Metabolism-Based Resistance of a Wild Mustard (*Sinapis arvensis* L.) Biotype to Ethametsulfuron-methyl

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Under controlled-environment conditions, ethametsulfuron-methyl doses that inhibited growth by 50% (ED_{50}) were >100 and <1 g of active ingredient (ai) ha^{-1} for ethametsulfuron-methyl-resistant (R) and -susceptible (S) wild mustard, respectively. There were no differences between the two biotypes with regard to absorption and translocation of the herbicide. Three days after treatment, approximately 90, 5, and 2% of the applied [^{14}C]ethametsulfuron-methyl was found in the treated leaf, foliage, and roots of each biotype, respectively. Acetolactate synthase extracted from the two biotypes was equally sensitive to both ethametsulfuron-methyl and chlorsulfuron. These results indicate that resistance was not due to differences in the target site, absorption, or translocation. However, ethametsulfuron-methyl was metabolized more rapidly in the R than the S biotype. Approximately 82, 73, 42, 30, and 17% of the recovered radioactivity remained as ethametsulfuron-methyl in R wild mustard 3, 6, 18, 48, and 72 h after treatment, respectively. Conversely, 84, 79, 85, and 73% of the ^{14}C was ethametsulfuron-methyl in the S biotype 12, 24, 48, and 72 h after treatment, respectively. On the basis of these results, it is proposed that resistance is due to enhanced metabolism of ethametsulfuron-methyl in the R biotype.

Keywords: *Sulfonylurea; absorption; translocation; acetolactate synthase*

INTRODUCTION

The herbicide ethametsulfuron-methyl acts by inhibiting the enzyme acetolactate synthase (ALS), the key enzyme involved in the biosynthesis of branched-chain amino acids (LaRossa and Schloss, 1984). The unique aspect of this herbicide is its use to selectively remove Brassicaceae weeds such as wild mustard (*Sinapis arvensis* L.) from Brassicaceae crops (Buchanan et al., 1990a,b; Hall et al., 1992). Currently, ethametsulfuron-methyl is registered in Canada for use in canola (*Brassica napus*) and commercial brown mustard (*Brassica juncea*), and registration is being pursued for other Brassicaceae crops such as rutabaga (Van Eerd and Hall, 2000). There are conflicting reports on the effectiveness of ethametsulfuron-methyl at different stages of plant growth and development. For example, in 1987, Parsons reported that herbicidal activity was independent of the growth stage of wild mustard, whereas several authors (Swanton and Chandler, 1989; Blackshaw and Derksen, 1992; Blackshaw, 1989a,b) found that the efficacy of ethametsulfuron-methyl decreased after the six-leaf stage of weed development. Wild mustard can lead to yield losses, due to weed competition, of ≥36% in canola crops (Blackshaw et al., 1987; McMullen et al., 1994). The amount of herbicide required to effectively control wild mustard in canola has been shown to be as low as 0.005 kg of active ingredient (ai) ha^{-1} when applied at the two−three-leaf stage of development (Swanton and Chandler, 1989; Blackshaw, 1989a,b). It has been shown that the repeated use of sulfonylurea herbicides leads to the development of resistance based on altered target site (Saari et al., 1994).

The basis for crop tolerance (oilseed rape, rutabaga, and commercial brown mustard) to ethametsulfuron-methyl is not due to sulfonylurea-insensitive ALS; it is the result of rapid metabolism of ethametsulfuron-methyl (Hall et al., 1992; Van Eerd and Hall, 2000; Saari et al., 1994; Lichtner et al., 1995). Recently, Jeffers et al. (1996) observed that a resistant (R) biotype of wild mustard was 48-fold more resistant to ethametsulfuron-methyl than a susceptible (S) wild mustard. Furthermore, on the basis of the lack of cross-reactivity of this biotype to other sulfonylurea herbicides, they suggested that resistance of this biotype to ethametsulfuron-methyl might be due to enhanced metabolism. Recently, researchers have shown resistance to the sulfonylurea herbicides in the grass weeds *Lolium rigidium* and *Alopecurus myosuroides* is due to increased metabolism (Menendez et al., 1997; Preston et al., 1996).

On the basis of this information (Jeffers et al., 1996; Sunderland et al., 1995; Menendez et al., 1997; Preston et al., 1996) and our experience that differences in absorption and translocation do not usually account for the development of resistance to herbicides, we hypothesize that metabolism is the basis for resistance of the

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R biotype of wild mustard. If confirmed, this would be the first confirmation of altered metabolism of a sulfonylurea herbicide being the basis for resistance of a broadleaf weed population. The objectives of this study were to (i) compare the absorption, translocation, and metabolism of ethametsulfuron-methyl in the R and S biotypes and (ii) determine the sensitivity of ALS from both biotypes to the herbicide.

MATERIALS AND METHODS

Source of Plant Materials. Resistant and susceptible wild mustard seeds were collected in Vegreville, Alberta, and Manitoba, respectively, from the same seed source used by Jeffers et al. (1996).

Plant Growth Conditions. Seeds of R and S wild mustard biotypes were germinated in the dark on 0.6% agar for the dose−response experiments. Seedlings were transplanted to 10-cm-diameter pots containing soil and placed in a controlled-environment growth room maintained at 25/21 °C day/night temperatures with a 16-h photoperiod and average day/night relative humidities of 64/70%. The soil in each pot was fertilized twice a week with a nutrient solution consisting of 20−20−20 (N−P−K) soluble fertilizer (20 g L^{-1}) containing micronutrients. A combination of fluorescent and incandescent lighting supplied an average light intensity of 500 μeinstein m^{-2} s^{-1} (photon flux density). Plants of uniform size were selected for experiments.

Inhibition of ALS Activity. ALS was partially purified from leaves of R and S wild mustard as described by Devine et al. (1991) and Lichtner et al. (1995). Briefly, plants at the three−four-leaf stage of development were harvested and homogenized in extraction buffer [100 mM KH_2PO_4 buffer (pH 7.5) containing 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM TPP (thiamin pyrophosphate), 10 μM FAD (flavin adenine dinucleotide), and 10% (v/v) glycerol]. Proteins were precipitated with 60% (v/v) saturated ammonium sulfate and centrifuged, and the pellet was collected and resuspended in extraction buffer. The resuspended pellet was desalted by chromatography on a PD-10 Sephadex G-25 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with elution buffer (100 mM potassium phosphate containing 20 mM sodium pyruvate and 0.5 mM $MgCl_2$) and the fraction containing ALS collected.

The crude enzyme fraction, assay buffer (83.3 mM potassium phosphate, pH 7.0, containing 167 mM sodium pyruvate, 16.7 mM $MgCl_2$, 1.67 mM TPP, and 16.6 μM FAD), and herbicide solutions of either chlorsulfuron or ethametsulfuron-methyl were added (1:3:1 v/v/v) to a microtiter plate and incubated for 30 min. The enzyme reaction was stopped by adding 20 μL of 6 N H_2SO_4 and incubated for 15 min. Creatine and α-naphthol were added to the solutions and incubated for an additional 15 min, after which time the absorbance was measured at 540 nm. A standard curve of acetoin was used to quantify reaction products, and protein concentrations were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as a standard. Experiments contained eight replicates and were repeated three times. ALS activity was expressed as a percentage of the control treatment, which contained no herbicide. The data from all of the experiments were pooled.

Uptake and Translocation. Radiolabeled [*phenyl*-(U)-^{14}C]-ethametsulfuron-methyl (605.9 MBq $mmol^{-1}$, purity = 97.2%) was dissolved in ethanol and added to 0.1 M phosphate buffer (pH 7.5) containing 0.5% Tween 20 (oxysorbic 20−polyoxyethylene sorbitan monolaurate) to a final ethanol concentration of 10% and 563 μM (specific activity of 340 Bq μL^{-1}) herbicide. Ten 1-μL drops of the ^{14}C-labeled herbicide solution were applied to the adaxial surface of the second fully expanded leaf of R and S wild mustard at the three−four-leaf stage of development. Both R and S plants were harvested 0, 12, 24, 48, and 72 h after application, and the unabsorbed herbicide was washed from the treated leaf with 30 mL of aqueous wash solution [10% ethanol, 0.1% Tween (v/v)] as described by Van Eerd and Hall (2000). Radioactivity in the leaf wash was determined by liquid scintillation spectroscopy (LSS). Each plant was divided into the treated leaf, remaining foliage, and root. The samples were dried, and the ^{14}C content was determined by combustion in a Harvey OX biological sample oxidizer (Harvey Instrument Co.) and analyzed by LSS.

Each experiment was repeated at least twice, and each treatment time consisted of four replicates. Uptake and translocation were determined for each plant and expressed as a percent of the ^{14}C recovered. Data from all experiments were pooled, and means and standard errors were determined.

Metabolism of Ethametsulfuron-methyl. A field dose of 15 g of ai ha^{-1} was prepared using a commercial formulation of ethametsulfuron-methyl, Muster (75 DF), mixed with water and 0.2% Agral 90. The herbicide solution was applied with a motorized hood sprayer equipped with a flat-fan nozzle (SS8002E) calibrated to deliver 200 L ha^{-1} of spray solution at 275 kPa. Wild mustard plants at the three−four-leaf stage of development were treated within 2 h of the start of the photoperiod. Immediately after spraying, the third leaf was treated with 10 μL of [^{14}C]ethametsulfuron-methyl (3.4 kBq) as described above. The R and S biotypes were harvested 3, 6, 12, 18, 24, 48, and 72 h after treatment (HAT) and 12, 24, 48, and 72 HAT, respectively.

Each [^{14}C] treated leaf was homogenized in 1 mL of 7:3 acetonitrile/water (v/v). The homogenizer was washed with 3 mL of the same solution and combined with the leaf homogenate to give a final volume of 4 mL. This crude extract was passed through a nylon filter (0.45 μM) prior to high-performance liquid chromatography (HPLC) analysis as described by Van Eerd and Hall (2000). The Shimadzu model LC-6A HPLC was equipped with an ODS Spherex column (250 × 10 mm; 5-μm film thickness; Phenomenex, Torrance, CA). The sample (100 μL) was injected and chromatographed using a nonlinear concave no. 2 gradient of 5−100% B (acetonitrile) for 30 min and 100% B for 5 min at a flow rate of 2 mL min^{-1} (solvent A, 0.1% aqueous phosphoric acid). [^{14}C]Ethametsulfuron-methyl and its radiolabeled metabolites were detected and quantified using a Flo-one\Beta A-250 radioactivity flow detector (Radiomatic Instruments and Chemical Co. Inc., Tampa, FL).

Involvement of cytochrome P450 in the metabolism of ethametsulfuron-methyl was investigated using R wild mustard plants. Within 2 h of the start of the photoperiod, at the four-leaf stage, the third or fourth leaf was excised from a plant and the petiole of each leaf was placed into 20 mL of 0.25 strength fertilizer solution (5 g L^{-1}; Plant Products, Burlington, ON) contained in a 22-mL vial. After 24 h, the petiole of each leaf was placed in PBS containing piperonyl butoxide (PBO; 50 mM; Kwon and Penner, 1996) for 7 h, after which time, it was transferred to a PBS solution containing [^{14}C]ethametsulfuron-methyl ([110] nM; 0.66 kBq) for a further 18 h. The leaf tissues were removed, extracted, and analyzed on the HPLC to determine ethametsulfuron-methyl metabolism.

RESULTS AND DISCUSSION

ALS Experiments. Under controlled-environment conditions, ED_{50} values for the R and S biotypes were >100 and <1 g of ai ha^{-1}, respectively (data not shown). Enzyme activity of extracted ALS from both the R and S biotypes was inhibited to the same extent by chlorsulfuron and ethametsulfuron-methyl (Figure 1). This result is not in keeping with the results of other researchers, who found that sulfonylurea herbicide resistance in broadleaf plants is based on altered ALS. Jeffers et al. (1996) and Lichtner et al. (1995) speculated that the mechanism of weed resistance to sulfonylureas is not likely to be the same in all species. Therefore, on the basis of our results and those of Jeffers et al. (1996), we hypothesized that differences in ethametsulfuron-methyl absorption, translocation, and/or metabolism between R and S biotypes may be responsible for

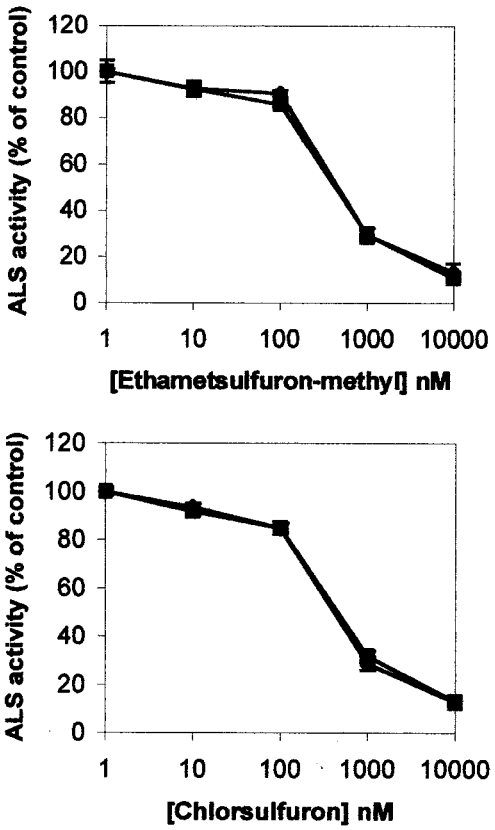

Figure 1. Inhibition by ethametsulfuron-methyl and chlorsulfuron (1−10000 nM) of ALS activity extracted from S (■) and R (◆) biotypes of *S. arvensis*. Data are expressed as a percentage of ALS activity in the absence of herbicide. Bars represent standard errors of the means. Where bars are not visible, they are smaller than the symbol at each concentration.

resistance. Menendez et al. (1997) found that concentrations of other sulfonylurea herbicides required to inhibit the ALS enzyme by 50% (I_{50}) did not vary significantly, leading them to conclude that resistance of *Alopercurus myosuroides* was not due to altered ALS. Our hypothesis is further supported by the results of Hall et al. (1992), who found no differences among the sensitivities of ALS from tolerant (commercial brown mustard) and sensitive (wild mustard) species. In these cases, differences in selectivity of the sulfonylureas were metabolism-based.

Absorption and Translocation. Uptakes of ethametsulfuron-methyl were similar at all harvest times in both R and S wild mustard. For example, 72 HAT approximately 83 and 78% of recovered ethametsulfuron-methyl was absorbed by the treated leaf of the R and S biotypes, respectively (Figure 2), indicating that, selectivity, differences were not due to differences in herbicide uptake. There was no difference in translocation between the two biotypes at all times except 72 HAT. At this time (72 HAT), more radioactivity translocated from the treated leaf of the R (14%) than the S (8%) biotype (Figure 2). Less translocation may occur in the S than the R biotype because of the phytotoxicity of the herbicide to the S as opposed to the R biotype. Hall et al. (1992) reported that when tolerant commercial brown mustard and susceptible wild mustard were compared, only 10% of the recovered [^{14}C]ethametsulfuron-methyl was translocated from the treated leaf 3 days after treatment in the tolerant species. Furthermore, Sunderland et al. (1995) reported similar results with tolerant tall morningglory (*Ipomoea purpurea*) and susceptible entireleaf morningglory (*Ipomoea hederacea*)

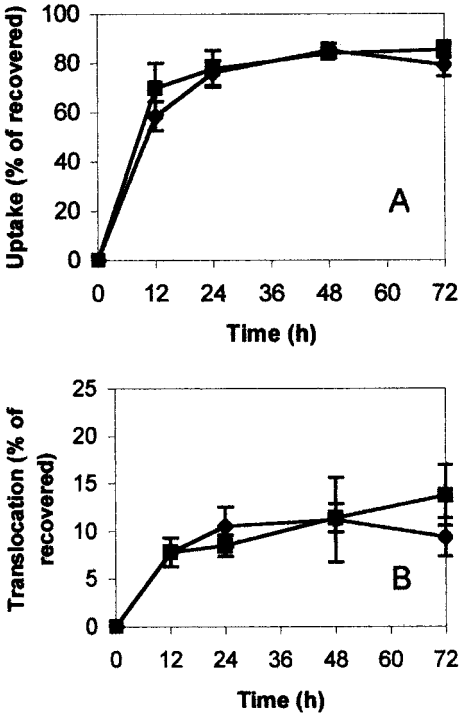

Figure 2. Uptake (A) and translocation (B) of ^{14}C following treatment with [^{14}C]ethametsulfuron-methyl of S (■) and R (◆) biotypes of *S. arvensis* (0−72 h after application). Data are expressed as a percentage of recovered ^{14}C. Bars represent standard errors of the means. Where bars are not visible, they are smaller than the symbol at each concentration.

using DPX-PE350; 90% of the herbicide remained in the treated leaf at all harvest times.

Metabolism. Regardless of the biotype, the recovery of [^{14}C]ethametsulfuron-methyl was >90%. There was no qualitative difference in the metabolites found in both biotypes (Figures 3 and 4); however, there was a quantitative difference between the biotypes. These results agree with those of Hall et al. (1992) and Lichtner et al. (1995), who observed no qualitative difference between metabolites found in tolerant and susceptible species. Ethametsulfuron-methyl and a short-lived intermediate eluted at 31 and 29 min, respectively. This intermediate metabolite was further metabolized to two stable metabolites with retention times of 22 and 21 min (Figure 3). Van Eerd and Hall (2000) used the same HPLC conditions to separate the metabolites and determined that the two latter metabolites were *O*-desethylethametsulfuron-methyl and *N*-desmethyl-*O*-desethylethametsulfuron-methyl by mass spectroscopy. Previously, Lichtner et al. (1995) determined that *O*-desethylethametsulfuron-methyl was nontoxic breakdown products of ethametsulfuron-methyl and rapid conversion of ethametsulfuron-methyl to these metabolites confers tolerance to oilseed rape.

More than 70% of the ^{14}C remained as ethametsulfuron-methyl 72 HAT of the S biotype. Furthermore, there was no difference in the extent of metabolism in the S biotype at each harvest time (Figure 5). In contrast, the quantity of [^{14}C]ethametsulfuron-methyl decreased from 82% (3 HAT) to 17% (48 HAT) the R biotype (Figure 5). Our findings agree with those of Hall et al. (1992) and Lichtner et al. (1995), who reported rapid metabolism of ethametsulfuron-methyl in tolerant commercial brown mustard and oilseed rape as opposed to S wild mustard. Furthermore, the rate of metabolism in sensitive weed species (e.g., susceptible wild mustard)

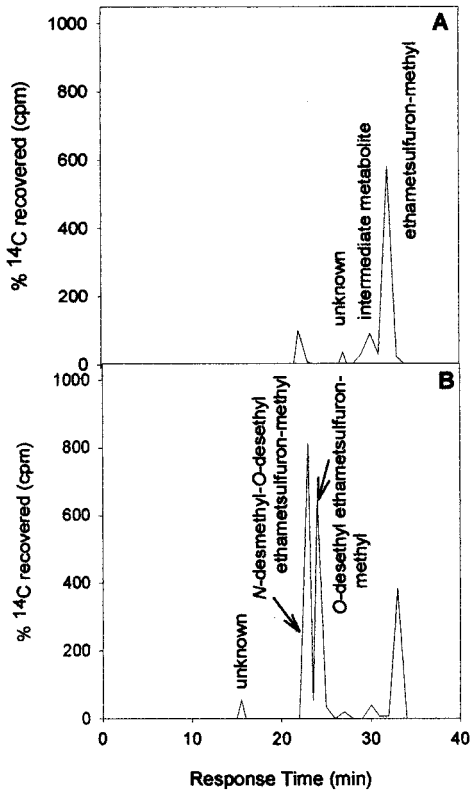

Figure 3. HPLC profile of [^{14}C]ethametsulfuron-methyl metabolism by an R biotype of *S. arvensis* (A) 12 and (B) 72 h after application of the herbicide.

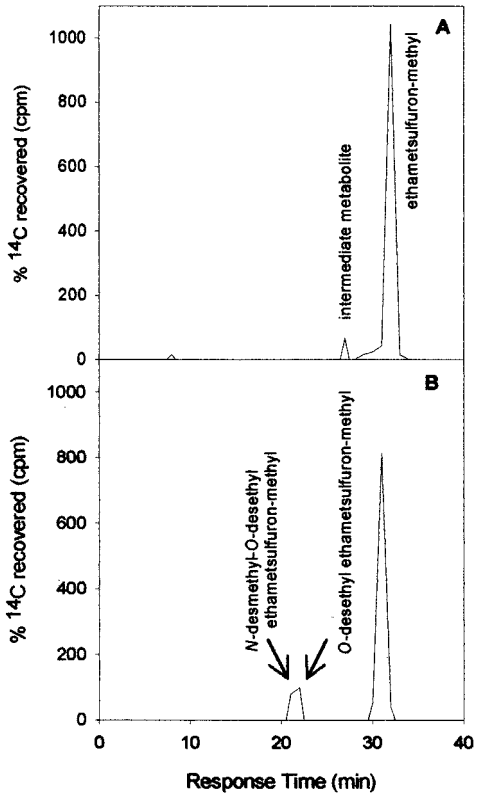

Figure 4. HPLC profile of [^{14}C]ethametsulfuron-methyl metabolism by an S biotype of *S. arvensis* (A) 12 and (B) 72 h after application of the herbicide.

has been shown to be 2−14 times slower than the rates in tolerant oilseed rape (Lichtner et al., 1995) and commercial brown mustard (Hall et al., 1992). Similarly, Menendez et al. (1997) found no qualitative difference

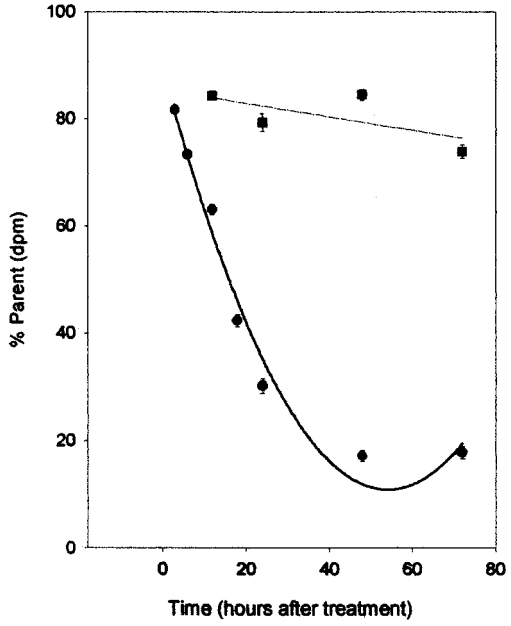

Figure 5. Percentage of recovered radioactivity remaining as [^{14}C]ethametsulfuron-methyl in R (●) and S (■) biotypes of *S. arvensis*. Bars represent standard errors of the means. Where bars are not visible, they are smaller than the symbol at each concentration.

between the metabolites produced by R and S *Alopercurus myosuroides*; however, the rate of metabolism in the R species was greater. In contrast, Sunderland et al. (1995) found one less metabolite of the sulfonylurea herbicide DPX-PE350 in susceptible entireleaf morningglory than tolerant tall morningglory.

PBO, an inhibitor of cytochrome P450, decreased the rate of metabolism of ethametsulfuron-methyl in the R biotype. After 18 h, 58 ± 2.9 and 37 ± 3.6% ($P < 0.001$; $F_{1,21} = 18.62$) of the recovered radioactivity remained as [^{14}C]ethametsulfuron-methyl in PBO-treated and control (no PBO) leaves, respectively. Furthermore, after 18 h, 36 ± 2.7 and 49 ± 4.0% ($P < 0.009$; $F_{1,21} = 8.28$) of the recovered ^{14}C were found as the two major metabolites in PBO-treated and control (no PBO) leaves, respectively. On the basis of these results, it is likely that a cytochrome P450-mediated enzyme is responsible for the rapid metabolism of ethametsulfuron-methyl in the R biotype. Due to the slow rate of metabolism in the S biotype, the effects of PBO on metabolism of ethametsulfuron-methyl could not be determined. It is not clear whether metabolism in both biotypes of wild mustard is due to an innate P450 system specific for ethametsulfuron-methyl metabolism, which is enhanced in the R biotype, or whether this P450 system is specific to only the R biotype. Our results are supported by those of Preston et al. (1996), who demonstrated that malathion (a P450 inhibitor) inhibited the metabolism of the sulfonylurea herbicide chlorsulfuron in *L. rigidum*. However, these authors concluded that the resistance of this *L. rigidum* biotype was not limited to a cytochrome P450-mediated enzyme detoxification because this species has multiple resistance (two or more genetic-based types of resistance) to several herbicide families with entirely different modes of action.

In conclusion, differences between the susceptibilities of the two biotypes of wild mustard to ethametsulfuron-methyl are not due to altered absorption, translocation, or sensitivity of ALS. However, ethametsulfuron-methyl was metabolized more rapidly in the R than in the S

biotype. This is the first report of metabolism-based resistance in a broadleaf weed to a sulfonylurea herbicide. Furthermore, metabolism in the R biotype of wild mustard may be mediated, at least partially, by a cytochrome P450-dependent enzyme system as demonstrated by using the cytochrome P450 inhibitor PBO. Whether the P450 system is also operational at lower levels in the S biotype is not known. These findings are crucial for understanding metabolism-based resistance to sulfonylurea herbicides.

ABBREVIATIONS USED

ALS, acetolactate synthase; BSA, bovine serum albumin; DPM, disintegrations per minute; EC_{50}, effective dosage at which 50% of the population is affected; HAT, hours after treatment; HPLC, high-performance liquid chromatography; LSS, liquid scintillation spectroscopy; PBO, piperonyl butoxide; PBS, phosphate-buffered saline; R, resistant; S, susceptible; cpm, counts per minute.

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