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Cross-Resistance to Herbicides in Annual Ryegrass (*Lolium rigidum*)[1]

II. Chlorsulfuron Resistance Involves a Wheat-Like Detoxification System

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ABSTRACT

***Lolium rigidum* Gaud. biotype SLR31 is resistant to the herbicide diclofop-methyl and cross-resistant to several sulfonylurea herbicides. Wheat and the cross-resistant ryegrass exhibit similar patterns of resistance to sulfonylurea herbicides, suggesting that the mechanism of resistance may be similar. Cross-resistant ryegrass is also resistant to the wheat-selective imidazolinone herbicide imazamethabenz. The cross-resistant biotype SLR31 metabolized [*phenyl*-U-^{14}C]chlorsulfuron at a faster rate than a biotype which is susceptible to both diclofop-methyl and chlorsulfuron. A third biotype which is resistant to diclofop-methyl but not to chlorsulfuron metabolized chlorsulfuron at the same rate as the susceptible biotype. The increased metabolism of chlorsulfuron observed in the cross-resistant biotype is, therefore, correlated with the patterns of resistance observed in these *L. rigidum* biotypes. During high performance liquid chromatography analysis the major metabolite of chlorsulfuron in both susceptible and cross-resistant ryegrass coeluted with the major metabolite produced in wheat. The major product is clearly different from the major product in the tolerant dicot species, flax (*Linium usitatissimum*). The elution pattern of metabolites of chlorsulfuron was the same for both the susceptible and cross-resistant ryegrass but the cross-resistant ryegrass metabolized chlorsulfuron more rapidly. The investigation of the dose response to sulfonylurea herbicides at the whole plant level and the study of the metabolism of chlorsulfuron provide two independent sets of data which both suggest that the resistance to chlorsulfuron in cross-resistant ryegrass biotype SLR31 involves a wheat-like detoxification system.**

There are currently at least 100 weed biotypes which have become resistant to herbicides (11). In most cases resistance has developed following exposure to one chemical, or chemicals of similar structure and mode of action, in several consecutive years. These biotypes are often resistant to other chemicals of similar structure and mode of action but are not resistant to chemicals of different structure which have different modes of action (15). In insects the development of resistance to chemicals of widely different chemical structure and mode of action has been well documented (10). This phenomenon is termed cross-resistance. Until recently there were no reports of cross-resistance to herbicides in plants. There are now at least two weed species which display cross-resistance: they are biotypes of annual ryegrass (*Lolium rigidum*) in Australia (5–7) and black grass *Alopecurus myosuroides* (14) in the United Kingdom and the Federal Republic of West Germany.

Biotypes of *L. rigidum* resistant to the selective graminicide diclofop-methyl[2] have been reported from throughout the wheat growing regions of Australia. The first report of diclofop-methyl resistance in *L. rigidum* was in 1982 following the use of diclofop-methyl in four consecutive years (8). It was subsequently found that some diclofop-methyl resistant ryegrass biotypes can be cross-resistant to the sulfonylurea herbicide chlorsulfuron (6), a herbicide which is chemically unrelated and has a different mode of action. This development was the first report of herbicide cross-resistance. Herbicide cross-resistance in *L. rigidum* is of economic concern as diclofop-methyl and chlorsulfuron are the only herbicides registered in Australia for selective postemergent control of this serious annual grass weed in cereal crops. Diclofop-methyl resistant *L. rigidum* biotypes from different regions differ in the range and levels of herbicides to which resistance is displayed. Not all biotypes are cross-resistant to chlorsulfuron

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[2] Abbreviations: diclofop-methyl, (±)-methyl 2-(4-(2,4-dichlorophenoxy)phenoxy)propanoate; chlorsulfuron, 2-chloro-*N*-((((4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino)carbonyl)benzene)sulfonamide; 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, a mixture of methyl 6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*m*-toluate and methyl 6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*p*-toluate); imazapyr (±)-2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl)-3-pyridinecarboxylic acid; fluazifop-butyl (±)-butyl 2-(4-((5-(trifluoromethyl)-2-pyridinyl)oxy)phenoxy)propanoate; haloxyfop-methyl methyl 2-(4-((3-chloro-5-(trifluoromethyl)-2-pyridinyl)oxy)phenoxy)propanoate; ACC, acetyl coenzyme A carboxylase; ALS, acetolactate synthase; MFO, mixed function oxidase.

(7). *L. rigidum* SLR31, the subject of this study, is a diclofop-methyl resistant biotype which is cross-resistant to chlorsulfuron.

Diclofop-methyl is one of the aryloxyphenoxypropionate herbicides which inhibit the enzyme ACC (EC 6.4.1.2) involved in lipid synthesis (16). Diclofop-methyl also disrupts the electrochemical membrane potential of plant cells (18) and, therefore, has at least two sites of action. There are no large differences in the target enzyme ACC between susceptible and cross-resistant ryegrass (12). It is unlikely that differential uptake, translocation or activation of the herbicide or a less sensitive target enzyme are major factors in the diclofop-methyl resistance (JAM Holtum, JM Matthews, RE Häusler, DR Liljegren, SB Powles, manuscript in preparation). Root tip cells of cross-resistant ryegrass are able to recover from a certain level of herbicide induced membrane depolarization but in the susceptible they cannot (18). The present working hypothesis for resistance to diclofop-methyl is that the combined effect of the slightly lower levels of diclofop-acid accumulated and the ability of the membranes to recover in the cross-resistant biotype SLR31 could explain the level of resistance observed (JAM Holtum, JM Matthews, RE Häusler, DR Liljegren, SB Powles, manuscript in preparation).

Chlorsulfuron is one of the sulfonylurea herbicides which inhibit the enzyme ALS (EC 4.1.3.18) an enzyme involved in the synthesis of the branched chain amino acids valine, leucine, and isoleucine (3). A second group of herbicides, the imidazolinones, also inhibit ALS activity (17). The extractable activity of ALS is similar in the resistant and susceptible ryegrass and the enzyme is equally inhibited by chlorsulfuron *in vitro* (12). There is no differential induction of ALS activity when plants are pretreated with chlorsulfuron (12). Cross-resistance to chlorsulfuron is, therefore, not due to a less sensitive target enzyme.

Differential metabolism of sulfonylurea herbicides is involved in their selectivity between species (3). Chlorsulfuron is a selective pre- or postemergent herbicide used in wheat for control of dicot weeds and certain annual grasses. Wheat is tolerant because it has the capacity to rapidly detoxify chlorsulfuron (19). The initial step in the detoxification is hydroxylation of the phenyl ring followed by rapid conjugation with glucose. The initial hydroxylation is thought to be mediated by a mixed function oxidase system (2, 19). A second detoxification mechanism is present in tolerant dicots. The initial step in this process is hydroxylation of the methyl substituent of the triazine ring (9) (Fig. 1). Evolutionary relationships would suggest that any mechanism or detoxification in cross-resistant ryegrass is more likely to be similar to wheat rather than tolerant dicots.

This study has investigated the mechanism of resistance to chlorsulfuron in the cross-resistant ryegrass biotype SLR31. As the characteristics of the target enzyme ALS are similar in the resistant and the susceptible biotypes (12) it appeared likely that resistance could be endowed by an increased ability to metabolize chlorsulfuron by a mechanism similar to that in wheat. Two independent lines of investigation were followed. (a) The spectrum of sulfonylurea herbicide resistance of the cross-resistant ryegrass was compared to the spectrum of tolerance in wheat. If a similar detoxification system endows both chlorsulfuron resistance in cross-resistant ryegrass and tolerance in wheat then the spectra of resistance/tolerance to sulfonylurea herbicides should also be similar. (b) Studies

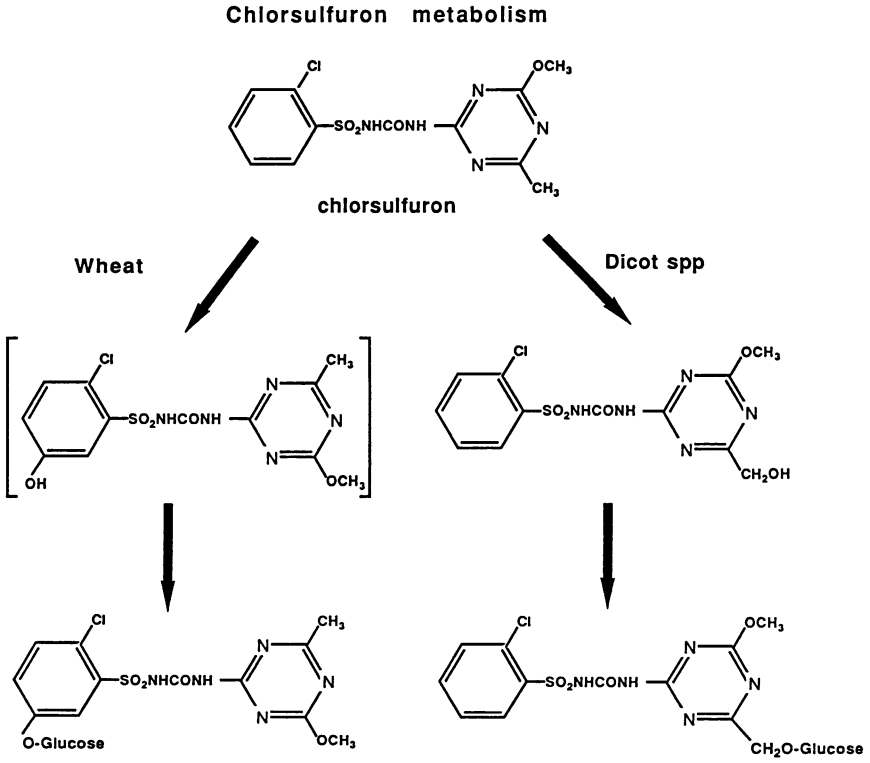

Figure 1. Mode of chlorsulfuron metabolism proposed for wheat and tolerant dicots. In wheat the initial step is hydroxylation of the phenyl ring. The product is then rapidly conjugated to glucose to form a herbicidally inactive glucoside. In resistant dicots the initial step is the oxidation of the 4-methyl group of the triazine ring followed by conjugation with glucose again forming a herbicidally inactive glucoside (adapted from Georghiou [10] and Sweester [20]).

of [*phenyl*-U-^{14}C]chlorsulfuron uptake, translocation, and metabolism were conducted to determine whether the cross-resistant ryegrass metabolizes chlorsulfuron at a faster rate than the susceptible and whether the products of any such metabolism are similar to those produced either by wheat or tolerant dicots.

MATERIALS AND METHODS

Plant Material

Three biotypes of *Lolium rigidum* Gaud. were used: (a) the susceptible biotype VLR1 which is susceptible to diclofop-methyl and chlorsulfuron at normal field rates, (b) biotype VLR6 which is resistant to diclofop-methyl but susceptible to chlorsulfuron, and (c) the cross-resistant biotype SLR31 which is resistant to both diclofop-methyl and chlorsulfuron. Wheat (*Triticum aestivum* L.) cultivar machete and flax (*Linium usitatissimum* L.) were used as examples of chlorsulfuron tolerant monocot and dicot crop plants.

Dose Response to Herbicides

Seeds of resistant and susceptible ryegrass were germinated in plastic dishes containing a 0.5 cm layer of 0.6% agar and maintained for 7 d in a growth room 22°C, 14 h, 20–30 μmol photons $m^{-2}s^{-1}$ light period/15°C, 10 h dark period. Seedlings, 2 cm high, were transplanted into 2 L pots containing potting soil at 12 seedlings per pot (4 cm spacings). Wheat seed was directly sown at the time of transplanting the ryegrass so that the two species were at the same growth stage when treated. The plants were grown outdoors during the normal autumn-winter growing season for this species. Plants were treated 7 to 14 d after transplanting when at the two leaf stage and 5 cm in height. This is the growth stage when plants are most susceptible and when treatment is recommended under agricultural conditions.

Herbicides were applied in a laboratory spraying cabinet. The herbicide was delivered via two 110° hydraulic nozzles in a total volume of 113 L/ha at a pressure of 250 kPa. Herbicides were applied as commercial formulations supplied by the manufacturer with 0.2% v/v Agral 600 surfactant added. Twenty four plants were used for each treatment. Plants were harvested 4 to 8 weeks after spraying. Each experiment was performed at least twice at different times. Absolute levels of resistance varied between experiments performed at different times due to environmental conditions. The results shown are the results of one typical experiment.

Metabolism Studies

Ryegrass seed was germinated on 0.6% agar (as described above) and the plants transferred after 7 d into potting soil in 40 × 30 × 12 cm trays at a spacing of 2 cm. Plants were then placed in a glass house for 5 d before being transferred to a growth room 2 d before treatment. Growth room conditions were 17°C, 12 h, 110 to 180 μmol photons $m^{-2}s^{-1}$ light period/13°C, 12 h dark period. At the time of treatment the plants were 14 d old, two leaf stage and similar in appearance to field grown plants. Plants were gently removed from the soil and the stems excised under water at a point just above the coleoptile (corresponding roughly to the point of emergence from the soil).

[^{14}C]Chlorsulfuron Treatment

[*phenyl*-U-^{14}C]Chlorsulfuron (11.5 μCi/mg) was dissolved in double distilled water to a final concentration of 6 μg/mL and 200 μL aliquots dispensed into Eppendorf tubes. Into each tube either five ryegrass or two wheat excised seedlings were placed for a period of 2 or 3 h. A total of 30 ryegrass or 12 wheat seedlings were used per treatment. After treatment the base of the plant stems were washed by dipping in two aliquots of double distilled water. Plants were either harvested immediately or placed into tubes containing double distilled water for a further chase period before being harvested. Plants were weighed at the time of harvest, frozen in liquid N_2 and then stored at −80°C until extraction.

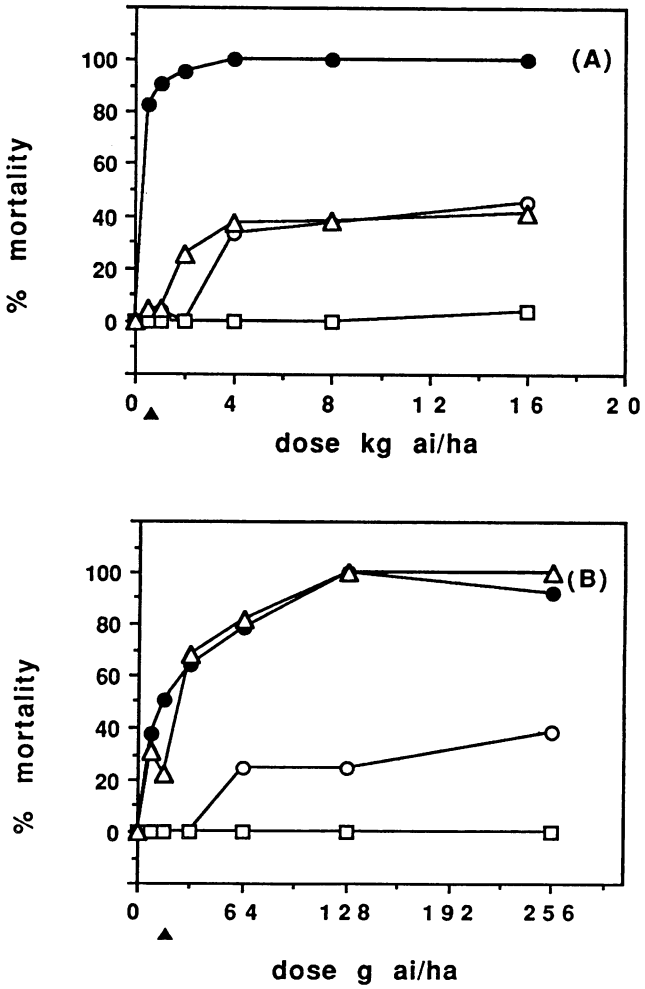

Figure 2. The survival of wheat (□), susceptible VLR1 (●), cross-resistant SLR31 (○), and VLR6 (△) biotypes of *L. rigidum* grown in pots treated with (A) diclofop-methyl or (B) chlorsulfuron. Plants were treated at the two leaf stage. The normal field dose for control of ryegrass is indicated by the arrow (▲).

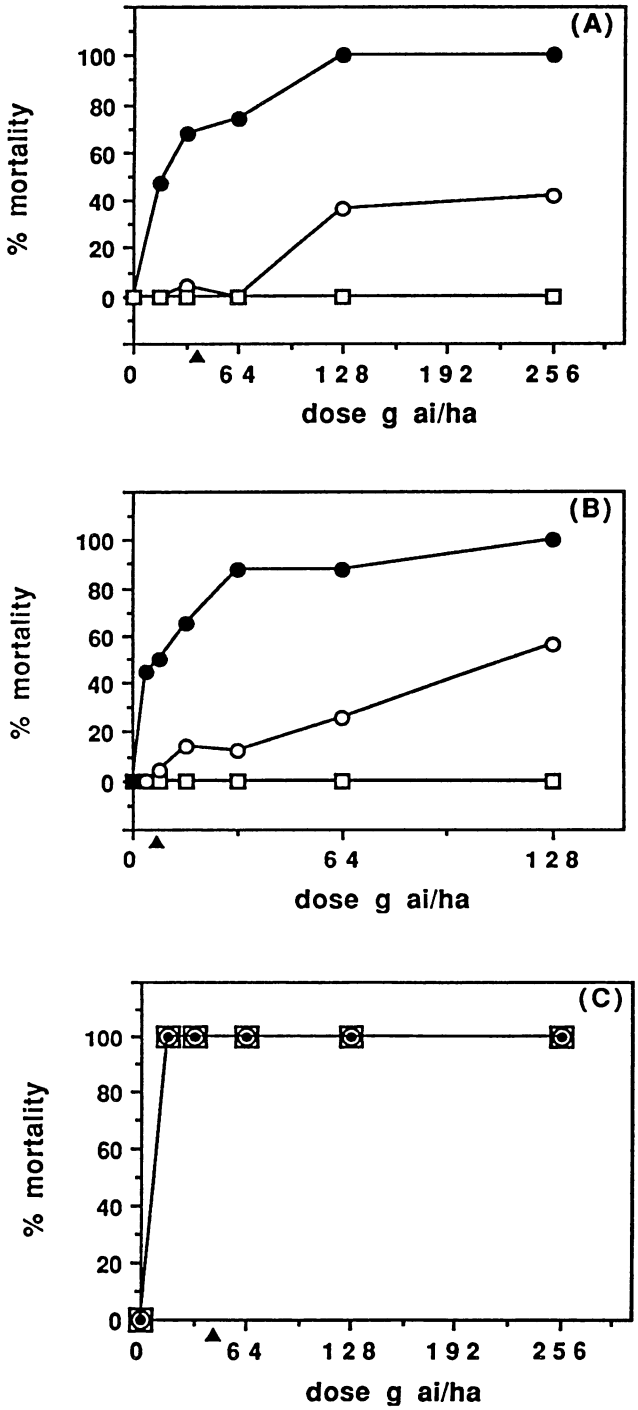

Figure 3. The survival of wheat (□) and susceptible VLR1 (●) and cross-resistant SLR31 (○) biotypes of *L. rigidum* grown in pots treated with (A) triasulfuron, (B) metsulfuron-methyl, or (C) sulfometuron methyl. Plants were treated at the two leaf stage. The normal field dose is indicated by the arrow (▲).

Extraction

Plants were ground in a mortar and pestle with sand, liquid nitrogen and chilled 80% methanol (2 mL). The mortar and pestle were rinsed with 80% methanol (2 mL) and the two fractions pooled. The plant material was sedimented by centrifugation for 20 min at 10,000*g*. The pellet was extracted with a further two aliquots (2 mL each) of chilled 80% methanol. Controls which were spiked with [^{14}C]chlorsulfuron at the time of extraction showed that this method of extraction gave >95% recovery (data not shown). Radioactivity recovered from the dose remaining in solution, stem washings, and plant extracts was >78% of that applied. Most recoveries were in the range of 85 to 95%.

HPLC

Extracts were evaporated to near dryness at reduced pressure and resuspended in double distilled water for loading onto the HPLC column. Separation was performed on an ODS 250 × 4.6 mm 5 μm C18 Brownlee Labs column using

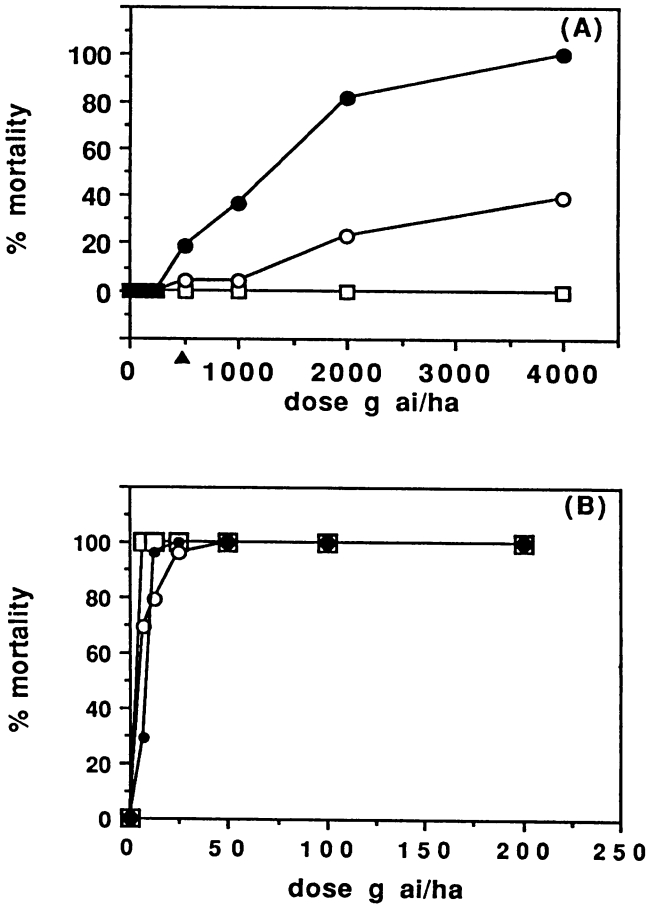

Figure 4. The survival of wheat (□), susceptible VLR1 (●), and cross-resistant SLR31 (○) biotypes of *L. rigidum* grown in pots sprayed with (A) imazamethabenz or (B) imazapyr. Plants were treated at the two leaf stage. The normal field dose for imazamethabenz is indicated by the arrow (▲). The normal application rate of imazapyr is greater than 500 g active ingredient/ha.

a gradient from 5 to 40% acetonitrile in 20 min followed by a gradient from 40 to 95% acetonitrile in 15 min with a constant final concentration of 0.2% acetic acid. The flow rate was 1.5 mL/min throughout with a column temperature of 39°C. [^{14}C]Chlorsulfuron and metabolites were detected using a Berthold LB 504 HPLC radiation monitor fitted with a Z2000–4 homogeneous counting flow cell.

RESULTS AND DISCUSSION

Resistance to Sulfonylurea and Imidazolinone Herbicides

The cross-resistant ryegrass is resistant to diclofop-methyl and exhibits similar levels of resistance to the wheat-selective sulfonylurea herbicides chlorsulfuron, metsulfuron-methyl and triasulfuron (Figs. 2, A and B, and 3, A and B). At normal field rates, which give good control of susceptible ryegrass, control of the cross-resistant ryegrass is negligible while application rates many times field rates are required to cause 50% mortality. The cross-resistant ryegrass is not resistant to the nonselective herbicide sulfometuron-methyl (Fig. 3C). The cross-resistant ryegrass is resistant to the wheat-selective imidazolinone herbicide imazamethabenz but not the nonselective herbicide imazapyr (Fig. 4). This is the first report of cross-resistance to an imidazolinone herbicide in *L. rigidum*.

Wheat is tolerant to the sulfonylurea herbicides chlorsulfuron, metsulfuron-methyl, and triasulfuron which it can rapidly metabolize (3) (Fig. 5). Chlorsulfuron and metsulfuron-methyl are detoxified by hydroxylation of the phenyl group probably involving a mixed function oxidase enzyme system (1, 19) (Fig. 1). TLC analyses of the products of triasulfuron metabolism suggest that a similar mode of detoxification is probably involved (13). Wheat is susceptible to the nonselective sulfonylurea herbicide sulfometuron-methyl which it cannot rapidly detoxify (20) (Fig. 5). The observation that cross-resistant ryegrass is resistant to the wheat selective sulfonyureas chlorsulfuorn, metsulfuron-methyl, and triasulfuron but not to the non-selective sulfometuron-methyl supports the hypothesis that the resistance mechanism in cross-resistant ryegrass and the tolerance mechanism in wheat are similar.

Metabolism of Chlorsulfuron

The rates of uptake of [^{14}C]chlorsulfuron in wheat and three ryegrass biotypes of varying susceptibility were similar (Table I).

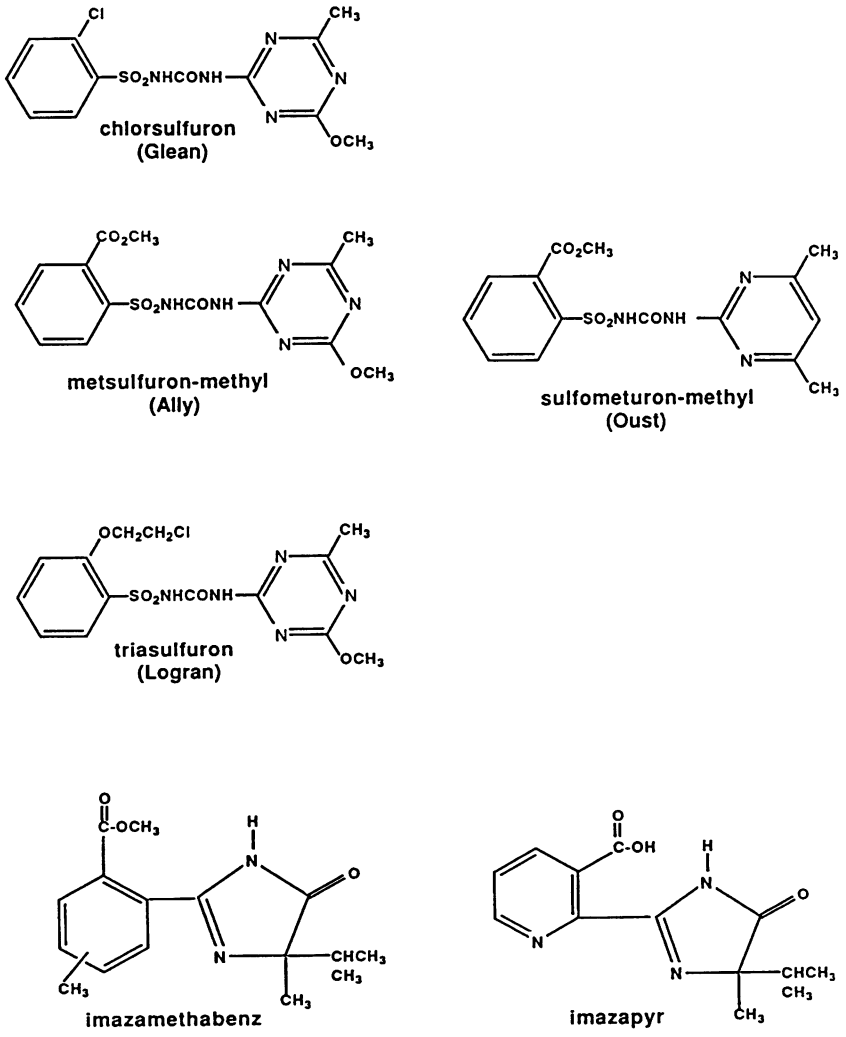

Figure 5. Structures of three wheat selective sulfonylureas: chlorsulfuron, metsulfuron-methyl, and triasulfuron and the nonselective sulfonylurea, sulfometuron-methyl plus the wheat selective imidazolinone, imazamethabenz, and the nonselective imidazolinone imazapyr.

Table I. *Uptake of [^{14}C]Chlorsulfuron over 3 h*

Each value is the average of four replicate experiments plus or minus standard error except in the case of VLR6 which is the average of two experiments.

Wheat	Ryegrass		
	Cross-Resistant	Susceptible	VLR6
	ng/mg fresh wt		
2.85 ± 0.28	2.94 ± 0.31	3.06 ± 0.40	2.85 ± 0.05

Cross-resistant ryegrass SLR31 metabolized chlorsulfuron at a rate approximately twice that of the susceptible biotype (Fig. 6A). While the cross-resistant biotype required 6 h to metabolize 50% of the chlorsulfuron taken up the susceptible required in excess of 12 h. The increased rate of detoxification of chlorsulfuron in the cross-resistant ryegrass is specifically related to chlorsulfuron resistance and not simply a metabolic side effect of diclofop-methyl resistance as biotype VLR6, which is susceptible to chlorsulfuron but resistant to diclofop-methyl, metabolizes chlorsulfuron at the same rate as the chlorsulfuron and diclofop-methyl susceptible biotype VLR1 (Fig. 6B). Wheat degrades chlorsulfuron at a much faster rate than any ryegrass biotype tested, taking 2 h to degrade 50% (Fig 6B). The rates of degradation of chlorsulfuron are correlated with the levels of tolerance observed at the whole plant level, with wheat being more tolerant than cross-resistant ryegrass SLR31 which is more tolerant than the two chlorsulfuron susceptible biotypes VLR1 and VLR6.

The major metabolite of chlorsulfuron in wheat and ryegrass exhibited a similar elution profile when separated using reverse-phase HPLC (Fig. 7). When extracts from wheat and ryegrass were combined and chromatographed a single major peak was eluted (data not shown). Sweetser *et al.* (19) identified the major metabolite of wheat as the glycosylated derivative of chlorsulfuron hydroxylated in the phenyl ring (Fig. 1).

The products of metabolism of chlorsulfuron in the chlorsulfuron-tolerant dicot, flax, are not the major metabolites produced in ryegrass (Fig. 7). Two minor metabolites produced by ryegrass exhibit similar retention profiles to the metabolites produced by flax. The major metabolite produced by flax is most likely that formed following oxidation of the 6-methyl substituent of the triazine ring (9) (Fig. 1). It is important to note that there was no difference in the metabolite profiles from susceptible and resistant ryegrass except that at any given time a larger proportion of activity was present as metabolites in the cross-resistant than in the susceptible (Fig. 7). It was the rate of metabolism that differed rather than the nature of the metabolites. The cross-resistant ryegrass appears to utilize existing, wheat-like metabolic pathways to detoxify chlorsulfuron at twice the rate of the susceptible biotype (Fig. 7).

The mechanisms of resistance to the other wheat-selective sulfonylureas and to the wheat-selective imidazolinone imazamethabenz in cross-resistant ryegrass remain to be elucidated, although metabolism is strongly suspected. It is possible that the 4-methoxy-6-methyl-1,3,5-triazine ring that is common to the wheat-selective sulfonylurea herbicides may be involved in the recognition between the herbicide molecules and detoxifying enzymes that confer resistance in wheat and cross-resistant ryegrass (Fig. 5). Sulfometuron-methyl, which is nonselective and kills both wheat and ryegrass, has a 4,6-dimethyl-pyrimidine in place of the 4-methoxy-6-methyl-1,3,5-triazine ring (Fig. 5).

Wheat tolerates the imidazolinone herbicide imazamethabenz by detoxification resulting from rapid hydroxylation of the methyl constituent of the phenyl ring followed by conjugation to glucose (4). In contrast the nonselective imazapyr is not rapidly detoxified. Although the imidazolinone herbicides are structurally distinct from the sulfonylurea herbicides and might be expected to be detoxified by a mechanism that differs from that involved in sulfonylurea detoxification, both the imidazolinones and sulfonylureas bind and inhibit the enzyme ALS and it is possible that they may expose similar binding sites to detoxifying oxidative enzymes (Fig. 5).

Mechanism of Cross-Resistance

The data presented here do not identify how exposure of population SLR31 to diclofop-methyl has selected not only for resistance to diclofop-methyl but also for cross-resistance to chlorsulfuron. Although the patterns of susceptibility and tolerance of sulfonylurea and imidazolinone herbicides are similar for ryegrass and wheat, the patterns of susceptibility and resistance to aryloxyphenoxypropionate herbicides are not. Wheat tolerates diclofop-methyl but is killed by haloxyfop-methyl and fluazifop-butyl while the cross resistant ryegrass is resistant to all three (data not shown). Wheat is tolerant to diclofop-methyl and chlorsulfuron because of its ability to rapidly degrade both herbicides using MFO enzymes. Although cross-resistant ryegrass exhibits an enhanced ability to degrade chlorsulfuron (Fig. 6), possibly via a MFO-

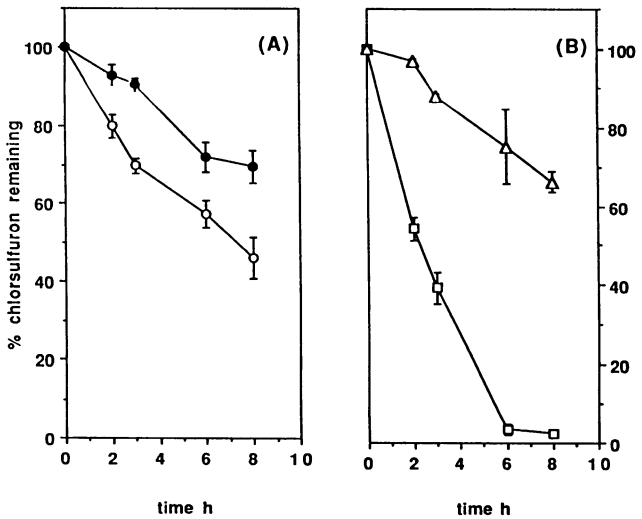

Figure 6. The amount of [^{14}C]chlorsulfuron remaining in extracts from excised seedlings harvested at various times after the commencement of uptake: (A) susceptible (●) and cross-resistant ryegrass (○) and (B) wheat (□) and ryegrass biotype VLR6 (△). Values are the average of four repeat experiments except in the case of VLR6 which is the average of two experiments. Error bars indicate standard error of the mean.

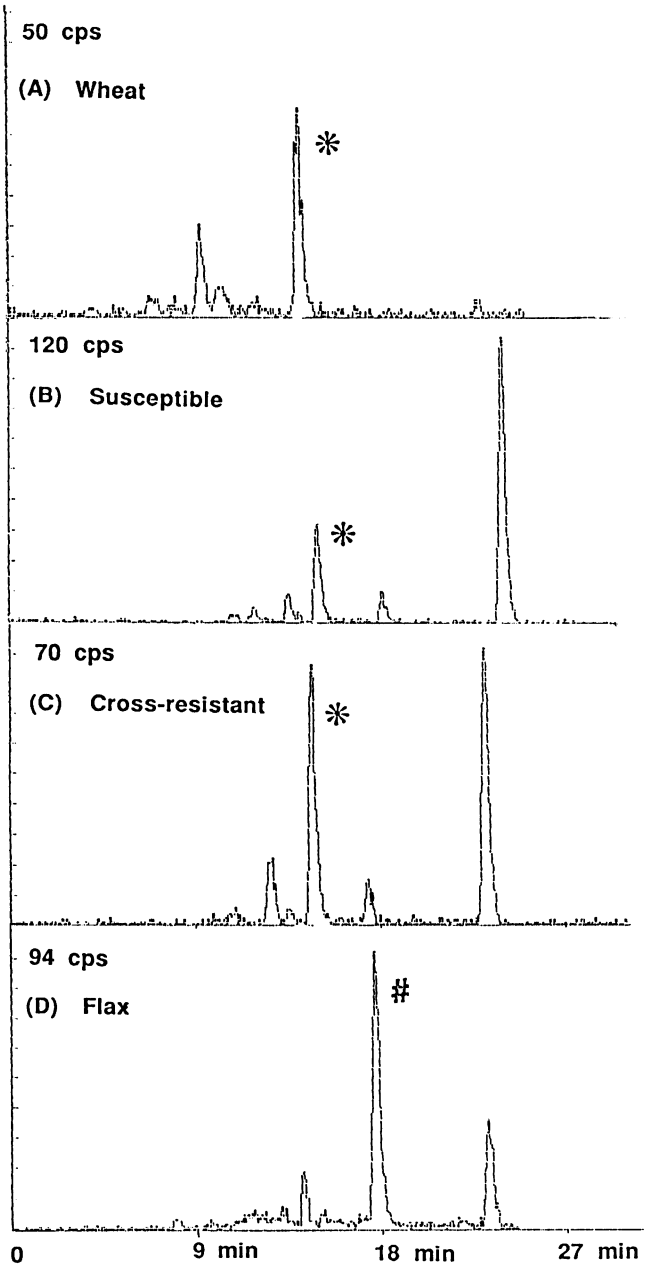

Figure 7. HPLC chromatograms of ^{14}C-labeled chorsulfuron and metabolites in extracts from (A) wheat, (B) susceptible and (C) cross-resistant *L. rigidum*, and (D) flax. Excised seedlings were allowed to take up the dose for 3 h and then transferred to water for 5 h (8 h total). Maximum values for the *y*-axis are given in counts per second. The major metabolite of wheat and both *L. rigidum* biotypes (*) has the same retention time but the major metabolite of flax (#) does not. [^{14}C]Chlorsulfuron is the last compound to elute at approximately 23 min.

catalyzed mechanism, there is little evidence to suggest enhanced MFO activity is involved in the breakdown of diclofop-methyl in cross-resistant ryegrass (JAM Holtum, JM Matthews, RE Häusler, DR Liljegren, SB Powles, manuscript in preparation).

The ability of wheat and of the cross-resistant ryegrass to recover from diclofop-methyl induced membrane depolarization has been suggested as a mechanism of resistance to the aryloxyphenoxypropionate herbicides (JAM Holtum, JM Matthews, RE Häusler, DR Liljegren, SB Powles, manuscript in preparation). However, chorsulfuron does not depolarize membranes at physiologically meaningful concentrations. Chlorsulfuron at micromolar concentrations can depolarize plant membranes but even at its limit of solubility chlorsulfuron depolarization is only partial and the mode of recovery of membrane potential does not correlate with resistance. Thus it is highly unlikely that membrane effects are a major factor in chlorsulfuron resistance (R Häusler, personal communication).

Another possible mechanism for cross-resistance is differential compartmentalization of the herbicides away from the target enzymes. This possibility remains to be investigated.

CONCLUSION

The investigation of the dose response to sulfonylurea herbicides at the whole plant level and the study of the metabolism of chlorsulfuron provide two independent sets of data, both of which suggest that the resistance to chlorsulfuron in cross-resistant ryegrass biotype SLR31 involves a wheat-like detoxification system.

ACKNOWLEDGMENTS

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