JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 3653–3660 3653
DOI:10.1021/jf8039999

Mechanism of Resistance to Penoxsulam in Late Watergrass [*Echinochloa phyllopogon* (Stapf) Koss.]

Hagai Yasuor,*,† Maria D. Osuna,‡ Aida Ortiz,§ Néstor E. Saldain,# James W. Eckert,† and Albert J. Fischer†

† Weed Science Program, Department of Plant Sciences, University of California, Davis, California, 95616, ‡ Departamento de Hortofruticultura, Centro de Investigación "Finca La Orden", 06781 Guadajira, Badajoz, Spain, § Universidad Central de Venezuela, Maracay, Estado de Aragua, Venezuela, and # Instituto Nacional de Investigación Agropecuaria, Treinta y Tres, Uruguay

Late watergrass [*Echinochloa phyllopogon* (Stapf.) Koss.] is a major weed of California rice that has evolved P450-mediated metabolic resistance to multiple herbicides. Resistant (R) populations are also poorly controlled by the recently introduced herbicide penoxsulam. Ratios (R/S) of the R to susceptible (S) GR_{50} (herbicide rate for 50% growth reduction) ranged from 5 to 9. Although specific acetolactate synthase (ALS) activity was 1.7 higher in R than in S plants, the enzyme in R plants was about 6 times more susceptible to the herbicide. R plants exhibited faster (2.8 times) oxidative [^{14}C]-penoxsulam metabolism than S plants 24 h after treatment. Addition of malathion (P450 inhibitor) enhanced herbicide phytotoxicity and reduced penoxsulam metabolism in R plants. Tank mixtures with thiobencarb (can induce P450) antagonized penoxsulam toxicity in R plants, suggesting penoxsulam may be broken down by a thiobencarb-inducible enzyme. These results suggest *E. phyllopogon* resistance to penoxsulam is mostly due to enhanced herbicide metabolism, possibly via P450 monooxidation.

KEYWORDS: *Echinochloa phyllopogon*; herbicide resistance; cytochrome P450 monooxygenases; malathion; acetolactate synthase; thiobencarb; enzyme overexpression

INTRODUCTION

Herbicides that inhibit the acetolactate synthase (ALS) enzyme [also known as acetohydroxyacid synthase (AHAS); EC 4.1.3.18] comprise one of the largest and most relevant herbicide classes. ALS inhibitors are used in many cropping systems because of their broad weed control spectrum, crop selectivity, safety to humans, and relatively low usage rates (*1*). These herbicides are grouped into five structurally diverse chemical classes: sulfonylurea, imidazolinone, triazolopyrimidine sulfonamide, pyrimidinylbenzoate, and sulfonylaminocarbonyltriazolinone herbicides (*1*). The ALS/AHAS enzyme catalyzes two different reactions: either condensation of two molecules of pyruvate to form acetolactate or condensation of α-ketobutyrate and pyruvate to form acetohydroxybutyrate in the biosynthetic pathway of the branched-chain amino acids valine, leucine, and isoleucine (*2*).

Penoxsulam (2-(2,2-difluoroethoxy)-*N*-(5,8-dimethoxy [1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl)-6-(trifluoromethy)benzenesulfonamide)) is a new triazolopyrimidine sulfonamide herbicide used in California for postemergence broad-spectrum weed control in rice (*Oryza sativa* L.) (*3*). Phytotoxicity of selective herbicides such as penoxsulam depends not only on their inhibitory potency upon the target site but also on the rate of herbicide metabolism in plants (*4*−*6*).

*Corresponding author [telephone (530) 754-2144; fax (530) 752-4604; e-mail hyasuor@ucdavis.edu].

Weed resistance to ALS inhibitors was first reported in 1987 (*7*, *8*), five years after the introduction of the first sulfonylurea herbicide. There are now more than 90 weed biotypes with resistance to these herbicides (*9*). Most resistance cases to ALS-inhibiting herbicides result from specific mutations causing alterations at the herbicide binding site on the ALS that render the enzyme less sensitive to the herbicide (target-site resistance) (*1*). However, several weed biotypes have an enhanced ability to detoxify ALS-inhibiting herbicides, which often endows them with moderate cross-resistance (R/S ratio of <10) to herbicides with various modes of action (*1*). Unlike target site mediated resistance, metabolic resistance to ALS inhibitors can be selected for by herbicides not belonging to this chemical family (*10*−*12*).

The California rice culture system is heavily dependent on herbicides for weed control. More than 40 years of thiocarbamate use for grass control led to the evolution of herbicide resistance in late watergrass (*Echinochloa phyllopogon* (Stapf) Koss.), which is a major weed of rice in the Sacramento Valley of California. Populations of this species exhibit simultaneous resistance to herbicides from different chemical groups and with different modes of action: molinate and thiobencarb (thiocarbamates), fenoxaprop-ethyl and cyhalofop (aryloxyphenoxy propionate), byspyribac-sodium (pyrimidinyl benzoate), bensulfuron-methyl (sulfonylurea), and clomazone (isoxazolidinone) (*13*, *14*). An earlier study using inhibitors of cytochrome P450 monooxygenases (EC 1.14.14.1, P450s) suggested that P450-mediated herbicide degradation was

 Published on Web 3/26/2009

involved in this multiple-herbicide resistance (*15*). Recently, Yun et al. (*16*) showed that a multiple herbicide resistant *E. phyllopogon* biotype from California exhibited greater P450 contents and monooxygenating activity toward these herbicides than a susceptible biotype. P450 isozymes belong to the most important phase I enzymatic system involved in herbicide metabolism by weeds and crops, including that of several ALS inhibitors (*17*, *18*). The involvement of P450 monooxygenases in metabolic resistance has often been detected when specific P450 inhibitors, such as organophosphate insecticides (malathion), enhance injury to resistant biotypes when applied in conjunction with the herbicide (*19*, *20*). Inhibition by malathion occurs when atomic sulfur released from the oxygenated organophosphate inhibits the P450 apoprotein (*17*). There are many and diverse P450 isozymes in plants, which are encoded by a large family of genes (*17*). P450s vary in species and substrate specificity and can be selectively induced or inhibited by various compounds. Thiocarbamate herbicides can undergo phase I metabolism as P450 substrates and can be P450 inducers or inhibitors (*16*, *21*, *22*), which can affect their interactions with other herbicides, thus the observed antagonistic and synergistic effects of thiobencarb upon the ability of bispyribac-sodium to control *E. phyllopogon* (*23*). Resistance to bispyribac-sodium in other biotypes of this weed is due to enhanced P450 metabolism (*23*, *24*).

Penoxsulam selectivity in rice results from O-dealkylation of the heterocyclic methoxy group of the parent compound, leading to the formation of 5-OH-penoxsulam (*25*), which is a reaction typically catalyzed by P450 (*17*). Penoxsulam degradation rates by indica and japonica rice varieties are 7- and 3-fold higher than by *E. crus-galli*, respectively. Penoxsulam is subject to microbial degradation, and in flooded rice soils its half-life ranges between 2 and 123 days depending on soil composition (*26*). The major metabolites found in soil 7 days after penoxsulam application were 5-OH-penoxsulam (2-(2,2-difluoroethoxy)-*N*-(5,6-dihydro-8-methoxy-5-oxo[1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl)-6-(trifluoromethyl)-benzenesulfomamide); (3-[[[2-(2,2-difluoroethoxy)-6-(trifluoromethyl) phenyl]sulfonyl]amino]-1*H*-1,2,4-triazole-5-carboxylic acid (BSTCA), and its transient intermediate BSTCA-methyl (methyl 5-[[[2-(2,2-difluoroethyoxy)-6-(trifluoromethyl)phenyl]sulfonyl]amino], 1*H*-1,2,4-triazole-3-carboxylic acid) (*26*). Microbial degradation of penoxsulam can also yield other minor products: 2-amino-TP (5,8-dimethoxyl[1,2,4]triazolo-[1,5-*c*]pyrimidin-2-amine); 5,8-diOH-penoxsulam (2-(2,2-difluoroethoxy)-6-trifluoromethyl-*N*-(5,8-dihydroxy-[1,2,4] triazolo[1,5-*c*]pyrimidin-2-yl)benzenesulfonamide); and BST (2-(2,2-difluoroethyoxy)-*N*-1*H*-1,2,4-triazol-3-yl-6-(trifluoromethyl)benzenesulfonamide) (*26*) (Dow AgroSciences, personal communication). The product TPSA (5,8-dimethoxyl [1,2,4]triazolo-[1,5-*c*]pyrimidin-2-ylsulfamic acid) can be found in water as a result of penoxsulam photolysis in aquatic systems (Dow AgroSciences, personal communication).

Although penoxsulam has been commercially available in California only since 2005, resistance to this herbicide has already been detected in *E. phyllopogon* populations (*3*). Understanding the mechanisms of this resistance will be relevant toward the rational deployment of this new herbicide and the management of herbicide resistance in California rice.

We conducted plant and enzymatic studies to (i) quantify resistance to penoxsulam in a multiple herbicide resistant *E. phyllopogon* biotype; (ii) determine if penoxsulam resistance in this R biotype is endowed by reduced ALS sensitivity to the herbicide; and (iii) detect if enhanced P450-mediated metabolism is involved in resistance by assessing penoxsulam interactions with malathion and thiobencarb and by evaluating [^{14}C]-penoxsulam metabolic profiles using HPLC separation.

MATERIALS AND METHODS

Plant Material and Growing Conditions. A resistant (R) *E. phyllopogon* accession was collected in rice fields of the Sacramento Valley of California (39° 40′ N/122° 09′ W latitude/longitude) with a history of repeated use of the thiocarbamate herbicides molinate and thiobencarb as reported by Fischer et al. (*13*). This R population has shown resistance to molinate, thiobencarb, fenoxaprop-ethyl, bispyribac-sodium (*13*), and clomazone (*14*). A herbicide-susceptible (S) control accession also originated from a Sacramento Valley rice field (39° 27′ N/121° 43′ W). Mass-collected seeds (spikelets) of the R and S accessions were used in dose−response studies, and three-times-selfed inbred strains derived from single seeds of these R and S accessions were used in the assay of mechanisms of resistance to ensure genetic uniformity. Relative responses to molinate, thiobencarb, fenoxaprop-ethyl, and bispyribac-sodium, plant morphology, AFLP fingerprinting, and P540 activity of these strains have been described in detail elsewhere (*16*, *27*).

For all experiments, seeds were germinated for 5 days on a wet Whatman no. 1 paper in a growth chamber set at 26/10 °C day/night temperature and a 16 h photoperiod under 500 μmol/m^2/s photsynthetic photon flux density (PPFD) delivered by a mixture of incandescent and fluorescent lights; for whole-plant response and ALS activity experiments, uniform R and S seedlings with ~1 mm long radicle and coleoptile were transplanted into plastic pots (8 × 8 cm) filled with Yolo clay loam (fine-silty, mixed, non acid, thermic Typic Xerorthents, 1.02% organic matter) and placed in a greenhouse where natural light was supplemented by 900 μmol/m^2/s PPFD; average temperatures ranged from 22 to 31 °C, and day length was 16 h. Emerged seedlings were thinned to five uniform and equidistantly spaced plants per pot. Pots were partially immersed in water and fertilized after establishment with 12.6 kg/ha nitrogen (ammonium sulfate). For other experiments, the young seedlings were transplanted to either Petri dishes or hydroponics tubes as will be described in the following sections.

Chemicals. Commercial penoxsulam 217 g/L SC (Granite SC), [^{14}C]-penoxsulam (XDE-638-Het-2-^{14}C, 30.0 mCi/mmol, 99% chemical purity) (**Figure 1**), and reference standards (98−99% pure) for the transformation products 5-OH-penoxsulam, 2-amino TP, 8-OH-penoxsulam (2-(2,2-difluoroethoxy)-*N*-(8-hydroxy-5-methoxy [1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl)-6-(trifluoromethyl)benzenesulfonamide) (purity not available) BST, BSTCA, BSTCA-methyl, and TPSA were provided by Dow AgroSciences LLC (Indianapolis, IN). Commercial thiobencarb 840 g/L EC (Abolish 8 EC) was obtained from Valent USA Corp. Agricultural Products (Walnut Creek, CA). A commercial formulation (500 g/L, EC) of the P450 inhibitor malathion was purchased from Ace Hardware Corp. (Oak Brook, IL).

Dose−Response Experiments. Dose−response experiments were conducted to quantify the extent of resistance to penoxsulam in the R accession and to detect if P450 metabolism was involved as a mechanism of resistance by evaluating penoxsulam interactions with the P450 inhibitor malathion and with thiobencarb (a P450 substrate).

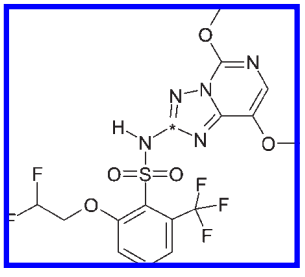

Figure 1. Chemical structure of penoxsulam; the asterisk indicates the location of the [^{14}C].

Responses to Penoxsulam and Malathion. Seedlings of the R accession were treated at the three-leaf stage of growth with either 0 or 1000 g/ha malathion. After approximately 3 h, plants of both R and S accessions were sprayed with penoxsulam (0, 1.25, 2.5, 5, 10, 20, or 40 g/ha) using a commercial formulation (Granite SC). All treatments were applied using a cabinet sprayer equipped with an 8001E-VS (Spraying Systems, Wheaton, IL) flat-fan even-spray nozzle to deliver 380 L/ha at 275 kPa pressure. Plants were put back in the greenhouse for further development, and 1 day after penoxsulam had been sprayed, the immersion water was raised to 3 cm above the soil surface. Aboveground fresh weight per pot was measured 15 days after spraying.

In a different experiment also involving malathion, seeds from both accessions were germinated as described earlier, and five seedlings were placed in 55 mm Petri dishes lined with two Whatman no. 1 paper disks. Various penoxsulam solutions (0, 4.34, 43.4, 434, or 4340 μg/L) were prepared in deionized water, and 4 mL of each was spread onto the paper in each Petri dish. In addition, Petri dishes containing both R and S seedlings were also treated with either 0 or 300 mg/L malathion, which was added to the penoxsulam solutions. Each Petri dish bottom plate was placed without its lid within a transparent 250 mL plastic cup covered with a thin Saran plastic wrap to contain evaporation and prevent chemical contamination between treatments. All cups were placed in a growth chamber as described earlier. The coleoptile length of each seedling was measured in each Petri dish 10 days after treatment.

Data from both foliar and Petri dish experiments were expressed as percent of the untreated control. Experimental treatments (combinations of penoxsulam rates, P450 inhibitor, and *E. phyllopogon* accessions) were replicated four (foliar treatment experiment) or six (Petri dish experiment) times, and each experiment was conducted twice.

Effect of Thiobencarb on Penoxsulam Toxicity. Experiments were conducted in the greenhouse, sprayed, and evaluated as described in the previous section. Six penoxsulam rates (0, 15, 30, 45, 60, and 75 g/ha) were applied in tank mixtures with four thiobencarb rates (0, 1120, 2240, and 4480 g/ha). Treatments (a factorial combination of penoxsulam and thiobencarb rates) were replicated four times, and each experiment was conducted twice.

ALS Activity Studies. The ALS enzyme activity was estimated by quantifying the product acetolactate after conversion to acetoin by decarboxylation in the presence of acid according to the method of Ray (*2*). For this experiment, R and S *E. phyllopogon* inbred strain plants were used, which had been established and grown in the greenhouse as described earlier. At the 3−4-leaf stage of growth, 4 g of leaf tissue from each strain was freshly harvested for total protein extraction and to assay ALS activity in response to a range of penoxsulam concentrations (0, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1.0, and 10 μM), which were added to the incubation mixture following the procedure of Osuna et al. (*24*). The possible production of acetoin from other sources was not considered (*28*). The experiment was repeated three times (biological replicate), and the enzyme activity for each herbicide concentration was determined in triplicate.

[^{14}C]-Penoxsulam Metabolism. Plants of R and S inbred strains were grown hydroponically in a growth chamber after placing one newly germinated seedling of each accession in a 6 mL glass tube containing 2 mL of half-strength Hoagland's solution. Growing conditions were as described earlier for growth chamber experiments. Commercially formulated penoxsulam was mixed with [^{14}C]-penoxsulam to prepare a dilution with a specific activity of 0.94 Bq/μL and a total penoxsulam concentration of 34 μg/L, which corresponds to the penoxsulam concentration resulting when a 50 g/ha field rate is applied to a conventionally flooded (15 cm depth) rice field (*3*). When plants reached the 2−3-leaf stage of growth, 2 mL of radiolabeled penoxsulam solution (1.87 kBq of [^{14}C]-penoxsulam) was added to each plant. In addition, plants were treated with either 0 or 33 mg/L malathion added to the growth medium to elucidate the role of cytochrome P450 in penoxsulam metabolism. Preliminary experiments showed that this malathion concentration synergized penoxsulam effects upon *E. phyllopogon* and had no effect on plants when applied alone (data not shown). Plants were harvested at 24, 48, and 96 h after [^{14}C]-penoxsulam was added to the growing medium and kept at −20 °C until further extraction. Unabsorbed [^{14}C]-penoxsulam was removed from the roots' surface by washing with 2 mL of 80% methanol, which removed detectable ^{14}C residues (data not shown). Treatments (combinations of penoxsulam, malathion, plant harvest time, and R or S *E. phyllopogon* inbred strains) were replicated three times, and the experiment was conducted twice.

Frozen plant samples were ground to powder in liquid nitrogen using a mortar and pestle. Ground tissue was subjected to extraction with 2 mL of 90% acetonitrile, and the homogenate was centrifuged at 27000*g* for 15 min. The pellet was washed four times with 90% acetonitrile (vortex and recentrifugation as described) until ^{14}C was no longer extracted. The supernatants from all washes were combined and evaporated to a final volume of 1 mL under a stream of N_2 at 10 kPa. The remaining solution was filtered through an Acrodisc 0.45 μm membrane syringe filter (Pall Corp., Ann Arbor, MI), and extractions were stored at −20 °C until HPLC analysis. Parent penoxsulam and its metabolites were separated by injecting 50 μL samples of the extract from each plant into a Hewlett-Packard model 1090 integrated HPLC instrument (Palo Alto, CA) with a diode array detector (230 nm) connected to a fraction collector. A 250 mm × 4.6 mm i.d., 5 μm Econosil C18 5U column (Alltech Associates, Deerfield, IL) was used with a gradient mobile phase of 0.01% acetic acid/acetonitrile (95:5 v/v for 10 min, changed to 75:35 over 5 min, changed to 65:35 over 5 min, changed to 55:45 over 5 min, held for 10 min, and returned to 95:5 over 2 min at a constant flow rate of 1.0 mL/min). The HPLC eluate was collected in scintillation vials with a fraction collector at 1.25 min intervals, and radioactivity in each fraction was assayed by liquid scintillation spectrometry 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. Analytes in the fractions were cochromatographed with analytical grade nonlabeled standards using HPLC as described. In addition, samples from the growth medium at each incubation time were subjected to HPLC separation to account for possible penoxsulam breakdown products due to microbial activity during the incubation period. Recovered radioactivity was calculated as the sum of that extracted from plant tissues (residual absorbed radioactivity), the radioactivity remaining in the growth medium, and that present in the root washes and was expressed as percent of the radioactivity present in the growth medium at the initiation of the experiment. Radioactivity not recovered in this procedure was either possibly still present in plant tissues (not quantified, because a sample oxidizer was not available) or lost to evaporation (not quantified). Data are presented as percentages for each fraction present in the residual absorbed radioactivity detected in each plant extraction.

Statistical Analysis. Treatments were arranged in a completely randomized design. Data from repeated experiments were pooled for analysis, and inspection of error distributions suggested that assumptions of normality held reasonably well. Four- and three-parameter log−logistic regressions (*29*, *30*), a model accounting for hormetic effects (*31*), and three-parameter hyperbolic or exponential decay models were used to describe dose−response data. The regression models chosen in each case were those with the lowest mean square errors and the highest significance in their coefficients.

Herbicide rates to inhibit plant growth and/or ALS activity by 50% with respect to the untreated control (GR_{50} and I_{50}, respectively) were calculated, and a resistance index (R/S ratio) was computed as $GR_{50}(R)/GR_{50}(S)$. Values of GR_{50} or I_{50} were considered to be statistically different when their respective 95% confidence intervals did not overlap. Regression analysis was conducted using SigmaPlot (version 10.0, 2006) statistical software (Systat Software, Inc., San Jose, CA). For the ALS-specific activity (in the absence of the herbicide) means were separated using Student's paired *t* test. For the [^{14}C]-penoxsulam metabolism study, penoxsulam metabolites were quantified as percent of the total radioactivity recovered in each sample, data were subjected to analysis of variance, and means were separated using Tukey's

HSD test or paired t tests with $\alpha = 0.05$. Analyses were conducted using JMP software (version 7, 2007 Academic, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Dose−Response Experiments. Dose−response experiments conducted on whole plants and seedlings treated with penoxsulam confirmed the resistance to this herbicide in the R *E. phyllopogon* accession; R/S ratios of the GR_{50} were about 5 and 9 in the greenhouse and Petri dish assays, respectively (**Table 1** and **Figure 2**). Previous studies (*24*) with other R and S *E. phyllopogon* accessions from California had found resistance to the ALS inhibitors bispyribac-sodium (R/S = 9) and bensulfuron-methyl (R/S = 26). In those cases, resistance was due to enhanced P450-mediated metabolism. However, most cases of resistance to this group of herbicides involved reduced sensitivity at the target site, whereas differential herbicide metabolism, absorption, and translocation between R and S biotypes have been less frequently associated with resistance or found to play a minor role (*1*). Therefore, to elucidate the mechanism(s) endowing penoxsulam resistance to these *E. phyllopogon* accessions, we conducted ALS activity assays and evaluated penoxsulam interactions with the P450 inhibitor malathion and the herbicide thiobencarb, which is known to be a P450 substrate in R and S *E. phyllopogon* (*14*). In addition, we used HPLC and [^{14}C]-penoxsulam to establish if biotypes differed in their ability to metabolically detoxify penoxsulam.

ALS Activity. As observed for the bispyribac-sodium and bensulfuron-methyl R and S *E. phyllopogon* accessions evaluated by Osuna et al. (*24*), the ALS extracted from R plants was not less sensitive to penoxsulam than that extracted from S plants (**Table 2** and **Figure 3A**). Therefore, we conclude that the observed *E. phyllopogon* resistance to penoxsulam is not due to reduced sensitivity of the target ALS enzyme. Besides herbicide insensitivity resulting from structural alterations on the ALS enzyme, overexpression of a sensitive ALS could also lead to resistance; however, ALS overexpression has not yet been documented as a mechanism of resistance in weeds (*32*). In this study, the specific ALS activity in the absence of herbicide was 1.7 times higher ($P < 0.05$) in R than in S plants, and the specific ALS activity of R plants was also higher under the effects of penoxsulam concentrations $\leq 10^{-2}$ μM (**Table 2** and **Figure 3B**). However, because the ALS enzyme in R plants is about 6 times more susceptible to penoxsulam than that of S plants (**Table 2**), it is doubtful this possible ALS overexpression in R plants could account for the 5−8-fold penoxsulam resistance factor found in whole plants in this study (**Table 1** and **Figure 2**). ALS overexpression can result from gene amplification effected by a highly active promoter (*33*), from naturally occurring alterations of the ALS gene (*34*, *35*), or from some type of posttranscriptional regulation in vitro (*33*). *E. phyllopogon* is an allotetraploid (*27*), and it is conceivable that more than one copy of the ALS gene could be expressed as found by Ouellet et al. (*36*) in *Brassica napus*. Whether found in vitro or naturally occurring, ALS overexpression seems to make only a moderate or minor contribution toward resistance, as recently found by Calha et al. (*37*) and Figueroa et al. (*38*) in *Alisma plantago-aquatica* biotypes with target-site resistance to bensulfuron-methyl. Therefore, our next step was to investigate

Table 1. Herbicide Rates Required for 50% Reduction (GR_{50}) of Aboveground Fresh Biomass or Seedlings' Coleoptile Length and Ratios (R/S) of the GR_{50} Values of Resistant (R) to Susceptible (S) *Echinochloa phyllopogon* Plants Treated with Various Rates of Penoxsulam Alone or in Combination with Malathion[a]

	fresh weight		coleoptile length	
biotype	GR_{50} (95% CI) (g/ha)	R/S	GR_{50} (95% CI) (μg/L)	R/S
S	1.87 (1.61−2.18)		192.7 (89.9−385.7)	
R	9.36 (7.31−12.67)	5.01	1698.0 (962.4−2985.8)	8.81
R + malathion	4.20 (3.74−4.79)	2.24	2.9 (1.6 −5.8)	0.02

[a] Penoxsulam was applied at 0, 1.25, 2.5, 5, 10, 20, or 40 g/ha and at 0, 4.34, 43.4, 434, or 4340 μg/L in greenhouse and Petri dish experiments, respectively, with or without 1000 g/ha or 300 mg/L malathion; GR_{50} values were calculated from regression curves presented in **Figures 1** and **2**; values in parentheses are the 95% confidence interval of the mean.

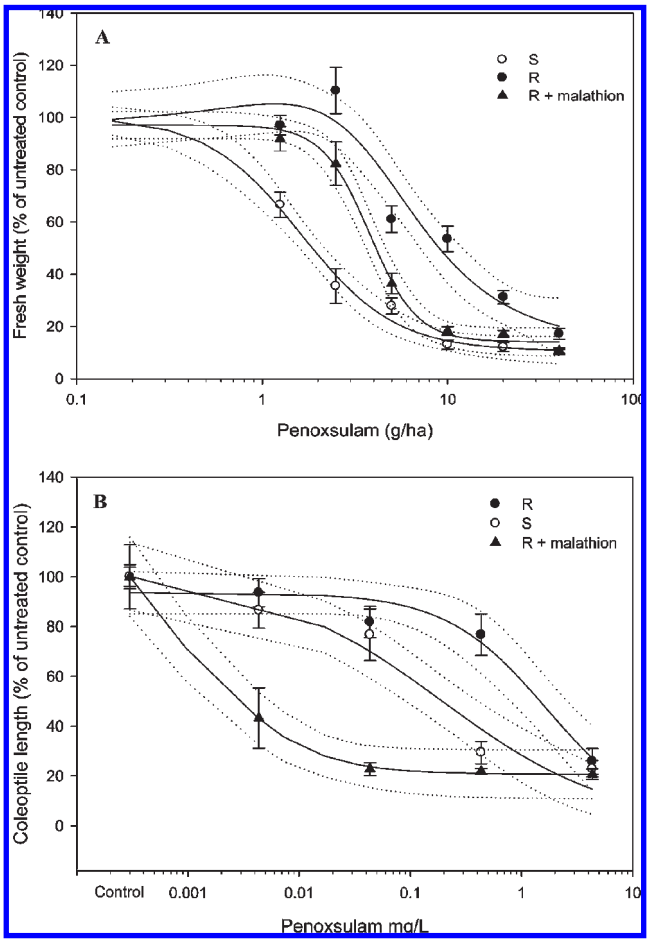

Figure 2. Penoxsulam dose−response assays on susceptible (S, ○) and resistant (R, ●) *Echinochloa phyllopogon* plants; R plants were treated with penoxsulam alone or with malathion (▲). (**A**) Foliar application of penoxsulam alone or after pretreatment with 1000 g/ha malathion to three-leaf stage plants in the greenhouse; data are aboveground fresh weights expressed as percent of the mean untreated control; each data point is the mean ± SE ($n = 8$). Fitted equations are as follows: Y (○) = 10.6 + ((100.3 − 10.6)/((1 + (X/1.6)$^{1.7}$); Y (●) = 11.9 + (((97.3 − 11.9) + (14.3X))/((1 + (X/4.0)$^{1.9}$))); Y (▲) = 14.1 + ((97.1 − 14.1)/((1 + (X/3.8)$^{3.4}$). (**B**) Seedling dose−response assay with penoxsulam alone or in mixture with 300 mg/L malathion conducted in Petri dishes; data are coleoptile lengths expressed as percent of the mean untreated control; each data point is the mean ± SE ($n = 12$). Fitted equations are as follows: Y (○) = 100.2/(1 + exp(1.3 × (log(X) − log(0.2)))); Y (●) = 93.6/(1 + exp(2.2 × (log(X) − log(1.7)))); Y (▲) = 20.7 + (79.3 × 0.0017)/(0.0017 + X); $P < 0.001$; Dotted lines represent 95% CI; calculated GR_{50} values and R/S ratios are presented in **Table 1**.

Table 2. ALS-Enzyme Specific Activity in the Absence of Herbicide, and I_{50} Values Obtained in ALS-Enzyme Activity Assays with Extracts of Resistant (R) and Susceptible (S) *Echinochloa phyllopogon* Plants Incubated with Various Concentrations of Penoxsulam

biotype	ALS activity[a] (nmol of acetoin/mg of protein/h)	R/S	I_{50} (95% CI)[b] (nM)	R/S
S	2.6 ± 0.2		20.5 (19.7−21.3)	
R	4.4 ± 0.3	1.7	3.2 (2.8−3.6)	0.155

[a] ALS specific activity found in the absence of penoxsulam; values (means ± SE) are statistically different ($P < 0.001$) according to a Student's paired t test; R/S are ratios of the ALS specific activity of R to S plants. [b] ALS-enzyme activity in the presence of penoxsulam (0, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1.0, and 10 μM); I_{50} values were calculated from regression curves presented in **Figure 3**; R/S are ratios of I_{50} values of R to S plants; values in parentheses are the 95% confidence interval of the mean.

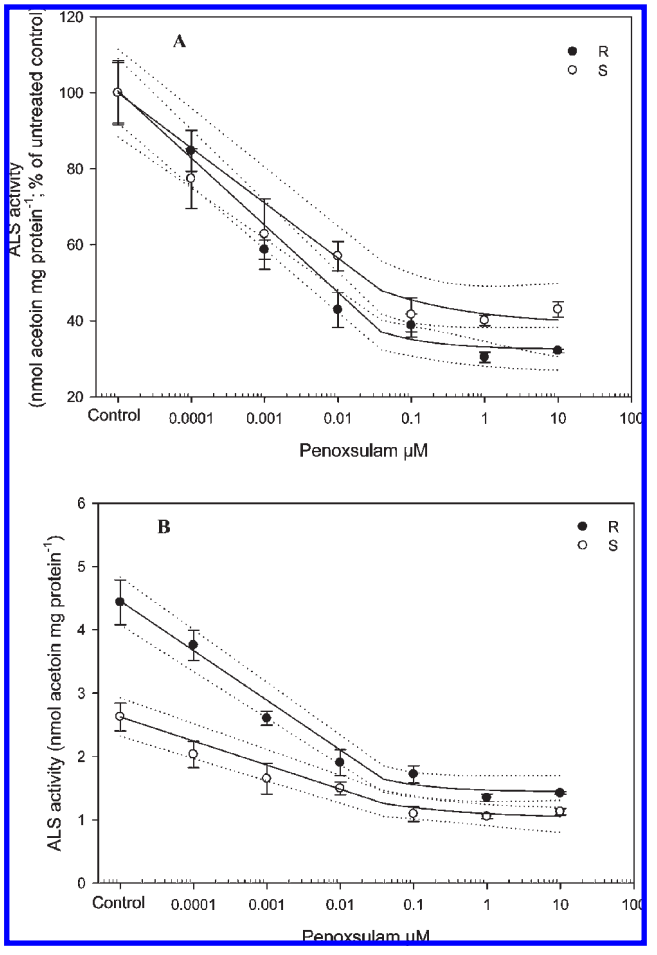

Figure 3. ALS specific activity (nmol of acetoin/mg of protein/h) measured in susceptible (S, ○) and resistant (R, ●) *Echinochloa phyllopogon* plant extracts; data are presented as percent of no herbicide control (**A**) or as absolute specific activity values (**B**). Leaf extracts were incubated with a range of penoxsulam concentrations including no-herbicide controls. Each point is the mean ± SE ($n = 6$). Fitted equations are as follows: (**A**) Y (●) = 32.5 + (((100.5 − 32.5))/((1 + (X/0.0006)$^{0.6234}$))); Y (○) = 39.0 + (((100.0 − 39.0))/((1 + (X/0.0004)$^{0.3848}$))); (**B**) Y (●) = 1.44 + (((4.45 − 1.44))/((1 + (X/0.0006)$^{0.6250}$))); Y (○) = 1.02 + (((2.62 − 1.02))/((1 + (X/0.0004)$^{0.3848}$))). Dotted lines represent 95% CI; calculated I_{50} values and R/S ratios are presented in **Table 2**.

the role of P450-mediated metabolism as a mechanism of penoxsulam resistance in the R biotype.

P450 Interaction with Malathion and Thiobencarb. *Effect of Malathion*. Malathion (1000 g/ha) had no effect on R *E. phyllopogon* growth, but plants sprayed with malathion prior to treatment with penoxsulam suffered enhanced toxicity by this herbicide and had lower fresh weights compared to plants treated with penoxsulam alone (**Figure 2A**). Values of GR_{50} for the R accession were 9.36 and 4.20 g/ha without and with malathion pretreatment, respectively, and malathion reduced the R/S ratio from about 5 to 2 (**Table 1**). Even stronger synergistic effects were observed when malathion was applied with penoxsulam to R *E. phyllopogon* seedlings growing in Petri dishes (**Table 1** and **Figure 2B**). Herbicide-inducible P450 activity can be high in young tissues (*39*), which critically depend on intense ALS activity (*40*). Blocking P450-mediated detoxification would make young seedlings particularly vulnerable to an ALS-inhibiting herbicide, thus the stronger synergism observed with malathion on penoxsulam-treated coleoptiles compared to older R *E. phyllopogon* plants (**Table 1** and **Figure 2**). The strong inhibition of penoxsulam detoxification by malathion suggests that malathion and penoxsulam are oxidized by the same P450 isoform (*41*). Malathion had already been used by Fischer et al. (*15*) and by Osuna et al. (*24*) to detect P450-mediated metabolic resistance to bispyribac-sodium and bensulfuron-methyl in *E. phyllopogon*. Much of the evidence of P450 involvement in herbicide resistance has been derived from in vivo experiments using P450 inhibitors (*42*). Our earlier work (*16*) assayed P450 monooxygenase contents and activity in microsomal fractions isolated from shoots of these same R and S biotypes. Both P450 contents and specific activities as determined by the degradation rates of herbicides used as substrates (thiobencarb, fenoxaprop-ethyl, and bispyribac-sodium) were higher in R than in S plants. P450-mediated metabolism has also been reported as a mechanism of resistance to ALS inhibitors in other plant species (*11*, *12*). Therefore, elimination of resistance by malathion would suggest a possible involvement of P450-mediated herbicide metabolism in R *E. phyllopogon* resistance to penoxsulam.

Effect of Thiobencarb. Thiobencarb (1120 g/ha) had no effect on R *E. phyllopogon* growth but reduced penoxsulam toxicity (**Figure 4**), causing a 3-fold increase in the GR_{50} (from 14 to 39.7 g/ha). Higher doses of thiobencarb (2240 and 4480 g/ha) caused similar antagonism (data not shown). In previous studies, tank mixtures with low rates of thiobencarb had antagonized bispyribac-sodium control of *E. phyllopogon* (*23*). The mechanism of this antagonism is not clear, but Yun et al. (*16*) demonstrated that pretreating seedlings of these R and S *E. phyllopogon* accessions with thiobencarb induced greater P450 activity, and the effects were stronger in R than in S plants. Therefore, the reduced sensitivity of R *E. phyllopogon* to penoxsulam observed in our experiment after pretreatment with thiobencarb suggests penoxsulam is presumably being metabolized by a thiobencarb-inducible P450 isozyme. This would also support the idea that repeated use of thiocarbamate herbicides (molinate and thiobencarb) for many years selected for these multiple-herbicide-resistant *E. phyllopogon* biotypes in California (*13*).

The lack of evidence supporting target-site-based resistance and the observed synergistic and antagonistic interactions when penoxsulam was combined with either malathion or thiobencarb, respectively, strongly support the idea that a P450-mediated enhanced detoxification mechanism confers penoxsulam resistance to R plants. To confirm this, we compared metabolic profiles in tissue extracts of [^{14}C]-penoxsulam-treated R and S plants as affected by the presence or absence of malathion.

HPLC [^{14}C]-Penoxsulam Metabolism. Penoxsulam is oxidatively metabolized in rice, which is a penoxsulam-tolerant crop, via O-dealkylation of one of the heterocycle methyl groups. It is also metabolized by anaerobic soil microbes through a complex multistep oxidative biotransformation with 5-OH-penoxsulam as the first P450-mediated product followed by the generation of the intermediate metabolite BSTCA-methyl (*25*, *26*). Radioactivity recovered did not differ ($P > 0.05$) between R (82 ± 2.0%) and S (86 ± 2.5%) plants. According to the HPLC elution profiles of extracts from R and S *E. phyllopogon* plants following a 48 h root exposure to [^{14}C]-penoxsulam, R plants were clearly able to convert the parent herbicide into polar metabolites (I and II) with two distinct peaks that coeluted with the 5-OH-penoxsualm and BSTCA-methyl standards, respectively. These compounds are known major penoxsulam degradation products (*25*, *26*). Peaks for metabolites I and II were only minimally expressed in eluates of S plant extracts (**Figures 5A**,**B**). Elution of other minor peaks in the chromatograms did not match that of any other penoxsulam metabolites used as standard in this study. Therefore, we infer that R and S plants differ in their abilities to convert penoxsulam into the two major polar metabolites I and II (presumably 5-OH-penoxsualm and BSTCA-methyl). Further precise analysis (e.g., LC-MS/MS) would be required for conclusive identification of these metabolites. Addition of the P450 inhibitor malathion to the growth medium strongly reduced penoxsulam metabolism by R plants, and their elution profile resembled that of S plants (**Figure 5C**). Because plants were continuously exposed to [^{14}C]-penoxsulam uptake via roots for the duration of the experiment (up to 96 h), the peaks corresponding to the parent compound remained similarly high in all cases. Although penoxsulam was metabolized to some extent by the S biotype, the biotransformation rates were greater for R plants, which by 48 h after initial exposure to [^{14}C]-penoxsulam had accumulated considerably more of the two polar metabolites I and II than S plants (**Table 3**). By 96 h after treatment, R plants had accumulated twice as much of metabolite I than S plants (**Table 3**). At all times, malathion reduced penoxsulam metabolites in R plants to levels similar to those found in S plants; thus, we infer the strong reduction of penoxsulam metabolism by malathion suggests *E. phyllopogon* resistance involves

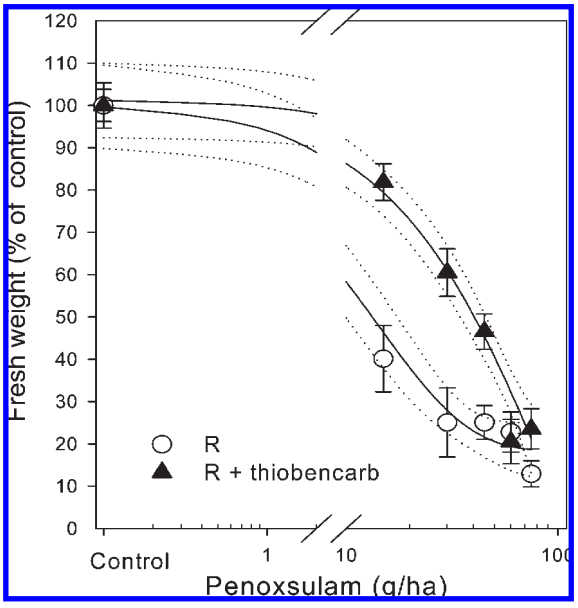

Figure 4. Effect of thiobencarb (1120 g/ha) on resistant *Echinochloa phyllpogon* whole plants treated with different doses of penoxsulam applied alone (○) or in mixture with thiobencarb (▲). Each point is the mean ± SE (n = 8). Fitted equations are as follows: Y (▲) = −27.4 + (127.5 × exp(−0.012X)); Y (○) = 22.0 + (78.0 × exp(−0.075X)).

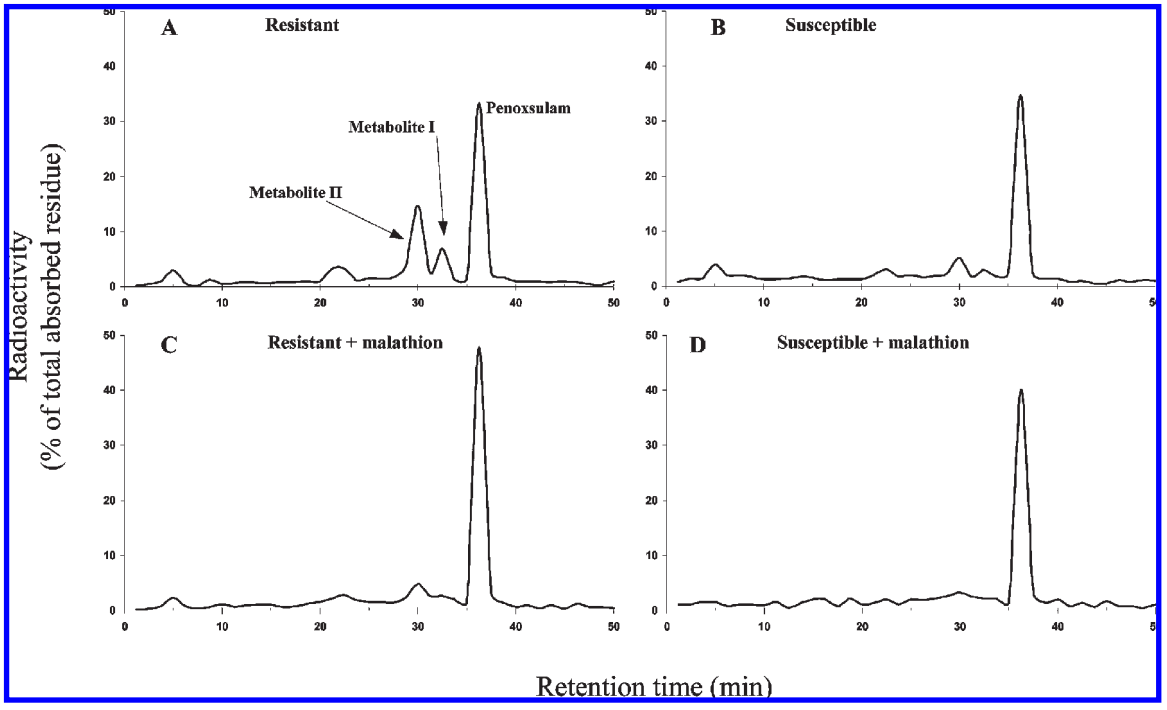

Figure 5. HPLC chromatograms of [^{14}C]-penoxsulam (XDE-638-Het-2-^{14}C) and its metabolites extracted from resistant (**A**, **C**) and susceptible (**B**, **D**) *Echinochloa phyllpogon* inbred plants after 48 h of root exposure to [^{14}C]-penoxsulam alone (**A**, **B**) or in mixture with 33 mg/L malathion (**C**, **D**). Metabolites I and II were coeluates with 5-OH-penoxsulam and BSTCA-methyl analytical standards, respectively, according to their retention times. Data are presented as percent of total absorbed residue; identified peaks were cochromatogrammed with nonradioactive standards.

Table 3. Percentage of the Residual Absorbed Radioactivity Represented by Penoxsulam and Its Metabolites Detected by HPLC in Extracts of Resistant (R) and Susceptible (S) *Echinochloa phyllopogon* Plants in the Presence and Absence of Malathion[a,b,c]

			radioactivity (% of total extracted)			
			R		S	
HAT	RT (min)	compound	penoxsulam	penoxsulam + malathion	penoxsulam	penoxsulam + malathion
24	32.5	I	6.2 ± 0.9a	2.1 ± 0.5b	2.0 ± 0.6b	1.4 ± 0.6b
	30	II	8.5 ± 2.0a	1.5 ± 0.9a	3.3 ± 0.7a	4.6 ± 3.7a
	36.25	penoxsulam	19.8 ± 2.3a	31.8 ± 8.1a	24.9 ± 7.3a	27.0 ± 3.8a
48	32.5	I	6.8 ± 0.5a	2.6 ± 0.7b	2.9 ± 0.7b	2.1 ± 0.1b
	30	II	14.6 ± 1.1a	4.9 ± 2.1b	5.1 ± 1.1b	3.3 ± 1.3b
	36.25	penoxsulam	33.3 ± 3.8a	47.8 ± 5.7a	34.7 ± 6.4a	40.1 ± 2.7a
96	32.5	I	10.8 ± 0.8a	4.7 ± 0.7b	5.5 ± 0.7b	2.2 ± 0.8b
	30	II	20.8 ± 3.4a	6.6 ± 3.1b	10.2 ± 0.9ab	5.1 ± 1.7b
	36.25	penoxsulam	30.7 ± 3.2a	32.0 ± 6.3a	23.7 ± 3.9a	37.1 ± 3.7a

[a] Parent penoxsulam and its metabolites were assayed for 24, 48, and 96 h after treatment (HAT) with [^{14}C]-penoxsulam applied to the hydroponic medium either alone or in combination with 33 mg/L malathion; compounds were separated using an HPLC instrument equipped with an Econosil C18 5U column employing a mobile phase of 0.01% acetic acid/acetonirtile gradient; RT is HPLC retention time in minutes. [b] Only metabolites I and II, which were coeluates with the 5-OH-penoxsulam and BSTCA-methyl analytical standards, respectively, are included in the statistical analysis. Within rows, means accompanied by the same letter do not differ according to Tukey's HSD test with $P = 0.05$. [c] Recovered radioactivity (83.5 ± 1.6%) = [radioactivity extracted from plant tissues (residual absorbed radioactivity) + radioactivity remaining in the growth medium + radioactivity present in the root washes]/radioactivity in the growth medium at the initiation of the experiment × 100.

P450-mediated penoxsulam metabolism. A low extent of penoxsulam metabolism was observed in S plants (**Table 3** and **Figure 5**); thus, R plants exhibit an enhanced expression of a metabolic ability already present in wild-type *E. phyllopogon*. Similar effects of P450 inhibitors on the metabolism of the ALS inhibitor propoxycarbazone-sodium have been reported earlier for *Bromus tectrum* (*12*) and *Lolium rigidum* (*20*). Although microbes in flooded soils can further degrade penoxsulam to other polar metabolites (*26*) and other triazolopyrimidine herbicides can undergo phase II conjugation with glucose and homoglutathione (*25*), we found no evidence of other major metabolites within the duration our study (**Figure 5**). Therefore, results of these experiments explain the synergistic effects of malathion on penoxsulam toxicity to R *E. phyllopogon* seedlings and plants (**Table 1** and **Figure 2**) and further support the idea that *E. phyllopogon* resistance to penoxsulam is due to enhanced P450-mediated detoxification. Differential rates of herbicide metabolism have also allowed explaining resistance and selectivity for other ALS-inhibiting herbicides in weeds and crops (*12*, *20*, *25*).

We conclude from this study that an *E. phyllopogon* accession with evolved resistance to multiple herbicides is also cross-resistant to penoxsulam to a level that can explain control failures in the field. We demonstrated that resistance is mainly conferred by an enhanced ability of R plants to detoxify penoxsulam via malathion-sensitive monooxygenases and that the higher expression of penoxsulam-sensitive ALS activity in R plants compared to S plants may not play a significant role in the overall resistance. The existence of more than one mechanism mitigating herbicide toxicity will complicate herbicide resistance management in rice. Furthermore, the presence of similar penoxsulam biotransformation processes in rice and in one of its major weeds suggests that metabolic resistance might jeopardize the ability of new herbicides to control weeds in rice for which selectivity is P450-mediated.

ACKNOWLEDGMENT

We thank Dow AgroSciences LLC for providing [^{14}C]-penoxsulam and analytical standards and Prof. Dr. Ron Tjeerdema for the use of his laboratory facilities and for his help throughout the study.

LITERATURE CITED

(1) Tranel, P. J.; Wright, T. R. Resistance of weeds to ALS-inhibiting herbicides: what have we learned?. *Weed Sci*. **2002**, *50*, 700–712.

(2) Ray, T. B. Site of action of chlorsulfuron. *Plant Physiol*. **1984**, *75*, 827–831.

(3) Fischer, A. J.; Eckert, J. W.; Boddy, L.; Marchesi, C.; Merotto, A.; Osuna-Ruiz, M. D.; Yasuor, H.; Schaal B. Weed Control in Rice—Annual Report Comprehensive Rice Research (Jan 1–Dec 31, 2007); available at http://www.carrb.com/07rpt/2007%20Fischer%20RP-1.pdf, 2007, accessed Aug 28, 2008.

(4) Hodges, C. C.; De Boer, G. J.; Avalos, L. Uptake and metabolism as mechanisms of selective herbicidal activity of the 1,2,4-triazolo[1,5-*a*] pyrimidines. *Pestic. Sci*. **1990**, *29*, 365–378.

(5) Gerwick, B. C.; De Boer G. J.; Schmitzer, P. R. Mechanisms of tolerance to triazolopyrimidine sulfonanilide herbicides. In *Chemistry of Plant Protection—Herbicide Inhibiting Branched Amino Acid Biosynthesis*; Statter, J., Ebing, W., Eds.; Springer-Verlag: Berlin, Germany, 1994; Vol. *10*, pp 145–160.

(6) DeBoer, G. J.; Thornburgh, S.; Her, R. J. Uptake, translocation and metabolism of the herbicide florasulam in wheat and broadleaf weeds. *Pest Manag. Sci*. **2006**, *62*, 316–324.

(7) Mallory-Smith, C. A.; Thill, D. C.; Dial, M. J. Identification of sulfonylurea herbicide-resistant prickly lettuce (*Lactuca serriola*). *Weed Technol*. **1990**, *4*, 163–168.

(8) Primiani, M.; Cotterman, M. J. C.; Saari, L. L. Resistance of kochia (*Kochia scoparia*) to sulfonylurea and imidazolinone herbicides. *Weed Technol*. **1990**, *4*, 169–172.

(9) Heap, I. The International Survey of Herbicide Resistant Weeds, available at http://www.weedscience.org/in.asp, 2008, accessed Aug 28, 2008.

(10) Christopher, J. T.; Powles, S. B.; Liljegren, D. R.; Holtum, J. A. M. Cross-resistance to herbicide in annual raygrass (*Lolium rigidum*). II. Chlorsulfuron resistance involves a wheat-like detoxification system. *Plant Physiol*. **1991**, *95*, 1036–1043.

(11) Hidayat, I.; Preston, C. Cross-resistance to imazethapyr in a fluazifop-P-butyl-resistant population of *Digitaria sanguinalis*. *Pestic. Biochem. Physiol*. **2001**, *71*, 190–195.

(12) Park, K. W.; Fandrich, L.; Mallory-Smith, C. A. Absorption, translocation, and metabolism of propoxycarbazone-sodium

in ALS-inhibitor resistant *Bromus tectorum* biotypes. *Pestic. Biochem. Physiol*. **2004**, *79*, 18–24.
(13) Fischer, A. J.; Ateh, C. M.; Bayer, D. E.; Hill, J. E. Herbicide-resistant early (*Echinochloa oryzoides*) and late (*E. phyllopogon*) watergrass in California rice fields. *Weed Sci*. **2000**, *48*, 225–230.
(14) Yasuor, H.; TenBrook, P. L.; Tjeerdema, R. S.; Fischer, A. J. Responses to clomazone and 5-ketoclomazone by *Echinochloa phyllopogon* resistant to multiple herbicides in Californian rice fields. *Pest Manag. Sci*. **2008**, *64*, 1031–1039.
(15) Fischer, A. J.; Bayer, D. E.; Carriere, M. D.; Ateh, C. M.; Yim, K. Mechanisms of resistance to bispyribac-sodium in an *Echinochloa phyllopogon* accession. *Pestic. Biochem. Physiol*. **2000**, *68*, 156–165.
(16) Yun, M. S.; Yogo, Y.; Miura, R.; Yamasue, Y.; Fischer, A. J. Cytochrome P-450 monooxygenase activity in herbicide-resistant and susceptible late watergrass (*Echinochloa phyllopogon*). *Pestic. Biochem. Physiol*. **2005**, *83*, 107–114.
(17) Werck-Reichhart, D.; Hehn, A.; Didierjean, L. Cytochrome P450 for engineering herbicide tolerance. *Trends Plant Sci*. **2000**, *5*, 1360–1385.
(18) Siminszky, B. Plant cytochrome P450-mediated herbicide metabolism. *Phytochem. Rev*. **2006**, *5*, 445–458.
(19) Gressel, J. Synergizing herbicides. *Rev. Weed Sci*. **1990**, *5* 49–82.
(20) Christopher, J. T.; Preston, C.; Powles, S. B. Malathion antagonizes metabolism-based chlorsulfuron resistance on *Lolium rigidum*. *Pestic. Biochem. Physiol*. **1994**, *49*, 172–182.
(21) Devine, M.; Duke, S. O.; Fedtke, C. *Physiology of Herbicide Action*; Prentice Hall: Englewood Cliffs, NJ, 1993; pp 95−112, 376−394.
(22) Usui, K. Metabolism and selectivity of rice herbicides in plants. *Weed Biol. Manag*. **2001**, *1*, 137–146.
(23) Fischer, A. J.; Cheetham, D. P.; Vidotto, F.; De Prado, R. Enhanced effect of thiobencarb on bispyribac-sodium control of *Echinochloa phyllopogon* (Stapf) Koss. in California rice (*Oryza sativa* L.). *Weed Biol. Manag*. **2004**, *4*, 206–212.
(24) Osuna, M. D.; Vidotto, F.; Fischer, A. J.; Bayer, D. E.; De Prado, R.; Ferrero, A. Cross-resistance to bispyribac-sodium and bensulfuron-methyl in *Echinochloa phyllopogon* and *Cyperus difformis*. *Pestic. Biochem. Physiol*. **2002**, *73*, 9–17.
(25) Johanson, T. C.; Mann, R. K.; Schmitzer, P. R. Triazolopyrimidines. In *Modern Crop Protection Compounds*; Krämer, W., Schirmer, U., Eds.; Wiley-VCH Verlag: Weinheim, Germany, 2007; pp 93−114.
(26) Jabusch, T. W.; Tjeerdema, R. S. Microbial degradation of penoxsulam in flooded rice field soils. *J. Agric. Food Chem*. **2006**, *54*, 5962–5967.
(27) Tsuji, R.; Fischer, A. J.; Yoshino, M.; Roel, A.; Hill, J. E.; Yamasue, Y. Herbicide-resistant late watergrass (*Echinochloa phyllopogon*): similarity in morphological and amplified fragment length polymorphism traits. *Weed Sci*. **2003**, *51*, 740–747.
(28) Forlani, G.; Mantelli, M.; Nielsen, E. Biochemical evidence for multiple acetoin-forming enzymes in cultured plant cells. *Phytochemisty* **1999**, *50*, 255–262.
(29) Seefeldt, S. S.; Jensen, J. E.; Furest, E. P. Log−logistic analysis of herbicide dose−response relationships. *Weed Technol*. **1995**, *9*, 218–227.
(30) Knezevic, S.; Streibig, J. C.; Ritz, C. Utilizing R software package for dose-response studies: the concept and data analysis. *Weed Technol*. **2007**, *21*, 840–848.
(31) Brain, P.; Cousens, R. An equation to describe dose responses where there is stimulation of growth at low doses. *Weed Res*. **1989**, *29*, 93–96.
(32) Duggleby, R. G.; McCourt, J. A.; Guddat, L. W. Structure and mechanism of inhibition of plant acetohydroxyacid synthase. *Plant Physiol. Biochem*. **2008**, *46*, 309–324.
(33) Odell, J. T.; Caimi, P. G.; Yadav, N. S.; Mauvais, C. J. Comparison of increased expression of wild-type and herbicide-resistant acetolactate synthase genes in transgenic plants, and indication of posttranscriptional limitation on enzyme activity. *Plant Physiol*. **1990**, *94*, 1647–1654.
(34) Boutsalis, P.; Karotam, J.; Powles, S. B. Molecular basis of resistance to acetolactate synthase-inhibiting herbicides in *Sisymbrium orintale* and *Brassica tournefortii*. *Pestic. Sci*. **1999**, *55*, 507–516.
(35) Yu, Q.; Nelson, J. K.; Zheng, M. Q.; Jackson, M.; Powles, S. B. Molecular charaterisation of resistance to ALS-inhibiting herbicides in *Hodeum leporium* biotypes. *Pest Manag. Sci*. **2007**, *63*, 918–927.
(36) Ouellet, T.; Rutledge, R. G.; Miki, B. L. Members of the acetohydroxyacid synthase multigene family of *Brassica napus* have divergent patterns of expression. *Plant J*. **1992**, *2*, 321–330.
(37) Calha, I. M.; Osuna, M. D.; Serra, C.; Moreira, I.; De Prado, R.; Rocha, F. Mechanism of resistance to bensulfuron-methyl in *Alisma plantago-aquatica* biotypes from Portuguese rice paddy fields. *Weed Res*. **2007**, *47*, 231–240.
(38) Figueroa, R.; Gebauer, M.; Fischer, A. J.; Kogan, M. Resistance to bensulfuron-methyl in water plantain (*Alisma plantago-aquatica*) populations from Chilean paddy fields. *Weed Technol*. **2008**, *22*, 602–608.
(39) Persans, M. W.; Schuler, M. A. Differential induction of cytochrome P450-mediated triasulfuron metabolism by naphthalic anhydride and triasulfuron. *Plant Physiol*. **1995**, *109*, 1483–1490.
(40) Singh, B. K. Biosynthesis of valine, leucine, and isoleucine. In *Plant Amino Acids*; Singh, B. K., Ed.; Dekker: New York, 1999; pp 227−247.
(41) Baerg, R. J.; Barrett, M.; Polge, N. D. Insecticide and insecticide metabolite interactions with cytochrome P450-mediated activities in maize. *Pestic. Biochem. Physiol*. **1996**, *55*, 10–20.
(42) Barrett, M. The role of cytochrome P450 enzymes in herbicide metabolism. In *Herbicides and Their Mechanisms of Action*; Cobb, A. H., Kirkwood , R. C., Eds.; CRC Press: Boca Raton, FL, 2000; pp 25−37.

Received for Review December 23, 2008. Accepted March 11, 2009. Revised manuscript received March 9, 2009. This research was partially funded by grants from the California Rice Research Board.