 5 biotypes of late watergrass. The R biotypes included EPR1, EPR2 and EPR3, while the S biotypes were EPS1 and EPS2. Soil, flood depth, seedling stage and greenhouse conditions were all as described for the P450 inhibitor experiments except that average greenhouse temperature in 2003 was 22°C. In 2003 plants (4 per pot) were treated with Cerano® (ai clomazone; 0, 42, 84, 168, 337 and 673 g ai ha^{-1}). Granular Cerano® was powderized using a mortar and pestle and uniformly spread over the water in each basin. In 2005 plants (10 per pot) were treated with Command® (ai clomazone; 0, 84, 168, 337, 673 and 1346 g ai ha^{-1}) using a cabinet sprayer as described above. Treatments were arranged in a completely randomized design (n = 5 in 2003; n = 3 in 2005). Plants were harvested 21 DAT and above-ground fresh weight was recorded. The 2003 experiment was conducted once, while the 2005 experiment was repeated.

Confirmation of test solution concentrations

For the laboratory experiment, additional test solutions were prepared for extraction to confirm test solution concentrations. Each solution (40 mL) was placed in a 60-mL screw-cap tube. Samples were acidified to 0.1% with acetic acid and then 5 mL methanol were added prior to extraction with 3 x 5 mL hexane. After each hexane addition, samples were shaken for 1 h on a wrist-action shaker then were allowed to separate for a few minutes before drawing off the organic layer. The three hexane extracts were combined in a 15 mL test tube and were volume reduced to 1 mL under N_2.

Extracts were analyzed using a Hewlett-Packard model 5890 gas chromatograph (GC) coupled to a Hewlett-Packard model 5971 mass selective detector (MSD; Agilent Technologies, Palo Alto, CA). A ZB-50 column capillary column was used (30 x 0.25 mm ID, 25-μm film thickness; Phenomenex, Torrance, CA) with helium as the carrier gas (0.6 mL min^{-1}). A Hewlett-Packard model 7673 autosampler (Agilent Technologies) was used to make 10-μL splitless injections. Injector and detector temperatures were both at 290°C. The GC oven was initially at 40°C and was raised to 300°C over 16 minutes. The mass spectrometer was operated in scan mode (5-550 amu) as follows and analytes were quantitated on specific ions as follows: (1) clomazone, ion *m/z* 125; (2) disulfoton, ion *m/z* 88; (3) demeton-S, ion *m/z* 88; and (4) PBO, ion *m/z* 176. Recoveries for clomazone, PBO, disulfoton and demeton-S were 87, 105, 81 and 53%, respectively, and reported concentrations have been corrected accordingly.

β-Carotene analysis

For laboratory experiments, whole plants were extracted, while for greenhouse experiments, shoots were extracted. All replicates of all treatments were analyzed for β-carotene in the laboratory study; treatments with clomazone alone, clomazone plus demeton-S at 106 and 212 g ai ha^{-1}, and clomazone plus disulfoton at 848 g ai ha^{-1} were analyzed in the greenhouse study.

Pigment analysis was accomplished according to previously described methods with some modification (Takaichi 2000, Helrich 1990, TenBrook & Tjeerdema 2005). Plants were homogenized (Tekmar tissue homogenizer, model TP 18/10 S1, P.O. Box 429576 Cincinnati, OH 45242-9576) in 12.5 mL 7:2 acetone:methanol solution. The tissue probe was rinsed for 30 s with approx. 10 mL 7:2 acetone:methanol. Rinsate was added to homogenate, which was then centrifuged for 5 min at 1500 rpm (495 g). Supernatant was transferred to a 100-mL round bottom flask. Solids were resuspended in extraction solvent, vortexed for 1 min, centrifuged again and supernatant was added to the round bottom flask. Ethanol (a few mL) was added to aid in water removal, and then the extract was evaporated to dryness at 40°C under vacuum and redissolved in 3 mL hexane. Pigment extracts were loaded onto 500-mg silica SPE columns (Alltech Associates, Inc., Deerfield, IL) and ß-carotene was eluted with 6 mL of hexane. Extracts were volume-reduced under N_2 gas to a final volume of 4.5 mL. Acetone (0.5 mL) was added to bring the final solvent ratio to 9:1 (hexane:acetone). Absorbance at 436 nm was recorded and the extinction coefficient of 0.196 mL μg^{-1} (Helrich 1990) was used to

calculate ß-carotene concentrations; extraction efficiency was 100 ± 6.2% as previously reported (TenBrook & Tjeerdema 2005).

Statistics

Data were expressed as percentages of the controls (groups with no clomazone applied) and the R-bioassay package was used to fit non-linear regression curves (R Development Core Team 2004, Ritz & Streibig 2005). Table 8 shows the equations for the models used. Parameters generated with R (shown in Table 9) were used to calculate clomazone rates that inhibited plant growth by 25, 50 or 75% (GR_{25}, GR_{50} and GR_{75},

Table 8. Dose-response models described by Ritz and Streibig (2005); $c = 0$ in 3-parameter models.

Model	Equation
Logistic 3 and 4 parameter	$f(x(b,c,d,e)) = c + \frac{d-c}{1+\exp\{b(\log(x)-\log(e))\}}$ b = relative slope around e c = lower limit of $f(x)$ d = upper limit of $f(x)$ e = dose producing response half-way between c and d; for $c = 0$ and $d = 100$, $e = ED_{50}$[a]
Brain-Cousens 3 and 4 parameter	$f(x(b,c,d,e)) = c + \frac{d+fx-c}{1+\exp\{b(\log(x)-\log(e))\}}$ b = relative slope around e c = lower limit of $f(x)$ d = Limiting dose of stimulation[b] e = dose producing response half-way between c and d f = initial rate of increase in $f(x)$ in cases of hormesis[b]

[a] ED_{50} = dose causing 50% reduction in response compared to control.
[b] Brain and Cousens (1989)

respectively; GR = growth reduction). The curve-fitting model was then used to generate standard errors for GR values. Comparisons between GR_{25}, GR_{50}, and GR_{75} values among curves were made using the sensitivity index (SI) function in R. Data from inhibitor experiments were analyzed by ANOVA (Excel v. X) followed by Fisher's Protected LSD. All data were tested for normality and homogeneity of variance and were Box-Cox transformed as needed (Quinn and Keough 2002).

RESULTS

Greenhouse experiments with P450 inhibitors

Results of treatments of late watergrass biotype EPR1 with disulfoton in combination with clomazone are shown in Table 9. At 4-d, clomazone reduced growth by 26 and 27% in experiments 1 and 2 (Exp. 1 and Exp. 2), respectively. Pre-treatment with disulfoton increased 4-d growth by as much as 66% compared to treatment with clomazone alone (848 g ai ha^{-1} disulfoton; $p < 0.05$). Clomazone reduced 20-d growth by 91 and 86% in Exps. 1 and 2, respectively. Disulfoton pre-treatment increased growth by as much as 5.3-fold (Exp. 1; 848 g ai ha^{-1} disulfoton; $p < 0.05$), but no disulfoton treatment improved growth to the level of the untreated control. Levels of β-carotene were reduced 37% by treatment with clomazone alone and were further reduced by treatment with disulfoton (Exp. 2; 848 g ai ha^{-1}; $p < 0.05$).

Effects of clomazone alone and in combination with oxydemeton methyl on late watergrass biotype EPR1 show similar trends and are shown in Table10. Growth at 4 d was reduced 27% by clomazone treatment compared to the untreated control in Exp. 2 ($p < 0.05$). Compared to treatment with clomazone alone, treatment with clomazone plus

oxydemeton methyl at 848 g ai ha^{-1} improved 4-d growth by 57% in Exp. 1 and by 36% in Exp. 2, while oxydemeton methyl at 106 g ai ha^{-1} improved 4-d growth by 41% in Exp. 2 ($p < 0.05$). Clomazone treatment alone reduced 20-d growth by 92 and 86% in Exps. 1 and 2, respectively ($p < 0.05$). Pre-treatment with oxydemeton methyl increased 20-day growth by as much as 6-fold (Exp. 1; 106 g ai ha^{-1} oxydemeton methyl; $p < 0.05$). In Exp. 2 pre-treatment with oxydemeton methyl at 212, 424, 848 and 1696 g ai ha^{-1} increased growth by 64-300% compared to treatment with clomazone alone ($p < 0.05$). Growth (20-d) did not improve to the level of the untreated control in either experiment. Levels of β-Carotene were reduced 38% by treatment with clomazone in Exp. 2 and pre-treatment with oxydemeton methyl caused further reduction ($p < 0.05$).

Laboratory P450 inhibitor experiments

Results of laboratory exposures of early watergrass biotype EOS1 to clomazone alone and in combination with disulfoton, demeton-S or PBO are shown in Table 11 with measured laboratory concentrations converted to units of g ai ha^{-1}, assuming a 10-cm flood. Treatment with clomazone alone at 660 g ai ha^{-1} reduced growth and β-carotene levels by 26 and 72%, respectively ($p < 0.05$). Treatment with PBO at 6800 g ai ha^{-1} increased both growth and β-carotene levels (by 28 and 48%, respectively) compared to clomazone alone. Treatment with disulfoton at 60 g ai ha^{-1} increased growth and β-carotene levels by 46 and 52%, respectively, compared to treatment with clomazone alone. Demeton-S treatments did not increase growth compared to clomazone-only treatments, but β-carotene levels improved by 48% from treatment with 320 g ai ha^{-1} demeton-S together with 660 g ai ha^{-1} clomazone.

Table 9. Effect of disulfoton on clomazone toxicity to late watergrass (EPR1); data are from 2005 greenhouse study (mean; n = 3).

		4-d Growth (% of control)		20-d Growth (% of control)		β-carotene (% of control)	
Clomazone	Disulfoton	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
(g ai ha^{-1})	(g ai ha^{-1})	(%)	(%)	(%)	(%)	(%)	(%)
0	0	100	100	100	98	90	98
673	0	74[a]	73[a]	9[a]	14[a]	52	62[a]
673	106	98[b]	96[b]	13	46[b]	NA	NA
673	212	94	106[b]	19	16	NA	NA
673	424	99[b]	79	28	20	NA	NA
673	848	80	121[b]	48[b]	22	54	25[b]
673	1696	105[b]	82	38[b]	30[b]	NA	NA

[a] Significantly reduced from untreated control; Fisher's Protected LSD (0.05).
[b] Significantly different from treatment with clomazone alone; Fisher's Protected LSD (0.05).

Table 10. Effect of oxydemeton methyl on clomazone toxicity to late watergrass (EPR1); data are from 2005 greenhouse study (mean; n = 3).

		4-d Growth (% of control)		20-d Growth (% of control)		β-carotene (% of control)	
Clomazone	Oxydemeton methyl	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
(g ai ha^{-1})	(g ai ha^{-1})	(%)	(%)	(%)	(%)	(%)	(%)
0	0	100	100	100	98	95	98
673	0	74	73[a]	8[b]	14[a]	53	61[a]
673	106	86	103[b]	48[b]	18	65	42[b]
673	212	94	88	22	36[b]	38	34[b]
673	424	80	93	22	42[b]	NA	NA
673	848	116[b]	99[b]	32[b]	24[b]	NA	NA
673	1696	68	73	25	23[b]	NA	NA

[a] Significantly reduced from untreated control; Fisher's Protected LSD (0.05).
[b] Significantly different from treatment with clomazone alone; Fisher's Protected LSD (0.05).

Table 11. Effect of P450 inhibitors on clomazone toxicity to early watergrass (EOS1); data are from laboratory study (mean; n = 4-6).

		Parameter (% of control)			Parameter (% of control)			Parameter (% of control)	
Clomazone	PBO	4-d Growth	β-carotene	Disulfoton	4-d Growth	β-carotene	Demeton-S	4-d Growth	β-carotene
--------(g ai ha^{-1})--------		----------(%)----------		(g ai ha^{-1})	----------(%)----------		(g ai ha^{-1})	----------(%)----------	
0	0	100	97	0	100	97	0	100	97
660	0	74[a]	27[a]	0	74[a]	27[a]	0	74[a]	27[a]
660	1900	62	30	60	108[b]	41[b]	320	69	40[b]
660	6800	95[b]	40[b]	420	73	32	NA	NA	NA

[a] Significantly reduced from untreated control; Fisher's Protected LSD (0.05).
[b] Significantly different from treatment with clomazone alone; Fisher's Protected LSD (0.05).

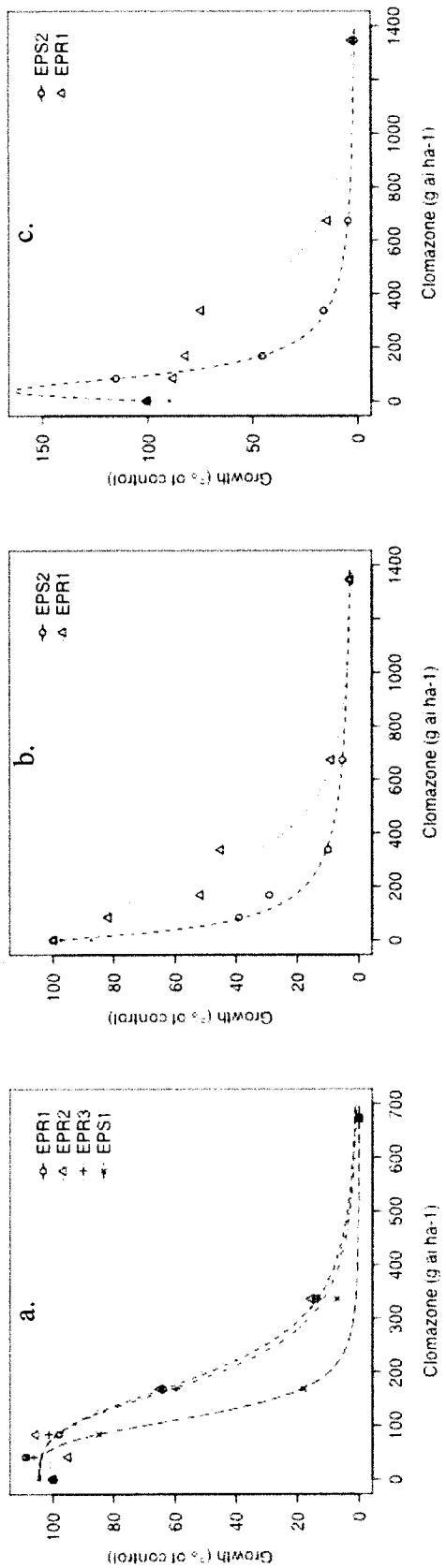

Figure 8. Clomazone dose-response curves for resistant watergrass biotypes EPR1, EPR2 and EPR3 compared to susceptible biotypes EPS1 and EPS2. a. 2003 greenhouse study (logistic 3-parameter model; n=5). b. 2005 greenhouse study, experiment 1 (logistic 3-parameter model; n = 3). c. 2005 greenhouse study, experiment 2 (Brain-Cousens 3-parameter model; n = 3). Points represent mean responses. Model equations and parameters appear in Tables 8 and 12.

Table 12. Curve-fit parameters for biotype comparison.

Year (Exp)[a]	Biotype[b]	b	*p*[c]	d	*p*	e	*p*	f	*p*	Model[d]	BoxCox λ[e]	*p*	Lack of fit *p*[f]
2003	EPS1	4.3	< 0.01	105	< 0.01	118	< 0.01	NA[g]	NA	Logistic 3	NA	NA	0.48
	EPR1	3.4	< 0.01	104	< 0.01	192	< 0.01	NA	NA	Logistic 3	NA	NA	
	EPR2	3.8	< 0.01	101	< 0.01	202	< 0.01	NA	NA	Logistic 3	NA	NA	
	EPR3	3.5	< 0.01	105	< 0.01	185	< 0.01	NA	NA	Logistic 3	NA	NA	
2005 (1)	EPS2	1.1	< 0.01	98	< 0.05	49	0.11	NA	NA	Logistic 3	0.1	< 0.01	0.55
	EPR1	2.1	< 0.01	94	< 0.01	240	<0.01	NA	NA	Logistic 3	0.1	< 0.01	
2005 (2)	EPS2	2.6	< 0.01	100	< 0.01	66	0.07	2.7	0.47	Brain-Cousens 3	0.2	< 0.01	0.32
	EPR1	3.3	< 0.01	90	< 0.01	393	< 0.01	0.038	0.56	Brain-Cousens 3	0.2	< 0.01	

[a] Exp = experiment number.
[b] See text for descriptions.
[c] $p < 0.05$ indicates that parameter value is significantly different from 0.
[d] As described by Ritz and Streibig (2005) and shown in Table 1.
[e] BoxCox transformation of variable $Y = (Y^{\lambda} - 1)/\lambda$ for $\lambda \neq 0$; $\ln(Y)$ for $\lambda = 0$ (Quinn & Keough 2002).
[f] $p > 0.05$ indicates acceptable fit for all curves in experiment.
[g] NA = Not Applicable.

Resistance

Figure 8 shows dose-response curves generated for each experiment and Table 13 gives comparisons of GR_{25}, GR_{50}, and GR_{75} values calculated from parameters shown in Table 12. In all experiments, the S biotypes (EPS1 and EPS2) had lower GR values compared to the R biotypes (EPR1, EPR2 and EPR3; $p < 0.05$).

Table 13. Comparision of growth reduction (GR) values from dose-response curves for R vs. S biotypes of late watergrass; (mean; * significantly different from S biotype, $p < 0.05$).

Year (Exp)[a]	Biotype[b]	GR_{25}[c] (clomazone, g ai/ha)	GR_{50}[c] (clomazone, g ai/ha)	GR_{75}[c] (clomazone, g ai/ha)
2003	EPS1	91	118	152
n = 5	EPR3	136*	185*	253*
	EPR2	151*	202*	271*
	EPR1	140*	192*	265*
2005 (1)	EPS2	18	49	137
n = 3	EPR1	143*	240*	402*
2005 (2)	EPS2	132	158	211
n = 3	EPR1	297*	405*	562*

[a] Exp = experiment number.
[b] See text for descriptions.
[c] GR_{25}, GR_{50}, and GR_{75} = application rate of clomazone causing 25, 50, and 75% reduction in response, respectively.

DISCUSSION

In both greenhouse and laboratory experiments, effects of clomazone alone were more severe as measured by β-carotene levels vs. growth. Reductions in 4-d growth were similar in all experiments (means of 26-27%) with clomazone treatments at 660-673 g ai ha^{-1}, while reductions in β-carotene levels were 38 and 72% in EPR1 and EOS1, respectively. Similar results were reported in experiments with rice and R and S biotypes of early and late watergrasses (TenBrook & Tjeerdema 2005). As discussed, the non-mevalonate isoprenoid synthetic pathway produces precursors to both gibberellins and β-carotene, but through different branches via the common precursor geranyl-geranyl diphosphate (GGPP; Sponsel 2002). Clomazone, or rather an active metabolite, inhibits the first step in the synthetic pathway (Vencill *et al.* 1990) upstream from GGPP. Thus, the gibberellin and carotenoid synthesis branches would have to compete for limited GGPP. Gibberellins are C_{20} molecules, whereas carotenoids are C_{40} molecules so it makes sense that carotenoid production would suffer more under conditions of reduced GGPP. Further, it is possible that gibberellins have an alternative source of precursors (Taiz and Zeiger 1998), which could explain the difference between growth and β-carotene sensitivity. Direct measurement of gibberellins after clomazone exposure would enhance understanding of these two different clomazone effects.

Clomazone activation by P450 enzymes has been confirmed by this study, but P450 involvement in clomazone detoxification is also suggested. The P450 inhibitors PBO, disulfoton and oxydemeton methyl applied in combination with clomazone demonstrated protective effects with respect to growth in both EPR1 and EOS1, but only

4-day growth improved to the level of the untreated control. Protection from bleaching was also observed for EOS1 treatments with PBO, disulfoton and demeton-S, but not for EPR1, which suffered further damage from application of disulfoton and oxydemeton methyl. These antagonistic and synergistic effects of P450 inhibitors on clomazone response indicate P450 enzymes are involved in activation of clomazone, as well as in its detoxification. P450 inhibitors have been shown to be isozyme-specific (Barrett *et al.* 1997), so whether an herbicide is synergized or antagonized by a P450 inhibitor depends on whether or not the herbicide requires activation, which isozymes are affected by the inhibitor, and what happens to the relative levels of activation and detoxification isozyme activity. Further work is needed to directly measure P450 isozyme activity in plants exposed to clomazone alone and in combination with P450 inhibitors. The role, if any, of glutathione conjugation in clomazone detoxification and herbicide resistance in watergrasses also needs further study.

Growth is a physiological phenomenon that integrates the effects of numerous environmental factors. As such, the effects of clomazone on growth could be a consequence of reduced β-carotene levels and it would be expected that if plant growth is protected from clomazone by P450 inhibitors, then β-carotene levels should also be protected. However, the effects of various clomazone/P450 inhibitor combinations on growth observed in this study were not mirrored in β-carotene levels, suggesting that clomazone affects these parameters independently. In the greenhouse study with R late watergrass (EPR1) β-carotene levels were further reduced by application of P450 inhibitors, indicating synergism between clomazone and the inhibitors. Conversely, in the

laboratory study with S early watergrass (EOS1) PBO, disulfoton and demeton-S all increased β-carotene levels compared to treatments with clomazone alone, indicating antagonism between clomazone and inhibitors. These results suggest that growth and β-carotene levels are affected independently by clomazone, which could be due to competition for limited GGPP, or could be the result of multiple molecular targets for clomazone.

Interpretation of the results of this study and others that utilize P450 inhibitors to discern mechanisms of susceptibility and resistance in plants is difficult, particularly when working with a metabolically activated compound such as clomazone. The actions of the inhibitors themselves are not straightforward. For example, PBO is a suicidal inhibitor of P450 enzymes, which can, after about 24 h of exposure, act as a P450 inducer (Hodgson & Levi 1998). Demeton-S and oxydemeton methyl are products of oxidation of disulfoton, and are known cholinesterase inhibitors, but are also P450 inhibitors with oxidizable sulfur groups. As esterase inhibitors, it is possible that these demeton compounds could inhibit the hydrolysis of 5-ketoclomazone, which is a cyclic ester. As discussed in the introduction, it is not clear whether such a hydrolysis step would detoxify 5-ketoclomazone, or further activate it.

Consider a possible herbicide resistance mechanism that is consistent with this and other studies. The S and R biotytpes used in the current study have been designated as such due to susceptibility and resistance to thiocarbamates, aryloxyphenoxy-propionates and bispyribac-sodium (Fischer *et al.* 2000a). Given the evidence discussed previously indicating that clomazone is metabolically oxidized by P450 enzymes to its

active form, resistance to clomazone would not be expected if the resistance mechanism in the R biotypes is elevated P450 activity. In fact, toxicity would be expected to be higher with higher P450 activity. Contrarily, the S biotypes (EPS1 and EPS2) in this study had consistently lower GR values than the R biotypes (EPR1, EPR2 and EPR3). Seeing the same pattern of tolerance in herbicides that do not require metabolic activation as in those that do suggests either that the resistance mechanism is not related to P450 activity or that it is due to differential activity of P450 isozymes.

Another possibility is that the R and S biotypes differ in their abilities to form glutathione conjugates. Molinate, thiobencarb, fenoxaprop-ethyl and clomazone are all capable of forming glutathione conjugates in various species of plants and animals (Pena-Llopis *et al.* 2001, Tjeerdema & Crosby 1988a, 1988b, 1987, Pena *et al*, 2000, Edwards & Cole 1996, Tal *et al.* 1995, 1993). However, the structure of bispyribac-sodium does not suggest the possibility of glutathione conjugation and no evidence for this detoxification pathway was found in the literature. This leads to the more likely explanation that differential activity of P450 isozymes (activating vs. detoxifying) is the difference between R and S biotypes. Plants with higher levels of activation vs. detoxification P450 isozyme activity would be susceptible to clomazone, thiobencarb, molinate, fenoxaprop ethyl and bispyribac sodium, while plants with higher levels of detoxification vs. activation activity would be resistant to all of these. This is plausible given that differential levels and activities of P450 enzymes in watergrasses have been reported (Yun *et al.* in press).

Two areas for future research are 1) the possibility that clomazone directly affects gibberellin synthesis; and 2) the possibility that clomazone acts on more than one target. Gibberellic acid (GA) is applied to crops, including rice, to promote seed germination, enhance growth, and induce flowering and ripening (Taiz & Zeiger 1998). Studies have shown that application of GA to pea (*Pisum sativum* L.) and corn (*Zea mays* L.) reduced growth-inhibiting effects of clomazone (Sandmann and Böger 1986; Vencill *et al.* 1989). It is important to know if application of clomazone might reduce the effectiveness of GA applications in rice. Finally, only one clomazone molecular target has been identified to date (Mueller *et al.* 2000), but the independence of growth and β-carotene effects observed in this study suggests the possibility of multiple targets. For development of successful clomazone safening techniques, molecular mechanisms of clomazone action and selectivity need further study.

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Section V: Summary and conclusions

Clomazone toxicity and biotransformation were studied in rice and watergrasses (*Echinochloa* spp.). Clomazone resistance and the effects of P450 inhibitors on clomazone toxicity were studied and compared in early and late watergrasses (EWG and LWG, respectively). Clomazone effects on watergrass biotypes that have shown resistance (R) or susceptibility (S) to thiocarbamates, aryloxyphenoxy-propionates and bispyribac-sodium were also studied. Rice growth was unaffected by clomazone in 7-d laboratory exposures (No Observed Effect Concentration (NOEC) = 7.9 μM) while both EWG and LWG growth were reduced (NOEC = 0.21 and 0.46 μM, respectively, with no difference between R and S biotypes based on the NOEC). Rice and watergrasses suffered severe bleaching from clomazone exposure. Measurement of β-carotene levels produced NOEC values of 0.21, < 0.08, < 0.08, 0.08 and 0.46 μM clomazone for rice, R and S EWG, and R and S LWG, respectively. Of particular note is that LWG and rice responses to clomazone were similar with respect to β-carotene levels indicating that it would be difficult to use clomazone to control this weed without damaging the rice crop. Although no differences in growth between R and S biotypes were observed based on NOEC values, and the NOEC for S LWG was higher than for R LWG, resistance to clomazone at field-relevant rates was observed for growth in R EWG and for β-carotene levels in R LWG (R/S IC_{50} ratios of1.8 and > 4.5, respectively; IC_{50} = concentration of clomazone causing 50% inhibition compared to the control).

Having found rice to be relatively clomazone-tolerant, and EWG to be relatively sensitive, these two species were exposed to ^{14}C-labeled clomazone to determine differences in accumulation, biotransformation and mass balance. With regard to

accumulation on a nmol g^{-1} basis, there was no significant difference between the two species ($p > 0.05$). In terms of extractable ^{14}C residues, the concentration in EWG was significantly higher (4.2 ± 0.5 vs. 1.8 ± 0.1 nmol g^{-1} in EWG vs. rice, respectively; $p < 0.01$). More metabolized residue was measured in EWG compared to rice (84.1% vs. 67.9%; $p < 0.01$). Both species produced hydroxylated forms of clomazone, as well as β-D-glucoside conjugates, and several other unidentified polar metabolites, but EWG generally produced higher metabolite concentrations. The suspected active metabolite, 5-ketoclomazone, was found in both rice and EWG, comprising < 1% of total metabolites in both species, although the concentration was significantly higher in EWG vs. rice (21 ± 2 vs. 5.7 ± 0.5 pmol g^{-1}, respectively; $p < 0.01$). Consistent with the action of a metabolically activated herbicide, the more sensitive EWG qualitatively and quantitatively metabolized more clomazone than the more tolerant rice.

To further explore the questions of metabolic activation and detoxification of clomazone, as well as resistance mechanisms and potential safening techniques, R and S biotypes of early and late watergrasses (*Echinochloa* spp.) were exposed to clomazone alone or in combination with P450 inhibitors piperonyl butoxide (PBO), disulfoton, demeton-S and oxydemeton methyl in laboratory and greenhouse experiments. Clomazone GR_{25}, GR_{50}, and GR_{75} values were lower for S biotypes than for R biotypes (18-132 vs. 136-297, 49-158 vs. 185-405, and 137-211 vs. 253-562 g ai ha^{-1}, respectively; $p < 0.05$). The P450 inhibitors PBO, disulfoton and oxydemeton methyl applied in combination with clomazone demonstrated protective effects with respect to growth in both R LWG and S EWG. Protection from bleaching was also observed for S EWG for

treatments with PBO, disulfoton and demeton-S, but not for R LWG, which suffered further damage from application of disulfoton and oxydemeton methyl. Considering this combination of responses with application of P450 inhibitors together with the clomazone resistance seen in R LWG biotypes presents a bit of a puzzle that may be explained, in part, by either P450 isozyme activity or by the possibility that clomazone has multiple molecular targets. Also, it is possible that clomazone affects gibberellin and carotenoid synthesis independently or differentially via competition for the common precursor GGPP.

The biochemistry of clomazone action is extremely complex. The work presented here has shown that clomazone use in rice culture could be problematic due to the similar sensitivities of rice and LWG and that clomazone resistance will be a problem for control of watergrasses. However, this work has provided confirmation that clomazone requires P450 activation to a toxic metabolite. Thus, a possible means of safening rice from clomazone is suggested: P450 inhibitors could be applied to rice (via seed treatments, for example) to reduce susceptibility to clomazone, a process that has been successful in rice with disulfoton (Jordan *et al.* 1998), but has not been tested with other inhibitors in rice. Further understanding of P450 isozymes involved in clomazone activation and detoxification could allow development of safeners based on the higher level of clomazone metabolism observed in the more sensitive EWG compared to rice. For example, inducers of activating P450 isozymes could be applied to weeds to increase clomazone toxicity, or inducers of detoxifying isozymes could be applied to rice to decrease toxicity.

Future clomazone research is needed in several areas. First is to determine whether activation and detoxification of clomazone are due to the action of different P450 isozymes, and whether different levels of isozymes among biotypes is what imparts resistance or susceptibility. Studies have shown that P450 enzymes are responsible for resistance and selectivity to other herbicides in watergrasses (Yun *et al.*, in press; Fischer *et* al. 2000). The interaction of clomazone activating and detoxifying isozymes will be challenging to resolve. Concomitant with this is the need to determine the role, if any, of glutathione conjugation in clomazone detoxification and herbicide resistance in watergrasses. While clomazone will react with glutathione (Vencill *et al.* 1990), this reaction, and any subsequent metabolic processing that may occur *in vivo*, has not been studied.

Another important avenue of research is whether clomazone inhibits gibberellin synthesis directly and whether this is the primary means by which clomazone reduces growth. While the one molecular target that has been identified for clomazone (Mueller *et al.* 2000) has the potential to affect gibberllin synthesis it is possible that growth is affected as a result of action on a different molecular target.

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