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Mechanisms of resistance to herbicides which inhibit acetolactate synthase in annual ryegrass (*Lolium rigidum*)

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Abstract

There are at least two mechanisms of resistance to ALS inhibitors in *L.rigidum* populations. Biotype SLR31 is cross-resistant to some crop-selective ALS inhibitors but is not cross-resistant to non-selective ALS inhibitors. The population has herbicide-sensitive ALS but excised seedlings of SLR31 metabolised [^{14}C]chlorsulfuron at approximately twice the rate of a susceptible biotype in the tissue closest to the meristems. Population WLR1 is resistant to crop-selective and non-selective ALS inhibitors and has ALS which is less-sensitive to inhibition by both crop-selective and non-selective herbicides. Thus, herbicide-insensitive ALS may cause resistance to both selective and non-selective sulfonylurea and imidazolinone herbicides in *L.rigidum*. A second resistance mechanism exists, however, which dose not cause resistance to non-selective ALS inhibitors and involves increased chlorsulfuron metabolism.

The major metabolite of [^{14}C]chorsulfuron in *L.rigidum* coeluted with that of wheat suggesting that the major pathway of metabolism in *L.rigidum* may be the same as that in wheat. Similar HPLC elution profiles for resistant and susceptible *L.rigidum* biotypes suggest that the pathway of metabolism is the same in all biotypes. There were no differences in root or leaf uptake between intact resistant and susceptible plants which could explain resistance.

Some mixed function oxidase inhibitors slightly increase chlorsulfuron phytotoxicity toward biotype SLR31 but combined treatment caused less damage to SLR31 than chlorsulfuorn alone applied to a susceptible biotype. Conversly, the crop safener NAA reduced the phytotoxic effect of chlorsulfuron toward wheat but not toward *L.rigidum* biotype SLR31. Darkness, after commencement of [^{14}C]chlorsulfuron treatment, caused metabolism in excised seedlings to slow dramatically. Whole SLR31 plants placed in darkness immediately following chlorsulfuorn treatment suffered increased phytotoxic effects, but damage

was less than that suffered by a susceptible biotype. These results suggest that chlorsulfuron metabolism in *L.rigidum* does not involve mixed function oxidase enzymes and/or that other resistance mechanisms may exist in population SLR31.

In population SLR31, diclofop-methyl and chlorsulfuron resistance are both nuclear encoded dominant or semi dominant characters and at least one gene controlling diclofop-methyl resistance in the absence of chlorsulfuron resistance is present. In population WLR1, sulfometuron-methyl and chlorsulfuron resistance are both nuclear encoded and, probably, dominant traits. At least one gene controlling chlorsulfuron resistance in the absence of sulfometuron-methyl resistance is present.

Different herbicide treatments in the field may lead to resistance to ALS inhibitors in populations of *L.rigidum*. Four diclofop-methyl applications may cause cross-resistance to chlorsulfuorn but diclofop-methyl treatment is unlikely to select for herbicide-insensitive ALS. Three applications of chlorsulfuron may cause chlorsulfuron resistance while five applications may select for herbicide-insensitive ALS. Chlorsulfuron appears less likely than diclofop-methyl, to select for cross-resistance to herbicides from different chemical groups with different modes of action.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made.

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J.T.Christopher

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Members of the Weed Science Research Group at the Waite Institute, December 1992, with visiting academic Dr. Rick Roush. From top left:- Jack Christopher, Hassan Alizadeh, Dr. Rick Roush, Dr. François Tardif, Dr. Steve Powles, John Matthews, Jo Richter, Peter Boutsalis, Ali Mansooji, Dr. Chris Preston, Fiona McAlister, Dr. Joe Holtum, Chanya Maneechote, Edison Purba and Dr. Linda Hall.

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Abbreviations

ABT, 1-aminobenzotriazole;

ALS, acetolactate synthase (EC 4.1.3.18)

ACCase, acetyl coenzymeA carboxylase (EC 6.4.1.2)

Amino Acids Codes

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Asn or Asp	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamate	Glu	E
Gln or Glu	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Ther	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

biteranol, 1-(biphenyl-4-yl-oxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol;

chlorotoluron, N'-(3-chloro-4-methylphenyl-N,N-dimethylurea;

chlorsulfuron, 2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide;

diclofop-methyl, (±)-methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate;

diquat, 6,7-dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium ion;

diuron, N'(3,4-dichlorophenyl)-N,N-dimethylurea;

EDTA, ethylenediaminetetaracetic acid

fluazifop-butyl (±)-butyl 2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoate;

g ai ha^{-1}, grams active ingredient per hectare;

glyphosate, *N*-(phosphonomethyl)glycine;

GR_{25}, herbicide required to reduce growth by 25% with respect to untreated plants;

haloxyfop-methyl methyl 2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoate;

I_{50}, herbicide required to reduce *in vitro* ALS activity 50% with respect to control assays minus herbicide;

imazamethabenz, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-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;

imazathepyr, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid;

LD_{50}, dose of herbicide required to kill 50% of plants;

linuron, N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea:

metribuzin, 2-[[[[(4-methoxy-6-1,3,5-trazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoic acid

metsulfuron-methyl, methyl-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate;

paraquat, 1,1'-dimethyl-4,4'-bipyridinium ion;

PBO, piperonylbutoxide, 5-[2-(-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxile;

PCR, polymerase chain reaction;

pendimethalin, N-(1-ethoxypropyl)-3,4-dimethyl-2,6-dinitrobenzinamine;

RFLP, restriction fragment length polymorphism;

sethoxydim, 2-[1-(ethoxyimino)butyl]-5-[2(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one;

simazaine, 6-chloro-N,N'-diethyl-1,3,5-trazine-2,4-diamine;

sulfometuron-methyl, 2-[[[[(4,6- dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoate;

tebuconazole, (RS)-1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol;

triadimefon, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)butanone;

tridimenol, (1RS,2RS:1RS,2SR)-1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol;

tridiphane, 2-(3,5-dichlorophenyl-2-(2,2,2-trichloroethyl)oxirane;

thifensulfuron, 3-[[[[(4-methoxy-6-methyl-1,3,5-trazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylic acid;

triasulfuron, 2-(2-chloroethoxy)-N-[[(4-methoxy-6-methyl)-1,3,5-triazin-2-yl]carboxyl]benzenesulfonamide;

trifluralin, 2,6-dinitro-N,N-dipropyl-4-(trifluromethyl)benzenamine;

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Chapter 1

Introduction

1.1 Herbicide resistance

1.1.1 The development of resistance

Xenobiotic resistance occurs when the response of a population of organisms to a xenobiotic is reduced due to selection following repeated application of the xenobiotic to the population. For the purpose of this document, the definition of herbicide resistance is; a resistant weed is one that survives and grows normally at the usually effective dose of herbicide (for that species) (LeBaron and Gressel 1982). The term "tolerance" will be used when referring to the naturally occurring ability of a species to survive a xenobiotic without prior selection by the xenobiotic. Cross-resistance will be used to denote the phenomenon whereby a population exposed to one xenobiotic compound becomes resistant to one or more compounds of different chemical structure and different mode of action. Multiple-resistance will be used to denote the phenomenon whereby a population develops resistance to several compounds from different chemical classes with different modes of action following exposure to all of those compounds (or compounds of similar structure and mode of action).

Resistance to xenobiotics has been recorded in populations of arthropods, bacteria, plant pathogens, mammals, nematodes and weeds (Georgiou 1986). The development of insecticide resistance since the introduction of synthetic insecticides in the 1940s has been, possibly, the most spectacular example (Georgiou 1986). The rapid development of herbicide resistance in weed populations has occurred more recently, but since 1980 the number of new cases of herbicide resistance reported annually has roughly equalled the number of new reports of insecticide and fungicide resistance (Georgiou 1986). By March 1992 there were herbicide resistant biotypes of 86 plant species reported. The majority of these occurred in North America and Europe (Anon 1992).

Resistance to some of the earlier and most widely used herbicide groups has become most widespread. Triazine resistance was the first type of herbicide resistance, initially reported in the 1960's, and is still the most

numerous example, with resistant biotypes of 58 species reported to March 1992 (Table 1.1). The triazines were released in 1955 (reviewed in LeBaron and Gressel 1982). Resistance to the pyridyliums, released in 1960, is the next most numerous example with resistant biotypes of 18 species reported. The number of reported cases of resistance to the substituted ureas, released in 1951, and the hormone-like phenoxyacetic acids, first released in the 1940s, also rank highly (Table 1.1). However, the time since the commercial introduction of a chemical is not generally a good predictor of the number of cases of resistance. Resistance to two of the most recently commercialised herbicide groups, acetolactate synthase (ALS) inhibitors, and the grass-selective aryloxyphenoxyalkanoates rank more highly than the much older dinitroanilines, for example (Table 1.1).

Table 1.1 Numbers of species for which herbicide resistant biotypes were reported , March 1992.

Herbicide		Number of species		
Group	Examples	Broadleaf	Grasses	Total
Triazines	(Atrazine)	41	17	58
Pyridyliums	(paraquat, difenzoquat)	12	6	18
ALS inhibitors	(sulfonylureas)	5	5	10
Substituted ureas	(chlorotoluron, diuron)	5	2	7
Phenoxys	(2,4,-D)	7	-	7
Aryloxyphenoxy alkanoates	(diclofop)	-	6	6
Dinitroanlines	(trifluralin)	1	3	4
Carbamates	(triallate)	2	1	3
Amides	(propanil)	-	2	2
Cyclohexane -diones	(sethoxydim)	-	2	2
Triazoles	(amitrole)	-	2	2
Urocils	(bromacil)	2	-	2
Acetanilides	(alachlor)	1	1	1
Arsenical	(MSMA)	1	1	1
Nitriles	(bromoxynil)	1	-	1
Picolinic acid	(picloram)	1	-	1
Pyridazinones	(pyrazon)	1	-	1

From Anon. 1992. Ciba Geigy North Carolina

Many factors affect the rate at which resistance develops. The number of susceptible plants which survive herbicide application will be dependent on the phytotoxicity of the chemical and the period for which the chemical remains active. The persistence of the weed species in the seed bank is also important. Susceptible individuals will quickly be eliminated if a herbicide with high phytotoxicity and a long period of activity is used on a weed population with limited persistence in the seed bank (reviewed in Gressel and Segel 1982, Holt and LeBaron 1990). The best documented example of this is the extensive and repeated use of triazine herbicides in of North America which has led to herbicide resistance in many weed species.

Crop-selectivty of herbicides can be due to rapid degradation of the herbicide molecule in tolerant species. If the chemical structure of a herbicide makes it susceptible to degradation by enzymes which already exist in some plant populations it is more likely that populations of initially susceptible weed species will also contain individuals which posses such enzymes. Similarly, if the target process affected by the herbicide can be easily modified while still remaining functional, this will also favour the development of resistance. These factors have probably contributed to the relatively rapid appearance of resistance to the grass-selective herbicides and the ALS inhibitors (discussed below). Clearly, the more genetic variability there is in a weed population, the more likely it will be to contain individuals with characteristics favouring survival. This is probably a major factor in the development of resistance to many different types of herbicides in, the highly variable, *L.rigidum* in Australia (Powles and Matthews 1992).

Until recent years, herbicide resistance has been of relatively minor concern to farmers. Although examples of triazine resistance were numerous and widespread in North America, the weeds could be easily controlled using alternative herbicides at a reasonable cost (LeBaron and McFarland 1989). Biotypes resistant to other herbicide groups have also, in general, been easily controlled by alternative herbicides and have been much

less widespread. In contrast, the rapid development of resistance to the more recently introduced grass-selective herbicides and ALS inhibitors has become a major management problem for farmers and chemical manufacturers (Holt and LeBaron 1990, Powles and Matthews 1992).

Resistance to the aryloxyphenoxypropionate herbicide diclofop-methyl is the most serious and widespread example of resistance to the grass-selective aryloxyphenoxypropionate and cyclohexanedione herbicides. Since 1982, hundreds of populations of *L.rigidum* in Australia have developed resistance to diclofop-methyl (Heap and Knight 1990). Many of these populations have cross-resistance and multiple resistance to herbicides with different modes of action. Populations of *Alopecurus myosuroides* in Britain, which became resistant to the photsystem II inhibitor chlorotoluron, have also developed cross-resistance to diclofop-methyl (Moss 1990). Populations of Italian ryegrass (*Lolium multiflorum*) resistant to diclofop-methyl have arisen in Washington state (Gronwald *et al.* 1989). More recently populations of wild oats in Canada and Australia have developed resistance to aryloxyphenoxypropionate and cyclohexanedione herbicides (Joseph *et al.* 1990, Mansooji *et al.* 1992). The number of reports of resistance in wild oats in Canada and in wild oats and *L.rigidum* in Australia are expected to increase in coming years (Morrison *et al.* 1992, Powles and Matthews 1992).

Resistance to herbicides which inhibit ALS has become widespread in parts of North America and Australia In North America this development has led to changes in label recommendations for certain herbicides, including the withdrawl of some of the more persistant sulfonylureas (McKinley 1990). The development of resistance to ALS inhibitors is discussed in section 1.3.5.

From being unknown thirty years ago herbicide resistance has become a widespread and rapidly increasing problem to farmers. One particularly concerning aspect of the development of resistance has been the rapid occurence of resistance to the recently introduced, selective herbicide

groups. The development of resistance in Australia in some ways reflects the experience in other countries but the level of multiple resistance and cross-resistance in populations or *L.rigidum* gives added complexity to the situation.

1.1.3 Herbicide resistance in Australia

Herbicide resistance has occurred in populations of seven weed species in Australia. Biotypes with resistance to nine major chemical groups of herbicides with at least six different sites of action are known.

Several populations of *Hordeum glaucum* have developed resistance to the pyridilium herbicides paraquat and diquat (Powles 1986). In one particular field three other species with resistance to these herbicides have also been detected, *Hordeum leporinum*, *Arctotheca calendula* and *Vulpia bromoides* (Table 1.2) (Purba *et al.* 1993). The populations are easily controlled by alternative herbicides.

Resistance to the aryloxyphenoxypropionate herbicides and the cyclohexanedione herbicides in the wild oat species *Avena sterilis* and *Avena fatua* have the potential to become a much greater management problem. Herbicide resistance has so far been confirmed in only a few populations of wild oats in Australia (Mansooji *et al.* 1992) but experience in Canada suggests that the number of cases could increase rapidly in coming years (Morrison *et al.* 1992).

Herbicide resistance in one species, *Lolium rigidum*, presents the biggest management problem to farmers and is by far the most widespread example of herbicide resistance in Australia. The number of farms in cereal growing regions with herbicide-resistant ryegrass has been estimated to be approaching 3000 (R.Davis DuPont Aust. Ltd. pers. comm.). In Western Australia, the largest state, 10% of cereal farms may be affected (R.Davis DuPont Aust. Ltd. pers. comm.). The ability of this species to develop resistance and cross-resistance to herbicides of distinct chemical classes with different modes of action is not matched by any other species. By 1989 populations of *L.rigidum* had developed resistance to herbicides from 8

distinct chemical groups which have 6 different sites of action (Table 1.2). The spectrum of resistance to herbicides from different chemical groups varies greatly between populations (Heap and Knight 1990). Biotypes resistant to nearly all selective herbicides used for *L.rigidum* control in Australia have been reported.

Table 1.2 Herbicide resistance in Australia

Herbicide class* (site of action)	Species	Reference
Bipyridiliums (photosystem I)	*Hordeum leporinum*	Tucker and Powles 1991
	Hordeum glaucum	Powles 1986
	Vulpia bromoides	Purba *et al.* 1993
	Arctotheca calendula	Powles *et al.* 1989
Dinitroanalines (microtubule formation)	*L.rigidum*	Heap and Knight 1990
Aryloxyphenoxy-propionates (ACCase**)	*L.rigidum*	Heap and Knight 1986
	Avena sterilis	Mansooji *et al.* 1993
	Avena fatua	Mansooji *et al.* 1993
Cyclohexanediones (ACCase)	*L.rigidum*	Heap and Knight 1990
Sulfonylureas (ALS**)	*L.rigidum*	Heap and Knight 1986
Phenylureas (photosystem II)	*L.rigidum*	Burnet *et al.* 1991
Triazines (photosystem II)	*L.rigidum*	Burnet *et al.* 1991
Triazinones (photosystem II)	*L.rigidum*	Burnet *et al.* 1991
Triazoles (pigment biosynthesis)	*L.rigidum*	Burnet *et al.* 1991

* Populations of the species have been confirmed resistant to at least one herbicide within the class. (Sites of action discussed in section 1.2)

**ALS= acetolactate synthase, ACCase= acetylcoenzyme A carboxylase

1.2 Mechanisms of herbicide resistance

1.2.1 Potential mechanisms of herbicide resistance

A herbicide must be able to affect some vital target process within a plant to a sufficient degree, and for a sufficient time, to kill the plant. To achieve this, the herbicide must enter the plant, reach the site of action within the plant, remaining in the active form, at a high enough concentration, for a sufficient period of time. Clearly, when exposed to selection pressure by herbicides, plants might evolve changes in any number of processes which could lead to resistance.

Behavioural adaptations may also lead to reduced herbicide efficacy by avoidance of the herbicide. For example, delayed or staggered germination would allow annuals to avoid non-residual herbicides. Plants may have changes to the leaf cuticle or trichomes which reduce herbicide uptake or, may limit herbicide entry via the roots. The herbicide could be prevented from reaching the target tissue by changes in translocation. Once in the target tissue, the herbicide may be prevented from entering the cell by binding to the cell wall or exclusion at the plasmalemma. It may then be excluded from the target site, within the cell, by exclusion from some particular cell compartment or organelle, or by sequestration in the vacuole. The herbicide may be metabolised within the cell to a herbicidally inactive product. Conversely, some compounds require conversion within the plant to become herbicidally active. Changes reducing the rate of activation could reduce the activity of such a compound. Changes at the target site may reduce the effect of a herbicide if it reaches the target site intact. Where the herbicidal effect is caused by binding with an enzyme, a mutation of the gene encoding the enzyme protein could alter the affinity for the herbicide. The damage caused by a herbicide may result from the build up of toxic agents as a result of the interaction with the target process. Thus mechanisms to negate the affect of such toxic agents could also provide resistance. Examples of herbicide resistance due to many of these possible causes have been documented.

1.2.2 Resistance to inhibitors of photosystem II.

The triazine and substituted urea herbicides act by interacting with the Q_B-binding protein of photosystem II blocking the flow of electrons between the electron acceptors Q_A and Q_B (VanRensen 1989). Most cases of triazine resistance are due to reduced herbicide binding at the Q_B-binding protein (Pfister and Arntzen 1979) but the resistant biotypes do not exhibit resistance to substituted ureas which also bind to the Q_B-binding protein (Table 1.3). The mutation causes a ten fold decrease in the rate of electron flow from Q_A to Q_B which is thought to be involved in lowering the rate of photosynthesis in some resistant biotypes under certain conditions, leading to their comparative lack of fitness in the field (VanRensen 1989).

Studies of the molecular genetics of herbicide-resistant mutants of *Clamydomonas reinhardtii, Euglina gracilis, Anacysitis hybridus, Anacyistus nidulans, Solanum nigrum and Brassica campestris* have revealed that mutations occurring at five sites of the psbA gene can cause herbicide resistance (Table 1.3) (Mazur and Falco 1989). Most of the mutations occur at serine 264 which is the site of the mutation in resistant weeds. Mutations at four other sites in the proteins from algae and cyanobacteria cause various levels of resistance to triazine herbicides as well as varying levels of cross-resistance to structurally distinct substituted ureas, phenol-type herbicides and metribuzin (Table 1.3). Analysis of mutations, along with X-ray crystallographic data have been used in development of a model of the herbicide binding site of the Q_B-binding protein. The mutations are all at the stroma side of helices 4 and 5 of the Q_B-binding protein (Trebst and Draber 1986). The site of binding of both the triazine and phenylurea herbicides are partially overlapping while the phenol-type herbicide binding site overlaps the phenylurea herbicide binding site but not that of the triazines (VanRensen 1989, Trebst 1986). The binding site of phenol-type herbicides also overlaps the site of binding of quinone type inhibitors (Trebst and Draber 1986). Thus, structurally dissimilar compounds can affect plants in a similar manner by binding to partially overlapping sites of the

same protein. The finding that the quinone binding site of a plant protein may bind compounds of dissimilar structure suggests that such sites are more likely to be identified in herbicide screening programs. Studies of the interaction of herbicides with the enzyme acetolactate synthase have supported this hypothesis (section 1.2.2).

Table 1.3. Q_B-binding protein mutations causing herbicide resistance

Amino acid change	Species	Relative resistance			
		Atrazine	Metribuzin	Diuron	Bromoxynil
219 Val → Ilue	*C.reinhardtii*	2	15	1	1
251 Ala → Val	*C.reinhardtii*	25	1000	5	
255 Phe → Tyr	*C.reinhardtii*	15		0.5	1
264 Ser → Gly	*A.hybridus*	1000	200	1	
→ Gly	*S.nigrum*	1000		1	20
→ Gly	*B.campestris*	600	50	1	
→ Ala	*C.reinhardtii*	100		10	
→ Ala	*E.gracilis*				
→ Ala	*A.nidulans*	17	5000	150	300
275 Leu → Phe	*C.reinhardtii*			5	4
264 Ser → Ala 255 Phe → Tyr	*A.nidulans*	360	200	300	320
264 Ser → Ala 255 Phe → Leu	*A.nindulans*	2.5	175	2650	35

Mazur and Falco 1989

Triazine resistance may also be caused by increased metabolism of the herbicide. Increased metabolism of atrazine has been demonstrated in forage grasses (Weimer *et al.* 1988) and velvetleaf (*Abutilon theophrasti*) (Anderson and Gronwald 1991). In resistant velvetleaf, metabolism is enhanced *via* conjugation to glutathione (Anderson and Gronwald 1991).

In *L.rigidum* biotypes triazine resistance can also involve increased detoxification (Burnet 1991, Burnet 1993b). *L.rigidum* biotypes WLR2 and VLR69 are resistant to triazine herbicides but photosystem II of

thylakoids isolated from these plants remains sensitive to herbicides. Differences between these biotypes and a sensitive *L.rigidum* biotype in uptake and translocation of the triazine herbicide, simazine, were not sufficient to explain resistance. The resistant biotypes have the ability to detoxify simazine at twice the rate of a susceptible biotype. When the mixed function oxidase inhibitor aminobenzotriazole was applied to the roots of resistant plants it increased the phytotoxic effects of simazine toward the plants and also decreased the rate of metabolism of the herbicide. Increased metabolism by a mixed function oxidase system, catalysing dealkylation of simazine is suggested as the mechanism of resistance in both these *L.rigidum* populations (Burnet 1993b).

Substituted urea resistance due to mutation of the Q_B-binding protein has not been demonstrated in higher plants although mutant enzymes resistant to diuron have been isolated from other organisms (Table 1.3). Resistance to linuron resulting from reduced translocation has been reported in carrot (*Daucus carota* L.) and parsnip (*Pasinaca sativa* L.) (Lawrence 1987). Increased metabolism of chlorotoluron involving arylhydroxylation, mediated by a mixed function oxidase system has been demonstrated in microsomal preparations from wheat (*Triticum aestivum*) (Cabanne *et al.* 1987, FonnePfister and Kreuz 1990). Mixed function oxidase mediated detoxification is also suspected in chlorotoluron resistant *L.rigidum* biotypes WLR2 and VLR69 (Burnet 1991, Burnet 1993b). Increased metabolism of chlorotoluron via a mixed function oxidase system has also been suggested for chlorotoluron resistant biotypes of *Alopecurus myosyroides* (Moss 1990)

1.2.3 Resistance to herbicides interacting with photosystem I

The bipyridylium herbicides paraquat and diquat are the most commonly used herbicides which interact with photosystem I (Dodge 1989). These herbicides do not directly interact with a specific protein, instead they act by capturing electrons form the iron sulfur centres A/B which would normally be passed to ferredoxin (Dodge 1989). Reduction of bipyridilium molecules causes the formation of radical cations which can react with molecular oxygen to form the superoxide anion. The bipyridylium cation is reoxidised in the process, acting as a cyclic catalyst. Superoxide then reacts with hydrogen peroxide to form highly destructive hydroxyl radicals in a redox reaction catalysed by iron. Superoxide, hydrogen peroxide and hydroxyl radicals are normally scavenged within the plant by superoxide dismutase and the ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase system (Dodge 1989).

The mechanism(s) of resistance to pyridylium herbicides are not fully understood. Increased activity of the scavenging enzymes has been proposed as the mechanism for resistance in *Conysa bonariensis* (Shaaltiel and Gressel 1986) although other workers have failed to confirm this (Vaughn and Fuerst pers. comm.). Increased scavenging enzyme systems were also postulated to be responsible for a decrease in photoinhibition (Jansen *et al.* 1989) although, again, this has been disputed (Preston *et al.* 1991). Decreased translocation of the herbicide through the plant and decreased transport across the plasmalemma have been suggested as possible mechanism of paraquat resistance in other weed biotypes and in methylviologen resistant biotypes of bacteria (Preston *et al.* 1992 and 1993, Morimyo *et al.* 1992).

Although several weed species resistant to bipyridilium herbicides are known in Australia there have been no reports of resistance in *L.rigidum.* These herbicides remain useful tools for controlling populations of *L.rigidum* which have developed resistance to herbicides of other chemical groups.

1.2.4 Resistance to herbicides which inhibit lipid synthesis.

Carbamothioate, chloroacetamide and pyridazolinone herbicides inhibit lipid biosynthesis in plants at various points in the pathway but also affect other processes within the plant. The relative importance of their effect on lipid synthesis is as yet unclear (reviewed in Gronwald 1991).

In contrast, there is clear evidence that the most important mode of action for the aryloxyphenoxyalkanoate and cyclohexanedione herbicides is inhibition of the plastidic enzyme acetyl coenzymeA carboxylase (ACC, E.C.6.4.1.2) (reviewed in Gronwald 1991). This enzyme catalyses the formation of malonyl coenzymeo A from acetyl coenzyme A and bicarbonate, an important step in lipid biosynthesis and other important synthetic processes (Secor *et al.* 1989). There is also evidence that a second mode of action leading to irreversable depolarisation of membranes may be important in some cases (Shimabukuro 1990b, Hausler *et al.* 1991).

The aryloxyphenoxypropionate herbicide, diclofop-methyl enters the plant in the form of the methyl ester which is hydrolysed to the herbicidally active diclofop-acid by esterases within the plant. Diclofop-methyl is selective in wheat because wheat has the ability to rapidly detoxify the herbicide by arylhydroxylation mediated by mixed function oxidase system(s) (Gorecka *et al.* 1981). In susceptible wild oats diclofop is converted to the neutral glycoester which can be hydrolysed back to the active form (Shimabukuro *et al.* 1979).

Diclofop-methyl resistance in *L.multiflorum* is due to a mutant form of ACCase which is less sensitive to inhibition by the herbicide (Burton et al 1991). The trait was found to be inherited as a single semidominant character (Betts *et al.* 1992). The mechanism of diclofop-methyl resistance in *Alopecurus* remains to be elucidated but detoxification involving mixed function oxidase enzymes is suspected (Moss 1990). Diclofop-methyl resistance in wild oat populations involves herbicide insensitive ACCase in some cases (Mansooji *et al.* 1992) but a diclofop resistant biotype from Canada with a herbicide sensitive ACCase has been described (Devine 1992).

Resistance in this biotype is correlated with rapid recovery in membrane potential (Devine 1992).

Diclofop-methyl resistance in resistant *L.rigidum* biotype SLR3 is due to an insensitive form of ACCase (Tardif 1992). The mechanism of diclofop resistance in *L.rigidum* biotypes with herbicide-sensitive ACCase remain to be elucidated (see section 1.4).

Mechanisms of herbicide resistance can, therefore, be many and varied. There may be more than one mechanism of resistance to herbicides of a given chemical class. Different mechanisms of resistance to a single compound may occur in different species or in different populations of a single species.

Populations of *L.rigidum* are able to develop resistance to chemicals of many different classes with different modes of action. Target-site mutations may lead to resistance, as in diclofop-methyl resistant biotypes WLR96 and SLR3 (Tardif 1992, Holtum pers. comm.). Increased metabolism may occur, as in the substituted urea and triazine resistant biotype WLR2 (Burnet 1993a, Burnet 1993b). Other mechanisms of resistance exist in populations which are diclofop-methyl resistance but have herbicide sensitive ACCase. It is likely that further mechanisms, causing resistance to other groups of herbicides, remain to be identified.

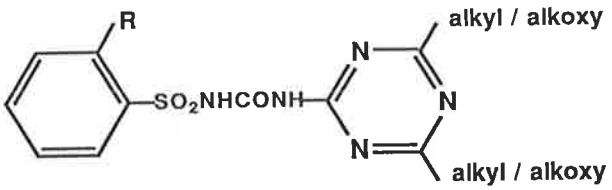
1.3 Plant interactions with herbicides which inhibit acetolactate synthase (ALS)

1.3.1 Herbicides which inhibit acetolactate synthase

Several chemically distinct groups of herbicides are known to inhibit the first key enzyme in the synthesis of the branched chain amino acids in plants, acetolactate synthase (ALS). The most important groups are sulfonylureas and imidazolinones (Ray 1984, Shaner *et al.* 1984). Several other chemical classes have more recently become available or are being investigated. The triazolopyrimidine sulfonanilides (Gerwick *et al.* 1990, Kleschick et al. 1992, Subramanian et al. 1989) and pyridimidyl-oxybenzenoic acid (Hawkes 1989), and vinyl sulfonylureas (McFadden and Huppatz 1992) are structurally related to the sulfonylureas. The sulfonylcarboxamide (Crews *et al.* 1989), and phthalylvaline anilides (Huppatz and Casida 1985) are structurally related to the imidazolinones (reviewed in Stidham 1991) (Figure 1.1).

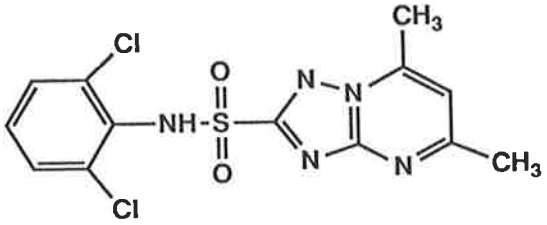
Sulfonylureas consist of an aryl group linked to a nitrogen-containing heterocycle by a sulfonylurea bridge (Figure 1.1). Ortho substituents, except for carboxyl and hydroxyl groups, favour herbicidal activity in compounds where a phenyl group is the aryl component. Other aryl groups also give activity including thiophene (as in thifensulfuron), furan, pyridine and naphthalene (Beyer *et al.* 1988). Different heterocyclic nitrogen-containing substituents give activity although symmetrical triazine and pyrimidine rings with alky substituents have the highest activity (Beyer *et al.* 1988). These compounds do not show any obvious structural similarity with any of the cofactors, intermediates or end products of the ALS reaction. The first member of this group to be commercialised was chlorsulfuron, introduced by DuPont in 1982. Presently at least 16 sulfonylurea herbicides produced by a number of companies are registered for use in a variety of crops (Hartley and Kidd 1983).

Generalised sulfonylurea

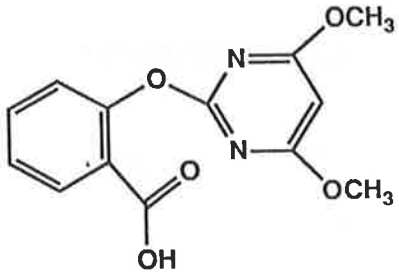


Aryl | Sulfonylurea bridge | Heterocycle

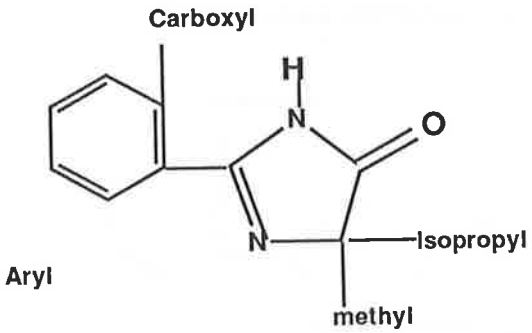
Triazolopyrimidine



Pyrimidyl-oxybenzoic acid

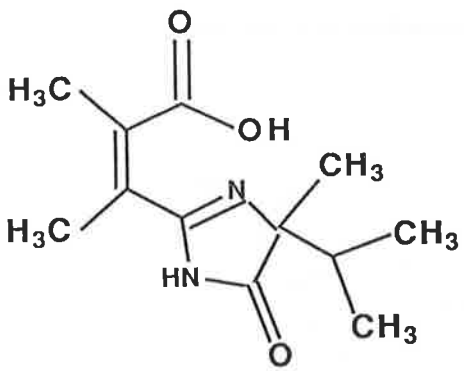


Generalised imidazolinone



Imidazolinone ring

Non-aromatic imidazolinone



Sulfonylcarboximide

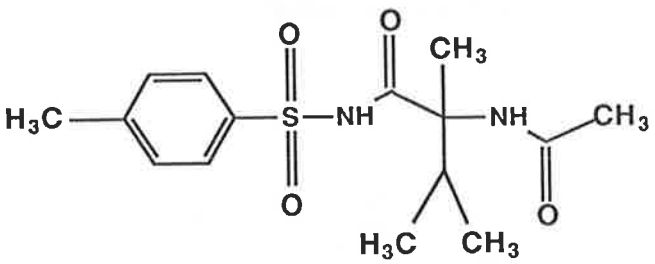


Figure 1.1 Chemical structures of herbicides which inhibit acetolactate synthase. (Beyer 1988, Los 1986, Stidham 1991)

The structure of the imidazolinones is quite distinct from that of the sulfonylureas. The basic features required for herbicidal activity are an imidazoline ring substituted by a methyl and an isopropyl group, attached to an aromatic ring with an ortho carboxyl substituent (Figure 1.1) (Los 1986). The imidazolinones show structural similarity to valine, a feedback inhibitor of ALS (Huppatz and Casida 1985, Brown *et al.* 1987).

Since the discovery of these two basic structures many different derivatives of each have been shown to have herbicidal activity. Compounds which only loosely resemble the basic sulfonylurea structure such as the triazolopyrimidines and pyrimidyl-oxy-benzoic acid show activity (Fig. 1.1) (Stidham 1991). Similarly widely-varying structures with broad similarity to the imidazolinones such as derivatives which lack the aromatic ring, or the even more divergent sulfonylcarboximide, also show activity (Stidham 1991). The herbicide binding site of ALS is therefore able to bind herbicides of widely varying structures.

Herbicidal ALS inhibitors act by inhibiting cell division in meristematic tissue. Studies with bacteria revealed that growth inhibition of bacteria by sulfonylurea herbicides was due to inhibition of the synthesis of the branched chain amino acids valine, leucine and isoleucine by inhibition of the enzyme acetolactate synthase (LaRossa and Schloss 1984). This was confirmed by studies of herbicide resistant tobacco mutants selected in tissue culture (Chaleff and Ray 1984).

Several properties make ALS inhibitors highly desirable for use as herbicides. The branched chain amino acids are among the 14 amino acids essential to the diet of animals, as animals lack the biosynthetic pathway for their production (Lehninger 1975). Thus, even though some herbicides, such as sulfometuron-methyl, are extremely potent inhibitors of ALS, they have very low toxicity to animals (Beyer *et al.* 1988, Shaner et al 1982, Gerwick *et al.* 1990, Kleschick 1992). The potency of the compounds as enzyme inhibitors leads to a second advantageous feature; efficacy at low application rates. Sulfonylurea herbicides are effective in many cases at

application rates of 2 to 75 grams active ingredient per hectare (Brown 1990). Imidazolinones generally require slightly higher application rates. Many compounds are also selective in various crops adding to their utility (see 1.3.4).

ALS inhibitors, particularly the sulfonylurea and imidazolinones, have proved to be effective and versatile herbicides. This finding has given increased impetus to the study of the target enzyme.

1.3.2 **Acetolactate synthase, the target enzyme**

Acetolactate synthase (ALS) (E.C.4.1.3.18), also called acetohydroxyacid synthase, is the first key enzyme in the synthetic pathway for the branched chain amino acids in prokaryotes, fungi and plants. ALS catalyses the condensation of two molecules of pyruvate to form acetolactate leading to synthesis of L-valine and L-leucine or the condensation of pyruvate with α–ketobutyrate leading to the synthesis of L-isoleucine (Fig. 1.2)

The enzymes involved in branched chain amino acid synthesis are found in a single multi-enzyme complex in the mitochondria of fungi (Bergquist *et al.* 1974, Squires *et al.* 1985, Ryan 1974). The active complex occurs *in vitro* only when mitochondria are supplied with the necessary substrates for respiration and oxidative phosphorylation (Bergquist *et al.* 1974). In plants ALS activity is probably confined to the plastids. Most, if not all, of the ALS activity in spinach leaves occurs in the chloroplasts (Miflin 1974) while ALS activity in pea protoplasts occurs entirely in the chloroplast (Jones *et al.* 1985). The synthesis of branched chain amino acids in spinach chloroplasts is light dependant and can be almost completely inhibited by valine or isoleucine (Miflin 1974) .

ALS activity in plants is highest in young tissue where rapid cell division is occurring. In the leaves of *Phaseolus lunatus* (Lima bean) total ALS activity increased rapidly until age of 5 days but remained relatively constant thereafter (Singh *et al.* 1990). In Black Mexican Sweetcorn (BMS)

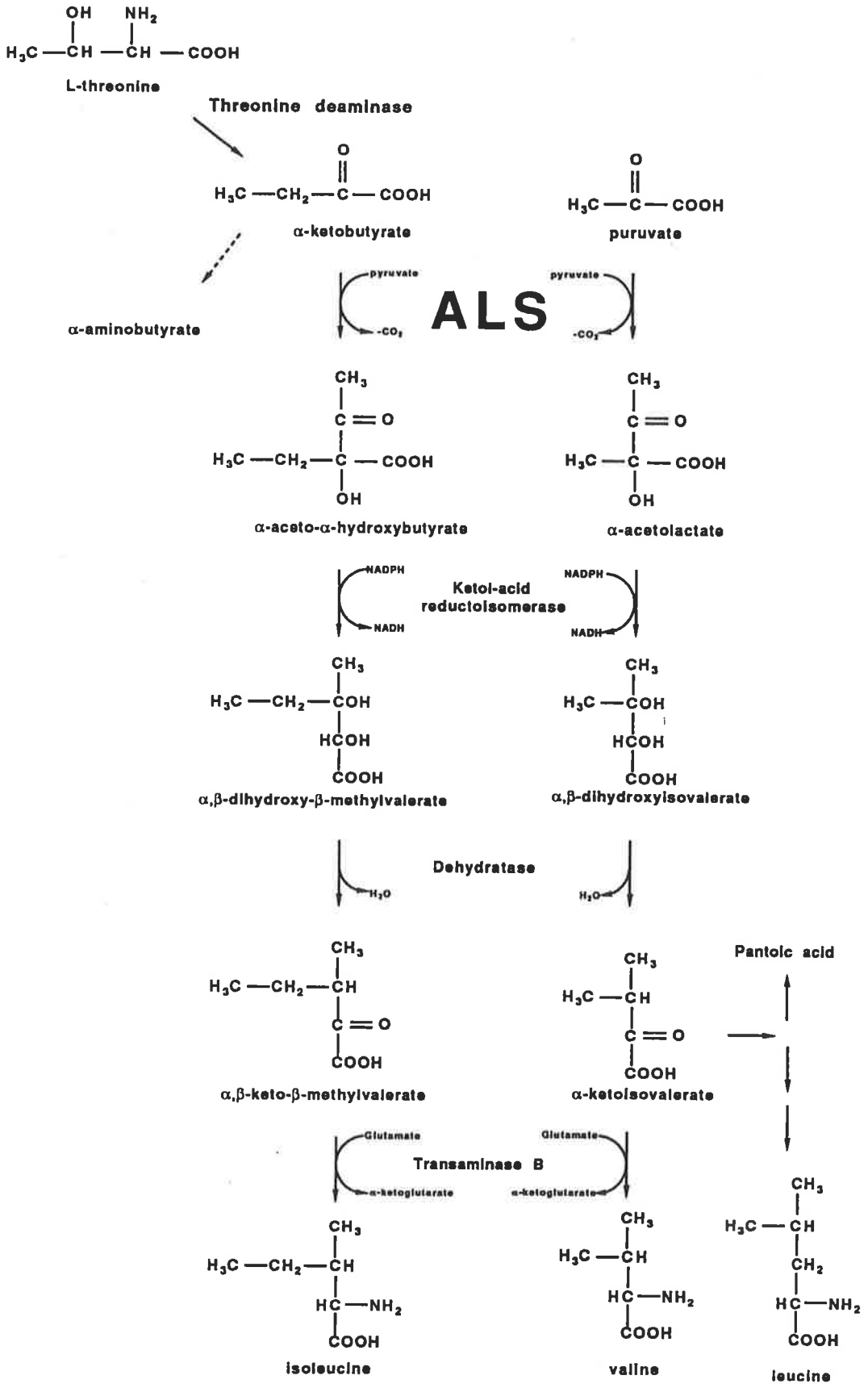


Figure 1.2 Branched chain amino acid synthesis.

Adapted from DeFelice et al 1974.

cell cultures ALS activity was greatest for cultures in logarithmic growth phase (Singh *et al.* 1990). Levels of transcription of ALS sequence in shoots of *Brassica napus*. where highest in young leaf tissue and floral buds (Bekkaoui *et al.* 1991). Similarly the highest expression in *B.napus* root tissue occurred in the young tips and in callus cultures expression was highest when cells were rapidly dividing (Wiersma *et al.* 1989). However, transcripts were not abundant in any *B.napus* tissue, only moderate levels of ALS mRNA were detected even in rapidly growing tissue (Wiersma et al. 1989,. As ALS is not abundant in plant tissue and has proved labile during extraction, understanding of the plant enzyme has lagged behind knowledge of the bacterial and fungal enzymes until the last three to four years.

Studies of ALS from bacteria are the most detailed, providing information about the evolution of ALS and how the enzyme interacts with herbicides. In wild type *Escherichia coli* up to six ALS genes have been proposed, some cryptic (not expressed) (Schloss *et al.* 1985). Three active gene products have been isolated and characterised. These are ALSI, ALSII and ALS III encoded by the *ilv* BN, *ilv* GMEDA and *ilv* IH operons respectively (Friden *et al.* 1985, reviewed inWek *et al.* 1985). Each isozyme consists of a tetramer of two large and two small subunits (Eoyang and Silverman 1984, Schloss *et al.* 1985, Wek *et al.* 1985). The large subunits encoded by the *ilv*B, *ilv*G, and *ilv*I genes respectively carry the catalytic activity while the small subunits are required for maximum catalysis and confer sensitivity to feed back inhibition by the end products valine, leucine and isoleucine (Eoyang and Silverman 1984, Lawther *et al.* 1981, Friedberg and Seijffers 1990). Promoter and regulation sequences are upstream (5') of those encoding the large subunit in each operon while genes encoding the small subunits of the three isozymes (*ilv*N, *ilv*M and *ilv*H respectively) are down stream (3') (Squires *et al.* 1983, Schloss *et al.* 1985, DeFelice *et al.* 1974). Besides the *ilv*M and *ilv*G genes encoding ALSII the *ilv*GMEDA operon also encodes the enzymes transaminase B (*ilv*E), dihydroxyacid dehydrase (*ilv*D) and threonine deaminase (*ilv*A) (Figure 1.2). Thus, in bacteria, many of the

enzymes of the branched chain amino acid pathway are under common transcriptional control. Regulation of the *ilv*NB and *ilv*GMEDA operons involve transcription attenuation regulated by the levels of aminoacylation of $tRNA^{Val}$, $tRNA^{Ilue}$ and $tRNA^{Lue}$ (Friden *et al.* 1982, Lawther and Hatfield 1980). Sequences in the promoter, regulator region alter the conformation of the transcribed RNA so that when levels of isoleucyl-, leucyl- or valyl-tRNA are adequate for cell function, transcription is attenuated. When levels of these aminoacyl-tRNAs are limiting, however, the conformation of the transcribed RNA does not cause attenuation so that transcription and translation are able to proceed (Lawther and Hatfield 1980). Transcription of *ilv*B is also positively regulated by cyclic AMP and cyclic AMP receptor protein (Friden *et al.* 1982). Regulation of the *ilv*IH operon differs from that of the other 2 isozymes (Wek *et al* . 1985).

The three isozymes vary in susceptibility to feedback inhibition by branched chain amino acids and inhibition by herbicides. ALSI and ALSIII from *E.coli* are subject to feedback inhibition by valine while ALSII is not (Lawther *et al.* 1981) (Table 1.4). ALSIII is also subject to feedback inhibition by leucine (DeFelice and Levinthal 1977). ALSI is not inhibited by sulfometuron-methyl while ALSII and III are subject to inhibition (Squires *et al.* 1983).

Table 1.4 Bacterial ALS isozymes

Isozyme	Genetic Locus	Feedback Inhibitors	Sulfometuron Inhibition	Reference
ALSI	*ilv*BN	val	(-)	Lawther *et al* 1981
ALSII	*ilv*GMEDA		(+)	Grimminger & Umbarger 1979
ALSIII	*ilv*IH	val leu	(+)	Squires *et al* 1983

The DNA and amino acid sequences of the large and small subunits of the three isozymes share considerable homology (Squires *et al.*

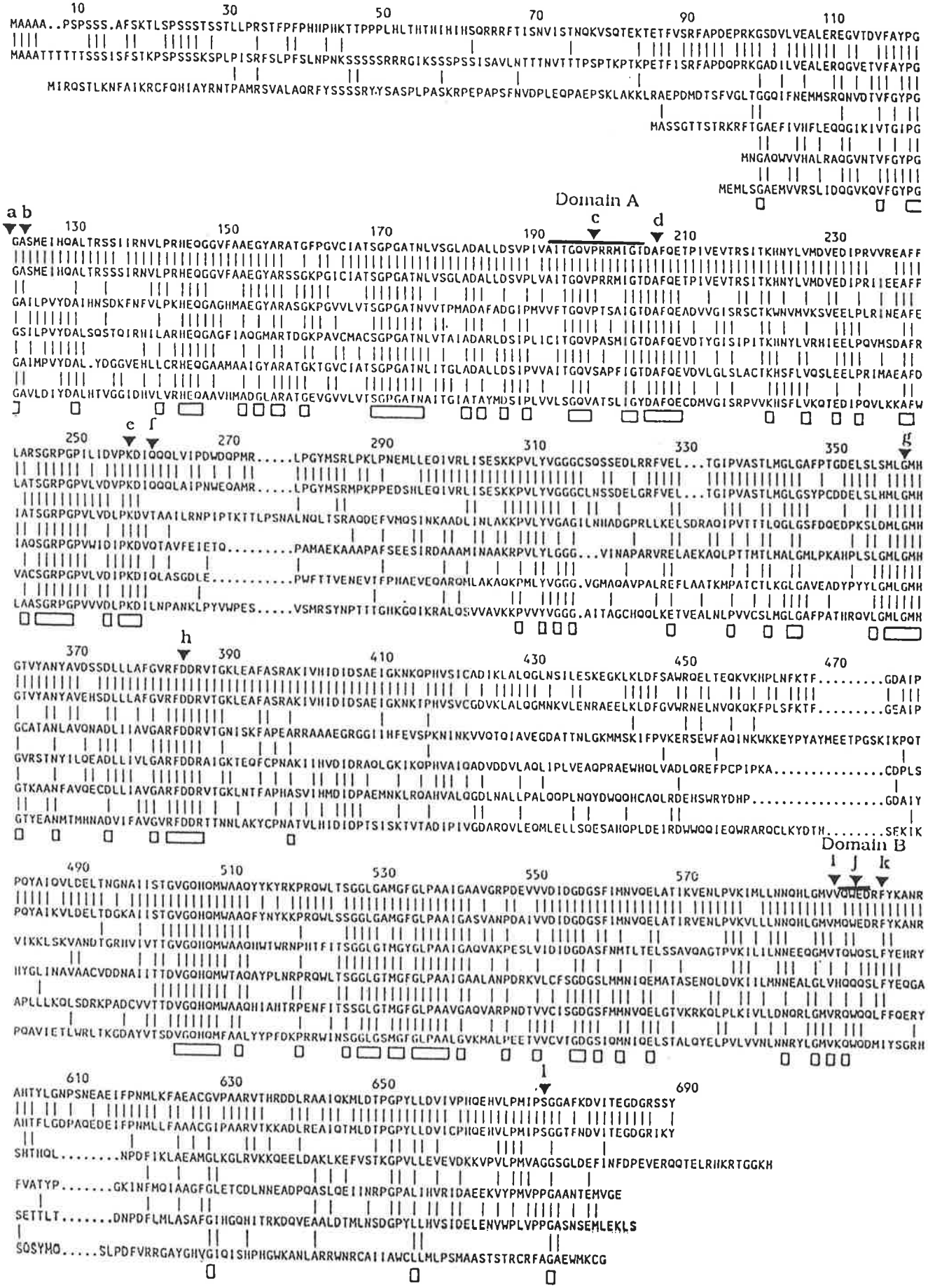
1983, Wek *et al.* 1985) (Figure 1.3). The large subunits of ALSI and II have 46% amino acid homology, II and III have 41% homology while I and III have 38% homology (Wek *et al.* 1985). The amino acid sequences of the large subunits share three regions of high homology interspersed by less similar regions. Isoenzymes I and II also shared considerable homology in the hydroxy terminal sequence not shared by isozyme III (Figure 1.3.). The hydrophobicity of the various regions of the proteins is even more highly conserved with the three conserved regions being hydrophobic and the interspersed regions of lesser homology being hydrophilic (Squires *et al.* 1983, Wek *et al.* 1985) (Figure 1.3. and 1.4). This suggests that the primary structure of the proteins is highly conserved. On the basis of their nucleotide and amino acid sequence homology and their regulation mechanisms ALSI and II are more similar to each other than they are to ALSIII (Wek *et al.* 1985).

Wild type *Salmonella tymphimurium* has two functional ALS isozymes corresponding to ALSI and ALSII of *E.coli* (Shaw and Berg 1980). ALS III is only expressed when a frame shift mutation in the *ilvI* gene occurs (Squires *et al.* 1983). Some of the first evidence that sulfonylurea herbicides act by inhibition of ALS came from studies of *Salmonella* ALS (LaRossa and Schloss 1984). LaRossa and Schloss showed that sulfometuron-methyl was able to inhibit the growth of *Salmonella tymphimurium* when grown on medium containing valine. Growth inhibition was due to inhibition of ALS isozyme II and only occurred when valine was present in the medium, as valine inhibits ALS isozyme I, which is less sensitive to inhibition by sulfometuron-methyl.

Purified ALS II from *S.tymphimurium* consisted of two large and two small subunits of 59300 Da and 9700 Da respectively (Schloss et al. 1985), the large subunit being similar in size and amino acid sequence to that of *E.coli* ALSII and ALSI (Schloss *et al.* 1985, Lawther *et al.* 1981, Grimminger and Umbarger 1979) and the smaller subunit being similar to that of *E.coli* isozyme II (Schloss *et al.* 1985, Lawther *et al.* 1981).

In yeast the single ALS isozyme is encoded by the ILV2 gene which maps to chromosome X111 (Falco and Dumas 1985). The amino acid sequence of the enzyme shows considerable homology with the large subunits of *E.coli* ALS enzymes II and III with 40% of amino acids identical for all three enzymes (Squires *et al.* 1985) (Figure 1.3). Three regions of high homology between the three enzymes interspersed by regions of low homology correspond to three homologous regions of the *E.coli* proteins (Squires et al. 1985, Wek *et al* 1985) (Figure 1.4). The gene is also similar to the bacterial gene in that it has none of the intervening sequences typical of eucaryotic genes. The sequence differs from *E.coli* , however, by possessing an extra ninety amino acid peptide at the amino terminal which is similar to yeast mitochondrial transit sequences. This sequence probably directs the transfer of the protein to its site of action in the mitochondria (Squires *et al.* 1985, Wek *et al* 1985). Only one gene locus for ALS (ILV2) has been described in yeast suggesting that eucaryotes do not have the multiplicity of isozymes found in bacteria (Falco and Dumas 1985).

Comparison of the nucleotide sequence of sulfonylurea herbicide resistant bacteria and yeast revealed that a single base pair substitution can cause the difference between a herbicide-sensitive and an insensitive ALS (Yadav *et al.* 1986 ref 635). Spontaneous mutants of yeast and *E.coli* were selected using sulfometuron-methyl in cell culture (Yadav *et al.* 1986). Molecular analysis of the *ilv*G gene of an *E.coli* mutant and the ILV2 gene of a yeast mutant revealed that both had a single base pair substitution causing an amino acid substitution in the 160 residue amino-terminal conserved region of the protein (Figure 1.3). The yeast mutation resulted in the substitution of serine for proline at amino acid 192 which caused greatly reduced sensitivity to sulfometuron-methyl (Yadav *et al.* 1986). The *E.coli* mutation resulted in substitution of valine for alanine at residue 26 of the protein causing greatly reduced sensitivity to


Domain A
Domain B
a
b
c
d
e
f
g
h
i
j
k
l

tobacco

Arabidopsis

yeast

***E.coli* ALSI**

***E.coli* ALSII**

***E.coli* ALSIII**

Figure 1.3 (Opposite) Comparison of ALS amino acid sequences from tobacco, *Arabidopsis*, yeast, *E.coli* isozyme I, *E.coli* isozyme II and *E.coli* isozyme III. Vertical lines indicate amino acids conserved between adjacent sequences and boxes indicate amino acids conserved in all sequences.

Positions where mutation of the yeast sequence causes herbicide resistance are marked by arrows with the letters a, b, c, d, e, f, g, h, i, j, k which represent yeast amino acids 116, 117, 192, 200, 251, 254, 379, 583, 586, and 590 respectively (see also Table 1.5).

The conserved motif, AITGQVPRRMIGT of the plant conserved region, Domain A, is marked by a horizontal line. The crucial proline in this domain corresponds to the proline at yeast position 192 and the point of mutation of arabidopsis mutant GH50 and tobacco mutants C3 and S4. The substitution of proline for other amino acids at this position in *E.coli* isoenzymes II and III is involved in reduced herbicide sensitivity.

The conserved motif, QWED of plant conserved region, Domain B, is also marked by a horizontal line. The crucial tryptophan in this sequence corresponds to yeast amino acid position 586 and it is mutation at this residue which causes increased resistance in tobacco mutant S4/Hra. Substitution of glutamine for tryptophan in *E.coli* isozyme I at this position is involved in reduced herbicide sensitivity. The arrow and letter l indicate the position of asparagine for serine substitution in the imidazolinone resistant arabidopsis mutant GH90.

Amino acid sequence is from Mazur et al 1987. Single letter codes for amino acids are given on in abbreviations.

sulfometuron-methyl but also 75% reduction in ALS activity (Yadav *et al.* 1986). In another study where yeast were selected on minimal agar containing sulfometuron-methyl 50 out 66 resistant mutants isolated were dominant or semidominant characters which map to the ILV2 locus (Falco and Dumas 1985). Many (but not all) of these ALS mutants had reduced levels of ALS activity and the level of ALS inhibition caused by 800nM sulfometuron-methyl ranged from 0% to 63% between mutants (Yadav *et al.* 1986). These data suggest that the ALS mutants selected may represent many different mutations of the ILV2 gene although no sequence information is reported.

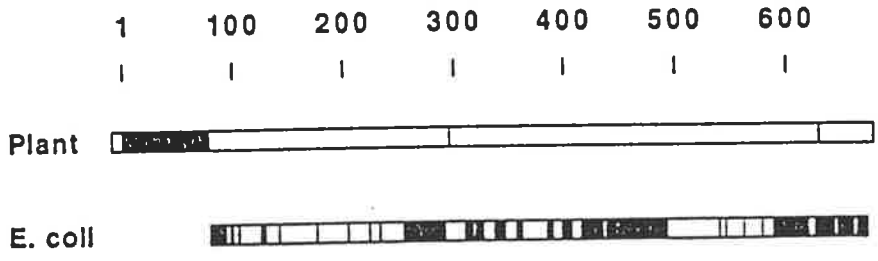


Figure 1.4 Conserved amino acid sequence of ALS *E.coli* isoenzymes I, II and III and the two plant species tobacco and Arabidopsis. Conserved regions are shown in white. The three conserved regions between E.coli isoenzymes extending approximately from amino acid positions 100 to 250, 300 to 400 and 500 to 600 correspond to conserved hydrophobic regions in all five sequences. (From Hartnett *et al.* 1990)

Spontaneous mutations and site directed mutagenesis of the **yeast ILV2 gene have revealed that many mutations occurring at ten** different sites can lead to herbicide insensitive ALS (Table 1.5). Some amino acids can be substituted by a range of other residues to produce catalytically-active, herbicide-insensitive enzyme while at other residues only a few changes are possible (Mazur and Falco 1989). Confirmation that these sites contribute to herbicide sensitivity in other organisms was found by site directed mutagenesis of ALS from *E.coli*. ALS isozyme II from *E.coli* is less sensitive to sulfometuron than that of yeast and plants (Falco *et al.*

1987). The isozyme has a serine residue at the analogous situation to the proline at position 192 in yeast (Figure 1.3). When the serine was substituted for proline, herbicide sensitivity of ALS II from *E.coli* was increased. Similarly *E.coli* isozyme I is insensitive to sulfometuron. The amino acid sequence of *E.coli* isozyme I differs form that of *E.coli* isozyme II by having a glutamine residue in place of tryptophan (Figure 1.3, position j). When tryptophan was substituted for this glutamine in *E.coli* isozyme I, herbicide sensitivity was increased (Mazur and Falco 1989).

Table 1.5 Amino acid substitutions in ALS from herbicide resistant yeast mutants

Amino acid position	Wild type	Herbicide resistant mutants
116	G	S N
117	A	PSTILVNQDEKRHWFY M
192	P	A S V Q E R W Y
200	A	T V DE R W YC
251	K	P T N DE
354	M	V K
379	D	G PS VN E W
583	V	A N YC
586	W	GA S ILVN EKRH YC
590	F	G L N R C

* see abbreviations for single letter code for amino acids from Mazur & Falco 1989

The most detailed molecular information concerning plant ALS comes from studies using *Arabidopsis thaliana* and *Nicotiana tabacum*.. DNA probes of the ILV2 gene from yeast were used to isolate the native ALS gene from *Arabidopsis* and tobacco ALS (Mazur *et al* 1987). *Arabidopsis* and tobacco genes had a high level of homology with 73% nucleotide homology and 84% amino acid homology (Figure 1.3). The three regions of homology in bacterial and yeast isozymes were maintained but there was also considerable homology between the two plant sequences even in the regions corresponding to the non-conserved regions of bacteria and yeast

isozymes (Figure 1.3 & 1.4). The ALS proteins all shared regions of hydrophobicity and hydrophylicity which may be important in primary structure. Both plant genes lack introns, an unusual feature for a eucaryotic gene (Mazur *et al.* 1987). The fact that the ALS sequence is highly conserved between *Arabidopsis* (family *Cruciferae)*) and tobacco (family *Solonacae*) suggests that the sequence may be highly conserved among plants in general.

Although tobacco and *Arabidopsis* ALS amino acid sequences were highly conserved in the structural region, the putative transit sequences were not highly conserved, having only 27% amino acid homology. This suggests that more variation between taxa may be expected in the transit peptide sequences of plant ALS (Mazur 1987).

Plants with herbicide-insensitive ALS have now been isolated from several species. Sulfonylurea and imidazolinone resistant lines of *Arabidopsis* have been isolated (Haughn and Somerville 1986 and 1990). A sulfonylurea resistant mutant (GH50) was isolated by selection of ethyl methane sulfonate (EMS) treated seed on minimal agar medium containing chlorsulfuron (Haughn and Somerville 1986). Similarly an imidazolinone resistant mutant (GH90) was identified on minimal agar containing imazapyr (Sathasivan *et al.* 1991, Haughn and Somerville 1990). ALS from mutant line GH50 was 300 fold less sensitive to chlorsulfuron than that of the wild type but was only 5 fold less sensitive to imazapyr (Haughn and Somerville 1986). In contrast ALS from GH90 was >100 fold less sensitive to imazapyr but only 5 fold less sensitive to chlorsulfuron (Haughn and Somerville 1990). Both mutations were inherited as dominant nuclear encoded genes mapping to a single locus (CSR1) on chromosome 3 (Haughn and Somerville 1986 and 1990). The mutant genes of GH50 and GH90 were designated *csr*1 and *csr*2 respectively.

Comparison of the nucleotide sequence of the *csr*1 gene encoding ALS in mutant GH50 with the sequence from herbicide sensitive *Arabidopsis* revealed a single base pair substitution causing the amino acid

substitution of serine for proline at position 198. This is the analogous protein position to the proline 192 to serine substitution causing resistance in yeast (Figure 1.3) (Haughn *et al.* 1988). In contrast the *csr*2 gene had a single base pair substitution leading to the amino acid substitution of asparagine for serine at amino acid 632 of the protein (Sathasivan *et al.* 1990). This is a region of homology between tobacco and *Arabidopsis* which corresponds to a non-homologous region of the bacterial and yeast proteins (Mazur *et al.* 1987, Wek *et al.* 1985). This mutation has only been shown for *Arabidopsis*.

The *Arabidopsis* gene *csr*1 was successfully cloned into *E.coli* yielding large quantities of purified protein (Smith et al 1989). The bacteria were able to cleave the transit peptide encoded by the plant sequence to yield a mature plant protein which appears to have only one subunit (Smith *et al.* 1989).

When the *csr*1 gene was introduced into tobacco, chlorsulfuron resistant plants were recovered (Haughn *et al* 1988). As with *Arabidopsis* mutant GH50, tobacco transformed with the *csr*1 gene was not highly cross-resistant to imidazolinone herbicides (Gabard *et al.* 1989). In transformed tobacco *csr*1 encoded ALS exhibited resistance to the sulfonylurea herbicides chlorsulfuron, metsulfuron-methyl, DPX-L5300 (Express) and DPX-M6316 (Harmony) but did not exhibit resistance to the imidazolinone herbicides imazapyr, imazathapyr or imazaquin nor did it exhibit resistance to the sulfonylurea herbicide triasulfuron (Gabard *et al.* 1989). The lack of resistance to triasulfuron is unexpected as the structure is very similar to that of chlorsulfuron and metsulfuron-methyl (Figure 3.1, Chapter 3). The interaction of the enzyme with the ortho substituent of the phenyl ring must be affected by this substitution.

Herbicide resistant mutants of *Nicotiana tabacum* were isolated from tissue culture using ethylnitrosourea mutagenesis followed by selection on chlorsulfuron or sulfometuron-methyl-containing agar. Linkage analysis of six mutants showed that all were alleles of two loci. All mutants were

nuclear inherited as dominant or semi dominant traits (Chaleff and Ray 1984). The phenotypes of the different mutations varied in the level of resistance to, and cross-resistance between, sulfometuron-methyl and chlorsulfuron (Chaleff and Ray 1984). Further investigation revealed that the mutants C3 and S4 (both highly resistant to chlorsulfuron and sulfometuron) have a herbicide-insensitive ALS (Chaleff and Bascomb 1987). Each mutation alone caused only 50% of ALS activity in tobacco to be resistant. It was concluded that C3 and S4 mutations encoded mutations for two different structural genes in the allotetraploid genome of tobacco. The loci were designated SuRA and SuRB respectively (Chaleff and Bascomb 1987). A second mutation at the SuRB locus of tobacco additional to that of the mutant S4 was found to increase chlorsulfuron resistance (Creason and Chaleff 1988). This mutant (SR4/Hra) was found to be inherited as a single dominant gene at or near the same genetic locus SuRB.

N.tabacum is an allotetraploid which has arisen by hybridisation of *N.tomentosiformis* and *N. sylvestris.* Restriction fragment length polymorphism (RFLP) analysis of ALS encoding sequences from *N.tabacum* revealed that the class I ALS gene (locus SuRA) of tobacco is contributed by the parent *N.sylvestris* while the classII ALS gene (locus SuRB) is contributed by *N.tomentosiformis.*

Comparison of the nucleotide sequence encoding ALS for the sulfonylurea resistant tobacco mutant C3 with that of herbicide sensitive ALS from tobacco reveals that resistance results from a single base pair substitution causing a proline to glycine substitution at residue 196 the classI ALS gene (Figure 1.3) (Mazur *et al.* 1987, Lee *et al.* 1988). This amino acid substitution is at an analogous position in the protein to that described for yeast and *Arabidopsis* (Figure 1.3). Resistance in mutant S4/Hra results from two single base pair mutations in the classII ALS gene. One results in substitution of alanine for the proline at residue 196 and a second results in a tryptophan to leucine substitution at position 573 (Figure 1.3) (Lee *et al.* 1988). This tryptophan is at a position analogous to

the guanine of *E.coli* enzyme I discussed above (Mazur and Falco 1989). The involvement of these *Arabidopsis* genes in resistance was confirmed by transformation of susceptible tobacco cells.

ALS from *Brasica napus* was shown to have a very high level of homology with that of *Arabidopsis* and tobacco differing in only 88 and 134 respectively out of the total 576 amino acids (Wiersma *et al.* 1989) . The motif AITGQVPRRMIGT surrounding herbicide resistant mutations at proline of *Arabidopsis* mutation GH50 and tobacco mutation C3 and S4 (Figure 1.3) and the motif QWED surrounding the Hra mutation of tobacco are conserved between all three plant sequences (Sathasivan *et al.* 1991). These motifs were designated conserved Domain A and conserved Domain B respectively (Figure 1.3).

The N-terminus chloroplast transit peptide shows little homology between the three plant genomes. *B.napus* is thought to be a hybrid between *B.campestris* and *B.olearacea.* RFLP analysis revealed that the DNA sequence encoding the transit peptide was unique to the *B.campestris* parent and differed from that of *B.olearacae* (Wiersma *et al.* 1989). A second sequence cloned from *B.napus* had 92% amino acid homology with the first sequence but the N-terminal transit peptide differed (Figure 1.3). This may be the gene originating from the *B. olearacae* parent.

A specific polymerase chain reaction (PCR) technique was used to amplify DNA from Domain A of the ALS encoding sequences of three weed species. Biotypes of kochia (*Kochia scoparia*), prickly lettuce (*Lactuca serriola*), and Russian thistle (*Salsola iberica*) had developed resistance to sulfonylurea herbicides (Guttieri *et al.* 1993). The sequences showed that in the resistant *Lactuca* the proline of Domain A had been replaced with histidine, in the *Kochia* biotype the proline was substituted by threonine. Even though two distinct sequences for Domain A were detected in the resistant *Salsola* biotype, both retained proline in common with most herbicide sensitive ALS sequences. A resistant *Salsola* biotype is known to posses a herbicide insensitive ALS (Saari *et al.* 1992). It is not clear whether

the *Salsola* material with herbicide resistant ALS (Saari *et al.* 1992) is the same as that used to determine the sequence of Domain A (Guttieri *et al.* 1993) although both were collected in Washington state. If the material is the same it appears that the ALS mutation causing resistance is not at the proline of Domain A. Comparison of 6 resistant *Kochia* biotypes showed a good correlation between resistant phenotype and RFLPs generated by mutation of the proline residue of Domain A. However, one resistant biotype did not have a mutation at this point. Thus mutations of ALS in weeds, like those of laboratory selected biotypes and other organisms, can occur at different sites.

The molecular data on ALS indicate that mutations in at least four different positions of the ALS nucleotide sequence can lead to amino acid substitutions causing the enzyme in bacteria, yeast and plants to become insensitive to herbicides (Table 1.6). However, ten different sites where certain amino acid substations can cause herbicide resistance are known in yeast. In view of the many similarities between ALS from different organisms, it is likely that the amino acids at all ten sites known to be involved in herbicide resistance in yeast ALS may also be important in plant ALS.

The proline of Domain A of plants and tryptophan of Domain B are known to be important in all groups of organisms. Virtually any substitution for the proline of Domain A causes insensitivity to sulfonylurea herbicides. The α–imino heterocycle of proline imposes a unique constraint upon the conformation of the peptide bond of this residue compared with other protein amino acids. Substitution for virtually any other amino acid may, therefore, have profound effects on the primary conformation of the protein. That, this residue affects the interaction of the enzyme with the ortho, aryl substituent of some sulfonylureas is demonstrated by the lack of cross-resistance to triasulfuron in the *csr*1 mutant of *Arabidopsis* (Haughn *et al.* 1988). None of the substitutions for the proline of Domain A have been reported to cause cross-resistance to imidazolinones alone, although ALS

from the S4/Hra mutant of tobacco which also has a second substitution at amino acid 573 is resistant to herbicides from both groups. The imidazolinone cross-resistance in this case may be due to the substitution in Domain B, although the effect on imidazolinone sensitivity of the Domain B substitution alone is not known. Conversely the mutation at residue 653 of the *Arabidopsis* mutant GH50 causes imidazolinone resistance with only low levels of resistance to sulfonylureas.

Table 1.6 Herbicide-resistant ALS mutations

Species/ mutant	Amino acid substitution	Resistance	Reference
Mutations affecting Domain A			Satasivan et al 1991
S.cerevisiae/ILV2-410	Pro → Ser	SU*	Yadav et al 1986
A.thaliana/GH50	Pro → Ser	SU	Haughn et al 1988
N.tabaccum			
Class I ALS/C3	Pro → Gln	SU	Lee et al 1988
ClassII ALS/S4	Pro → Ala	SU	Creason&Chaleff 1976
L.serriola	Pro → His	SU	Guttieri et al 1992
Kochia scoparia	Pro → Thr	SU	Guttieri et al 1992
Mutations at other positions			
E.coli/pB1SUR10	Val (26#) → Ala	SU	Yadav et al 1986
A.thaliana/GH90	Ser(653) → Asn	Im	Sathasivan et al 1991
N.tabaccum/Hra	Trp(573) → Leu	SU&Im	Lee et al 1988

* SU=sulfonlyureas, Im=imidazolinones

** spectrum of resistance of mutants S4 and Hra together

amino acid position

In the absence of X-ray crystallographic data to elucidate the three dimensional structure of the ALS protein it is impossible to say which residues actually make up the herbicide binding site but it is clear that residues at all ten positions discussed must be either part of the binding site or, that alterations of these residues can affect the conformation of the binding site. Clearly the proline of domain A of plant ALS is important in the binding of sulfonylurea herbicides. Whether the proline of Domain A affects binding of imidazolinones is unclear. The serine at position 653 of the *Arabidopsis* protein is clearly more important in binding of imidazolinones than sulfonylureas. The results suggest that, like the herbicide binding site of the Q_B-binding protein of photosystem II (section1.2.2), the binding site of ALS is able to bind molecules of different structure at partially overlapping regions.

Studies of resistant phenotypes with herbicide-insensitive ALS which have been selected from many different species in the laboratory also suggest that many different mutations of the ALS sequence can lead to herbicide resistance. The spectrum of herbicide cross-resistance between different chemical groups, the level of resistance to specific herbicides and level of ALS activity of herbicide resistant ALS mutants can vary greatly. Molecular analysis of more of these mutants in the future has the potential to greatly increase our knowledge on the interactions of herbicides with plant ALS.

Mutants selected using sulfonylurea or imidazolinone cause varying phenotypes of varying cross-resistance between groups. Four sulfometuron-resistant mutants of *Chlamydomonas reinhardtii* had ALS I_{50} values for sulfometuron from 240nM to 1900nM compared to 2.2nM for the unselected line (Hartnett *et al.* 1987). ALS activities in the absence of herbicide for two of these lines were similar to the parent line while two mutant lines showed a considerable decrease in activity (Hartnett *et al.* 1987). In a second study twenty one chlorsulfuron and imazaquin resistant lines of Chlamydomonas were isolated (Winder and Spalding 1988).

Only one of thirteen imazaquin resistant lines was resistant to chlorsulfuron while one imazaquin resistant mutant was hypersensitive to chlorsulfuron (Winder and Spalding 1988). Of eight chlorsulfuron resistant lines, six showed slightly increased imazaquin tolerance (Winder and Spalding 1988). Chlorsulfuron or sulfometuron selected cultures of the solanaceous weed *Datura innoxia* were shown to have varying levels of cross resistance to imidazolinone herbicides comparing I_{50} of cell growth in culture (Saxena and King 1988). Three mutants were highly resistant to both sulfonylureas and imidazolinones, one was sulfonylurea resistant but imidazolinone-sensitive and one was sulfonylurea resistant but hypersensitive to imidazolinones (Saxena and King 1988). Three imidazolinone resistant corn mutants isolated from BMS cell suspension cultures, XA17, AI12 and QJ22 differed widely in the level of ALS inhibition caused by imazapyr, imazaquin and sulfometuron (Singh *et al.* 1990).

ALS from some leguminous species differs from that of other plant families (Saari *et al.* 1991). Differences are also seen in the phenotypes of mutants selected. Soybean mutant W20, selected using chlorsulfuron, had ALS resistant to chlorsulfuron, chlorimuron-ethyl, DPX-L5300 and DPX-M6316 (Sebastian *et al.* 1989) but level of resistance is low compared to many other herbicide resistant ALS enzymes with the I_{50} for chlorsulfuron being only two fold that of the wild type. Similarly, three resistant lines of *Lotus corniculatus*, a forage legume, selected using DPX-M6316 had a less sensitive ALS but the I_{50} was only two fold that of the susceptible progenitor (Pofelis *et al.* 1992).

Selection of mutants using triazolopyrimidine herbicides has been shown to cause various levels of resistance to sulfonylurea and imidazolinone herbicides and pyrimidyl-benzoic acid. One triazolopyrimidine resistant mutant of tobacco and two cotton mutants varied in the level of cross-resistance to the four types of ALS-inhibiting herbicides in both cell culture growth and ALS I_{50} (Subramanian *et al.* 1990). One mutant had ALS which was less-sensitive to feed-back inhibition by branched chain

amino acids (Subramanian *et al.* 1990). One mutant had slightly reduced growth rate while the other mutants did not. In another study of fifteen tobacco and soybean mutants resistant to triazolopyrimidine herbicides all showed cross-resistance to chlorsulfuron while all but one were cross-resistant to imazaquin (Subramanian *et al.* 1989). These results support the hypothesis that the four types of ALS-inhibiting herbicides bind to an overlapping site.

Molecular studies of more of these mutants should greatly increase our understanding of the comparative binding of the herbicides from different chemical groups. Although the physical topography of the binding site is not yet known, studies of ALS from bacteria do provide understanding of the mechanism of action and of how the enzyme and herbicides may interact.

Bacterial ALS II has considerable homology with the sequence of pyruvate oxidase (Grabau and Cronan 1986). Pyruvate oxidase uses FAD to catalyse the redox reaction by which pyruvate is oxidised with reduction of ubiquinone-40 (Koland *et al.* 1984) (Figure 1.5). Sulfometuron-methyl, imazaquin and 2-NO_2-6-Me-sulphonanilide and Ubiquinone-0 compete for the same binding site of ALS (Schloss et al. 1988). On the basis of these observations it was suggested that ALS has evolved from a pyruvate oxidase ancestral gene. The herbicide binding site being an evolutionary vestige of the quinone binding site of ancestral enzyme. The proposed mechanism of action of ALSII is compared with the mechanism of pyruvate oxidase in (Figure 1.4) (Schloss *et al.* 1988). In both reactions the first molecule of pyruvate is decarboxylated by reaction with thiamine pyrophosphate (TPP), complexed to ALS, forming a carboxyanion intermediate (2,2' Figure 1.5). In the pyruvate oxidase reaction the carboxyanion then reacts with the isoalloxazine ring of FAD which allows

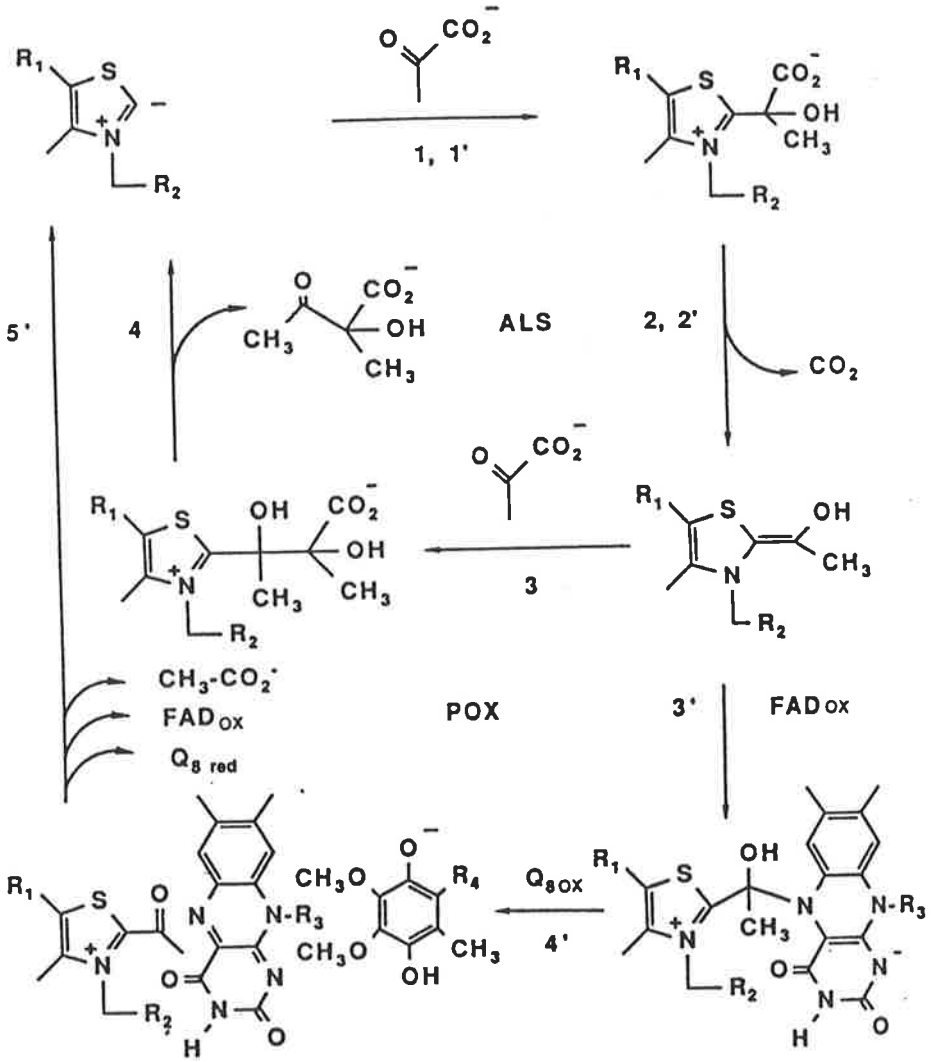


Figure 1.5 Comparison of the proposed reaction schemes ALS (steps 1-4) and pyruvate oxidase (POX) (steps 1' to 4'). Steps 1 and 1' are the same as are steps 2 and 2'. R_1 and R_2, respectively, represent the diphosphoethyl and pyridyl groups of thiamine pyrophosphate. R_3 represents the adenosinediphosphate ribitoyl group of FAD and R_4 represents the eight isoprene units of Q_8. (Schloss *et al.* 1988)

binding of oxidised ubiquinone-40 (Q_8) (4' Figure 1.5). This complex allows transfer of electrons from Q_8 to the carboxyanion intermediate with the consequent release of acetic acid. In the proposed mechanism for ALS, the isoalloxazine ring of FAD acts as an electrophile for the carboxyanion intermediate, helping to shield the carboxyanion from the solvent preventing its protonation. Evidence for this interaction comes from a change of absorbance by enzyme-bound FAD between 370 and 530 nm during conditions of enzyme turn over (Schloss 1984). The ALS reaction cannot proceed if the carboxyanion is protonated (Schloss *et al.* 1988), thus there is selective pressure for the FAD binding site to be maintained despite the lack of a redox function for FAD in the reaction. Thus, the ubiquinone binding site formed by the ALS-Mg-TPP-(decarboxylated)pyruvate-FAD complex is at least partially retained as is demonstrated by the binding of ubiquinone-0. This has been proposed as the herbicide binding site (Schloss *et al.* 1988).

Further evidence for the proposed mechanism of action comes from the time dependent nature of inhibition of ALS by ubiquinone-O and sulfometuron-methyl (Schloss 1984, Schloss *et al.* 1988). The inhibitors initially bind in an easily reversible manner followed by tighter binding after a period of enzyme turnover (Schloss *et al.* 1988, Ray 1986, Muhitch *et al.* 1987, Durner and Boger 1990). The affinity of ALS for sulfometuron-methyl was ten fold higher in the presence of pyruvate under conditions of turnover (Schloss *et al.* 1988) while TPP binding and sulfometuron binding were closely correlated (Schloss *et al.* 1988). These data suggest that the herbicide binds more tightly to the ALS-Mg-TPP-(decarboxylated)pyruvate-FAD complex which also agrees with the theory that the herbicide binding site and the quinone binding site have a common ancestry. After the formation of this tight binding complex under conditions of turnover, dissociation of the enzyme-herbicide complex becomes very slow (Schloss 1988). Thus, although initial binding of the herbicides is slow, the herbicides are still potent enzyme inhibitors because the rate of dissociation following formation of the tight complex is even slower (Schloss 1985).

These findings further confirm the suggested similarities between the binding of herbicides with ALS and binding of herbicides with the Q_B-binding protein of photosystem II. Both sites are quinone binding sites and can bind structurally different herbicide molecules. This suggests that quinone binding sites of plant enzymes are more likely to be inhibited by compounds in random screening programs than other classes of enzymes (Schloss *et al.* 1988).

The evidence suggests that genes coding for ALS occur at only one locus in the diploid plant genome and that there is only one subunit in the plant enzyme. ALS activity and herbicide interactions are affected by the specific amino acid sequence of the subunits and also by the level of aggregation. The stability of the enzyme during extraction and purification and the level of aggregation also depend on the presence or absence of cofactors.

Purification of the enzyme was greatly assisted by the discovery that *in vitro* bacterial ALSII complexed with all cofactors (thiamine pyrophosphate, FAD and magnesium) suffers oxygen-dependant loss of activity while the enzyme-FAD complex in the absence of thiamine pyrophosphate and metal ions is not rapidly inactivated (Schloss *et al.* 1985). Deactivation may be due to oxidation of the thiamine pyrophosphate to thiazole. This oxidised form of thiamine pyrophosphate will inhibit the activity of ALS by preventing initial step of the reaction (Figure 1.5) (Schloss *et al.* 1985, Schloss and Aulabaugh 1989). FAD was also required to stabilise ALS activity from maize and barley (Muhitch *et al.* 1987, Durner and Boger 1990) but pyruvate required to maintain activity during gel filtration (Muhitch *et al.* 1987).

ALS in Black Mexican Sweet corn cultures has been shown to have two forms (Singh *et al.* 1990, Singh *et al.* 1988, Singh and Schmitt 1989). ALS extracted from BMS could be separated into 2 fractions by gel filtration (Singh and Schmitt 1989) or anion exchange chromatography on Mono Q (Singh *et al.* 1988). Separation by either system produced 2 peaks,

form I representing 90% of activity with an estimated wt of 193000Da and form II represented 10% of activity and had an estimated molecular wt of 55,000 Da (Singh *et al.* 1988). The two forms differed in pH optima and inhibition kinetics by both branched chain amino acids and imidazolinone herbicides (Singh *et al.* 1990, Singh *et al.* 1989). Form I was virtually insensitive to inhibition by 1mM concentrations of branched chain amino acids isoleucine, leucine or valine either together or in combination but form II was inhibited by all three amino acids alone and more inhibited by combinations. ALS form I was slightly more sensitive to the herbicides imazapyr than ALS form II. Form II had a distinct pH optimum at pH 7 whereas ALSI had a broad pH optimum range between pH6 and pH 7 (Singh et al. 1988). Both forms had similar affinity for pyruvate. FAD caused an increase in the ALS form I over ALS form II. It was suggested that the two forms represent different aggregation states of the enzyme, form I being the tetramer and form I the individual subunits; (Singh *et al.* 1988) however further data is required to confirm this hypothesis (Singh *et al.* 1990, Singh *et al.* 1989). Purified ALS from barley also contained forms of different molecular weights believed to be 58000 Da monomer oligomeric species of 220000Da and 440000Da (Durner and Boger 1990). Aggregation depended upon both FAD and pyruvate concentration with the larger species being predominant in the presence of both (Durner and Boger 1990). The different forms were all susceptible to feedback inhibition by valine, leucine, isoleucine and sulfonylurea herbicides (Durner and Boger 1990).

Neither chlorsulfuron nor imazaquin compete with pyruvate for the catalytic site of ALS from barley (Durner *et al.* 1991). Chlorsulfuron is a noncompetitive inhibitor (it does not affect the affinity of the enzyme for the substrate) while imazaquin is uncompetative (binding is remote from the catalytic site but affinity for the substrate is altered allosterically). The uncompetetive interaction of imidazolinones has been reported in crude corn ALS (Shaner *et al.* 1984) but noncompetitive behaviour has been reported in pea and bacteria (Hawkes 1989, Schloss *et al.* 1988). Equilibrium dialysis

showed competition between imazaquin and chlorsulfuron for the same binding sites, again suggesting that the binding sites are at least partially overlapping. Herbicides did not affect the aggregation state of the enzyme (Durner *et al.* 1991).

In summary, it appears that the the ALS enzymes in bacteria, fungi and plants have probably evolved from a common pyruvate oxidase-like ancestral enzyme. Three isozymes of this enzyme have evolved in bacteria but only one isozyme has been reported in the eucaryotic fungi and plants. The eucaryotic enzymes show most similarity with bacterial ALSII. Plant, fungal and bacterial isozyme ALSII are all inhibited by sulfonylurea, imidazolinone and triazolopyrimidine herbicides. Herbicides of different chemical structure all bind at a single partially overlapping binding site which may an evolutionary vestige of the quinone binding site of a pyruvate oxidase-like ancestral enzyme. It is believed that during enzyme turnover the reaction of the first molecule of pyruvate followed by interaction with FAD gives rise to the vestigial quinone binding site in a conformation which has highest affinity for the herbicides. This leads to the slow, tight inhibition kinetic observed.

Mutants of ALS with reduced herbicide binding have been observed in bacteria, fungi and plants. The wide variation in the level of resistance and the spectra of cross-resistance to different herbicides as well as variations in total ALS activity in some cases suggest that many different mutations at the herbicide binding site can occur. There is molecular evidence at the molecular level for at least ten different sites where amino acid substitutions can occur giving rise to enzymes of varying herbicide-resistance spectra.

1.3.3 **Phytotoxicity**

Although it is clear that certain herbicides act by inhibiting ALS the process by which ALS inhibition kills plants is not fully understood. It has been suggested that by inhibiting ALS these compounds starve the plants of the essential amino acids valine, leucine and isoleucine (Singh *et al.* 1990, Beyer *et al.* 1988, Stidham 1991).

Singh *et al.* (1990) propose that, as imidazolinones are accumulated in the meristematic regions of plants (where ALS activity is greatest) all ALS activity in this region is inhibited preventing synthesis of branched chain amino acids. This lack of amino acids would prevent synthesis of further ALS. The plant would be unable to grow and would suffer acute shortage of branched chain amino acids leading to death (Singh *et al.* 1990). Such arguments also hold for sulfonylurea herbicides (reviewed in Beyer 1988).

In corn root tips inhibition of DNA synthesis by imazapyr could be alleviated by addition of 1mM valine, leucine and isoleucine and total protein synthesis rates remained constant 24h after herbicide application. However, soluble protein levels dropped 40% and the total amino acid pool increased 32% suggesting that inhibition of synthesis of some critical proteins, brought about by limiting levels of branched chain amino acids, may be the cause of phytotoxicity (Shaner and Reider 1986).

However, other studies where levels of branched chain amino acids and protein synthesis have been measured in herbicide treated plants have raised doubts about whether branched chain amino acid starvation is a direct cause of phytotoxicity. In excised pea root tips treated with imazapyr or chlorsulfuron, branched chain amino acid levels did not correlate with rate of cell division (Rost *et al.* 1990). When root tips were treated for 8h at herbicide levels which completely inhibit cell division, pools of branched chain amino acids were reduced by only 50% while levels of herbicide which do not fully inhibit root growth cause a similar reduction in branched chain amino acid pools (Rost *et al.* 1990). This suggests that reduction of

branched chain amino acid pools is not the crucial factor for inhibition of cell division. When pea root tips were cultured in 28 μM chlorsulfuron for 28h, no quantitative differences were seen in protein synthesis although four protein products detected on two dimensional polyacrylamide gels appeared to be peculiar to treated tissue (Clayton and Reynolds 1991). The nature and function of these proteins is not known. The proteins may be produced simply in response to the stress caused by inhibition of cell division (Clayton and Reynolds 1991).

In the synthetic pathway leading to isoleucine production, ALS catalyses the condensation of pyruvate with α–ketobutyrate which comes from deamination of threonine (Figure 1.2). It has been reported that build up of this intermediate is toxic to *E.coli*, and *Salmonella* (Shaw and Berg 1980, Primerano and Burns 1982). *Salmonella* mutant strain DU501 has an inactive *ilv*G gene encoding ALSII. This mutation caused build up of α–ketobutyrate because the ALSI isozyme of *Salmonella* is less efficient at using this substrate than ALSII (Shaw and Berg 1980). It was proposed that transaminase B (Primerano and Burns 1982) was then able to catalyse the transamination of α–ketobutyrate and isoleucine to form α–aminobutyrate and α–keto-β-methylvalerate (Figure 1.2). This reduced the level of isoleucine, removes the normal feedback inhibition of threonine deaminase by isoleucine and caused further build up of α–ketobutyrate. As α–ketobutyrate is competative with the normal substrate of heptapantoate hydroxymethyltransferase, the first step in the pantothenate synthesis pathway, this pathway was also inhibited (Figure 1.2) (Primerano and Burns 1982. Pantothenate is required for production of succinyl coenzyme A, required for methionine synthesis, so mutant DU501 also had a requirement for pantothenate or methionine (Primerano and Burns 1982). The autotoxicity of α–ketobutyrate in strain DU501 could be mimiced by exogenous application of α–ketobutyrate to wild type *Salmonella* (Primerano and Burns 1982).

Other mutants of *Salmonella* have demonstrated that build up of α–ketobutyrate can have further pleiotropic effects. Mutant SMS409 had a defective aspartate aminotransferase enzyme due to mutation of the encoding aspC gene. The mutant was sensitive to sulfometuron-methyl and hypersensitive to exogenous α–ketobutyrate (VanDyk and LaRossa 1986). Aspartate aminotransferase catalyses the transamination of oxaloacetic acid (OAA) from the Kreb's cycle to aspartate. Culture of mutant SMS409 on sulfometuron containing medium caused approximately double the accumulation of α–ketobutyrate in SMS409 mutant *Salmonella* as in the wild type. It was proposed that sulfometuron inhibition led to a build up the substrate α–ketobutyrate which is known to cause a drop in OAA levels. Lower OAA levels would lead to reduction of aspartate production additional to that caused by the aspC mutation. This would lead to extreme shortage of aspartate (VanDyk and LaRossa 1986). Thus the build up of α–ketobutyrate has detrimental pleiotropic effects on metabolic pathways other than branched chain amino acid biosynthesis.

Exogenous addition of a-ketobutyrate affects several metabolic processes including, valine and pantothenate biosynthesis, flux through the tricarboxylic acid cycle and aspartate synthesis (reviewed in VanDyk and LaRossa 1986). Accumulation of a-ketobutyrate in wild type yeast was ten fold more rapid when grown in the presence of valine (which inhibits ALSI) (LaRossa *et al.* 1987). The accumulation of a-ketobutyrate was suppressed by isoleucine, which suppresses the enzyme threonine deaminase, reducing the growth inhibiting effect of sulfometuron. In a mutant with threonine deaminase which is insensitive to feedback inhibition by isoleucine, growth inhibition by sulfometuron was not inhibited by addition of isoleucine (LaRossa *et al.* 1987). The level of sensitivity of several aspC mutants of *Salmonella* was correlated with the ability to convert a-ketobutyrate to other (presumably less toxic) products (LaRossa *et al.* 1987). These data suggest that a-ketobutyrate accumulation is directly involved in toxicity.

If feedback regulation of threonine deaminase in plants is similar to that in bacteria the toxic effects of a-ketobutyrate described above

could also occur in plants. In fact, the situation could be worse in plants as they appear to lack the bacterial ALS isozymes which are less-sensitive to inhibition by herbicides and could divert at least some of the build up of α–ketobutyrate.

Inhibition of ALS does lead to accumulation of the substrate α–ketobutyrate in the plant *Lemna minor* (Rhodes *et al.* 1987). The α–ketobutyrate can be converted to the toxic metabolite α–amino-n-butyrate (Rhodes *et al.* 1987). Valine, leucine, isoleucine, proline and threonine accumulated in fronds of *Lemna minor* when glutamine synthase was inhibited using methionine sulfoximine (Rhodes *et al.* 1987). Labelling studies indicated that the source of these amino acids was protein turnover rather than *de novo* synthesis (Rhodes *et al.* 1986). In contrast the amino acids glutamine, glutamate, alanine, aspartate, asparagine and serine declined rapidly, suggesting that these amino acids are rapidly catabolised within the plant whereas, valine, leucine, isoleucine, proline and threonine are not (Rhodes *et al.* 1986). Chlorsulfuron did not inhibit the methionine sulfoximine-induced accumulation of valine, leucine and isoleucine (Rhodes *et al.* 1986). Thus, protein turnover may be responsible for the fact that branched chain amino acid pools are not totally depleted even at a chlorsulfuron level of 10^{-4} M, much higher than that required to inhibit growth (10^{-8} M). The amino acid α–amino-n-butyrate was detected and represented up to 2.44% of the amino acid pool after 24h when chlorsulfuron was applied at 10^{-4} M . It was suggested that chlorsulfuron inhibition of ALS leads to accumulation of α–ketobutyrate which is transaminated by a constitutive transaminase to α–amino-n-butyrate using glutamate or alanine as α–amino-*N* donors. The accumulation of α–amino-n-butyrate was inhibited by the transaminase inhibitor aminooxyacetate, supporting this hypothesis (Brunk and Rhodes 1988).

Circumstantial evidence that accumulation of α–ketobutyrate may be involved in phytotoxicity also comes from studies of potent inhibitors of another enzyme in the pathway for branched chain amino acid

biosynthesis. Inhibition of the second common enzyme in the pathway ketol-acid reductoisomerase (Fig 1.2), which would not cause accumulation of α–ketobutyrate, is not as phytotoxic as inhibition of ALS (Schloss and Aulbaugh 1990).

There is no direct evidence for phytotoxicity of α–ketobutyrate or α–aminobutyrate towards plants (Clayton and Reynolds 1991). In the absence of such information it is impossible to say whether the build up of these compounds reported in plants is sufficient to cause the types of toxic effects observed in bacteria. It is clear that branched chain amino acid pools are not completely diminished by herbicide inhibition of ALS but it appears that the pools are maintained by protein turnover, meaning that no net increase in protein can occur. Growth inhibition in plants is probably brought about by a combination of the inhibition of protein synthesis due to changes in pools of several amino acids which are brought about by the many changes in amino acid synthesis and catabolism which inhibition of ALS can cause. Accumulation of toxic intermediates and metabolites caused by these same changes may also contribute to toxicity.

If pleiotropic effects caused by inhibition of ALS are important for phytotoxicity, then mutations of many enzymes may affect the level of damage incured. In bacteria it has been shown that mutations causing inactivation of the enzymes aspartate and tyrosine aminotransferase can increase the pleiotropic effects caused by ALS inhibition (VanDyk and LaRossa 1986). Conversly, mutations reducing the rate of transamination of α–ketobutyrate to α–aminobutyrate or increasing the rate of metabolism of α–ketobutyrate by alternative pathways would be expected to reduce the level of damage.

1.3.4 **Selectivity**

A useful feature of many ALS inhibiting herbicides is selectivity between plant species. Sulfonylurea herbicides selective in wheat, barley, corn, soybeans and rape are now commercialised while compounds with selectivity in cotton, potatoes and sugar beet have been reported (Brown 1990). Imidazolinones selective in wheat and soybeans are now available and selective triazolopyrimidines are nearing release (Brown *et al.* 1987a, Kleschick *et al.* 1992).

Many lines of crop species with herbicide resistance have been produced in the laboratory by *in vitro* selection or by transformation with genes encoding herbicide-resistant ALS but none of these have, so far, been used commercially. The *csr*1 gene of *Arabidopsis* has been used transform chicory, lettuce, flax, *Helianthus*, and *Dendrantema* among other species in addition to those discussed in section 1.3.2 (Vermeulen *et al.* 1992). *In vitro* selection has also been used to select imidazoline resistant wheat lines with herbicide insensitive ALS (Newhouse et al. 1992).

The selectivity of commercial sulfonylurea and imidazoline herbicides is not due to differences in herbicide sensitivity of ALS between species. The level of extractable ALS and the I_{50} of ALS for imazapyr, imazathapyr and sulfometuron vary between species (Table 1.7) (Singh *et al.* 1990, Brown 1990) but there is little correlation between either the level of ALS activity, or the I_{50} for herbicides, with the level of resistance observed at the whole plant level (Singh *et al.* 1990, Brown 1990). A significant difference in growth response due to the sulfonylurea DPX-N8189 is due to a naturally less-sensitive ALS in soybeans and some other legume crop species when compared to certain weed species (Saari *et al.* 1991). However, no commercial herbicides, selective due to differential enzyme sensitivity, have been reported.

Similarly, differences in uptake and translocation cannot account for selectivity. Both sulfonylurea herbicides and imidazolinones accumulate in plants by the mechanism of ion trapping of weak acids

caused by preferential movement of the protonated species from the more acidic apoplast to the less acid symplast where they tend to dissociate (Brown 1990, VanEllis and Shaner 1988). The small differences measured in uptake and translocation between species do not account for selectivity (Brown 1990).

Table 1.7 Acetolactate synthases from different plant species

Species	Specific ALS activity		I_{50}		
	µmoles acetoin mg protein^{-1}h^{-1}	µmoles acetoin g fresh wt^{-1} h^{-1}	Imazapyr (µM)	Imazathapyr (µM)	Sulfometuron (nM)
Maticaria	0.04	0.2	9.5	7.9	74.1
Flax	0.06	0.3	2.8	1.7	50.1
Sunflower	0.07	0.6	4.7	2.9	31.3
Arabidopsis	0.12	0.7	5.1	1.8	7.3
Ragweed	0.07	0.7	14.1	5.4	21.9
Barley	0.09	0.9	7.6	4.0	38.0
Wheat	0.08	0.9	17.8	5.7	32.4
Rape	0.08	0.9	2.9	1.3	20.5
Amaranthus	0.19	1.3	3.5	2.0	21.0
Spinach	0.31	1.7	1.4	0.4	4.0
Tobacco	0.34	1.7	3.0	0.8	7.9
Sorghum	0.28	1.8	5.9	2.1	27.6
Soybean	0.22	2.1	19.4	51.3	95.5
Mustard	0.42	2.1	2.6	1.5	17.8
Corn	0.37	2.8	4.0	1.2	23.4
Lima bean	0.26	2.9	7.5	18.4	160.0
Pea	0.50	5.9	31.7	83.2	53.7
BMS cells	5.36	16.4			

From Singh *et al* 1990

In all reported instances, selectivity of commercial herbicides which inhibit ALS results from differences between species in the rate of metabolism of the herbicides. The mechanisms by which the herbicides are

metabolised differ widely between species and between herbicide analogues.

The sulfonylurea herbicides chlorsulfuron, metsulfuron-methyl and triasulfuron are selective in wheat. Although ALS from wheat is sensitive to inhibition by these compounds, the plant is tolerant because it has the capacity to rapidly detoxify them (Sweetser *et al.* 1982, Anderson *et al.* 1989, Meyer and Muller 1989). The initial step in the detoxification of these three compounds is aryl hydroxylation of the phenyl ring mediated by a cytochrome P450-dependant mixed function oxidase system (MFO) (Sweetser 1985, Frear *et al.* 1991) followed by rapid conjugation with glucose (Figure 1.6). A second detoxification mechanism for chlorsulfuron is present in tolerant dicots. The initial step in this process is hydroxylation of the methyl substituent of the triazine ring (Hutchison *et al.* 1984) (Fig. 1.6). Detoxification of metsulfuron-methyl also involves alternative pathways in wheat and barley (Brown 1990). In contrast to chlorsulfuron, metsulfuron-methyl and triasulfuron, wheat is sensitive to sulfometuron-methyl which is not rapidly metabolised (Sweetser 1985). The slower rate of sulfometuron-methyl metabolism compared to metsulfuron metabolism in wheat is due to both a reduction in the rate of hydroxylation and a reduction of sugar conjugation of the primary metabolite (Anderson and Swain 1992).

Similarly the imidazolinone herbicide imazamethabenz is selective in wheat due to rapid metabolism (Brown *et al.* 1987). The first step in metabolism is oxidation, possibly mediated by an MFO system, followed by glucose conjugation (Fig 1.7). In contrast imazapyr is lethal to wheat, as it is not rapidly detoxified. Lack of a metabolically labile alkyl substituent on the phenyl ring is thought to play a crucial role in lack metabolism of imazapyr (see Chapter 3, Figure 3.1 for structure of imazapyr).

Thifensulfuron is also rapidly metabolised by wheat but the thiophene ring is not as susceptible to oxidation as the phenyl rings of some other sulfonylureas. Instead the compound is metabolised by a combination of de-esterification, hydrolysis of the urea or sulfonamide bonds of the

bridge and to a lesser extent O-demethylation of the methoxy substituent of the triazine ring (Brown 1990).

O-demethylation is also the mechanism by which rice detoxifies bensulfuron methyl (Levitt *et al.* 1981). In contrast chlorimuron ethyl is metabolised in soybeans by conjugation to homoglutathione (Brown and Neighbors 1987). The imidazolinones imazaquin and imazathapyr are selective in soybeans due to differential metabolism (Shaner and Robson 1985).

Triazolopyrimidines are also selective between species due to enhanced metabolism (Hodges 1990, Kleschick *et al.* 1992). Metabolism of triazolopyrimidine herbicides involves methyl hydroxylation or hydroxylation of the aniline ring followed by glucose conjugation in both monocot and dicot species, the rate of the reaction being the determinate of resistance (Hodges 1990).

Thus, crop selectivity of herbicides which inhibit ALS is due to rapid detoxification in tolerant species. There are many different mechanisms by which ALS inhibitors can be metabolised but it is the rate of detoxification which determines resistance. Typically, sulfonylurea-tolerant species metabolise the herbicide with a half life of from one to five hours while sensitive species require greater than twenty hours (Brown 1990).

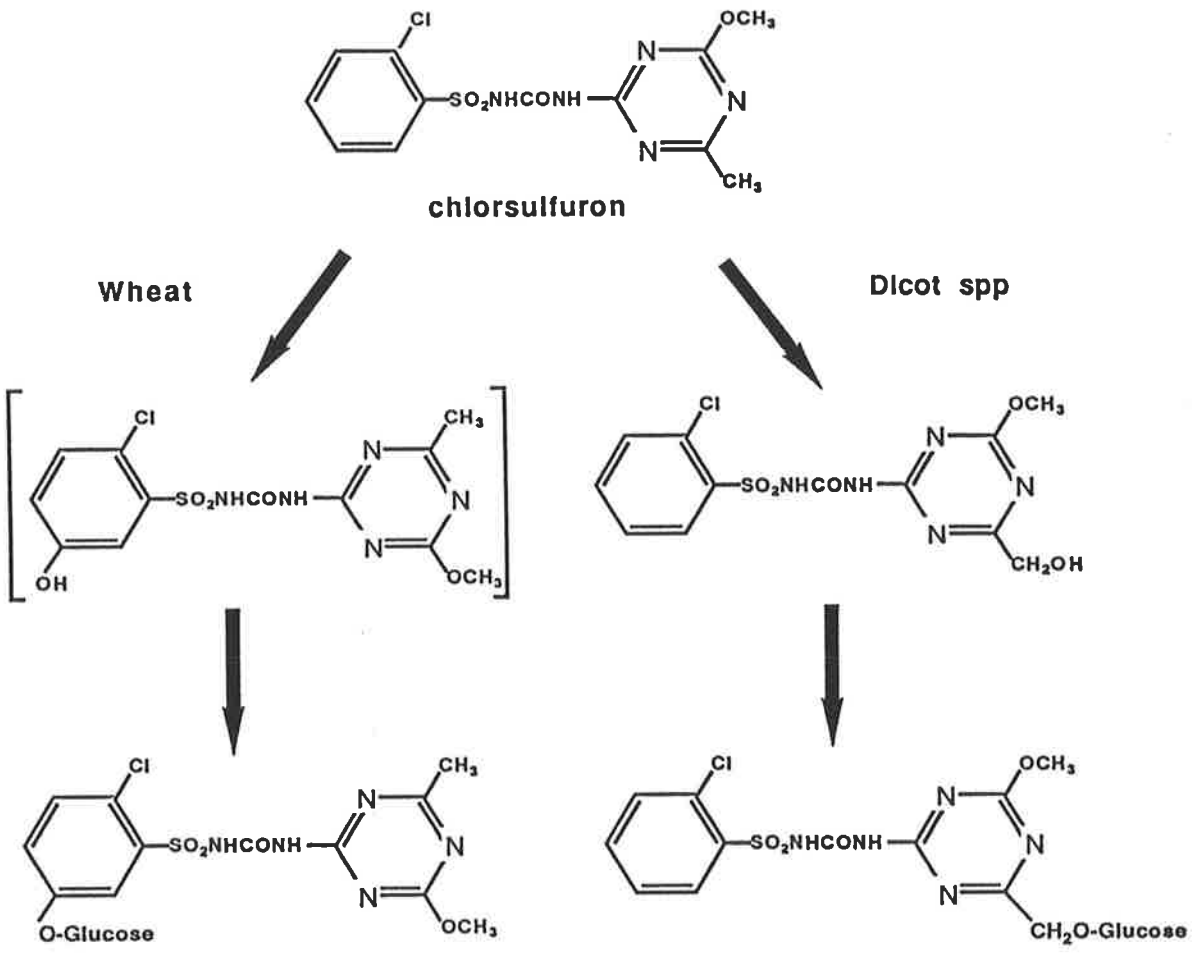


Figure 1.6 Chlorsulfuron metabolism in wheat and resistant dicots. From Sweetser *et al.* 1982 and Hutchison *et al.* 1984.

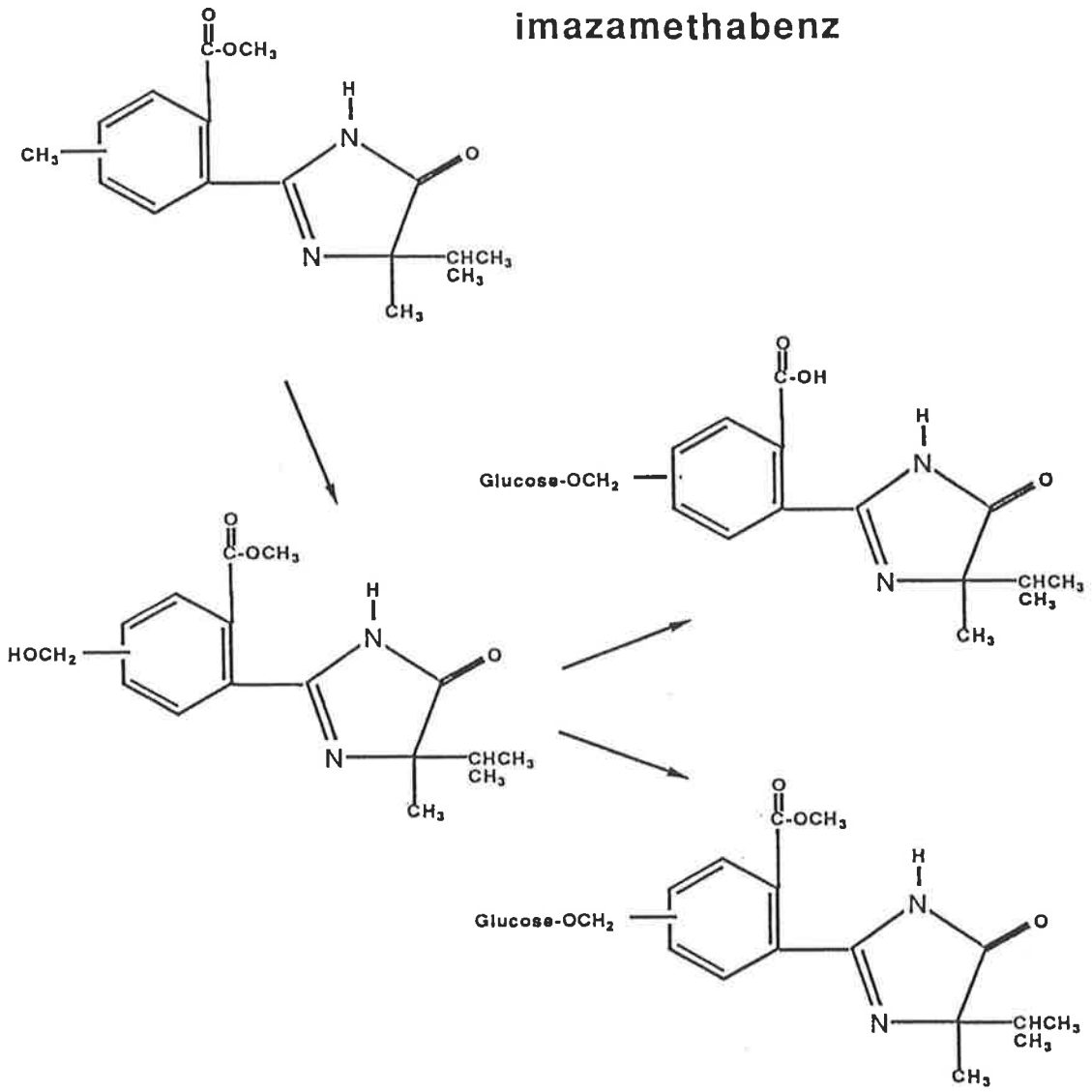


Figure 1.7 Imazamethabenz metabolism in wheat. From Brown *et al.* 1987.

1.3.5 Modes of resistance to ALS inhibiting herbicides

It is clear from studies of herbicide-resistance obtained by selection in the laboratory and herbicide-selectivity between weed and crop species, that plants may avoid the toxic effects of ALS inhibition by at least two mechanisms. They may avoid toxicity by mutation of the enzyme itself or they may be able to rapidly metabolise the herbicide.

Other, independent mechanisms of resistance have been observed in yeast (Falco and Dumas 1985). Of sixty six sulfometuron-resistant mutants fifty were found to be ALS mutants but ten did not have herbicide-insensitive ALS. These non-ALS mutations caused a lower level of herbicide resistance and were inherited at two separate loci. Seven mutations were found to be allelic with the *pdr*1 locus of yeast which encodes for pleiotropic drug resistance. Mutations at this locus confer drug resistance by affecting the permeability of the cell to inhibitors (Falco and Dumas 1985). Mutation at this locus in yeast was shown to confer cycloheximide resistance as well as sulfometuron resistance. This locus maps to yeast chromosome VII while the ILV2 gene encoding ALS is on chromosome XIII. A third type of low level resistance inherited at an unknown locus on chromosome XV was also identified. The mechanism of this third type of resistance is unknown (Falco and Dumas 1985).

If the pleiotropic effects of ALS inhibition are important to the phytotoxicity of herbicides then mutations of the other enzymes involved would also affect the level of damage (see section 1.3.3).

1.3.6 Resistance in weed species to ALS inhibiting herbicides

Herbicides which inhibit ALS have been widely used in major cereal growing areas since the introduction of chlorsulfuron in the early 1980s. The first confirmed report of resistance came in 1986 with the discovery of chlorsulfuron-cross resistance in a biotype of *L.rigidum.*. Since then many populations of seven weed species have developed resistance to ALS-inhibiting herbicides throughout the cropping regions of Australia and North America.

The persistent use of sulfonylureas in areas of continuous cropping and in industrial areas and easements in North America has resulted in the appearance of resistant weed biotypes. The first 2 confirmed cases of sulfonylurea resistance following selection by chlorsulfuron were reported in *Kochia scoparia* L. and *Lactuca serriola* L. (Primiani *et al.* 1990, Mallory-Smith *et al.* 1990). Since then chlorsulfuron resistance has been confirmed in *Salsola iberica* , *Stellaria media* and *Lolium perenne* at many locations in the USA and Canada (McKinley 1990). In all reported instances resistance is due to herbicide-insensitive ALS (Hall and Devine 1990, Saari *et al.* 1990 and 1992)

In contrast all reports of sulfonylurea–resistance in Australia, prior to commencement of this study in 1989, were instances of cross-resistance in *L. rigidum* following development of resistance to the selective aryloxyphenoxypropionate herbicide diclofop-methyl (Heap and Knight 1990).

1.4 Herbicide cross-resistance in *Lolium rigidum*

The first confirmed report of herbicide resistance in *L.rigidum* came with the report of diclofop-methyl resistance in 1982 only 4 seasons after that compound had been introduced to the Australian market (Heap and Knight 1982). More surprising was the finding that the population was also cross-resistant to the sulfonylurea herbicide chlorsulfuron which was not registered for use in Australia until later that year (Heap and Knight 1986). Although cross-resistance to insecticides had been widely reported (Georgiou 1986) this was the first confirmed report of cross-resistance to herbicides from different chemical groups with different modes of action. The level and spectrum of cross-resistance varies greatly between biotypes (Heap and Knight 1990). By 1990 biotypes of ryegrass were known to have resistance to herbicides of six different modes of action. Few cases of such wide herbicide cross-resistance have been reported. Cross-resistance in *Alopecurus myosuroides* is the only comparable example (Moss 1990).

In the diclofop-methyl resistant, chlorsulfuron cross-resistant biotype SLR31 metabolism of diclofop is slightly greater than that of a susceptible biotype but this factor alone is not considered likely to explain the level of resistance observed (Holtum *et al.* 1991). Diclofop resistance in *L.rigidum* biotypes correlates with the ability to recover from membrane depolarisation caused by diclofop (Hausler *et al.* 1991). In biotypes with herbicide-sensitive ACCase, resistance may be due to a combination of factors, including slightly enhanced detoxification, the ability of the membrane to recover from depolarisation and possibly some other factor such as sequestration of the herbicide away from the chloroplast (Holtum *et al.* 1991). Cross-resistance to chlorsulfuron in biotype SLR31 was not due to a herbicide insensitive ALS (Matthews *et al.* 1990).

1.5 Objectives of this project

The aim of this project was to determine the mechanism or mechanisms by which biotypes of *L.rigidum* can be resistant to herbicides which inhibit ALS.

Studies of the chlorsulfuron cross-resistant, diclofop-methyl resistant biotype SLR31, had failed to reveal a mechanism which might fully explain resistance to either herbicide. Determination of the mechanism of chlorsulfuron resistance in biotype SLR31 might elucidate some common feature of the mechanism which could explain the development of chlorsulfuron cross-resistance. If the mechanisms of resistance to the two herbicides were unrelated, however, it might be simply a matter of chance that both mechanisms have occurred in some biotypes.

During the course of this study suspected chlorsulfuron-resistant biotypes of *L.rigidum* were reported from fields which had been exposed to chlorsulfuron in several growing seasons. Studies were undertaken to determine whether these biotypes were resistant and, if so, whether the mechanism of resistance in these biotypes was similar to that of the chlorsulfuron cross-resistant biotypes already known.

Chapter 2

Materials and Methods

In this section general methods will be described. Modifications to these methods and specific techniques are detailed in the relevant chapters.

2.1 Materials

2.1.1. Plant material

Lolium rigidum (Gaud.) biotypes

VLR1 Collected in a pasture field with no prior herbicide history in Victoria near Bordertown. Susceptible to all herbicides registered for control of ryegrass in Australia.

VLR4 Collected in 1989 from a field near Lake Bolac in Victoria which received four treatments with chlorsulfuron, three treatments with metolochlor, two treatments with alachlor and two treatments with paraquat+diquat from 1984 to 1989. .

VLR6 Collected from a field in Victoria which received five treatments with diclofop-methyl, and three treatments with simazine and . The biotype is resistant to diclofop-methyl but susceptible to chlorsulfuron (Heap and Knight 1990)

SLR2 Collected from a pasture field near Bordertown in South Australia with no prior herbicide history. The biotype is susceptible to all herbicides known to control *L. rigidum* (Heap and Knight 1990).

SLR19 Collected in 1991 from a field near Yorktown, South Australia treated with one application of trifluralin, three applications of diuron + MCPA, one application of metsulfuron-methyl and five applications of chlorsulfuron from 1983 to 1991

SLR31 Collected from a field near Bordertown, South Australia treated with twelve applications of trifluralin, four applications of diclofop-methyl. The biotype is resistant to aryloxyphenoxypropionate herbicides and cross-resistanat to chlorsulfuron (Heap and Knight 1986).

NLR12 Collected in 1986 from a field in New South Wales treated with 4 applications of trifluralin and 7 applications of diclofop-methyl. The biotype is resistant to aryloxyphenoxypropionate herbicides and cross-resitant to chlorsulfuron. (Heap and Knight 1990).

WLR1 Collected in 1989 from a field near Jerramungup in Western Australia treated with chlorsulfuron in 7 consecutive years.

Number of herbicide applications			
Biotype	diclofop	chlorsulfuron	Other herbicides *
SLR2	0	0	
VLR1	0	0	
VLR6	5	0	3 simazine
SLR31	4	0	12 trifluralin
NLR12	7	0	4 trifluralin
VLR4	0	4	3 metolachlor 2 alachlor 2 paraquat+diquat
SLR19	0	5	1 trifluralin 3 diuron + MCPA
WLR1	0	7	

* chemical names listed in abbreviations

Other species

Wheat (*Triticum aestivum* L.) cultivar Machete

Wheat is resistant to chlorsulfuron due to the ability to rapidly detoxify the herbicide despite having ALS which is sensitive to herbicides (Sweetser *et al.* 1982).

Flax (*Linium usaitatissimum* L.)

Flax is also resistant to chlorsulfuron due to its ability to rapidly metabolise the herbicide despite having ALS which is sensitive to herbibicides (Hutchison *et al.* 1984)

2.1.2 **Chemicals**

Herbicides used for dose response experiments. Commercial formulations registered for use in Australia were used where available.

Common Name*	Proportion Active Ingredient	Trade Name	Manufacturer
chlorsulfuron	750 g ai kg^{-1}	Glean	DuPont Australia Ltd
diclofop-methyl	375 g ai L^{-1}	Hoegrass	Hoesht Australia Ltd.
imazamethabenz	300 g ai L^{-1}	Assert ##	American Cyanamid Aust. Ltd.
imazapyr	200 g ai L^{-1}	Arsenal #	American Cyanamid Aust. Ltd.
imazathapyr	200 g ai L^{-1}	Pursuit ##	American Cyanamid Aust. Ltd.
metsulfuron-methyl	750 g ai kg^{-1}	Ally ##	DuPont Australia Ltd
sethoxydim	117 g ai L^{-1}	Sertin	Schering Austraia Ltd.
sulfometuron-methyl	750 g ai kg^{-1}	Oust #	DuPont Australia Ltd
triasulfuron	710 g ai kg^{-1}	Logran	Ciba-Geigy Australia Ltd.

* chemical names listed in abbreviations

\# Non selective herbicide

selective herbicide not registered for *L.rigidum* control

Surfactant

Agral 600, (ICI Australia Ltd.), used at 2 ml L^{-1} unless otherwise indicated.

Triton X100, (BDH Chemicals Australia Ltd.)

Technical grade reagents used in ALS enzyme inhibition studies.

Common name *	Purity	Manufacturer
chlorsulfuron	98%	DuPont Ltd
imazamethabenz	97.8%	American Cyanamid Ltd
imazapyr	98.9%	American Cyanamid Ltd
imazathatpyr	98.8%	American Cyanamid Ltd.

* chemical names listed in abbreviations

Radioisotopes

[phenyl-U-^{14}C]-chlorsulfuron, 98% radiopurity, 11.5 μCi mg^{-1}, DuPont Ltd

2.2 Plant culture

2.2.1 Germination

Seeds of ryegrass were germinated in dishes containing a 0.5cm layer of 0.6% agar and maintained for 7 days in a growth chamber under conditions of 22C, 14h, 20-30 μE $m^{-2}s^{-1}$ light period/ 15C, 10h dark period. Seedlings were transplanted when 2cm in size approximately 7 days after commencement of germination. Plants of even size and vigour where chosen for transplanting into the final culture medium.

Wheat seed was directly sown into potting medium at the time of transplanting of ryegrass so that the two species reached the two leaf stage at the same time allowing herbicide treatments to be carried out at an equal stage of development. Flax was also directly sown.

2.2.2 Pot culture

Germinated seedlings were transfered to 2L pots containing potting soil based on a mix of sand and peat, either 12 plants per pot (4cm spacings) or 21plants per pot (3 cm spacings) at a depth of 1 cm. Plants were grown outdoors during the normal autumn-winter growing season for this species (unless otherwise specified).

2.2.3 Hydroponic culture

HydropOnic culture in 250 ml vials.

Seedlings germinated as above where transfered to 250mL polycarbonate vials containing nutrient medium based on that of Hoagland (1938) (see below). Tweny one plants per vial (1.5cm spacings) were supported by fiberglass mesh so that the roots passed through to the nutrient medium and the shoots extended outward through 0.5 cm holes in the blackenned plastic lid (Fig. 2.2.3.(I)). Vials where wrapped with aluminium foil to prevent light reaching the roots. Nutrient solution was changed at the time of treatment (when plants were at the two leaf stage) after which the solution was regularly replenished to replace transpiration losses.

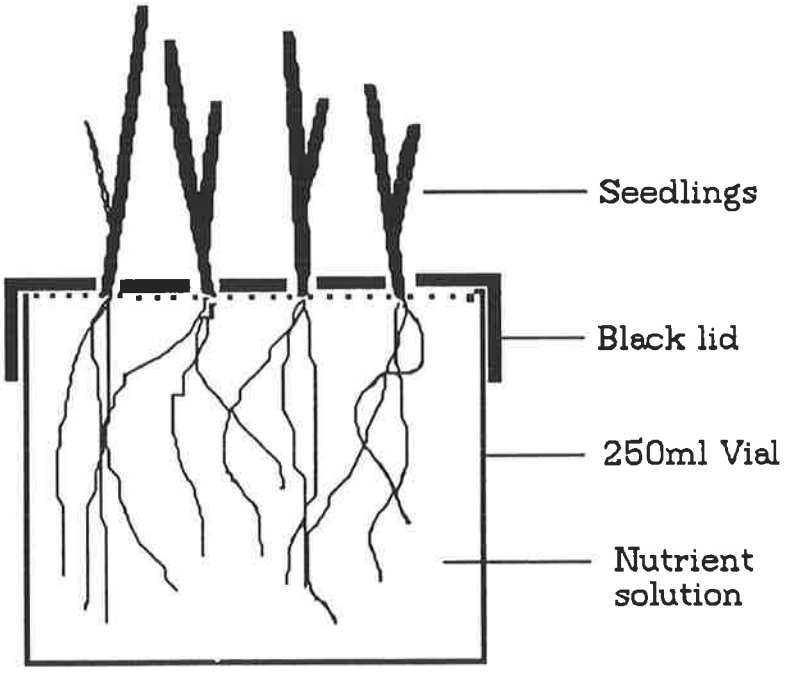


Figure 2.2.3.(I) Hydroponic culture of ryegrass in 250mL vials

Hydroponic culture in 4L trays.

Seedlings germinated on agar (2.2.1) were transferred to styrofoam trays containing 63 individual cells 2.5 x 2.5cm which were floated on nutrient medium in trays of 40 x 30 x 12cm. Each seedling.was supported at the base within each cell by black polyacrylic beads suspended on fibre glass mesh through which the roots passed into the nutrient solution. Light was excluded from the trays using black plastic sheet.

Hydroponic nutrient medium (based on Hoagland 1938)

Nutrient Source	Final Concentration (μM)
KH_2PO_4	500
K_2SO_4	400
$MgSO_47H_2O$	1000
$Ca(NO)_24H_2O$	1670
KNO_3	1670
EDTA* Na_22H_2O	64
$FeSO_47H_2O$	72
$CaSO_42H_2O$	800
$Na_2MoO_42H_2O$	0.25
$CuSO_45H_2O$	0.16
$ZnSO_47H_2O$	0.38
$MnCl_24H_2O$	4.60
H_3BO_3	23.00

* chemical names listed in abbreviations

2.3 Herbicide treatment

Plants were treated with herbicide 7 to 14 days after transplanting when at the two leaf stage and 5cm in height. This is the growth stage when plants are most susceptible and when treatment is recommended under agricultural conditions.

Herbicides were applied with a laboratory cabinet sprayer. The herbicide was delivered *via* two 110° hydraulic nozzles in a total volume of 113L ha^{-1} at a pressure of 250kPa. They were applied as commercial formulations supplied by the manufacturer with 0.2% v/v Agral 600 surfactant added. Twenty four plants (two pots of 12 plants each) or 21 plants (one pot) were used for each treatment. Plants were harvested 4 to 8 weeks after spraying allowing surviving plants to make noticable growth before harvest. Plants which were green, had live meristems and had increased in size beyond the 2 leaf stage where classed as live plants and were harvested at ground level. Material from live plants (unless otherwise stated) was dried at 80C for 48h and dry weight determined.

Chapter 3

Differences in the response to herbicides of two *L.rigidum* biotypes suggest two resistance mechanisms.

3.1 Introduction

The response to herbicides was compared between two *L.rigidum* biotypes which had been exposed to different herbicide treatment in the field. Firstly, *L.rigidum* biotype SLR31 which became cross-resistant to chlorsulfuron following exposure to diclofop-methyl (Heap and Knight 1986, reviewed in section 1.4). Secondly, *L.rigidum* biotype WLR1, which was collected from a field which had been treated with chlorsulfuron in seven consecutive years, but had not been exposed to diclofop-methyl. Studies of the response of these two biotypes to a range of herbicides were conducted to elucidate the most likely mechanisms of resistance in the cross-resistant biotype SLR31 and the putative resistant biotype WLR1. This information was required to direct further studies into the mechanism of resistance and to assist farmers to devise strategies for management of resistant *L.rigidum* populations.

Cross-resistant biotype SLR31 became resistant to the aryloxyphenoxypropionate herbicide diclofop-methyl following exposure to the herbicide in four consecutive years (Heap and Knight 1982). This

biotype was subsequently found to be cross-resistant to the sulfonylurea herbicide chlorsulfuron which acts by inhibition of ALS (reviewed in section 1.3) (Heap and Knight 1986). Aryloxyphenoxypropionate herbicides act by inhibition of the plastidic enzyme acetyl coenzymeA carboxylase which is involved in lipid synthesis and other synthetic pathways (reviewed in section 1.2.4). These herbicides do not inhibit ALS and are chemically dissimilar from herbicides which do. As biotype SLR31 had not been exposed to herbicides which inhibit ALS, individuals with herbicide-insensitive mutations of this enzyme should not have been favoured by the herbicide treatment. ALS from this biotype remains sensitive to inhibition by chlorsulfuron (Matthews *et al.* 1990). Thus, cross-resistance to ALS inhibiting herbicides in this biotype is not due to the chance occurrence of herbicide-insensitive ALS.

The crop-selectivity of sulfonylurea and imidazolinone herbicides is due to rapid detoxification in tolerant species (reviewed in section 1.3.4). Like *L.rigidum* biotype SLR31, wheat has herbicide-sensitive ALS. Wheat is tolerant to chlorsulfuron because it can rapidly detoxify the herbicide (Sweetser *et al.* 1982). In contrast wheat is sensitive to sulfometuron-methyl which it cannot rapidly metabolise (Sweetser *et al.* 1985). Similarly, wheat is tolerant to the imidazolinone herbicide imazamethabenz which it can rapidly metabolise, but is sensitive to imazapyr which it cannot (Brown *et al.* 1987) (see section 1.3.4 for pathways). It is, therefore, possible that resistance to chlorsulfuron in *L.rigidum* biotype SLR31 could be due to rapid detoxification, as has been documented for wheat. If chlorsulfuron resistance in *L.rigidum* biotype SLR31 and chlorsulfuron tolerance in wheat are both due to increased metabolism, the spectrum of resistance to herbicides in SLR31 should be similar to that in wheat.

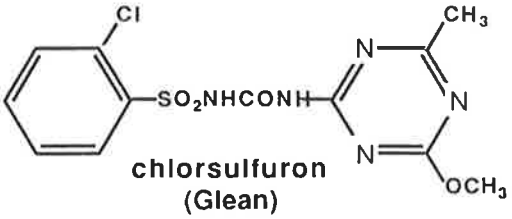
L.rigidum biotype WLR1 was collected from a field which had been treated with chlorsulfuron in seven consecutive seasons (section 2.1). Chlorsulfuron failed to control this population in 1989. This was the first, putative, report of sulfonylurea resistance resulting from the use of a

sulfonylurea herbicide in Australia. Cases of sulfonylurea resistance following the use of sulfonylureas had been reported in North America (reviewed in section 1.3.6). Resistance in these North American biotypes is due to a mutant form of ALS which causes resistance to both crop-selective and non-selective sulfonylureas (Saari *et al.* 1990 and 1992). As *L.rigidum* biotype WLR1 was selected by the use of a sulfonylurea herbicide the mechanism of resistance may be similar to that of similarly selected weed biotypes from North America. If this were the case the spectrum of resistance to herbicides of biotype WLR1 would differ from that of wheat.

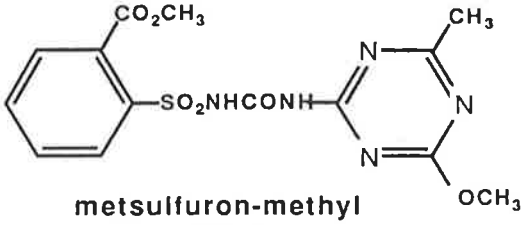
To test these questions, experiments were conducted to determine the spectrum of resistance of *L.rigidum* biotypes SLR31 and WLR1 to herbicides which inhibit ALS. The crop-selective herbicides, chlorsulfuron, triasulfuron, metsulfuron-methyl and imazamethabenz were tested (Figure 3.1.). These herbicides are rapidly detoxified by tolerant crop species (Sweetser *et al.* 1982, Anderson *et al,* 1989, Meyer & Muller 1989) . The non-selective herbicides sulfometuron-methyl and imazapyr, which are not rapidly metabolised, were also tested (Figure 3.1).

Previously reported instances of chlorsulfuron resistance in biotypes of *L.rigidum* have all been cases of cross-resistance which have arisen following selection with diclofop-methyl (Heap and Knight 1990). This observation raises the question of whether the opposite may also occur. Could treatment of *L.rigidum* populations with a sulfonylurea herbicide lead to cross-resistance to dissimilar herbicides with different mode of action, such as diclofop-methyl? To test whether this had occurred in biotype WLR1 the herbicide response of this biotype was compared to that of biotype SLR31 for herbicides from several different chemical groups, which do not inhibit ALS.

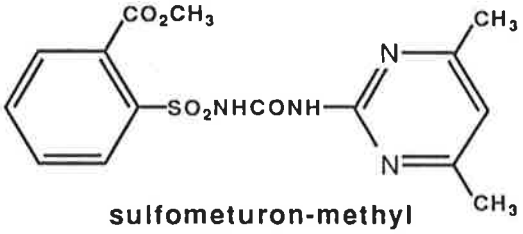
The response of biotype WLR1 was tested for herbicides to which biotype SLR31 is known to exhibit resistance. Herbicides from two chemical groups, the aryloxyphenoxypropionates and cyclohexanediones, which act by



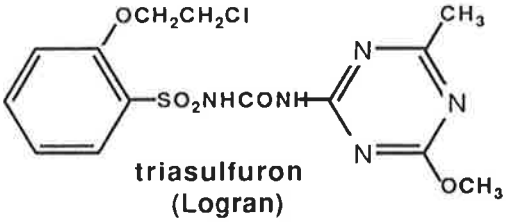
chlorsulfuron
(Glean)



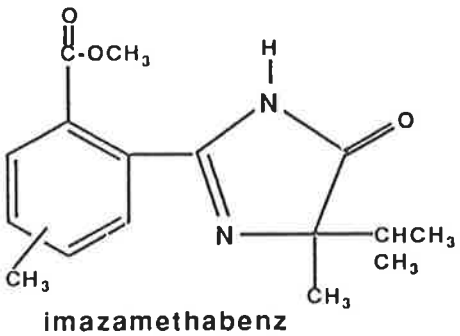
metsulfuron-methyl
(Ally)



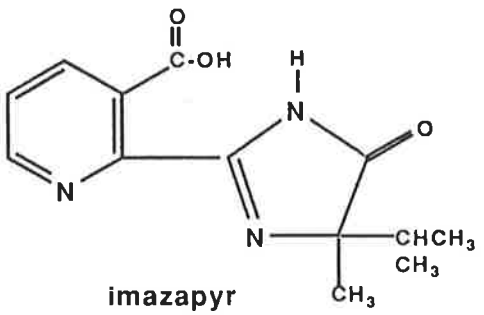
sulfometuron-methyl
(Oust)



triasulfuron
(Logran)



imazamethabenz



imazapyr

Figure 3.1 Structures of three crop-selective sulfonylureas: chlorsulfuron, metsulfuron-methyl, and triasulfuron and the non-selective sulfonylurea, sulfometuron-methyl plus the crop-selective imidazolinone, imazamethabenz, and the non-selective imidazolinone, imazapyr.

inhibiting ACCase were tested. These included diclofop-methyl, fluazifop-butyl, haloxyfop-ethoxyethyl and sethoxydim. The dinitroanaline herbicides trifluralin and pendimethlin, which act by inhibition of microtubule formation were also tested.

Herbicides to which biotype SLR31 does not exhibit resistance were also studied. Herbicides tested included the photosynthesis inhibitors diuron and metribuzin. Glyphosate, which inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase, and paraquat , which acts by intercepting electron flow in photosystem I, were also tested.

3.2 Materials and Methods

3.2.1 Sulfonylurea and imidazolinone herbicides

Wheat and *L.rigidum* biotypes SLR31, WLR1 and the susceptible VLR1, were grown in pots in the field, treated with herbicides and harvested as described in sections 2.2 and 2.3. Twenty four plants per treatment were used. As 50% growth reduction could not be achieved with some biotypes using some herbicides the amount of herbicide required to reduce dry matter by 25% compared to untreated plants was calculated. Experiments using chlorsulfuron, triasulfuron, metsulfuron-methyl, imazamethabenz, imazapyr and diclofop-methyl were repeated three times in the autumn-winter seasons of 1989, 1990, and 1991.

3.2.2 Herbicides which do not inhibit ALS

Experiments using haloxyfop-ethoxyethyl, fluazifop-butyl, sethoxydim, diuron, metribuzin, glyphosate and paraquat were conducted using plants grown in pots in the field as described in section 2.2 and 2.3. using 24 plants per treatment.

A different technique was employed for experiments using the pre-emergent herbicides trifluralin and pendimethalin. Potting medium was placed in 2L pots to a level 5 cm below the rim of the pot. A measured volume containing approximately 200 seeds of *L.rigidum* biotypes VLR1, SLR31 or WLR1 was evenly spread on the soil surface of each pot before the seed was covered with a 1 cm layer of potting medium. Herbicide was applied to the surface of the potting medium as described in section 2.3 and covered with a further 1 cm layer of potting medium. Pots were watered and kept in the field until harvest 28 days after treatment. Shoots where harvested at soil level and the dry weight determined. Total dry weight of material from each pot was compared to that of untreated pots.

3.3 Spectra of herbicide resistance

3.3.1 Crop-selective sulfonylurea and imidazolinone herbicides

The dose response to crop-selective sulfonylurea and imidazolinone herbicides of susceptible *L.rigidum* biotype VLR1, wheat and *L.rigidum* biotypes SLR31 and WLR1 are shown in Figures 3.2 to 3.5. *L.rigidum* is normally controlled by the crop-selective herbicides, chlorsulfuron, triasulfuron and metsulfuron-methyl at application rates of 16, 35 and 8 g ai ha^{-1} respectively as demonstrated by the response of susceptible biotype VLR1 (Figures 3.2 to 3.4). Similalry, wheat is tolerant to these herbicides (Figures 3.2 to 3.4). In contrast to the susceptible biotype VLR1, biotype SLR31 survives high levels of these herbicides (Figures 3.2 to 3.4, Table 3.1). At the normal field rates there was little mortality of biotype SLR31. Although the crop-selective imidazolinone herbicide imazamethabenz is not registered for *L.rigidum* control, at doses greater than 500 g ai ha^{-1} this herbicide showed activity against the susceptible biotype VLR1 (Figure 3.5). However, there was little control of biotype SLR31 or wheat even at application rates up to 4000 g ai ha^{-1} (Figure 3.5). Thus, biotype SLR31 is resistant to all the wheat-selective ALS inhibitors tested.

Biotype WLR1 is also resistant to the selective sulfonylurea and imidazolinone herbicides (Figures 3.2 to 3.5, Table 3.1). Thus, biotype WLR1 is the first reported example of sulfonylurea resistance in *L.rigidum* caused by exposure to a sulfonylurea herbicide. However, biotype WLR1 does not exhibit the same response to these herbicides as that observed for biotype SLR31. Biotype WLR1 is more resistant to these herbicides than is biotype SLR31. Mortality of biotype WLR1 is less than or equal to that of SLR31 at all doses of each herbicides (Figures 3.2 to 3.5).

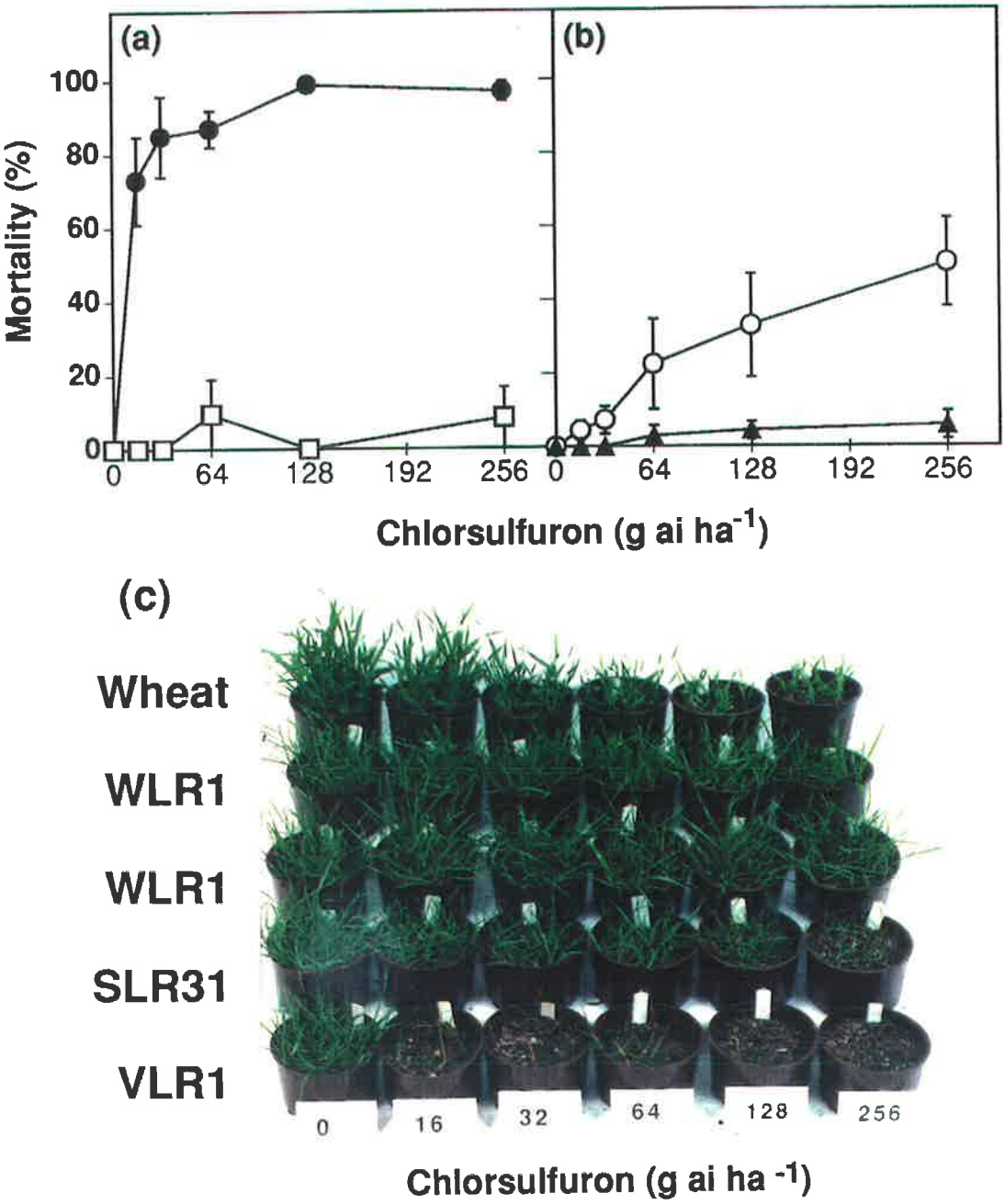


Figure 3.2 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the cereal-selective sulfonylurea herbicide, chlorsulfuron. Application rates of 8 to 16 g ai ha^{-1} are recommended for *L.rigidum* control in Australia. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

Plants from one replicate experiment of those represented above, photographed at the time of harvest (c).

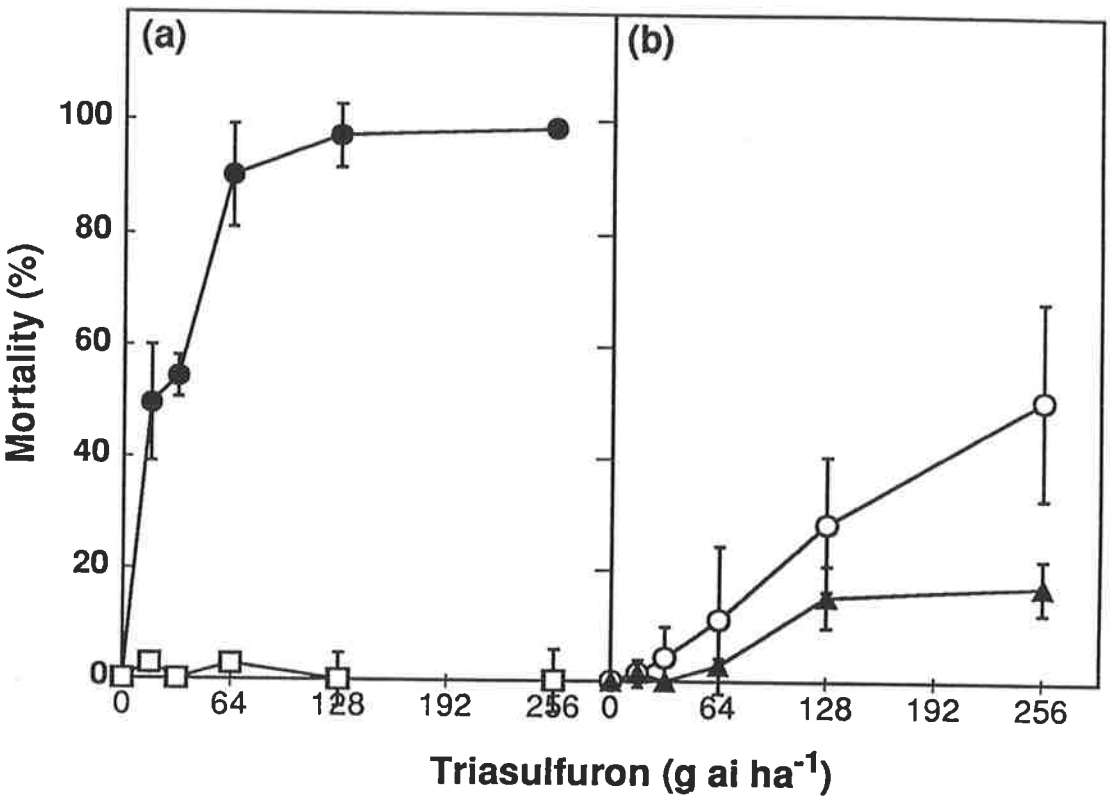


Figure 3.3 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the cereal-selective sulfonylurea herbicide, triasulfuron. The application rate of 35 g ai ha^{-1} is recommended for *L.rigidum* control in Australia. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

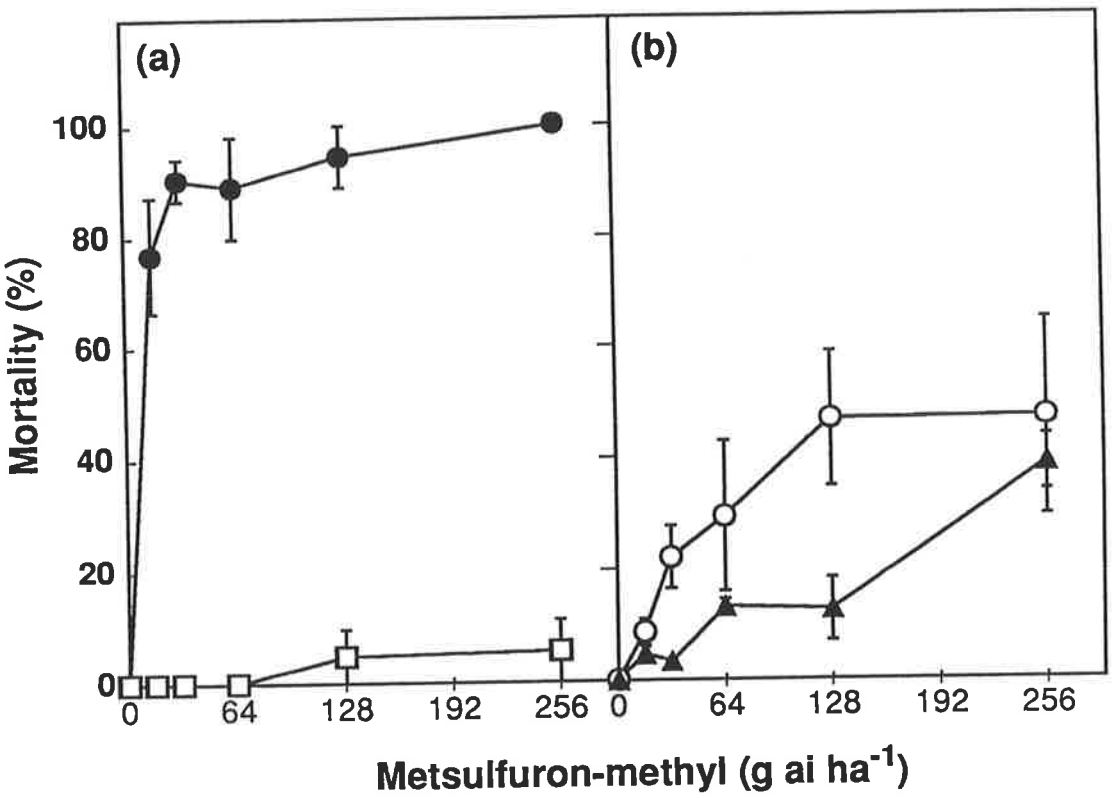


Figure 3.4 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the cereal-selective sulfonylurea herbicide, metsulfuron-methyl. This herbicide is not recommended for *L.rigidum* control in Australia but application rates of 4 to 8 g ai ha^{-1} are used to control broadleaf weeds in cereal crops. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

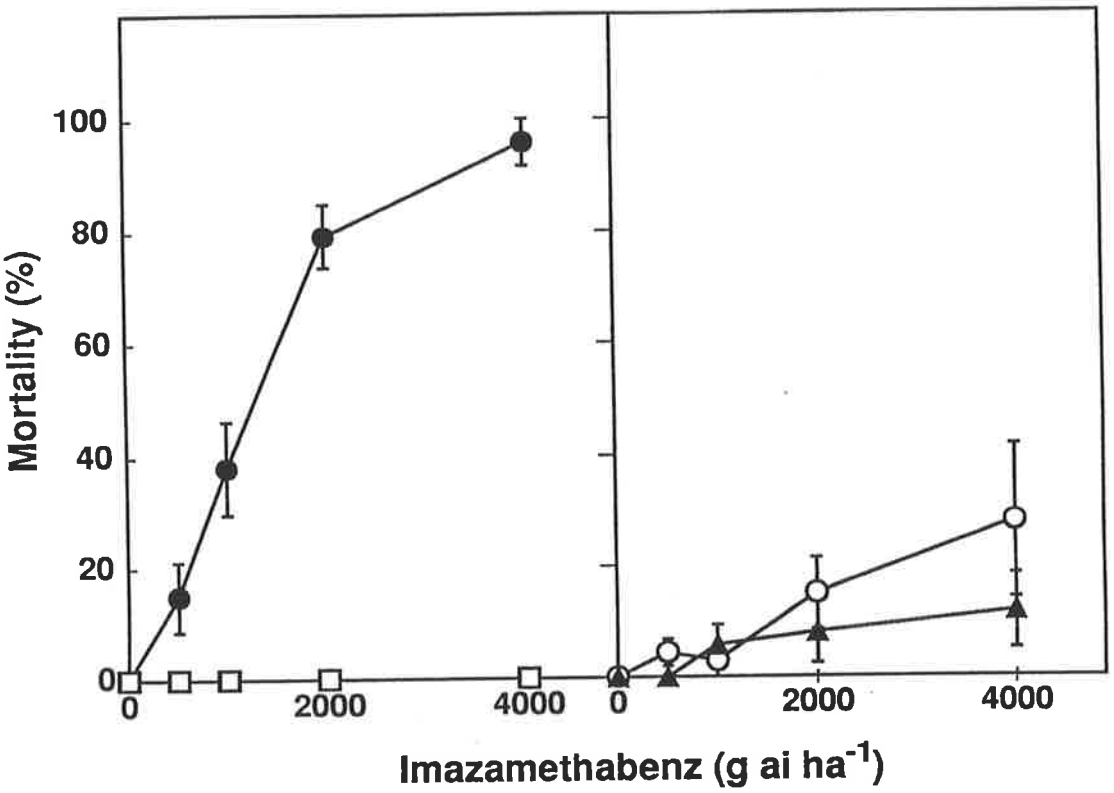
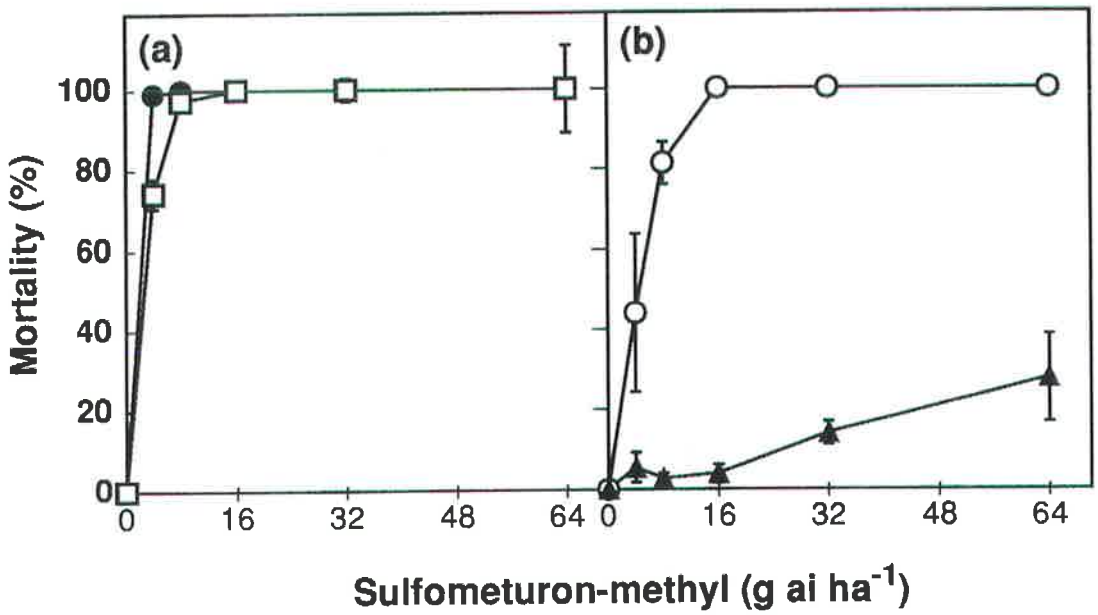


Figure 3.5 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the cereal-selective imidazolinone herbicide, imazamethabenz. This herbicide is not registered for use in Australia; it is used at the rate of 550 g ai ha^{-1} for control of weeds in cereal crops in North America. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

3.3.2 **Non-selective sulfonylurea and imidazolinone herbicides**

The non-selective herbicides sulfometuron-methyl and imazapyr control *L.rigidum* but these herbicides also kill wheat (Figures 3.6 and 3.8, Table 3.1). In contrast to the wheat-selective sulfonylurea and imidazolinone herbicides (Figures 3.2 to 3.5), the response of biotype SLR31 to non-selective herbicides sulfometuron-methyl and imazapyr is similar to that of the susceptible biotype VLR1 (Figures 3.6 & 3.8, Table 3.1). Sulfometuron-methyl killed all VLR1, SLR31 and wheat plants at doses greater than 16 g ai ha^{-1} (Figure 3.6). Similarly, at doses greater than 50 g ai ha^{-1} imazapyr all VLR1, SLR31 and wheat plants were killed (Figure 3.8).

The dose response of *L.rigidum* biotype WLR1 to the non-selective herbicides sulfometuron-methyl and imazapyr differs markedly from that of biotypes SLR31, VLR1 and wheat (Figure 3.6 & 3.8. Table 3.1). Biotype WLR1 is resistant to these non-selective ALS inhibitors as well as being resistant to their crop-selective analogues. In contrast to SLR31, VLR1 and wheat, some individuals of biotype WLR1 survived application of 512 g ai ha^{-1} (Figures 3.6 & 3.7). Similarly, some WLR1 plants survived at up to 200 g ai ha^{-1} of imazapyr (Figure 3.8).



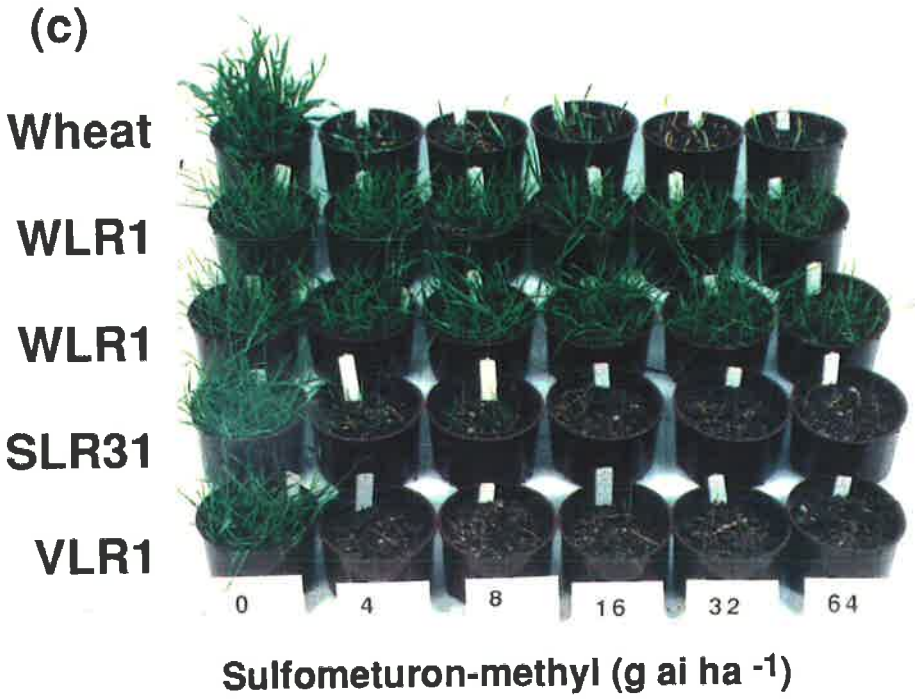


Figure 3.6 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the non-selective sulfonylurea herbicide, sulfometuron-methyl. This herbicide is used for industrial weed control at application rates from 8 g ai ha^{-1}. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

Plants from one replicate experiment of those represented above, photographed at the time of harvest (c).

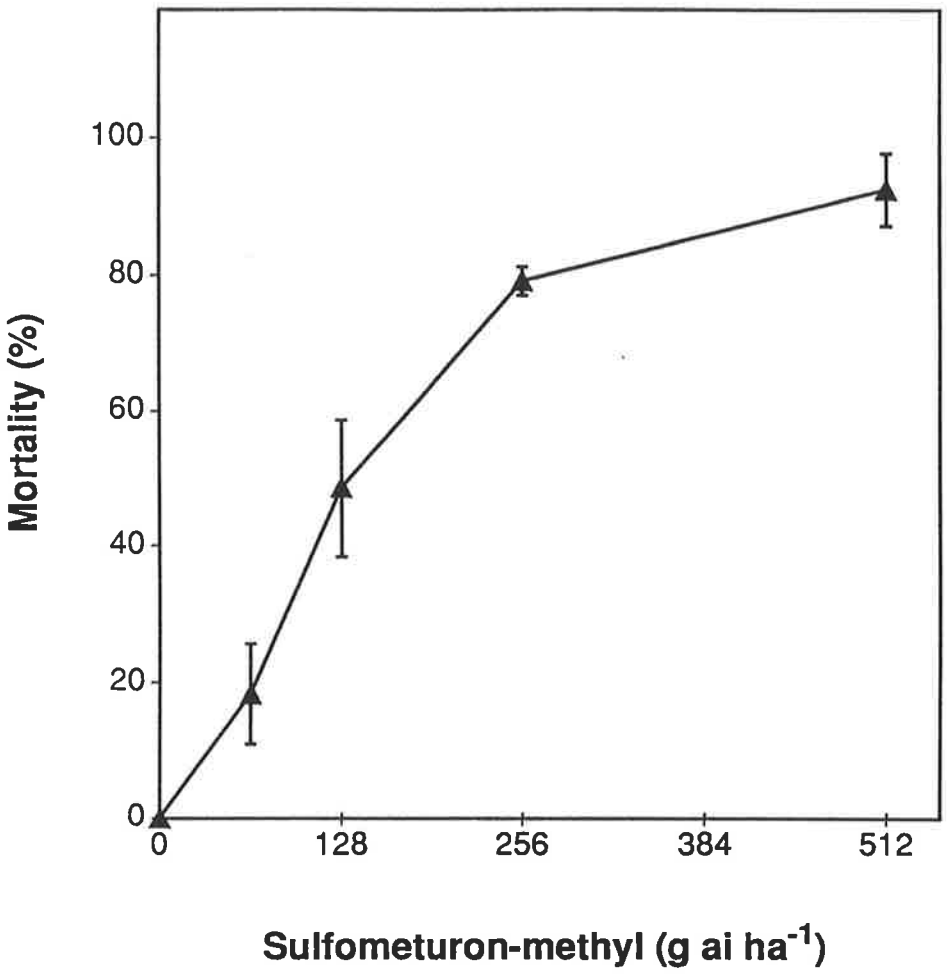


Figure 3.7 Mortality of *L.rigidum* biotype WLR1 (▲) cultured in pots and treated with the non-selective sulfonylurea herbicide, sulfometuron-methyl. This herbicide is used for industrial weed control at application rates from 8 g ai ha^{-1}. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

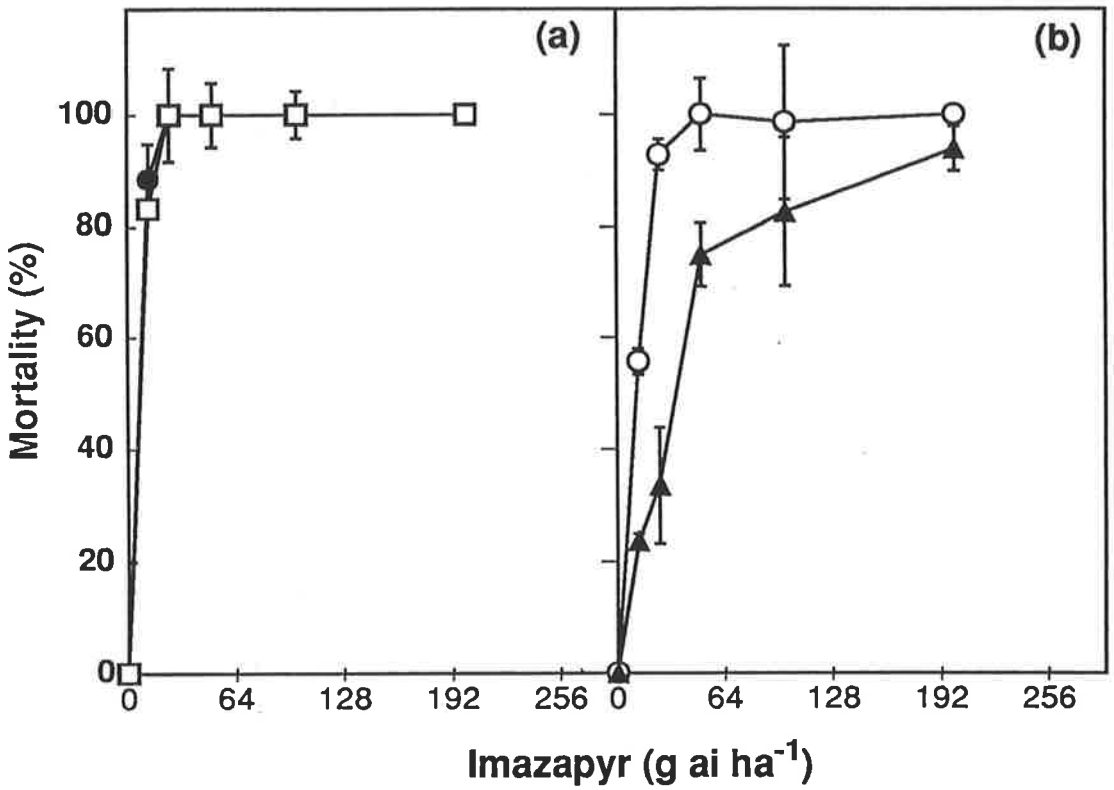


Figure 3.8 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the non-selective imidazolinone herbicide, imazapyr. This herbicide is used for industrial weed control at rates from 500 g ai ha^{-1}. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

Table 3.1 Growth response of three ryegrass biotypes to herbicides which inhibit ALS. Values are GR_{25} values in g ai ha^{-1} ± S.E. of 3 experiments.

	VLR1	SLR31	WLR1
Selective herbicides			
Chlorsulfuron	3.8 ± 0.2 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
Non-selective 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

Values followed by different letters in the same line are significantly different ($P<0.05$)

3.3.3 **Response to other groups of herbicides**

L.rigidum is normally controlled by the crop-selective aryloxyphenoxypropionate herbicide diclofop-methyl at application rates from 375 to 560 g ai ha^{-1}, as demonstrated by the response of susceptible biotype VLR1 (Figure 3.9). Wheat is tolerant to this herbicide (Figure 3.9). Like wheat, biotype SLR31 is resistant to diclofop-methyl (Figure 3.9). However, biotype WLR1 remains sensitive to this herbicide exhibiting similar dose response to that of susceptible biotype VLR1 (Figure 3.9).

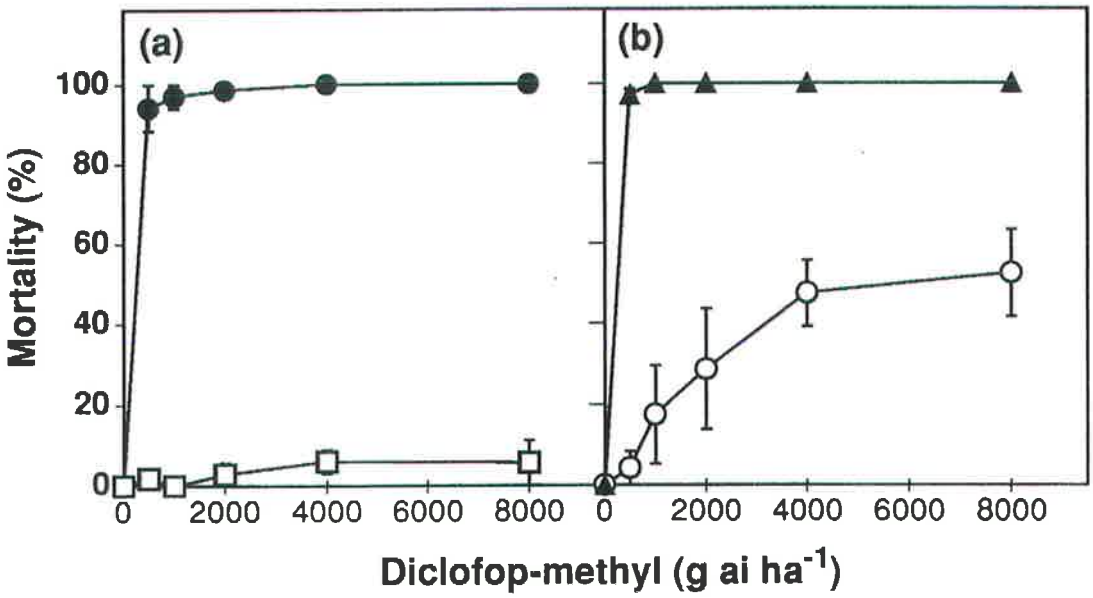
L.rigidum is normally controlled by the aryloxyphenoxyalkanoate and cyclohexanedione herbicides fluazifop-butyl, haloxyfop-ethoxyethyl and sethoxydim, which are not selective in wheat (Figure 3.10). The data in Figure 3.10 indicate that biotype SLR31 is also less-sensitive to these ACCase inhibiting herbicides. A proportion of individuals from biotype SLR31 survive high doses of the herbicides (Heap and Knight 1990) (Figure 3.10). In contrast, biotype WLR1 exhibits dose response similar to that of the susceptible biotype VLR1 for all these herbicides. WLR1 is susceptible to these herbicides and, therefore, differs from biotype SLR31.

L.rigidum is controlled by the crop-selective dinitroanaline herbicides trifluralin and pendimethalin at normal application rates of 400 and 1800 g ai ha^{-1} respectively, as demonstrated by the response of biotype VLR1 (Figure 3.11). Biotype SLR31 and wheat are resistant the dinitroanaline herbicides trifluralin and pendimethalin (Figure 3.11). In contrast, the response of biotype WLR1 is indistinguishable from that of the susceptible biotype VLR1 (Figure 3.11).

These results are important for the management of *L.rigidum* population WLR1 as they mean that cereal-selective and non-selective herbicides from these groups may still be used for control of this population. This would not have been predicted from previous knowledge of chlorsulfuron cross-resistant biotypes.

Both biotypes SLR31 and WLR1 are sensitive to several other groups of herbicides. Response to the photosynthetic inhibitors diuron and

metribuzin for biotypes WLR1 and SLR31 is similar to that of the susceptible biotype (Figure 3.12). Similarly, both biotypes SLR31 and WLR1 are susceptible to the non-selective herbicides glyphosate and paraquat (Figure 3.13). These herbicides may, therefore, be considered for use in management of either resistant *L.rigidum* population.



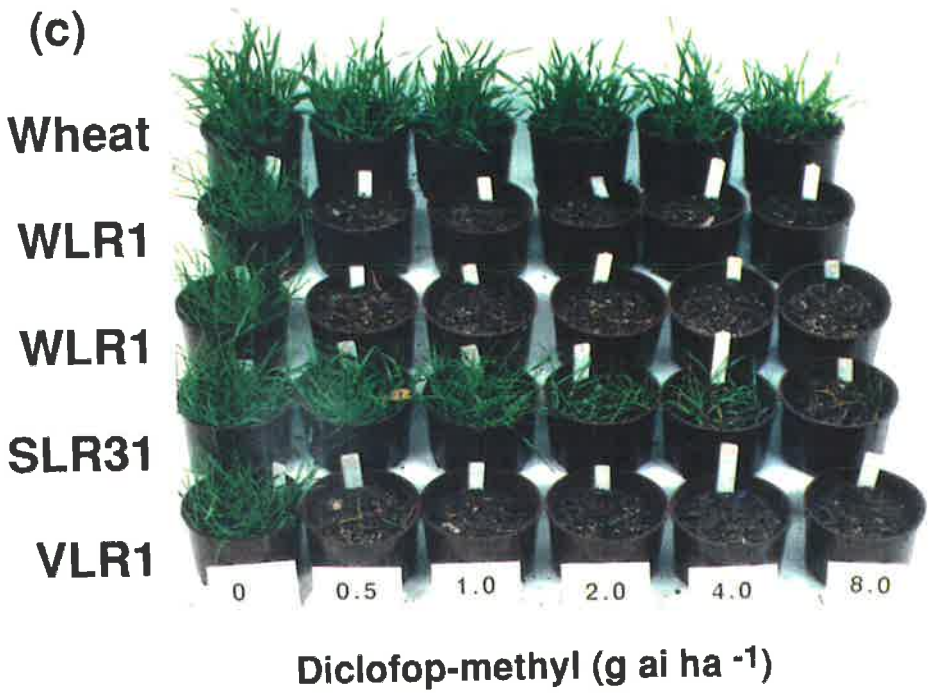
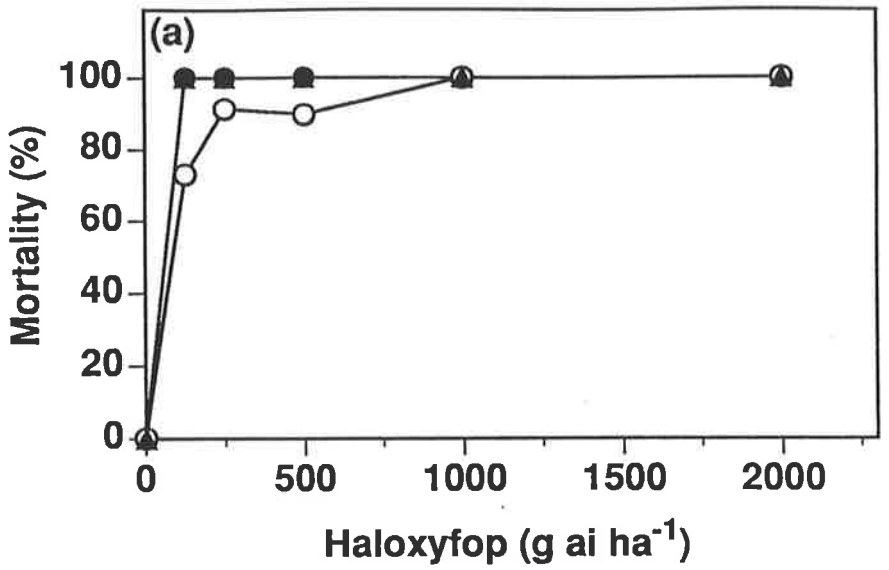
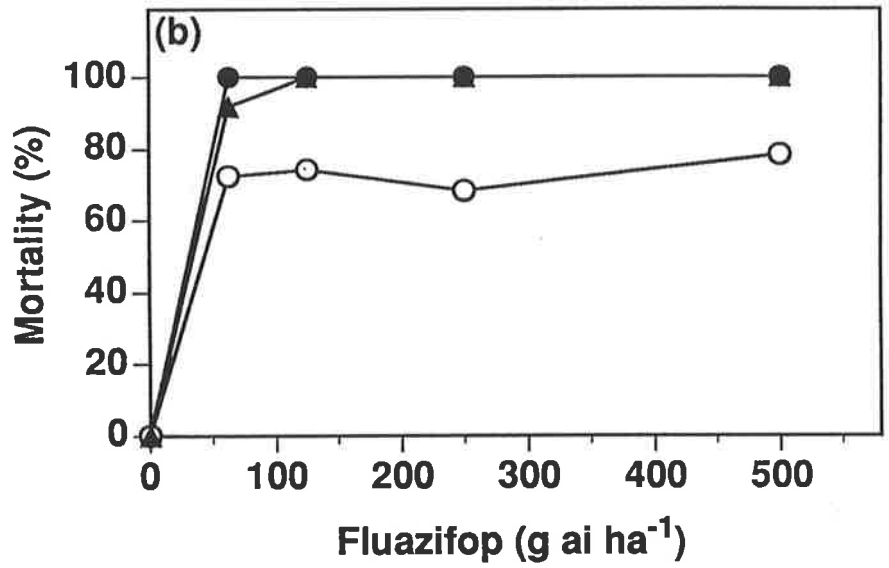


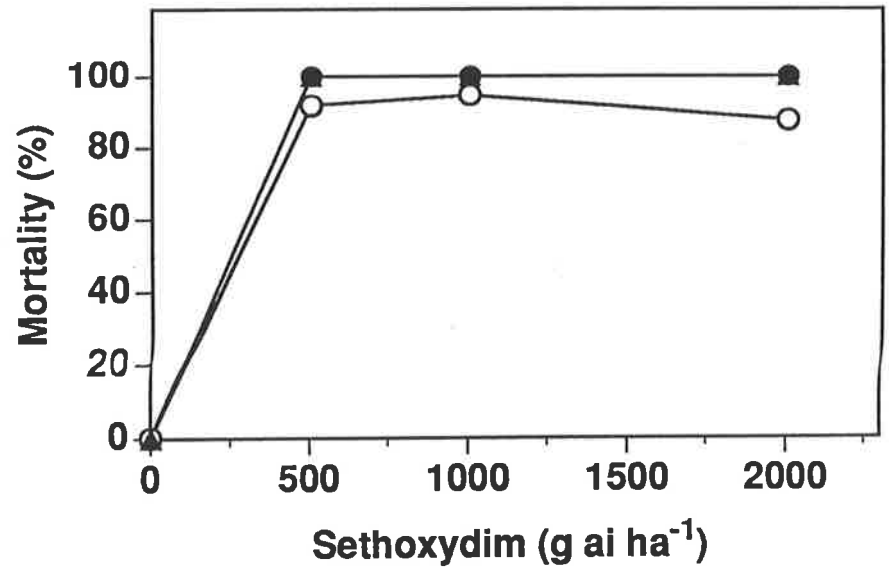
Figure 3.9 Mortality of, (a) *L.rigidum* biotype VLR1 (●) and wheat (□), and, (b) *L.rigidum* biotypes SLR31(○) and WLR1 (▲), cultured in pots and treated with the cereal-selective aryloxyphenoxypropionate herbicide, diclofop-methyl. Application rates of from 375 to 560 g ai ha^{-1} are recommended for *L.rigidum* control in Australia. Data are the means of three replicate experiments. Vertical bars represent the standard errors of the means.

Plants from one replicate experiment of those represented above, photographed at the time of harvest (c).

Figure 3.10Mortality of *L.rigidum* biotypes SLR31(○) and WLR1 (▲), and VLR1 (●), cultured in pots and treated with the aryloxyphenoxypropionate herbicides, (a) haloxyfop-ethoxyethyl, (b) fluazifop-butyl and (c) the cyclohexanedione herbicide, sethoxydim. Application rates of 104 g ai ha^{-1} for haloxyfop-ethoxyethyl, 106 g ai ha^{-1} for fluazifop-butyl and 186 g ai ha^{-1} for sethoxydim are recommended for *L.rigidum* control in Australia. Data are the means of two replicate experiments without error bars.


(a)
Mortality (%)
100
80
60
40
20
0
0
500
1000
1500
2000
Haloxyfop (g ai ha-1)


(b)
Mortality (%)
100
80
60
40
20
0
0
100
200
300
400
500
Fluazifop (g ai ha-1)


Mortality (%)
100
80
60
40
20
0
0
500
1000
1500
2000
Sethoxydim (g ai ha-1)

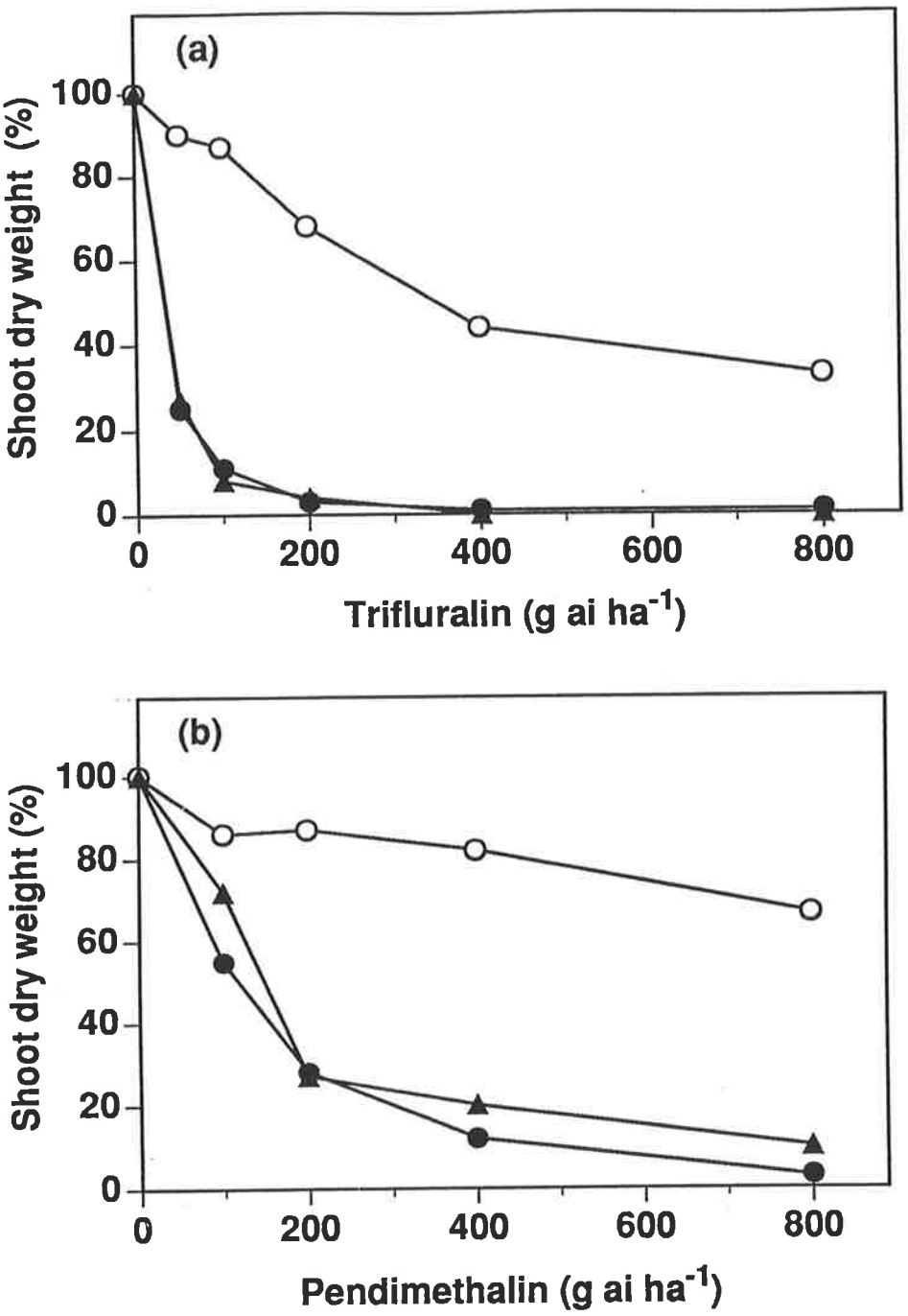


Figure 3.11 Relative dry weight (%), of shoots of *L.rigidum* biotypes SLR31(○) and WLR1 (▲), and VLR1 (●), cultured in pots and treated with the herbicides, (a) trifluralin and (b) pendimethalin, compared to untreated controls. Application rates of 400 g ai ha^{-1} for trifluralin and from 1200 to 1800 g ai ha^{-1} for pendimethalin are recommended for *L.rigidum* control in Australia. Data are the means of two experiment using 24 plants per treatment shown without error bars.

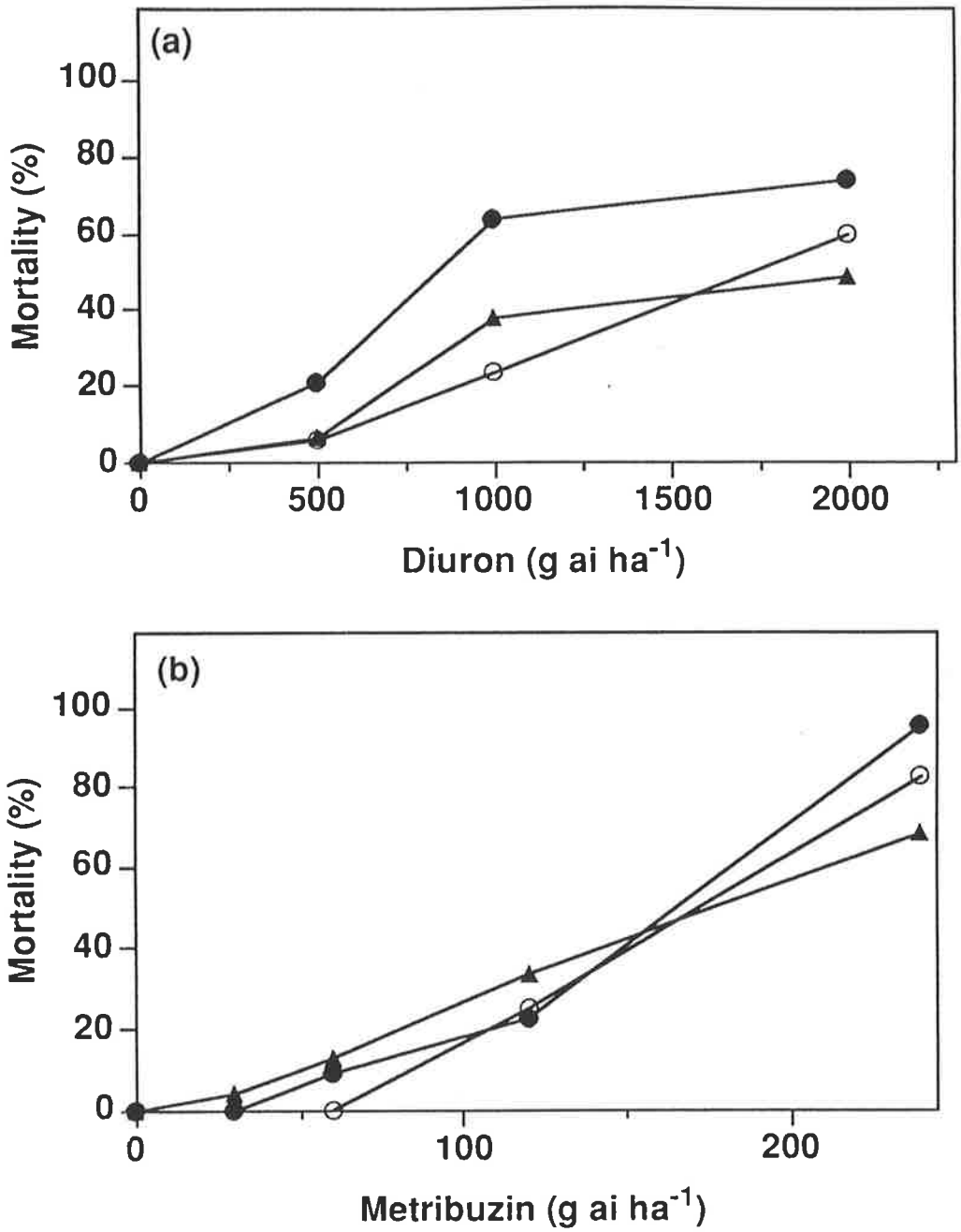


Figure 3.12Mortality of *L.rigidum* biotypes SLR31(○) and WLR1 (▲), and VLR1 (●), cultured in pots and treated with herbicides which inhibit photosynthesis, (a) diuron and (b) metribuzin. Application rates of from 700 to 7000 g ai ha^{-1} for diuron and from 300 to 1000 g ai ha^{-1} for metribuzin are recommended for *L.rigidum* control in Australia. Diuron data are the means of two replicate experiments without error bars. Metribuzin data are from a single experiment using 24 plant per treatment.

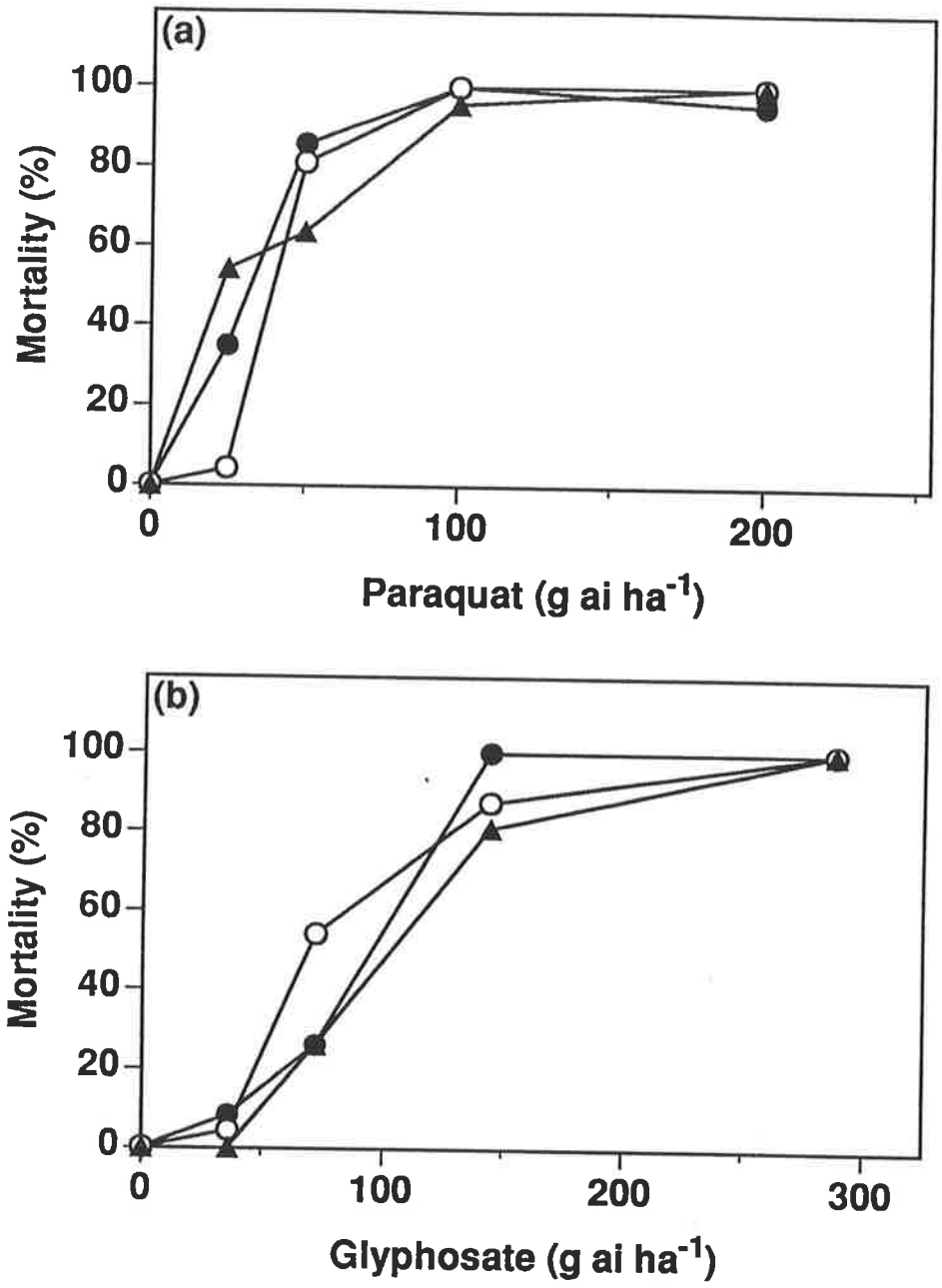


Figure 3.13 Mortality of *L.rigidum* biotypes SLR31(○) and WLR1 (▲), and, VLR1 (●), cultured in pots and treated with the non-selective herbicides, (a) paraquat and (b) glyphosate. Application rates of 200 g ai ha^{-1} for paraquat and 72 g ai ha^{-1} for glyphosate are recommended for *L.rigidum* control in Australia. Data are from one experiment using 24 plants per treatment.

3.4 Discussion

Cross-resistant biotype SLR31 is resistant to the crop-selective sulfonylurea and imidazolinone herbicides chlorsulfuron, triasulfuron, metsulfuron-methyl and imazamethabenz. Wheat is also resistant to these herbicides because it can rapidly metabolise them to inactive products (reviewed in section 1.3.4). SLR31 is not resistant to the non-selective herbicides sulfometuron-methyl and imazapyr. Wheat is also susceptible to sulfometuron-methyl and imazapyr because it cannot rapidly metabolise them (Sweetser 1985, Brown 1987). SLR31 and wheat both have herbicide sensitive ALS (Matthews *et al.* 1990, Sweeter *et al.* 1982). This data is consistent with the hypothesis that resistance in biotype SLR31 is due to increased metabolism of the herbicide, as it is in wheat.

In contrast to biotype SLR31, biotype WLR1 is resistant to both crop-selective and non-selective sulfonylurea and imidazolinone herbicides. This biotype is, therefore, similar to the sulfonylurea resistant weed biotypes of North America, which have been shown to have a herbicide-insensitive ALS (Saari *et al.* 1990 and 1992).

Biotype WLR1 also differs from biotype SLR31 in response to herbicides which do not inhibit ALS. Biotype SLR31 exhibits resistance to a wide range of chemically different herbicides with different modes of action (Heap and Knight 1986 and 1990). In contrast, WLR1 does not exhibit cross-resistance to any herbicides which do not inhibit ALS. This suggests that biotype SLR31 may have many mechanisms of resistance to different herbicides, however, resistance to ALS inhibitors in biotype WLR1 could be entirely due to a herbicide-insensitive ALS.

Treatment of the *L.rigidum* population WLR1 with chlorsulfuron in seven consecutive seasons, therefore, has not led to selection of cross-resistance to the aryloxyphenoxypropionate or cyclohexanedione herbicides. This demonstrates that treatment of a population of *L.rigidum* with a sulfonylurea herbicide does not necessarily result in cross-resistance to these groups of herbicides.

Thus, there are clear differences in the spectra of herbicide resistance between biotypes SLR31 and WLR1. These differences suggest that the mechanisms of resistance to sulfonylurea and imidazolinone herbicides differ between the two biotypes.

Chapter 4

Chlorsulfuron resistance in *L.rigidum* can involve increased metabolism

4.1 Introduction

Whole plant studies comparing the spectrum of herbicide resistance of *L.rigidum* biotype SLR31 and wheat suggest that resistance to some cereal-selective sulfonylurea and imidazolinone herbicides in this biotype may be due to increased metabolism, as it is in wheat. The herbicide resistance spectrum of biotype WLR1, however, suggests that increased metabolism is not the main determinant of resistance in this biotype.

To determine whether the cross-resistant biotype SLR31 can metabolise chlorsulfuron at a faster rate than the susceptible biotype VLR1 studies of [^{14}C-U-phenyl]chlorsulfuron uptake and metabolism were conducted. Chlorsulfuron is metabolised by different pathways in wheat and tolerant dicots (see 1.3.5). Studies were also conducted to determine whether the products of chlorsulfuron metabolism in *L.rigidum* are similar to those produced either by wheat, or the tolerant dicot species, flax.

The resistant response of biotype WLR1 to non-selective sulfonylurea and imidazolinone herbicides suggests that the mechanism in this biotype may be herbicide-insensitive ALS (Chapter 3). If this is the case, biotype WLR1 may be resistant to chlorsulfuron without any increase in the rate of metabolism. Hence, the uptake and metabolism of [^{14}C]chlorsulfuron where investigated in this biotype.

4.2 Materials and methods

4.2.1 [^{14}C]Chlorsulfuron metabolism studies: Method A

Treatment of excised plants

Excised seedlings of *L.rigidum* biotypes SLR31, VLR1, VLR6, wheat and flax (*Linium usitatissimum L.*) were cultured and treated with [^{14}C]chlorsulfuron by Method A as described below. Biotype VLR6 is resistant to diclofop-methyl but susceptible to chlorsulfuron (Christopher *et al* 1991, Heap and Knight 1990).

After germination on agar, *L.rigidum* plants were transferred to 40 x 30 x 12cm trays at a spacing of 2cm as described in section 2.2. Trays were placed in a glass house for 5 days before being transferred to a growth room 2 days prior to treatment. Growth room conditions were 17C, 12h, 110 to 180 $\mu E\ m^{-2}s^{-1}$ light period/ 13C, 12h dark period. At the time of treatment the plants were 14 days 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). Wheat plants were directly sown into potting medium at the time that *L.rigidum* plants were transferred (as described in section 2.2).

[^{14}C-U-phenyl]chlorsulfuron (11.5 μCi/mg) was dissolved in double distilled water to a final concentration of 6 $\mu g\ ml^{-1}$ and 200 μl aliquots dispensed into Eppendorf tubes. Into each tube either 5 ryegrass or 2 wheat excised seedlings were placed for a period of 2 or 3 hours. A total of 30 ryegrass or 12 wheat seedlings were used per treatment. After 3 hours

treatment the base of the plant stems were washed by dipping in 2 aliquots of double distiled 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.

Extraction

Plants were ground in a mortar with sand, liquid nitrogen and chilled 80% methanol (2ml). The mortar and pestle were rinsed with 80% methanol (2ml) and the two fractions pooled. The plant material was sedimented by centrifugation for 20 minutes at 10,000 g. The pellet was extracted with a further 2 aliquots (2ml each) of chilled 80% methanol. Controls spiked with [^{14}C]chlorsulfuron at the time of extraction showed that this method of extraction gave >95% recovery. 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 analysis

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 250x4.6mm 5μm C18 Brownlee Labs column using 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^{-1} throughout, with a column temperature of 39C. [^{14}C]chlorsulfuron and metabolites were detected using a Berthold LB 504 HPLC radiation monitor fitted with a Z2000-4 homogeneous counting flow cell.

4.2.2 [^{14}C]Chlorsulfuron metabolism studies: Method B

Plants of *L.rigidum* biotypes VLR1, SLR31 and WLR1 were cultured as above except that growth chamber conditions of 22C, 14h, 280-320 μE m^{-2} s^{-1} light period, 15C 10h dark period, produced plants with similar appearance to field grown plants which did not need pre-conditioning in the glass house.

Treatment conditions were as described above except that all plants were treated for three hours and kept constantly illuminated from the commencement of treatment. At harvest the leaf lamina 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 as described above except that the gradient used the two phases A= 90% H_2O, 1% (v/v) acetic acid, B= acetonitrile, 0.2% (v/v) acetic acid with a linear gradient from 40% B to 100% B in 10 min. ^{14}C was detected using a Radiomatic series A100 radiation monitor fitted with a yttrium silicate solid scintillant cell of 250µL void volume.

4.2.3 Acetolactate synthase assays

ALS activity in leaf lamina and culms was assayed as described in section 5.2.

4.3 [^{14}C]chlorsulfuron metabolism in excised shoots of biotype SLR31.

Studies using Method A, showed that *L.rigidum* biotype SLR31 metabolised chlorsulfuron at an initial rate approximately twice that of the susceptible biotype (Figure 4.1). Biotype SLR31 had metabolised 50% of the [^{14}C]chlorsulfuron within six hours but the susceptible biotype VLR1 required in excess of twelve hours (Figures 4.1 & 4.3). The increased rate of detoxification of chlorsulfuron in SLR31 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, metabolised chlorsulfuron at the same rate as the chlorsulfuron and diclofop-methyl susceptible biotype VLR1 (Figure 4.1). Wheat degrades [^{14}C]chlorsulfuron at a faster rate than any of these *L.rigidum* biotypes, taking 2 hours to metabolise 50% of the herbicide (Figure 4.1). The rates of degradation of chlorsulfuron are correlated with the levels

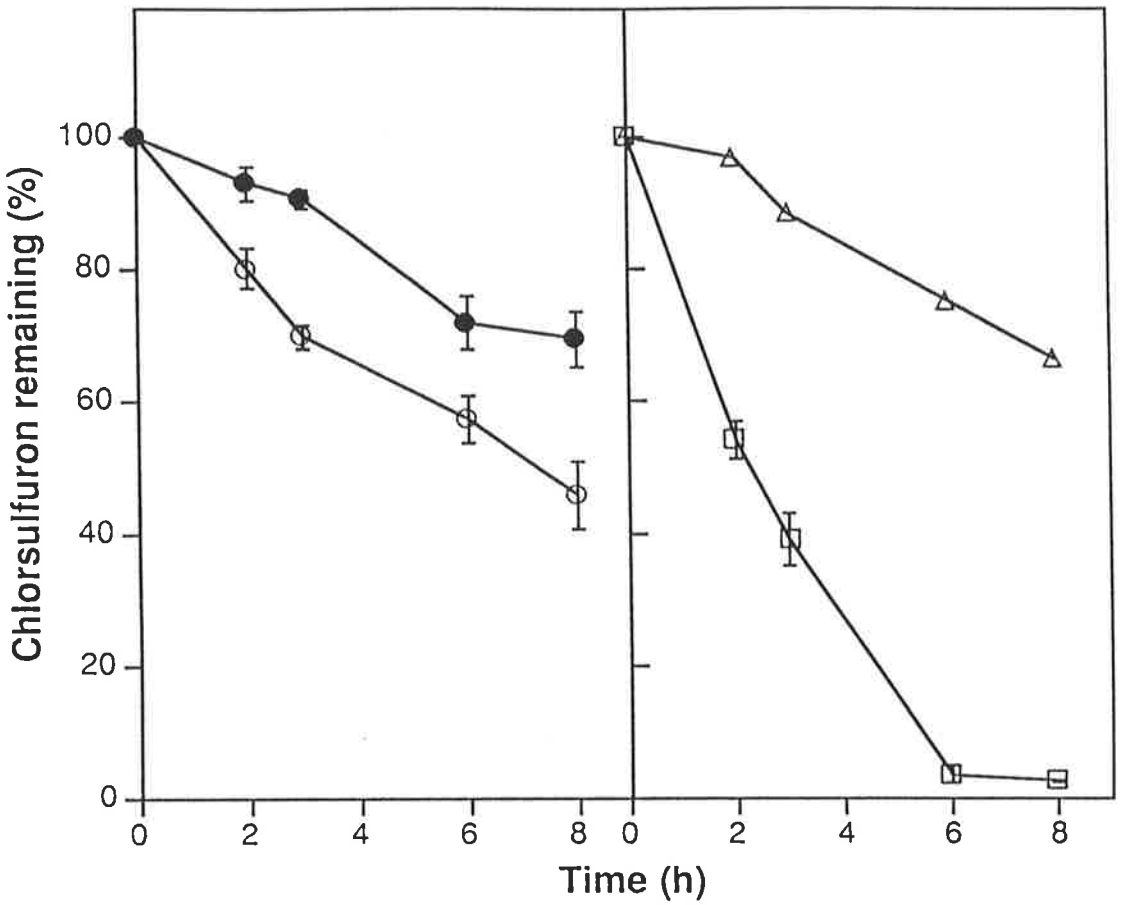


Figure 4.1 The amount of [^{14}C]chlorsulfuron remaining unmetabolised in extracts from excised seedlings harvested at various times after the commencement of herbicide treatment: (a) *L.rigidum* biotypes VLR1 (●) and SLR31 (○) and (b), *L.rigidum* biotype VLR6 (△) and wheat (□). Values for VLR1, SLR31 and wheat are the means of four replicate experiments. Vertical bars indicate standard error of the mean. Values for VLR6 are the means of two experiments (no error bars).

of resistance observed at the whole plant level, with wheat being more tolerant than cross-resistant *L.rigidum* SLR31 which is more resistant than chlorsulfuron susceptible biotypes VLR 1 and VLR6 (Chapter 3).

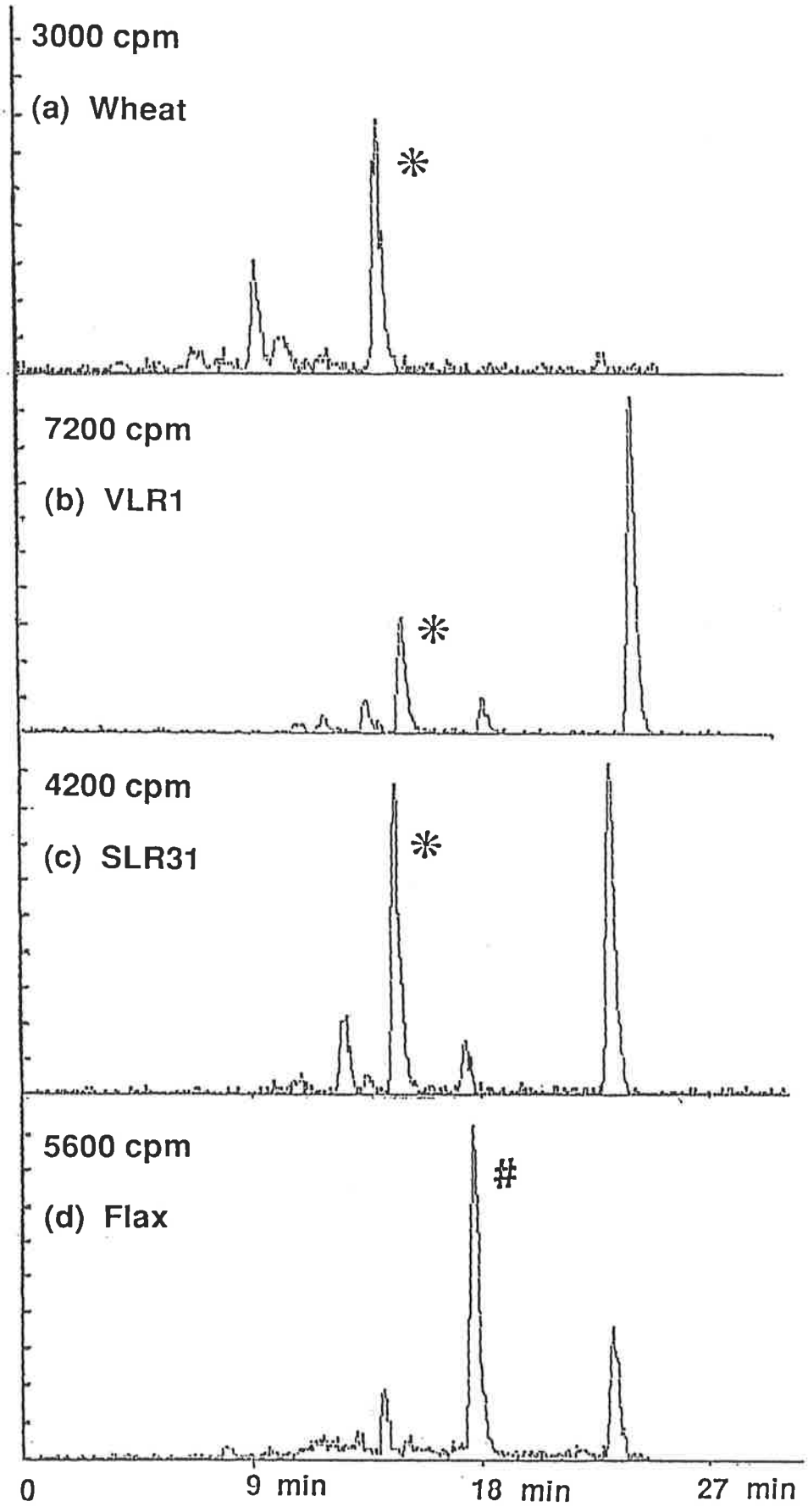
The major metabolite of chlorsulfuron in wheat and *L.rigidum* exhibited a similar elution profile when separated using reverse-phase HPLC, method A (Figure 4.2). When extracts from wheat and *L.rigidum* were combined and chromatographed a single major peak was eluted (data not shown). Sweetser, *et al* (1982) identified the major metabolite of wheat as the glycosylated derivative of chlorsulfuron hydroxylated in the phenyl ring (see Figure 1.6, Chapter 1).

The products of metabolism of chlorsulfuron in the chlorsulfuron tolerant dicot, flax, are not the major metabolites produced in *L.rigidum* (Figure 1.6). Two minor metabolites produced by *L.rigidum* exhibit similar retention profiles to the metabolites produced by flax. The major flax metabolite is most likely that formed following oxidation of the 6 methyl substituent of the triazine ring (Hutchison *et al.* 1984) (Figure 1.6).

It is important to note that there were no observed differences between the metabolite profiles from susceptible and resistant *L.rigidum* except that, at any given time, a larger proportion of activity was present as metabolites in the cross-resistant than in the susceptible plants (Figure 4.2). It was the rate of metabolism that differed rather than the nature of the metabolites. The cross-resistant *L.rigidum* appears to utilise existing, wheat-like metabolic pathways to detoxify chlorsulfuron at twice the rate of the susceptible biotype (Figure 4.2).

Although the initial rate of metabolism was clearly faster in biotype SLR31 there was still a substantial proportion of [^{14}C]chlorsulfuron remaining after twelve hours (Figure 4.3). The level of unmetabolised chlorsulfuron remaining after twelve hours did not decrease substantially even when plants were harvested up to forty eight hours after the commencement of treatment (Figure 4.3). Twelve hours after the

Figure 4.2 HPLC chromatograms of [^{14}C] labelled chlorsulfuron 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 three hours and then transferred to water for five hours (8 hours total). [^{14}C]chlorsulfuron and metabolites were separated by HPLC analysis method A. Maximum values for the "Y" axis are given in counts per minute. 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 23min.


3000 cpm
(a) Wheat
*
7200 cpm
(b) VLR1
*
4200 cpm
(c) SLR31
*
5600 cpm
(d) Flax
#
0
9 min
18 min
27 min

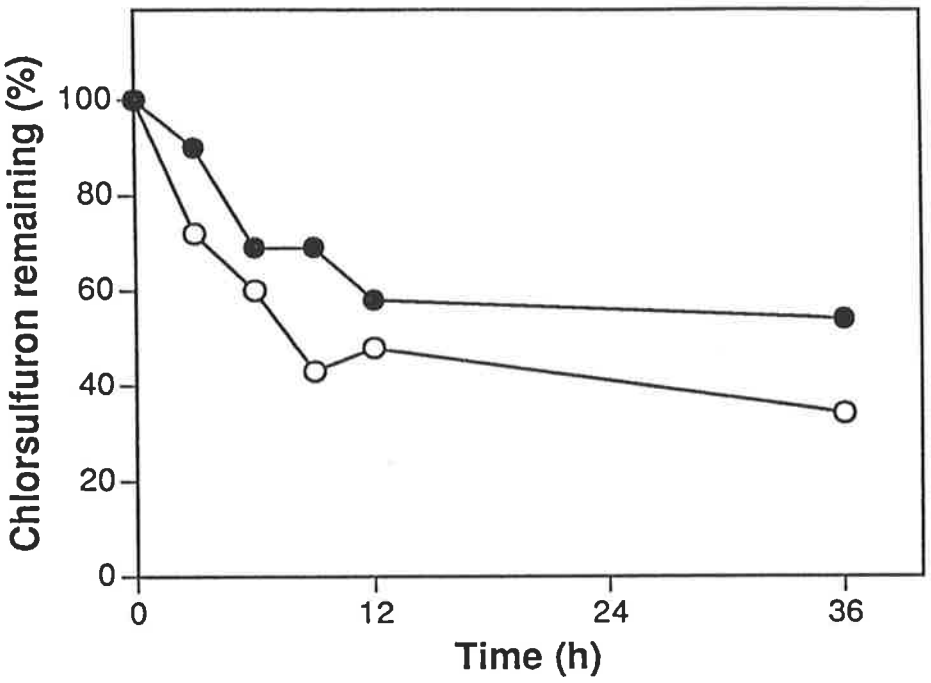


Figure 4.3 The amount of [^{14}C]chlorsulfuron remaining unmetabolised in extracts from excised seedlings harvested at various times after the commencement of uptake of *L.rigidum* biotypes VLR1 (●) and SLR31 (○). Values are the means of two replicate experiments (no error bars).

commencement of treatment corresponded to the end of the light period. This suggested that periods of darkness may affect chlorsulfuron metabolism in *L.rigidum* (see Chapter 8). Further studies were needed to determine whether the unmetabolised chlorsulfuron remaining was able to inhibit the growth of either resistant or susceptible plants.

4.4 [^{14}C]chlorsulfuron metabolism in excised shoots of biotype SLR31 using method B

Subsequent studies of [^{14}C]chlorsulfuron where conducted using a slightly modified procedure, method B. The growth chamber was constantly illuminated after the commencement of feeding to avoid any affect which dark periods may have on metabolism. To determine whether the unmetabolised chlorsulfuron was in the region near the meristem, plants were dissected at the time of harvesting. The differentiated leaf lamina tissue was separated from the culm tissue of the seedling. The culm tissue of excised seedlings is the tissue adjacent to the meristem prior to excission. ALS activity is greatest in regions of rapid cell division and is relatively low in fully differentiated plant tissue (reviewed in section 1.3.2). Measurements confirmed that ALS activity, per mg protein of the enzyme extract, is 3 to 4 times higher for the culms than for the leaf lamina (Table 4.1).

In studies using method B, uptake of [^{14}C]chlorsulfuron by excised shoots was similar for the susceptible and resistant biotypes (Table 4.2) as it was using method A (Chrisopher 1991). In biotypes SLR31 and VLR1 approximately 67 and 71% respectively of ^{14}C accumulated in the leaf lamina, with the remaining ^{14}C in the culms. Clearly, a substantial amount of the ^{14}C accumulates in tissue which is remote from the meristems. The accumulation of ^{14}C in the leaf lamina may have been influenced by the method used to treat these plants. The herbicide was given *via* the severed xylem vessels of the cut stems and it might be expected that accumulation of

Table 4.1 ALS activity in the leaf lamina and culms of 3 to 4 leaf seedlings of three *L.rigidum* biotypes, (nanomoles acetolactate produced mg protein^{-1}).

	Biotype		
	VLR1	WLR1	SLR31
Leaf	57 ± 24	63 ± 39	95 ± 21
Culms	219 ± 61	288 ± 79	283 ± 40

Values are the means of three replicates ± SE. Means within each tissue compared using t-test are not significantly different between biotypes (P>0.05).

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Table 4.2 Total ^{14}C accumulation in leaf lamina and culms of *L.rigidum* plants after 3 hours treatment expressed as equivalents of ng [^{14}C]chlorsulfuron per plant.

	Biotype		
	VLR1	WLR1	SLR31
Leaf	33.6 ± 4.9	38.3 ± 3.5	28.9 ± 2.3
Culms	14.2 ± 1.3	14.1 ± 1.4	14.0 ± 1.0
Total	47.9 ± 6.3	52.4 ± 4.2	42.9 ± 3.4

Values are the means of four replicates ± SE. Means within each tissue compared using t-test are not significantly different between biotypes ($P>0.05$).

^{14}C should be directly proportional to transpiration, while this might not be so if the herbicide were applied to the leaf cuticle or roots of intact plants (see Chapter 6).

Both susceptible biotype VLR1 and resistant biotype SLR31 exhibited an ability to degrade chlorsulfuron. In each biotype chlorsulfuron was more rapidly degraded in the culms (Figure 4.4), the site of greatest ALS activity (Table 4.1). However, the rate of chlorsulfuron metabolism in the culms differed between biotypes. SLR31 metabolized chlorsulfuron in the culms more rapidly than the susceptible biotype VLR1. Metabolism of 50% of the chlorsulfuron in the culms required 3.7±0.3 h in biotype SLR31 versus 7.1±0.6 h in biotype VLR1 (n=4).

There was also a difference in the level to which the resistant and susceptible biotypes could reduce chlorsulfuron in the culms over longer time periods. Biotypes SLR31 was able to metabolise over 95% of chlorsulfuron in the culms after 24 hours. In contrast 18 ± 6% of chlorsulfuron remained unmetabolised in the culms of biotype VLR1 after 24 hours and this did not decrease significantly after 48 hours (Figure 4.4). This suggests that the after 24 hours the susceptible biotype may be too damaged to continue metabolising the herbicide.

In contrast to the culms, the ^{14}C in the leaf lamina remained mostly as unmetabolised chlorsulfuron. After 12 h, less than 20% of the chlorsulfuron was metabolised (Figure 4.4). This pattern was observed in both resistant and susceptible biotypes.

As 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.

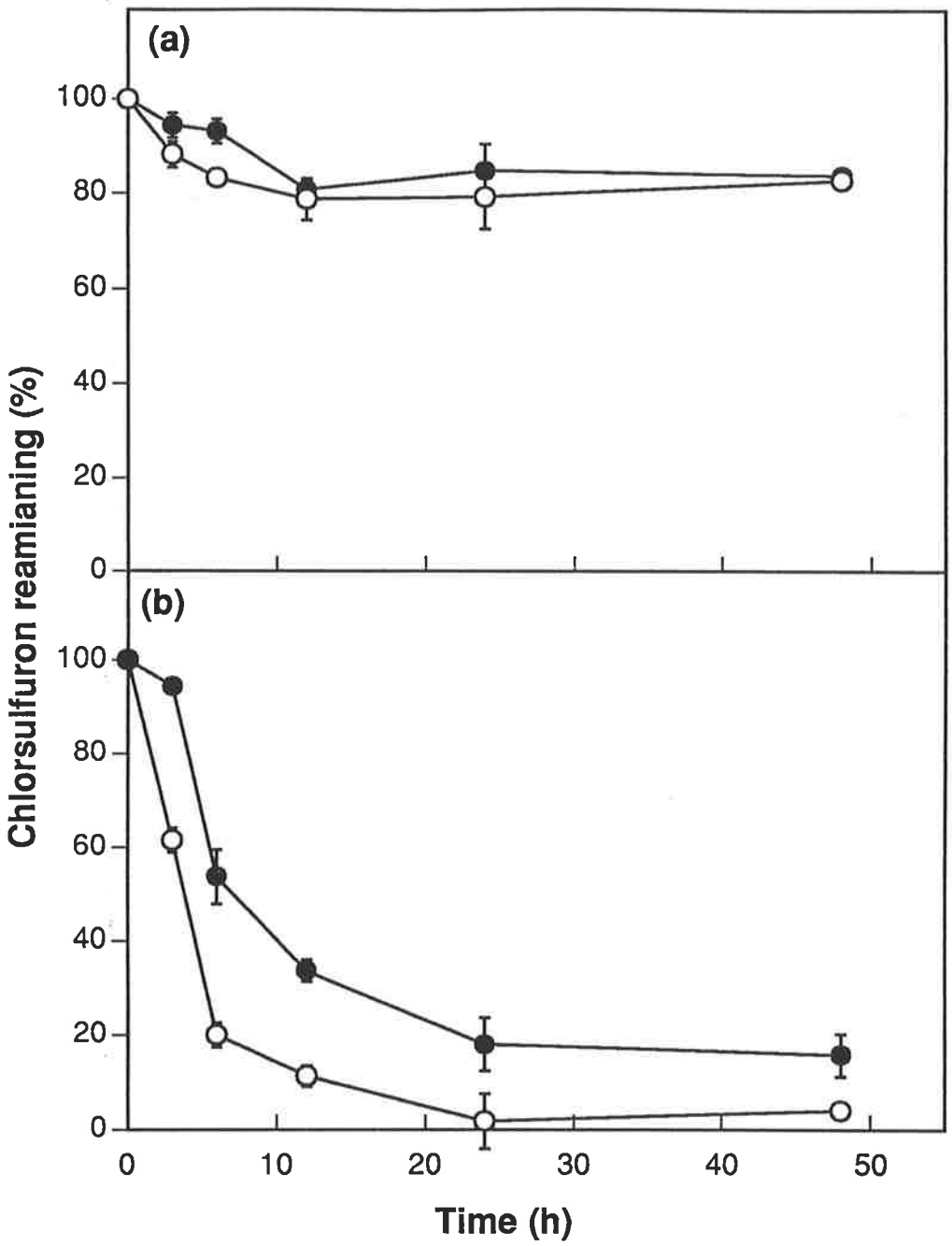


Figure 4.4 The amount of [^{14}C]chlorsulfuron remaining unmetabolised in extracts from leaf lamina (a) and culms tissue (b) from excised seedlings of *L.rigidum* biotypes VLR1 (●) and SLR31 (○), harvested at various times after the commencement of herbicide teatment. Values are the average of four repeat experiments. Vertical bars indicate standard error of the mean.

4.4 [^{14}C]chlorsulfuron metabolism in excised shoots of biotype WLR1 using method B

Uptake and metabolism of [^{14}C]chlorsulfuron in excised stems of *L.rigidum* biotype WLR1 were studied using method B. The uptake of [^{14}C]chlorsulfuron of biotype WLR1 was similar to that of the cross-resistant biotype SLR31 and susceptible biotype VLR1 (Table 4.2). Approximately 52% of ^{14}C accumulated in the leaf lamina tissue.

In common with the other *L.rigidum* biotypes, metabolism of chlorsulfuron in biotype WLR1 was much more rapid in the culm tissue than in the leaf lamina (Figure 4.5). The time taken to metabolise 50% of chlorsulfuron in the culms tissue was 5.1 ± 0.6 h which is intermediate between the cross-resistant biotype SLR31 and the susceptible biotype VLR1. Like biotype SLR31 biotype WLR1 was able to metabolise almost all of the chlorsulfuron in the culms after forty eight hours while VLR1 could not (Figure 4.5). ALS activity was also greater in the culm tissue of WLR1 than in the leaf lamina (Table 4.1) again suggesting that the rate of metabolism of chlorsulfuron in the culm tissue is more important than the rate of metabolism in the leaf lamina.

HPLC elution profiles of ^{14}C-labelled chlorsulfuron and metabolites from the leaf lamina and culms of WLR1 were similar to those of VLR1 (Figure 4.6) suggesting that the pathway of metabolism is the same in all biotypes.

Increased metabolism of chlorsulfuron in the culm region of excised seedlings of *L.rigidum* correlates with the response to herbicides observed for whole plants of *L.rigidum* biotypes SLR31 VLR1 and wheat (Chapter 3). In contrast, the level of metabolism of chlorsulfuron in biotype WLR1 cannot explain the response observed at the whole plant level. The GR_{25} of biotype WLR1 for chlorsulfuron is higher than that of SLR31 (Table 3.1, Chapter 3) but WLR1 dose not metabolise chlorsulfuron more rapidly (Figure 4.5). This suggests that increased metabolism is not the only mechanism of chlorsulfuron resistance in biotype WLR1.

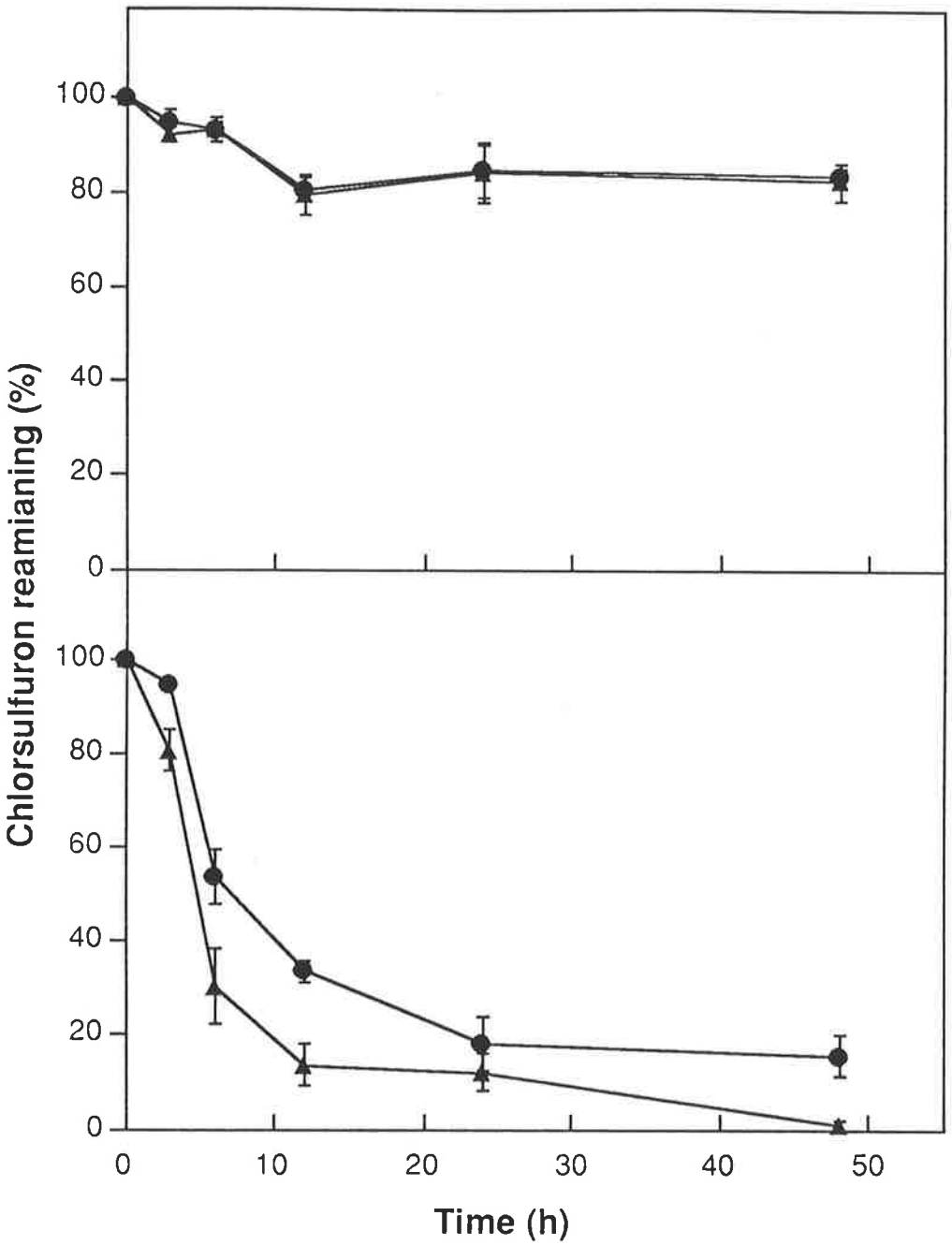


Figure 4.5 The amount of [^{14}C]chlorsulfuron remaining unmetabolised in extracts from leaf lamina (a) and culms tissue (b) from excised seedlings of *L.rigidum* biotypes VLR1 (●) and WLR1 (▲), harvested at various times after the commencement of herbicide teatment. Data are from the same experiments represented in Figure 4.5. Values are the average of four repeat experiments. Vertical bars indicate standard error of the mean.

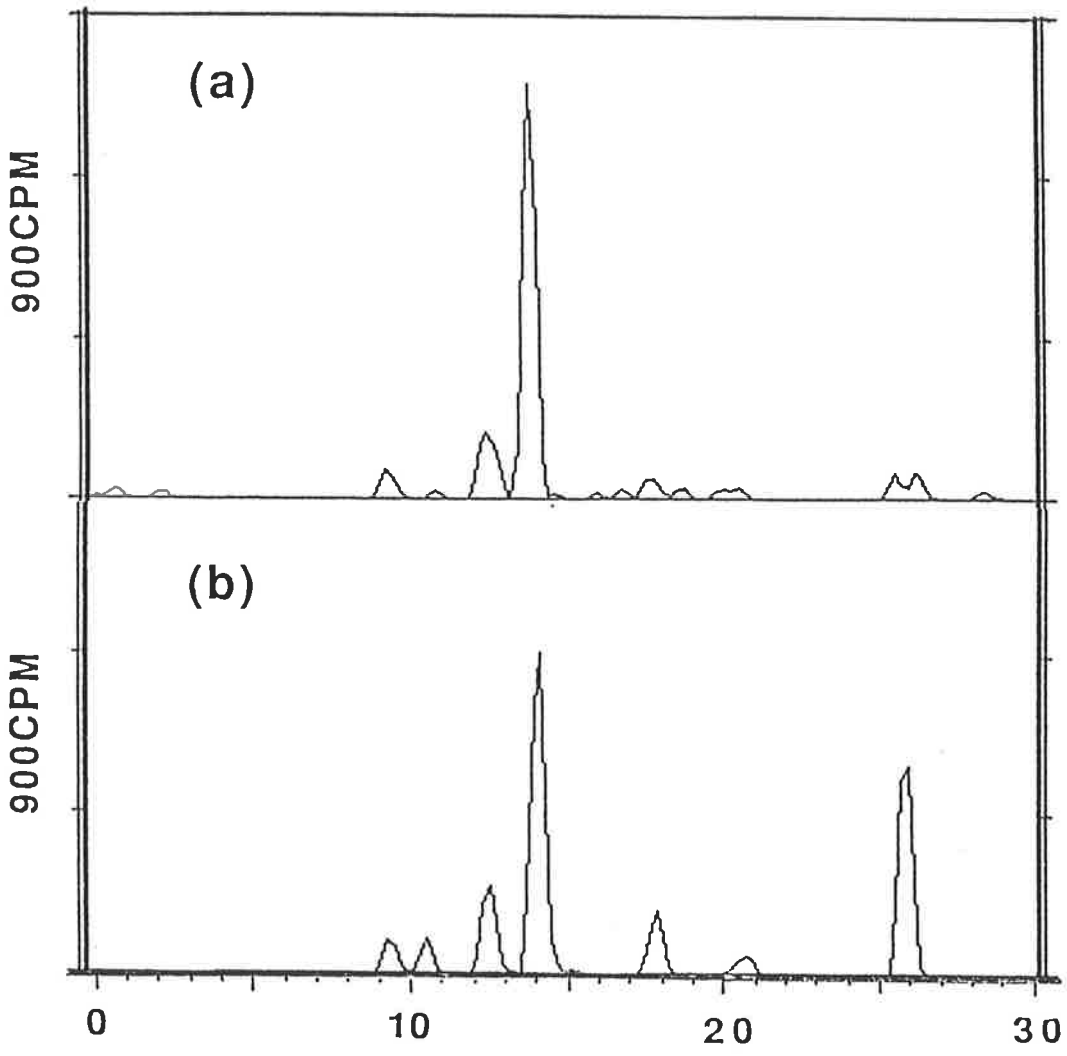


Figure 4.6 HPLC chromatograms of [^{14}C] labelled chlorsulfuron and metabolites in extracts from (a) susceptible *L.rigidum* biotype VLR1 and (b) resistant *L.rigidum* WLR1. Excised seedlings were allowed to take up the [^{14}C]chlorsulfuron for three hours and then transferred to water for nine hours (12 hours total). Maximum values for the "Y" axis are given in counts per minute. [^{14}C]chlorsulfuron and metabolites were separated by HPLC analysis method B.

4.5 Discussion

Taken together, the evidence from studies of the whole plant response to herbicides and studies of [^{14}C]chlorsulfuron metabolism in *L.rigidum* biotype SLR31 indicate that chlorsulfuron resistance in this biotype involves the ability to metabolise the herbicide more rapidly than a susceptible biotype. The results of HPLC analysis suggest that the metabolic pathway in *L.rigidum* is the same in both resistant and susceptible biotypes and is the same pathway occurring in wheat (Figure 4.2). Studies by Cotterman and Saari confirm that the rate of chlorsulfuron metabolism is greater in the chlorsulfuron cross-resistant *L.rigidum* biotype SR4/84 than in the susceptible biotype SLR2 (Cotterman and Saari 1992). These authors demonstrated that the major metabolite produced in *L.rigidum* can be hydrolised using the enzyme β-glucosidase to give a product which coelutes with an authenticated standard of hydroxychlorsulfuron (Cotterman and Saari 1992). This is further evidence that the pathway of chlorsulfuron metabolism in *L.rigidum* may be the same as that in wheat.

The mechanisms of resistance to the other wheat-selective sulfonylureas and to the wheat-selective imidazolinone, imazamethabenz in biotype SLR31 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 SLR31 (Figure 3.1, Chapter 3. Sulfometuron-methyl, which is non-selective and kills both wheat and *L.rigidum*, has a 4,6-dimethyl-pyrimidine in place of the 4-methoxy-6-methyl-1,3,5-triazine ring (Figure 3.1).

Wheat tolerates the imidazolinone herbicide imazamethabenz by detoxification resulting from rapid hydroxylation of the methyl substituent of the phenyl ring followed by conjugation to glucose (Brown 1987, see section 1.3.5). In contrast the non-selective 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 acetolactate synthase. It is possible that they may expose similar binding sites to detoxifying oxidative enzymes.

The time taken to degrade 50% of the chlorsulfuron in the culms of resistant *L.rigidum* biotypes SLR31 and WLR1 are within the range of from one to five hours found for tolerant crop species (Brown 1990). The time taken for sensitive weed species to metabolise sulfonylurea herbicides is generally more than twenty hours (Brown 1990). However, the time taken in the culms of sensitive *L.rigidum* is only seven hours. This suggests that only a small increase in the rate of metabolism is needed to cause the failure of *L.rigidum* control using chlorsulfuron.

In contrast to biotype SLR31 the lack of correlation between the rate of chlorsulfuron metabolism and whole plant response of biotype WLR1 provide further evidence that increased metabolism is not the only mechanism of chlorsulfuron resistance in this biotype. This observation agrees with the hypothesis that resistance to ALS inhibitor in biotype WLR1 may involve a herbicide-insensitive ALS.

Chapter 5

Herbicide resistance in *L.rigidum* can involve herbicide-insensitive ALS

5.1 Introduction

The response of biotype WLR1 to herbicides which inhibit ALS suggests that the mechanism of resistance in this biotype is similar to that of sulfonylurea resistant weed biotypes from North America. In common with these weed biotypes, WLR1 is resistant to both crop-selective and non-selective sulfonylurea herbicides (Chapter 3, section 1.3.5). Resistance in North American weed biotypes is caused by a mutant, herbicide-insensitive, ALS. It is, therefore, possible that resistance in *L.rigidum* biotype WLR1 may be caused by a mutant form of ALS.

Studies of [^{14}C]chlorsulfuron metabolism in biotype WLR1 indicate that, although the rate of metabolism is greater than that of susceptible *L.rigidum* biotype VLR1, it is not greater than that of resistant biotype SLR31 (Chapter 4). However, plants of biotype WLR1 suffer less mortality and less growth reduction from chlorsulfuron treatment than do plants of biotype SLR31 (Chapter 3). Thus increased metabolism alone can not explain the relative degree of resistance of biotype WLR1 when compared to biotype SLR31.

Studies of ALS from biotype WLR1 were conducted to determine whether ALS from this biotype is less sensitive to inhibition by herbicides and, if so, whether the level of herbicide resistance of the enzyme correlates with the level of resistance observed at the whole plant level.

5.2 Materials and Methods

5.2.1 Enzyme preparation

Ryegrass seedlings were pot cultured (2.2.2) in 40 x 30 x 12cm trays under growth cabinet conditions of 22C 14h, 280 to 320 μE $m^{-2}s^{-1}$ light period, 15C, 10h dark period, resulting in seedlings which resembled field-grown plants. Shoot material was harvested at soil level 21 or 28 days after transplanting when at the 3 to 4 leaf stage, frozen in liquid nitrogen and stored at -80C until extraction.

The method of enzyme extraction was based on Ray (1984) and Huppatz and Casida (1985). Frozen shoot material (12g) was ground in a mortar in 2 volumes of buffer containing 0.2M K_2HPO_4, 0.2M KH_2PO_4, 0.5M sodium pyruvate, 100mM thiamine pyrophosphate, 10mM $MgCl_2$ and 1mM FAD. Cellular material was removed by centrifugation (27000g, 20min). Protein was precipitated using 50% $(NH_4)SO_4$ for 30min followed by centrifugation (27000g, 30min). The protein was resuspended in 2.5ml of buffer containing 0.2M K_2HPO_4, 0.2M KH_2PO_4 and 10mM $MgCl_2$ and desalted on a Sephadex G25 column. Enzyme preparations were used immediately. Protein was determined by the Bradford method using commercial reagents supplied by Biorad Ltd.

5.2.2 Enzyme Assay conditions

ALS assays were as described by Ray (1984) except that 139 mM pyruvate was used and assay time was 30 min unless otherwise specified. The assays were performed in a final volume of 500 μL of pH 7 buffer which contained 0.2 M K_2HPO_4, 0.2 M KH_2PO_4, 139 mM sodium pyruvate, 100 mM thiamine pyrophosphate, 10m M $MgCl_2$ and 1 mM FAD to which herbicides had been added in 25 μl of double distilled water. Assays were initiated by addition of enzyme preparation (75 μl) containing from 0.8 mg to 1.7 mg protein. After incubation for 60 min at 37C assays were stopped by addition of 6N H_2SO_4 (100μM).

Acetolactate was converted to acetoin which was determined by the method of Westerfield (1945). The assay mixtures were incubated at 55C

for 15 min immediately after sulfuric acid addition after which creatine (0.5 ml, 5g L^{-1}) and 1-naphthol (0.5 ml, 40 mg ml^{-1} in 2.5N NaOH) were added prior to a further 15 min at 55C. After cooling for 15 min, absorbance at 530 nm was determined. Acetolactate production was calculated by the method of LaRossa and Schloss (1984).

5.3 Acetolactate syntahse activity

The sensitivity of ALS extracted from ryegrass biotype WLR1, to a range of herbicides, differed markedly from that of ALS from biotypes VLR1 and SLR31 (Figure 5.1, Table 5.1). The level of ALS activity and pyruvate dependence were similar in extracts from the three biotypes (Figure 5.2 and 5.3). The concentration of chlorsulfuron required to inhibit ALS activity by 50% (the I_{50} values) for ALS from VLR1 and SLR31 were similar at 50 ± 15 nM and 38 ± 12 nM respectively (n=3). In contrast, the ALS from WLR1 exhibited an I_{50} for chlorsulfuron of greater than 1600 nM a value greater than 30 times that of the enzymes from the other two biotypes (Figure 5.1). The I_{50} values of ALS from WLR1 are 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 5.1).

The ratio of I_{50} values for ALS from biotype WLR1 versus the I_{50} value for ALS from the susceptible biotype VLR1 are similar to those reported for sulfonylurea resistant *Lolium perenne* (Saari *et al* 1992). These authors report that ALS extracted from sulfonylurea resistant *Lolium perenne* had I_{50} values greater than those of the susceptible by a factor of 35, 50 and 7 times for the herbicides chlorsulfuron, sulfometuron-methyl and imazapyr, respectively. These values are similar to those for *L.rigidum* biotype WLR1 (Table 5.2). The I_{50} ratios differ from those reported for sulfonylurea-resistant biotypes of *Stellaria media*, *Salsola iberica* and *Kochia scoparia* (Saari *et al* 1992)). These results suggest that the ALS mutation(s) involved in sulfonylurea reistance in these two *Lolium* species may be similar but differ from those in the other species. Molecular studies are required to clarify this point.

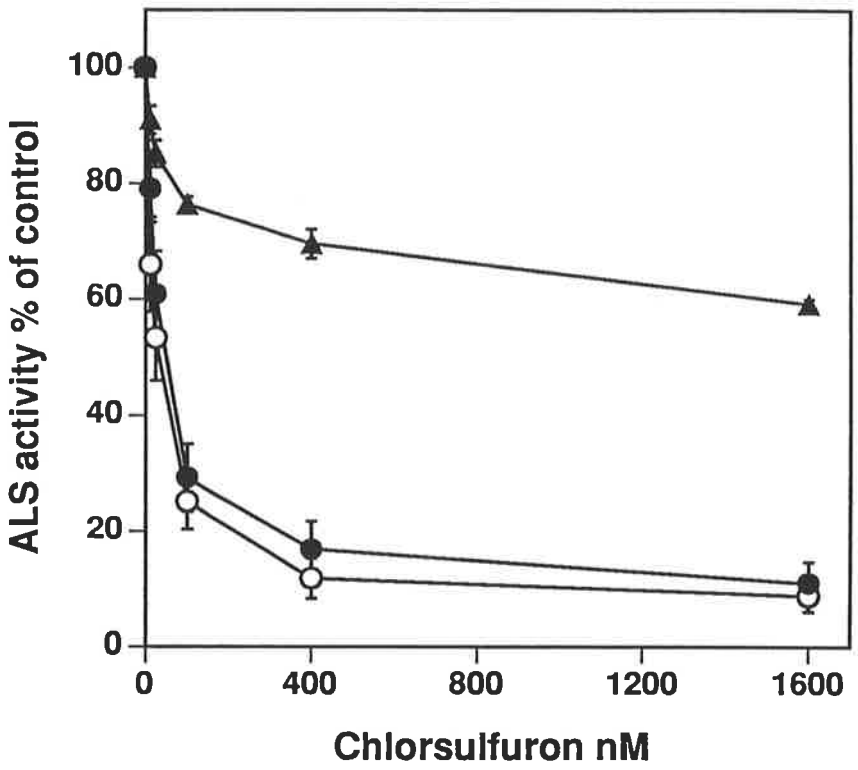


Figure 5.1 Chlorsulfuron inhibition of ALS activity extracted from resistant ryegrass biotypes WLR1 (▲), SLR31 (O) and susceptible biotype VLR1 (●) in 30 min assays using 139 mM pyruvate. ALS activity is expressed as % of activity of control assays without herbicide. Activity in control assays was similar for all biotypes (compare Figure 5.2 and 5.3). Data are means of 3 replicates. Vertical bars represent standard errors.

Table 5.1 I_{50} values to different herbicides for ALS from three ryegrass biotypes. Values are the mean ± S.E. of 3 experiments.

Herbicide	VLR1	SLR31	WLR1
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

Values followed by different letters in the same line are significantly different ($P<0.05$).

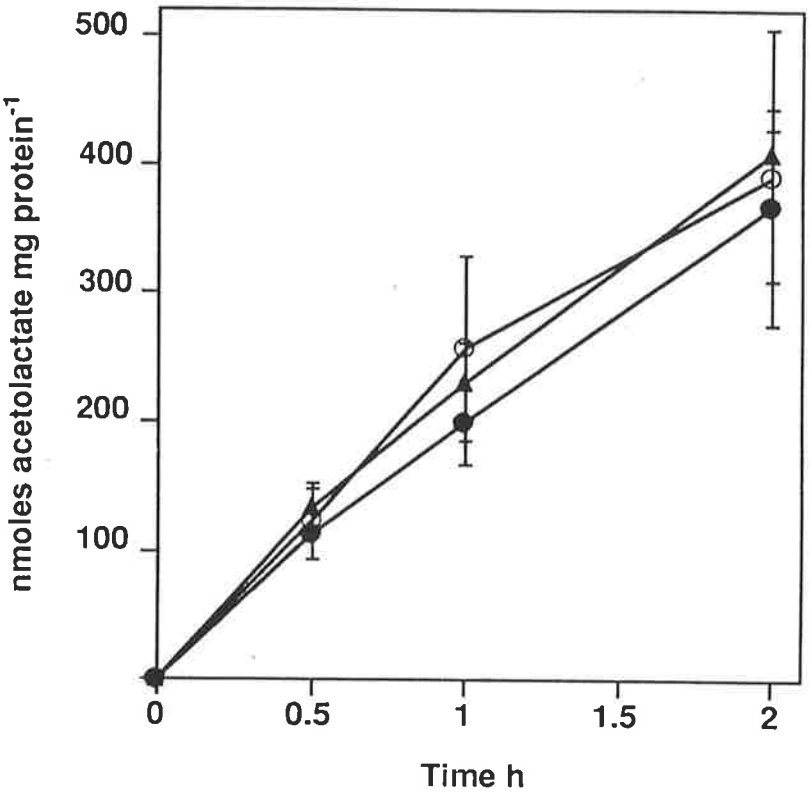


Figure 5.2 ALS activity from ryegrass biotypes WLR1 (▲), SLR31 (O) and VLR1 (●) in nmoles acetolactate mg protein^{-1}. Data are means of 3 replicates. Vertical bars represent standard errors.

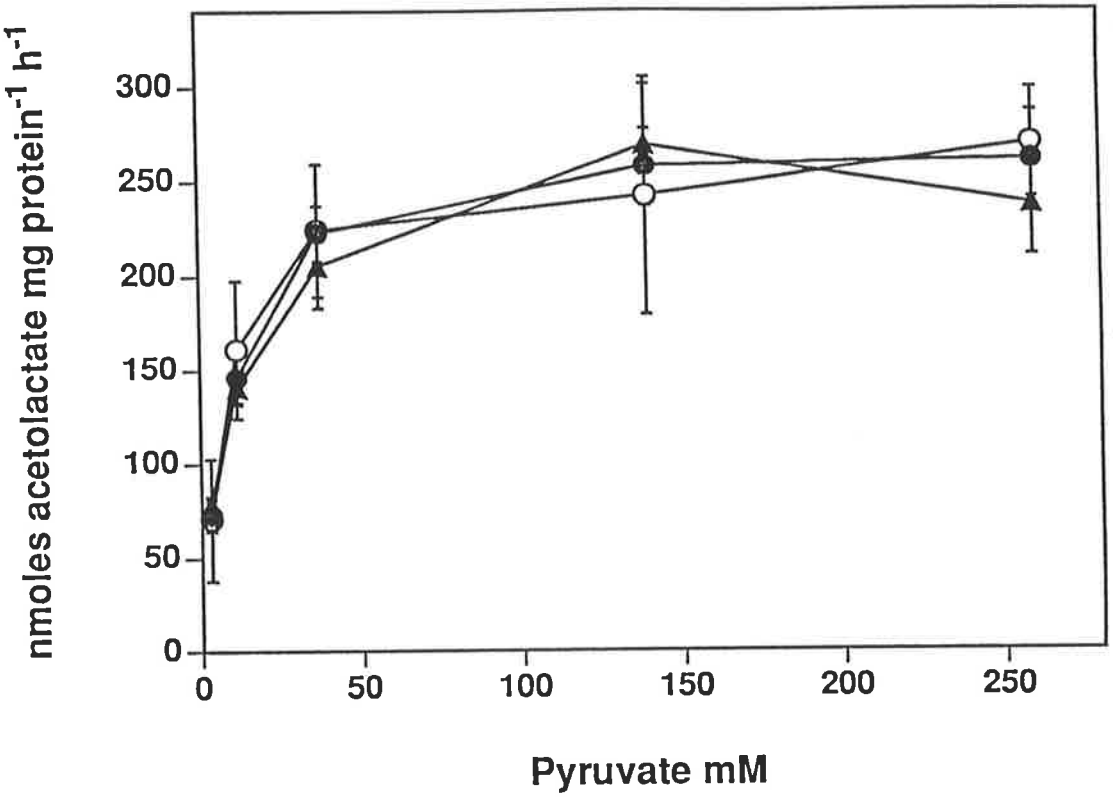


Figure 5.3 Pyruvate dependence of ALS activity from ryegrass biotypes WLR1 (▲), SLR31 (○) and VLR1 (●) in nmoles acetolactate mg protein^{-1} h^{-1} in 30 min assays. Data are means of 3 replicates. Vertical bars represent standard errors.

WLR1 has a mutant ALS which is less-sensitive to inhibition by sulfonylurea and imidazolinone herbicides (Figure 5.1, Table 5.1). This is probably the basis of the difference in the spectrum of resistance observed between the biotypes WLR1 and SLR31 (Chapter 3). The difference between the GR_{25} of pot grown plants of WLR1 and SLR31 for sulfometuron-methyl and imazapyr (Table 3.1) reflect the differences in I_{50} of the ALS from these two biotypes (Table 5.1). *L. rigidum* biotype WLR1 is resistant to both cereal-selective and non-selective sulfonylurea and imidazolinone herbicides and has ALS which is less-sensitive to these herbicides. Biotype WLR1 differs from the sulfonylurea cross-resistant biotype SLR31 which has an increased ability to metabolise chlorsulfuron (Figure 4.4) but has a herbicide-sensitive ALS (Figure 5.1). Biotype SLR31 is resistant to the wheat-selective sulfonylurea and imidazolinone herbicides chlorsulfuron, triasulfuron, metsulfuron-methyl and imazamethabenz but is not resistant to the non-selective herbicides sulfometuron-methyl and imazapyr (Table 3.1). Therefore, the increased ability to metabolise cholrsulfuron in the absence of a herbicide-insensitive ALS does not confer resistance to the non-selective herbicides sulfometuron-methyl and imazapyr. WLR1 also has an increased ability to detoxify chlorsulfuron (Figure 4.5) but resistance to the non-selective herbicides is probably due to a less-sensitive ALS. Clearly resistance to ALS inhibitors in *L. rigidum* can involve at least two mechanisms 1) increased ability to metabolise the herbicide and 2) a mutant form of the target enzyme ALS which is less sensitive to herbicides. These two mechanisms may be present in a single biotype.

Chapter 6

Uptake, translocation and metabolism of chlorsulfuron in intact plants.

6.1 Introduction

Reduction of herbicide uptake is one of the potential resistance mechanisms discussed in section 1.2. Uptake of herbicides absorbed through the roots could be reduced by the development of exclusion mechanisms within the root. Similarly, modifications to the leaf cuticle or epidermis could change the rate of uptake of herbicides which are absorbed through the leaf. Chlorsulfuron is absorbed through both the root and leaf tissue (Brown 1990).

Studies discussed in Chapter 4, using [^{14}C]chlorsulfuron applied *via* the xylem vessels of excised plants, circumvent any possible exclusion mechanisms at the leaf cuticle or roots (2.4). Therefore, further experiments to measure root and leaf uptake of ^{14}C-chlorsulfuron were conducted. Herbicide was applied at a level where differences in growth response between resistant and susceptible plants is observed.

6.2 Materials and Methods

6.2.1 Root uptake of chlorsulfuron

Seedlings of resistant ryegrass biotypes SLR31, WLR1 and susceptible biotype VLR1, were grown in hydroponics in 250 mL vials as described in section 2.2.3. When seedlings were at the two leaf stage, nutrient solution was replaced with fresh medium containing 82 nM [^{14}C]chlorsulfuron. Transpiration was measured by comparing the weight loss of vials containing plants and vials which did not. Plants were harvested 6h, 24 h, 48 h, and 72 h after the commencement of treatment or placed in fresh nutrient minus herbicide for a further 96 h. At harvest, nutrient solution was damped from the roots using paper towel, roots separated from shoots and the leaf lamina separated from the culm tissue. Fresh weight was recorded, the material frozen in liquid nitrogen and stored at -20C until extraction. The samples were extracted and analysed by HPLC following Method B described in section 4.2.2. The experiment was replicated 4 times using one vial containing 21 plants for each treatment.

6.2.2 Leaf uptake of chlorsulfuron

Initial tests with non-radioactive chlorsulfuron were used to determine the amount of herbicide which can be applied to differentially effect the growth of resistant and susceptible *L.rigidum* biotypes. Plants of resistant *L.rigidum* biotype SLR31 and susceptible biotype SLR2 where grown in a growth chamber in potting soil in 40 x 30 x 12 cm trays at a spacing of 3 cm as described in section 2.2. Growth chamber conditions were, 22C, 14 hr, 150 to 250 $\mu E\ m^{-1}\ s^{-1}$ light period, 10 h 15C dark period. Chlorsulfuron was placed on the leaves of two leaf plants in 1µl drops of 0.2% Triton X100, using from 7 to 12 plants per treatment. After 14 days the dry weight of treated plants was compared with controls treated with surfactant only. These studies revealed that applications of 120 or 240 ng chlorsulfuron in a single drop on the upper surface of the first leaf of 2 leaf *L.rigidum* seedlings caused greater reduction of dry matter accumulation in the susceptible biotype SLR2 than in the resistant biotype SLR31 (Table 6.1).

Table 6.1 Relative dry weight (%) of *L.rigidum* biotypes 14 days after treatment with non-radioactive chlorsulfuron applied in a 1µl drop to the upper surface of the first leaf, compared to untreated plants. Untreated controls plants of biotype SLR31 and SLR2 had an average dry weight of 50.3 ± 6.4 mg per plant and 46.2 ± 4.4 mg per plant respectively.

Biotype Chlorsulfuron applied	SLR31	SLR2
100ng	86 ± 4	40 ± 4
200ng	66 ± 4	32 ± 6

Data are the means of three experiments ± standard error.

Application of 120 or 240 ng of non-radioactive chlorsulfuron in a single drop in the axil between the first and second leaf, or divided between five drops applied to the upper surface of the first leaf, caused similar differences in dry matter accumulation between resistant and susceptible biotype (data not shown).

In subsequent experiments, herbicide uptake was measured by application of [^{14}C]chlorsulfuron to the leaf surface of *L.rigidum* plants grown as described above. Drops of 1µl containing either 100 ng or 200 ng of [^{14}C]chlorsulfuron in 0.2% Triton X 100 where applied to 2 leaf plants. Twenty four hours after treatment the plants were harvested, the leaf surface washed and the tissue stored at -20C prior to extraction in 80% MeOH and liquid scintillation counting as usual (2.4.2). Control plants treated with [^{14}C]chlorsulfuron and washed immediately prior to storage and extraction in the same manner, were used to correct for herbicide adhering to the cuticle but not inside the leaf tissue.

6.3 Root uptake and whole plant response to chlorsulfuron

[^{14}C]chlorsulfuron was applied to the roots of hydroponically grown *L.rigidum* seedlings to determine whether there was any evidence for reduced root uptake in resistant biotypes when herbicide was applied at a concentration which caused differential growth response between resistant and susceptible biotypes. In the absence of herbicide, growth in hydroponics was similar for each biotype (Figure 6.1). However, when 82 nM [^{14}C]chlorsulfuron was added to the nutrient medium of biotype VLR1, growth was completely inhibited and 20% of plants were necrotic within 72 h of treatment (Figure 6.1). Growth of the resistant biotype SLR31 was reduced but growth of WLR1 was similar to that of untreated plants (Figure 6.1). Thus, the same pattern of growth reduction is observed in this system as was observed for pot grown plants treated by spray application, the level of resistance following the order VLR1 < SLR31 < WLR1 (Chapter 3).

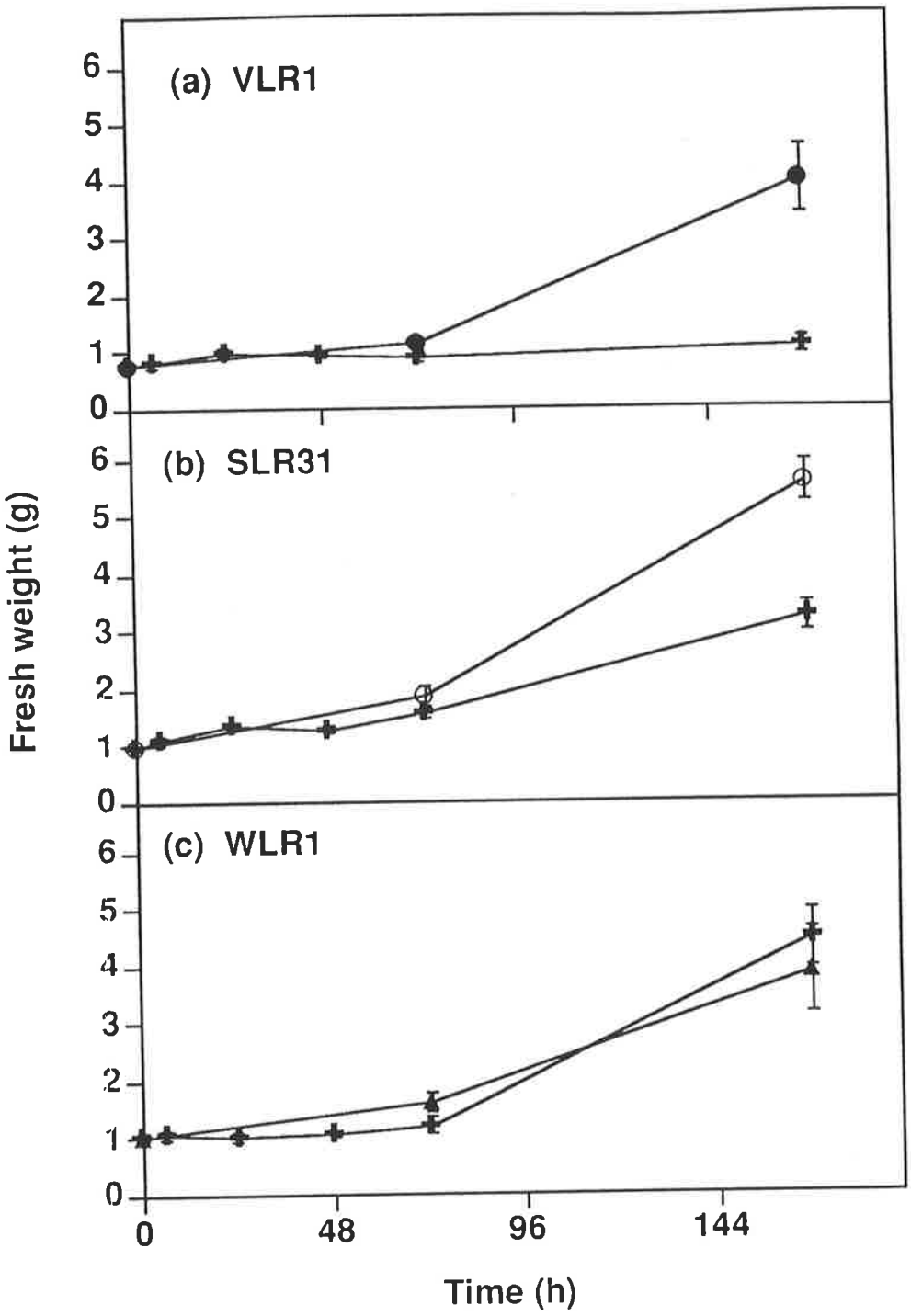


Figure 6.1 Fresh weight of hydroponically grown plants of ryegrass biotypes (a) VLR1 in the absence of herbicide (●) or with 82nM [^{14}C]chlorsulfuron (✚), (b) SLR31 in the absence of herbicide (○) or with 82nM [^{14}C]chlorsulfuron (✚), and (c), WLR1 in the absence of herbicide (▲) or with 82nM [^{14}C]chlorsulfuron (✚). Plants were harvested at various times after addition of herbicide to the nutrient solution. The maximum period of herbicide exposure was 72h. Vertical bars are standard error of the mean of 4 replicates

The differential growth between resistant and susceptible biotypes evident in Figure 6.1 is not the result of reduced entry of chlorusulfuron into the resistant biotypes. Biotypes SLR31 and WLR1 took up similar amounts of [^{14}C]chlorsulfuron. However, the susceptible biotype VLR1 took up less ^{14}C in total and less in the culms, than the resistant biotypes (Figure 6.2, Table 6.2). Accumulation of ^{14}C in the roots and leaf lamina of the resistant biotypes was either greater than or equal to that of the susceptible in all cases. There is no suggestion that the resistant biotypes exclude chlorsulfuron from the roots, nor that there is any difference in translocation to the shoots. The reduced uptake in the susceptible biotype VLR1 is probably due to the decreased growth and reduced transpiration caused by the herbicide. After the 72 h treatment period, none of the plants transferred to herbicide-free nutrient solution lost significant levels of herbicide (Figure 6.2).

The amount of ^{14}C which accumulated in the culms of these intact plants was less than that in experiments using excised seedlings, where differential rates of metabolism were observed (Table 4.2, Chapter 4). After the maximum treatment period (72 h) the amount of ^{14}C accumulated in the culms of each biotype was less than the amount which accumulated in excised stems exposed to herbicide for 3 h (Table 6.2). Insufficient ^{14}C accumulated in whole plant experiments to allow determination of the proportion of ^{14}C which remained as unmetabolised chlorsulfuron.

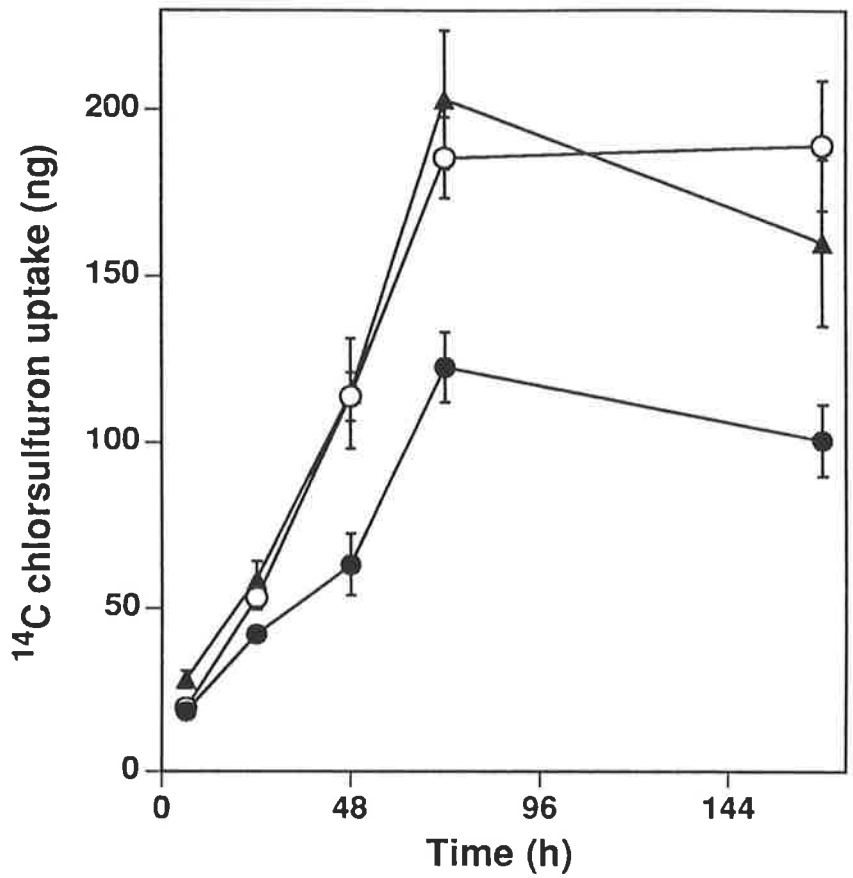


Figure 6.2 Total accumulation of ^{14}C in the leaves and culms of ryegrass biotypes VLR1 (●), SLR31 (○) and WLR1 (▲) treated with 82nM [^{14}C]chlorsulfuron in nutrient solution. Vertical bars represent the standard error of the mean of 4 replicates.

Table 6.2 Accumulation of ^{14}C in culms of *L.rigidum* given herbicide *via* cut stems with 3 h exposure (from Table 4.2) or given to intact plants via nutrient solution with 72 h exposure (equivalent of ng [^{14}C]chlorsulfuron per plant).

Biotype	VLR1	SLR31	WLR1
via nutrient solution	3.0 ± 0.3[a]	5.0 ± 0.4[b]	4.2 ± 0.6[b]
via cut stems	14.2 ± 1.3[a]	14.0 ± 1.0[a]	14.1 ± 1.4[a]

Data are the means ± standard error of 4 experiments. Values followed by the same letter in the same line are not significantly different (P>0.05).

Therefore, differential metabolism of chlorsulfuron can be measured in excised resistant and susceptible plants containing higher amounts of herbicide than are required to cause differential growth response in whole plants. This would suggest that differential metabolism can occur at least as rapidly in plants treated in the field as it does in excised seedlings.

6.4 Leaf uptake of chlorsulfuron in intact plants

[^{14}C]chlorsulfuron was applied to the leaf surface of *L.rigidum* seedlings at an application rate which causes differential growth response between resistant and susceptible biotypes (Table 6.1). Only small amounts of chlorsulfuron were taken up *via* the leaf cuticle leading to variability in the results of ^{14}C labelling studies. However, there was no evidence for reduced leaf uptake of chlorsulfuron in resistant *L.rigidum* biotypes (Table 6.3). In a single experiment when plants were treated with 100 ng [^{14}C]chlorsulfuron in 5 x 1µl drops applied to the upper surface of the first leaf, followed by 2 washes in 50% MeOH, the uptake of [^{14}C] after 24 hr was

equivalent to 15 ng [^{14}C]chlorsulfuron per plant for susceptible biotype SLR2 and 31 ng per plant for SLR31 (Table 6.3). Uptake was also similar between biotypes when plants were treated with 177 ng [^{14}C]chlorsulfuron placed as a single drop in the axil between the first and second leaf. In one experiment, plants were washed 3 times in 10% EtOH after 24 h and

Table 6.3 Accumulation of ^{14}C in seedlings of *L.rigidum* 24 h after application in two experiments (equivalent of ng [^{14}C]chlorsulfuron per plant).

	Susceptible biotypes			Resistant biotypes	
	SLR2	VLR1	SLR31	WLR1	VLR4
Experiment 1	15	-	31	-	-
Experiment 2	-	52	46	71	63

Experiment 1: 100 ng [^{14}C]chlorsulfuron in 5 x 1µl drops applied to the surface of the first leaf followed by two washes in 50% MeOH.
Experiment 2: 177 ng [^{14}C]chlorsulfuron in 1 x 1µl drop applied to the axil between the first and second leaves followed by three washes in 10% EtOH.

extracted as usual. [^{14}C] uptake was equivalent to 52 ng [^{14}C]chlorsulfuron in susceptible biotype VLR1, 46 ng for biotype SLR31, 71 ng for biotype WLR1 and 63 ng for biotype VLR4 (Table 6.3). These results do not suggest that uptake of chlorsulfuron is reduced in resistant biotypes of *L.rigidum*. However, replicated data are required to determine whether there is any small but consistent difference between uptake in resistant and susceptible biotypes.

6.5 Discussion

There is no evidence that reduced root or leaf uptake in resistant biotypes of *L.rigidum* is involved in chlorsulfuron resistance. This data is in agreement with the work of Cotterman and Saari (Cotterman and Saari 1992) who show that chlorsulfuron uptake via the leaf cuticle was highly variable but did not differ significantly between the resistant *L.rigidum* biotype SLR4/84 and the susceptible biotype SLR2. These authors also report that in excised roots of larger (three to four leaf) plants of resistant ryegrass biotype SLR4/84, metabolism of chlorsulfuron was more than four times that of susceptible biotype SLR2 when treated with 28μM [^{14}C]chlorsulfuron for a period of two hours.

Thus, increased metabolism of chlorsulfuron in the roots of resistant *L.rigidum* can be observed when the roots are treated with a significantly higher concentration of the herbicide than that needed to completely inhibit growth and cause significant mortality of the susceptible biotype VLR1. Similarly, increased metabolism of the herbicide in the culms of resistant plants can be observed when chlorsulfuron concentration is greater than that found in dying susceptible plants.

Chapter 7

Are mixed function oxidases involved in chlorsulfuron metabolism in *L.rigidum* ?

7.1 Introduction

As discussed in Chapter 4, chlorsulfuron resistance in *L.rigidum* biotype SLR31 involves increased herbicide metabolism by a mechanism similar to that operating in wheat. Biotype SLR31 exhibits herbicide-sensitive ALS (Figure 5.1). In a resistant biotype with a herbicide-sensitive ALS, factors effecting the rate of metabolism should effect the level of resistance. In this section, studies using compounds which have the potential to inhibit or induce known herbicide detoxification mechanisms in plants are discussed. Compounds which inhibit chlorsulfuron metabolism, in addition to providing an indication of the type of detoxification process(es) involved, could lead to the development of herbicide synergists to increase the effect of herbicides against *L.rigidum*. Conversely, herbicide antagonists could also prove a valuable aid to investigating the mechanism of herbicide detoxification.

HPLC analysis showed the major metabolite of chlorsulfuron metabolism in *L.rigidum* to coelute with the major metabolite of chlorsulfuron produced by wheat (Chapter 4). This has been previously identified as the glycosylated derivative of chlorsulfuron hydroxylated in the

phenyl ring (Sweetser *et al.* 1982). Cotterman and Saari (1992) confirmed that the major product of chlorsulfuron metabolism in *L.rigidum* could be hydrolysed using the enzyme β-glucosidase to give a product which coeluted with an hydroxy-chlorsulfuron standard. The production of an aryl-hydroxylated intermediate is a reaction typical of xenobiotic metabolism mediated by cytochrome P450-dependant mixed function oxidase enzyme systems (MFOs), also called monooxygenases (Hatzios and Penner 1982). Mixed function oxidase enzyme systems capable of metabolising herbicides have been isolated from plants. Microsomal MFOs from wheat which have the capacity to metabolise diclofop-methyl, chlorotoluron, 2,4-dichlorophenoxyacetic acid, chlorsulfuron and triasulfuron have been previously reported (Mc Fadden *et al.* 1989, Frear & Marauas 1990, Frear *et al.* 1991, Zimmerlin and Durst 1990, Zimerlin and Durst 1992). Evidence suggests that the MFO catalysing diclofop-methyl hydroxylation in wheat may be the same as that which catalyses lauric acid hydroxylation (Zimmerlin 1992).

MFOs are membrane bound enzyme systems containing heme coordinated divalent cations which facilitate the oxidation of a wide range of substrates using energy from NADH or NADPH (Riviere and Cabanne 1987). They are required for many metabolic processes and are involved in the detoxification of xenobiotics in the mammalian liver, insects, fungi and plants (reviewed in Hatzios and Penner 1982). In insects, MFOs are particularly important in detoxification of insecticides and enhanced levels of MFO-mediated detoxification are involved in many cases of insecticide resistance and cross-resistance (Brattsten *et al.* 1986). Inhibitors of insect MFOs have been widely used to increase the efficacy of certain insecticides (Hodgson 1985).

Many different MFO isozymes occur in the various tissues and organs of animals and plants (Riviere and Cabanne 1987) as well as being differentiated between the organelles of individual cells (Donaldson 1991). There is increasing evidence for the occurrence of many different isozymes of

MFOs in plant tissues which differ in level and range of substrate specificity and level of inducibility by exogenous chemicals (Donaldson 1991).

The MFO-inhibitors piperonyl butoxide (PBO), aminobenzotriazole (ABT) and tetcyclasis can enhance the phytotoxicity of chlorotoluron toward wheat (Cabanne *et al.* 1987, Mougin *et al.* 1991). PBO, tetcyclasis and paclobutrazol by microsomal P450s from wheat (Frear *et al.* 1991); ABT also increases the phytotoxic effect of chlorotoluron towards chlorotoluron resistant biotypes of *L.rigidum* (Burnet *et al.* 1993a). MFO inhibitors have also been suggested to increase the phytotoxic effects of herbicides toward herbicide resistant populations of *Alopecurus* (Kemp *et al.* 1988). Increased injury of maize by the sulfonylurea herbicide primisulfuron may be caused by MFO inhibition caused by soil applied insecticides (Kreuz and Fonne-Pfister 1992).

Conversely, compounds which stimulate the detoxification of herbicides have been used to reduce the phytotoxic effects of herbicides upon certain crop species (reviewed in Hatzios 1989). Such compounds are termed herbicide safeners. Naphthalic anhydride (NAA) treatment of seed has been shown to decrease the phytotoxic effects of certain sulfonylureas toward wheat (Sweetser 1985). Recently, herbicide safeners have been commercially released for safening cereal crops against the grass-selective aryloxyphenoxypropionate herbicides (Amerein *et al.* 1989, and Bieringer *et al.* 1989). These compounds are thought to increase the rate of detoxification of certain aryloxyphenoxypropionate herbicides although the enzymes systems involved are not reported (Amerein *et al.* 1989, and Bieringer *et al.* 1989).

The MFO isozymes involved in herbicide metabolism in wheat and maize occur at low levels in the plant tissue. Activity of these isozymes is usually undetectable in tissues which have not been pretreated with inducers to increase their activity. Tissue is commonly treated with compounds such as phenobarbitol, ethanol or NAA to induce MFOs responsible for metabolism of herbicides (Mougin 1991, Zimmerlin 1992).

Identifying inducers of chlorsulfuron metabolism in *L.rigidum* would, therefore, be valuable for *in vitro* studies of any MFO system(s) involved. Additionally, if chlorsulfuron resistance in resistant *L.rigidum* biotypes with herbicide-sensitive ALS is based on MFO-dependant detoxification it may be possible to increase the level of resistance using inducers of MFOs and to reduce it using MFO inhibitors.

Experiments were conducted to test the effects of MFO inhibitors and inducers on the level of phytotoxicity of chlorsulfuron toward biotype SLR31. Mixed function oxidase inhibitors PBO, ABT, tetcyclasis and five fungicidal sterol biosynthesis inhibitors where tested. Similarly, the MFO inducers NAA, CGA 185072 and Hoe 070452 where tested.

7.2 Materials and Methods

7.2.1 MFO inhibitors (herbicide synergists)

To demonstrate synergistic or antagonistic effects between compounds, it is necessary to measure the effects of several different levels of each compound in a replicated experiment of factorial design (Green & Bailey 1987). To facilitate quick screening of many chemicals for potential synergistic or antagonistic effects without the need for such extensive tests, pilot experiments were used to determine the maximum levels of each inhibitor/inducer which could be applied without causing a primary effect. The identified levels of compounds were used in tests with the expectation that any interactions would be most evident at high levels of application.

Piperonylbutoxide (PBO)

L.rigidum biotypes VLR1, SLR31, WLR2 and WLR1 and wheat were cultured in pots placed in the field, 21 plants per pot (2.2.2). Plants were treated at the 2 leaf stage in a laboratory spray cabinet (2.3). PBO was applied in two applications of 3 L ai ha^{-1} in 113 L ha^{-1} water to give a total application of 6 L ai ha^{-1} in a total volume of 226 L ha^{-1}. Herbicides, were applied, were added to this solution. Two replicates experiments were

conducted. Plants were harvested at soil level, 24 days after treatment before mortality and dry weight were determined.

Aminobenzotriazole (ABT)

L.rigidum biotypes VLR1, WLR1 and SLR31 were cultured hydroponically in 250 mL vials (2.2.3). At the 2 leaf stage, nutrient solution was replaced with solution containing 70 μM ABT, 100 nM chlorsulfuron, or 70 μM ABT + 100 nM chlorsulfuron. Control vials received fresh nutrient solution only. Mortality, dry weight of roots and shoots were determined 21 days after treatment. Four replicate experiments were conducted. Data were analysed by two way analysis of variance.

Tetcyclasis

Ryegrass biotypes VLR1, SLR31, WLR1 and WLR2 were cultured using hydroponics in 250 mL vials (2.2.3). The nutrient solution was replaced when plants were at the 2 leaf stage with solution containing 3.7 nM tetcyclasis, herbicide or 3.7 nM tetcyclasis + herbicide. For biotypes VLR1, SLR31 and WLR1, 100 nM chlorsulfuron herbicide was used. For biotype WLR2 2 μM chlorotoluron was used. Each treatment consisted of one vial containing 21 plants. The experiment was replicated 3 times. Mortality, dry weight of roots and dry weight of shoots were determined 21 days after treatment and data were analysed by two way analysis of variance.

7.2.2 Crop safeners

Dose response to chlorsulfuron in the presence and absence of safeners was determined using pot cultured plants, 24 plants per treatment (2.3). Experiments for each safener were conducted once only. Seed of *L.rigidum* biotypes VLR1, SLR31 and wheat were mixed with 0.25 g naphthalic anhydride before being germinated on agar. Safeners, Hoe 070452 and CGA 185072 were added to the herbicide mixture during herbicide treatment in the laboratory spray cabinet (2.3).

7.3 Effectors of mixed function oxidases

7.3.1 MFO inhibitors (herbicide synergists)

Piperonylbutoxide

Piperonylbutoxide is a methylenedioxyphenyl-containing compound. Many compounds containing methylenedioxyphenyl substituents are known to inhibit MFOs (Sacher et al 1971). Piperonylbutoxide is widely used as an insecticide synergist because of the ability to inhibit MFO-based detoxification of certain insecticides (Kulkarni and Hodgson 1976) while carbamate insecticides and plant alkaloids containing MDP substituents have been shown to be auto synergists (23). Methylenedioxyphenyl-containing compounds have not been found to be so widely inhibitory to MFOs in plants but PBO has been shown to increase the phytotoxicity of chlorotoluron in wheat (Mougin 1991).

Application of 6 L ai ha^{-1} PBO alone had no detectable phytotoxic effect upon *L.rigidum* seedlings (Figure 7.1). The fact that PBO application can affect the interaction of plants and herbicides is demonstrated by the response of biotype WLR2. Growth reductions due to 500 g ai ha^{-1} chlorotoluron were 45% and 49% for each of two replicate experiments respectively and when chlorotoluron was applied in combination with 6 L ai ha^{-1} PBO, the growth reductions increased to 82% and 87% for each replicate respectively this result confirms the work of Burnet (Burnet 1992) (Figure 7.1).

In contrast to chlorotoluron treated WLR2, the interactive affects between PBO and chlorsulfuron applied to other *L.rigidum* biotypes, were small. Chlorsulfuron treatment at 4 g ai ha^{-1} caused approximately 50% reduction in shoot dry weight of the susceptible biotype VLR1 but addition of PBO did not increase the effect (Figure 7.1). Dry weight of cross-resistant biotype SLR31 was unaffected by the

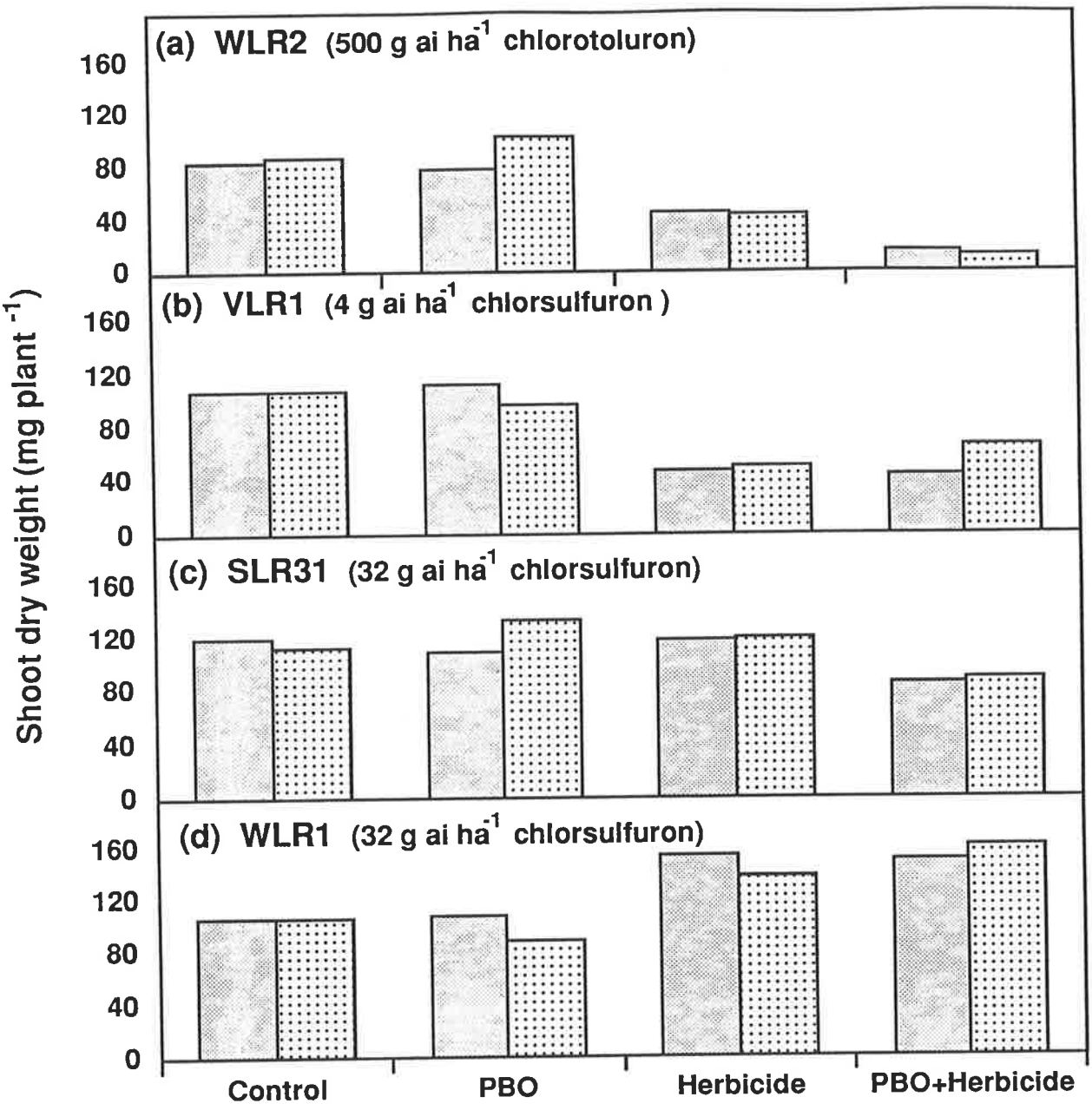


Figure 7.1 Shoot dry weight 24 days after treatment of pot cultured ryegrass of biotypes, (a) WLR2, (s) VLR1, (c) SLR31, and (d) WLR1; untreated (control), treated with PBO 6 L ha^{-1} (PBO), treated with herbicide only, treated with herbicide + PBO 6 L ha^{-1}. Mean for each of 2 replicate experiments are represented by stippled bars for each treatment.

application of 32 g ai ha^{-1} chlorsulfuron. Treatment of biotype SLR31 with 32 g ai ha^{-1} chlorsulfuron with addition of PBO caused a slight reduction in shoot dry weight but this weight reduction was less than observed for biotype VLR1 treated at a much lower rate of herbicide alone.

There was no reduction in growth due to herbicide in resistant biotype WLR1 either with or without PBO in fact, some growth enhancement may be evident (Figure 7.1). A synergistic effect between PBO and chlorsulfuron on WLR1 would not be expected, given that herbicide-insensitive ALS is the major determinant of chlorsulfuron resistance in biotype WLR1 and not enhanced metabolism. Thus, the small differential effect caused by PBO is only observed in a biotype for which increased chlorsulfuron metabolism is thought to be a major factor in resistance (biotype SLR31) but is not observed in the sensitive biotype VLR1 or in biotype WLR1, where herbicide-insensitive ALS is more important. These results suggest that the interactive phytotoxic effect of PBO and chlorsulfuron toward biotype SLR31 may be due to an effect on herbicide metabolism rather than a small phytotoxic effect of PBO alone which is not detected in the absence of the herbicide.

When 30 μM PBO was applied to the roots of SLR31 plants grown hydroponically in 4L trays (described in section 2.2.3) in the presence of 100 nM chlorsulfuron, shoot dry weight of plants treated with PBO and chlorsulfuron, 15 days after treatment, was reduced 49% compared to the untreated controls (Table 7.1). When the more soluble PBO analog methylenedioxyphenolic acid was applied to SLR31 plants using the same system, those treated with inhibitor plus chlorsulfuron exhibited a growth reduction of 49%, (14 days after treatment) compared to untreated plants (Table 7.1). In metabolism studies where [^{14}C]chlorsulfuron was fed to excised shoots (2.4.1), metabolism of [^{14}C]chlorsulfuron over 3 h did not decrease with addition of up to 10μM PBO (data not shown). Thus the results of experiments using several different experimental systems

consistently suggest that PBO has a small effect on the phytotoxicity of chlorsulfuron towards ryegrass biotype SLR31.

Table 7.1 Shoot dry weight (mg) of *L.rigidum* biotype SLR31 grown in nutrient solution (4 L). Treated with 100 nM chlorsulfuron plus or minus mixed funtion oxidase inhibitor, piperonyl butoxide (PBO 30 μM) or methylenedioxyphenolic acid (MDPa 600 nM).

MFO Inhibitor	Control	Inhibitor only	Chlorsulfuron only	Chlorsulfuron + inhibitor
PBO	76	71	62	39
MDPa	74	74	54	38

Data are from one experiment for each inhibitor and represent the mean of 27 plants per treatment for MDPa and the mean of 5 plants harvested 15 days after treatment for PBO.

Aminobenzotriazole (ABT)

The results of experiments using ABT applied *via* the nutrient medium of hydroponically grown plants exhibit a similar pattern to the results obtained using PBO applied to the foliage. Firstly, the results of Burnet *et al.* demonstrated that, in a similar hydroponic system, 70 μM ABT plus chlorotoluron had a similar phytotoxic effect upon the resistant biotype WLR2 to that of chlorotoluron alone applied to the susceptible biotype VLR1 (Burnet *et al.* 1993a).

As with foliar applied PBO (Figure 7.1), ABT applied alone had no detectable phytotoxic effect for the three *L.rigidum* biotypes (Figure 7.2). There was some interaction between the effect of chlorsulfuron and hydroponically applied ABT upon *L.rigidum* biotype SLR31. However, the interactive effect of ABT and chlorsulfuron toward biotype SLR31 was not on shoot dry weight but mortality. Shoot dry weight of surviving plants

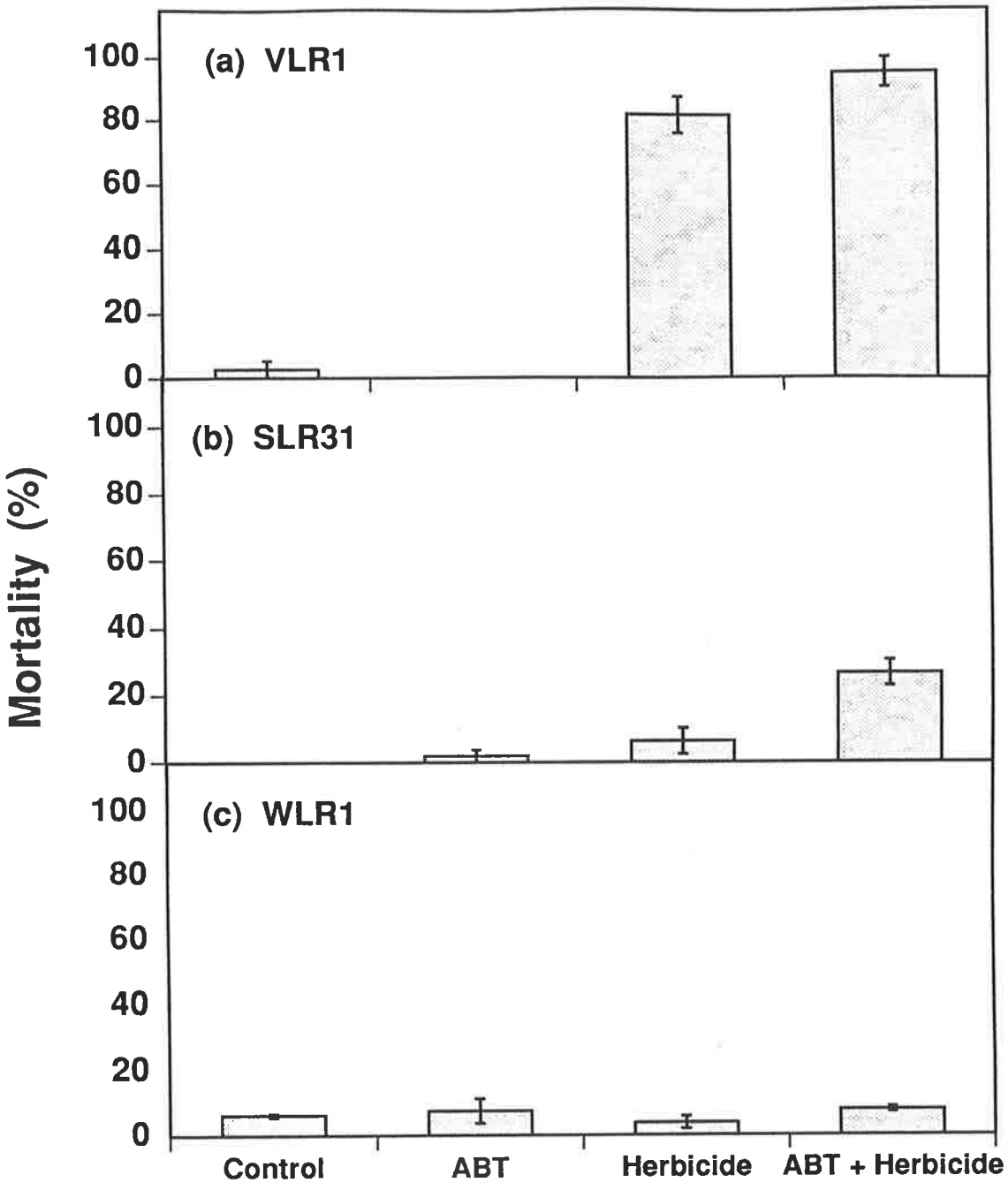


Figure 7.2 Mortality of hydroponically grown *L.rigidum* biotypes, (a) VLR1, (b) SLR31, and (c) WLR1 in nutrient solution only (control), nutrient + 70 μM ABT (ABT), nutrient + 100 nM chlorsulfuron (Herb), and nutrient + 70 μM ABT +100nM chlorsulfuron (ABT + Herb) harvested 21 days after treatments. Vertical bars represent the standard error of the mean of 3 replicates.

was not significantly effected ($p<0.05$) while there was a small, but significant, interactive effect on mortality caused by ABT plus chlorsulfuron applied to biotype SLR31 ($P<0.05$, Figure 7.2). Like the effect of foliar applied PBO on shoot dry weight, the increased mortality caused by ABT plus chlorsulfuron applied to SLR31 (27 ± 6.6 %), was less than the mortality caused by the effect of chlorsulfuron alone applied to the susceptible biotype VLR1 (82 ± 5.7 %) (Figure 7.2).

The interaction between the effects of chlorsulfuron and ABT on mortality of biotypes VLR1 and WLR1 was not significant. Nor were the effects on shoot or root dry weight ($P<0.05$). This is also similar to the results achieved by foliar application of PBO (Figure 7.1).

Tetcyclasis

Tetcyclasis was also able to increase the phytotoxic effect of chlorotoluron toward biotype WLR2 when applied *via* the hydroponic nutrient medium ($P<0.05$, Figure 7.3). However, there were no significant increases in the phytotoxic effects of chlorsulfuron, due to addition of tetcyclasis, upon the root or shoot dry weight or mortality of *L.rigidum* biotypes VLR1, SLR31 or WLR1 ($P>0.05$, Figure 7.3). Thus, tetcyclasis does increase the phytotoxicity of chlorotoluron toward biotype WLR2 but does not effect the phytotoxicity of chlorsulfuron toward biotype SLR31.

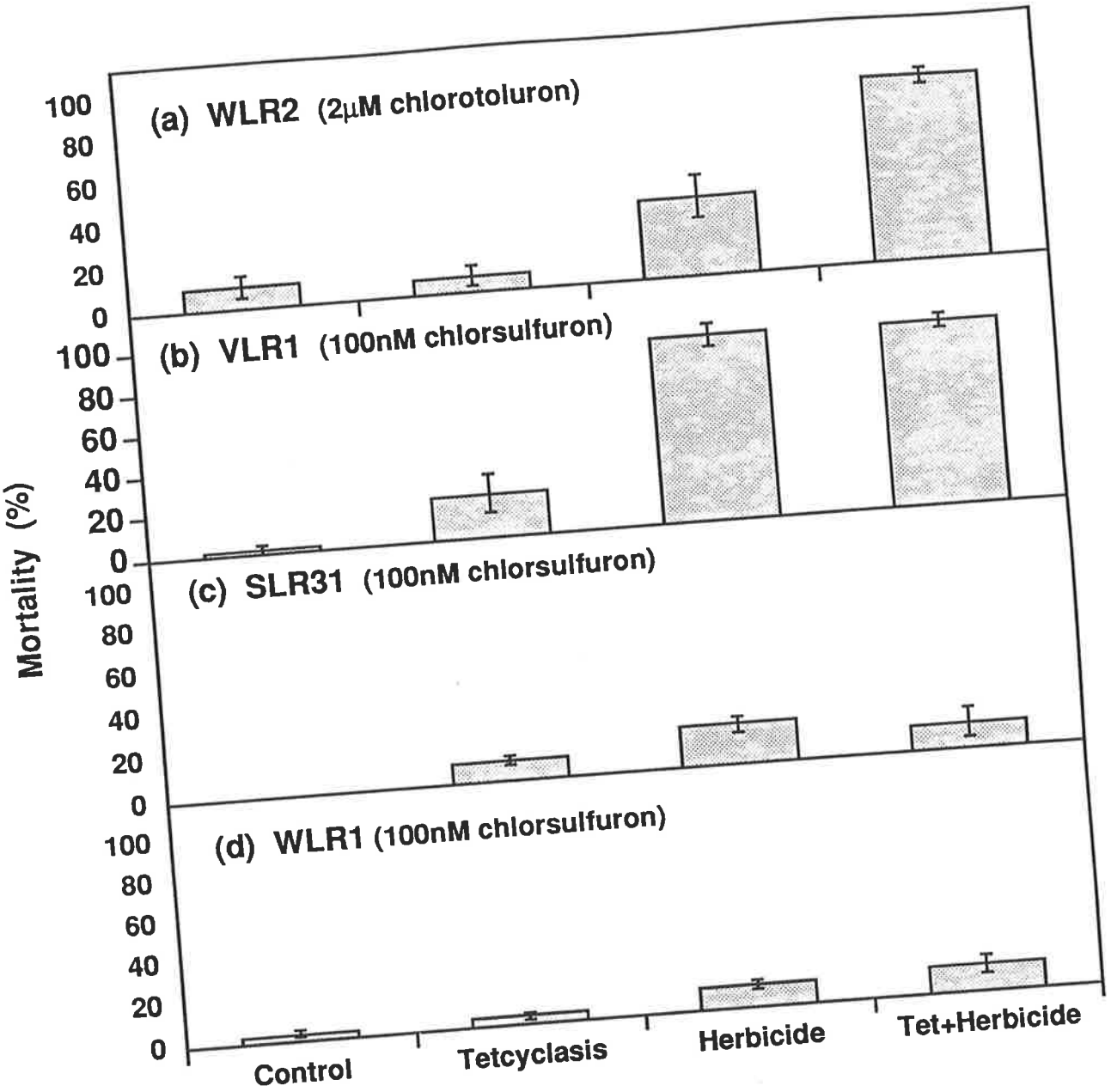


Figure 7.3 Mortality of hydroponically grown ryegrass biotypes, (a) WLR2, (b) VLR1, (c) SLR31 and (d) WLR1; in nutrient solution only (control), nutrient + 3.7 nM tetcyclasis, nutrient + herbicide, and nutrient + 3.7 nM tetcyclasis + herbicide . For biotype WLR2 2 μM chlorotoluron herbicide was used while 100 nM chlorsulfuron herbicide was used for all other biotypes. Vertical bars represent the standard error of the mean of 3 replicates.

Sterol biosynthesis inhibitors

Systemic triazole and pyridine fungicides which act by inhibition of MFOs involved in sterol biosynthesis in fungi (Lenton 1987) were also tested by addition to the nutrient medium of SLR31 plants growing in hydroponic culture in 4L trays (2.2.3). Of four such compounds tested only bitertanol was found to have a small effect on shoot dry weight similar to that observed with PBO in hydroponics (Figure 7.4). The fungicides tebuconazole, tridimefon and tridimenol had no interactive effect upon mortality, shoot dry weight or root dry weight.

7.3.2 Crop safeners

Naphthalic anhydride pretreatment of wheat seed has been shown to increase the rate of chlorsulfuron metabolism (Sweetser 1985). The results in Figure 7.5 indicate that pretreatment of wheat seed with 0.25 g NAA per 100 g seed can reduce the phytotoxic effect of chlorsulfuron toward wheat when grown in pots in the field. However, dose response to chlorsulfuron of *L.rigidum* biotypes VLR1 and SLR31 grown from seed pre-treated with naphthalic anhydride was similar to that of plants grown from untreated seed (Figure 7.5) and similar to that observed in previous experiments (Chapter 3). This suggests that, although NAA seed treatment can increase chlorsulfuron metabolism in wheat (Sweetser 1985), it has little effect upon *L.rigidum*.

Similarly when chlorsulfuron dose response was determined in single experiments using safeners Hoe 070542 (120 g ai ha^{-1}) and CGA 185972 (1 L ha^{-1}), levels in excess of those recommended for safening cereals against aryloxyphenoxypropionate herbicides, little difference in dose response between herbicide treated plants plus or minus safener was observed (Table 7.2).

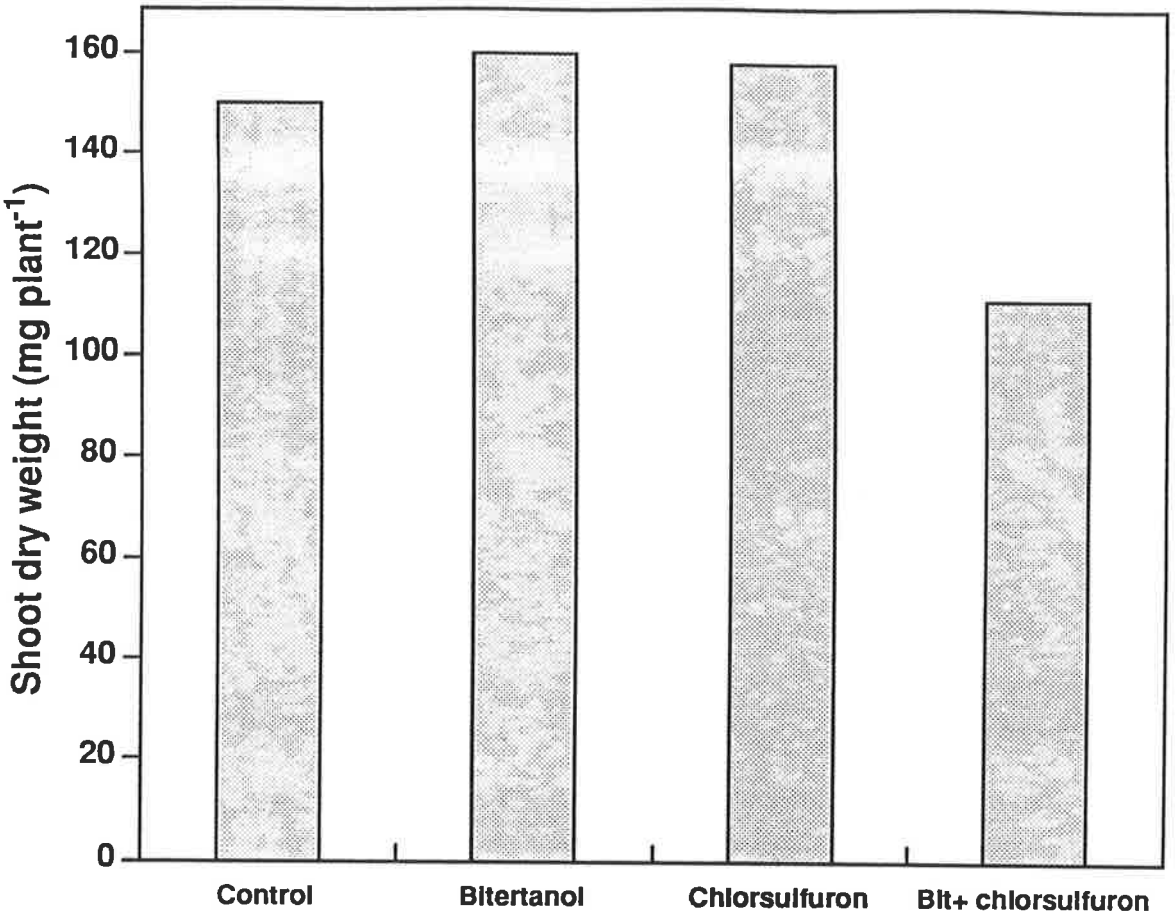


Figure 7.4 Shoot dry weight of hydroponically grown *L.rigidum* biotype SLR31 in nutrient solution only (control), nutrient + 3.4 μg L^{-1} bitertanol (bitertanol), nutrient + 100 nM chlorsulfuron (Herbicide), and nutrient + 3.4 μg L^{-1} bitertanol + herbicide (Bit+Herbicide). Data represent the means for one experiment using 28 plants per treatment.

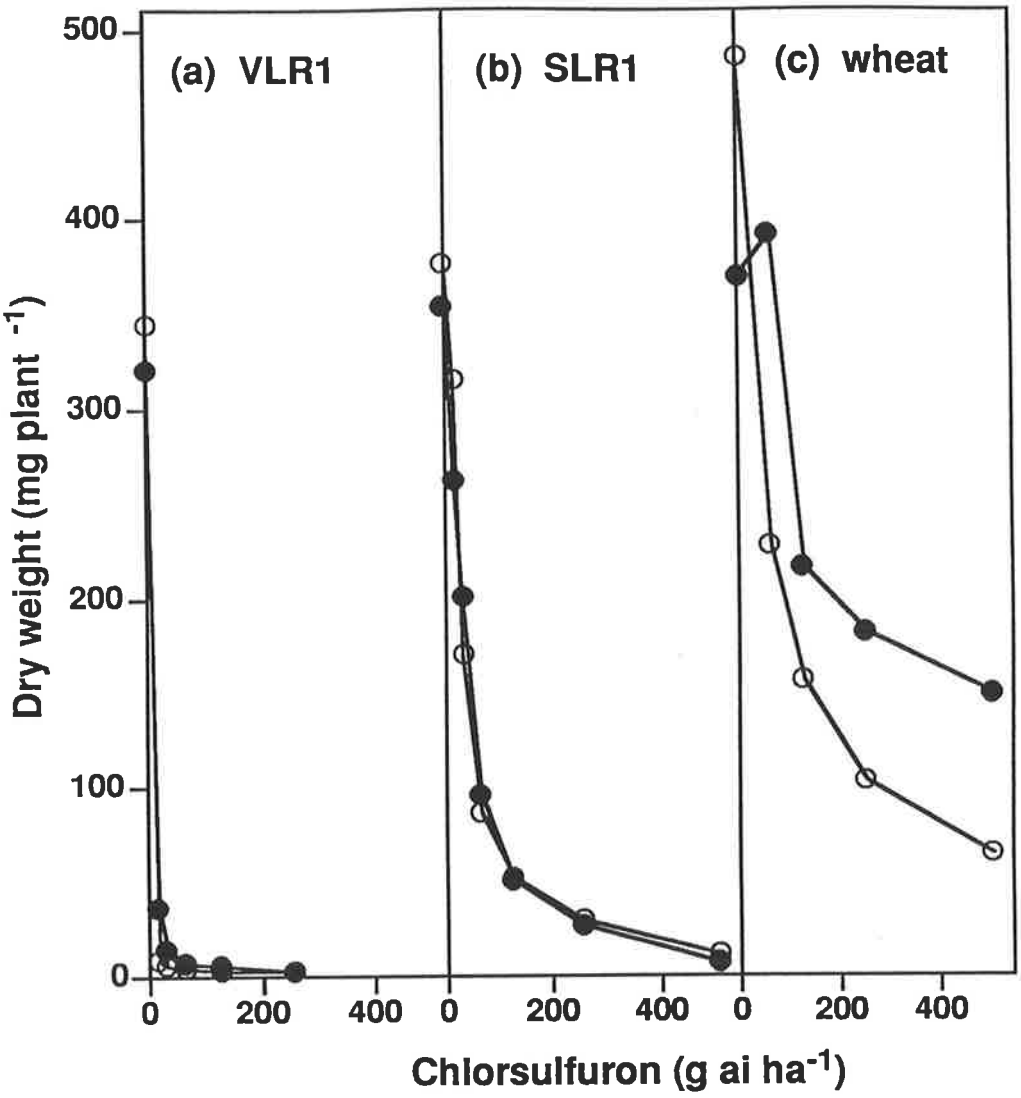


Figure 7.5 Dry weight of *L.rigidum* biotypes (a) VLR1, SLR31 (b) and wheat (c), grown from seed treated with (●), or without (○), 0.25 g per 100 g seed NAA. Plants were treated with chlorsulfuorn at the two leaf stage and harvested 42 days after treatment. Data are the means from one experiment using 24 plants per treatment.

Table 7.2 Mortality (%) of *L.rigidum* biotypes grown in pots in the field and treated with chlorsulfuron, chlorsulfuron plus Hoe 070542 (120 g ai Ha^{-1}) or chlorsulfuron plus CGA 185972 (1 L ha^{-1}).

Biotype	VLR1	SLR31	WLR1
Chlorsulfuron (g ai ha^{-1})	16	256	256
Chlorsulfuron only	67	55	0
plus Hoe 07542	67	42	0
Chlorsulfuron only	33	58	8
plus CGA 185972	42	59	0

7.4 Discussion

The results represented in Figures 7.1 and 7.3 in conjunction with those of Burnet *et al.* 1993 demonstrate that MFO inhibitors can have a synergistic effect with the herbicide chlorotoluron upon the cholotoluron resistant biotype WLR2. When PBO, ABT and tetcyclasis were applied with the herbicide chlorotoluron to the chlorotoluron resistant biotype WLR2 the phytotoxic effects approached those of chlorotoluron applied to the susceptible biotype VLR1. The increased phytotoxic effect of chlorotoluron toward biotype WLR2 in the presence of these MFO inhibitors suggests that the compounds can enter *L.rigidum* plants and effect herbicide metabolism. Studies using [^{14}C]chlorotoluron confirm that ABT can reduce the rate of of metabolism in this biotype (Burnet *et al.* 1993a)

High levels of the MFO inhibitors PBO and ABT slightly increased the phytotoxic effect of chlorsulfuron towards biotype SLR31 (Figures 7.1 and 7.2). However, the level of damage caused to SLR31 by these compounds in combination with chlorsulfuron was less than the level of damage caused to VLR1 by chlorsulfuron alone. Thus, it appears, the

compounds do not inhibit metabolism of chlorsulfuron in resistant *L.rigidum* biotype SLR31 to the same extent that they inhibit chlorotoluron metabolism in biotype WLR2. If the compounds do not inhibit chlorsulfuron metabolism, chlorsulfuron metabolism may involve an MFO isozyme which is insensitive to them or alternatively, the process may involve some other oxidative system mediated by such agents as FAD or Cu^{++} containing monooxygenases (Mahler & Cordes 1971) or hydroxamic acids (Zuniga *et al.* 1983, Niemeyer 1988). These studies are far from exhaustive, however, as several groups of chemicals thought to inhibit or induce MFOs have not been tested. Organophosphate insecticides have been recently been reported to inhibit primisulfuron degradation in maize which is thought to be due to an MFO enzyme system (Kreuz and Fonne-Pfister 1992) while many other plant growth regulators and herbicide synergists such as paclobutrazole and tridiphane could also be tested.

There is no evidence that the MFO inducer NAA or the crop safeners CGA 18507 or HOE 070452 can reduce the phytotoxicity of chlorsulfuron toward ryegrass, despite the fact that NAA was able to safen wheat against chlorsulfuron treatment. Therefore, these compounds do not appear to enhance chlorsulfuron metabolism in *L.rigidum*.

Thus, either the MFO inhibitors and herbicide safeners tested do not effect metabolism of chlorulfuron in biotype SLR31, or increased metabolism is not the only mechanism of resistance in this biotype. The data presented in this chapter are consistent with at least three hypotheses.

(1) Chlorsulfuron resistance in biotype SLR31 involves an oxidative system other than an MFO and is therefore insensitive to MFO inhibitors.

(2) Chlorsulfuron resistance in biotype SLR31 involves MFO(s) with a different specificity to those involved in metabolism of chlorotoluron in biotype WLR2 which are less-sensitive to inhibition by compounds which inhibit the MFO isozyme(s) causing chlorotoluron metabolism in WLR2.

(3) Chlorsulfuron resistance in SLR31 involves an MFO system which is inhibited by the compounds tested but also involves another, unidentified, mechanism which is not effected

Further data are required to differentiate betweeen these hypotheses. Studies using [^{14}C]chlorsulfuron are required to determine whether the MFO inhibitors tested can reduce chlorsulfuron metabolism in SLR31. Experiments are also needed to determine the nature of the catalytic system facilitating chlorsulfuron metabolism in *L.rigidum* and to determine whether increased metabolism is the only mechanism involved in chlorsulfuron resistance in biotype SLR31. Future research needs are discussed in the light of these and other results in Chapter 13.

Chapter 8

Effect of light period following herbicide application on chlorsulfuron resistance

8.1 Introduction

Studies of chlorsulfuron metabolism in excised *L.rigidum* seedlings using method A revealed that the rate of metabolism slowed markedly or ceased, approximately twelve hours after commencement of herbicide application (Figure 4.3, Chapter 4). This coincided with the switch from light to dark period. Metabolism did not recommence when illumination resumed in the next light period. The level of chlorsulfuron remaining unmetabolised after thirty six hours was similar to that after twelve hours (Figure 4.3). In subsequent studies using method B, illumination was continuous following commencement of herbicide treatment. Metabolism of chlorsulfuron in these studies continued for more than twelve hours after the commencement of treatment (Figure 4.4, Chapter 4).

These findings raised the question of whether continuous light is needed for chlorsulfuron metabolism. Interruption of the light period may

cause cessation of chlorsulfuron metabolism. If metabolism in ex plants does cease when illumination ceases, this may also occur in intact plants. Alternatively, the arrest of chlorsulfuron metabolism observed in Figure 4.3 may be due to the rapid metabolism in the culms nearing completion, leaving a proportion of chlorsulfuron unmetabolised in the leaf lamina. To address these issues, studies were initiated to determine whether seedlings remain capable of metabolising chlorsulfuron in the culms tissue for more than twelve hours after excision. The effect of an interruption of the light period during the period of rapid chlorsulfuron metabolism in the culms, was also investigated.

If chlorsulfuron metabolism in intact plants were to cease with the onset of darkness and not recommence thereafter, it follows that limiting the light period immediately after herbicide treatment should limit the amount of herbicide which can be metabolised. If increased chlorsulfuron metabolism is a major cause of resistance in *L.rigidum* biotype SLR31, limiting the light period immediately following chlorsulfuron treatment should increase the herbicide phytotoxicity for this population. Such a finding would not only help to elucidate the mechanisms of resistance to chlorsulfuron in *L.rigidum* but might lead to improvements in the efficacy of the herbicide by applications late in the day.

The effect of light period following herbicide treatment upon phytotoxicity of chlorsulfuron toward *L.rigidum* biotypes was investigated. As chlorsulfuron metabolism is greater in cross-resistant biotype SLR31 than in the susceptible biotype VLR1 (Chapter 4), a greater increase in phytotoxicity due to reduced metabolism would be expected in biotype SLR31 than in VLR1. Chlorsulfuron resistant biotype WLR1 also has a higher rate of chlorsulfuron metabolism than biotype VLR1 but this biotype has herbicide insensitive-ALS (Chapter 5). Therefore, a reduction in metabolism should not greatly increase the phytotoxic effect of chlorsulfuron on biotype WLR1. However, if light period were to effect the phytotoxicity of chlorsulfuron towards *L.rigidum* for reasons unrelated to metabolism then

biotype WLR1 should also be effected. To test these hypotheses, plants of the three *L.rigidum* biotypes were treated with herbicide followed by various periods of illumination. Experiments were conducted in the growth chamber for optimum control of environmental conditions. Further tests were conducted using pots kept in the field to determine if any effects were apparent under conditions more closely related to those encountered in agriculture.

8.2 Materials and Methods

8.2.1 [^{14}C]chlorsulfuron metabolism by excised seedlings in response to darkness or light.

In a single experiment, *L.rigidum* seedlings of biotypes SLR31 and VLR1 were grown, excised and treated with [^{14}C]chlorsulfuron as described in section 4.2.2., method A. For all treatments, 36 seedlings were exposed to [^{14}C]chlorsulfuron solution for 3 h, harvested immediately or removed to vials containing distilled water for a further 3 h, then harvested 6 h after the commencement of treatment. Most treatments were maintained in the light throughout the experiment but plants of one treatment of each biotype, after 3 h exposure to [^{14}C]chlorsulfuron solution, were immediately placed in darkness by covering with a light excluding, ventilated box before harvesting 6 h after the commencement of treatment. [^{14}C]chlorsulfuron and metabolites were extracted using 80% MeOH and analysed by HPLC using method A, as described in section 4.2.2.

In a second experiment plants, of *L.rigidum* biotypes SLR31, VLR1 and VLR6 were excised, exposed to [^{14}C]chlorsulfuron using method A, as described in section 4.2.2 using 36 seedlings per treatment. Plants for one treatment of each biotype were harvested immediately prior to herbicide treatment, exposed to [^{14}C]chlorsulfuron solution for 3h, transferred to distilled water for a further 5 h and harvested 8 h after the commencement of treatment. Plants for a second treatment of each biotype had been excised 24h prior to the commencement of herbicide application and kept in vials

containing water only. These plants experienced approximately 12 h of illumination, 10 h of darkness followed by a further 2 h of illumination, prior to commencement of herbicide treatment. Plants of each biotype freshly excised and excised 24 h beforehand were treated and harvested at approximately the same time. [^{14}C]chlorsulfuron and metabolites were extracted using 80% MeOH and analysed by HPLC using method A, as described in section 4.2.2.

8.2.2 Response of whole plants to light period following chlorsulfuron application

Growth cabinet studies

L.rigidum biotypes VLR1, SLR31 and WLR1 were pot cultured, as described in section 2.2.2., using 21 plants per pot and placed in a growth chamber, (22C, 14h, 300 μE m^{-2} s^{-1} light period, 15C, 10h dark period). At the two leaf stage plants were treated with chlorsulfuron in the laboratory spray cabinet, as described in section 2.3, at different times of the day then immediately returned to the growth chamber such that they received 13 h, 3 h or 0 h of 300 μE m^{-2} s^{-1} light before the commencement of the next dark period. To maximise the likelihood of detecting differences between treatments, herbicide levels were chosen to cause levels of mortality approaching 50% of each biotype (where possible). Thus, susceptible biotype VLR1 was treated with 4 g ai ha^{-1} chlorsulfuron, cross-resistant biotype SLR31 with 64 g ai ha^{-1} chlorsulfuron and resistant biotype WLR1 with 256 g ai ha^{-1} chlorsulfuron. There were 4 replicates of 21 plants for each treatment. Mortality and shoot dry weight were determined at harvest 17 days after treatment. Data were analysed by analysis of variance.

Studies with pots in the field

L.rigidum biotypes VLR1, SLR31 and WLR1 were pot cultured outdoors in the field throughout the experiment. Plants were treated with herbicide and harvested 21 days after treatment as described in section 2.2 and 2.3. Plants of each biotype were treated with herbicide at levels known to cause near 50% mortality, either at sunset or 5 h before sunset. One 2 L

pot containing 21 seedlings was used for each treatment. Four replicates of each experiment were conducted concurrently. Susceptible *L.rigidum* biotype VLR1 was treated with 4 g ai ha^{-1}, SLR31 with 64 g ai ha^{-1} and WLR1 with 256 g ai ha^{-1} chlorsulfuron on 21st of march 1992. Temperature at the time of spraying was 16C. Plants treated 5 h before sunset, were placed in the sun, light intensity of 870 $\mu E\ m^{-2}\ s^{-1}$, immediately following treatment.

In a second study *L.rigidum* biotypes VLR1, SLR31 and WLR1 were treated with 125, 4000 or 125 g ai ha^{-1} diclofop-methyl respectively on the 24th of April 1992. Temperature at the time of spraying 5 h before sunset was 30C. Plants treated 5 h before sunset were placed in the sun, light intensity of 1450 $\mu E\ m^{-2}\ s^{-1}$, at the time of treatment.

8.3 [^{14}C]chlorsulfuron metabolism in response to darkness or light

Excised *L.rigidum* seedlings, constantly illuminated following commencement of [^{14}C]chlorsulfuron treatment, steadily metabolised the herbicide over a period of 6 h. After 6 h, 72 and 54% of [^{14}C]chlorsulfuron remained unmetabolised after six hours in seedlings of biotypes VLR1 and SLR31 respectively (Figure 8.1), which is similar to the results previously obtained (Figure 4.1). However, when seedlings were placed in the dark after a 3 h light period and harvested after a further 3 h darkness, there was little or no herbicide metabolism during the 3 h dark period. The amount of [^{14}C]chlorsulfuron remaining unmetabolised after 3 h in seedlings of biotype VLR1 was 82% but after a further 3 h in darkness 83% was still unmetabolised. Similarly in biotype SLR31, 77% of [^{14}C]chlorsulfuron remained after 3 h but after a further 3 h in darkness 72% still remained. It appears that little metabolism of chlorsulfuron occurred following the cessation of illumination in excised seedlings of either the cross-resistant or the susceptible biotype (Figure 8.1).

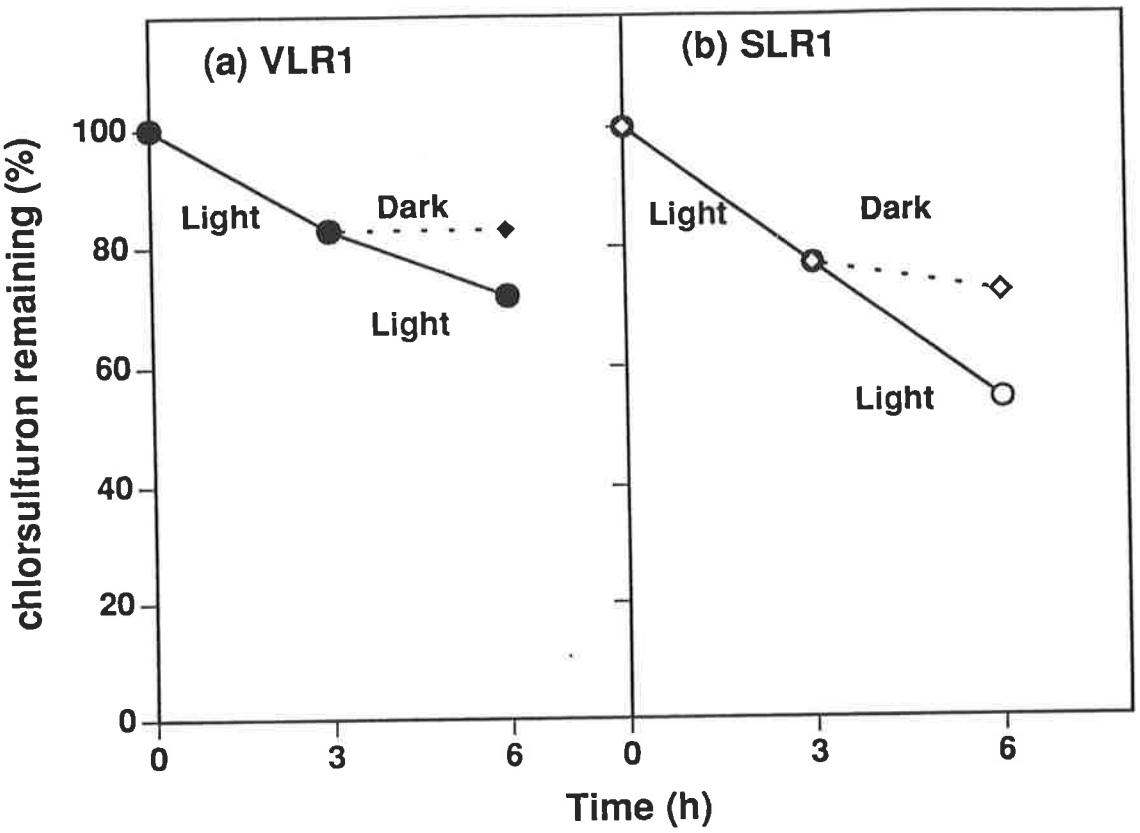


Figure 8.1 [^{14}C]chlorsulfuron remaining unmetabolised in excised seedlings of *L.rigidum*, (a) biotype VLR1 or (b) biotype SLR31 when constantly illuminated following commencement of herbicide treatment (●,○), or placed in the dark 3h after the commencement of treatment (◆,◇). Data are from one experiment using 36 seedlings per treatment.

Plants excised twenty four hours before [^{14}C]chlorsulfuron treatment retained the ability to metabolise the herbicide (Table 8.1). In *L.rigidum* biotypes VLR1 and VLR6, which are susceptible to chlorsulfuron, 70 and 69% of [^{14}C]chlorsulfuron applied to freshly excised seedlings remained after three hours (Table 8.1). When seedlings of VLR1 and VLR6 were excised twenty four hours prior to herbicide treatment 80 and 75% of [^{14}C]chlorsulfuron remained after three hours respectively. For biotype SLR31, 39% of [^{14}C]chlorsulfuron remained after three hours in freshly excised seedlings and 67% remained after three hours in seedlings excised twenty four hours prior to treatment. Thus, although the rate of metabolism was reduced, seedlings from all biotypes excised twenty four hours prior to treatment, retained the ability to metabolise chlorsulfuron.

Table 8.1 [^{14}C]chlorsulfuron remaining (%) in excised seedlings 8 h after commencement of treatment

Biotype	VLR1	VLR6	SLR31
Excised immediately before treatment	70	69	39
Excised 24h before treatment	80	75	67

Data are from one experiment using 36 seedlings per treatment.

Thus, in excised *L.rigidum* seedlings, chlorsulfuron metabolism is dramatically reduced, or ceases, in the dark, while plants kept constantly illuminated are able to continue metabolism. Secondly, plants excised twenty four hours before herbicide treatment retain the ability to metabolise chlorsulfuron at a reduced rate. These findings indicate that the cessation of chlorsulfuron metabolism after twelve hours observed in Chapter 4 is due to an effect of darkness and is not due to the effects of seedling excision.

8.4 Response of whole plants to light period following chlorsulfuron application

Growth cabinet studies

The absence of a light period following chlorsulfuron treatment did increase the phytotoxicity of the herbicide for biotype SLR31. When treated with 64 g ai ha^{-1} and immediately placed in the dark for ten hours before the commencement of the light period, population SLR31 suffered 25 ± 4 % mortality (Figure 8.2). If treated plants were immediately returned to the growth room under 300 μE m^{-2} s^{-1} light for three hours, mortality was reduced to 6 ± 2 % (Figure 8.2). Two way analysis of variance indicates that the interaction between time of spraying and mortality for biotype SLR31 is significant ($P>0.05$).

In contrast to biotype SLR31, mortality of biotypes VLR1 and WLR1 was unaffected by a three hour light period prior to the dark period (Figure 8.2). There was a slight reduction in mortality of biotypes VLR1 and WLR1 when sprayed thirteen hours before the dark period although the interaction between time of spraying and mortality was not significant ($P>0.05$) (Figure 8.2). Biotype VLR1 has a slower rate of chlorsulfuron metabolism than the two resistant biotypes (Chapter 3). Herbicide-insensitive ALS is more important than increased metabolism for chlorsulfuron resistance in resistant biotype WLR1 (Chapter 4). Thus, the results suggest that the increased mortality of biotype SLR31 when placed in darkness immediately following chlorsulfuron treatment is probably due to a reduction of chlorsulfuron metabolism, rather than a general phytotoxic effect of reduced light period following chlorsulfuron treatment. Biotype SLR31 appears able to metabolise enough chlorsulfuron within the first three hours to reduce mortality while the susceptible biotype VLR1 requires more than thirteen hours.

The results represented in Figure 8.2 indicate that light period between chlorsulfuron treatment and the commencement of the first dark period can affect mortality of biotype SLR31, but the maximum level of

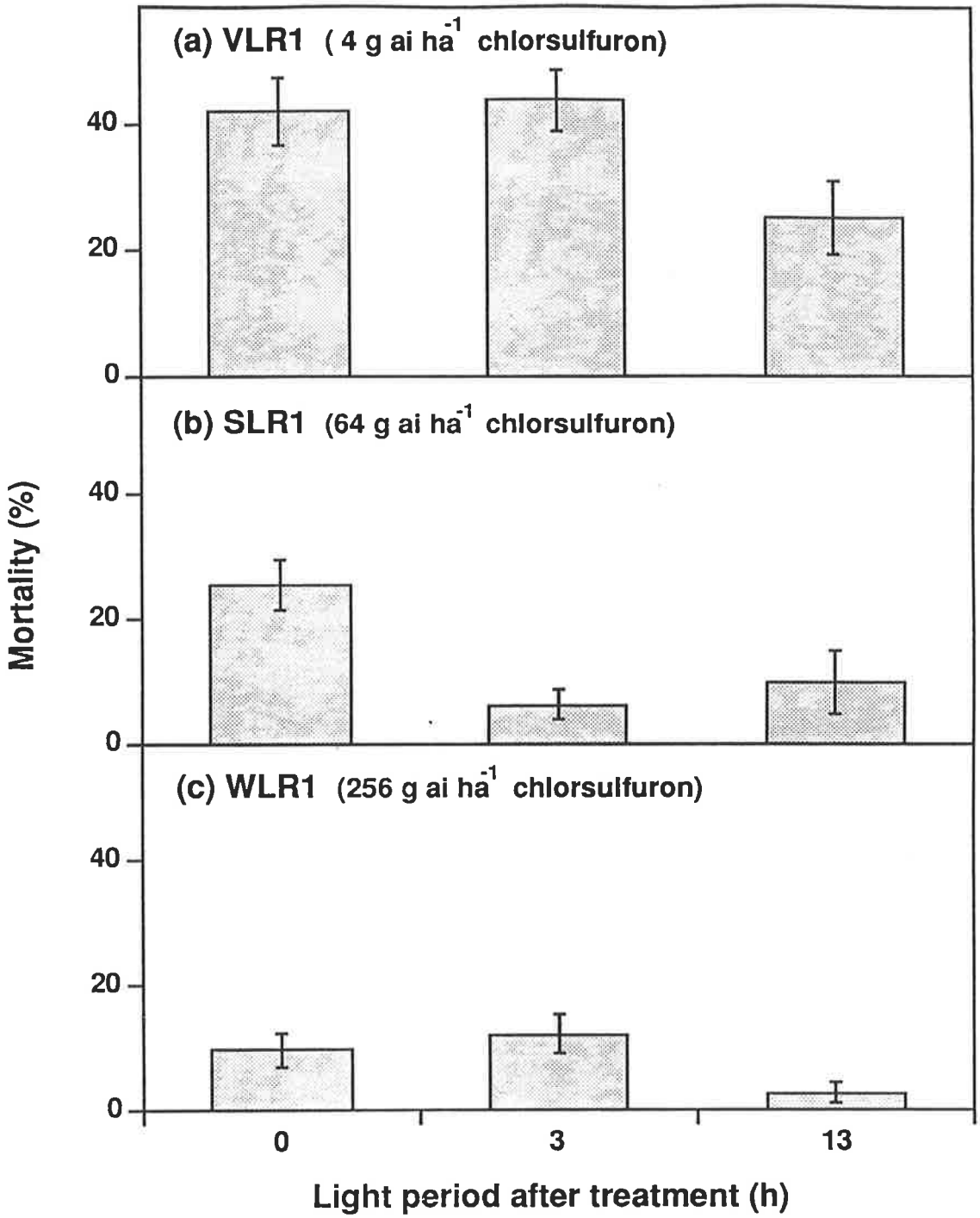


Figure 8.2 Mortality of pot cultured plants of *L.rigidum* biotypes (a) VLR1 treated with 4 g ai ha^{-1} chlorsulfuron, (b) biotype SLR31 treated with 64 g ai ha^{-1} chlorsulfuron and (c) WLR1 treated with 256 g ai ha^{-1} chlorsulfuron. Plants were cultured in a growth cabinet and treated at various times such that they had 0 h, 3 h or 13 h light period at 300 $\mu Em^{-2}s^{-1}$ prior to the commencement of the first dark period. Data are the means of four replicate experiments. Vertical bars represent the standard error of the mean.

mortality (when treated at the commencement of the dark period) was only 25 %, even though the herbicide was applied at 64 g ai ha^{-1} (Figure 8.2). An application of 64 g ai ha^{-1} chlorsulfuron would be expected to kill 100% of individuals from a susceptible biotype such as biotype VLR1 (see Chapter 3). Thus, reduction of the light period following chlorsulfuron treatment does not cause the response of biotype SLR31 to revert to the response of a susceptible biotype.

Studies with pots in the field

The results of experiments using pot cultured plants maintained in the field confirm and extend those obtained in growth cabinet studies. Plants of SLR31 treated with 64 g ai ha^{-1} chlorsulfuron five hours before sunset suffered 9 ± 1 % mortality while those treated at sunset suffered the significantly greater mortality of 23 ± 3 % ($P<0.05$) (Figure 8.3). In contrast, biotype VLR1 treated with 4 g ai ha^{-1}, and biotype WLR1 treated with 256 g ai ha^{-1}, did not exhibit increased mortality when treated at sunset compared to treatment five hours before sunset ($P>0.05$) (Figure 8.3). These results suggest that the onset of darkness soon after chlorsulfuron application may reduce the amount of herbicide metabolised both in the growth cabinet conditions and in the field, where light intensity and other environmental conditions are more variable. The increase in phytotoxicity of chlorsulfuron caused by reduced light period is only significant for biotype SLR31.

8.5 Response of whole plants to light period following diclofop-methyl application

A common mechanism is yet to be elucidated to explain the development of chlorsulfuron cross-resistance following diclofop-methyl treatment in *L.rigidum* biotypes such as biotype SLR31. One possibility is that detoxification of both herbicides is mediated by a common system. However, it has been suggested that metabolism of diclofop-methyl in cross-resistant biotype SLR31 is unlikely to be the major factor causing diclofop-methyl resistance (Holtum et al. '91 ref 195). If this is so then, even if the

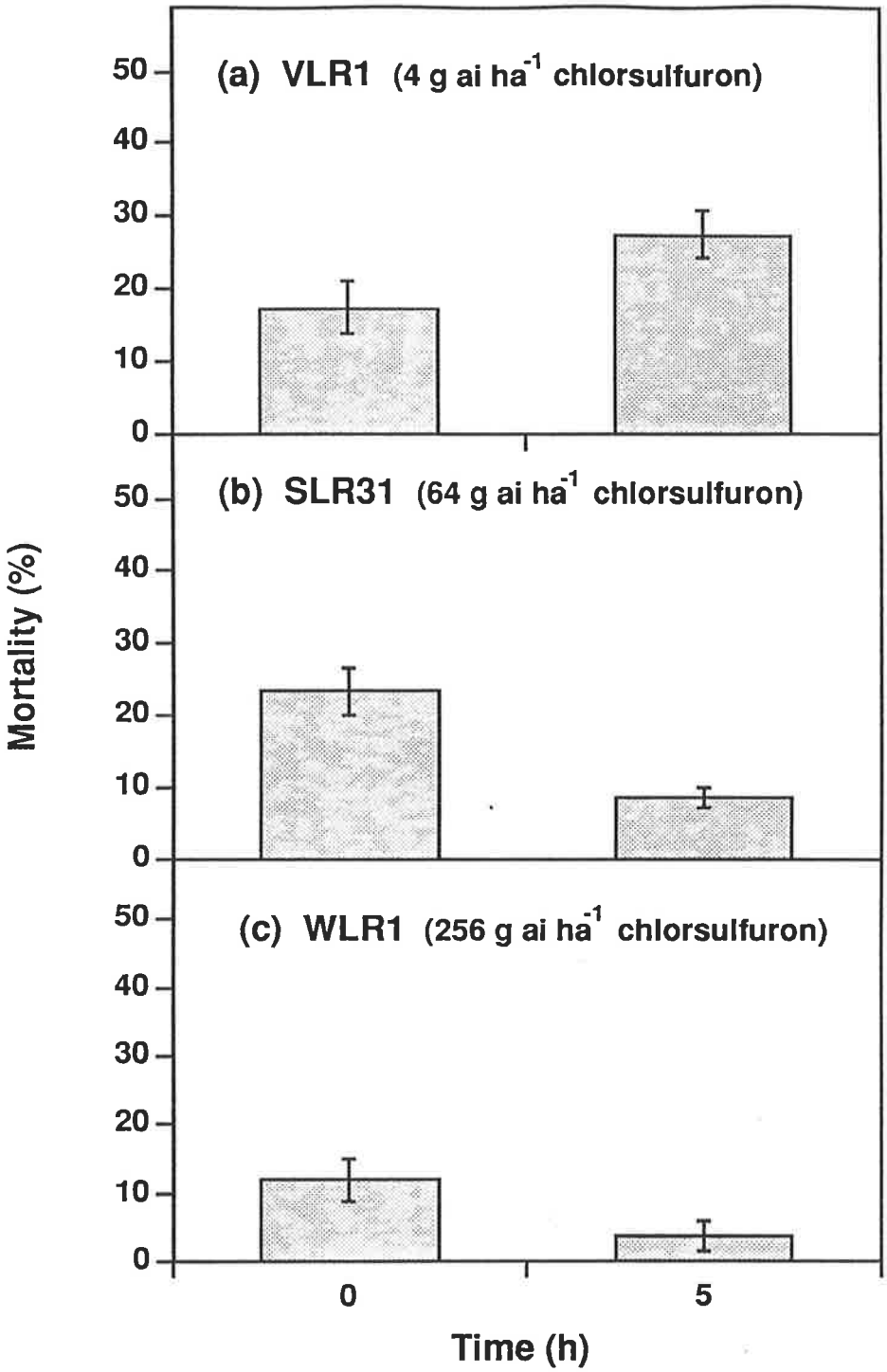


Figure 8.3 Mortality of pot cultured plants of *L.rigidum* biotypes (a) VLR1 treated with 4 g ai ha^{-1} chlorsulfuron, (b) SLR31 treated with 64 g ai ha^{-1} chlorsulfuron, and (c) WLR1 treated with 256 g ai ha^{-1} chlorsulfuron. Plants were cultured in the full sun in the field and treated at sunset or 5 h prior to sunset. Data are the means of four replicate experiments. Vertical bars represent the standard error of the mean.

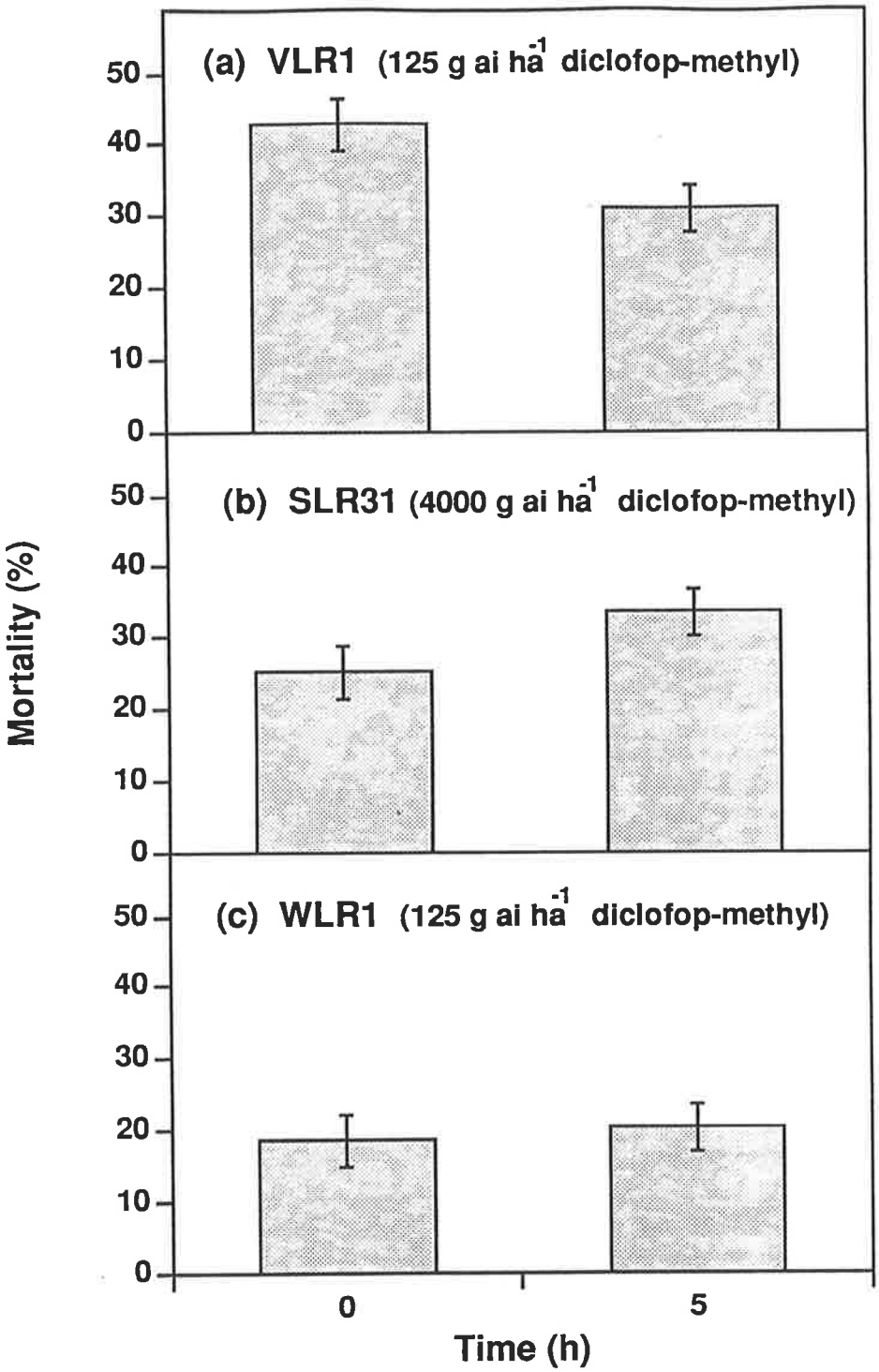


Figure 8.4 Mortality of pot cultured plants of *L.rigidum*, (a) susceptible biotype VLR1 treated with 125 g ai ha^{-1} diclofop-methyl, (b) diclofop-methyl resistant, chlorsulfuron cross-resistant biotype SLR31 treated with 4000 g ai ha^{-1} diclofop-methyl, and, (c) chlorsulfuron resistant, diclofop-methyl susceptible biotype WLR1 treated with 125 g ai ha^{-1} diclofop-methyl. Plants were cultured in the full sun in the field and treated at sunset of 5h prior to sunset. Data are the means of four replicate experiments. Vertical bars represent the standard error of the mean.

chlorsulfuron detoxification system can also detoxify diclofop-methyl, the light period following herbicide application might not affect the level of phytotoxicity of diclofop-methyl towards biotype SLR31. Conversly, if light period following treatment did affect the phytotoxicity of diclofop-methyl toward biotype *L.rigidum* SLR31, this would suggest that the chlorsulfuron detoxification system could be the common mechanism causing cross-resistance. Experiments were conducted with diclofop-methyl using pots kept in the field to examine these possibilities.

There were no significant differences between plants treated five hours before sunset and those treated at sunset with diclofop-methyl (Figure 8.4). This indicates that the system causing increased chlorsulfuron metabolism in biotype SLR31 may not be the common mechanism leading to diclofop-methyl, chlorsulfuron cross-resistance. Alternatively, the chlorsulfuron metabolism system may be able to mediate diclofop-methyl metabolism but is less important for diclofop-methyl resistance than it is for chlorsulfuron resistance. This is consistent with the suggestion that diclofop-methyl metabolism is not important for diclofop-methyl-resistance in biotype SLR31 (Holtum *et al.* '91). The implications of these observations will be further discussed in later sections.

8.6 Discussion

Metabolism of [^{14}C]chlorsulfuron in excised *L.rigidum* seedlings slows dramatically, or ceases irreversibly, after plants are placed in the dark (Figure 8.1). Limiting the light period between chlorsulfuron treatment and the first dark period increases the phytotoxic effect of chlorsulfuron towards biotype SLR31 when plants are grown in the growth chamber and in the field (Figures 8.2 and 8.3). The increased phytotoxicity of chlorsulfuron caused by reduced light period is probably the result of reduced chlorsulfuron metabolism with this biotype.

The increased phytotoxicity for biotype SLR31 caused by limiting light period following chlorsulfuron treatment does not reach the same level

as chlorsulfuron toxicity for susceptible biotype VLR1. This result shows similarities with the effects of chlorsulfuron applications in conjunction with MFO inhibitors. The increased phytotoxicity caused by chlorsulfuron application in conjunction with MFO inhibitors to biotype SLR31 does not reach the level of phytotoxicity caused by chlorsulfuron applied alone to biotype VLR1 (Chapter 7). Further studies to determine whether MFO inhibitors, or reduction in the light period following herbicide application, can completely inhibit chlorsulfuron metabolism in intact plants are required to determine whether increased metabolism is the only mechanism of chlorsulfuron resistance in biotype SLR31.

As herbicide metabolism is dramatically reduced in the absence of light (Figure 8.1), it is possible that synthesis of the enzymes involved may be suppressed in tissue which is not exposed to light. This would have implications for investigations of the detoxification system if mixed function oxidases are involved. Traditionally, etiolated tissue has been used for the study of plant microsomal mixed function oxidase systems. This is true for studies which demonstrated that MFO's in wheat are capable of metabolising triasulfuron and diclofop-methyl (Frear and Mauraus 1990, Frear *et al.* 1991, Zimmerlin and Durst 1990 and 1992). It is not known whether chlorsulfuron metabolism occurs in etiolated tissue of *L.rigidum* and, if so, whether there is increased metabolism in etiolated tissue of resistant versus susceptible plants.

The data do not indicate that practical treatment with chlorsulfuron or diclofop-methyl at sunset can increase the efficacy of the herbicides in agricultural situations. There was no increase in the efficacy of either herbicide for control of the susceptible biotype VLR1. The increased control of cross-resistant biotype SLR31, using chlorsulfuron applied at sunset, did not approach acceptable levels even though applied at 64 g ai ha^{-1}, a rate from four to eight times the recommended rate for *L. rigidum* control.

Chapter 9

Does selection with diclofop-methyl necessarily lead to chlorsulfuron resistance?

9.1 Introduction

The first confirmed case of chlorsulfuron resistance was not due to selection pressure from the herbicide chlorsulfuron but occurred in a population of *L.rigidum* which had been treated with diclofop-methyl in four consecutive seasons (Heap and Knight 1986). Seed was collected from the field before the first ALS inhibiting herbicide, chlorsulfuron, became available for commercial use in Australia (Heap and Knight 1986). Since that time many populations of *L.rigidum* have been reported to be cross-resistant to sulfonylurea herbicides following diclofop-methyl treatment in the field (Heap and Knight 1990 Gurjeet Gill pers. comm.). Suspected herbicide resistant populations from Western Australia were tested for resistance to the aryloxyphenoxyalkanoic acid, diclofop-methyl and the sulfonylurea, triasulfuron. Of nine populations exposed to from five to ten applications aryloxyphenoxyalkanoic acids, but not exposed to sulfonylureas, four were found to show partial resistance to triasulfuron (Gurjeet Gill pers.comm.). It has also been demonstrated that selection of the susceptible biotype VLR1 with diclofop-methyl under controlled experimental conditions can lead to significant levels of chlorsulfuron resistance in as little as two generations (Matthews *et al.* 1992). These

results indicate that some populations of *L.rigidum* are able to develop chlorsulfuron cross-resistance following diclofop-methyl treatment.

L.rigidum biotype VLR6 had developed resistance to diclofop-methyl following diclofop-methyl treatment in five seasons, but did not exhibit cross-resistance to chlorsulfuron (Heap and Knight 1990). The response of this population to further selection using diclofop-methyl was studied to determine whether this biotype has the ability to develop chlorsulfuron cross-resistance. As normal application rates of from 375 to 560 g ai ha^{-1} diclofop-methyl had failed to produce chlorsulfuron cross-resistance, selection pressure was increased by treatment with a higher application rate of 4000 g ai ha^{-1} diclofop-methyl.

9.2 Materials and methods

9.2.1 Selection procedure

Seed of biotype VLR6 was sown in a field at the Waite Agricultural Research Institute near Adelaide in South Australia in June of 1989. When at the 2 to 3 leaf stage, plants were treated with 4000 g ai ha^{-1} diclofop-methyl using a 2 m hand-held boom sprayer. Approximately 39% of plants survived herbicide application (Table 9.1). Surviving plants were allowed to grow, cross pollinate and produce seed in the field. Pollen from outside the plot area was excluded by a 2 m high screen. The seed collected was called selected generation one. In 1990, seed of selected generation one was germinated and the seedlings pot cultured before treatment with 4000 g ai ha^{-1} diclofop-methyl in the laboratory spray cabinet as described in sections 2.2 and 2.3. Approximately 72% of plants survived herbicide application (Table 9.1). After 28 days surviving plants were transferred to 10 L pots and placed in the field to produce seed. Plants in pots were surrounded, as a group, with a 1 m polythene screen to exclude outside pollen so that cross-fertilisation occurred only amongst the surviving plants. The seed collected was called selected generation two. In 1991, seed of selected generation two was germinated and the seedlings pot cultured,

treated with 4000 g ai ha^{-1} diclofop-methyl. Approximately 78% of plants survived herbicide application (Table 9.1). Surviving plants were transferred to 10 L pots placed in the field, surrounded by polythene sheet, to produce seed, called selected generation three.

Table 9.1 Survival of the original population, VLR6, and selected generations, pot cultured and treated with 4000 g ai ha^{-1} diclofop-methyl in 1992.

Original	Selected 1	Selected 2	Selected 3
38.8 ± 7.0	71.8 ± 2.6	77.5 ± 8.3	90.2 ± 2.6

Data are the means of three replicate experiments ± standard error.

9.2.2 **Testing of progeny**

In autumn of 1992 seed of ryegrass biotypes VLR6 and the three selected generations were pot cultured and placed in the field, 21 plants per pot as described in section 2.2. Seedlings were treated with 8, 16, 32, 64, 128 and 256 g ai ha^{-1} chlorsulfuron or 4, 8, 16 and 32 kg ai ha^{-1} diclofop-methyl, maintained in the field and harvested 28 days after treatment as described in section 2.3. Susceptible biotype VLR1, used as a control, responded as in previous experiments (Chapter 3). One pot of 21 plants was used for each treatment and the experiment was replicated 3 times. Regression analysis of mortality upon log dose was used to determine the LD_{50} for each biotype for each replicate. Regression analysis, of the mean LD_{50} values obtained for each generation against the number of generations of selection using 4000 g ai ha^{-1} diclofop-methyl, was used to determine whether the increase in LD_{50} with each generation was significantly greater than zero.

9.3 Effect of diclofop selection upon chlorsulfuron response of biotype VLR6.

Selection pressure using 4000 g ai ha^{-1} diclofop-methyl caused a substantial increase in the LD_{50} for diclofop-methyl over three generations despite the fact that the original population, VLR6, was already resistant to this herbicide. This is illustrated by the dose response to diclofop-methyl of the original population and selected generation three plotted in Figure 9.1. The LD_{50} for diclofop-methyl increased from 2800 ± 1000 g ai ha^{-1} in the original VLR6 population to 25,500 ± 6000 g ai ha^{-1} in selected generation three, a 9.1 fold increase (Figure 9.2). Regression analysis indicates that the increase in mean LD_{50} between generations is statistically significant ($P<0.05$) (Figure 9.2).

The LD_{50} increase for chlorsulfuron was much smaller than the LD_{50} increase for diclofop-methyl as can be seen from the dose response to chlorsulfuron of the original population and selected generation three plotted in Figure 9.3. LD_{50} for chlorsulfuron increased from 19.5 ± 3.1 g ai ha^{-1} in the original VLR6 population to 33.2 ± 2.5 g ai ha^{-1} in selected generation three, a 1.7 fold increase (Figures 9.4). Regression analysis indicates that the increase in chlorsulfuron LD_{50} for each generation is not significantly greater than zero ($P>0.05$) (Figure 9.4).

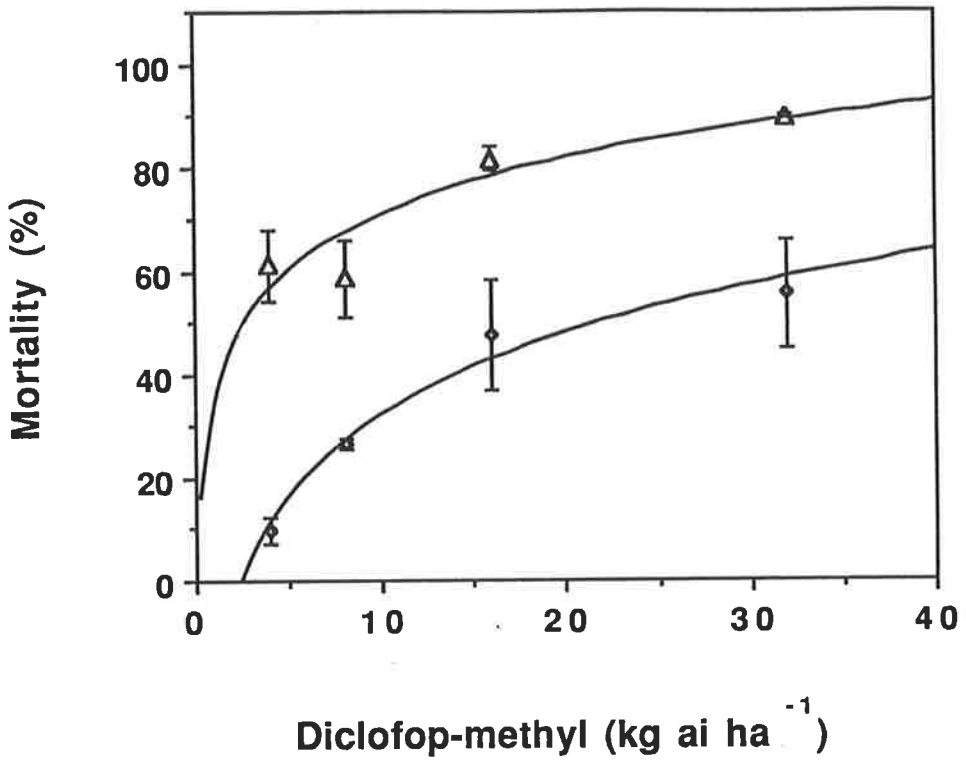


Figure 9.1 Mortality of *L.rigidum* biotype VLR6, original population (△) and VLR6, selected generation three (◇), pot cultured in the field and treated with diclofop-methyl. Lines represent best fit of mean mortality in each generation against log dose. Data are the mean of three replicate experiments. Vertical bars represent the standard error of the mean.

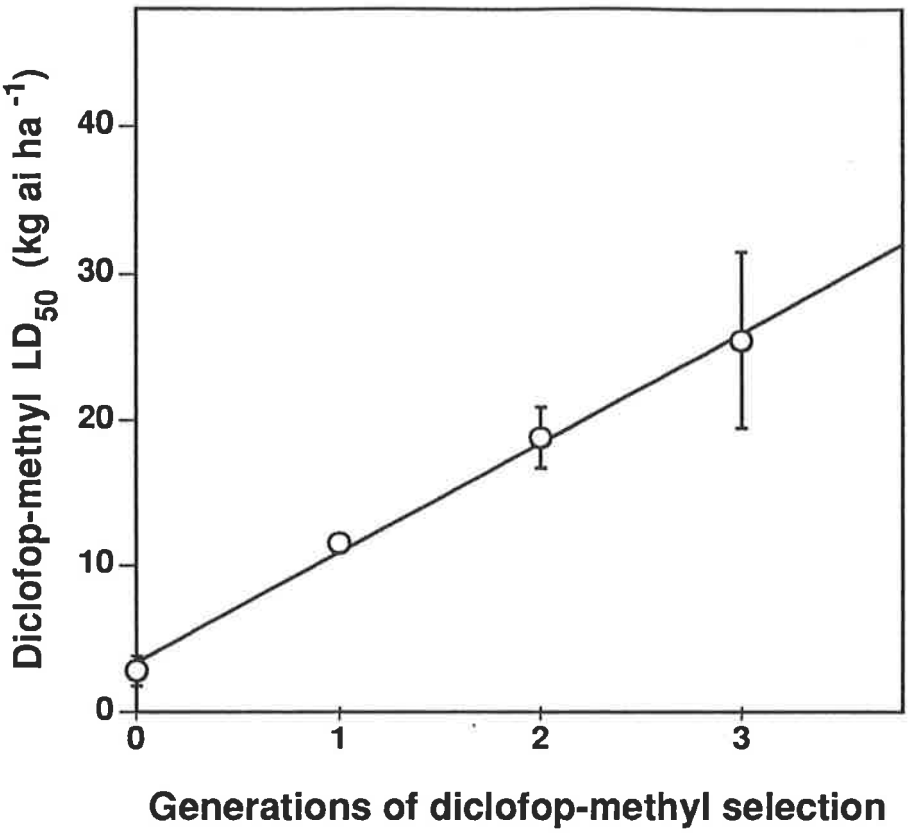


Figure 9.2 Diclofop-methyl LD_{50} of *L.rigidum* biotype VLR6 and progeny, plotted against the number of generations of selection using 4000 g ai ha^{-1} diclofop-methyl. Data represent the mean LD_{50} value for each generation from three replicate experiments. Vertical bars represent the standard error of the mean.

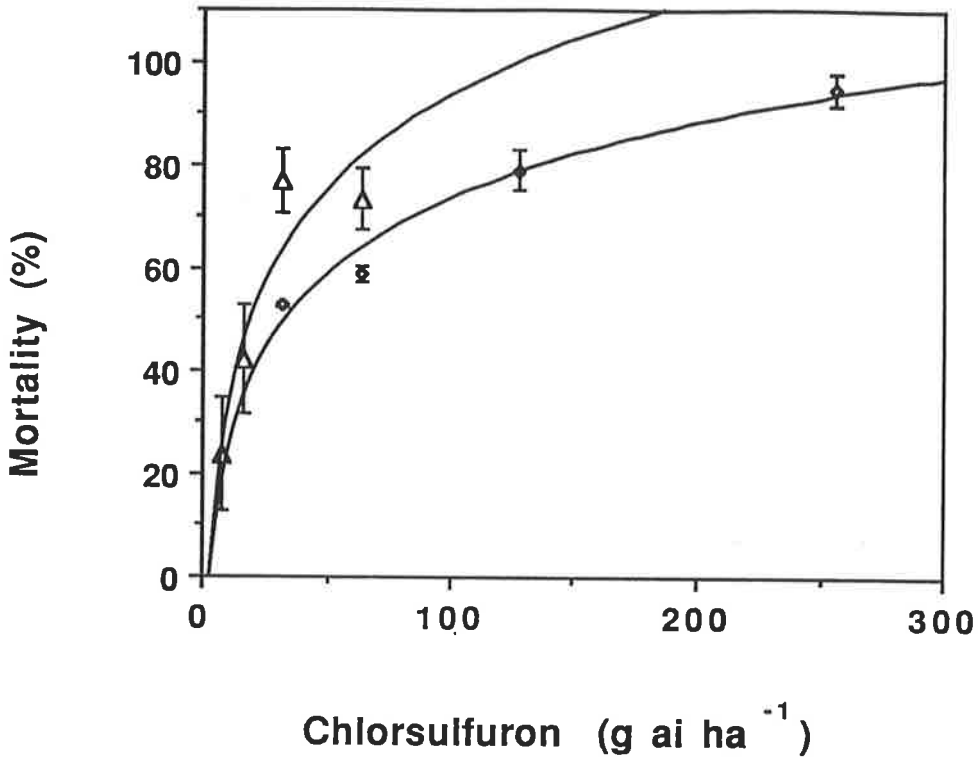


Figure 9.3 Mortality of *L.rigidum* biotype VLR6, original population (△) and VLR6 selected generation three (◇), pot cultured in the field and treated with chlorsulfuron. Lines represent the best fit of mean mortality against log dose. Data are the mean of three replicate experiments. Vertical bars represent the standard error of the mean.

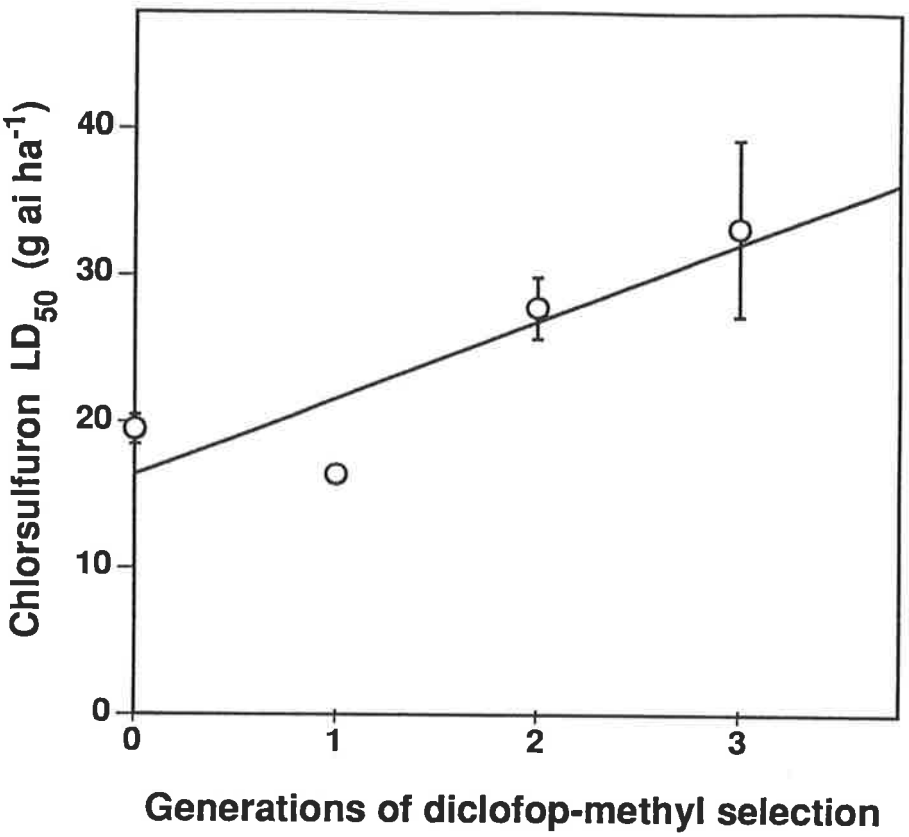


Figure 9.4 Chlorsulfuron LD_{50} of *L.rigidum* biotype VLR6 and progeny, plotted against the number of generations of selection using 4000 g ai ha^{-1} diclofop-methyl. Data represent the mean LD_{50} value for each generation from three replicate experiments. Vertical bars represent the standard error of the mean.

9.4 Discussion

The results represented in Figures 9.1 and 9.2 demonstrate that selection with a high rate of diclofop-methyl has caused a substantial increase in the level of resitance to diclofop-methyl in populaion VLR6, despite the fact that the population was already resistant. However, the level of chlorsulfuron resistance in population VLR6 increased only slowly, if at all, in response to treatment with 4000 g ai ha^{-1} diclofop-methyl (Figure 9.3, 9.4). Thus, the dramatic increase in diclofop-methyl resistance was not accompanied by a concomitant increase in chlorsulfuron cross-resistance. (Figure 9.1, 9.2).

The response to diclofop-methyl treatment in this population is different from that of the susceptible population VLR1. Biotype VLR1 plants treated with diclofop-methyl at the recommended application rate of 375 g ai ha^{-1}, in two generations exhibited an increase in chlorsulfuron LD_{50} from approximately 24 to 120 g ai ha^{-1} (Matthews *et al.* 1992). The LD_{50} for diclofop-methyl increased from 105 to >3000 g ai ha^{-1} (Matthews et al. '92, ref 423). Thus, treatment of biotype VLR1 with the recommended rate of diclofop-methyl in two generations led to a large increase in LD_{50} for both diclofop-methyl and chlorsulfuron. The chlorsulfuron LD_{50} of 120 g ai ha^{-1}, for progeny of VLR1 after two generations selection using the recommended rate of diclofop-methyl, is greater than the chlorsulfuron LD_{50} of 33.2 ± 2.5 g ai ha^{-1} for VLR6 selected generation three (Figure 9.4). VLR6 selected generation three had been exposed to five treatments of diclofop-methyl at recommended application rates in the field followed by selection with 4000 g ai ha^{-1} in three subsequent generations. This result suggests that population VLR1 has a greater ability to develop chlorsulfuron cross-resistance, following selection using diclofop-methyl, than does population VLR6.

It should be noted that selection using different rates of herbicide may favour different resistance mechanisms. For example, selection with 4000 g ai ha^{-1} diclofop-methyl may kill individuals having

mechanisms causing low levels of diclofop-methyl resistance but favour individuals with mechanisms causing high levels of diclofop-methyl resistance, such as ACCase mutants (Table 9.1). If a common mechanism causing both diclofop-methyl and chlorsulfuron resistance only causes a low level of diclofop-methyl resistance, selection using 4000 g ai ha^{-1} diclofop-methyl may kill individuals which have this mechanism only. However, population VLR6 was exposed to five applications of diclofop-methyl at recommended rates prior to selection using 4000 g ai ha^{-1}. This suggests that VLR6 cannot rapidly develop chlorsulfuron cross-resistance in response to either high or low rates of diclofop-methyl.

Selection at the normal field rate followed by severe selection with high rates of diclofop-methyl resulted in only a small increase in chlorsulfuron cross-resistance in one population of *L.rigidum*, VLR6. In contrast, as little of two treatments with recommended application rate of diclofop-methyl can cause cross-resistance to chlorsulfuron in another *L.rigidum* population, VLR1 (Matthews et al '92 ref 423). It has been demonstrated that a proportion of *L.rigidum* populations have the ability to develop chlorsulfuron cross-resistance in response to diclofop-methyl selection in under five generations (Matthews *et al.* 1992, Heap and Knight 1986 and 1990, Gurjeet Gill pers. comm.). The results of this study indicate that other populations may take longer to develop such cross-resistance or, may lack individuals with the required mechanism(s) of cross-resistance. The possible implications of this result are discussed in section 12.

Chapter 10

Inheritance of sulfonylurea resistance with herbicide-sensitive ALS.

10.1 Introduction

L.rigidum biotype SLR31 is chlorsulfuron cross-resistant following treatment with diclofop-methyl. However, ALS from this biotype remains sensitive to chorsulfuron (Matthews *et al.* 1990). Initial studies of the inheritance of diclofop-methyl resistance in *L.rigidum* biotype SLR31 demonstrated that the trait is nuclear encoded and is probably not controlled by a single gene (McCarthy 1988). Since then new evidence suggests the presence of more than one mechanism of diclofop-methyl resistance in biotype SLR31. Mechanisms may include, a slightly increased rate of diclofop-methyl metabolism, a mechanism related to the ability of membranes to recover from diclofop induced depolarisation and, possibly, a third mechanism of sequestering the herbicide away from the active site (Hausler *et al.* 1991, Holtum *et al.* 1991). If SLR31 has more than one

mechanism of diclofop-methyl resistance, it is likely that resistance is controlled by genes at more than one locus. Conversely, only one mechanism of chlorsulfuron cross-resistance has been identified in biotype SLR31 which is increased metabolism of the herbicide (Chapters 3, 4, 5). Whether this is the only chlorsulfuron resistance mechanism in biotype SLR31 is not known.

The response of biotype SLR31 to the herbicides chlorsulfuron and diclofop-methyl show similarities (compare Figures 3.2 and 3.9, Chapter 3). SLR31 suffers approximately 40% mortality at application rates of 128 g ai ha^{-1} chlorsulfuron or 4000 g ai ha^{-1} diclofop-methyl which are approximately ten times those recommended for ryegrass control (Figures 3.2 and 3.9). At application rates of 256 g ai ha^{-1} chlorsulfuron or 8000 g ai ha^{-1} diclofop-methyl, rates which are approximately twenty times those recommended for ryegrass control, mortality of SLR31 is approximately 50%. The proportion of individuals surviving high rates of chlorsulfuron and diclofop-methyl are similar, but it is not known whether the individuals which survive chlorsulfuron are the same individuals which survive diclofop-methyl. To address this question, studies were conducted to determine whether individuals of SLR31 surviving high rates of either chlorsulfuron or diclofop-methyl are able to pass resistance to the other herbicide on to their progeny.

Investigations of inheritance of herbicide resistance traits in *L.rigidum* are made difficult by both, the strict out crossing mechanism of *Lolium* species (Feron *et al.* 1983, Cornish *et al.* 1980), and the wide range of genetic variation found within the species. Thus, phenotypic effects due to the genetic variation of the parents are always present in the progeny in addition to the phenotypic effects caused by herbicide resistance traits. Extensive studies of the inheritance of herbicide resistant traits in many individual families over several generations are required to determine the number of genes involved in resistance, the correlation between each gene with resistance to various herbicides and the dominance characteristics of

each trait. Such studies are beyond the scope of this work. Studies of aspects of the inheritance of chlorsulfuron cross-resistance which can be investigated at the population level and others which can be investigated by studies of a limited number of families are described in this section.

Studies were undertaken to determine whether the gene(s) controlling chlorsulfuron resistance are nuclear encoded or maternally inherited and whether there is any evidence that inheritance may be controlled by a single gene. Also addressed, was the question of whether chlorsulfuron-resistance co-segregates with diclofop-methyl resistance in all resistant individuals from population SLR31 or whether it can segregate independently.

Resistant parents selected from population SLR31, using a high rate of either chlorsulfuron or diclofop-methyl, were crossed with susceptible individuals to determine whether these resistant parents are capable of passing both chlorsulfuron and diclofop-methyl resistance to the progeny, whether the characters are dominant or recessive, and whether chlorsulfuron resistance is maternally inherited. The progeny of these first generation crosses were intercrossed to produce a second generation allowing study of the segregation of chlorsulfuron resistance inherited from the selected resistant parents. To determine whether chlorsulfuron resistance and diclofop-methyl resistance are always under common genetic control, further studies were conducted to test individual families for diclofop-methyl and chlorsulfuron resistance.

10.2 Materials and methods

10.2.1 Crossing procedure

Lolium species have a very strict self-incompatibility mechanism thought to involve more than one loci (Feron *et al.* 1983, Cornish *et al.* 1980). A common allele at any of these loci causes an incompatible reaction. This makes self-pollination virtually impossible in *Lolium* species. Crosses

were conducted by placing the two parent plants in a 10 L pot containing potting medium and excluding outside pollen using a 1 m x 0.3 m polythene tube placed around the plants and pot. The tube was open at the top to allow air movement which is necessary for pollination and healthy plant growth. Plants were grown in the field during the normal growing season. When grown singly in this manner plants did not set viable seed, confirming that the tubes were capable of excluding external pollen and that the self-incompatibility mechanism of ryegrass ensures that seed produced results from cross-pollination only.

The amount of seed resulting from each individual cross varied widely. Although all plants used in crossing experiments were germinated at the same time and cultured under the same conditions, some individual crosses failed to produce viable seed. Asynchronous flowering or incompatibility of the individual plants are likely causes. Asynchronous flowering also resulted in low numbers of seeds being produced from some individual crosses.

As *L.rigidum* spikes do not shatter, seed from each individual plant may be easily separated by harvesting each plant whole before threshing the seed from the spike. Seed was stored for at least 2 months after harvest to ensure high levels of germination.

10.2.2 Selection of resistant parents and first generation crosses

Resistant parents were selected from population SLR31 using high rates of either chlorsulfuron or diclofop-methyl. In one set of experiments SLR31 seedlings were grown in pots and treated at the 2 leaf stage with 128 g ai ha^{-1} chlorsulfuron. The selected SLR31 parents which survived 128 g ai ha^{-1} chlorsulfuron were called the $SLR31^{C}$ population. In a second set of experiments resistant parents were similarly selected using 4000 g ai ha^{-1} diclofop-methyl. The selected SLR31 parents which survived 4000 g ai ha diclofop-methyl were called the $SLR31^{D}$ population.

The genotype of resistant parents selected in this manner is uncertain. Approximately 40% of the SLR31 population survive selection with either

chlorsulfuron or diclofop-methyl at these application rates. The selection procedure would be expected to enrich the selected parent population for individuals which are homozygous for herbicide resistance. However, for dominant herbicide resistance traits some heterozygotes may also survive.

After herbicide treatment, SLR31 survivors were allowed to grow until approximately 15 to 20 tillers had formed before the plants were carefully teased from the soil and divided into 2 equal clones. Plants of the susceptible parent, biotype VLR1, were grown and divided similarly but without herbicide testing, again producing two clones of each individual. One clone of each selected resistant parent was placed into a 10 L pot with one clone from a susceptible plant. The second clone of each selected resistant plant was placed into a 10 L pot with the second clone of another selected resistant plant. Similarly, the second clone of each susceptible plant was placed in a 10 L pot together with the second clone of one other susceptible plant. Each individual pot was enclosed by a plastic sheath immediately prior to flowering as described above (10.2.1). The experimental design allows the response of the progeny of crosses between selected resistant and susceptible plants to be compared with progeny from the actual resistant parents used for the crosses in addition to the original unselected SLR31 population. Similarly, the progeny of actual susceptible parents used to make the crosses may be tested. The procedure also ensures that progeny tested are from seed produced under similar environmental conditions. Progeny from crosses between selected resistant plants were pooled. Progeny of the crosses between unselected susceptible parents were pooled. The seed from reciprocal crosses between selected resistant and susceptible plants were pooled such that seed from crosses having resistant female parent were pooled, and seed from crosses having susceptible female parent were pooled. For ease of discussion crosses between selected resistant parents and VLR1 were called the F1, even thought the genotype of the resistant parents is not certain.

In experiments using chlorsulfuron selected parents, 10 $SLR31^C$ and 10 VLR1 plants were crossed as described above. For experiments using diclofop-methyl selected parents 8 $SLR31^D$ and 8 VLR1 plants were used.

The response to the herbicides chlorsulfuron and diclofop-methyl of plants from the first generation were tested in the following year using pot cultured plants grown in the field. For crosses using chlorsulfuron selected resistant parents first generations progeny were tested with 0, 16, 32, 64, 128, 256 g ai ha^{-1} chlorsulfuron or 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 kg ai ha^{-1} diclofop-methyl as described in sections 2.2 and 2.3. For crosses using diclofop-methyl selected resistant parents the first generation progeny were tested with the same application rates of diclofop-methyl but with chlorsulfuron at the rates of 0, 19, 37, 74, 148 and 296 g ai ha^{-1}. In each case, the experiment was conducted once only using 24 plants per treatment. The first generation progeny were further tested in 1992 using plants grown in pots in the field and treated with 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl in three replicate experiments using 24 plants per replicate. These rates of herbicide are just sufficient to kill susceptible plants. The progeny of the susceptible parents used in the crosses showed similar herbicide response to the unselected VLR1 population (Figure 10.1), therefore, only VLR1 was used in replicated trials in 1992.

10.2.3 **Second generation crosses (F2)**

To test the segregation of herbicide resistant traits, plants from F1 crosses were intercrossed in the following year to produce a second generation. Thus, six untested plants from the first generation crosses between $SLR31^C$ and VLR1 (three from each of the pooled reciprocal crosses) were grown in 10 L pots surrounded by a common polythene screen allowing pollination between all six plants. For ease of discussion, the pooled progeny were called the F2. Similarly, nine untested individuals of first

generation crosses between SLR31^D and VLR1 were intercrossed to produce the F2.

In 1992 plants from unselected VLR1 and SLR31 populations, from each of the first and the second generations were cultured in 40 cm by 30 cm by 12 cm trays at 2 cm spacing. Trays were cultured in the field as described in section 2.2, treated with 16 g ai ha^{-1} chlorsulfuron as described in section 2.3 and mortality was scored 28 days after treatment.

10.2.4 **Experiments using individual families**

To test whether chlorsulfuron and diclofop-methyl resistance are always inherited together, individual families from SLR31^C and SLR31^D parents were tested with both herbicides. In experiments using SLR31^C parents, further first generation crosses between SLR31^C and VLR1 were made to provide seed for this. In experiments using SLR31^D individual backcross families were used.

Individual F1 crosses using SLR31^C parents

To provide sufficient seed for testing individual families a further eight SLR31^C plants were crossed with susceptible VLR1 plants in 1991 as described above for pooled F1 crosses. Seed from reciprocal crosses were pooled for each pair of plants. In 1992 plants from each of the individual crosses were pot cultured in the field as described in section 2.2 and tested using 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl as described in section 2.3. These rates of herbicides are just sufficient to kill susceptible plants. Plants were harvested 28 days after treatment. Dry weight and mortality were determined.

Individual backcross families from SLR31^D progeny

In the year following the original crosses, twelve individual F1 plants from the crosses between SLR31^D and VLR1 plants (six form each of the pooled reciprocal crosses) were backcrossed to susceptible VLR1 plants. In the third year following the original crosses, plants from each backcross family were pot cultured in the field and treated with either 250 g ai ha^{-1} diclofop-methyl or 16 g ai ha^{-1} chlorsulfuron as described in sections 2.2

and 2.3. These rates of herbicides are just sufficient to kill susceptible plants. Plants were harvested 28 days after treatment allowing mortality and dry weight to be determined.

10.3 Inheritance of resistance from SLR31 parents which survive chlorsulfuron.

Pooled first generation crosses

Results of experiments in which resistant parents SLR31^{C} were crossed with susceptible VLR1 plants, provide the first information on the inheritance of chlorsulfuron resistance in *L.rigidum* populations. Tests of the first generation progeny reveal that reciprocal crosses of SLR31^{C} with VLR1 exhibit a similar response to chlorsulfuron (Figure 10.1). Comparison of the mean mortality of subsequent, replicated experiments confirm that the response of the reciprocal crosses between SLR31^{C} and VLR1 to 16 g ai ha^{-1} chlorsulfuron are not statistically different ($P>0.05$) (Table 10.1). This indicates that the resistance is nuclear encoded.

The responses of both reciprocal F1 crosses were intermediate between the progeny of the resistant parents and the progeny of the susceptible parents (Figure 10.1). This indicates that the character is not a recessive trait. If the selected resistant parents were homozygous for resistance, this result would indicate that the character is semi-dominant. Alternatively, the result may indicate that the trait is fully dominant but that the selected resistant parents were not homozygous.

The response to chlorsulfuron of original SLR31 and VLR1 populations were similar to that previously observed (Chapter 3). However, the progeny of the resistant parents exhibit significantly less mortality than the unselected SLR31 population and both reciprocal first generation crosses ($P<0.05$) (Table 10.1). Thus, selection using high rates of chlorsulfuron is able to increase the level of chlorsulfuron resistance in population SLR31.

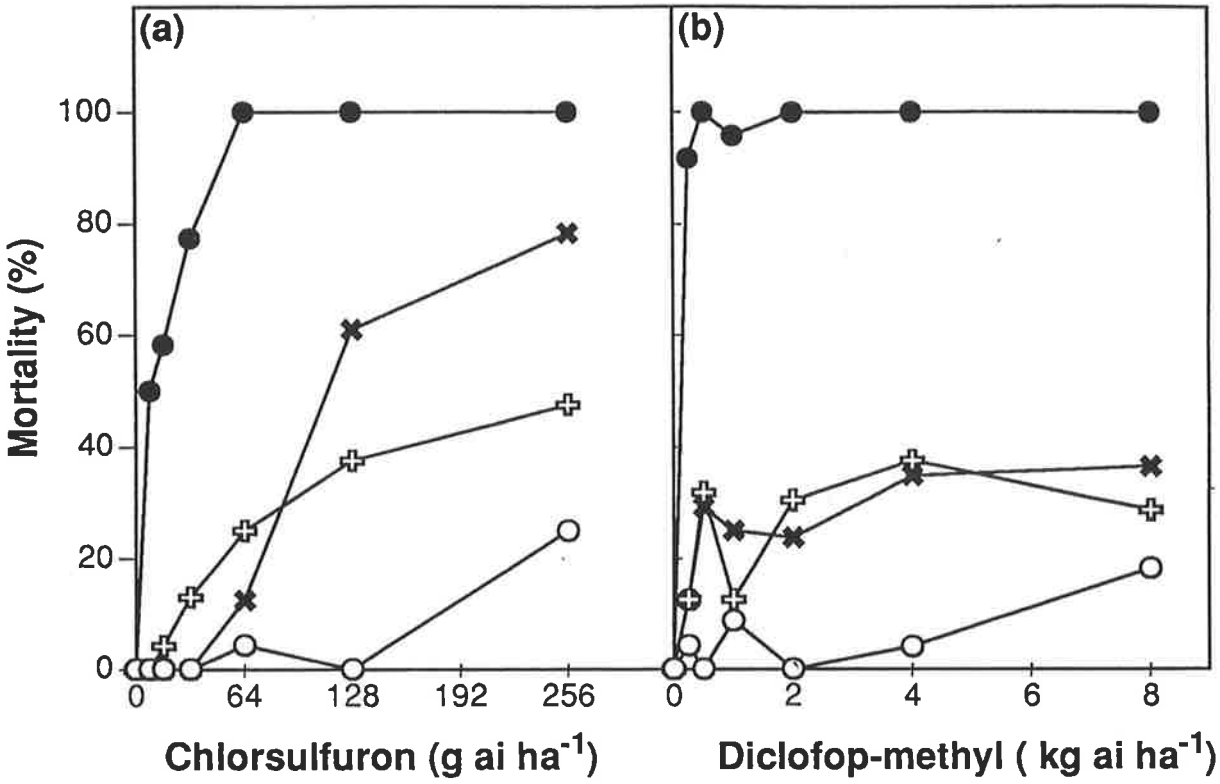


Figure 10.1 Mortality of progeny from, the susceptible VLR1 parents (●), the resistant SLR31^{C} parents (○), F1 crosses of SLR31^{C} with VLR1 plants, having susceptible female parents (✖), and F1 crosses of SLR31^{C} with VLR1, having SLR31^{C} female parent (✙). Plants were pot cultured and treated with (a) chlorsulfuron or (b) diclofop-methyl as described in sections 2.3 and 2.3 using 24 plants per treatment in 1990. (The resistant SLR31^{C} parents are plants from population SLR31 which survive 128 g ai ha^{-1} chlorsulfuron.)

Table 10.1 Mortality (%) of *L.rigidum* populations treated with 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl in 1992. R = resistant $SLR31^C$ parents, S = susceptible VLR1 parents.

	Original populations			First generation crosses	
	VLR1	SLR31	RXR	F1 RXS (R female)	F1 SXR (S female)
Chlorsulfuron	88.9[a] ± 3.2	29 2[b] ± 6 1	4.8 [c] ± 2.8	44.7 [b] ± 8.7	42.7 [b] ± 2.3
Diclofop-methyl	100 [a] ± 0	30.4 [bc] ± 9.6	6.7 [c] ± 1.7	49.5 [b] ± 8.1	42.8 [b] ± 4.9

$SLR31^C$ parents are plants from population SLR31 which survived 128 g ai ha^{-1} chloruslfuron treatment.

Data are the mean of three replicate experiments ± standard error of the mean.

[a] Means followed by different letters in the same row are significantly different ($P<0.05$).

The progeny of the first generation crosses were also tested with the aryloxyphenoxyproionate herbicide diclofop-methyl (Figure 10.1, Table 10.1). Tests of the first generation using diclofop-methyl demonstrate that SLR31 parents, selected with a high rate of chlorsulfuron, can pass both chlorsulfuron and diclofop-methyl resistance on to their progeny (Figure 10.1, Table 10.1). Reciprocal crosses between selected resistant parents and susceptibles show similar dose response to diclofop-methyl confirming previous findings that diclofop-methyl resistance is nuclear encoded (Figure 10.1) (McCarthey 1988). Comparison of the mean mortality of susequent, replicated experiments treated at 250 g ai ha^{-1} diclofop-methyl confirm that mortality of the reciprocal crosses of selected resistant parents with susceptibles are not significantly different ($P>0.05$) (Table 10.1).

The response of the reciprocal crosses is intermediate between that of the progeny of the resistant parents and progeny of the susceptible parents (Figure 10.1). This indicates that diclofop-methyl resistance is inherited either as a semi-dominant trait or that the trait is dominant and the selected resistant parents were not homozygous for resistance.

Segregation of chlorsulfuron resistance inherited from $SLR31^C$ in the second generation

To test for segregation of chlorsulfuron resistance traits, the F1 crosses of $SLR31^C$ with VLR1 were intercrossed to produce the F2. Plants from all populations were treated with 32 g ai ha^{-1} chlorsulfuron and mortality scored 28 days after treatment. The ratio of resistant to susceptible individuals in the F2 is approximately 3:1 (Table 10.2). Chi squared analysis indicates that the data agree with this hypothesis ($P>0.05$). The simplest hypothesis which fits this model is that chlorsulfuron resistance trait is controlled by a single dominant gene occuring mainly in the homozygous state in the $SLR31^C$ population (Table 10.2). However, it must be noted that this is the result of a single experiment representing the pooled results of 10 individual crosses using parents of uncertain genotype.

Table 10.2 Mortality of *L.rigidum* populations treated with 32 g ai ha^{-1} chlorsulfuron in 1992. R = resistant $SLR31^C$ parents, S = susceptible VLR1 parents.

	Live	Dead	Total	χ^2
Original populations				
VLR1 (susceptible)	7	25	32	
SLR31 (resistant)	31	2	33	
First generation crosses				
SXS	5	29	34	
RXR	74	0	74	
F1 crosses RXS (reciprocals pooled)	56	5	61	
F2	63	13	76	
expected (3:1)	57	19		0.6297 (P>0.05)

$SLR31^C$ parents are plants from population SLR31 which survived 128 g ai ha^{-1} chloruslfuron treatment.

Within the present data there are inconsistencies with the theory that chlorsulfuron resistance is controlled by a single dominant gene occurring in the homozygous state in the selected resistant parents. Firstly, if the parents were homozygous for a single dominant gene the F1 should be uniformly heterozygous for the trait and would display a resistant phenotype. The presence of some susceptible individuals in the F1 crosses between SLR31^{C} and VLR1 suggests that this is not the case (Table 10.2). Secondly, the survival of some susceptible plants at a dose of 32 g ai ha^{-1} chlorsulfuron (Table 10.2), which killed susceptible individuals in previous experiments (Figure 10.1, Chapter 3), also complicates the interpretation of the results. Either the growth of plants in trays at a close spacing of 2 cm, or the unusually wet conditions immediately after spraying may have reduced the efficacy of the herbicide. If this is the case then the number of resistant plants may have been overestimated. This problem did not occur for concurrent experiments using the herbicide sulfometuron (Table 11.2, Chapter 11).

Individual F1 crosses using SLR31^{C}

In 1991, a further eight SLR31^{C} plants were selected and used to make a further eight individual crosses with VLR1 plants, allowing progeny from individual crosses to be tested. The progeny of each of these crosses were tested using 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl (Table 10.3).

The simplest of the possible hypotheses to explain chlorsulfuron and diclofop-methyl cross-resistance would be that resistance to both herbicides is controlled by a single dominant gene and that selection of the resistant parents using 128 g ai ha^{-1} chlorsulfuron has allowed survival of only the homozygous resistant individuals. This would lead to the expectation that the first generation progeny of crosses between SLR31^{C} and VLR1 would be heterozygous for resistance and exhibit uniform resistance to both herbicides when tested at a rate of herbicide just adequate to kill the

Table 10.3. Mortality of individual families of F1 crosses between SLR31^C with VLR1, treated with 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl in 1992.

	Chlorsulfuron			Diclofop-methyl		
Cross	Live	Dead	χ^2(1:1)	Live	Dead	χ^2(1:1)
1	11	0	9.090*	7	4	0.182
2	12	0	10.083*	7	4	0.182
3	10	0	8.100*	4	6	0.100
4	12	4	3.063	6	11	0.471
5	9	1	4.900*	9	2	4.900*
6	21	0	14.087*	15	6	3.048
7	19	0	23,529*	8	4	0.750
8#	3	0		1	2	

SLR31^C parents are plants from population SLR31 which survived 128 g ai ha^{-1} chlorsulfuron treatment.

* χ^2 analysis indicates that the number of live plants is significantly greater than that expected for a ratio of live to dead of 1:1 ($P<0.05$).

Insufficient seed of this family was available to allow χ^2 analysis of the results.

homozygous susceptible phenotype. The results in Table 10.3 reveal that all crosses were resistant to 16 g ai ha^{-1} chlorsulfuron with only two families, 4 and 5, recording any mortality. This would suggest that six of the eight resistant parents may be homozygous for at least one major gene controlling chlorsulfuron resistance. Mortality in families 4 and 5 is less than 50% (Table 10.3), which suggests that the trait may be controlled by more than one gene. Alternatively, the mortality in these two families may be due to differences in the genetic background of the parents. If this were the case, all eight families may be derived from resistant parents homozygous for chlorsulfuron resistance.

When the individual first generation crosses were tested with 250 g ai ha^{-1} diclofop-methyl, a different response was observed (Table 10.3). The data for most families fit the hypothesis that resistant and susceptible individuals occur in the ratio of 1:1. This is what would be expected if diclofop-methyl resistance were controlled by a single dominant gene and the selected resistant parents are mostly heterozygous for the trait. Alternatively, diclofop-methyl resistance may be a semi-dominant trait.

In contrast to the other families, mortality due to diclofop-methyl in family 5 is significantly less than would be expected if the resistant and susceptible phenotypes were in the ratio of 1:1 (Table 10.3). This would suggest that diclofop-methyl resistance may be controlled by more than one gene in this family. The selected resistant parent of family 5 may have had resistant alleles of at least two genes for diclofop-methyl resistance. Alternatively, diclofop-methyl resistance may be controlled by a single dominant gene for which the selected resistant parent of family 5 was homozygous while the resistant parents of the other families were heterozygous.

Mortality data for chlorsulfuron and diclofop-methyl in the eight individual F1 crosses (Table 10.3), suggest the hypothesis that the selected resistant plants were homozygous for a single gene which causes chlorsulfuron resistance to be inherited as a dominant character and

diclofop-methyl resistance as a semi-dominant character. The resistant parent of family 5, however, may have had at least one further gene causing diclofop-methyl resistance. Backcrosses made from each individual first generation family, tested with chlorsulfuron and diclofop-methyl would be needed to confirm this hypothesis. Larger numbers of seed in each family would also be needed to test for different ratios of resistant to susceptible phenotypes in each family.

10.3 Inheritance of resistance from SLR31 parents which survive diclofop-methyl.

Pooled first generation crosses using SLR31^{D}

Experiments were conducted to test whether resistant parents from population SLR31 which survived 4000 g ai ha^{-1} diclofop-methyl can pass chlorsulfuron and diclofop-methyl resistance traits to their progeny in the same manner as resistant parents which survive 128 g ai ha^{-1} chlorsulfuron.

Reciprocal crosses between SLR31^{D} and VLR1 exhibit similar response to chlorsulfuron (Figure 10.2, Table 10.4) and, therefore, chlorsulfuron resistance is not maternally inherited from the SLR31^{D} population. The response to chlorsulfuron of both reciprocal crosses was intermediate between that of progeny from the selected resistant parents and progeny from the susceptible parents (Figure 10.2, Table 10.4). Thus, the trait is not recessive and may be partially dominant. Alternatively, the trait may be completely dominant but the selected resistant parents were not homozygous for resistance.

The response to chlorsulfuron of F1 progeny from SLR31^{D} parents was similar to that of the original, unselected SLR31 population. Mortality of the F1 progeny from SLR31^{D} plants is not significantly different from the original SLR31 population ($P>0.05$) (Table 10.4). This contrasts with the F1 progeny from SLR31^{C} plants, described above, which were less-sensitive to chlorsulfuron than the original SLR31 population.

The response to diclofop-methyl of the F1 progeny display similarities with their response to chlorsulfuron. The response of the F1 progeny of reciprocal crosses between SLR31^{D} and susceptibles was similar (Figure 10.2). However, there is inconsistency between the result represented in Figure 10.2 and the result of replicated experiments using 250 g ai ha^{-1} diclofop-methyl (Table 10.4). In the replicated experiment the response of the reciprocal crosses was significantly different (Table 10.4, $P<0.05$). The reason for this discrepancy is unclear, however, in the

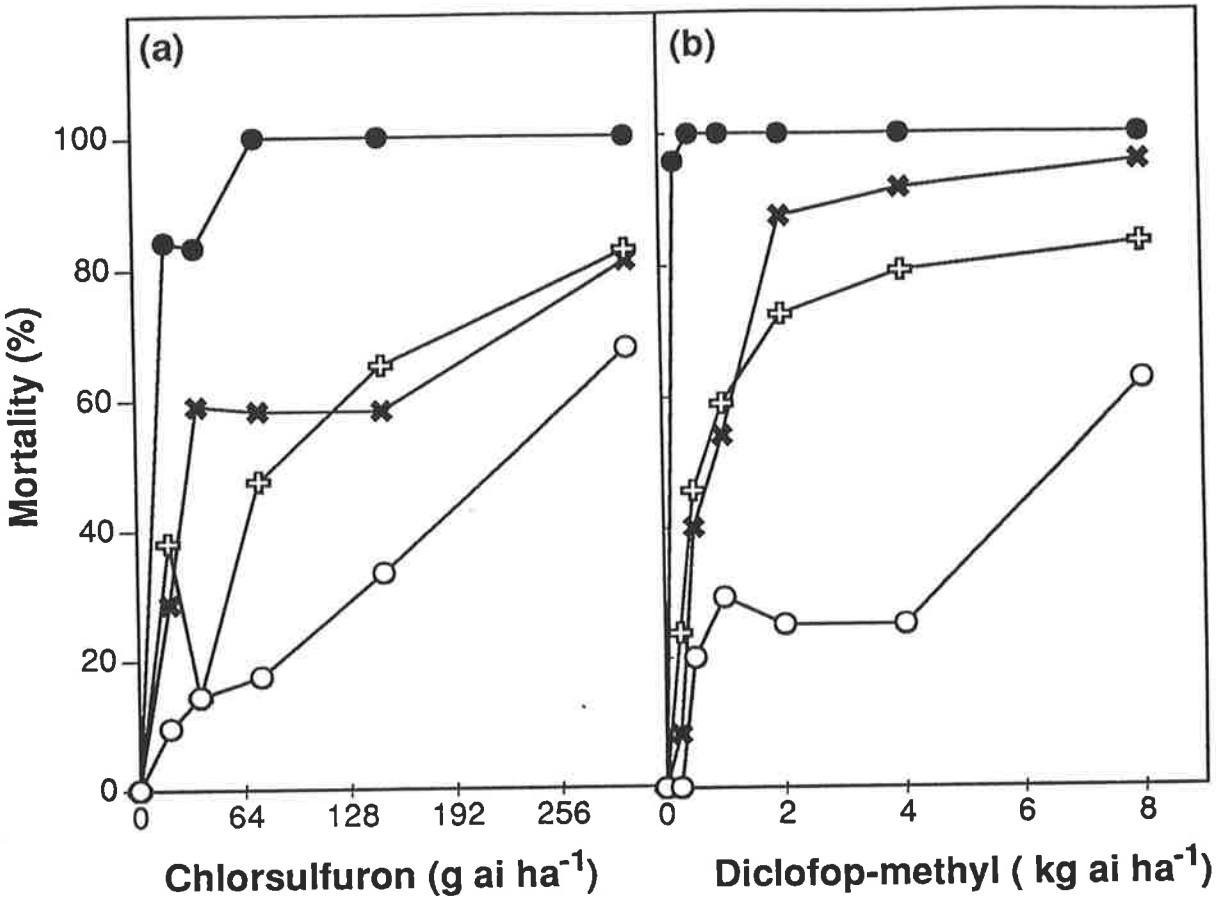


Figure 10.2 Mortality of progeny from, the susceptible VLR1 parents (●), the resistant SLR31^{D} parents (○), F1 crosses of SLR31^{D} with VLR1 plants, having susceptible female parents (✖), and F1 crosses of SLR31^{D} with VLR1, having SLR31^{D} female parent (✜). Plants were pot cultured and treated with (a) chlorsulfuron or (b) diclofop-methyl as described in sections 2.3 and 2.3 using 24 plants per treatment in 1991. (The resistant SLR31^{D} parents are plants from population SLR31 which survive 4000 g ai ha^{-1} diclofop-methyl.)

Table 10.4 Mortality (%) of *L.rigidum* populations treated with 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl, in 1992. R = $SLR31^D$ parents, S = susceptible VLR1 parents.

	Original populations		First generation crosses		
	VLR1	SLR31	RXR	F1 RXS (R female)	F1 SXR (S female)
Chlorsulfuron	88.9 [a] ± 3.2	29.2 [b] ± 6.1	24.2 [b] ± 3.6	40.8 [b] ± 2.0	33.4 [b] ± 3.6
Diclofop-methyl	100 [a] ± 0	30.4 [bc] ± 9.6	8.1 [b] ± 4.2	46.0[c] ± 4.1	14.9 [b] ± 5.1

$SLR31^D$ parents are plants from population SLR31 which survived 4000 g ai ha^{-1} diclofop-methyl treatment.

Data are the mean of three replicate experiments ± standard error of the mean.

[a] Means followed by different letters in the same line are significantly different ($P<0.05$).

replicated experiments there was less mortality due to diclofop-methyl in crosses having susceptible female parents than those having resistant female parents (Table 10.4). This would not be the case if the character were maternally inherited. Thus, despite the discrepancy, the data confirm that diclofop-methyl resistance is nuclear encoded.

The response to diclofop-methyl of both reciprocal crosses is intermediate between the response of progeny of the selected resistant parents and that of the susceptible parents (Figure 10.2). This indicates that the trait is partially dominant.

Segregation of chlorsulfuron resistance inherited from SLR31^{D} in the second generation

Untested F1 progeny from crosses between SLR31^{D} and VLR1 were intercrossed to produce the F2. Plants from all populations were treated with 32 g ai ha^{-1} chlorsulfuron and mortality was scored 28 days after treatment (Table 10.5).

Chi squared analysis indicates that the data in Table 10.5 agree with the hypothesis that susceptible and resistant plants in the second generation are in a ratio of 3:1 (Table 10.5). The simplest hypothesis which would fit this model is that the resistant parents were homozygous for the chlorsulfuron resistance trait which is controlled by a single dominant gene (Table 10.5). However, it must be noted that this is the result of a single experiment representing the pooled results of ten individual crosses using parents of uncertain genotype.

The data in Table 10.5 are inconsistent with the hypothesis that the SLR31^{D} population are homozygous for chlorsulfuron resistance in some respects as are the data for SLR31 chlorsulfuron survivors in Table 10.1, previously discussed, and therefore, the numbers of resistant plants may have been over estimated.

Table 10.5 Mortality of *L.rigidum* populations treated with 32 g ai ha^{-1} chlorsulfuron, in 1992. R = resistant $SLR31^D$ parents, S = susceptible VLR1 parents.

	Live	Dead	Total	χ^2
First generation crosses				
RXR	110	0	110	
SXS	13	18	34	
F1 crosses RXS (reciprocals pooled)	96	45	141	
F2				
	55	13	68	
expected (3:1)	51	17		0.961 (P>0.05)

This experiment was conducted concurrently with that reported in Table 10.1, where results for original populations SLR31 and VLR1 are given.

$SLR31^D$ parents are plants from population SLR31 which survived 4000 g ai ha^{-1} diclofop-methyl treatment.

Individual backcrosses from first generation crosses between SLR31^{D} and VLR1

Nine individual plants from the first generation crosses of SLR31^{D} with VLR1 were, individually, backcrossed to susceptible VLR1 plants to produce nine backcross families. Plants from each backcross family were tested with either 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl and the mortality determined. The response of each family is shown in Table 10.6.

The F1 data indicate that both chlorsulfuron resistance and diclofop-methyl resistance traits are dominant or semi-dominant (Table 10.4). As discussed above for the SLR31^{C} experiments, the simplest possible hypothesis would be that resistance to both herbicides is encoded by a single fully dominant gene, and selection of, in this case the SLR31^{D}, parents has selected for homozygous individuals. However, backcross families rather than F1 crosses were used in SLR31^{D} experiments. In the backcross progeny, the hypothesis would lead to an expectation of heterozygous individuals displaying resistant phenotype and susceptible individuals in the ratio 1:1. Chi squared analysis of the results in Table 10.6 indicate that the mortality data for families 2 and 3 fit this model for mortality due to both chlorsulfuron and diclofop-methyl. This suggests that the F1 parent of each family carried a single dominant gene for both chlorsulfuron and diclofop-methyl resistance, or one single dominant gene for each.

However, the data for the other families do not agree with the hypothesis that resistance to both herbicides could be controlled by one dominant gene. Data for family 1 indicate that the first generation parent of this family, like families 2 and 3, carried genes causing both chlorsulfuron and diclofop-methyl resistance. Mortality due to chlorsulfuron observed in family 1 fits the 1:1 hypothesis for a single dominant gene, but mortality due to diclofop-methyl is significantly less than expected ($P<0.05$) (Table 10.5). This suggests that the resistant parent of family 1 may have had resistant

Table 10.6. Mortality of individual backcross families from crosses $SLR31^D$ and VLR1, backcrossed with VLR1. Plants were treated with 16 g ai ha^{-1} chlorsulfuron or 250 g ai ha^{-1} diclofop-methyl.

	Chlorsulfuron			Diclofop-methyl		
Cross	Live	Dead	χ^2 (1:1)	Live	Dead	χ^2 (1:1)
1	13	7	1.25	15	5	4.06#
2	7	11	0.50	12	5	2.88
3	7	11	0.50	8	10	0.06
4	3	13	5.06*	0	21	21.04*
5	3	17	8.46*	12	8	0.90
6	5	16	6.26*	4	17	8.52*
7	6	15	5.26*	3	17	8.45*
8	0	21	21.04*	11	9	0.05
9	1	19	14.46*	12	8	0.35

$SLR31^D$ parents are plants from population SLR31 which survived 4000 g ai ha^{-1} diclofop-methyl treatment.

*χ^2 analysis indicates that the mortality for these families is significantly greater than would be expected if resistant and susceptible phenotypes were present in the ratio 1:1 (P>0.05).

#χ^2 analysis indicates that the mortality for this family is significantly less than would be expected if resistant and susceptible phenotypes were present in the ratio 1:1 (P>0.05).

allele of more than one gene for diclofop-methyl resistance. Alternatively, the low mortality due to diclofop-methyl in family 1 may be due to differences in the genetic background of the parent plants.

Families 5, 8 and 9 differ from families 1, 2 and 3. Observed mortality caused by diclofop-methyl in families 5, 8 and 9 fit the 1:1 ratio expected for a single dominant gene, however, chlorsulfuron mortality does not (Table 10.6). Greater than 85 % mortality due to chlorsulfuron occurred in families 5, 8 and 9. This level of mortality is similar to that of susceptible plants. This suggests that the first generation parent of each family carried genes for diclofop-methyl resistance but lacked genes encoding chlorsulfuron resistance.

The data for families 4, 6 and 7 differ from all the other families. Observed mortality caused by both herbicides is greater than 71%, and does not fit the expected 1:1 ratio for either (Table 10.6). This suggests that the individuals from these families lack genes coding for high levels of resistance to either herbicide. This implies that the first generation parent of each family was homozygous susceptible for resistance to both herbicides.

There are no families which exhibit only 50% chlorsulfuron mortality but high levels of mortality due to diclofop-methyl (Table 10.6). This implies that the there were no first generation parents carrying chlorsulfuron resistance in the absence of diclofop-methyl resistance. This is due to selection of the resistant SLR31^{D} parents using a high rate of diclofop-methyl.

Thus the phenotypic response to herbicides of the individuals from crosses between SLR31^{D} and VLR1 are not uniform. Some individuals from the first generation crosses had genes causing both chlorsulfuron and diclofop-methyl resistance, some had genes causing diclofop-methyl resistance but not chlorsulfuron resistance, while others did not have genes for either resistance trait. This indicates that the SLR31^{D} population, were not genetically uniform for either resistance trait. The intermediate response to both herbicides of the first generation progeny is, therefore, the

integration of the response of several different genotypes and is not due to a uniform response caused by a semi-dominant gene. Further studies of the response to herbicides of individual first generation families, as previously discussed for individuals families using chlorsulfuron selected parents, would be needed to determine the genotypes of the diclofop-methyl selected resistant parents.

Individual backcross families 5, 8 and 9 appear to have arisen from a first generation parent with at least one gene for diclofop-methyl resistance but not having chlorsulfuron resistance. Indicating that at least one gene causing diclofop-methyl resistance without chlorsulfuron resistance is present in the population. Families derived from parents with chlorsulfuron resistance but not diclofop-methyl resistance are lacking.

The results of the individual backcrosses demonstrate that diclofop-methyl and chlorsulfuron resistance are not always under the control of a common gene in biotype SLR31. Diclofop-methyl resistance can occur independently of chlorsulfuron resistance. The diclofop-methyl resistance gene which segregates separately from chlorsulfuron resistance may, or may not, be the same gene which controls diclofop-methyl resistance coincident with chlorsulfuron resistance. Thus, the present data would support two alternative hypotheses. Firstly, chlorsulfuron and diclofop-methyl resistance may be controlled by independent genes. Secondly, there may be at least one gene present in population SLR31 which controls both chlorsulfuron and diclofop-methyl resistance and one which controls diclofop-methyl resistance only.

10.5 Discussion

These experiments using, biotype SLR31, provide the first information on the inheritance of chlorsulfuron resistance in *L.rigidum*. Biotype SLR31 has herbicide-sensitive ALS (Matthews *et al.* ,90) but has increased ability to detoxify chlorsulfuron (Chapter 4). Therefore, these studies provide the first information on the genetic control of the chlorsulfuron resistance mechanism in *L.rigidum* which involves increased metabolism.

Data from F1 crosses of resistant parents selected with either chlorsulfuron or diclofop-methyl indicate that inheritance of both diclofop-methyl and chlorsulfuron resistance in biotype SLR31 are controlled by nuclear encoded genes (Figures 10.2 & 10.3, Tables 10.1 & 10.4). Neither trait is recessive, both are dominant or semi-dominant characters.

The data from the F1 clearly illustrate that the selected resistant plants can pass on both chlorsulfuron and diclofop-methyl resistance to their progeny, whether selected using high levels of diclofop-methyl or chlorsulfuron (Tables 10.1 & 10.4). The progeny of SLR31 individuals which survived chlorsulfuron, exhibit a higher level of chlorsulfuron resistance than the unselected SLR31 population (Table 10.1). However, this was not true for the progeny of SLR31 individuals which survived diclofop-methyl (Table 10.4).

The F2 from crosses of parents selected with either diclofop-methyl or chlorsulfuron have chlorsulfuron resistant and susceptible phenotypes in the ratio 3:1 (Table 10.2, Table 10.5). This suggests that the trait is controlled by a single, largely dominant, gene. Further tests are needed to confirm this. Evidence from subsequent individual crosses of chlorsulfuron selected parents with susceptibles suggest that the parents may all be homozygous for chlorsulfuron resistance (Table 10.3) .

Evidence from backcrossed families made from the first generation progeny of $SLR31^{D}$ crossed with VLR1, demonstrate that at least one gene for diclofop-methyl resistance can segregate independently of

chlorsulfuron resistance. This indicates that diclofop-methyl and chlorsulfuron resistance in SLR31 are not exclusively controlled by one gene. Chlorsulfuron and diclofop-methyl resistance may be controlled by independent genes. Alternatively, there may be one gene controlling chlorsulfuron and diclofop-methyl resistance and another gene controlling diclofop-methyl resistance in the absence of chlorsulfuron resistance. The occurrence of at least one gene causing both diclofop-methyl and chlorsulfuron resistance in biotype SLR31 is suggested by the development of chlorsulfuron cross-resistance following diclofop-methyl treatment in this biotype.

It is clear that studies using a large number of families tested individually in several generations will be required to determine how many genes control each trait, the dominance relationship of each and their occurrence in population SLR31.

Chapter 11

Inheritance of sulfonylurea resistance involving herbicide-insensitive ALS

11.1 Introduction

Resistance to sulfonylurea and imidazolinone herbicides in *L.rigidum* biotype WLR1, involves a herbicide-insensitive ALS (Chapter 5). In particular, resistance to the non-selective, sulfonylurea herbicide sulfometuron-methyl is probably the result of herbicide-insensitive ALS in this biotype (Chapter 5). In another *L.rigidum* population, VLR69, the majority of individuals are resistant to chlorsulfuron but only a subset of approximately 4% are resistant to sulfometuron-methyl (Burnet *et al.* 1993c). This sulfometuron-methyl resistant subset of population VLR69 have herbicide-insensitive ALS (Burnet *et al.* 1993c). Weed biotypes from North America, which are resistant to sulfometuron-methyl, have herbicide insensitive ALS (Saari *et al.* 1990 and 1992). Tolerance to sulfometuron-methyl in some grasses is due to increased metabolism (cited in Anderson and Swain 1992). Although sulfometuron-methyl metabolism in *L.rigidum* has not been measured, sulfometuron-methyl resistance in *L.rigidum* correlates with herbicide-insensitive ALS. Plant genes encoding for

herbicide-insensitive ALS were inherited as dominant or semidominant traits at a single locus in all cases reported (reviewed in section 1.5.3).

Biotype WLR1 also has the ability to metabolise chlorsulfuron more rapidly than a susceptible biotype (Chapter 4). If increased metabolism is important for chlorsulfuron resistance in biotype WLR1, it is likely that chlorsulfuron resistance in this biotype is controlled by genes at a minimum of two loci. At least one gene controlling increased metabolism and another gene encoding herbicide-insensitive ALS would be anticipated.

In this section the inheritance of resistance to sulfonylurea herbicides was studied using biotype WLR1. Investigations of the inheritance of sulfometuron-methyl resistance were used to indicate the inheritance of herbicide-insensitive ALS. Investigations of the inheritance of chlorsulfuron resistance were also undertaken to determine whether more than one mechanism of chlorsulfuron resistance exists in biotype WLR1.

11.2 Materials and methods

First generation crosses

To test the dominance relationships of chlorsulfuron and sulfometuron-methyl resistance traits and to determine whether either character is maternally inherited, crosses between plants of biotype WLR1 and VLR1 were made in 1990. The crossing procedure was similar to that described for first generation crosses in section 10.2.2, except that the resistant WLR1 parents were not tested with herbicide prior to crossing. Thus, eight unselected plants of biotype WLR1 and eight of biotype VLR1 were grown in pots kept in the field until they had 10 to 15 tillers. Plants were removed from the soil and divided into two clones. One clone of each resistant plant was placed in a 10 L pot with one clone of a susceptible plant and one clone was placed in a pot with a clone of one other resistant plant. Similarly, the second clone from each susceptible plant was placed in a 10 L pot with a second clone from one other susceptible plant. Each two plants in a 10 L pot were surrounded with a polythene sheath prior to flowering as

described in section 10.2.1. The seed from the resistant WLR1 parents were pooled as were the seed of the susceptible VLR1 parents. Seed of the reciprocal crosses between WLR1 and VLR1 were pooled separately such that, progeny having WLR1 female parent were pooled and progeny having VLR1 female parent were pooled. For ease of discussion the progeny of crosses between WLR1 and VLR1 will be called the F1, even though the genotype of the parents is not certain.

In 1991 the progeny of first generation crosses were pot cultured in the field and treated with herbicides as described in sections 2.2 and 2.3. Response to chlorsulfuron was determined in a single experiment using 24 plants per treatment. In 1992, the reciprocal crosses were further tested at a single rate of 16 g ai ha^{1} chlorsulfuron and 4 g ai ha^{-1} sulfometuron-methyl using 24 plants per treatment in experiments replicated three times.

Segregation of chlorsulfuron resistance in the second generation

In 1991, progeny of the F1 crosses were allowed to intercross to produce a second generation. Eighteen plants (9 from each of the pooled reciprocal crosses) where cultured in 10L pots, surrounded by a common polythene screen allowing cross-pollination between all plants. All the seed produced was collected in a single sample which was called the F2. In 1992 plants of the parent populations WLR1 and VLR1, the first generation crosses and the second generation were cultured in 40 cm by 30 cm by 12 cm trays in the field at 2 cm spacing and tested with either 32 g ai ha^{-1} chlorsulfuron or 4 g ai ha^{-1} sulfometuron-methy as described in sections 2.2 and 2.3.

Individual backcross families

To allow testing of chlorsulfuron and sulfonylurea resistance in individual families, backcrosses where made in 1991. Twelve plants from the F1 crosses between WLR1 and VLR1 (6 from each of the pooled reciprocal crosses) were individually crossed with VLR1 plants as described in section 10.2.1. Seed from both parents were pooled within each backcross family. Plants from each backcross family were pot cultured in

the field and treated with herbicide in 1992 as described in sections 2.2 and 2.3. Plants from each family plus the original populations VLR1 and WLR1 were treated with chlorsulfuron 16 g ai ha^{-1}, sulfometuron-methyl 4 g ai ha^{-1} or kept as untreated controls.

11.3 Inheritance of chlorsulfuron resistance in biotype WLR1.

First generation crosses

The response to chlorsulfuron of the progeny of WLR1 crossed with VLR1 was similar for reciprocal crosses (Figure 11.1). Replicated tests of mortality caused by 16 g ai ha^{-1} chlorsulfuron confirm that mortality for the reciprocal crosses are not significantly different from each other ($P<0.05$) (Table 11.1). This indicates that chlorsulfuron resistance is nuclear encoded. The mortality of the reciprocal crosses is not significantly different from that of the original WLR1 population (Table 11.1). This indicates that the character is inherited as a dominant trait.

Table 11.1 Mortality (%) of *L.rigidum* populations treated with 16 g ai ha^{-1} chlorsulfuron, or 4 g ai ha^{-1} sulfometuron-methyl in 1992.

	Parent populations		First generation crosses	
	S	R	WLR1 X VLR1	
	VLR1	WLR1	(WLR1 female)	(VLR1 female)
Chlorsulfuron	88.9[a] ± 3.2	21.1 [b] ± 6.1	22.2 [b] ± 1.6	12.7 [b] ± 5.7
Sulfometuron	100 [a] ± 0	16.1 [b] ± 1.5	56.8 [c] ± 4.1	57.7 [c] ± 7.8

Data are the mean of three replicate experiments ± standard error of the mean.

[a] Means followed by different letters in the same line are significantly different ($P<0.05$).

Segregation of chlorsulfuron resistance in the second generation

To allow study of the segregation of resistance traits, a second generation was made by intercrossing plants from the F1 crosses. Individual plants from the parent populations WLR1 and VLR1, progeny of all the first generation crosses, and the F2 progeny were tested using 32 g ai ha^{-1} chlorsulfuron (Table 11.2).

Data from the first generation crosses indicate that chlorsulfuron resistance is inherited as a nuclear encoded, dominant character (Table 11.1). One of the simplest models which would fit this data is that most individuals of population WLR1 are homozygous for a single dominant chlorsulfuron resistance allele. This model would lead to the expectation that resistant and susceptible phenotypes would occur in the second generation in the ratio of 3:1. Chi squared analysis indicates that the data are in agreement with the hypothesis that resistant and susceptible individuals occur in the ratio of 3:1 in the second generation progeny ($P>0.05$) (Table 11.2). However, it must be noted that the results represent the pooled data for several crosses using parents of uncertain genotype. The experiment was conducted concurrently with those reported for biotype SLR31 in Tables 10.2 and 10.4 and has the same problem of reduced herbicide efficacy leading to possible over-estimation of the number of resistant plants (Table 11.2).

If at least one dominant or semi-dominant gene for increased metabolism and one dominant or semi-dominant gene for herbicide-insensitive ALS occur in biotype WLR1, the ratio of resistant to susceptible individuals in the second generation would not be expected to fit the ratio of 3:1. However, the fact that mortality due to sulfometuron-methyl is higher than mortality due to chlorsulfuron in the progeny of both the first and second generations suggests that there are two genes for chlorsulfuron resistance in biotype WLR1 (compare Tables 11.1 and 11.3).

Table 11.2 Mortality of *L.rigidum* populations treated with 32 g ai ha^{-1} chlorsulfuron in 1992.

	Live	Dead	Total	χ^2
Parent populations				
VLR1 (susceptible)	7	25	32	
WLR1 (resistant)	81	0	81	
First generation				
Progeny of WLR1 parents	73	3	77	
Progeny of VLR1 parents	13	18	31	
F1 WLR1 by VLR1 crosses (reciprocals pooled)	136	6	142	
F2	60	25	85	
expected (3:1)	63.75	21.25		0.6628 (P>0.05)

To determine the number of genes involved in chlorsulfuron resistance in biotype WLR1 further studies using individual families over a number of generations are required.

11.4 Inheritance of sulfometuron-methyl resistance

First generation

Tests of crosses between WLR1 and VLR1 revealed that mortality caused by 4 g ai ha^{-1} sulfometuron-methyl does not differ significantly between reciprocal crosses ($P<0.05$), indicating that sulfometuron-methyl resistance is nuclear encoded. Mortality of the reciprocal crosses due to sulfometuron-methyl is significantly greater than that of the original WLR1 population ($P>0.05$) (Table 11.1). This indicates that the character may be semi-dominant. Alternatively, the character may be dominant occurring mainly in the heterozygous state in the WLR1 parents.

Table 11.3 Mortality of *L.rigidum* populations treated with 4 g ai ha^{-1} sulfometuron in 1992.

	Live	Dead	Total	χ^2
Parent populations				
VLR1(susceptible)	0	43	43	
WLR1 (resistant) 83	13		96	
First generation				
Progeny of VLR1 parents	0	3	33	
Progeny of WLR1 parents	73	30	93	
F1 WLR1 by VLR1 crosses (reciprocals pooled)	34	57	81	
Expected				
Model 1 (0.5 : 0.5)	40.5	40.5		7.209 (0.005<P<0.01)
Model 2 (0.65 : 0.35)	52.7	28.4		34.088 (P< 0.005)
Moedl 3 (0.432 : 0.568) 35	46			5.547 (0.01<P<0.025)
F2	32	69	101	

Segregation of sulfometuron-methyl resistance in the second generation

Data for mortality due to sulfometuron-methyl in first and second generation progeny are presented in Table 11.3. All plants from the original susceptible population VLR1 were killed by the treatment as were the progeny of the actual susceptible parents used in the first generation crosses (Table 11.3). If we assume that sulfonylurea resistance results from a herbicide-insensitive ALS and that genetic control of ALS in *L.rigidum* is at a single locus, this would indicate that the VLR1 population is homozygous recessive for sulfometuron-methyl resistance.

The original WLR1 population is clearly not homozygous for sulfometuron-methyl resistance as 13 of the 96 individuals were killed by the low dose of 4 g ai ha^{-1} sulfometuron-methyl. This suggests that these 13 individuals were homozygous susceptible. The proportion of homozygous resistant and heterozygous individuals amongst the 83 survivors is not known.

Three models were proposed to explain the present data. The first model proposed that after seven generations of selection using chlorsulfuron the gene for sulfometuron-methyl resistance and the susceptible gene had reached equilibrium within the population so that the number of homozygous resistant individuals was equal to the number of homozygous susceptibles. Thus, of the 96 original WLR1 plants tested 13 individuals were homozygous recessive and 13 individuals homozygous resistant, leaving 70 individuals heterozygous. Crossing these plants with homozygous susceptibles from population VLR1 would lead to the expectation that the proportion of heterozygotes and homozygous susceptibles would be equal $(13 + (1/2 \times 70))/96 = 0.5$. Chi squared analysis of the data in Tables 11.3 the data for the progeny of the WLR1 parents and the crosses between WLR1 and VLR1 do not agree with this hypothesis ($0.005 < P < 0.01$). This model was not further tested.

The second model proposes that the increase in the proportion of susceptibles in the progeny of crosses between WLR1 and VLR1 compared to the parent population was due to the presence of heterozygous individuals in the resistant parent population. Thus, the proportion of heterozygotes in the WLR1 population can be calculated by subtracting the proportion of susceptibles in the parent population from the proportion of susceptibles in the F1 crosses. The proportion of susceptibles in the parent population is 13/96 = 0.135 (Table 11.3). The proportion of susceptibles in the F1 crosses is 57/101 = 0.564 (Table 11.3). Therefore the proportion of heterozygotes in the original WLR1 population is 0.564 - 0.135 = 0.429. Therefore, the proportion of homozygous resistant individuals in the WLR1 population is 1-0.429 - 0.135 = 0.435. The proportion of homozygous resistant, heterozygous and homozygous susceptibles are estimated as O.435 : 0.429 : 0.135. Thus, the WLR1 population would produce resistant gametes in the proportion of 0.435 + (1/2 x 0.429) = 0.650 and susceptible gametes in the proportion 0.135 + (1/2 x 0.429) = 0.350. Crossing WLR1 plants with homozygous susceptible plants from biotype VLR1 should produce heterozygote (of resistant phenotype) and homozygous susceptibles in the ratio of 0.650 : 0.350. Chi squared analysis reveals that the data do not fit this hypothesis ($P < 0.005$). This model was not further tested.

A third model was proposed which assumes that homozygous resistant individuals are missing from the WLR1 population and that almost all the individuals are heterozygous for sulfometuron-methyl resistance. This would mean that the WLR1 population tested in Table 11.3 would have consisted of 83 heterozygotes and 13 homozygous susceptibles. The population would produce resistant gametes in the proportion 1/2 x 83/96 = 0.432 and susceptible gametes in the proportion (1/2 x 83/96) + 13/96 = 0.568. If this population is crossed with homozygous susceptibles from population VLR1 the expected ratio of heterozygotes to homozygous susceptibles would be 0.432 : 0.568. Chi squared analysis indicates that the data in Table 11.3 do not fit this hypothesis ($0.01 < P < 0.025$),

however, of the three models tested model 3 is closest to fitting the data. It is suggested that the sulfometuron-methyl resistant individuals from population WLR1 are mainly heterozygous.

11.5 Inheritance of chlorsulfuron and sulfometuron-methyl resistance in individual backcross families

Individual backcross families were made from the progeny of F1 crosses. Twelve plants from the F1 were individually backcrosses to VLR1 plants to produce twelve individual backcross families. The following year, progeny from each backcross family were tested using 16 g ai ha^{-1} chlorsulfuron, or 4 g ai ha^{-1} sulfometuron-methyl (Table 11.3 and 11.4). If both traits were controlled by a common, dominant allele occurring in the homozygous state in the resistant parents we would expect heterozygotes (displaying resistant phenotype) and homozygous recessive individuals in the ratio 1:1 in the backcross. Chi squared analysis of the data in Tables 11.3 and 11.4 indicate that mortality in most families fit this hypothesis. Families 1, 2 4, 5, 6, 9, 10, and 12 have resistant and susceptible phenotypes present in the approximate ratio of 1:1. This would suggest that the F1 parent of each could have been heterozygous for a single dominant gene for both chlorsulfuron and sulfometuron-methyl resistance.

Two families, 3 and 11, exhibit significantly greater than 50% mortality due to chlorsulfuron (Table 11.4). Mortality for these families is similar to that expected for susceptible plants (see Table 11.1). Examination of the response to sulfometuron-methyl (Table 11.5) reveals that these two families are also susceptible to this herbicide. Thus, the first generation parent of these two families lacked genes for resistance to either herbicide.

A different response is seen in families 7 and 8 which exhibit approximately 50% mortality to chlorsulfuron (Table 11.4) but 100% mortality when treated with sulfometuron-methyl (Table 11.5). This would suggest that the first generation parent of these families carried the

Table 11.4 Mortality of *L.rigidum* individual backcross families treated with 16 g ai ha^{-1} chlorsulfuron in 1992.

Family	Live	Dead	Total	χ^2 (1:1)
1	12	7	19	0.84
2	14	6	20	2.45
3	4	17	21	6.86*
4	6	10	16	0.56
5	5	13	18	2.72
6	13	6	19	1.89
7	11	9	20	0.05
8	8	9	17	0.06
9	8	10	18	0.06
10	4	9	13	1.23
11	1	18	19	19.06*
12	13	7	20	1.25

*χ^2 analysis indicates that the ratio of live plants to dead plants is significantly less than 1:1 for these families (P>0.05).

Table 11.5 Mortality of *L.rigidum* individual backcross families treated with 4 g ai ha^{-1} sulfometuron-methyl in 1992.

Family	Live	Dead	Total	χ^2 (1:1)
1	14	6	20	2.45
2	5	12	17	2.12
3	0	21	21	19.05*
4	7	9	18	0.06
5	9	11	20	0.05
6	6	10	16	0.45
7	0	21	21	19.05*
8	0	21	21	19.05*
9	11	10	21	0.05
10	7	9	16	0.06
11	0	17	17	15.06*
12	11	9	20	0.05

*χ^2 analysis indicates that the ratio of live plants to dead plants is significantly different from 1:1 (P>0.05).

chlorsulfuron resistance trait but not the sulfometuron-methyl resistance trait.

Thus, the F1 parents of the individual backcross families probably included plants which were homozygous susceptible for both chlorsulfuron and sulfometuron-methyl resistance and plants which were heterozygous for resistance to both herbicides. The F1 also included individuals which were heterozygous for chlorsulfuron resistance but homozygous susceptible for sulfometuron-methyl resistance. This data indicates that at least two mechanisms for chlorsulfuron resistance exist in population WLR1, one of which does not cause sulfometuron-methyl resistance.

It is not possible to distinguish families arising from parents which have herbicide-insensitive ALS but lack the second chlorsulfuron resistance mechanism as the mutant ALS is highly resistant to chlorsulfuron.

11.6 Discussion

The data for the F1 indicate that both chlorsulfuron and sulfometuron-methyl resistance are nuclear encoded. Chlorsulfuron resistance is inherited as a dominant trait. Sulfometuron-methyl resistance is either inherited as a semi-dominant trait or as a dominant trait occurring in the heterozygous state in some individuals.

In the second generation chlorsulfuron resistant and susceptible plants occur in the approximate ratio of 3:1 (Table 11.2). This result is not what would be expected if chlorsulfuron resistance is controlled by at least two, independantly segregating, dominant or semi-dominat genes occurring together in most of the individual parents. Results from individual backcross families confirm that chlorsulfuron resistance in the absence of sulfometuron-methyl resistance can occur in population WLR1. Studies of individual families of the F1 will be needed to determine the cause of the

apparent 3:1 ratio of chlorsulfuron resistant to susceptible phenotypes in the F2.

Segregation of sulfometuron-methyl resistance in the second generation shows a different pattern to that of chlorsulfuron resistance. This is further evidence that the two traits are not always under common genetic control.

Data for mortality due to sulfometuron-methyl do not fit any of three models analysed but come closest to fitting a model which proposes that individuals homozygous for sulfometuron-methyl resistance are absent from the WLR1 population. The results from first generation crosses suggest that sulfometuron-methyl resistance is semidominant character or that the character is dominant but occurs largely in the heterozygous state in the WLR1 population. Evidence from previous studies would suggest that the second hypothesis is more likely.

ALS from biotype WLR1 has an I_{50} for sulfometuron-methyl which is greater than thirty times that of ALS from biotypes with susceptible ALS (Chapter 5). In whole plant studies, population WLR1 exhibits little mortality at doses up to 16 g ai ha^{-1} sulfometuron-methyl (Figure 3.6, Chapter 3). Thus, WLR1 individuals with mutant ALS might be expected to display a resistant phenotype when tested at the relatively low dose of 4 g ai ha^{-1} sulfometuron-methyl. Secondly, the level of ALS activity in extracts from WLR1 appears to approach a level of approximately 50% of that for untreated controls when high concentrations of sulfometuron-methyl are added to the assay (Figure 5.1, Chapter 5). In contrast, ALS activity from susceptible biotypes appears to approach zero (Figure 5.1, Chapter 5). This suggests that approximately 50% of extractable ALS activity from the WLR1 population is highly resistant to sulfometuron-methyl but the remainder of activity remains sensitive. Taken together these data suggest sulfometuron-methyl resistance in biotype WLR1, caused by a mutant ALS, is inherited as a dominant trait but most sulfometuron-methyl resistant individuals are heterozygous.

The presence of approximately 13% sulfometuron-methyl susceptible individuals in the parent population, indicate that the population is not genetically uniform for sulfometuron-methyl resistance. Further experiments, using individual families from first generation crosses and are need to estimate of the proportion of the different genotypes in the WLR1 population.

The data for individual backcross families indicate that chlorsulfuron resistance can occur independent from sulfometuron resistance in progeny of WLR1. This is consistent with the hypothesis that resistance to ALS inhibitors in WLR1 is controlled by at least two genes. There is probably at least one gene controlling chlorsulfuron resistance in the absence of sulfometuron-methyl resistance. There is also at least one gene encoding herbicide-insensitive ALS which causes both chlorsulfuron and sulfometuron resistance. It appears that most of the approximately 13% of WLR1 individuals homozygous susceptible for sulfometuron-methyl resistance may be chlorsulfuron resistant due to the second mechanism of chlorsulfuron resistance as the population exhibits little mortality when treated with 32 g ai ha^{-1} chlorsulfuron (Table 11.2).

The proportion of WLR1 individuals with genes for increased chlorsulfuron metabolism is not known. Chlorsulfuron metabolism in excised seedlings of population WLR1 is almost as rapid as that of biotype SLR31 which has herbicide-sensitive ALS (Figure 4.5, Chapter 4). This may indicate that the majority of WLR1 individuals also have increased ability to detoxify chorsulfuron when compared to susceptible biotypes. As biotype WLR1 remains susceptible to diclofop-methyl the mechanism causing increased chlorsulfuron metabolism in this biotype does not appear to cause diclofop-methyl cross-resistance (Figure 3.9, Chapter 3). HPLC data suggest that the metabolic pathway for chlorsulfuron is similar for all biotypes (Figures 4.2 and 4.6, Chapter 4). This evidence, therefore, suggests that the mechanism causing increased metabolism of chlorsulfuron in some biotypes of *L.rigidum* does not necessarily correlate with diclofop-methyl resistance.

However, as the majority of WLR1 individuals have herbicide-insensitive ALS the difference between the rate of metabolism in population WLR1 and susceptible population VLR1 could be due, at least in part, to the fact that VLR1 plants are dying but WLR1 plants are protected by the mutant enzyme.

Chapter 12

Comparison of herbicide response of *L.rigidum* biotypes with different herbicide treatment history.

12.1 Introduction

Resistance to herbicides which inhibit ALS has been shown to involve at least two mechanisms in biotypes of *L.rigidum* (Chapters 3 & 4). The mechanisms of resistance to ALS inhibiting herbicides vary in three biotypes of *L.rigidum* which arose following widely different herbicide treatment regimes. Biotype SLR31 arose following treatment with diclofop-methyl in four consecutive seasons. This biotype has herbicide-sensitive ALS but increased ability to metabolise the wheat-selective herbicide, chlorsulfuron (Chapter 4). The mechanism of resistance in this biotype causes resistance to some selective sulfonylurea and imidazolinone herbicides but does not cause resistance to the non-selective herbicides such as sulfometuron-methyl (Chapter 3). In contrast, biotype WLR1 has a herbicide-insensitive form of ALS which appears to cause resistance to both selective and non-selective sulfonylurea and imidazolinone herbicides (Chapter 5). A third biotype, VLR69, became resistant to ALS inhibiting herbicides following treatment with a wide range of herbicides differing in mode of action (Burnet *et al.* 1993c) (Table 12.1). The majority of individuals from this population were resistant to chlorsulfuron but only a small proportion, approximately 4%, were also resistant to sulfometuron-methyl (Burnet *et al.* 1993c). The sulfometuron-methyl resistant plants had a herbicide-insensitive ALS, whereas the majority of the population did not. The population, as a whole, had an increased ability to metabolise chlorsulfuron (Burnet *et al.* 1993c). Thus, in biotypes, WLR1 and VLR69,

resistance to sulfometuron-methyl is correlated with an herbicide-insensitive ALS. The proportion of individuals with herbicide-insensitive ALS appears to differ widely between populations with different herbicide treatment history.

Biotypes SLR31 and VLR69 are also resistant to the aryloxyphenoxypropionate herbicide diclofop-methyl to which both have been exposed. In contrast, biotype WLR1 has not been exposed to aryloxyphenoxypropionate herbicides and is not resistant to them (Chapter 3).

In this section the response to herbicides of three biotypes of *L.rigidum* arising from fields with different herbicide treatment history are compared with the three biotypes already discussed. The diclofop-methyl resistant, chlorsulfuron cross-resistant biotypes SLR31 and NLR12 had been exposed to four and seven treatments of diclofop-methyl respectively but had not been treated with herbicides which inhibit ALS (Heap and Knight 1990). Two putative chlorsulfuron resistant biotypes, VLR4 and SLR19, had been exposed to chlorsulfuron but had not been exposed to aryloxyphenoxypropionate herbicides. Biotypes VLR4, SLR19 and WLR1 had been exposed to four, five and seven treatments of chlorsulfuron respectively (Table 12.1)

The effect of the imidazolinone herbicide, imazathapyr, on resistant and susceptible *L.rigidum* biotypes was also tested. Imazathapyr is used for control of broadleaf weeds in peas in some cereal growing areas of Australia at application rates of from 75 to 100 g ai ha^{-1}. Although imazathapyr is not recommended for *L.rigidum* control, it is proposed that the use of this herbicide in the legume phase of a crop rotation will select for ALS-inhibitor resistance in *L.rigidum*.

12.2 **Materials and methods**

Five *L.rigidum* biotypes were pot cultured in the autumn-winter season of 1992, treated with herbicides and harvested as described in section 2.2 . Three replicate experiments were conducted concurrently using

two pots of twelve plants per treatment except for sulfometuron-methyl where 108 plants were used for each treatment. Chlorsulfuron treatments of biotypes VLR1, SLR31 and WLR1 were performed once only.

Table 12.1 Herbicide treatment history of *L.rigidum* biotypes

Biotype	Number of herbicide applications		
	diclofop	chlorsulfuron	Other herbicides *
VLR1	0	0	
VLR69	2	5	17 diuron 5 atrazine 2 paraquat+diquat **
SLR31	4	0	12 trifluralin
NLR12	7	0	4 trifluralin
VLR4	0	4	3 metolachlor 2 alachlor 2 paraquat+diquat
SLR19	0	5	1 trifluralin 3 diuron + MCPA
WLR1	0	7	

* chemical names listed in abbreviations

** Burnet *et al* . 1993

Collection locations are given in section 2.1

12.3 Response to herbicides

Chlorsulfuron

Determinations of dose response to chlorsulfuron revealed two further *L.rigidum* biotypes which are resistant to ALS inhibiting herbicides (Figure 12.1). Biotypes VLR4 and SLR19 are resistant to chlorsulfuron (Figure 12.1). At the normal application rate of 16 g ai ha^{-1}, biotype VLR4 suffered only 16.0 ± 6.2 % mortality while biotype SLR19 suffered no mortality. Thus, the three biotypes VLR4, SLR19 and WLR1 have developed chlorsulfuron resistance following chlorsulfuron treatment.

The previously tested biotypes VLR1, SLR31 and WLR1 exhibited similar response to chlorsulfuron as was observed in previous experiments (Figure 12.1, Chapter 3). The response of biotype VLR4 to chlorsulfuron was most similar to that of biotype SLR31. The responses of biotypes NLR12 and SLR19 were more similar to WLR1. The biotypes may be ranked from most sensitive to most resistant in the order,

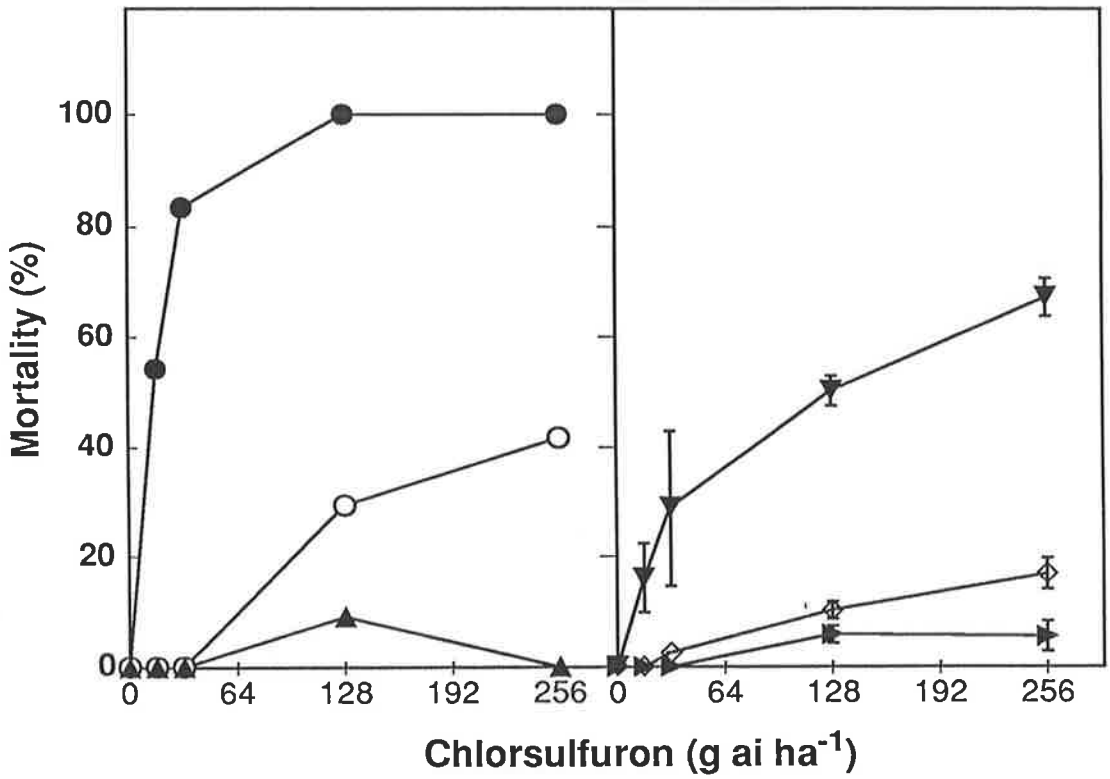


Figure 12.1 Mortality of, (a) *L.rigidum* biotypes VLR1 (●), SLR31(○) and WLR1 (▲), and (b), *L.rigidum* biotypes NLR12(◇), VLR4 (▼) and SLR19 (►), cultured in pots and treated with the crop-selective sulfonylurea herbicide, chlorsulfuron. Application rates of 8 to 16 g ai ha^{-1} are recommended for ryegrass control in Australia. Data for biotypes NLR12, VLR4 and SLR19 are the means of three replicate experiments. Vertical bars represent SE. Data for VLR1, SLR31 and WLR1 represent one replicate only, without error bars.

VLR1 > VLR4=SLR31 > WLR1=SLR19=NLR12. It should be noted that the degree of chlorsulfuron resistance in cross-resistant biotype NLR12, which has not been exposed to ALS inhibitors (Table 12.1), is similar to that of the most resistant biotypes SLR19 and WLR1, which have been treated with chlorsulfuron.

Imazathapyr

Response to imazathapyr varied greatly between biotypes, but the degree of resistance for each biotype did not correlate with the level of resistance to chlorsulfuron (Figures 12.2 & 12.3). The two cross-resistant biotypes, SLR31 and NLR12, were the most resistant to imazathapyr having LD_{50} values of 530 and >600 g ai ha^{-1} respectively (Figure 12.2). The chlorsulfuron selected biotypes SLR19, VLR4 and WLR1 had LD_{50} values of 120, 170 and 370 g ai ha^{-1} respectively (Figure 12.2). There seems to be little correlation between the number of chlorsulfuron treatments to which a biotype has been exposed and degree of imazathapyr resistance.

The susceptible biotype VLR1 has an LD_{50} of 50 g ai ha^{-1} (Figure 12.2) This indicates that use of imazathapyr at normal application rates (75 to 100 g ai ha^{-1}) will provide significant selection pressure toward the development of resistance in *L.rigidum* populations.

Sulfometuron-methyl

In order to detect small numbers of resistant individuals in some populations, resistance to sulfometuron-methyl was tested at a single dose using 108 plants for each replicate. The application rate was 16 g ai ha^{-1}, which is known to kill all plants from susceptible biotype VLR1 (Figure 12.3). The number of survivors in the other populations differed markedly. Biotypes which had been treated with chlorsulfuron at least five times had a large proportion of individuals which survived sulfometuron-methyl treatment. The proportion of individuals which survived sulfometuron-methyl in population SLR19, which had been previously exposed to 5 chlorsulfuron applications (Table 12.1), was 88.2 ± 0.9 % (Figure 12.3). In population WLR1, which had been previously exposed to 7 chlorsulfuron

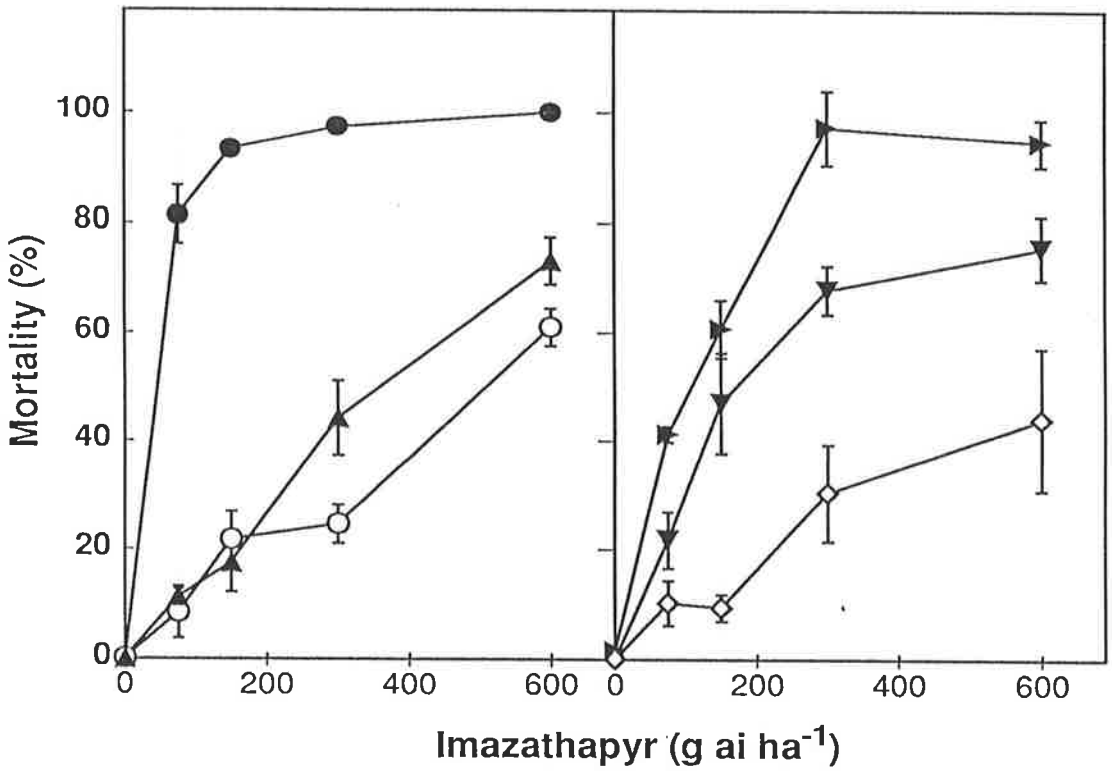
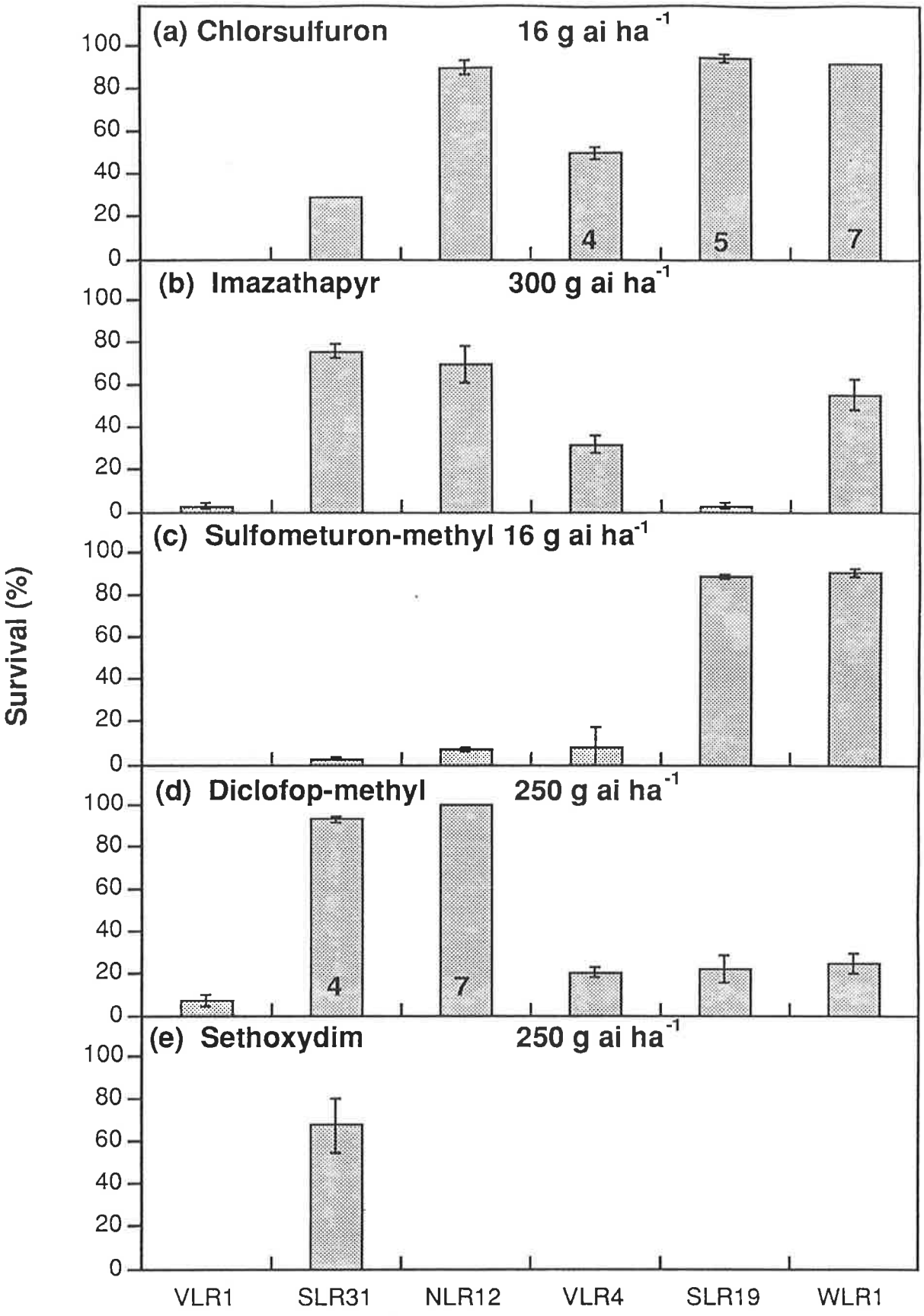


Figure 12.2 Mortality of, (a) *L.rigidum* biotypes VLR1 (●), SLR31(○) and WLR1 (▲), and (b), *L.rigidum* biotypes NLR12(◇), VLR4 (▼) and SLR19 (►), cultured in pots and treated with the crop-selective imidazolinone herbicide, imazathapyr. This herbicide is recommended for control of broadleaf weeds in peas at application rates from 75 to 100 g ai ha $^{-1}$ but is not used for *L.rigidum* control. Data are the means of three replicate experiments for all biotypes. Vertical bars represent SE.

Figure 12.3 Survival of five *L.rigidum* biotypes cultured in pots and treated with (a) chlorsulfuron 128 g ai ha^{-1}, (b) imazathapyr 300 g ai ha^{-1}, (c) sulfometuron-methyl 16 g ai ha^{-1}, (d) diclofop-methyl 250 g ai ha^{-1} and (e) sethoxydim 250 g ai ha^{-1}. Application rates of, 8 to 16 g ai ha^{-1} chlorsulfuron, 365 to 560 g ai ha^{-1} diclofop-methyl and 186 g ai ha^{-1} sethoxydim are recommended for *L.rigidum* control. Imazathapyr is not recommended for *L.rigidum* control but is used for broadleaf weed control in peas at 75 to 100 g ai ha^{-1}. Sulfometuron is used for non-selective weed control at rates above 70 g ai ha^{-1}. Data for biotypes VLR1, SLR31 and WLR1 treated with chlorsulfuron represent one replicate only, without error bars. Data for all other treatments are the means of three replicate experiments. Vertical bars represent SE. Data for chlorsulfuron and imazathapyr are from the same experiments represented in Figures 12.1 and 12.2.


(a) Chlorsulfuron 16 g ai ha-1
(b) Imazathapyr 300 g ai ha-1
(c) Sulfometuron-methyl 16 g ai ha-1
(d) Diclofop-methyl 250 g ai ha-1
(e) Sethoxydim 250 g ai ha-1
Survival (%)
0
20
40
60
80
100
4
5
7
VLR1
SLR31
NLR12
VLR4
SLR19
WLR1

applications (Table 12.1), the proportion of individuals surviving sulfometuron-methyl was 90.6 ± 2.0 % (Figure 12.3). There were less sulfometuron-methyl survivors in the cross-resistant biotypes, which had not been exposed to herbicides that inhibit ALS. Populations SLR31 and NLR12 had 2.8 ± 0.5 % and 7.1 ± 3.3% survival respectively, which are significantly greater than zero ($P<0.05$). This suggests that these populations, which have not been exposed to ALS inhibitors, may contain a small number of individuals with herbicide resistant ALS. Biotype VLR4 had 8.6 ± 8.6 % survivors (Figure 12.3). Despite the small numbers of sulfometuron-methyl survivors in some populations which have not been exposed ALS inhibitors, the data suggest that these populations are less likely to have herbicide insensitive ALS.

Aryloxyphenoxypropionate and cyclohexanedione herbicides.

The results of treatment with 250 g ai ha^{-1} diclofop-methyl are given in Figure 12.3. This application rate is slightly below the 375 to 560 g ai ha^{-1} recommended for *L.rigidum* control. However, all individuals of the susceptible population VLR1 were killed by the treatment (Figure 12.3). Only biotypes SLR31 and NLR12, which had been previously exposed to diclofop-methyl (Table 12.1), exhibit a high proportion of individuals which survived diclofop-methyl treatment (Figure 12.3). Only SLR31 has individuals which survive treatment with the cyclohexanedione herbicide sethoxydim (Figure 12.3). The proportion of individuals from populations which had not been exposed to aryloxphenoxyalkanoate herbicides, was much less than that of populations SLR31 and NLR12 (Table 12.1, Figure 12.3). It should be noted, however, that 22.1 ± 6.5 % and 25 ± 4.8 % of individuals from biotypes SLR19, and WLR1 respectively survived treatment with 250 g ai ha^{-1} diclofop-methyl. Although these small numbers of survivors at a low application rate of diclofop-methyl do not suggest that the populations are resistant, the numbers are significantly different from zero ($P<0.05$). This may suggest that treatment with chlorsulfuron can lead to

changes in diclofop-methyl sensitivity. However, there is considerable variation in herbicide sensitivity in untreated populations of *L.rigidum* (Matthews *et al.* 1992) and population SLR19 has been previously exposed to herbicides from other chemical groups which may have influenced its resistance status (Table 12.1). Controlled selection experiments, of the type described in Chapter 9, would be needed to test whether selection with chlorsulfuron can cause a large enough change in diclofop-methyl sensitivity to lead to diclofop-methyl resistance in populations of *L.rigidum*..

12.4 Discussion

Examination of these seven biotypes, demonstrates the variation in herbicide treatment patterns which may lead to resistance to ALS inhibiting herbicides in *L.rigidum* populations. The response to herbicides of these *L.rigidum* biotypes suggests that populations which are exposed to four treatments of either chlorsulfuron or of diclofop-methyl may develop resistance to chlorsulfuron. Populations exposed to at least five treatments of chlorsulfuron may also have a large proportion of individuals which survive sulfometuron-methyl treatment, suggesting that a large proportion of the population may have herbicide-insensitive ALS. The response to herbicides of biotypes SLR31 and NLR12 suggest that treatment with diclofop-methyl without exposure to herbicides which inhibit ALS, is less likely to select for resistance to sulfometuron-methyl. This suggests that mechanisms of chlorsulfuron resistance other than herbicide-insensitive ALS may be preferentially selected by diclofop-methyl treatment. Such mechanisms are most likely to involve increased metabolism as observed in biotype SLR31. None of the populations exposed to herbicides which inhibit ALS, but not exposed to aryloxyphenoxyalkanoate herbicides, exhibit resistance to diclofop-methyl. This suggests that treatment with herbicides which inhibit ALS may be less likely cause herbicide cross-resistance in *L.rigidum* than treatment with diclofop-methyl (discussed further in Chapter 13).

The studies using imazathapyr demonstrate that susceptible *L.rigidum* populations treated during the grain legume phase of a crop rotation will suffer considerable mortality. This would tend to hasten the development of resistance to herbicide which inhibit ALS in *L.rigidum* populations, especially those exposed to selective sulfonylurea herbicides in the cereal phase. Chlorsulfuron cross-resistant biotype SLR31, has chlorsulfuron-sensitive ALS but can rapidly metabolise the herbicide (Chapters 4 and 5). SLR31 also has imazathapyr-sensitive ALS (Table 5.1) but is resistant to the herbicide (Figure 12.2). This suggests that rapid metabolism may also be important for imazathapyr resistance in *L.rigidum*. However, measurement of the rate of metabolism of imazathapyr would be needed to confirm this.

Chapter 13

Conclusions and further research needs

13.1 Conclusions

The main aim of this project was to determine the mechanism, or mechanisms, by which *L.rigidum* can become resistant to herbicides which inhibit ALS. Wherever the mechanisms of resistance to herbicides which inhibit ALS has been reported in weed species other than *L.rigidum*, resistance has been caused by herbicide-insensitive mutants of the target enzyme (Saari *et al.* 1990, and 1992). However, the results of this study and those of Cotterman and Saari (1992) demonstrate that resistance to ALS inhibiting herbicides in *L.rigidum* is more complicated.

Studies with two *L.rigidum* biotypes, which had been exposed to different herbicide treatment in the field, led to the discovery that at least two mechanisms are involved in resistance to herbicides which inhibit ALS in populations of *L.rigidum*. Biotype SLR31 was resistant to aryloxyphenoxypropionate herbicides, and some herbicides from other chemical groups, following exposure to diclofop-methyl in the field, but had not been exposed to herbicides which inhibit ALS (Heap and Knight 1986). This biotype was cross-resistant to crop-selective sulfonylurea and imidazolinone herbicides but not to non-selective herbicides in these groups (Chapter 3). ALS from this biotype remained susceptible to inhibition by herbicides (Matthews et al. 1990, Table 5.1). These characteristics of biotype SLR31 are similar to wheat, which also has herbicide-sensitive ALS but has the ability to rapidly metabolise certain herbicides (Sweetser *et al.* 1982). Studies using [^{14}C]chlorsulfuron revealed that excised seedlings of biotype

SLR31 have the ability to metabolise chlorsulfuron at approximately twice the rate of seedlings from a susceptible biotype in the tissue closest to the meristem (Chapter 4).

A second *L.rigidum* biotype, WLR1, was found to be chlorsulfuron resistant following chlorsulfuron treatment in the field (Chapter 3). This was the first report of herbicide resistant *L.rigidum* selected by sulfonylurea herbicide treatment. The biotype was resistant to both crop-selective and non-selective sulfonylurea and imidazolinone herbicides but was not resistant to herbicides from other chemical groups with different modes of action (Chapter 3). This result suggested that resistance in biotype WLR1 was due to a herbicide-insensitive mutation of the target enzyme, ALS. ALS extracted from biotype WLR1 was less-sensitive to inhibition by both crop-selective and non-selective sulfonylurea and imidazolinone herbicides (Chapter 5). This biotype also had the ability to metabolise [^{14}C]chlorsulfuron more rapidly that a susceptible biotype (Chapter 4).

Studies of these two different biotypes indicated that herbicide-insensitive ALS may cause resistance in *L.rigidum* biotypes but there is a second mechanism of resistance in this species which involves an increased ability to metabolise chlorsulfuron. This second mechanism does not cause resistance to the non-selective sulfonylurea and imidazolinone herbicides sulfometuron-methyl or imazapyr.

Studies of intact plants given [14C]chlorsulfuron at application rates which caused differential growth response between resistant and susceptible biotypes, revealed no differences in uptake via the roots or leaf lamina which might explain resistance (Chapter 6). The total amount of herbicide found in the tissue of these intact plants was less than that in excised seedlings in which the increased chlorsulfuron metabolism of resistant biotypes had been observed (Table 6.2). This suggests that the differential rate of metabolism observed in excised seedlings also occurs in intact plants treated with application rates of chlorsulfuron which cause

differential growth response between resistant and susceptible biotypes. Cotterman and Saari, 1992, also report no significant differences in leaf or root uptake of [14C]chlorsulfuron when applied to larger *L.rigidum* plants at much higher concentrations

During HPLC analysis of [^{14}C]chlorsulfuron and its metabolites the major metabolite produced in *L.rigidum* coeluted with that of wheat (Figure 4.2). Cotterman and Saari, 1992, report that this metabolite can be hydrolysed to produce a compound which coelutes with hydroxy chlorsulfuron, providing further evidence that the major pathway of chlorsulfuron metabolism in *L.rigidum* may be the same as that in wheat. HPLC elution profiles are similar for all resistant and susceptible *L.rigidum* biotypes tested suggesting that the pathway of metabolism is the same in all biotypes (Figure 4.2 and 4.6). Only the rate of metabolism appears to differ between biotypes (Chapter 4).

Studies with several mixed function oxidase inhibitors revealed that some of these compounds can cause a slight increase in phytotoxicity of chlorsulfuron toward biotype SLR31 (Chapter 7). However, these compounds do not increase the phytotoxicity of chlorsulfuron toward biotype SLR31 to levels comparable with those suffered by a susceptible biotype (Chapter 7). MFO inhibitors can reduce the rate of chlorotoluron metabolism in chlorotoluron resistant biotype WLR2, causing the phytotoxic effects on WLR2 to approach those of chlorotoluron alone applied to a susceptible biotype (Burnet *et al.* 1993c). These same inhibitors cause a much smaller increase in chlorsulfuron phytotoxicity in SLR31 (Chapter 7). Similarly, the herbicide safener NAA can reduce the phytotoxic effect of chlorsulfuron toward wheat but does not do this for *L.rigidum* biotype SLR31 (Figure 7.5). These results suggest that MFO's may not be involved in detoxification of chlorsulfuron in *L.rigidum* and/or that metabolism of chlorsulfuron is not the only mechanism of resistance in biotype SLR31.

Experiments with [^{14}C]chlorsulfuron using excised seedlings revealed that periods of darkness, after the commencement of herbicide

treatment, caused chlorsulfuron metabolism to slow dramatically or cease (Figure 8.1). Whole plant studies revealed that placing seedlings in the dark immediately following herbicide treatment increased the phytotoxic effect of chlorsulfuron toward biotype SLR31 (Chapter 8). This increase was not detected in biotype VLR1 with a slower rate of metabolism than SLR31, nor in biotype WLR1, for which herbicide-insensitive ALS is a more important determinant of resistance than increased metabolism. This suggests that the increase in phytotoxicity in biotype SLR31 is due to an effect of darkness upon chlorsulfuron metabolism. As with the effect of MFO inhibitors, however, the increased phytotoxicity in biotype SLR31 does not approach the levels suffered by the a susceptible biotype. Again suggesting that increased metabolism may not be the only mechanism of resistance in biotype SLR31. Further studies of chlorsulfuron metabolism in SLR31 plants treated with MFO inhibitors or treated immediately prior to a dark period are required to determine whether these treatments can halt metabolism in intact plants.

Studies of the inheritance of chlorsulfuron and diclofop-methyl resistance in biotype SLR31 reveal that both are nuclear encoded dominant or semi dominant characters (Chapter 10). There is at least one gene controlling diclofop-methyl resistance in the absence of chlorsulfuron resistance occurring in population SLR31 (Chapter 10). The number of genes controlling resistance in individuals with both chlorsulfuron and diclofop-methyl resistance is not known.

Studies of inheritance of chlorsulfuron and sulfometuron-methyl resistance in biotype WLR1 reveal that both characters are nuclear encoded and both are, probably, dominant (Table 11.1). At least one gene controlling chlorsulfuron resistance in the absence of sulfometuron-methyl resistance occurs in population WLR1 (Table 11.4 and 11.5). As biotype WLR1 has the ability to metabolise chlorsulfuron more rapidly than a sucseptible biotype it appears likely that this gene may control increased metabolism. Biotype WLR1 displays little increase in diclofop-methyl resistance compared to a susceptible biotype (Chapter 3, Chapter 12). Therefore, this finding suggests

that the mechanism causing increased metabolism of chlorsulfuron in some *L.rigidum* biotypes may not be the cause of chlorsulfuron cross-resistance following diclofop-methyl treatment.

Studies with seven biotypes reveal that different herbicide treatments in the field may lead to resistance to ALS inhibitors in populations of *L.rigidum* (Chapter 12). The proportion of individuals which survive sulfometuron-methyl and chlorsulfuron treatment in such populations may vary greatly. Selection with the aryloxyphenoxypropionate herbicide, diclofop-methyl, can lead to cross-resistance to ALS inhibitors in as little as four applications but appears less likely to select for sulfometuron-methyl resistance (Chapter 12). This suggests that diclofop-methyl treatment is unlikely to select for herbicide-insensitive ALS. However, selection by diclofop-methyl did not lead to chlorsulfuron cross-resistance in biotype VLR6 (Chapter 9). This suggests that some *L.rigidum* populations may lack the ability to develop chlorsulfuron cross-resistance following diclofop-methyl treatment.

Selection of *L.rigidum* populations with chlorsulfuron may cause chlorsulfuron resistance in as little as three applications (Chapter 12). In populations with exposure to over five applications of chlorsulfuron, a large proportion of individuals may survive sulfometuron-methyl treatment suggesting that many individuals have herbicide-insensitive ALS (Chapter 12). Selection with chlorsulfuron appears less likely to cause resistance to herbicides of other chemical groups with different modes of action than does selection with diclofop-methyl (Chapter 12).

Studies with the crop-selective imidazolinone herbicide imazathapyr confirm that the use of this herbicide in the legume phase of a crop rotation may increase the rate of onset of resistance to ALS inhibiting herbicides in *L.rigidum* populations (Chapter 12).

13.2 Chlorsulfuron, diclofop-methyl cross resistance in *L.rigidum*

Previous studies have shown that treatment of *L.rigidum* populations with diclofop-methyl leading to diclofop-methyl resistance leads to chlorsulfuron cross-resistance in some populations (Heap and Knight 1990, Girjeet Gill pers comm.). Of nine populations from Western Australia which had been exposed to aryloxyphenoxyalkanoate herbicides but had not been exposed to sulfonylureas, four were found to be partially resistant to triasulfuron (Gurjeet Gill pers. comm). However, evidence from population VLR6 demonstrates that exposure to diclofop-methyl does not necessarily lead to chlorsulfuron cross-resistance in all populations (Chapter 9).

Resistance to both chlorsulfuron (this study) and diclofop-methyl (Tardif *et al.* 1993, Holtum *et al.* 1991) are known to involve more than one mechanism. The variation in the development of chlorsulfuron-resistance in different populations treated with diclofop-methyl could be explained if there are many mechanisms of resistance to both diclofop-methyl and chlorsulfuron present at varying frequency in the different populations and only one, or a few, of these cause resistance to both herbicides. The higher the proportion of individuals with the common mechanism(s) in any population, the more rapid will be the development of cross-resistance between the two herbicides. Treatment with chlorsulfuron would also select for such common mechanisms. However, there are no confirmed cases of this occurring. It is possible that the chlorsulfuron-treated, resistant populations in this study have a low initial frequency of individuals carrying such mechanisms. The small increase in survivors of low rates of diclofop-methyl application in populations SLR19 and WLR1 suggests that chlorsulfuron treatment may lead to changes in diclofop-methyl sensitivity (Chapter 12). Common mechanism(s) of diclofop-methyl and chlorsulfuron resistance may provide a different degree of protection against different herbicides.

The nature of a common mechanism of resistance to both chlorsulfuron and diclofop-methyl remains to be elucidated. Clearly,

mutations in either of the target enzymes, ACCase or ALS, are unlikely to cause cross-resistance. This is supported by the observation that population WLR1, which contains a large proportion of individuals with herbicide insensitive ALS, is not cross-resisitant to diclofop-methyl. Similarly, population SLR3, which contains a large proportion of individuals with herbicide insensitive ACCase, does not exhibit high levels of cross resistance to chlorsulfuron (Tardif*et al.* 1993).

A detoxifying enzyme system with affinity for both herbicides could be the cause of cross-resistance. If the degrading enzyme system had greater affinity for chlorsulfuron than for diclofop-methyl, treatment with diclofop-methyl leading to a 15% increase in the level of diclofop-methyl metabolism (Holtum *et al.* 1991) could lead to an almost 100% increase in the rate of chlorsulfuron metabolism, as observed in biotype SLR31 (Chapter 4). This type of situation has been observed in cases of insecticide resistance caused by mixed function oxidase enzymes (Brattsten *et al.* 1986) but evidence for the involvement of mixed function oxidase enzymes systems in chlorsulfuron or diclofop-methyl metabolism in *L.rigidum* is, so far, lacking (Chapter 7).

Change in the regulation of expression of detoxification mechanisms could also explain cross-resistance. A regulatory mechanism responding to a particular herbicide molecule, or simply responding to the stress imposed by herbicide treatment, might stimulate a greater level of expression of detoxification enzymes in resistant plants. Many enzymes varying in substrate specificity might be stimulated, the suite of enzymes varying between individuals. This model would not require that any single detoxification enzyme system have affinity for both herbicide molecules.

A membrane receptor protein able to influence the rate of influx or efflux of the herbicide would be another possible mechanism of herbicide cross-resistance. Differential uptake of sulfonylureas in selected yeast lines is known (Falco and Dumas 1985) as are differential effects of diclofop on the membrane potential of resistant, versus susceptible, *L.rigidum* biotypes

(Hausler *et al.* 1991, Holtum *et al.* 1991). Such a mechanism could lead to sequestration of herbicide away from the target enzyme.

Cross-resistance could also occur if there are separate mechanisms of resistance to chlorsulfuron and diclofop-methyl even if they were controlled by separated genes, if the genes controlling each mechanism were linked. It would then be possible that in some populations the rare individuals carrying a resistant allele at the diclofop-methyl resistance locus may also carry a resistance allele at the linked chlorsulfuron locus (eg. the ancestors of population SLR31) but in other populations the individuals having diclofop-methyl resistance may have a susceptible allele at the chlorsulfuron locus (eg. the ancestors of population VLR6).

13.3 Future research

(1) The catalytic system for chlorsulfuron metabolism in *L.rigidum*

The nature of the catalytic system causing chlorsulfuron metabolism in *L.rigidum* remains to be elucidated. The data suggest that MFO's may not be responsible for catalysis of chlorsulfuron metabolism in *L.rigidum.* Chlorsulfuron metabolism in *L.rigidum* may be catalysed by a different type of enzymic oxidase such as an FAD dependent or Cu^{++} coordinated oxidase (Mayer and Cordes 1971). Alternatively, chlorsulfuron metabolism in *L.rigidum* may be catalysed by hydroxamic acids (Niemeyer 1988). Further studies are required to characterise the catalytic system. Such understanding may lead to options for improving the efficiency of chlorsulfuron for *L.rigidum* control (and possibly other crop-selective herbicides). As metabolism appears to be reduced in darkness, studies of the catalytic system should start with green, rather than etiolated, material.

(2) Genetic control of resistance traits in *L.rigidum.*

It is clear that one of the major factors confounding research into herbicide resistance in *L.rigidum* , has been the use of populations of

unknown genotype in studies to date. The number of different genes controlling the known mechanisms of resistance yet to be determined and the existence of other mechanisms of resistance is suggested by the present data.

Biotype SLR31 has been shown to contain individuals with many different modes of resistance to a range of herbicides (Tardif *et al.* 1993, Holtum *et al.* 1990). This biotype could be used to identify a number of the genes involved in herbicide resistance in *L.rigidum* including some for resistance to ALS inhibitors. This population may be used to identify the genes controlling chlorsulfuron cross-resistance following exposure to diclofop-methyl.

Although very labour intensive, it appears that to determine the number of genes for herbicide resistance in biotype SLR31 it will be necessary to start with a large number of individual crosses between SLR31 individuals and a susceptible biotype. This would be followed by screening for resistance to a wide range of herbicides in each family. In the following year backcrosses to susceptible plants and F2 crosses between certain F1 plants could be used to further characterise the genotype of each family. Families with only a single mechanism of resistance may be identified in the backcross progeny, or further backcrosses may be needed. Only by identifying individuals which have a single mechanism of resistance can the number of genes involved be determined. The relationship between each gene and resistance to a variety of herbicides can then be studied.

Identification of individual families with only one mechanism of resistance will help to answer many questions about herbicide resistance. For example, further mechanisms of resistance to ALS inhibitors may be identified.

Once the classical genetics of herbicide resistance are understood, it will possible to investigate other genetic factors which may be involved. The wide range of genetic variability within the species *L.rigidum* suggests that mechanisms for generating genetic variability may exist within

the population. It is possible, for example, that transposons may be present in some families causing higher than normal rates of recombination and mutation. Such fundamental questions remain to be investigated.

(3) Other mechanisms of resistance to ALS inhibitors

Although two mechanisms of resistance to herbicides which inhibit ALS have been demonstrated in *L.rigidum* (Chapters 4 and 5), several some evidence suggests that these may not be the only mechanisms involved. Treatment with chlorsulfuron immediately prior to a dark period caused an increase in the phytotoxic effect of chlorsulfuron toward biotype SLR31 but the level of phytotoxicity was still less than that of chlorsulfuron applied to a susceptible biotype (Chapter 7 and 8). Preliminary evidence suggests that placing excised *L.rigidum* seedlings in the dark can dramatically reduce or stop chlorsulfuron metabolism (Figure 8.1). Further research is required to determine if this treatment can reduce the level of chlorsulfuron metabolism in biotype SLR31 to that of a susceptible biotype. If the rate of chlorsulfuron metabolism can be be reduced without rendering SLR31 plants susceptible, this would provide evidence that increased metabolism is not the only mechanism of chlorsulfuron resistance in this biotype.

It is also possible that an increased rate of chlorsulfuron metabolism can be observed in *L.rigidum* plants which have any of a number of chlorsulfuron resistance mechanisms. The increased rate of metabolism being due to the healthier physiological state of the resistant plant, versus a dying susceptible plant (Chapter 11). A method of inhibiting chlorsulfuron metabolism would be a valuable aid in experiments to clarify this point.

(4) Acetolactate synthase

One of the major findings of this work has been that herbicide-resistant mutations of ALS can cause resistance in *L.rigidum*, as they do in several other weed biotypes and in other groups of organisms (reviewed in

1.3). Specific polymer chain reaction techniques may now be used to rapidly determine the sequence of portions of the DNA sequence for acetolatcte synthase in plants (Guttieri *et al.* 1992). Thus, the DNA sequences from individuals of susceptible and resistant populations can be compared with known sequences from resistant and susceptible plants. This will assist in determining the number of separate mutations of ALS which occur in resistant *L.rigidum* populations. The number of different sequences occurring in an individual plant may also be determined. This will allow testing of the hypothesis that most individuals from the WLR1 population are heterozygous (Chapter 11).

(5) Studies of the pattern of occurrence of resistance to ALS inhibitors.

Investigation of a small number of *L.rigidum* populations indicates that resistance to ALS inhibitors can occur following widely different herbicide treatment in the field (Chapter 12). The data suggest that treatment with an ALS inhibitor is less likely to cause resistance to chemically different herbicides with different modes of application than treatment with the aryloxyphenoxypropionate herbicide diclofop-methyl. If true, this would be important for resistance management. It may be that using ALS inhibitors on *L.rigidum* populations before they are exposed to diclofop-methyl may delay the development of cross-resistance between aryloxyphenoxypropionate herbicides and ALS inhibitors. It must be noted, however, that the earlier commercial release of diclofop-methyl means that most *L.rigidum* populations have been exposed to more treatments of diclofop-methyl than of herbicides which inhibit ALS. This may be the cause for the apparent lack of populations exhibiting cross-resistance following treatment with ALS inhibitors. Further studies are required comparing the herbicide history of populations with the level of resistance to ALS inhibitors and the level of cross-resistance between ALS inhibitors and other herbicides.

As with many scientific investigation, therefore, this study has answered some questions but raised many new ones. It is clear from this work, however, that resistance to ALS inhibitors in *L.rigidum* is a complicated phenomenon involving more than one mechanism. Understanding of these mechanisms will be essential for the development of management strategies for this species which minimise the problem of herbicide resistance.

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