Plant Physiol. (1990) 94, 1180–1186
0032-0889/90/94/1180/06/$01.00/0

Received for publication December 13, 1989
Accepted June 14, 1990

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

I. Properties of the Herbicide Target Enzymes Acetyl-Coenzyme A Carboxylase and Acetolactate Synthase

John M. Matthews, Joseph A. M. Holtum*, David R. Liljegren, Barbara Furness, and Stephen B. Powles

Departments of Agronomy (J.M.M., J.A.M.H., S.B.P.) and Agricultural Biochemistry (D.R.L., B.F.), Waite Agricultural Research Institute, University of Adelaide, P.O. Bag 1, Glen Osmond, South Australia, 5064 Australia

ABSTRACT

***Lolium rigidum* biotype SR4/84 is resistant to the herbicides diclofop-methyl and chlorsulfuron when grown in the field, in pots, and in hydroponics. Similar extractable activities and affinities for acetyl-coenzyme A of carboxylase (ACCase), an enzyme inhibited by diclofop-methyl, were found for susceptible and resistant *L. rigidum*. ACCase activity from both biotypes was inhibited by diclofop-methyl, diclofop acid, haloxyfop acid, fluazifop acid, sethoxydim, and tralkoxydim but not by chlorsulfuron or trifluralin. Exposure of plants to diclofop-methyl did not induce any changes in either the extractable activities or the herbicide inhibition kinetics of ACCase. It is concluded that, in contrast to diclofop resistance in *L. multiflorum* and diclofop tolerance in many dicots, the basis of resistance to diclofop-methyl and to other aryloxyphenoxypropionate and cyclohexanedione herbicides in *L. rigidum* is not due to the altered inhibition characteristics or expression of the enzyme ACCase. The extractable activities and substrate affinity of acetolactate synthase (ALS), an enzyme inhibited by chlorsulfuron, from susceptible and resistant biotypes of *L. rigidum* were similar. ALS from susceptible and resistant plants was equally inhibited by chlorsulfuron. Prior exposure of plants to 100 millimolar chlorsulfuron did not affect the inhibition kinetics. It is concluded that resistance to chlorsulfuron is not caused by alterations in either the expression or inhibition characteristics of ALS.**

Biotypes of the grass weed *Lolium rigidum* (annual ryegrass) with resistance to the selective postemergent graminicide diclofop-methyl[1] have been identified in all major cereal and grain-legume cropping regions of mainland Australia (10, 11, 12, 21). In all cases identified to date, resistance has developed following exposure to diclofop-methyl. The diclofop-resistant biotypes are also resistant to a range of other aryloxyphenoxypropionate herbicides, the spectrum and extent of resistance being biotype-dependent (11). Some *Lolium* biotypes with resistance to diclofop-methyl are also resistant to herbicides of very different chemistry or modes of action. In some instances, such as probably for the majority of cases of resistance to the dinitroaniline herbicide trifluralin, populations have been exposed to these compounds, but in others there has been no exposure. This latter resistance phenomenon is termed cross-resistance. Herbicides against which there is cross-resistance include some, but not all, cyclohexanediones and sulfonylureas (11). Between them the biotypes of *L. rigidum* exhibit resistance to every selective postemergent graminicide registered for use in Australia and are resistant to several compounds which have not been released (11, 21).

When initially observed, the cross-resistance in *L. rigidum* was the first incidence of this phenomenon described for plants (12). Subsequently, cross-resistance has also been observed in *Alopecurus myosuroides* (black grass) in the Federal Republic of Germany and in the United Kingdom (17).

Apart from the potentially serious economic ramifications on cropping, the cross-resistance phenomenon is intriguing from a mechanistic viewpoint because some herbicides against which there is resistance have different modes of action. The aryloxyphenoxypropionates diclofop-methyl, diclofop acid, haloxyfop acid, quizalofop acid, and fluazifop acid and the cyclohexanediones sethoxydim, alloxydim, and tralkoxydim inhibit ACCase (E.C. 6.4.1.2) (2, 23, 24, 29) and, to a lesser extent, the pyruvate and α-ketoglutarate dehydrogenase complex (3). ACCase catalyses the acetyl CoA-, ATP- and HCO_3^--dependent synthesis of malonyl CoA, a precursor required in the synthesis of lipids, fatty acids, and in several pathways of secondary metabolism. The sulfonylureas chlorsulfuron, triasulfuron, and metsulfuron-methyl inhibit ALS (E.C. 4.1.3.18),

[1] Abbreviations: diclofop-methyl, methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate; ACCase, acetyl-CoA carboxylase; alloxydim, 2-(1-alloxyaminobutylidene)-5,5-dimethyl-4-methoxycarbonylcyclohexane-1,3-dione; a.i., active ingredient; ALS, acetolactate synthase; chlorazifop-proponyl, 2-proponyl 2-[4-[(3,5-dichloro-2-pyridinyl)oxy] phenoxy]propanoate; chlorsulfuron, 2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2yl)aminocarbonyl]-benzenesulfonamide; fenoxaprop-ethyl, (±)-ethyl 2-[4-[(6-chloro-2benzoxazolyl)oxy]phenoxy]propanoate; fluazifop-butyl, (±)-butyl 2-[4-[(5(trifluoromethyl)-2-pyridinyl)oxy]phenoxy]propanoate; glyphosate, *N*(phosphonomethyl)glycine; haloxyfop-methyl, (±) methyl-2-[4-((3-chloro-5(trifluoromethyl)-2-pyridinyl)oxy)phenoxy]propanoate; quizalofop, (±)-2[4[(6-chloro-2-quinoxalinyl)oxy]phenoxy] propanoic acid; sethoxydim, [1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-11-one; tralkoxydim, 2-[1-(ethoxyimino)propyl]-3-hydroxy-5-mesitylcyclohex-2-enone; trifluralin, α,α,α-trifluoro-2,6-dinitro-*N*-*N*-dipropyl-*p*-toluidine.

an enzyme involved in the synthesis of the essential, branched amino acids valine and isoleucine (1, 14, 22). The dinitroaniline trifluralin is an inhibitor of tubulin polymerization (16).

It is stressed that although herbicide-sensitive processes have been identified for the above-mentioned herbicides the possibility exists that a number of these herbicides interfere, perhaps in a biocidal manner, with more than one physiological process. For example, diclofop, haloxyfop, fluazifop, and sethoxydim can, to different extents, depolarize membranes (8, 26, 27, 30). The herbicidal and physiological activities of enantiomers of the active herbicides may not be the same (18, 24). For example, the R(+)enantiomer of diclofop acid inhibits its ACCase and has greater herbicidal activity but the S(−)enantiomer which does not inhibit ACCase also exhibits some herbicidal activity.

The biochemical mechanism, or mechanisms, responsible for cross-resistance in *L. rigidum* are not known. At least two general, not necessarily mutually exclusive, mechanisms could account for the phenomena. These are, first, the altered sensitivity of one or more herbicide target sites and, second, a reduction in the concentration of herbicides at their respective target sites. The latter phenomenon could be influenced by several factors including a reduction in the rates and amounts of herbicides which enter the resistant plants, reduced conversion of herbicides to their active forms, a reduction in the rates or amounts of herbicides translocated to their target sites, changes in the inter- or intracellular sequestration of herbicides or an increased capacity to detoxify the herbicides. Whatever the mechanism, or mechanisms, they must be general enough to account for resistance to a range of structurally distinct herbicides yet specific enough to account for the degree of herbicide specificity which is still observed.

Here we report the levels and characteristics of ACCase and ALS from biotypes of *L. rigidum* either susceptible or resistant to aryloxyphenoxypropionate, cyclohexanedione and sulfonylurea herbicides. The possibility that resistance may be due to either increased expression or reduced herbicide-sensitivity of ACCase and ALS is investigated and discarded.

MATERIALS AND METHODS

Plant Material

Susceptible *Lolium rigidum* biotype SRS2 and resistant biotype SR4/84 were collected and identified originally by Dr. I. M. Heap (Waite Agricultural Research Institute, University of Adelaide). The latter biotype exhibits resistance to, among other herbicides, the aryloxyphenoxypropionates diclofop-methyl, haloxyfop-methyl, fluazifop-butyl, quizalofop-ethyl, fenoxaprop-ethyl, and chlorazifop-proponyl; the cyclohexanediones sethoxydim, tralkoxydim, and alloxydim; the sulfoylureas chlorsulfuron, triasulfuron, and metsulfuron methyl; the dinitroaniline trifluralin; and the imidazolinone imazamethabenz. Population SR4/84 was collected from the same field, but 2 years subsequent to the diclofop and chlorsulfuron resistant population described by Heap and Knight (10). In the intervening period the farmer had applied fluazifop-butyl and sethoxydim at rates of 212 g a.i. ha^{-1} and 781 g a.i. ha^{-1}, respectively.

Pisum sativum cv Maori Dwarf was obtained locally (Kellys Seed Co., Adelaide). *Vicia faba* cv Fiord and *Triticum aestivum* cv Millewa are common Australian agricultural varieties.

Unless specified otherwise, *L. rigidum* and *T. aestivum* were harvested at the two-leaf stage. This is the stage at which they are exposed to herbicides under normal agricultural practice. The tissue used for enzyme assays was the stem or shoot tissue 2 to 3 cm immediately superior to the zone of root initiation. Diclofop-methyl, when applied as a postemergent spray, must contact this tissue if plant death is to occur.

Growth Conditions

Pisum, Vicia, and *L. rigidum* used in pot experiments were germinated on filter paper in Petri dishes before being transplanted into sterile potting soil and grown in 15 cm pots in a greenhouse.

For spraying experiments, herbicides were applied to plants in an enclosed laboratory sprayer which delivered the herbicide solutions through two flat-fan hydraulic nozzles (Tee-Jet, 001) at a nozzle pressure of 250 kPa. With a boom speed of 1 m s^{-1}, a volume of 113 L ha^{-1} was delivered at plant height 40 cm below the nozzles. For the estimation of mortality, plants were rated as survivors if healthy green blades were still emerging from the basal node 21 d after treatment.

In hydroponic experiments *Lolium* seeds were germinated and grown in a growth room in polystyrene trays floating on one-half strength Hoagland solution. Growth conditions were 20°C, 12 h, 330 μmol photons m^{-2} s^{-1} light period/16°C, 12 h dark period; 54 seedlings were grown per tray containing 10 L of nutrient solution. *Triticum* was grown in Perlite and nutrient solution under similar conditions. Herbicides were added to the nutrient solution as required.

Crude ACCase Extracts

All extraction and purification operations were performed at 0 to 4°C. Washed tissue was ground in a chilled mortar with three volumes of buffer A (100 mM Tris-HCl [pH 8.0], 20 mM β-mercaptoethanol, 1 mM EDTA, 0.5% [w/v] insoluble PVP, 0.5% [w/v] PVP-40, 10% [v/v] glycerol, and 2 mM isoascorbic acid) supplemented with 5 mM PMSF. The brei was centrifuged for 10 min at 27,000*g*. The supernatant was loaded onto a column containing Sephadex G-25 (PD-10 column, Pharmacia) previously equilibrated with buffer B (25 mM Tricine [pH 8.0], 20 mM β-mercaptoethanol, 1 mM EDTA, and 10% [v/v] glycerol) supplemented with 5 mM PMSF. The eluate was stored on ice and assayed for ACCase activity immediately.

$(NH_4)_2SO_4$ Purification

Tissue was ground in buffer A and centrifuged as described. ACCase activity was precipitated between 10% and 40% $(NH_4)_2SO_2$ saturation. The pellet was resuspended in 2.7 mL of buffer B; 2.5 mL was applied to a column of Sephadex G-25 previously equilibrated with the same buffer. The eluate was stored on ice and assayed for ACCase activity immediately.

ACCase Assay

ACCase activity was assayed by following the incorporation of ^{14}C from $NaH^{14}CO_3$ into acid-stable product at 30°C ±

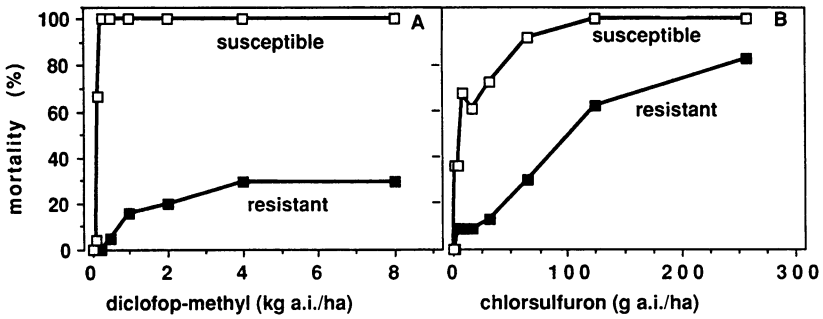

Figure 1. The survival of susceptible (□) and resistant (■) biotypes of *L. rigidum*, grown in pots, following exposure to diclofop-methyl (A) or chlorsulfuron (B). Twenty four plants of each biotype were sprayed at the two-leaf stage. The recommended rate for diclofop-methyl is 375 g a.i. ha^{-1}. The recommended rate for chlorsulfuron is 15 g a.i. ha^{-1}.

0.5°C (19). Assays contained, unless specified otherwise, in a volume of 200 μL, 50 mM Tricine (pH 8.0), 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 mM ATP, 10 mM $NaH^{14}CO_3$ (containing 0.2 μCi of ^{14}C), 0.3 mM acetyl-CoA, sample, and herbicide or H_2O as required. Reactions were initiated by the simultaneous addition of ATP and $NaH^{14}CO_3$ after 3 min incubation. Assay duration was 300 s for partially purified enzyme and 180 s for crude extracts. Reactions were terminated by the addition of 25 μL of glacial acetic acid. A fluted glass-fibre filter (Whatman GF-A) was added to each reaction tube and the volatile components evaporated at 70 to 80°C under a stream of dry air or N_2. Scintillation fluid (2 mL of 2:1 toluene:Triton X100 containing 4 g of 2,5-diphenyl oxazole and 100 mg 1,4-bis[5-phenyl-2-oxazolyl]-benzene L^{-1} toluene) was added and the acid-stable radioactivity determined. The output from the scintillation counter was corrected for background, counting efficiency and for acetyl-CoA- and ATP-independent incorporation of radioactivity.

ALS Extractions

The extraction of ALS was essentially that used by Ray (22). Modifications included an extraction buffer pH of 7.0 and a 0 to 50% $(NH_4)_2SO_2$ precipitation.

ALS Assay and Acetoin Determination

ALS activity was estimated from the differences in acetoin contents of sample and control mixtures (14, 22).

Herbicides

All herbicides were initially made up as 1 mM stock solutions in 70% (v/v) acetone. Stock solutions were diluted with H_2O prior to use such that the highest acetone concentration in any assay was 0.3% (v/v). The buffering capacities of the assay systems used were such that the addition of the unbuffered herbicides did not change the pH.

Diclofop-methyl and diclofop acid (50% S− and R+ isomers) were gifts from Hoechst Australia; fluazifop-butyl, fluazifop acid and tralkoxydim from I.C.I. Australia; haloxyfop-methyl and haloxyfop acid (50% S− and R+ isomers) from Dow Chemical Co., Walnut Creek; chlorsulfuron from Du Pont de Nemours Aust.; sethoxydim from Schering; and trifluralin from Nufarm Aust.

RESULTS AND DISCUSSION

Effects of Diclofop-Methyl and Chlorsulfuron on Growth

L. rigidum biotype SR4/84 survives exposure to concentrations of diclofopmethyl and chlorsulfuron far in excess of those used during common agricultural practice which normally provide control (Fig. 1). For pot-grown resistant plants the LD_{50} value, measured as the proportion of plants still surviving 21 d after spraying, for diclofop-methyl of >8 kg a.i. ha^{-1} was >80-fold that of the susceptible plants whereas for chlorsulfuron the LD_{50} of 100 g a.i. ha^{-1} was 20-fold that of the susceptible plants. On the basis of the preceding criteria the biotype SR4/84 has been defined as resistant.

Differential susceptibilities of biotypes of *L. rigidum* to diclofop-methyl and chlorsulfuron were also evident in plants grown in hydroponic culture (Fig. 2), a more convenient model cultivation system that was used for the experiments described in this study. In hydroponically cultured resistant plants the concentrations required to reduce the dry weights to 50% of those of the untreated controls after 7 d exposure to the herbicides were >5 μM for diclofop-methyl and >100 nM for chlorsulfuron. For susceptible plants the values were 1.1 μM and 50 nM, respectively.

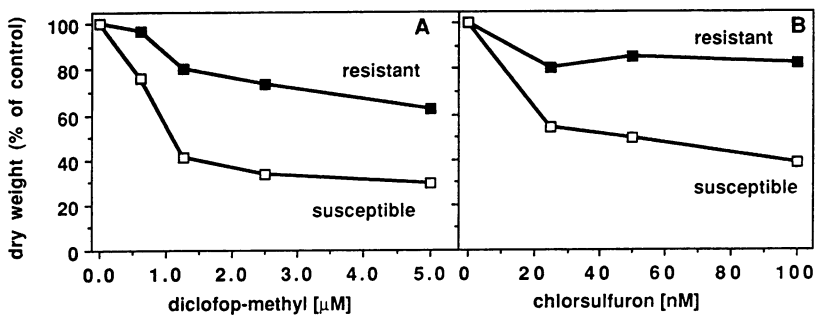

Figure 2. Dry weights of susceptible (□) and resistant (■) biotypes of *L. rigidum*, grown in hydroponic culture, following exposure to diclofop-methyl (A) or chlorsulfuron (B). The herbicides were added to the culture solution when plants were at the two-leaf stage. The plants were harvested 7 d subsequently. For the diclofop-methyl experiment each point is the mean dry weight of 36 plants, for the chlorsulfuron experiment each point is the mean dry weight of 28 plants.

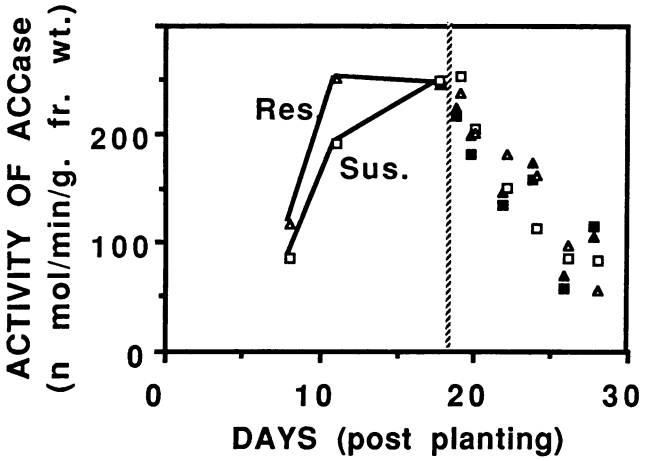

Figure 3. The activities of ACCase in crude extracts from susceptible (□, ■) and resistant (△, ▲) ryegrass during a 28 d developmental sequence. *Open symbols* are values for untreated plants, *closed symbols* are values for plants exposed to 500 nM diclofop-methyl at d 18 postemergence when plants were at the two-leaf stage. Day 18 postemergence is indicated by the *vertical, hatched line*.

ACCase from *L. rigidum*

It is conceivable that resistance against the aryloxyphenoxypropionate and cyclohexanedione herbicides is endowed by increased expression of the target enzyme ACCase. In susceptible and resistant biotypes of *Lolium* grown in half-strength Hoagland nutrient solution ACCase activity, defined as acetyl-CoA-, ATP-, and undenatured extract-dependent incorporation of ^{14}C from $H^{14}CO_3^-$, was detected in the plant tops which included the meristematic shoot region. The activity detected in the root extracts was extremely low and at the limits of detection of our assay system. The activities of ACCase extracted from the differentiating meristematic shoot region of two-leaved susceptible and resistant plants were similar at around 250 and 240 nmoles min^{-1} (g fresh weight)$^{-1}$, respectively (see also Fig. 3). A comparable activity of 199 nmol min^{-1} (g fresh weight)$^{-1}$ was observed for the enzyme extracted from wheat grown under an identical growth regime. The activities for wheat are comparable to those observed by others (9). Resistance within *L. rigidum* biotype SR4/84 to the aryloxyphenoxypropionate and cyclohexanedione herbicides is therefore not due to the increased expression of herbicidally sensitive ACCase.

The affinity of ACCase from the two biotypes for the substrate acetyl CoA was similar. Fifty percent enzyme activity was observed in the presence of 39 μM and 46 μM acetyl CoA in extracts from susceptible and resistant plants, respectively.

Inhibition of ACCase by Herbicides

ACCase from both biotypes was inhibited by low concentrations of all aryloxyphenoxypropionate and cyclohexanedione herbicides tested (Table I). The enzyme from both biotypes was not inhibited by the sulfonylurea herbicide chlorsulfuron nor by the dinitroaniline herbicide trifluralin although the susceptible biotype succumbs to the latter two herbicides (10). If observed, differences in the concentrations of herbicide required for 50% inhibition (I_{50}) of ACCase from the biotypes were small. We conclude that, in *L. rigidum* biotype SR4/84, the resistance to diclofop and to other aryloxyphenoxypropionate and cyclohexanedione herbicides is not invested in herbicide-insensitive ACCase.

ACCase from Species Tolerant to Diclofop-Methyl

The extent of inhibition of ACCase from crude extracts of *L. rigidum* by a range of ACCase-specific herbicides is species-dependent (Table I). With the exception of tralkoxydim, the herbicide inhibition kinetics of the enzymes from susceptible and resistant ryegrass are similar to those for the enzyme from wheat, a monocot species that is tolerant to both diclofop and tralkoxydim but which is susceptible to haloxyfop, fluazifop,

Table I. *Concentrations of Herbicides from Four Structural Classes Required to Inhibit by 50% ACCase from Susceptible and Resistant L. rigidum, T. aestivum cv Millewa, P. sativum cv Maori Dwarf, and V. faba cv Fiord*

Herbicides	*L. rigidum*		*T. aestivum*	*P. sativum*	*V. faba*
	Susceptible	Resistant			
			[μM]		
Aryloxyphenoxypropionates					
Diclofop-methyl	0.6 ± 0.13[a]	1.4 ± 0.34	0.3	3.0	5.9
Diclofop acid	0.2 ± 0.01	0.3 ± 0.07	1.1	4.3	>10
Fluazifop acid	0.6	1.75	2.0	>10	>10
Haloxyfop acid	0.4 ± 0.07	0.7 ± 0.1	4.2	>10	>10
Cyclohexanediones					
Sethoxydim	2.7 ± 0.6	2.5 ± 0.22	1.0	>10	6.5
Tralkoxydim	0.3 ± 0.09	0.4 ± 0.11	4.2	>10	>10
Dinitroaniline					
Trifluralin	>10	>10	—[b]	—	—
Sulfonylurea					
Chlorsulfuron	>10	>10	—	—	—

[a] Errors are SE of mean of three experiments. Other values are means of two experiments. [b] Not determined.

and sethoxydim. In comparison to ACCase from the monocots wheat and ryegrass, ACCase from the broad-leaved species *Pisum* and *Vicia* is less sensitive to inhibition by the aryloxyphenoxypropionates and cyclohexanediones. It has been proposed that selective graminicidal action of the aryloxyphenoxypropionate and cyclohexanedione herbicides is a direct result of the differential sensitivities of ACCase from monocot and broadleaved plants to these herbicides (2, 13, 23, 24).

Although there are large differences in the susceptibility of ACCase from different species to various herbicides, it is not yet clear whether these are the only differences in the capacity of the monocots and dicots studied to tolerate these herbicides. In the case of diclofop, many dicots have substantial capacities to detoxify the herbicide (13, 26) and there is evidence for mechanisms which negate the protonophoric activities known for a range of the aryloxyphenoxypropionate and cyclohexanedione compounds (8, 26, 27, 30). Tolerance of wheat to diclofop is thought to be due to the capacity of this species to detoxify the herbicide via a mixed-function oxidase (15, 26). Soybean, despite the possession of a relatively insensitive ACCase, also has a high capacity to detoxify diclofop-methyl (13, 26). The mechanism of tolerance in wheat to the cyclohexanedione tralkoxydim has not been reported.

It should be noted that resistance in *L. rigidum* to diclofop-methyl has probably developed *in situ* at most of the 50+ sites from which resistance has been documented. It is possible therefore that the mechanism of resistance will not be the same for all populations. Such a phenomenon, although uncommon, is not unknown. For example, resistance to sethoxydim in maize cell cultures has been ascribed to a sethoxydim-insensitive ACCase (20) whereas tolerance to diclofop-methyl in whole maize plants is inherited in a polygenic manner which suggests the involvement of more than one resistance mechanism (6). Resistance to sulfonylurea herbicides in different maize lines is due in some cases to insensitive ALS whereas in other cases ALS remains sensitive; in the latter cases increased detoxification of the herbicide is the most likely mechanism of resistance (5, 25). In contrast to the population of *L. rigidum* under study here, resistance in a diclofop-methyl insensitive sethoxydim-sensitive accession of the closely related species *L. multiflorum,* Italian ryegrass, has been ascribed to the diclofop-insensitive, sethoxydim-sensitive properties of ACCase (7, 28). To our knowledge, *L. multiflorum* does not exhibit crossresistance to the sulfonylurea herbicides or to trifluralin.

ACCase Activity during a Developmental Sequence

It is possible that resistance may be manifest only during a particular developmental stage or that it may be induced following the exposure of plants to a herbicide. In both resistant and susceptible ryegrass biotypes the extractable activity of ACCase in the 2 to 3 cm of tissue above the zone of root initiation changes with age (Fig. 3). Activity increases until about 10 d after planting and decreases after about 20 d post planting. The plants are at the one- to two-leaf stage, the stage at which they are normally sprayed in the field, between d 10 to 20 and tillering begins at around d 25. The total activity of ACCase from susceptible and resistant tissue at comparable stages of development, whether expressed on a fresh weight basis as in Figure 3 or on a protein basis, was not significantly different at any time during the first 30 d post planting. The variation in the extractable activity of ACCase during a developmental sequence is very similar to that observed for wheat (9) and presumably reflects the demands imposed by the high rates of fatty acid and lipid synthesis in differentiating, developing tissue.

The addition of 500 nM diclofop-methyl to the nutrient solution at d 18 after planting did not affect the extractable activities of ACCase from either biotype during the next 9 d (Fig. 3). During this period the sensitivity to diclofop acid of ACCase from treated and untreated susceptible and resistant biotypes did not change and was not different to that shown in Table I. It is concluded that in *L. rigidum* biotype SR4/84 resistance to the aryloxyphenoxypropionate herbicides and cross-resistance to the cyclohexanedione herbicides does not reflect the herbicide- or development-induced synthesis or expression of a herbicide-insensitive form of ACCase.

ALS from *L. rigidum*

Biotype SR4/84 is resistant to chlorsulfuron (Figs. 1 and 2) and a number of other sulfonylurea and imidazolinone herbicides (4, 10), a target site of which is the enzyme ALS (1). The extractable activities of ALS from two-leaved susceptible and resistant ryegrass which had not been exposed to chlorsulfuron were similar at 1.1 ± 0.14 and 1.2 ± 0.07 nmol acetolactate formed mg^{-1} protein min^{-1}. The sensitivities of ALS to inhibition by chlorsulfuron were also similar with I_{50} values of about 4 nm (Fig. 4). We therefore conclude that resistance to the sulfonylurea herbicide chlorsulfuron does not result from any intrinsic difference in the expression or herbicide sensitivity of ALS between susceptible and resistant biotypes.

ALS from *L. rigidum* Exposed to Chlorsulfuron

In order to test whether changes in the expression or herbicide sensitivity of ALS are induced following the exposure

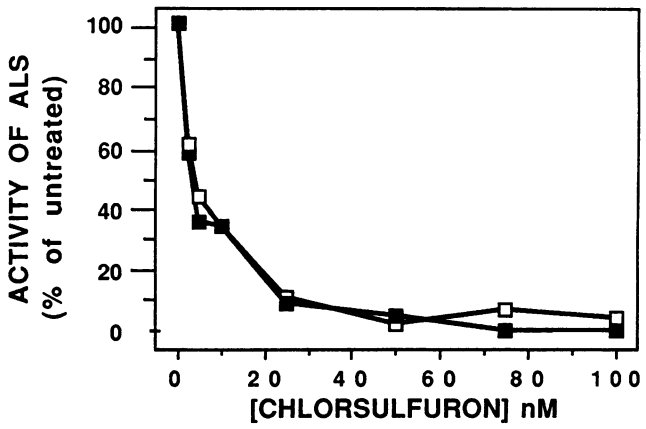

Figure 4. Chlorsulfuron inhibition of ALS from two-leaved *L. rigidum* plants susceptible (□) or resistant (■) to chlorsulfuron. For susceptible and resistant plants 100% ALS activity was 1.6 and 1.8 nmol mg^{-1} protein min^{-1}, respectively.

to chlorsulfuron, 100 nM chlorsulfuron was added to the nutrient solution for 7 d (Fig. 5). The extractable activity of ALS from both biotypes was less than that of the untreated controls during the first 4 d. The activity in the treated susceptible plants remained low throughout the experimental period. In contrast, the reduction in activity of ALS from the resistant biotypes was a transient phenomenon; activity recovered such that after 5 d the activities of ALS from treated and untreated resistant plants were comparable. After 7 d exposure, the average fresh weights of the resistant and susceptible plants were 78% and 40% respectively, of those estimated for the untreated controls.

The inhibition kinetics of ALS from susceptible and resistant biotypes following exposure to 100 nM chlorsulfuron for 2 d are similar to one another and similar to those for ALS from untreated plants, with 50% inhibition occurring at a herbicide concentration of 12 nM (Fig. 6). The differences in chlorsulfuron I_{50} values of 4 nM and 12 nM for ALS from untreated and treated plants (Figs. 4 and 6) are unlikely to be of importance to the resistance response because, apart from being small, the I_{50} values were similar for ALS from both the susceptible and resistant plants. We conclude that resistance to chlorsulfuron in biotype SR4/84 is not due to differences in either the expression or chlorsulfuron-sensitivity of ALS induced following exposure to the herbicide.

If resistance to chlorsulfuron is not due to the expression of chlorsulfuron tolerant ALS then what is the mechanism of resistance? At least three interpretations for the differential responses of ALS activity from the two biotypes to the exposure of chlorsulfuron are possible. The decrease in the activity

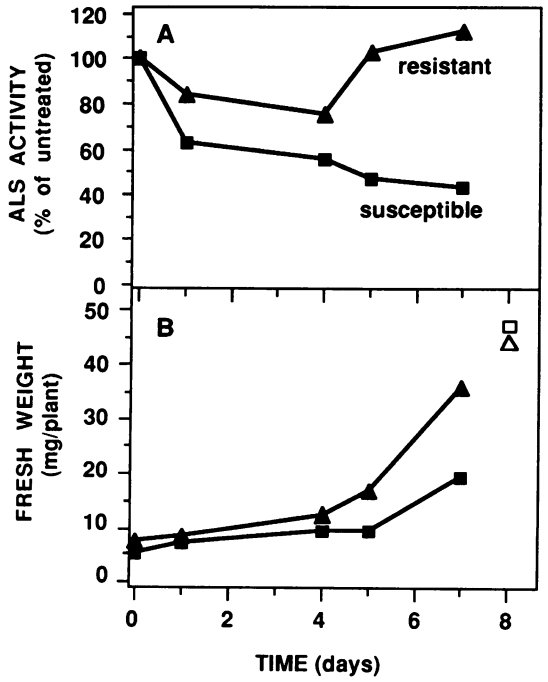

Figure 5. The activities of ALS in crude extracts (A) and the whole-plant fresh weights (B) of two-leaved susceptible (■) or resistant (▲) *L. rigidum* plants. Plants were untreated (□, △) or exposed continuously to 100 nM chlorsulfuron for 7 d (■, ▲).

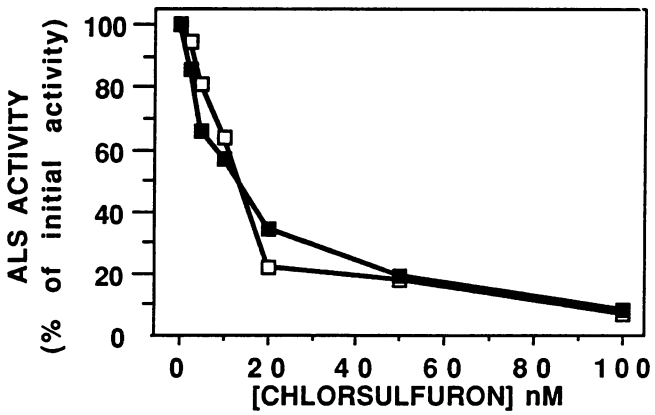

Figure 6. Chlorsulfuron inhibition of ALS from two-leaved *L. rigidum* plants, susceptible (□) or resistant (■) to chlorsulfuron, exposed to 100 nM chlorsulfuron for 24 h. For susceptible and resistant plants 100% ALS activity was 1.0 and 0.6 nmol mg^{-1} protein min^{-1}, respectively.

of the enzyme from susceptible tissue could be due to larger amounts of the herbicide entering the susceptible biotype and irreversibly inhibiting the enzyme. Alternatively, similar amounts of the herbicide could have entered both biotypes but the resistant biotype may be capable of more rapidly replacing ALS irreversibly inhibited by the herbicide. A third possibility is that equal amounts of the herbicide entered both biotypes but the resistant biotype has a greater capacity to metabolise the herbicide to a metabolically inactive form. Certainly the observation that the resistant biotype has the capacity to recover from the effects of the exposure to chlorsulfuron suggests that the herbicide is removed from the vicinity of the enzyme *in vivo*. Removal could be physical, as in transport, or chemical, as in detoxification. Preliminary experiments with ^{14}C-labeled chlorsulfuron support the possibility that the biotypes differ in the ability to oxidise chlorsulfuron to a biologically less active catabolite (4).

CONCLUSIONS

Multiple resistance against the aryloxyphenoxypropionate and cyclohexanedione graminicides in *L. rigidum* biotype SR4/84 is not due to a biotypical difference in the amount or the kinetic characteristics of the enzyme ACCase, an enzyme inhibited by aryloxyphenoxypropionate and cyclohexanedione herbicides. Cross-resistance to the sulfonylurea herbicides is not due to changes in the sensitivity or expression of the target enzyme ALS. Moreover, the characteristics of ACCase and ALS from susceptible and resistant *L. rigidum* are not dissimilar to the enzymes from other grass species which are either susceptible to the relavent herbicides or which exhibit tolerance purported to be based upon herbicide metabolism and detoxification (1, 22). It is therefore likely that the mechanism(s) of resistance against the aryloxyphenoxypropionate, cyclohexanedione and sulfonylurea herbicides in *L. rigidum* may involve changes in the sensitivities of as yet undescribed target sites (*cf.* refs. 8 and 26), the physical separation of the herbicides from their respective target sites and/or the detoxification of the biocidal compounds (4).

ACKNOWLEDGMENT

Partial support for this research was provided by a grant to J.A.M.H. and S.B.P. from the Australian Wheat Research Council.

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