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Cross-Resistance of a Chlorsulfuron-Resistant Biotype of *Stellaria media* to a Triazolopyrimidine Herbicide[1]

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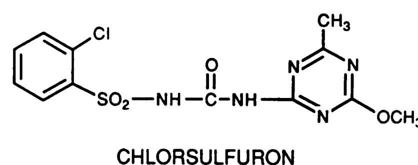
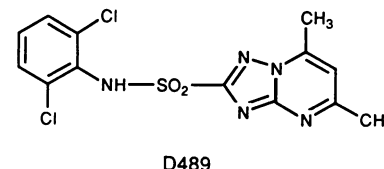
ABSTRACT

A biotype of *Stellaria media* (L.) Vill. has been identified that is highly resistant to the herbicide chlorsulfuron. Resistance is due to an altered acetolactate synthase (ALS) that is much less sensitive to chlorsulfuron than the ALS from the susceptible (S) biotype. The S biotype was extremely sensitive to D489 (*N*-[2,6-dichlorophenyl]-5,7-dimethyl-1,2,4-triazolo[1,5a]pyrimidine-2-sulfonamide), a member of a new class of triazolopyrimidine herbicides, while the chlorsulfuron-resistant biotype exhibited complete cross-resistance at both the whole plant and enzyme levels. ALS activity of the S biotype was reduced by approximately 90% in the presence of 0.1 micromolar D489, while that of the R biotype was reduced by less than 10%. This result suggests that the two herbicides share a common binding site on ALS. Only very slight cross-resistance at the ALS level was found to imazamethabenz, an imidazolinone herbicide.

ALS,[2] the first common enzyme in the biosynthesis of the branched chain amino acids valine, leucine, and isoleucine, is the principal site of action for three structurally diverse herbicide families (Fig. 1). The sulfonylureas, including chlorsulfuron and metsulfuron-methyl; the imidazolinones, including imazamethabenz; and the triazolopyrimidines, exemplified by the experimental compound *N*-[2,6-dichlorophenyl]-5,7-dimethyl-1,2,4-triazolo[1,5a]pyrimidine-2-sulfonamide (D489) all exhibit slow but tight binding to ALS (8, 11, 15).

Sulfonylurea-resistant mutants have been isolated in higher plants and unicellular organisms. Chlorsulfuron-resistant *Arabidopsis thaliana* (6), chlorsulfuron-resistant *Nicotiana tabacum* callus tissue (1, 2), and sulfometuron-methyl and chlorsufluron-resistant *Datura innoxia* haploid suspension cells (14) have been isolated following mutagenesis. Some chlorsulfuron and sulfometuron-methyl resistant lines of *N. tabacum* were selected without exposure to a mutagen (1). Unicellular organisms in which resistance to sulfonylurea herbicides has been identified include *Salmonella typhimurium* (9), *Escherichia coli* (22), *Synechococcus sp.* (4), *Saccharomyces cerevisiae* (3), and *Chlamydomonas reinhardtii* (5, 21). Sulfonylurea resistance is conferred by a single base pair





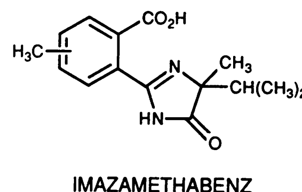


Figure 1. Structure of the three herbicides used in this study. Imazamethabenz is a mixture of the *m*- and *p*-isomers, as shown. The commercial product is formulated as the methyl ester rather than the free acid.

substitution in *A. thaliana* (6) and a one or two base pair substitution in *N. tabacum* (11); presumably, resistance is due to an altered binding site for which the herbicides have reduced affinity.

The relative ease with which sulfonylurea-resistant mutants can be obtained serves as an indication of the likelihood of resistance developing under conditions of repeated herbicide use in the field. Recently, metsulfuron-methyl-resistant *Lactuca serriola*, chlorsulfuron and metsulfuron-methyl-resistant *Kochia scoparia*, and *Salsola iberica* resistant to unspecified sulfonylurea herbicides were reported following repeated application of these herbicides (20). A chlorsulfuron-resistant strain of *Stellaria media* L. Vill. has been identified near Stony Plain, Alberta, Canada, in a field that had been treated with chlorsulfuron in 4 consecutive years (D Maurice, personal communication).

This study was undertaken to characterize the resistance of this biotype of *S. media* to chlorsulfuron, at the enzyme and

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[2] Abbreviations: ALS, acetolactate synthase (EC 4.1.3.18); FAD, flavin adenine dinucleotide.

whole plant level. In addition, the sensitivities of resistant (R) and susceptible (S) *S. media* biotypes to herbicides in the imidazolinone and triazolopyrimidine families were examined to assess cross-resistance to these structurally diverse ALS inhibitors.

MATERIALS AND METHODS

Seed Source

Seed of the R biotype of *Stellaria media* were collected at the Holborn Poultry Farm, Stony Plain, Alberta, Canada. Wild-type (S) seed, collected from a native population, were obtained from the Agriculture Canada Research Station, Lacombe, Alberta, Canada.

Whole Plant Resistance

Initial experiments were conducted to confirm resistance to chlorsulfuron in the Stony Plain *S. media,* and to assess cross-resistance at the whole plant level to herbicides from the other two families of ALS inhibitors.

Seed of S and R biotypes were germinated on moist filter paper before being transplanted to pots containing a 1:1:1 mixture of soil, peat, and vermiculite. The plants were placed in a controlled-environment chamber maintained at 16 °C with an 18-h photoperiod. After 14 d, the plants were sprayed with one of the following herbicide treatments: chlorsulfuron, at 0, 10, 50, or 100 g/ha; D489, at 0, 10, 50, or 100 g/ha; or imazamethabenz-methyl at 0, 100, 500, or 1000 g/ha. Chlorsulfuron and imazamethabenz-methyl were applied as the commercial formulations in water, and D489 was applied in 3% aqueous acetone (v/v) with 1% v/v Tween 20 as surfactant. Controls were sprayed with the same aqueous acetone/Tween 20 solution. After 21 d the plants were harvested and the fresh and dry weights determined. The data represent the average of two experiments with 12 plants per treatment in each experiment.

ALS Extraction and Assays

Seed of R and S biotypes were sown on the same medium as described above and placed in a greenhouse. Fresh tissue was harvested 10 to 20 d later for ALS assays.

Enzyme extraction and assay were modified from a procedure described by Ray (13) and Singh *et al.* (18). Leaf tissue was frozen in liquid nitrogen and powdered with 0.5 g PVP. Four mL of extraction buffer (100 mM potassium phosphate (pH 7.5) containing 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 10 mM FAD and 10% (by volume) glycerol) were added and the extract homogenized. The extract was then centrifuged at 27,000*g* for 30 min at 4 °C and saturated $(NH_4)_2SO_4$ added (30%, v/v). This was placed on ice for 30 min, then recentrifuged at 27,000*g* for 30 min, and saturated $(NH_4)_2SO_4$ added (50%, v/v). After an additional 30 min on ice the extract was recentrifuged at 27,000*g* for 30 min, and the pellet suspended in 200 μL extraction buffer. The extract was desalted on PD-1-Sephadex G-25 columns equilibrated with elution buffer (100 mM potassium phosphate containing 20 mM sodium pyruvate and 5 mM $MgCl_2$), and the crude enzyme preparation collected in 1.2 mL.

Fifty μL of crude enzyme fraction and 50 μL herbicide solution was added to 150 μL of assay buffer (83.3 mM potassium phosphate containing 33 mM sodium pyruvate, 6.6 mM $MgCl_2$, 50 mM TPP and 50 μM FAD) and the solution incubated at 37 °C for 30 min. The final concentration of herbicides in the assay solutions were 0, 0.01, 0.1, 1.0 and 3.0 μM (chlorsulfuron and D489) and 0, 0.1, 1.0, 10, and 50 μM (imazamethabenz-methyl and imazamethabenz). Fifty μL of a 1/50 dilution of 6 N H_2SO_4 were then added to stop the reaction and the solutions were incubated for 15 min at 60 °C. Following this incubation, 250 μL creatine solution (109 mg in 20 mL H_2O) and 250 μL naphthol solution (1090 mg in 20 mL 5 N NaOH) were added, and the solutions incubated at 60 °C for a further 15 min. The solutions were centrifuged for 10 min at 11,000*g*, and the absorbance read at 520 nm. Background absorbances, determined by adding 50 μl of a 1/50 dilution of 6 N H_2SO_4 before the enzyme preparation, were subtracted from the sample values. Enzyme activity was expressed as percent of an identical sample containing no herbicide.

Imazamethabenz-methyl was converted to imazamethabenz by a 12-h incubation with KOH in 10 mM phosphate buffer (pH 7.5). Combined data representing at least two experiments with three replicates each are presented in all instances. Standard errors are included as a measure of variability of the data. In many instances the standard errors are less than 5%, and are obscured by the data symbols in the figures.

RESULTS

Whole Plant Experiments

The R biotype was relatively unaffected by chlorsulfuron at all rates, whereas growth of the S biotype was significantly reduced by the two higher rates (Table I). Plants treated with the higher rates exhibited marked chlorosis at time of harvest.

Table I. *Influence of Topical Application of Chlorsulfuron, D489 and Imazamethabenz-Methyl on* S. media *Fresh Weight*

Plants were harvested 21 d after treatment.

Herbicide	Dose	Fresh Weight	
		R	S
	g/ha	*% of control*	
Chlorsulfuron	10	79 (6.6)[a]	67 (10.9)
	50	88 (9.8)	17 (6.7)
	100	65 (13.4)	11 (8.9)
D489	10	96 (8.0)	67 (8.4)
	50	101 (7.2)	24 (32.0)
	100	103 (8.8)	23 (10.1)
	100	68 (9.4)	93 (5.6)
Imazamethabenz-methyl	500	78 (14.2)	66 (13.5)
	1000	82 (11.5)	78 (12.1)

[a] Standard errors are given in parentheses.

The R biotype was completely resistant to D489 at all rates, whereas growth of the S biotype was moderately reduced by the low rate, and significantly reduced at the two higher rates (Table I). These plants exhibited symptoms similar to those of the S biotype treated with chlorsulfuron. There were no significant differences between the biotypes in response to imazamethabenz-methyl, both being unaffected at all rates of application. Dry weight values showed the same patterns of response in all instances (data not shown).

Inhibition of ALS Activity

ALS of the R biotype was inhibited much less by chlorsulfuron than that of the S biotype (Fig. 2). The ALS activity of the S biotype was inhibited approximately 80% by 0.1 μM chlorsulfuron, whereas the R biotype was inhibited only 10%. Control ALS activities of the R and S biotype were not significantly different.

The R biotype was almost completely resistant to D489, exhibiting only a slight reduction in ALS activity at 3.0 μM D489 (Fig. 2). In contrast, the S biotype was slightly more sensitive to D489 than to chlorsulfuron; ALS of the S biotype was inhibited slightly more (approximately 10%) by D489 than by chlorsulfuron, at both 0.01 and 0.1 μM.

Both R and S biotypes were tolerant of low levels of imazamethabenz-methyl, with differences between the two biotypes apparent only at 50 μM. At this concentration, ALS activities were reduced to 70% and 30% of controls for the R and S biotypes, respectively (Fig. 3). Because of the observed tolerance to imazamethabenz-methyl at the whole plant level, ALS assays were repeated with imazamethabenz. Both biotypes showed enhanced sensitivity to the biologically active (acid) form. Slight resistance to imazamethabenz was evident in the R biotype, but only at 1.0 μM; ALS activity was reduced to 41 and 19% of controls by 1.0 μM for the R and S biotypes, respectively (Fig. 3). At all other imazamethabenz concentrations, the inhibitory effects on ALS were equal in the R and S biotypes.

DISCUSSION

The results presented confirm resistance to chlorsulfuron at the whole plant level in *S. media,* and demonstrate that resistance is based on reduced sensitivity of the R biotype ALS to chlorsulfuron. Two other chlorsulfuron-resistant weeds, *Kochia scoparia* and *Salsola iberica,* have been reported recently, with resistance attributed to altered sensitivity of their ALS activity to chlorsulfuron (20).

The triazolopyrimidine herbicide, D489, is an extremely potent inhibitor of ALS (Fig. 2). The S biotype of *S. media* was sensitive to this herbicide, and its activity on ALS was slightly greater than that of chlorsulfuron. The R biotype, however, was even more resistant to D489 than to chlorsulfuron (see upper two lines in Fig. 2), indicating reduced affinity of the R ALS for D489 than for chlorsulfuron. This is the first report of a naturally occurring weed biotype that is resistant to a triazolopyrimidine herbicide, and suggests that other sulfonylurea-resistant weed species may also be resistant to triazolopyrimidine herbicides.

ALS from both the R and S biotypes was inhibited by imazamethabenz, with slightly reduced inhibition in the R biotype apparent only at 1.0 μM. However, neither biotype was affected by topical application of imazamethabenz-methyl, the commercially formulated compound, and the activity of this product at the ALS level was more than 50 times less than that of imazamethabenz (Fig. 3). Tolerance of imazamethabenz is based on lower levels of metabolism of the ester to the acid (the herbicidally active form) in tolerant species (17). Presumably, *S. media* does not metabolize this compound to the active form, which explains the high degree of tolerance observed in the whole plant experiments. Based on the data shown in Figure 3, it is likely that both the R and S biotypes would have been sensitive to the free acid. No cases of imidazolinone-resistant weeds in the field have been reported; however, imazaquin-resistant *C. reinhardtii* have been isolated following mutagenesis (21), suggesting that naturally occurring resistant biotypes also may occur.

The diverse structures of the three families of herbicides and common mode of action has generated considerable discussion concerning binding site(s). The herbicides binding to ALS are not similar in structure to the substrates, end-products or co-factors. The collected evidence suggests that the binding site(s) of these herbicides probably overlap. Imazaquin and a second triazolopyrimidine herbicide (1,2,4-triazolo[1,5a]-2,4-dimethyl-3-(*N*-sulfonyl-(2-nitro-6-methyl-aniline))-1,5-pyrimidine) have been shown to compete with labeled sulfometuron-methyl for a binding site on the 'ALS2 isozyme' from enteric bacteria (16). Herbicides likely bind close to the second substrate site, perhaps, in the case of the sulfonylureas, overlapping it. Sulfometuron-methyl is reported to exhibit competitive binding with respect to pyruvate in 'ALS isozyme II' of *Salmonella* (9) but not in ALS extracted from *Pisum sativum* tissue (8). Imidazolinones (11) and triazolopyrimidines (8) are reported to bind non-competitively

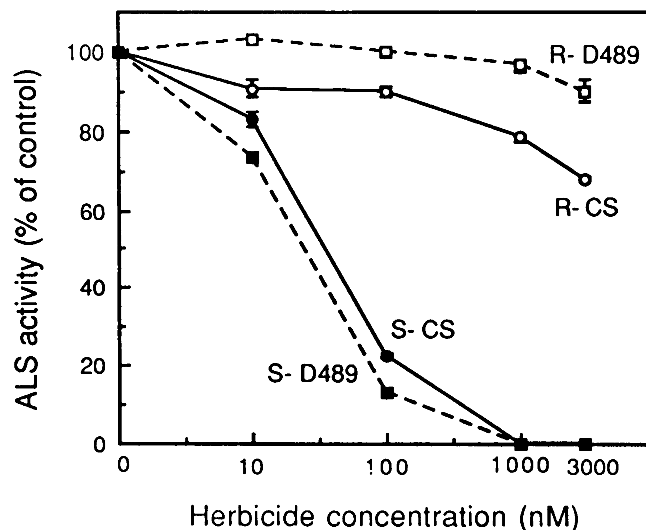

Figure 2. *In vitro* effects of chlorsulfuron and D489 on ALS activity from resistant and susceptible *S. media*. S, R = susceptible and resistant biotypes, respectively; CS = chlorsulfuron.

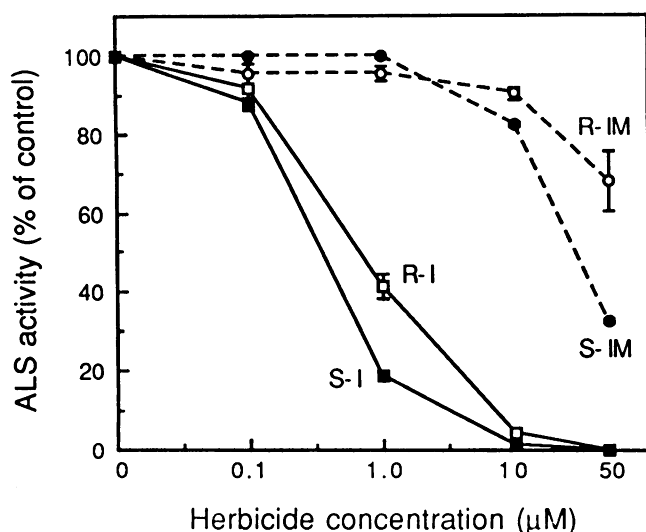

Figure 3. *In vitro* effects of imazamethabenz and imazamethabenz-methyl on ALS activity from resistant and susceptible *S. media*. S, R = susceptible and resistant biotypes, respectively; IM, I = imazamethabenz-methyl and imazamethabenz, respectively.

with respect to pyruvate in ALS extracted from *Zea mays* and *P. sativum* tissue, respectively. Herbicide binding is also reported to be proximal to the FAD binding site (8, 16).

The degree of overlap between the binding site(s) of different herbicides can also be inferred from studies with herbicide-resistant mutants. The *S. media* biotype examined in this study (Fig. 2) and a chlorsulfuron-resistant *A. thaliana* (data not shown) are highly cross-resistant to D489. In addition, 15 triazolopyrimidine-resistant tobacco and soybean culture mutants that are cross-resistant to chlorsulfuron have been isolated (19). However, some triazolopyrimidine-resistant mutants are not cross-resistant to all sulfonylurea herbicides, and triazolopyrimidine-resistant mutants vary widely in their cross-resistance to imidazolinones (MV Subramanian, personal communication). Cross-resistance between chemicals in the imidazolinone and sulfonylurea families varies among resistant biotypes and species. In this study, chlorsulfuron-resistant *S. media* exhibited only marginal cross-resistance to imazamethabenz. Chlorsulfuron-resistant *D. innoxia* was shown to be cross-resistant to imazapyr, imazaquin, and sulfometuron-methyl (14), while 6 of 8 strains of chlorsulfuron-resistant *C. reinhardtii* were cross-resistant to imazaquin (21). Chlorsulfuron-resistant *A. thaliana* was resistant to sulfometuron-methyl, but only marginally resistant to imazapyr (7) and to imazamethabenz (data not shown). However, of the 13 resistant strains of *C. reinhardtii* isolated on imazaquin, only one was cross-resistant to chlorsufluron (21).

The variety of patterns of cross-resistance between the imidazolinones and sulfonylureas suggests that the sites overlap but are not identical, and indicates that cross-resistance cannot be predicted. The degree of cross-resistance is likely a function of the location of the mutation, and the way in which it affects the binding sites of different herbicides. Although herbicide binding studies have not been conducted, it appears that the alteration in the R ALS decreases the binding affinity of chlorsulfuron and D489. However, this alteration does not influence imazamethabenz binding significantly. This does not preclude some overlapping of binding sites among these three classes of ALS inhibitors.

The rapid increase in populations of resistant weeds is of concern to those who use and regulate herbicides. The cross-resistance of chlorsulfuron-resistant *S. media* to D489 indicates a high probability that other triazolopyrimidine-resistant weeds may occur. This may pose some problems for the successful development of these herbicides. However, if sulfonylurea-resistant crops are successfully bioengineered (10, 12), triazolopyrimidines may be useful as alternate herbicides in these crops.

ACKNOWLEDGMENTS

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