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Resistance to Acetolactate Synthase-Inhibiting Herbicides in Annual Ryegrass (*Lolium rigidum*) Involves at Least Two Mechanisms[1]

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ABSTRACT

WLR1, a biotype of *Lolium rigidum* Gaud. that had been treated with the sulfonylurea herbicide chlorsulfuron in 7 consecutive years, was found to be resistant to both the wheat-selective and the nonselective sulfonylurea and imidazolinone herbicides. Biotype SLR31, which became cross-resistant to chlorsulfuron following treatment with the aryloxyphenoxypropionate herbicide diclofop-methyl, was resistant to the wheat-selective, but not the nonselective, sulfonylurea and imidazolinone herbicides. The concentrations of herbicide required to reduce in vitro acetolactate synthase (ALs) activity 50% with respect to control assays minus herbicide for biotype WLR1 was greater than those for susceptible biotype VLR1 by a factor of >30, >30, 7, 4, and 2 for the herbicides chlorsulfuron, sulfometuron-methyl, imazapyr, imazathapyr, and imazamethabenz, respectively. ALS activity from biotype SLR31 responded in a similar manner to that of the susceptible biotype VLR1. The resistant biotypes metabolized chlorsulfuron more rapidly than the susceptible biotype. Metabolism of 50% of [phenyl-U-^{14}C]chlorsulfuron in the culms of two-leaf seedlings required 3.7 h in biotype SLR31, 5.1 h in biotype WLR1, and 7.1 h in biotype VLR1. In all biotypes the metabolism of chlorsulfuron in the culms was more rapid than that in the leaf lamina. Resistance to ALS inhibitors in *L. rigidum* may involve at least two mechanisms, increased metabolism of the herbicide and/or a herbicide-insensitive ALS.

Sulfonylurea herbicides have been widely used in major cereal-growing areas since their introduction in the early 1980s. Their persistent use in North America has resulted in the appearance of resistant weed biotypes. The first two confirmed cases of sulfonylurea resistance following selection by chlorsulfuron[2] were reported in kochia (*Kochia scoparia* L.) and prickly lettuce (*Lactuca serriola* L.) (9, 14). Since then, chlorsulfuron resistance has been confirmed in Russian thistle (*Salsola iberica*), common chickweed (*Stellaria media*), and perennial ryegrass (*Lolium perenne*) at many locations in the United States and Canada (11). Until recently, the only documented cases of sulfonylurea resistance in Australia have been sulfonylurea cross-resistance in annual ryegrass (*Lolium rigidum* Gaud.) following development of resistance to the selective aryloxyphenoxypropionate herbicide diclofop-methyl (4, 5).

The sulfonylurea and the imidazolinone herbicides inhibit ALS, an enzyme involved in branched chain amino acid biosynthesis (reviewed in refs. 2 and 18). Resistant biotypes of *K. scoparia* and *L. serriola* have a less sensitive ALS and exhibit resistance to both wheat-selective and nonselective sulfonylurea and imidazolinone herbicides (9, 14, 16).

In contrast, sulfonylurea resistance in the Australian cross-resistant ryegrass biotype SLR31 is not due to herbicide-tolerant ALS (10). The mechanism of resistance, therefore, differs from that in North American sulfonylurea-resistant weed biotypes.

Species selectivity of some sulfonylurea herbicides is based upon the differential capacity of species to metabolize and detoxify the herbicides (reviewed in ref. 2). The resistance spectrum of the biotype SLR31 resembles that of wheat (4) in that both species are resistant to the selective sulfonylureas chlorsulfuron, triasulfuron, and metsulfuron-methyl, which wheat can rapidly metabolize (1, 12, 20), but are sensitive to the nonselective sulfometuron-methyl, which is not rapidly metabolized (19). Both have ALS that is sensitive to sulfonylurea herbicides (10, 20). Thus, selective resistance in wheat is due to a capacity to rapidly detoxify some selective sulfonylureas but not sulfometuron-methyl (1, 12, 19, 20). Increased metabolism is also involved in selective resistance in ryegrass biotype SLR31 (4). Similar resistance patterns for

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[2] Abbreviations: chlorsulfuron, 2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide; ALS, acetolactate synthase (EC 4.1.3.18); diclofop-methyl, (±)-methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate; metsulfuron-methyl, methyl-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]-carbonyl]amino]sulfonyl]benzoate; triasulfuron, 2-(2-chloroethoxy)-*N*-[[(4-methoxy-6-methyl)-1,3,5-triazin-2-yl]carboxyl]benzenesulfonamide; sulfometuron-methyl, 2-[[[[(4,6-dimethyl-2-pyrimidinyl)-amino]carbonyl]amino]sulfonyl]benzoate; imazamethabenz, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-4 (and 5)-methylbenzoic acid; imazapyr, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid; imazathapyr, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid; GR_{25}, herbicide dose required to reduce growth by 25% with respect to untreated plants; I_{50}, herbicide required to reduce in vitro ALS activity 50% with respect to control assays minus herbicide; g ai ha^{-1}, g of active ingredient per ha.

wheat and ryegrass biotype SLR31 are also observed for the imidazolinone herbicides. Both species are resistant to imazamethabenz but are sensitive to the nonselective herbicide imazapyr (4). Again, the selectivity of imazamethabenz in wheat has been shown to be due to differential metabolism (3).

In this study, we examined a ryegrass biotype, WLR1, which has a spectrum of resistance different from that of wheat and SLR31. Unlike SLR31, which became resistant to sulfonylureas following selection with diclofop-methyl, WLR1 had been exposed to chlorsulfuron in 7 consecutive years. WLR1 is the first case of sulfonylurea resistance in annual ryegrass that has occurred following selection with a sulfonylurea herbicide.

MATERIALS AND METHODS

Herbicides

Technical grade sulfonylurea and imidazolinone herbicides used in inhibition studies of ALS were supplied by DuPont Agricultural Products (Newark, DE) and American Cyanamid Co. (Princeton, NJ), respectively. Commercial formulations of herbicides used for whole-plant studies were supplied by DuPont (Australia) Ltd, Ceiba-Geigy Australia Ltd., and Cyanamid Australia Pty. Ltd.

Plant Material

Three biotypes of *Lolium rigidum* Gaud. were used: (a) biotype VLR1, which is susceptible to all herbicides registered for *L. rigidum* control in Australia at the manufacturer's recommended application rates, (b) biotype SLR31, which is resistant to diclofop-methyl and is cross-resistant to the wheat-selective sulfonylurea and imidazolinone herbicides chlorsulfuron, triasulfuron, metsulfuron-methyl, and imazamethabenz (4, 5), and (c) biotype, WLR1, collected near Jerramungup in Western Australia (33°57′ S 118°54′E) in a field that had been treated with chlorsulfuron during 7 consecutive years from 1983 to 1989, inclusive. The field had also been exposed to one or two applications of the nonselective herbicides *N*-(phosphonomethyl)glycine and 1,1′-dimethyl-4,4′-bipyridinium ion.

Dose Response of Pot-Grown Plants to Herbicides

Ryegrass seedlings were germinated on 0.6% (w/v) agar-solidified medium, transplanted at the one-leaf stage to 2-L pots containing potting soil, and kept outdoors during the normal autumn-winter growing season. Herbicides were applied in a laboratory spraying cabinet 7 to 14 d after transplanting when plants were at the two-leaf stage as previously described (4). Plants were harvested 4 to 8 weeks after treatment. Green plants with live meristems that had clearly increased in size since the time of spraying were harvested at soil level and dried at 80°C for 48 h before weighing. The GR_{25} was calculated. Growth reductions of 50% could not be achieved for resistant biotypes even at very high application rates of some herbicides. Twenty-four plants were used for each treatment, and each experiment was repeated three times (in 1990, 1991, and 1992) during the autumn-winter period.

ALS Activity and Chlorsulfuron Metabolism Studies

Ryegrass seedlings were germinated as above, transplanted to trays of potting soil at 2-cm spacings, and placed in a growth room. Growth room conditions were 22°C/14 h, 280 to 320 μmol photons m^{-2} s^{-1} light period and 15°C, 10-h dark period. The seedlings produced under these growing conditions resembled field-grown plants.

For ALS activity studies, ryegrass seedlings were harvested 21 or 28 d after transplanting when at the three- to four-leaf stage. Shoot material was harvested at soil level, frozen in liquid nitrogen, and stored at −80°C until extraction. Enzyme extraction was based on the methods of Ray (15) and Huppatz and Casida (6). The enzyme was precipitated in the presence of 50% saturated $(NH_4)_2SO_4$ solution. ALS assays were as described by Ray (15) except that 139 mM pyruvate was used and assay time was 30 min at 37°C unless otherwise specified. Acetolactate was determined by the method of Westerfield (21) and LaRossa and Schloss (8).

In metabolism studies, two-leaf-stage ryegrass seedlings were excised at soil level 14 d after transplanting, and the bases of the seedlings were placed in vials containing 6 μg/mL of [phenyl-U-^{14}C]chlorsulfuron (11.5 μCi/mg, supplied by DuPont). After 3 h, plants were either harvested or placed into vials of distilled water for various periods before harvest. Treatment conditions and extraction were as previously described (4) except that the leaf laminae were separated from the lower portion of the shoots (culms) for separate extraction and HPLC analysis. The culms consisted of the sheath of the first leaf and the section of the second leaf enclosed therein.

HPLC analysis was conducted at ambient temperature using an octadecaldimethylsilyl (C18) 250- × 4.6-mm, 5-μm Brownlee Labs column with a flow rate of 1.5 mL/min. Separation was achieved using two phases: A = 99% H_2O, 1% (v/v) acetic acid; B = acetonitrile, 0.2% (v/v) acetic acid with a linear gradient from 15% B to 40% B in 23 min followed by a linear gradient from 40% B to 100% B in 10 min. [^{14}C]Chlorsulfuron and metabolites were detected using a Radiomatic series A100 radiation monitor fitted with an yttrium silicate solid scintillant cell of 250-μL void volume.

RESULTS AND DISCUSSION

Dose Response to Wheat-Selective Herbicides

Ryegrass biotype WLR1 and the cross-resistant biotype SLR31 are both resistant to the wheat-selective, ALS-inhibiting herbicides chlorsulfuron, triasulfuron, metsulfuron-methyl, and imazamethabenz (Fig. 1, Table I). This is the first report of sulfonylurea resistance in Australia resulting from selection with a sulfonylurea herbicide.

Although WLR1 and SLR31 were both resistant to the wheat-selective herbicides tested, the level of resistance was not the same. WLR1 tolerated higher doses of these herbicides than did SLR31 (Fig. 1).

Dose Response to Nonselective ALS Inhibitors

Resistant ryegrass biotypes WLR1 and SLR31 differ markedly in their response to the nonselective ALS-inhibiting herbicides sulfometuron-methyl and imazapyr (Fig. 1). The

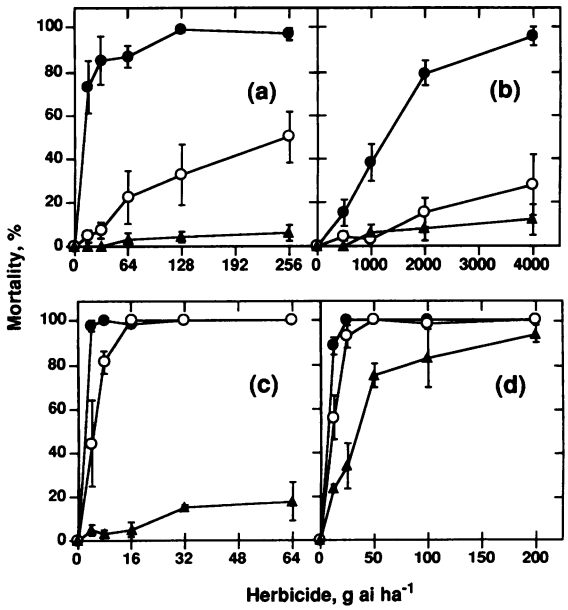

Figure 1. Mortality of resistant ryegrass biotypes WLR1 (▲) and SLR31 (○), and susceptible biotype VLR1 (●) grown in pots and treated with the wheat-selective herbicides chlorsulfuron (a) and imazamethabenz (b) and the nonselective herbicides sulfometuron-methyl (c) and imazapyr (d). Data are means of three replicate experiments. Vertical bars represent SE.

sulfonylurea sulfometuron-methyl controlled the susceptible biotype VLR1 and the cross-resistant biotype SLR31 at doses greater than 16 g ai ha^{-1} but gave little control of WLR1 even at 64 g ai ha^{-1}. Similarly, doses >50 g ai ha^{-1} of the imidazolinone herbicide imazapyr controlled VLR1 and SLR31 but gave only 75% control of WLR1.

Therefore, ryegrass biotype WLR1 exhibits resistance to both wheat-selective and nonselective sulfonylurea and imidazolinone herbicides, whereas the cross-resistant biotype SLR31 is resistant only to the wheat-selective compounds (Fig. 1, Table I). These differences in the spectra of resistance suggest that the mechanisms of herbicide resistance differ between the biotypes.

Table I. *Growth Response of Three Ryegrass Biotypes to Herbicides that Inhibit ALS*

Values are mean GR_{25} values in g ai ha^{-1} ± SE of three experiments.

	VLR1	SLR31	WLR1
Selective herbicides			
Chlorsulfuron	3.8 ± 0.2 a[a]	27.2 ± 6.2 b	110 ± 12 c
Triasulfuron	4.0 ± 0.2 a	31.3 ± 6.5 b	67.3 ± 20 b
Metsulfuron	4.2 ± 0.4 a	30.1 ± 7.4 b	47.8 ± 4.9 b
Imazamethabenz	322 ± 40 a	1311 ± 356 ab	2045 ± 713 b
Nonselective herbicides			
Sulfometuron	1.4 ± 0.4 a	1.5 ± 0.3 a	11.9 ± 1.8 b
Imazapyr	2.9 ± 0.5 a	4.8 ± 0.4 a	12.9 ± 2.1 b

[a] Values followed by different letters in the same horizontal line are significantly different (P = 0.05).

ALS Activity

The herbicide sensitivity of ALS extracted from ryegrass biotype WLR1 differed markedly from that of biotypes VLR1 and SLR31 (Table II). The I_{50} values of chlorsulfuron for ALS from VLR1 and SLR31 were similar at 50.7 ± 14.7 and 37.7 ± 12.4 nM, respectively (n = 3). In contrast, the ALS from WLR1 exhibited an I_{50} for chlorsulfuron of >1600 nM, a value >30 times that of the enzymes from the other two biotypes. The I_{50} values of ALS from WLR1 were greater than those for biotypes VLR1 and SLR31 by a factor of >30, 7, 4, and 2 for the herbicides sulfometuron-methyl, imazapyr, imazathapyr, and imazamethabenz, respectively (Table II). The K_m for pyruvate, V_{max}, and pH optima of in vitro ALS activity from resistant and susceptible biotypes were similar (data not shown).

The ratio of I_{50} values for ALS from biotype WLR1 versus those from the susceptible biotype VLR1 was similar to that reported for sulfonylurea-resistant *Lolium perenne*. Saari et al. (17) reported that ALS extracted from sulfonylurea-resistant *L. perenne* had I_{50} values greater than those of the susceptible plant by a factor of 35, 50, and 7 times for the herbicides chlorsulfuron, sulfometuron-methyl, and imazapyr, respectively. The I_{50} ratios differ from those reported for sulfonylurea-resistant biotypes of *Stellaria media*, *Salsola iberica*, and *Kochia scoparia* (14, 17). These results suggest that the ALS mutation(s) involved in sulfonylurea resistance in these two *Lolium* species may be similar but differ from those in the other species. Molecular studies are needed to clarify this point.

WLR1 possesses a mutant ALS that is less sensitive to inhibition by sulfonylurea and imidazolinone herbicides. This is probably the basis of the difference in the spectrum of resistance observed between the biotypes WLR1 and SLR31. The differences between the GR_{25} of pot-grown plants of WLR1 and SLR31 for sulfometuron-methyl and imazapyr (Table I) reflect the differences in I_{50} of the ALS extracted from these two biotypes (Table II).

Table II. *I_{50} Values to Different Herbicides for in Vitro ALS Activity from Three Ryegrass Biotypes*

Values are the means ± SE of three experiments.

Herbicide	VLR1	SLR31	WLR1
Chlorsulfuron (nM)	50.7 ± 14.7 a[a]	37.7 ± 12.4 a	>1600 b
Sulfometuron (nM)	23.1 ± 9.5 a	16.2 ± 2.2 a	>1600 b
Imazapyr (μM)	9.4 ± 1.7 a	9.4 ± 1.7 a	74.0 ± 0.6 b
Imazathapyr (μM)	2.7 ± 0.3 a	3.3 ± 0.3 a	14.5 ± 3.4 b
Imazamethabenz (μM)	197 ± 30 a	230 ± 36 a	420 ± 20 b

[a] Values followed by different letters in the same horizontal line are significantly different (P = 0.05).

Metabolism of Chlorsulfuron

Uptake of [^{14}C]chlorsulfuron by excised shoots was similar for the susceptible and both resistant biotypes (data not shown). In each biotype, approximately 70% of ^{14}C accumulated in the leaf lamina, with the remaining ^{14}C in the culms. The major accumulation of ^{14}C in the leaf lamina may have been influenced by the method used to treat these plants. The herbicide was fed via the severed xylem vessels of the cut stems, and it might be expected that accumulation of ^{14}C should be directly proportional to transpiration, whereas this might not be so if the herbicide were fed to intact plants.

Both susceptible and resistant biotypes exhibited an ability to degrade chlorsulfuron. In each biotype, chlorsulfuron was more rapidly degraded in the culms (Fig. 2), the site of greatest ALS activity (Table III). However, the rate of chlorsulfuron metabolism in the culms differed between biotypes. SLR31, the resistant biotype that has a sensitive ALS, metabolized chlorsulfuron in the culms more rapidly than the susceptible biotype VLR1. The rate of metabolism in the culms of WLR1 was intermediate. Metabolism of 50% of the chlorsulfuron in the culms required 3.7 ± 0.3 h in biotype SLR31, 5.1 ± 0.6 h in biotype WLR1, and 7.1 ± 0.6 h in biotype VLR1 (n = 4).

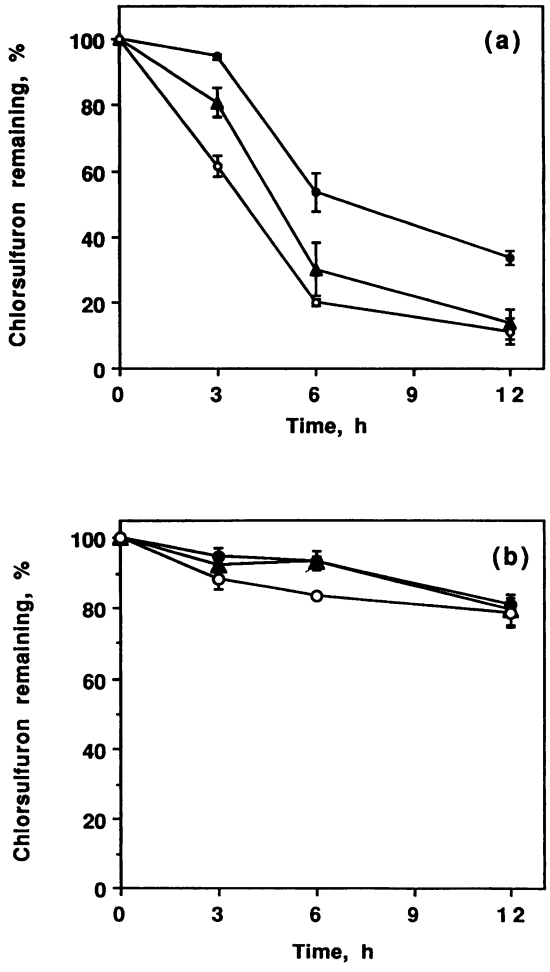

Figure 2. The percentage of ^{14}C remaining as unmetabolized [^{14}C]chlorsulfuron in extracts from (a) culms and (b) leaf lamina of excised seedlings of ryegrass biotypes WLR1 (▲), SLR31 (○), and VLR1 (●) harvested at various times following the commencement of treatment. Note that the total amount of ^{14}C in the culms and the leaf lamina differ (see text). Data are means of four replicates. Vertical bars represent SE.

Table III. *ALS Activity in the Leaf Lamina and Culms of Three- to Four-Leaf Seedlings of Three Ryegrass Biotypes in nmol Acetolactate Produced mg^{-1} of Protein h^{-1}*

Values are the means ± SE of three experiments.

Tissue	VLR1	SLR31	WLR1
Leaf lamina	57 ± 24[a]	63 ± 39	95 ± 21
Culms	219 ± 61	288 ± 79	283 ± 40

[a] Values in the same horizontal row are not significantly different (P = 0.05).

In contrast to the culms, the ^{14}C in the leaf lamina remained mostly as unmetabolized chlorsulfuron. After 12 h, <20% of the chlorsulfuron was metabolized (Fig. 2b). This pattern was observed in both resistant and susceptible biotypes.

HPLC elution profiles of ^{14}C-labeled chlorsulfuron and metabolites from the leaf lamina and culms of WLR1 were similar to those of VLR1 and SLR31 (data not shown), indicating that the differences in metabolism are quantitative but not qualitative. Christopher et al. (4) demonstrated that the elution patterns of VLR1 and SLR31 were similar and that the major metabolite coeluted with that of wheat but did not coelute with that of the resistant dicot flax. It was suggested that the metabolic pathway in ryegrass is not that found in flax (7) but may be that found in wheat (20). This is also true of ryegrass biotype WLR1.

Because the rate of metabolism of chlorsulfuron is highest in the culms, the region closest to the meristem, it is suggested that the concentration of chlorsulfuron in the culms is an important factor in determining whether a plant will exhibit resistance. This view is consistent with the observation that the culms contain 3 to 4 times more ALS activity than the leaf laminar tissue (Table III).

Clearly, the level of metabolism of chlorsulfuron in biotype WLR1 cannot explain the levels of resistance observed at the whole-plant level. The GR_{25} of biotype WLR1 for chlorsulfuron is higher than that of SLR31 (Table I), but WLR1 does not metabolize chlorsulfuron more rapidly (Fig. 2).

CONCLUSIONS

L. rigidum biotype WLR1, collected in a field following chlorsulfuron treatment, is resistant to both wheat-selective and nonselective sulfonylurea and imidazolinone herbicides. This biotype possesses an ALS that is less sensitive to these herbicides. Biotype WLR1 differs from the sulfonylurea cross-resistant biotype SLR31 selected using diclofop-methyl, which has an increased ability to metabolize chlorsulfuron but has a sensitive ALS. Biotype SLR31 is resistant to the wheat-selective sulfonylurea and imidazolinone herbicides chlorsulfuron, triasulfuron, metsulfuron-methyl, and imazamethabenz but is not resistant to the nonselective herbicides sulfometuron-methyl and imazapyr. The increased ability to

metabolize chlorsulfuron in the absence of a herbicide-insensitive ALS does not confer resistance to the nonselective compounds. Although increased metabolism may be involved in resistance to chlorsulfuron in biotype WLR1, resistance to the nonselective compounds is probably due to a less sensitive ALS. Clearly, resistance to ALS inhibitors in *L. rigidum* can involve at least two mechanisms, (a) increased ability to metabolize the herbicide and (b) a mutant form of the target enzyme ALS that is less sensitive to herbicides. These two mechanisms may be present in a single biotype. *L. rigidum* appears to have the potential to develop many different modes of resistance (13); therefore, the possibility of other physiological mechanisms being involved in resistance to ALS inhibitors cannot be excluded.

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