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Uptake and Metabolism of Clomazone in Tolerant-Soybean and Susceptible-Cotton Photomixotrophic Cell Suspension Cultures[1]

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ABSTRACT

Studies were conducted to determine the uptake and metabolism of the pigment synthesis inhibiting herbicide clomazone in tolerant-soybean (*Glycine max* [L.] Merr. cv Corsoy) and susceptible-cotton (*Gossypium hirsutum* [L.] cv Stoneville 825) photomixotrophic cell suspensions. Soybean and cotton on a whole plant level are tolerant and susceptible to clomazone, respectively. Preliminary studies indicated that I_{50} values for growth, chlorophyll (Chl), β-carotene, and lutein were, respectively, >22, 14, 19, and 23 times greater for the soybean cell line (SB-M) 8 days after treatment (DAT) compared to the cotton cell line (COT-M) 16 DAT. Differences in [^{14}C]clomazone uptake cannot account for selectivity since there were significantly greater levels of clomazone absorbed by the SB-M cells compared to the COT-M cells for each treatment. The percentage of absorbed clomazone converted to more polar metabolite(s) was significantly greater by the SB-M cells relative to COT-M cells at 6 and 24 hours after treatment, however, only small differences existed between the cell lines by 48 hours after treatment. Nearly identical levels of parental clomazone was recovered from both cell lines for all treatments. A pooled metabolite fraction isolated from SB-M cells had no effect on the leaf pigment content of susceptible velvetleaf (*Abutilon theophrasti* Medic.) or soybean seedlings. Conversely, a pooled metabolite fraction from COT-M cells reduced the leaf Chl content of velvetleaf. Soybean tolerance to clomazone appears to be due to differential metabolism (bioactivation) and/or differences at the site of action.

Clomazone [2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone] is a selective herbicide for use in soybean (24). A single preplant incorporated application of clomazone (dimethazone, FMC-57020, Command) controls many grasses and some broadleaf weeds. This compound reduces or stops the accumulation of plastid pigments in susceptible species by inhibiting an enzyme of the terpenoid pathway resulting in white, yellow, or pale green plants (7). The exact enzymatic site of clomazone action is currently unknown. Some of the suggested sites of action include: (a) isopentenyl pyrophosphate isomerase (5, 19); (b) prenyl transferases (5, 19); (c) enzymatic phytylation of chlorophyllide (5, 6); and (d) any enzyme of the terpenoid pathway after geranyl-geranylpyrophosphate production (1). The only report regarding the mechanism of clomazone selectivity suggested that the large differences in clomazone toxicity observed between tolerant bell pepper (*Capsicum annuum* L.) and susceptible tomato (*Lycopersicon esculentum* Mill.) seedlings could not be attributed solely to differential absorption, translocation, or metabolism (26).

Many researchers have reported the initiation of photoautotrophic and photomixotrophic cell suspensions (10, 12, 13, 27), and their potential use in herbicide mechanism of action and metabolism studies (3, 17, 20). Soybean and cotton photoautotrophic cell suspensions have been reported to behave as young expanding leaves with respect to pigment production, photosynthesis, and respiration (3, 17). The primary differences between the cell suspension and whole plant metabolism are a relatively low ribulose-1,5-bisphosphate carboxylase activity and a relatively high phospho*enol*pyruvate carboxylase activity in the cell suspension cultures (11).

Pigment production is inhibited in both cell cultures by the standard pigment synthesis inhibiting herbicide fluridone (data not shown). The enzymatic site of fluridone action is phytoene desaturase of the terpenoid pathway (2). The SB-M[2] and COT-M cell response to fluridone suggests that pigment synthesis in these cells occurs via the terpenoid pathway of whole plants. Consequently, the SB-M and COT-M cell suspensions appear to represent an effective system for physiology studies using a pigment synthesis inhibiting herbicide such as clomazone.

The objectives of this study were to: (a) compare the effects of clomazone on the growth and pigment content of soybean and cotton whole plant and cell suspension systems; (b) isolate, separate, quantify, and identify the levels of clomazone and clomazone metabolite(s) contained within the SB-M and COT-M cells; (c) determine the biological activity of the SB-M and COT-M pooled metabolite fractions on the Chl content of soybean and velvetleaf whole plants; and (d) ascertain the operative selectivity mechanism which conveys soybean tolerance to clomazone.

MATERIALS AND METHODS

Whole Plant Studies

Soybean (*Glycine max* (L.) Merr cv Corsoy 79) and cotton (*Gossypium hirsutum* (L.) cv Stoneville 825) seeds (Illinois

[1] Supported in part by funds from the Illinois Agricultural Experimental Station and FMC Corporation.

[2] Abbreviations: SB-M, soybean photomixotrophic cell line; COT-M, cotton photomixotrophic cell line; Rt, retention time; HAT, hours after treatment; DAT, days after treatment; DAS, days after subculture; KN, soybean cell medium; GR, cotton cell medium.

Foundation Seeds Inc., Champaign, IL and Stoneville Pedigreed Seed Co., Stoneville, MS) were planted into 0.95 L plastic pots which contained a Drummer silty clay loam (5% organic matter) treated with clomazone (commercial, technical, analytical, and radiolabeled forms of clomazone were all gifts from FMC Corp., Princeton, NJ) at rates of 0, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 kg/ha. Soil was treated by applying 50 mL of a clomazone solution to a 15.14 L bin containing 1.0 kg of soil and tumbled for 20 mins at 20 rpm. Each pot was then covered with 2 inches of untreated soil. Plants were grown in the greenhouse under supplemental incandescent/fluorescent lighting with a 15 h photoperiod. Six discs (10 mm diameter) from the first fully developed trifoliate soybean leaf (2 discs/leaflet) and the first fully developed unifoliate cotton leaf were harvested, weighted, and Chl extracted and measured spectrophotometrically (8).

The effects of clomazone and purified SB-M and COT-M cell clomazone metabolite fractions on the leaf pigment content of soybean and velvetleaf (*Abutilon theophrasti* Medic) seedlings was determined by placing plants into foam-rubber collared 50 mL Erlenmeyer flasks containing 55 mL of 25.0% Hoagland solution with the proper herbicide treatment. The molarity of the pooled metabolite fraction was determined using the percentage of recovered radioactivity of the pooled metabolite fraction relative to the initial total radioactivity level and molarity of parental clomazone used for each treatment. The velvetleaf treatment consisted of a 1.3 μM concentration (7.4 nCi) of [^{14}C]clomazone (aqueous) or a pooled [^{14}C]clomazone metabolite fraction (aqueous) whereas soybean seedlings were treated with a 6.5 μM concentration (36.8 nCi) of [^{14}C]clomazone or a pooled [^{14}C]clomazone metabolite fraction. Control flasks were utilized for all treatments. Velvetleaf and soybean seedlings were, respectively, in the newly emerged third leaf and first trifoliate leaf stage of development at the initiation of these greenhouse experiments. Plants were harvested 7 DAT and the weight and Chl levels of the third and fourth leaves of velvetleaf and the first trifoliate leaf of soybean were determined as previously, described. Attempts to determine the distribution of the radiolabeled clomazone and clomazone metabolites were unsuccessful. The low levels of radiolabeled metabolites (7.4 and 36.8 nCi) necessarily used in this study resulted in extremely variable and inconclusive data.

Photomixotrophic Culture Conditions

The soybean (cv Corsoy), and cotton (cv Stoneville 825), photomixotrophic cell suspensions used in this study were established in 1982 by Horn *et al.* (10), and 1985 by Blair *et al.* (3), respectively. SB-M cells (0.5 g) were subcultured into 50 mL of KN medium on a 14 d interval whereas COT-M cells (0.5 g) were subcultured into 50 mL of GR medium on a 28 d interval. KN (pH = 5.8) contains basic MS salts, NaFeEDTA (27.8 mg/L $FeSO_4 \cdot 7\ H_2O$ and 33.5 mg/L Na_2EDTA), thiamine (0.1 mg/L), NAA (1.0 mg/L), kinetin (0.2 mg/L), and 1% sucrose. GR medium (pH = 5.8) contains the basic MS salts, NaFeEDTA (as for KN), glycine (2.0 mg/L), thiamine (0.1 mg/L), nicotinic acid (0.5 mg/L), pyridoxine (0.5 mg/L), myo-inositol (0.1 g/L), picloram (121 μg/L), kinetin (1.5 mg/L), and 1% starch. The cells were cultured on an orbital shaker (130 rpm) under continuous light (200–250 $\mu E \cdot m^{-2} \cdot s^{-1}$) in a growth chamber (28 +/− 1°C) containing a 650 μL CO_2/L atmosphere.

Herbicide Application

All herbicides were added to the medium just prior to cell subculture. Technical grade clomazone (91% purity) prepared in 5 mM Mes (pH 5.8) was introduced into the medium by filter sterilization through a 0.22 μm membrane for growth/pigment analysis experiments. Methylene labeled [^{14}C]clomazone (31.14 mCi/mmol) and analytical grade clomazone (99% purity) dissolved in 100% ethanol were introduced directly into the medium for uptake and metabolism studies maintaining a final solvent concentration of 0.2%. Control flasks contained the final solvent concentration minus the herbicide.

Growth and Chl Analysis

Cells were harvested by vacuum filtration and weighed to determine cell fresh weight at 8 and 16 DAT, respectively, for the SB-M and COT-M cell lines. These harvest times were chosen to allow the time of cell exposure to the herbicide to be just over one-half the cell cycle period. Also, over 50% of the maximum cell Chl content (μg/g fresh weight) is attained by both cell lines within these time frames (data not shown). Chl was extracted from a 150 mg cell sample with 80% acetone and measured spectrophotometrically (15).

Carotenoid Extraction and Analysis

SB-M and COT-M cells were harvested 8 and 16 DAT, respectively. Since carotenoids are labile to combinations of light and oxygen (4), extraction procedures were conducted under a safe light and a nitrogen atmosphere whenever possible. Deionized water used for extraction was freshly boiled and placed on ice to remove all dissolved oxygen. Carotenoids were extracted, separated, and quantified using the procedures described previously (25). All samples were stored under nitrogen at −20°C until analysis using HPLC. Rts for β-carotene and lutein were 3.2 and 22.0 min, respectively.

[^{14}C]Clomazone Uptake and Metabolism Studies

Cell fresh weights were determined at 6, 24, and 48 HAT. Ethyl acetate (100 mL) was added to the medium fraction and vigorously shaken in a separatory funnel. The ethyl acetate fraction was reduced to dryness on a rotary evaporator and dissolved in 1 mL of 90% ethanol. Scintillation cocktail (16 mL) was then added to a sample (100 μL) to determine the total dpm in the medium fraction using liquid scintillation spectrometry. The aqueous fraction as discarded after the ethyl acetate wash because no appreciable radioactivity (<4%) remained in this fraction. The cell fraction was immediately ground in 90% ethanol (5 mL) for 1.5 min with a Polytron (5.5 setting) and the homogenate filtered through a 0.22 μm membrane. A sample (100 μL) of the filtrate was then used to determine the total dpm using liquid scintillation spectrometry. All samples were stored at −20°C until HPLC analysis.

Each cell extract sample was placed into a separatory funnel containing 1.5 mL hexane, mixed, and the two layers separated. The upper hexane layer was discarded and the extraction procedure repeated. The hexane extraction removed most of the Chl in the sample and contained 5% or less of the total radioactivity.

Separation and quantification of the [^{14}C]clomazone and metabolite(s) was accomplished using C-18 reversed phase HPLC (Beckman) coupled to a Flo-One/Beta model 1C radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc.). The 4.6 mm × 25 cm i.d. stainless steel column was prepacked with 5 μm Ultrasphere C-18 (Beckman) and was protected with an Adsorbosphere C-18 5 μm cartridge guard column. Known volumes of ethyl acetate and cell extract fractions were taken to dryness and resuspended in water:acetonitrile (60:40, v/v) and injected into the HPLC via a fully loaded 250 μL loop. Parental clomazone and total clomazone metabolite(s) were separated and quantified using a water:acetonitrile (60:40, v/v) mixture with a constant flow rate of 1.5 mL/min. The Rts for parental clomazone and metabolite(s) were 13.0 and 2.0 min, respectively.

Clomazone metabolite(s) were isolated using the HPLC conditions described previously, with the exception of the use of a 10 mm × 25 cm preparatory C-18 reversed phase stainless steel column. A water:acetonitrile (60:40, v/v) mixture with a flow rate of 3.0 mL/min separated parental clomazone from metabolite(s) with Rts of 31.6 and 3.4 min, respectively, for both cell lines. The clomazone metabolite(s) were isolated by collecting the HPLC eluate for 7 min immediately after sample injection. Isolated clomazone metabolite(s) were separated and quantified using a 3.0 mL/min flow rate of a water:acetonitrile (90:10, v/v) mixture for 2.0 min followed by a continuous gradient to water:acetonitrile (60:40, v/v) over a 45 min duration. Identification of clomazone metabolite(s) was accomplished using β-glucosidase (Sigma Chemical Co., St. Louis, MO) digestion (22) and cochromatography with the clomazone derivative 2-chlorobenzyl alcohol.

Statistical Analysis

All treatments were replicated 3 to 6 times, data averaged, and standard errors calculated.

RESULTS AND DISCUSSION

Whole Plant Sensitivity to Clomazone

Soybean plants (Corsoy 79) were 14.3 times more tolerant to clomazone relative to cotton plants (Stoneville 825) with respect to the inhibition of the accumulation of total Chl (mg/g fresh weight), (Fig. 1). The I_{50} values for Chl production in soybean and cotton leaves were, respectively, 2.14 and 0.15 kg/ha. Control treatments indicated that soybean leaves (3.8 mg Chl/g fresh weight) had 2 times the Chl level of cotton leaves (1.9 mg/g fresh weight). The soybean sensitivity to clomazone in the greenhouse studies was greater than that observed in field trials since the recommended field use rate of 1.3 kg/ha resulted in detectable leaf Chl reduction. This hypersensitive response is typical, however, of many herbicide dose-response studies performed in a greenhouse. Although

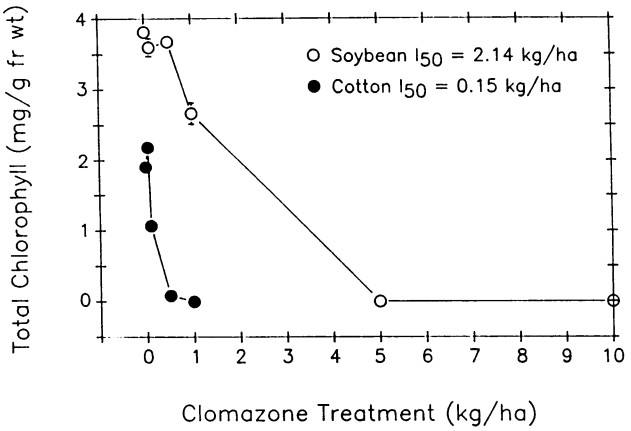

Figure 1. Effects of a soil-applied clomazone treatment on the Chl content of the first fully expanded trifoliate soybean and unifoliate cotton plant leaves. When not shown, the SE of the means from six replications are smaller than the symbol size and cannot be visualized.

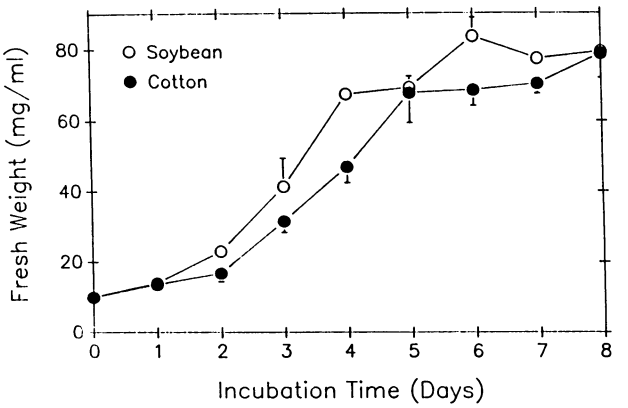

Figure 2. Growth cycle of the SB-M and COT-M cell suspension cultures. Growth is expressed as mg of cell fresh weight per mL of medium. The growth cycle was initiated by subculturing SB-M and COT-M cells (0.5 g) into 50 mL of freshly made KN and GR medium, respectively. The duration of the entire SB-M and COT-M cell growth cycles were 14 and 28 d, respectively.

cotton is sensitive to clomazone relative to soybean, corn (*Zea mays* L.), velvetleaf, and grass weeds are significantly more sensitive to clomazone than cotton (14, 24).

Clomazone Induced Cell Growth and Pigment Reduction

Although the growth curves for the SB-M and COT-M cell lines were similar, SB-M cells entered logarithmic growth between 1 and 2 DAS whereas COT-M cells initiated logarithmic growth between 2 and 3 DAS (Fig. 2). Consequently, the SB-M cell line maintained a higher density of cells (mg/mL) throughout the linear phase of the growth cycle relative to the COT-M cell line. SB-M and COT-M cells reached the stationary phase between 5 and 6 DAS. Although both cell lines attain the stationary phase of growth by 6 DAS, the Chl content continues to rise through 12 and 24 DAS for the SB-M and COT-M cell lines, respectively (data not shown). Consequently, the SB-M and COT-M cells are subcultured

every 14 and 28 d, respectively, to maintain the highest Chl content attainable.

Clomazone significantly reduced Chl levels (I_{50} = 47 μM) of the SB-M cells but had no effect on growth up to a 100 μM treatment (Fig. 3A). Conversely, clomazone inhibited both the growth (I_{50} = 4.2 μM) and Chl content (I_{50} = 3.2 μM) of the COT-M cells (Fig. 4A). Even though untreated SB-M cells (68.2 μg Chl/mL) have 4.6 times the Chl content of COT-M cells (14.9 μg Chl/mL), the I_{50} values for Chl content indicate that the SB-M cells are 14.7 times more tolerant than COT-M cells to clomazone. This value corresponds well to the 14.3-fold greater clomazone tolerance exhibited by soybean plants relative to cotton plants with respect to Chl reduction (Fig. 1). The remarkable similarity of soybean tolerance to clomazone relative to cotton on a whole plant and cultured cell suspension levels indicate that extrapolations from clomazone-induced responses observed in cell suspensions accurately reflect predicted responses on a whole plant level.

Carotenoid levels were significantly reduced by clomazone treatment in both cell lines (Figs. 3B and 4B). The I_{50}s for carotene (β-carotene) production were 46 and 2.4 μM in the SB-M and COT-M cells, respectively. The I_{50}s for xanthophyll (lutein) production were 48 and 2.2 μM for the SB-M and COT-M cells, respectively. Since the I_{50}s for Chl, β-carotene, and lutein production (Figs. 3 and 4) for the SB-M and COT-M cells are within a narrow range with respect to each cell line, it is possible that clomazone inhibits an enzymatic

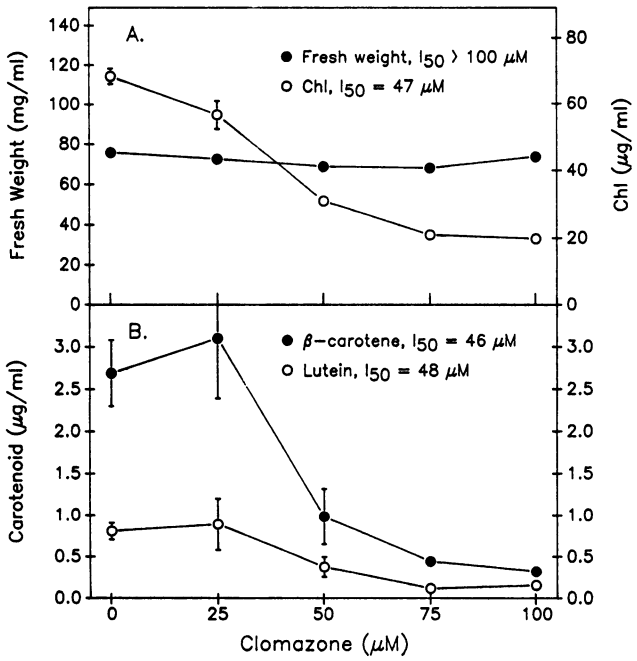

Figure 3. Effects of clomazone on SB-M cell growth and pigment content 8 DAT. Cells (0.5 g) were subcultured into 50 mL of KN medium containing the herbicide. Fresh weight and pigment content are expressed, respectively, as mg/mL and μg/mL of medium. Culture conditions were as described in "Materials and Methods." A, Fresh weight and Chl content; B, β-carotene and lutein content. Each point is the mean of three to four replications ± SE. When not shown, the SE is equal to or smaller than the symbol.

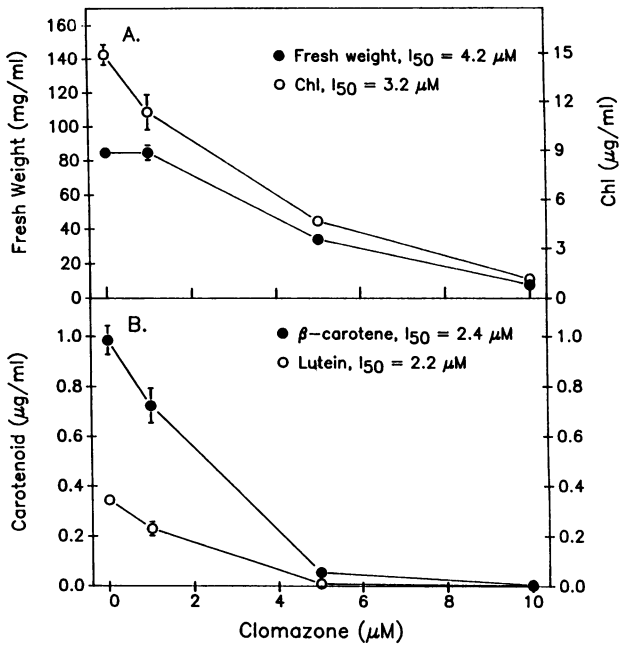

Figure 4. Effects of clomazone on the growth and pigment content of COT-M cells 16 DAT. Cells (0.5 g) were subcultured into 50 mL of GR medium containing the herbicide. Fresh weight and pigment content are expressed, respectively, as mg/mL and μg/mL of medium. Culture conditions were as described in "Materials and Methods." A, Fresh weight and Chl content; B, β-carotene and lutein content. Each point is the mean of three to four replications ± SE. When not shown, the SE is equal to or smaller than the symbol.

reaction common to all three of these pigment types. This conclusion is supported by a previous report which indicated that since the I_{50} values for Chl and carotenoid biosynthesis in autotrophically grown *Scenedesmus acutus* alga were identical (10 μM clomazone), the various effects of clomazone were due to a single enzyme target in the terpenoid biosynthetic pathway between acetate and geranylgeranyl pyrophosphate (18). The phytol component of Chl, carotenes, and xanthophylls are each directly derived from geranylgeranyl pyrophosphate of the terpenoid pathway. Clomazone does not inhibit the carotenogenic enzymes phytoene desaturase, ζ-carotene desaturase, or lycopene cyclase (19). Hence, the lack of Chl in clomazone treated leaves of susceptible species is not due to photooxidative processes resulting from a carotenoid deficiency.

[^{14}C]Clomazone Uptake and Translocation

Over 90% of the [^{14}C]clomazone was recovered for all treatments (data not shown). Uptake studies were conducted using a clomazone concentration of 0.44 μM because this treatment has no effect on the growth of either cell line (Figs. 3 and 4). Clomazone uptake by the SB-M cells increased dramatically at 6 and 24 HAT but leveled by 48 HAT (Fig. 5). Conversely, clomazone uptake by the COT-M cells increased gradually over the entire time course but to a much reduced level relative to the SB-M cells. The rates of clomazone uptake were nearly identical, however, by both cell lines

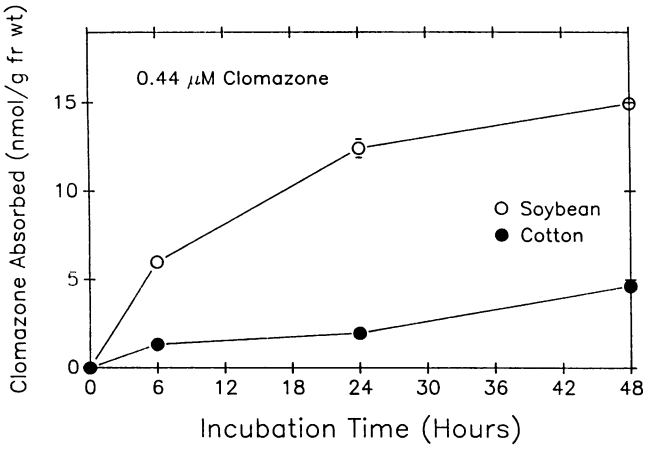

Figure 5. [^{14}C]Clomazone (0.70 μCi) uptake by SB-M and COT-M cells exposed to a 0.44 μM concentration. Culture conditions and application and recovery of clomazone were as described in "Materials and Methods." Each point is the mean of three to four replications ± SE. When not shown, the SE is equal to or smaller than the symbol.

between 24 and 48 HAT. Since, clomazone uptake (nmol/g fresh weight) was significantly greater by the SB-M cells compared to the COT-M cells for all three incubation times, decreased clomazone uptake is not the selectivity mechanism resulting in soybean cell tolerance to clomazone. This conclusion is consistent with previous reports which indicated that soybean plants grown hydroponically absorbed a greater percentage of [^{14}C]clomazone than more sensitive weed and crop species (14, 23). The mechanism resulting in the differential rates of clomazone uptake between the SB-M and COT-M cells is unknown.

Reports from three independent researchers support the hypothesis that differential translocation is not the selectivity mechanism that results in soybean tolerance to clomazone (14, 23, 26). Results from greenhouse studies using whole plants reported herein also demonstrate that soybean plants are sensitive to soil applied clomazone treatments equal to or greater than 1.0 kg/ha (Fig. 1), indicating that clomazone is translocated acropetally in soybean from root to shoot tissue.

[^{14}C]Clomazone Metabolism

The 1.0 and 6.5 μM clomazone concentrations used in the metabolism studies correspond to the I_{25} and I_{75} values, respectively, for Chl production in COT-M cells (Fig. 4). These concentrations were chosen in an effort to optimize the conditions necessary to determine if metabolism is the operative selectivity mechanism resulting in SB-M cell tolerance to clomazone. No conversion of parental [^{14}C]clomazone to any metabolite occurred in control flasks (no cells) of either medium by 48 HAT (data not shown).

Clomazone metabolism (percentage of clomazone absorbed) at 6 and 24 HAT was significantly greater in SB-M cells than COT-M cells for both the 1.0 and 6.5 μM treatments (Fig. 6, A and B). By 48 HAT, however, both cell lines converted over 90% of the absorbed parental clomazone (1 μM treatment) to a more polar metabolite(s) (Fig. 6A). The percentage of absorbed clomazone (6.5 μM treatment) converted to metabolite(s) by 48 HAT was 84 and 73% for SB-M and COT-M cells, respectively (Fig. 6B). A minor percentage of metabolite(s) was released into the medium by either cell line (Fig. 6, A and B).

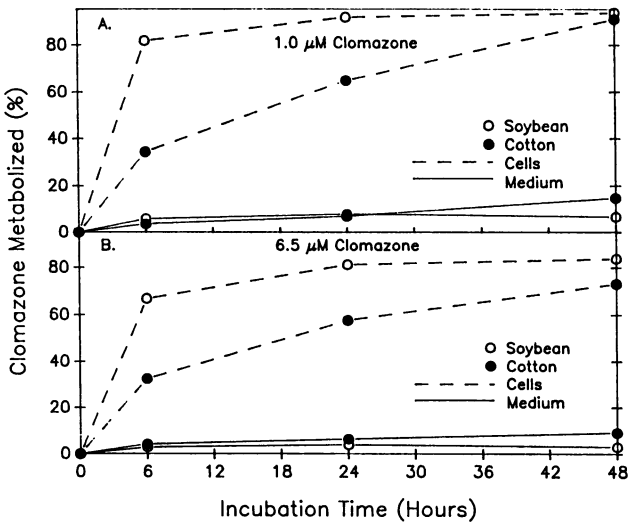

Figure 6. [^{14}C]Clomazone (0.70 μCi) metabolism by SB-M and COT-M cells exposed to a 1.0 or 6.5 μM concentration. Culture conditions, separation, and quantification of [^{14}C]clomazone and metabolites were as described in "Materials and Methods." The amount of clomazone metabolite(s) recovered from the cell and medium fractions is expressed as a percentage of the total radioactivity recovered from the respective fractions, A, 1.0 μM clomazone; B, 6.5 μM clomazone. Each point is the mean of three to six replications ± SE. When not shown, the SE is equal to or smaller than the symbol.

Table I. *Recovery of [^{14}C]Clomazone and [^{14}C]Metabolite(s) from SB-M and COT-M Cells*

Cells were subcultured into the medium containing 0.70 μCi [^{14}C] clomazone maintaining a final concentration of 1.0 or 6.5 μM. Culture conditions, and clomazone extraction, separation, and quantification procedures were as described in "Materials and Methods." Columns of numbers are the mean of 3 to 6 replications ± SE.

Clomazone	Time	Cell Extractable [^{14}C]Clomazone Recovered			
		Parent		Metabolite	
		SB-M	COT-M	SB-M	COT-M
μM	HAT	nmol/g fresh wt			
1.0	6	4.4 ± 0.2	3.9 ± 0.4	19.9 ± 0.9	2.0 ± 0.1
1.0	24	2.5 ± 0.2	2.6 ± 0.2	27.7 ± 0.8	4.8 ± 0.1
1.0	48	2.0 ± 0.1	1.7 ± 0.2	31.4 ± 2.3	17.5 ± 0.8
6.5	6	17.6 ± 0.5	18.5 ± 2.6	35.6 ± 1.7	9.0 ± 1.4
6.5	24	14.1 ± 0.3	13.6 ± 0.1	61.4 ± 2.1	18.7 ± 0.6
6.5	48	12.9 ± 0.4	12.3 ± 0.4	66.7 ± 1.3	33.5 ± 1.0

Although no significant differences in the level of cell extractable parental clomazone (nmol/g fresh weight) was detected between either cell line for each treatment, there was a significantly greater pool of clomazone metabolites within the SB-M cells relative to COT-M cells for all treatments (Table I). The higher rates of clomazone uptake and metabolism at 6 and 24 HAT by SB-M cells indicate that uptake may be related to metabolism.

The similar levels of absorbed clomazone converted to metabolites by 48 HAT and the nearly identical levels of parental clomazone recovered from both cell lines for all treatments indicate that differential metabolism may not be the operative selectivity mechanism that results in soybean tolerance to clomazone on a whole plant and cell suspension level. This hypothesis assumes that parental clomazone is the active form of the herbicide and that clomazone is converted to nontoxic metabolites by both cell lines. A previous report also indicated that metabolism alone may not account for the selectivity of clomazone observed between tolerant bell pepper and susceptible tomato varieties (26).

Identification and Quantification of Clomazone Metabolites

All of the clomazone metabolites (Rt = 3.4 min) extracted from the SB-M and COT-M cells using the isocratic HPLC solvent system were more polar than parental clomazone (Rt = 31.6 min) as evidenced by the marked decrease in Rt. The Rts of the primary clomazone metabolite(s) from SB-M (64.1%) and COT-M (57.9%) cells using the continuous gradient HPLC solvent system were 5.1 and 4.5 min, respectively (Table II). Cochromatography of a mixture of SB-M and COT-M cell metabolites indicate that these primary metabolites are different based on Rt. Since the β-glucosidase treatment had no effect on either the SB-M or COT-M cell major metabolite Rt profile, then none of the major metabolites are β-1,4 glycoside conjugates. The β-glucosidase treatment did result, however, in an increase of COT-M cell metabolite(s) with Rts of 6 to 8 min suggesting that up to 5.9% (8.7 − 2.8%) of COT-M cell metabolite(s) are β-glycosides. The β-glucosidase digestion of the SB-M cell clomazone metabolites also resulted in the emergence of a peak at 27.7 min which cochromatographed with the clomazone derivative 2-chlorobenzyl alcohol (Table II). This observation indicates that up to 5.4% of the clomazone metabolites from SB-M cells is a β-glycosyl-2-chlorobenzyl alcohol metabolite of parental clomazone.

Table II. *Quantification of Clomazone Metabolites before and after β-glucosidase Digestion*

Cells were cultured and [^{14}C]clomazone metabolites extracted and quantified as described in "Materials and Methods." Aliquots from the metabolite fraction were incubated with β-glucosidase and Rts derived from HPLC analysis were compared to the Rt profile derived from aliquots not treated with the enzyme. The Rt of 27.7 min corresponds to the clomazone derivative 2-chlorobenzyl alcohol.

Fraction Rt	Cell Extractable Clomazone Metabolite			
	SB-M		COT-M	
	β-Glucosidase Treatment			
	No	Yes	No	Yes
min				
0 to 2	0	0.1	0.2	0
2 to 4	2.4	1.7	22.8	21.1
4 to 6	64.1	62.4	57.9	57.4
6 to 8	0.4	0.1	2.8	8.7
8 to 10	6.4	5.9	11.4	10.2
10 to 12	13.8	14.3	0.3	0.3
12 to 14	5.9	4.2	0.1	0.3
27.7	0	5.4	0	0
Others	7.0	5.9	4.5	2.0
Total	100	100	100	100

Metabolite Activity on Whole Plants

The pooled metabolite fractions isolated from the SB-M and COT-M lines were tested for biological activity using hydroponically grown soybean and velvetleaf seedlings (Table III). The 1.3 μM clomazone treatment is 19 times greater than the reported clomazone I_{50} (0.07 μM) value for velvetleaf leaf Chl production using identical experimental conditions (14). The high metabolite concentration of 1.3 μM (based on the total pooled [^{14}C]metabolite fraction) was applied to sensitive velvetleaf seedlings in an effort to optimize the conditions necessary to determine if the pooled SB-M or COT-M cell metabolite fractions possess any toxicological properties. The 6.5 μM soybean treatment was the highest concentration attainable based on the total metabolite recovery from the SB-M and COT-M cell lines.

As expected, clomazone reduced leaf Chl levels of velvetleaf (1.3 μM) and soybean (6.5 μM) by 98.3 and 0%, respectively. The pooled metabolite fraction isolated from SB-M cells did not reduce the leaf Chl levels of either soybean or velvetleaf. Conversely, the pooled metabolite fraction isolated from COT-M cells resulted in a 51.3% reduction in leaf Chl levels of velvetleaf but had no effect on the Chl levels of soybean leaves. These data further support the previous conclusion that some portion of the metabolite pool produced by SB-M and COT-M cell lines are different. Based on the recovery of radiolabeled metabolites from the nutrient solution, soybean and velvetleaf seedlings absorbed a greater

Table III. *Effects of [^{14}C]Clomazone and [^{14}C]Clomazone Metabolites on the Leaf Chl Content of Soybean and Velvetleaf Seedlings*

Metabolites from SB-M and COT-M cells were extracted and separated from parental clomazone as described in "Materials and Methods." Experiments were initiated by placing velvetleaf (VLV, second leaf) and soybean (SOY, first trifoliate leaf) seedlings into 50 mL Erlenmeyer flasks containing 55 mL of 25.0% Hoagland solution and herbicide treatment. The 1.3 and 6.5 μM treatments contained 7.4 and 36.8 nCi, respectively. The leaf Chl content and radioactivity levels remaining in each flask were determined 7 DAT as described in "Materials and Methods." Data are the means from three to six replications ± SE.

Treatment		Species	Total Chl	Inh	[^{14}C] Recovered
	μM		μg/g fresh wt		%
Control	0	VLV	1932 ± 56		0
Clomazone	1.3	VLV	33 ± 10	98.3	25.6 ± 1.0
Metabolite					
SB-M	1.3	VLV	1971 ± 48	0	29.2 ± 2.7
COT-M	1.3	VLV	941 ± 357	51.3	48.6 ± 2.4
Control	0	SOY	2189 ± 38		0
Clomazone	6.5	SOY	2340 ± 108	0	5.8 ± 1.2
Metabolite					
SB-M	6.5	SOY	2411 ± 102	0	9.4 ± 0.3
COT-M	6.5	SOY	2418 ± 136	0	18.5 ± 0.5

percentage of the pooled SB-M cell metabolite fraction than the pooled COT-M cell metabolite fraction (Table III). These data indicate that soybean metabolizes clomazone to nonherbicidal metabolites whereas cotton and possibly other sensitive species such as velvetleaf, convert clomazone to metabolite(s) which possess herbicidal activity (bioactivation).

The structure of the COT-M cell metabolite(s) resulting in decreased leaf Chl levels in velvetleaf is unknown. Chlorosis could be the result of parental clomazone action if one or more of the metabolites is in the form of a conjugate that can undergo a reversible reaction liberating free parental clomazone. Since the distribution of the absorbed metabolites within soybean and velvetleaf whole plants is unknown (due to a limited quantity of radiolabeled metabolites), a herbicide bioactivation phenomenon cannot be excluded as a possible mechanism of selectivity. The probability that bioactivation represents the sole mechanism of selectivity appears questionable since the COT-M cell pooled metabolite fraction treatment (1.3 μM) caused a 51.3% reduction of the leaf Chl content in velvetleaf at a concentration 19 times greater the clomazone I_{50} value (0.07 μM). Furthermore, the major metabolites and analogs of clomazone applied postemergence to the tissue or hydroponically to seedlings showed less activity than parental clomazone (personal communication, D. Keifer, FMC Corp.) and a herbicide bioactivation mechanism of selectivity for soil applied herbicides is unprecedented.

The data presented herein do not conclusively identify either the active form of clomazone or the mechanism of soybean tolerance to clomazone. The mechanism of clomazone selectivity can be attributed to either a herbicide bioactivation phenomenon or to differences at the site of action. Assuming parental clomazone is an active form of the herbicide, a preponderance of indirect observations suggest that differences at the site of action may be an operative selectivity mechanism. These observations include: (a) differences in uptake (Fig. 5; 14, 23) and translocation (14, 23, 26) do not account for clomazone selectivity; and (b) similar rates of clomazone metabolism by both cell lines by 48 HAT (Fig. 6), the presence of nearly identical levels of parental clomazone in both cell lines for all treatments (Table I), and a previous report (26) all indicate that differential metabolism alone cannot account for clomazone selectivity. The lack of differential uptake, translocation, and/or metabolism as possible selectivity mechanisms indicate through a process of elimination that differences at the site of action may be an operative selectivity mechanism. Furthermore, some differences at the site of action most certainly exist since the 6.5 μM COT-M cell metabolite treatment (highly water soluble and hence very probable to be xylem mobile) had no effect on the leaf Chl content of soybean whereas only a 1.3 μM treatment resulted in significant leaf Chl reduction in velvetleaf (Table III). Also, based on the level of recovery of the pooled [^{14}C]metabolite treatments, soybean seedlings absorbed 81.5% of the applied radioactivity *versus* 51.4% by velvetleaf seedlings (Table III). The site of clomazone action in soybean is accessible since chlorosis can occur at high clomazone concentrations. According to this hypothesis, the enzymatic site in soybean must be sufficiently different from susceptible species resulting in a poor clomazone fit in soybean tissue.

CONCLUSIONS

Soybean were tolerant to clomazone relative to cotton on both a whole plant and a cell suspension culture systems. Consequently, the photomixotrophic cell suspensions appear to represent an effective system for physiology studies involving herbicides that inhibit or modify terpene biosynthesis.

Differences in uptake cannot be the operative selectivity mechanism since SB-M cells absorbed more clomazone than the COT-M cells at all three incubation times and at three different concentrations. The lack of acropetal clomazone translocation from root to shoot tissue in soybeans does not account for selectivity (14, 23, 26).

Metabolites produced in the SB-M and COT-M cell lines are different based on Rt and activity observed in velvetleaf leaves. Metabolites from COT-M cells reduced by one-half the leaf Chl content of velvetleaf but at a concentration approximately 19 times higher than that of parental clomazone. Neither species produced a major metabolite in the form of a β-glycoside.

The precise mechanism of clomazone selectivity and the active form of the herbicide remain unknown. The data reported herein suggest either a herbicide bioactivation phenomenon or differences at the site of action or a combination of these two processes as possible mechanisms of selectivity. Differences in sensitivity at the site of action has accounted for selectivity of other herbicides such as aryloxyphenoxypropionates (9, 21) and cyclohexadiones (16).

Future studies will be conducted using the photomixotrophic cell suspension system to: (a) follow the uptake, translocation, and distribution of SB-M and COT-M cell metabolites in soybean and velvetleaf seedlings; (b) identify the covalent structure of the metabolites(s) in an effort to characterize the molecular fate of clomazone in tolerant and susceptible species; and (c) more precisely determine the enzymatic site of clomazone action.

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