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Resistance to acetolactate synthase-inhibiting herbicides in *Sonchus oleraceus*, *Sisymbrium orientale* and *Brassica tournefortii*

Peter Boutsalis

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ABSTRACT

The development of resistance in dicot weed species to herbicides which inhibit acetolactate synthase (ALS), a key enzyme in branched-chain amino acid synthesis, is a serious problem in North American agriculture. In Australia biotypes from three weed species (*Sonchus oleraceus*, *Sisymbrium orientale* and *Brassica tournefortii*) have been confirmed resistant to three classes of herbicides which inhibit ALS (sulfonylurea, imidazolinone and triazolopyrimidine herbicides) but do not exhibit cross-resistance to herbicides with other modes of action.

Genetic crosses were made between susceptible and resistant biotypes of *Son. oleraceus* and *Sis. orientale*. F_2 and F_3 *Son. oleraceus* and F_2 *Sis. orientale* segregated when treated with 22.5 and 15 g ha^{-1} chlorsulfuron, respectively. ALS herbicide resistance was inherited as a single nuclear gene in all resistant biotypes of these species.

Resistance to ALS-inhibiting herbicides was due to the presence of a resistant form of ALS in resistant *Son. oleraceus*, *Sis. orientale* and *B. tournefortii* biotypes. ALS from the resistant *Son. oleraceus* biotype assayed *in vitro* was highly resistant to sulfonylurea herbicides and only slight resistant to imidazolinone and triazolopyrimidine herbicides. *Sis. orientale* ALS from three resistant biotypes was highly resistant to sulfonylurea, imidazolinone and triazolopyrimidine herbicides. In contrast, ALS from a resistant *B. tournefortii* biotype was highly resistant to sulfonylurea, slightly resistant to triazolopyrimidine and not resistant to imidazolinone herbicides. These different herbicide inhibition patterns for ALS from the various resistant biotypes prompted an investigation into the molecular basis for ALS herbicide resistance. Genomic DNA corresponding to four highly conserved regions of ALS, known as Domains A, B, C and D, were amplified from susceptible and resistant biotypes of *Sis. orientale* and *B. tournefortii* and sequenced. Within Domain A, nucleotide substitutions encoding Pro to Ile and Pro to Ala changes were identified for resistant biotypes of *Sis. orientale* and *B. tournefortii*, respectively. Two other resistant *Sis. orientale* biotypes did not possess a mutation at this position, but had a single

nucleotide substitution in Domain B encoding a Leu in place of a Trp. Substitutions at these two sites in the ALS gene are known to confer resistance to ALS-inhibiting herbicides in higher plants. No other amino acid differences were found between susceptible and resistant biotypes of either species. A second ALS gene, possibly inactive, was also identified in *B. tournefortii*.

An ecological study on *Son. oleraceus* and *Sis. orientale* revealed that seed of *Son. oleraceus* does not possess innate dormancy, whereas that of *Sis. orientale* possesses strong innate dormancy over summer. Buried seeds from both species germinated over 2.5 years suggesting burial enforces dormancy with no differences observed between susceptible and resistant biotypes. Furthermore, the seedbank life of seeds from resistant biotypes of both species is at least three years. These findings indicate that at least three years of seedset prevention of resistant biotypes of *Son. oleraceus* and *Sis. orientale* is required in order to reduce the seedbanks to manageable levels.

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 had been made.

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P. Boutsalis

CHAPTER 1

1.0 LITERATURE REVIEW

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1.1 DEVELOPMENT OF RESISTANCE

Resistance of organisms to pesticides is an example of selection of individuals with a particular trait(s) that helps them survive the selective agent. Pesticide resistance has occurred in insects, pathogens and more recently in weeds as a result of intense selection with insecticides, fungicides and herbicides, respectively. For the purpose of this thesis, herbicide resistance is defined as "the inherited ability of plants to withstand levels of herbicide that normally kill individuals that have not previously been exposed to the particular herbicide" (adapted from Gressel, 1990). Target site cross-resistance, investigated in this document, is defined as the expression of a herbicide resistant target site that endows the ability to withstand herbicides from different chemical classes that have the same mode of action.

1.2 HERBICIDE RESISTANCE IN AUSTRALIA

Herbicide resistance was first identified in Australia, when a biotype of *Lolium rigidum* Gaud. was confirmed resistant to the aryloxyphenoxypropionate (APP) herbicide, diclofop-methyl, near Naracoorte in South Australia (Heap and Knight, 1982). This species is regarded as the most serious herbicide resistance problem in Australia for two reasons. Firstly, *L. rigidum* is ubiquitous across most of the Australian cropping zone. Secondly because several biotypes have been shown to exhibit cross- or multiple-resistance (Heap and Knight, 1986; Matthews et al., 1990; Burnet et al., 1991; Table 1.1). A telephone survey in 1992 suggested that resistant *L. rigidum* was present on more than 2,000 Australian properties covering about 275,000 ha (Anon., 1992). A recent random survey of 200 paddocks in the mid-north of South Australia revealed that 40% of *L. rigidum* samples contained diclofop-methyl resistant individuals (R. Llewellyn, pers. comm.), with the likelihood of some of these samples being cross-resistant to other herbicide classes.

Following the outbreak of herbicide resistant *L. rigidum*, the next weed species to develop herbicide resistance in Australia was *Hordeum glaucum*, with biotypes resistant to the bipyridilium herbicides paraquat and diquat (Powles, 1986). A further three species

(*Hordeum leporinum*, *Arctotheca calendula* and *Vulpia bromoides*) resistant to bipyridilium herbicides have been confirmed, with one paddock containing resistant biotypes of all four species (Tucker and Powles, 1991; Powles et al., 1989; Purba et al., 1993; Table 1.1).

Wild oats (*Avena* spp.) have been described as potentially, the most serious weed of cropping (Holm et al., 1977). Herbicide resistance in wild oats to diclofop-methyl was first documented in 1990 in a population of diclofop-methyl resistant *Avena fatua* on a property near York, Western Australia (Boutsalis et al., 1990; Piper, 1990). Several biotypes of *A. fatua* and *A. sterilis* with varying degrees of resistance to APP and cyclohexanedione (CHD) herbicides have since been identified in South Australia, New South Wales, Victoria and Western Australia (Mansooji et al., 1992; Mansooji, 1993; Maneechote, 1995; G. Gill pers. comm.; Table 1.1). Wild oats collected during a random survey of the mid north of South Australia showed 4% of samples containing diclofop-methyl resistant individuals (B. Nietschke, pers. comm.).

The foregoing demonstrates that in terms of area affected, Australia has one of the most serious herbicide resistance infestations in the world. These herbicide resistance problems have been, in the past, dominated by grass weed species, particularly *L. rigidum*. The sulfonylurea (SU) herbicides, chlorsulfuron and triasulfuron (released in the early 1980's in Australia) provide excellent control of many dicot weeds and *L. rigidum* in cereal crops. Heavy reliance on these herbicide has imposed a strong selection pressure on both *L. rigidum* and dicot weeds. Consequently many *L. rigidum* biotypes resistant to SU herbicides have been identified (Christopher et al., 1992; Gill, 1995; Table 1.1). However, SU herbicides remained effective on dicot weeds until recently. Six species have now been identified by field trials to have resistance to SU herbicides in broadacre cropping systems (Table 1.4). Three of these species have been studied in detail here, *Sonchus oleraceus* L. (*Son. oleraceus*), *Sisymbrium orientale* L. (*Sis. orientale*) and *Brassica tournefortii* Gouan. (*B. tournefortii*).

Table 1.1. Herbicide resistance in Australia

Species	Herbicide group(s)	Reference
A. fatua and *A. sterilis*	APP	Mansooji et al., 1992
A. calendula	Bipyridiliums	Powles et al., 1989
H. glaucum	Bipyridiliums	Powles, 1986
H. leporinum	Bipyridiliums	Tucker and Powles, 1991
L. rigidum	Dinitroalanines	Heap and Knight, 1990 McAlister et al., 1995
	APP	Heap and Knight, 1986
	CHD	Heap and Knight, 1990
	SU	Heap and Knight, 1986 Christopher et al., 1992
	Phenylureas	Burnet et al., 1991
	Triazines	Burnet et al., 1991
	Triazinones	Burnet et al., 1991
	Triazoles	Burnet et al., 1991
V. bromoides	Bipyridiliums	Purba et al., 1993

1.3 WEED SPECIES INVESTIGATED

1.3.1 *Sonchus oleraceus*

Introduction

Son. oleraceus (Plate 1.1) is an annual diploid (2n=32) of the Compositae family (Anon., 1964). It is common in the temperate zones of both hemispheres, but is probably native to Europe and south-western Asia. It often occurs in disturbed sites and is a weed of arable crops and pastures (Jessop, 1986a).

Plate 1.1. *Sonchus oleraceus*

Son. oleraceus in Australia

In Australia, the common names for *Son. oleraceus* are common sowthistle and milkthistle. *Son. oleraceus* is not regarded as a serious agricultural weed, but occasionally causes problems in the summer rainfall cropping areas of southern Queensland and northern New South Wales, particularly in the summer fallow phase (S. Walker, pers. comm.). The germination pattern in this region begins from spring through to autumn, with a relatively dormant over-wintering period (Hutchinson et al., 1984).

Son. oleraceus forms a large taproot, and if uncontrolled can compete with crops for water and nutrients. In high densities it is a problem over the summer fallow period (the principle rainfall period for this region) because it depletes soil moisture intended for the following winter crop phase (S. Walker, pers. comm.).

Warm conditions ensure rapid achene development such that flowering to mature achene development can take as little as one week, with an average 6100 achenes per plant (Hutchinson et al., 1984). The pappus-borne achenes are liberated by wind and if favourable growing conditions follow, massive germination can occur in the next generation (Hutchinson et al., 1984). These features make *Son. oleraceus* a weed suited to summer rainfall areas. However, in southern Australia, with predominantly winter rainfall *Son. oleraceus* is not generally regarded as a serious weed problem.

Herbicide control

Many herbicide options are available for selective control of *Son. oleraceus* including photosystem II herbicides such as metribuzin and auxin analog herbicides such as MCPA and 2,4-D (Hutchinson et al., 1984). Non-selective herbicides including glyphosate and diquat offer excellent control for chemical fallow situations. The release of acetolactate synthase-inhibiting herbicides (hereafter referred to as ALS herbicides) which include the SU, has extended the chemical control options. Their high efficacy and broad target-weed spectrum has resulted in a heavy reliance upon them, particularly in minimum tillage systems, imposing strong selection pressure on weed populations.

1.3.2 *Sisymbrium orientale* and *Brassica tournefortii*

Introduction

Sis. orientale (Plate 1.2) and *B. tournefortii* (Plate 1.3) are diploid species (2n=14, 2n=20, respectively) belonging to the Brassicaceae family (Anon., 1976). Both species are native to Europe and Asia with *Sis. orientale* also native to North Africa. Like *Son. oleraceus*, these two species are also weeds of disturbed sites in high rainfall regions, but differ from the former in that their distribution extends into semi-arid regions (Jessop, 1986b).

Sis. orientale and *B. tournefortii* in Australia

In Australia, *Sis. orientale* and *B. tournefortii* infest not only the southern Queensland and northern New South Wales cropping zone but also the southern Australian agricultural belt (Plate 1.4). They are more vigorous competitors for light, water and nutrients than *Son. oleraceus*. Their normal growing pattern begins in autumn competing with winter growing crops. Anthesis for *B. tournefortii* occurs in July to October, earlier than the September to December pattern of *Sis. orientale*. Seeds are borne in siliqua in *Sis. orientale* and pods in *B. tournefortii* that shatter easily after maturity to replenish the seedbank. Innate dormancy in both species prevents seed germination over summer. Cooler autumn weather promotes germination of a large proportion of the previous year's seed (Wilson, 1985). However, both species have enforced dormancy that prevents a proportion of seeds from germinating in their first year in the seedbank.

Herbicide control

Generally, herbicides that control *Son. oleraceus* also control the two cruciferous weed species, such as photosystem II, auxin analog and ALS herbicides (Table 1.2). Furthermore, *Sis. orientale* and *B. tournefortii* are generally more sensitive to ALS herbicides than *Son. oleraceus* and numerous ALS herbicides are registered for their control (Table 1.2). Consequently, there is generally heavy reliance on ALS herbicides for weed control in agriculture imposing heavy selection for herbicide resistant individuals (Table 1.2).

Plate 1.2. *Sisymbrium orientale.*

Plate 1.3. *Brassica tournefortii.*

Plate 1.4. Australian cropping zone (cereal belt).

Table 1.2. ALS herbicides recommended for weed control of *Son. oleraceus*, *Sis. orientale* and *B. tournefortii* and crop selectivity.

Herbicide	Herbicide tolerant crops	Herbicide control recommendation		
		Sis. orientale	*B. tournefortii*	*Son. oleraceus*
Sulfonylureas				
Chlorsulfuron	Cereals	Yes	Yes	Yes
Sulfometuron	None	Yes	Yes	Yes
Triasulfuron	Wheat	Yes	Yes	Yes
Metsulfuron	Cereals	Yes	Yes	Yes
Imidazolinones				
Imazapyr	None	Yes	No	Yes
Imazethapyr	Beans, Peas	Yes	No	No
Triazolopyrimidines				
Flumetsulam	Wheat, Peas, Pastures	Yes	Yes	No
Metosulam	Cereals, Lupins	Yes	Yes	No

1.4 ALS-INHIBITING HERBICIDES

1.4.1 Chemistry

Introduction

Several classes of compounds are known to inhibit ALS of which four are commercially available, SU (Sauers and Levitt, 1984; Beyer et al., 1988; Blair and Martin, 1988), imidazolinones (IM) (Los, 1986), triazolopyrimidines (TP) (Kleschick et al., 1990; Kleschick et al., 1992) and pyrimidinyloxy-thiobenzoates (POB) (Takahashi et al., 1991) (Fig. 1.1). Sulfonylureas were the first class of ALS inhibitors discovered in the early 1970's (Sauers and Levitt, 1984), with chlorsulfuron the first SU herbicide commercially available (Ray, 1984).

Sulfonylureas

The structure of a typical SU is characterised by a sulfonylurea 'bridge' connecting two ring structures (Fig. 1.1). The ring attached to the sulphur atom has an *ortho* substituent, but otherwise can consist of benzene, pyridine or non-aromatic rings. At the other end of the bridge is a *meta*-substituted pyrimidine or triazine ring (Fig. 1.1).

Imidazolinones

This class of chemistry is characterised by an imidazolinone ring bonded to an aromatic ring at the 2 position (Fig. 1.1). High biological activity is achieved when the imidazolinone ring is substituted with a methyl and isopropyl group (Los, 1986). The aromatic ring contains a carboxylic acid group *ortho* to the imidazolinone ring. Several IM herbicides with differences at the aromatic ring have been developed (Los, 1986).

Triazolopyrimidines

This chemical class was developed from SU through rearrangement of the sulfonylurea bridge. (Kleschick et al., 1990). They differ from SU in that the pyrimidine ring has been

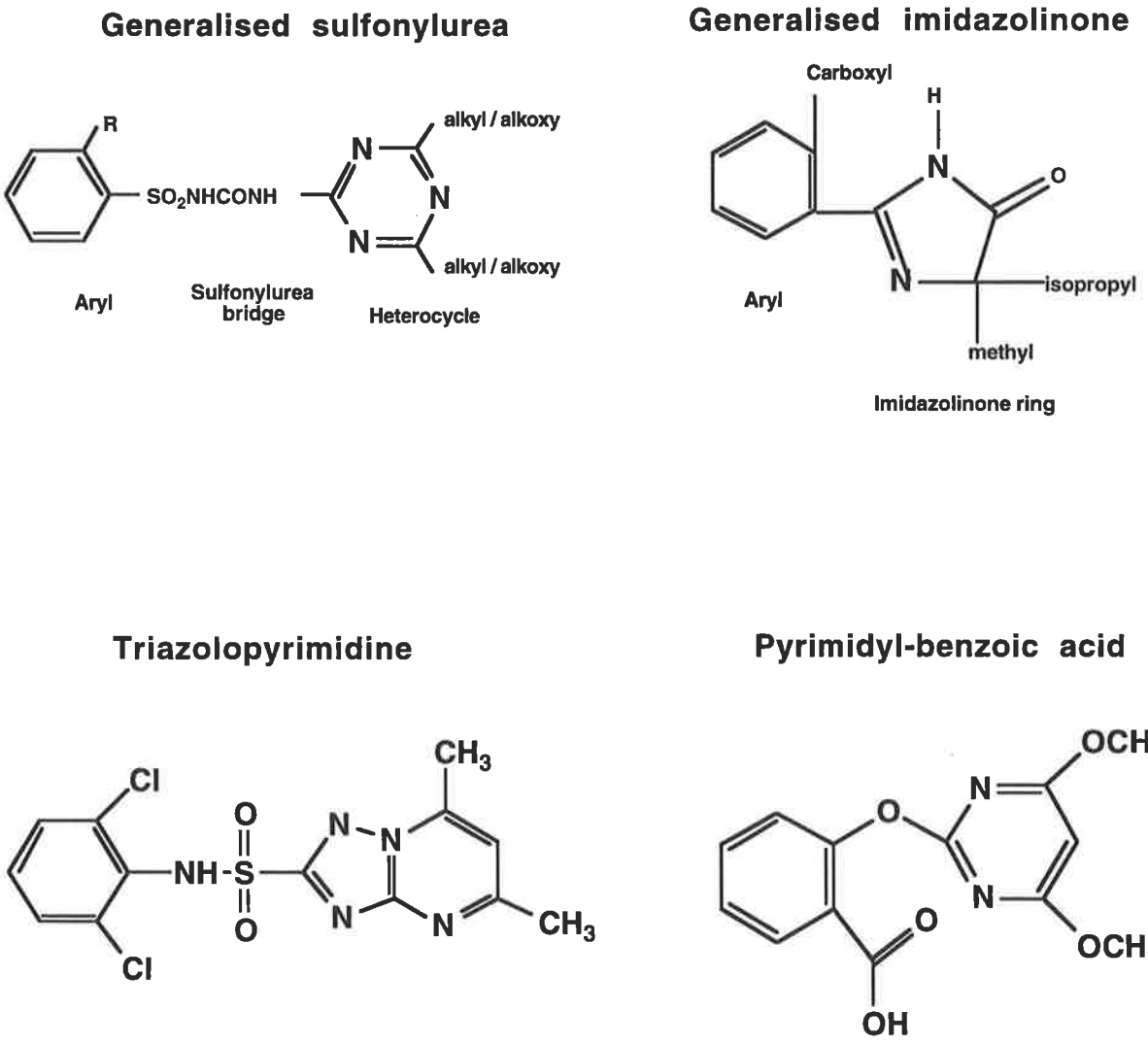


Figure 1.1. Chemical structures of four classes of ALS-inhibiting herbicides.

joined the bridge region of the SU structure, the sulfonyl moiety is moved adjacent to the TP rings, and the benzyl ring has two *ortho* substituents (Fig. 1.1).

Pyrimidyloxy-benzoic acids

This class of herbicides was also developed from the general sulfonylurea structure (Hawkes, 1989). Only the *meta*-substituted pyrimidine ring remains of the general sulfonylurea structure. The sulfonylurea bridge between the aromatic rings has been replaced by an ether linkage, and the second ring has an *ortho* carboxylic acid rather than an ester function (Fig. 1.1).

1.4.2 ALS inhibiting-herbicide usage in Australia and resistance

In Australia, the majority of registered ALS herbicides from the SU, IM and TP classes are for selective weed control in a variety of crops (Table 1.2). Currently many ALS herbicides are available for weed control in monocot and dicot crops, and therefore, there is the potential for application of ALS herbicides at several stages in the cropping rotation (Table 1.3).

To date, ten weed species in Australia have developed resistance through selection with SU herbicides (Table 1.4). In nearly all cases the SU resistant biotypes are also resistant to IM and TP herbicides (Christopher et al., 1992; Boutsalis and Powles, 1995). With the introduction of additional selective ALS herbicides from the IM and TP classes it is inevitable that increased selection pressure will lead to new outbreaks of resistant weeds.

1.4.3 Weeds resistant to ALS herbicides in North America and Europe

In North America, resistance to ALS herbicides was first reported in *Lactuca serriola* after selection with chlorsulfuron in a wheat field near Lewiston, Idaho (Mallory-Smith et al., 1990a). Since the first report, a further nine weed species have been confirmed resistant to ALS herbicides. In all cases but one (*Xanthium strumarium* selected with imazaquin,

Schmitzer et al., 1993), resistance has occurred through selection with SU herbicides (Table 1.5; reviewed in Saari et al., 1994). Furthermore, the mechanism of resistance in all biotypes tested has revealed a resistant ALS target site.

Table 1.3. ALS herbicides available in Australia

Chemical Class	Chemical name	Trade name	Distributor
Sulfonylureas	Bensulfuron-methyl	Londax	DuPont
	Chlorsulfuron	Glean	DuPont
		Siege	Nufarm
		Tackle 750	Farmoz Pty. Ltd.
		Chlorsulfuron	Davison Industries
	Sulfometuron-methyl	Oust	DuPont
	Thifensulfuron-methyl and Metsulfuron-methyl	Harmony M	DuPont
	Triasulfuron	Logran	Ciba Geigy
		Nugran	Nufarm
	Metsulfuron-methyl	Ally	DuPont
		Associate	Nufarm
		Brush off	DuPont
		Renovate	DuPont
		Lynx 600	Farmoz
Imidazolinones	Imazapyr	Arsenal	Cyanamid
	Imazethapyr	Spinnaker	Cyanamid
Triazolopyrimidines	Flumetsulam	Broadstrike	Dow Elanco
	Metosulam	Eclipse	Dow Elanco

Table 1.4. Weed species confirmed resistant following field application of ALS herbicides in Australia.

Species	Selective agent	Resistance mechanism	References
B. tournefortii	Cs	Resistant ALS	(1)
Cyperus difformis	Bm	unknown	(2)
Damasonium minus	Bm	unknown	(2)
Lactuca serriola	Ts	unknown	(3)
L. rigidum	Cs	Resistant ALS	(4)
Polygonum convolvulus	Cs	unknown	(5)
Raphanus raphanistrum	Cs	unknown	(6)
Sagittaria montevidensis	Bm	unknown	(2)
Sis. orientale	Cs/ Ts	Resistant ALS	(7)
Son. oleraceus	Cs	Resistant ALS	(7)

References: (1) Boutsalis et al., in preparation; (2) R. Graham pers. comm.; (3) C. Clarke, pers. comm.; (4) Christopher et al., 1992; (5) S. Atkins pers. comm.; (6) G. Gill pers. comm.; (7) Boutsalis and Powles, 1995.

Herbicide nomenclature:

Cs: chlorsulfuron

Bm: bensulfuron-methyl

Ts: triasulfuron

Table 1.5. Weed species confirmed resistant to field applied ALS herbicides in North America and Europe.

Species	Selective agent	Resistance mechanism	Reference
Amaranthus palmeri	Iz/Ts	unknown	(1)
Amaranthus rudis	Iz/Ts	unknown	(1)
Amaranthus retroflexus	Sm	Resistant ALS	(2)
Cyperus difformis	Bm	Resistant ALS	(3)
Kochia scoparia	Cs	Resistant ALS	(4); (5)
Lactuca serriola	Cs/ Mm	Resistant ALS	(6); (7)
Lolium perenne	Sm/ Cs	Resistant ALS	(8); (9)
Sagittaria montevidensis	Bm	Resistant ALS	(3)
Salsola iberica	Sm	Resistant ALS	(8)
Stellaria media	Cs	Resistant ALS	(8)
Xanthium strumarium	Iq	Resistant ALS	(10)

References: (1) Horak and Peterson, 1995; (2) Rubin et al., 1992; (3) Pappas-Fader et al., 1993; (4) Primiani et al., 1990; (5) Saari et al., 1990; (6) Mallory-Smith et al., 1990a; (7) Mallory-Smith, 1990; (8) Saari et al., 1992; (9) LeClair and Cotterman, 1992; (10) Schmitzer et al., 1993.

Herbicide nomenclature:

Cs: chlorsulfuron
Bm: bensulfuron-methyl
Mm: metsulfuron-methyl
Sm: sulfometuron-methyl
Ts: triasulfuron
Iq: imazaquin
Iz: imazethapyr

1.5 ACETOLACTATE SYNTHASE

1.5.1 Introduction

Acetolactate synthase (ALS; EC.4.1.3.18) catalyses the first committed reaction in the biosynthetic pathway leading to the synthesis of the branched-chain amino acids, leucine, isoleucine and valine (Fig. 1.2) (DeFelice et al., 1974; Jones et al., 1985; Miflin, 1974; Ray, 1984). This step involves the catalysis of two parallel reactions, the condensation of two pyruvate molecules to form α-acetolactate and condensation of one pyruvate molecule with one α-ketobutyrate molecule, forming α-aceto-α–hydroxybutyric acid. ALS is found in plants as well as in bacteria, archaebacteria, fungi and algae (Mazur and Falco, 1989). In plants, the highest ALS activity has been measured in actively growing tissues such as in young leaves of lima beans and tobacco (Singh et al., 1990; Keeler et al., 1993). ALS is presumed to be localised to the plastid in higher plants (Jones et al., 1985; Miflin, 1974), although in yeast and other fungi it is associated with the mitochondria (Ryan and Kohlaw, 1974). However, ALS is absent from animal cells consistent with the observation that ALS herbicides have very low mammalian toxicities (Levitt et al., 1981).

1.5.2 Structure of ALS

The quaternary structure of ALS from microorganisms has been thoroughly investigated. In *Escherichia coli* and *Salmonella typhimurium*, at least three multimeric isozymes have been identified (ALS I, II, III) each encoded by a different structural gene (Eoyang and Silverman, 1984; Grimminger and Umbarger, 1979; Schloss et al., 1985). Each bacterial ALS is a tetramer composed of two identical large catalytic (around 60 kDa) and two identical small regulatory (between 9 and 17 kDa) subunits, each unique to a given isozyme and encoded by a different structural gene (Eoyang and Silverman, 1984; Schloss et al., 1985). In plants however, the quaternary structure of ALS is potentially more complex since the apparent molecular mass of non-denatured ALS varies significantly (from 55 kDa to 440 kDa) in different species (Durner and Böger, 1988, 1990; Singh and Schmitt 1989; Singh et

al., 1988a; Bekkaoui et al., 1993). To date, the only ALS subunit that has been identified in plants is an approximately 60 kDa protein which when used in bacterial complementation studies can rescue the ALS minus phenotype (Smith et al., 1989; Wiersma et al., 1990). Those experiments confirm the functional homology between plant and bacterial 60 kDa subunits. As yet, no smaller subunits corresponding to the 9-17 kDa subunits of bacteria have been found for plant ALS.

Insights into the quaternary structure of native plant ALS have found that partially purified ALS from etiolated barley shoots appears to exist as two different forms, 440 kDa and 200 kDa at equilibrium with each other (Durner and Böger, 1990). In the presence of 50 μM FAD most of the ALS aggregated as a high molecular weight form (440 kDa) implying that FAD was involved in stabilising the quaternary structure of the protein and had no redox function (Durner and Böger, 1988, Schloss et al., 1988). Because there were negligible differences in feedback inhibition, affinity for pyruvate, acetoin production and inhibition by specific inhibitors the authors concluded that the 440 kDa and the 200 kDa forms are different aggregates of the basic 58 kDa subunit of barley ALS (Durner and Böger, 1990). However, it appears that these aggregates do not exist as isozymes in plant cells, but are formed by the conditions of chromatography.

In contrast, 50 μM FAD had no detectable effect on the native molecular mass of ALS from *Brassica napus* (Bekkaoui et al., 1993). Nevertheless, immunological characterisation of partially purified ALS from *B. napus* has also shown evidence for two forms, a homodimer of a 66 kDa subunit and a heterodimer of a 65 and 66 kDa subunits (Bekkaoui et al., 1993). The two forms displayed different sensitivity to feedback regulation by branched-chain amino acids, as well as sensitivity to chlorsulfuron suggesting they are true isozymes (Bekkaoui et al., 1993).

The low concentration of plant ALS and its instability once extracted has hindered the resolution of its quaternary structure. Until the native form of plant ALS has been purified its higher order structure will remain unknown, although the reports detailed above suggest that it exists in polymeric form (Durner and Böger, 1990; Bekkaoui et al., 1993).

Furthermore, there is no indication from the information discussed above whether one or more ALS genes are responsible for the various forms isolated (see Section 1.5.5 below).

1.5.3 Mode of action of ALS herbicides

ALS-inhibiting herbicides, as their name suggests, inhibit the enzyme acetolactate synthase. These herbicides have several features which have contributed to their widespread adoption. They are potent inhibitors of ALS which makes their registered field rates very low. In addition, they have excellent selectivity in many crops and have low mammalian toxicity (Beyer et al., 1988; Shaner et al., 1982; Gerwick et al., 1990).

Why inhibition of ALS should kill susceptible plants is not fully understood. It has been shown that ALS herbicides act by inhibiting cell division in meristematic tissues (Shaner and Reider, 1986; Höfgen et al., 1995; Shaner and Singh, 1993). The herbicidal effects of ALS herbicides may result from an accumulation of toxic substrates, from a direct depletion of the end products, or from depletion of later intermediates of the pathway. Any or all of these mechanisms may be responsible for plant death (Ray, 1984; Rhodes et al., 1987).

Some reports have related plant mortality to the accumulation of α-ketobutyrate as a result of ALS inhibition (Fig. 1.2). This intermediate product in the branched-chain amino acid pathway has several fates within the plant. It can be transaminated to α-aminobutyrate which is toxic to plants (Rhodes et al., 1987), enter the leucine pathway to form norleucine, or it may interfere with the pantothenate pathway by competing with α-ketoisovalerate for ketopantoate hydroxymethyl transferase, disrupting the production of acetyl-CoA (LaRossa et al., 1990). It has been reported that inhibition of ALS in bacteria causes accumulation of α-ketobutyrate leading to toxicity (Shaw and Berg, 1980; Primerano and Burns, 1982) as was seen following exposure of *S. typhimurium* to ALS herbicides (LaRossa et al., 1987). A similar observation was made in the higher plant *Lemna minor* treated with chlorsulfuron where the toxic transamination product, α-aminobutyrate accumulated rather than α-ketobutyrate (Rhodes et al., 1987). Based on these findings it was proposed that this accumulation plays a major role in the toxicity of ALS herbicides in plants (Van Dyk and LaRossa, 1986).

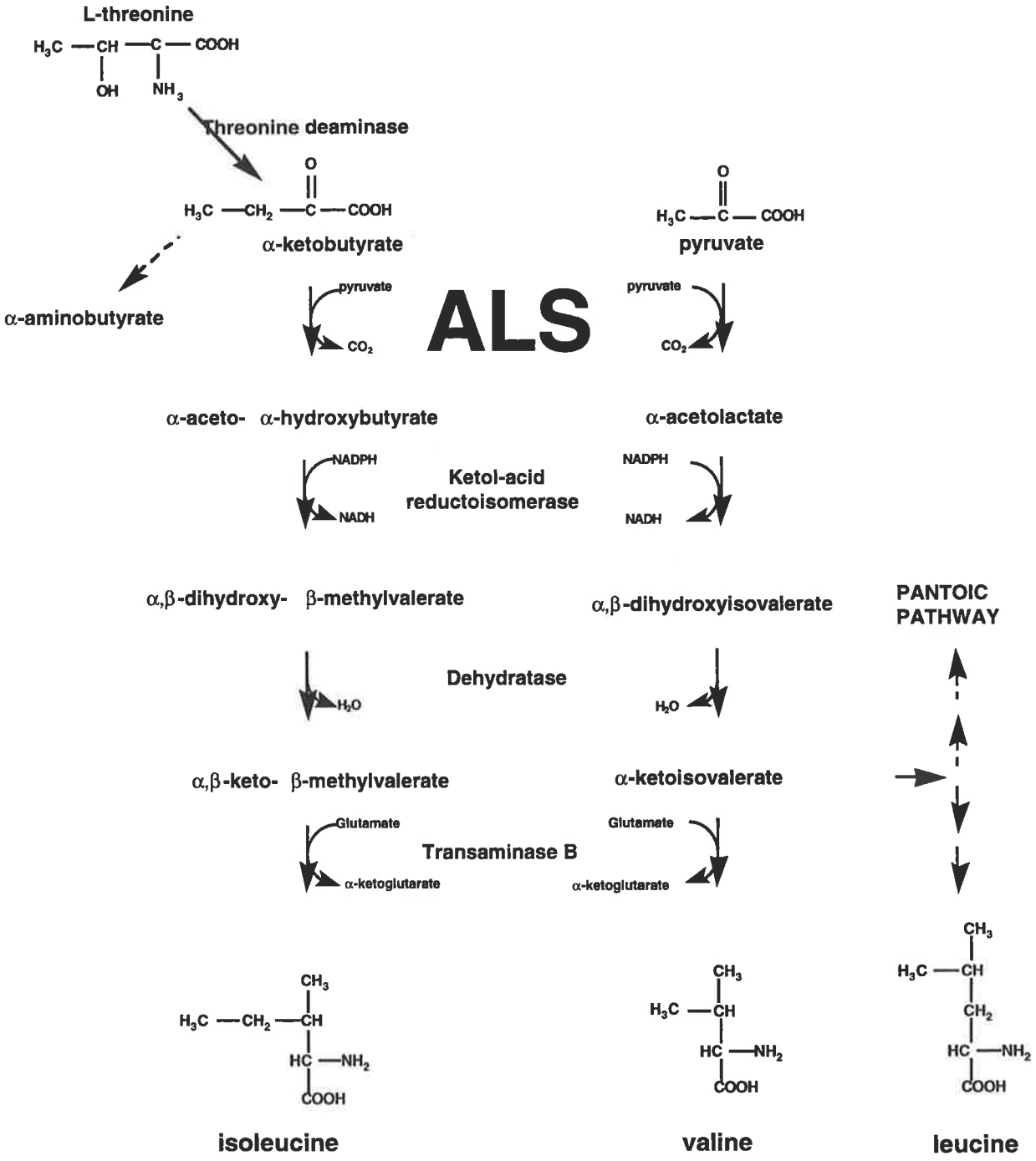


Figure 1.2. Branched-chain amino acid synthesis.

There is speculation as to the role α-ketobutyrate accumulation plays in ALS herbicide toxicity in plants. This compound is the product of threonine dehydratase, which is feedback-regulated by isoleucine (Stidham, 1991). If α-ketobutyrate was a major component of the toxicity of ALS herbicides, it should be possible to protect plants by supplementing with isoleucine (Shaner and Singh, 1993). However, isoleucine alone does not prevent imazaquin inhibition of corn growth. All three branched-chain amino acids are required to prevent accumulation of α-ketobutyrate (Shaner and Singh, 1993). Thus, accumulation of α-aminobutyrate or α-ketobutyrate causing plant death was excluded. This conclusion is supported by the antisense study of Höfgen et al. (1995). These authors constructed a chimeric ALS antisense gene and transformed *Solanum tuberosum* plants with this construct. Antisense plants showed severe growth retardation that mimicked the effects of ALS herbicides and contained reduced ALS mRNA and protein levels (Höfgen et al., 1995). No accumulation of α-aminobutyrate or α-ketobutyrate was observed in either antisense plants or in plants treated with IM herbicides (Höfgen et al., 1995).

Why are ALS herbicides such potent herbicides? Does ALS inhibition deplete levels of the end products leading to inhibition of both protein synthesis followed by cell division? Following chlorsulfuron treatment, addition of valine and isoleucine alleviated herbicide-induced growth inhibition of excised pea root cultures (Ray, 1984). The results of this study suggested that ALS herbicides might act by depletion of amino acid pools and hence inhibit protein synthesis. Further evidence for the protein depletion theory is presented by Shaner and Reider (1986). Following imazapyr treatment of corn root tips, DNA synthesis was inhibited but protein synthesis remained unchanged 24 h after herbicide exposure. However, a 40% reduction in soluble protein followed by a corresponding 32% increase in the amino acid pool was observed suggesting a cause for plant mortality (Shaner and Reider, 1986). Furthermore, these authors were able to reverse imazapyr-induced inhibition of DNA synthesis in corn root tips by the addition of the three branched-chain amino acids suggesting this herbicide depletes amino acid levels inevitably leading to plant death. Similarly, in imazethapyr treated soybean, ALS inhibition resulted in the reduction of soluble protein by

25% followed by a corresponding increase in the amino acid pool. However, if valine, leucine and isoleucine were pre-added no ALS inhibition observed (Scarponi et al., 1995).

A recent study employing an antisense ALS gene construct could find no decrease in the levels of the branched-chain amino acids following a reduction in ALS activity (Höfgen et al., 1995). Instead, the authors observed an increase in the total free amino acid pool size, with a dramatically altered balance of amino acids. From these findings they proposed that inhibition of ALS causes an imbalance in the free amino acid pool. This does not support the Shaner and Reidy theory, but might support an alternative proposal that imbalances in amino acid content have detrimental effects on plant growth. Furthermore, it has been shown that feeding of threonine and lysine inhibits growth of most plants, presumably by limiting methionine biosynthesis (reviewed in Bryan, 1980).

In light of these results it seems that reductions in soluble protein levels with corresponding increases in amino acid levels leads to a rapid decrease in DNA synthesis and mitosis which can be prevented or even reversed by addition of valine, leucine and isoleucine (Rost et al., 1990; Shaner and Singh, 1993). While the connection between mitosis and the branched-chain amino acid pathway has not been identified, prevention of cell division at the growing point would reduce plant growth and possibly lead to plant death. Furthermore, inhibition of ALS also causes a rapid decrease in the translocation of photosynthate to the meristematic regions of the plant (Hall and Devine, 1993) ensuring not only deprivation of branched-chain amino acids from the meristematic regions, but also general carbon depletion. Interaction of these factors is almost certainly why susceptible plants die after exposure to ALS herbicides (Shaner and Singh, 1993).

1.5.4 ALS herbicide binding site

No direct evidence has been presented that identifies the binding domain of ALS herbicides to ALS. Some reports suggest that ALS herbicide binding occurs in a site that is remote from the active site and is apparently not involved in catalysis or allosteric regulation (Schloss, 1990; Shaner, 1991). These findings are supported by investigations on three *A.*

thaliana mutants (GH50, GH90 and Tzp5) that each have a different single base change in the ALS gene, each conferring herbicide resistance. These changes affect the binding of herbicides to ALS without influencing the binding of substrates or feedback inhibitors such as L-valine, L-leucine and L-isoleucine (Mourad et al., 1995). In contrast, situations where laboratory and field selected ALS resistant species have altered feedback sensitivity to the branched-chain amino acids have been reported (Rathinasabapathi et al., 1990; Subramanian et al., 1990, 1991; Rathinasabapathi and King, 1991; Wu et al., 1994). Thus, current evidence indicates that the binding site of ALS herbicides may not be entirely separate but may overlap the regulatory or active sites.

Evidence for the origin of the binding site for ALS herbicides stems from the observation that the *E. coli* pyruvate oxidase gene, poxB, shares substantial sequence homology with ilvB, ilvG and ilvI, the genes coding for the large subunits of isozymes ALS I, ALS II and ALS III from *S. typhimurium* (Grabau and Cronan, 1986; Schloss, 1990). Detailed comparative investigations have been made of poxB and bacterial ALS II, the bacterial isozyme most closely resembling plant ALS (Schloss, 1990). Unlike ALS II, pyruvate oxidase uses its FAD for normal redox chemistry, and binds one additional co-factor *in vivo*, ubiquinone-40 (Q_8). Q_8 binds to pyruvate oxidase in a spatially and kinetically equivalent way that sulfometuron binds to ALS II, suggesting that the herbicide-specific site of ALS II may have a common evolutionary origin with the ubiquinone site of pyruvate oxidase (Schloss et al., 1988). Furthermore, only quinones with no or short isoprene tails such as ubiquinone-0 or ubiquinone-5 are bound by bacterial ALS and like SU, IM and TP herbicides, inhibit ALS.

Numerous examples of ALS resistant higher plants selected with ALS inhibitors have been reviewed in Saari et al. (1994). Biotypes resistant following selection with one ALS herbicide can exhibit resistance to more than one ALS inhibitor class, with the level of resistance varying on a case by case basis. This has been observed in laboratory plant mutants and field selected plants (Tables 1.5, 1.6). Moreover, it is evident that not only the different classes of ALS inhibitors but herbicides within each class probably bind differently

to ALS. Thus, an essential contributor to understanding how herbicides bind to ALS is the elucidation of these herbicide resistance patterns.

Despite the differing patterns of cross-resistance of ALS to herbicides, some trends have emerged. Three herbicide resistant mutants have been selected from the same strain of *A. thaliana* with different herbicides (Mourad and King, 1992). The mutant selected with chlorsulfuron (GH50) was cross-resistant to TP and the TP-resistant line (Tzp5) was cross-resistant to SU (Table 1.6). However, neither line was resistant to IM or POB. Conversely, an IM selected line GH90 was not resistant to SU or TP but was resistant to the POB. These patterns of resistance are perhaps not surprising since SU and TP have some chemical features in common (Fig. 1.1). In addition, *Gossypium hirsutum* (cotton) cells selected with a TP herbicide were cross-resistant to chlorsulfuron and similarly, chlorsulfuron-resistant *Stellaria media* was cross resistant to TP (Table 1.6; Subramanian et al., 1990; Hall and Devine, 1990). There are exceptions however, some IM-resistant *Chlamydomonas reinhardtii* and maize lines were resistant to IM only, while others showed cross-resistance to SU herbicides (Winder and Spalding, 1988; Newhouse et al., 1991).

In general, it appears that selection with IM herbicides results in IM and POB resistance with or without resistance to SU and TP (Table 1.6). On the other hand, selection with SU or TP results in resistance to these two classes but often no resistance to IM and POB (Table 1.6). It is therefore likely that SU and TP may share the same binding site, or if they have separate sites there are regions mutually inclusive to both herbicides and similarly, the IM and POB share a binding site. Numerous reports of specific mutations within the ALS gene that confer herbicide resistance are available. Analysis of these mutants could identify areas of the protein which are important for binding ALS herbicides (Table 1.6).

A number of mutations that endow herbicide resistance to ALS have been identified in higher plants (Tables 1.7 and 1.8) with the mutations occurring in one of five conserved domains of the ALS peptide sequence (Fig. 1.3). Mutations within each of these domains can independently confer a herbicide resistant ALS.

Table 1.6. Comparison of herbicide selected ALS resistant laboratory mutants and field-resistant weeds to the herbicide resistance spectrum at the ALS level.

Species	Selection agent	Resistant	Susceptible	Reference
Laboratory mutant				
Zea mays -XA17	IM	Sm, Iz, Iq	-	(1)
-X112	IM	low Iq, Iz	Sm	(1)
C. reinhardtii -IMR-13	Iq	Iq, Cs	-	(2)
-IMR-2	Iq	Iq	Cs	(2)
-CSR-5	Cs	low Iq; Cs	-	(2)
Datura innoxia -CSR-1	Cs	Cs, Sm, Ip, Iq	-	(3)
-SMR-1	Sm	mod Cs, Sm, Iq; Ip	-	(3)
-CSR-2	Cs	Cs, Sm	Ip, Iq	(3)
G. hirsutum -DO-2	TP	Cs, Iz, TP, POB	-	(4)
-PS-3	TP	Cs, TP; low POB	Iz	(4)
N. tabacum -KS-43	TP	TP, Iz, POB	-	(4)
A. thaliana -GH50	Cs	Cs, TP	Ip, POB	(5); (6)
-GH90	Ip	Ip, POB	Cs, TP	(7); (6)
Field selected species				
K. scoparia	Cs	Cs, Sm; low Ip, Ib, Iz	-	(8)
-Minot	Cs	Cs, Sm, Ip, Iq, Iz	-	(9)
-Power	Cs	Cs, Sm; mod Ip, Iq, Iz	-	(9)
-Bozeman	Cs	Sm; mod Cs, Ip, Iq, Iz	-	(9)
S. media -R2	Cs	Cs, Sm; low Ib	-	(10)
S. media	Cs	Cs, TP; low Ib	-	(11)
S. media	Cs	mod Cs, Sm; low Ip	-	(12)
L. perenne	Cs	Cs, Sm; low Ip	-	(12)
S. iberica	Cs	low Cs, Ip; mod Sm	-	(12)
X. strumarium	Iq	Iq	Cm, Fm	(13)

Herbicide abbreviations are chlorsulfuron (Cs), chlorimuron (Cm), sulfometuron (Sm), imazapyr (Ip), imazaquin (Iq), imazethapyr (Iz), imazamethabenz (Ib), unspecified IM (IM), flumetsulam (Fm), unspecified TP (TP), unspecified pyrimidyloxy-benzoate (POB).

Herbicide alone indicates high level resistance, 'mod' herbicide indicates intermediate level (I_{50} ratio 5-15) and 'low' herbicide indicates low resistance (I_{50} ratio <5).

References: (1) Newhouse et al., 1991; (2) Winder and Spalding, 1988; (3) Saxena and King, 1988; (4) Subramanian et al., 1990; (5) Haughn et al., 1988; (6) Mourad and King, 1992; (7) Haughn and Somerville, 1990; (8) Saari et al., 1990; (9) Sivakumaran et al., 1993; (10) Devine et al., 1991; (11) Devine et al., 1990; (12) Saari et al., 1992; (13) Schmitzer et al., 1993.

Unfortunately, responses to inhibition by the four ALS herbicide classes are not available for most of the ALS mutations presented in Table 1.7 and, therefore, a strong correlation between ALS inhibition and individual mutations cannot be made. However, even from the incomplete information available it seems that the five mutations cause different levels and spectra of resistance. Thus, do herbicides from the four classes of ALS herbicides bind to the same region? Even though the conserved domains are widely separated in the ALS polypeptide sequence, they may be adjacent in the folded, functional enzyme and in fact form a single herbicide binding domain (Sathasivan et al., 1991). If this is the case, the different herbicides must bind differently within this domain, with some herbicides overlapping into regulatory and enzyme catalytic sites.

Several reports show that branched-chain amino acids regulate ALS activity by feedback inhibition with various ALS mutations altering feedback inhibition (Miflin, 1969; Hawkes et al., 1988). However, ALS from TP resistant tobacco and cotton mutants along with ALS isolated from chlorsulfuron-resistant *K. scoparia* and *S. media* exhibited varying degrees of insensitivity to valine and/or leucine (Subramanian et al., 1990, 1991). Rathinasabapathi et al. (1990) reported a similar finding for ALS from chlorsulfuron-resistant *Datura innoxia* that was insensitive to feedback inhibition from all three branched-chain amino acids. These reports suggest that certain mutations may affect both herbicide binding and feedback regulation.

Mutations endowing resistance to ALS herbicides do not generally affect catalytic efficiency of ALS (reviewed in Saari et al., 1994). Two exceptions of resistant ALS mutants with increased K_m (apparent) have been reported suggesting the ALS mutation(s) alters herbicide binding such that there is some overlap into the active site (Rathinasabapathi and King, 1991; Wu et al., 1994). However, it is unlikely that resistant plants with reduced ALS catalytic efficiency would survive under field conditions.

Evidence suggests that the binding site of ALS inhibitors may not be entirely separate from the catalytic and/or regulatory domains with some herbicide overlap into these domains. However, until the X-ray crystal structure of ALS is determined the exact binding of herbicides will be a topic of speculation.

Table 1.7. Mutations identified in conferring ALS resistance in laboratory derived mutant plants (after Devine and Eberlein, 1996).

Species	Line	Selective agent	Amino acid changes	Mutation Domain	Resistance	Reference
A. thaliana	GH50	SU	Pro_{197} to Ser	A	SU/ TP not IM/ POB	(1); (2)
	GH90	IM	Ser_{653} to Asn	E	IM/ POB not SU/ TP	(3); (4); (5)
	Tzp5	TP	not Pro_{197} or Ser_{653} change	unknown	SU/ TP not IM/ POB	(2)
Brassica napus		-	Pro_{173} to Ser	A	SU	(6)
	AHAS3R	SU	Trp_{557} to Leu	B	SU 900/ IM 140 TP 1000	(7)
Lactuca sativa	ID-BR1	SU	Pro_{173} to His	A	SU	(8)
N. tabacum	SuRA-C3	SU	Pro_{196} to Gln	A	SU	(9)
	SU-27D5	SU	Pro_{196} to Ser	A	SU 780/ IM 150	(10)
		SU	Trp_{568} to Leu	B	SU	(11)
	SuRB-S4HRA	SU	Pro_{196} to Ala+ Trp_{573} to Leu	A + B	SU/ IM	(9)
		SU/IM	Pro_{197} to Ser+ Ser_{653} to Asn	A + E	SU/ IM	(12)
	GH90	IM	Ser_{653} to Asn	E		(3)
		SU	Ala_{199} to Asp	D	SU	(11)
Zea mays	Pioneer 3180 IR		Trp_{542} to Leu	B	SU 200/ IM 1000/ TP 2200/ POB 1100	(13)
	ICI 8532 IT	IM	Ala_{57} to Thr	C	SU 0.5/ IM 7/ TP 1.0 / POB 5	(13)

Resistance column indicates what herbicides the ALS is and is "not" resistant to. Numbers in this column indicate I_{50} ratios.

References: (1) Haughn et al., 1988; (2) Mourad and King, 1992; (3) Sathasivan et al., 1990; (4) Sathasivan et al., 1991; (5) Haughn and Somerville, 1990; (6) Wiersma et al., 1989; (7) Hattori et al., 1995; (8) Guttieri and Eberlein, pers. comm.; (9) Lee et al., 1988; (10) Harms et al., 1992; (11) Hartnett et al., 1990; (12) Hattori et al., 1992; (13) Bernasconi et al., 1995.

1.5.5 ALS mutations

All of the plant ALS genes sequenced to date lack introns, as confirmed by comparison of genomic and cDNA sequences from single biotypes (for example, Grula et al., 1995). The mRNAs predicted from all published plant ALS cDNA or genomic sequences are in the range of 1.9 to 2.1 kb and encode a 589-670 amino acid peptide. As plant ALS has been shown to be a nuclearly encoded, chloroplast localised enzyme, it is expected to include a chloroplast transit peptide in its primary amino acid sequence. Once directed to the chloroplast the transit peptide is cleaved, leaving a mature, functional enzyme. However, because the ALS protein has not yet been purified, the exact site of cleavage is unknown.

Nevertheless, there is a striking difference between the level of sequence conservation in the N-terminal region versus the rest of the peptide. The first 20-90 residues show little or no amino acid conservation among species (typical of chloroplastic transit peptides, von Heijne et al., 1989). In contrast, the remaining approximately 580 amino acids, are colinear and greater than 70% identical among species, and represent the mature ALS peptide. The latter is perhaps not surprising given the vital housekeeping function of the ALS enzyme, and is highlighted by the fact that ALS-like enzymes not only show considerable sequence conservation across bacteria, yeasts and higher plants but are functionally interchangeable (Smith et al., 1989). Studies on the molecular basis of ALS resistance in laboratory mutants of bacteria and yeast are numerous (reviewed in Saari et al., 1994). This review will concentrate on the molecular basis of ALS herbicide resistance of higher plants (Table 1.7).

Genetic and mutational studies have shown that the diploid species *A. thaliana* possesses only one form of the ALS enzyme, encoded by a single copy of the ALS gene (Haughn and Somerville, 1986; Falco and Dumas, 1985). Tobacco (*Nicotiana tabacum*), an allotetraploid derived from the hybridisation of *N. sylvestris* and *N. tomentosiformes*, exhibits two active ALS genes (one from each of the progenitor genomes) encoding two almost identical isoforms (99.3% similar at the amino acid level, Lee et al., 1988). This confirms earlier genetic studies which defined two unlinked loci contributing to ALS herbicide resistance in tobacco (Chaleff and Bascomb, 1987). However, the situation is more complex in two other

tetraploid species examined recently. Grula et al. (1995) have shown that the AD allotetraploid *Gossypium hirsutum* (cotton) possesses at least six copies of the ALS gene (three from each progenitor genome), four of which and possibly all six are actively transcribed. A single gene and a tandem pair of genes are encoded by each genome (and are all still present in diploid relatives *G. herbaceum* and *G. thurberi)*, the single genes (the only two for which sequence is published sharing greater that 95% amino acid identity) each encoding the constitutive housekeeping form of ALS found in *A. thaliana* and *N. tabacum*. The downstream gene of the tandem pair is also constitutive but expressed at much lower levels. The upstream genes are more divergent (85% identity to the other four) and are only expressed in floral tissues. In addition, certain *Brassica* species, both diploid (*B. campestris* and *B. oleracea*) and allotetraploid (*B. napus*), have been shown to encode several ALS genes, and to express two very distinct ALS isoforms, sharing only 85% amino acid identity within species (Rutledge et al., 1991; Bekkaoui et al., 1991; Ouellet et al., 1992).

At least five independent ALS loci (ALS I to V) are present in the genome of the allotetraploid *B. napus* (Rutledge et al., 1991). ALS I, II and III have been fully sequenced. ALS I and III (derived from the ancestral *B. oleracea* and *B. campestris* genomes, respectively) share 98% amino acid similarity (mature peptides only), are both constitutively expressed in a range of tissues and are thought to correspond to the isoform present in *A. thaliana* and *N. tabacum* (Rutledge et al., 1991: Ouellet et al., 1992). The mature peptide of *B. napus* ALS II differs in length from all other published plant sequences, which are otherwise colinear. ALS II exhibits one amino acid insertion and a two amino acid deletion with respect to all other sequences and is shorter by a further eight amino acids at its C-terminal end (Rutledge et al., 1991). ALS II also has a distinct pattern of expression from the constitutive, housekeeping ALS forms, and like the divergent form in *G. hirsutum*, is expressed only in floral tissues (Ouellet et al., 1992). ALS IV and V have interrupted coding regions and are thought to be pseudogenes (Rutledge et al., 1991). Although all three active *B. napus* genes can be mutated *in vitro* to produce herbicide resistant ALS enzyme (Wiersma et al., 1989; Bekkaoui et al., 1991), it is likely that only the constitutively expressed ALS I and ALS III forms are responsible for herbicide resistance in the field, ALS II is only

expressed in floral tissues, and is probably involved in seed development (Ouellet et al., 1992).

Amino acid substitutions at five sites (Domains A to E), endowing herbicide resistance, were initially characterised in laboratory generated mutants selected with ALS herbicides (Table 1.7), but those in Domains A, B and C have since also been identified in naturally occurring weed biotypes (Table 1.8; Section 5.1).

The first site identified was in a laboratory strain of *A. thaliana* (GH50) selected for resistance to SU herbicides, which had a single nucleotide substitution within the Pro_{197} codon of the 13 amino acid conserved Domain A (Fig. 1.3). This substitution resulted in a Pro to Ser substitution, the only amino acid difference between susceptible and resistant sequences. A second site conferring ALS resistance, in a region denoted Domain B, was identified in the ALS gene of a resistant strain of *N. tabacum* (Lee et al., 1988) in which substitution of Trp_{573} (in susceptible), for Leu (in resistant) also confers resistance to ALS herbicides. Bernasconi et al. (1995) have subsequently shown that of the four possible amino acid substitutions arising from a single nucleotide substitution in the Trp codon, only Leu produces active enzyme in laboratory generated *X. strumarium* mutants. A genetically engineered ALS resistant strain of corn (ICI 8532) was produced by introducing a single nucleotide change into the Domain C region resulting in a substitution of Ala_{123} (in susceptible sequences) for Thr in the resistant strain (Greaves et al., 1993; Siehl et al., 1996).

At a fourth site, a mutation involving substitution of Ala_{199} (in susceptible) for Asp (in resistant) in the six amino acid Domain D has been reported to encode an ALS resistant to SU herbicides in tobacco (Hartnett et al., 1990). Finally, a Ser_{653} (in susceptible) to Asn (in resistant) change was identified in an *A. thaliana* strain resistant to imazapyr (Sathasivan et al., 1991) existing within a five amino acid conserved domain (Domain E) (sections 1.4.4 and 1.4.5). Thus, there are at least five sites in resistant ALS enzymes that have been shown to independently confer resistance to ALS herbicides in higher plants.

DOMAIN	C	A	D	B	E
Peptide sequence	GY**A**R	AITGQV**P**RRMIGT	**A**FQETP	Q**W**ED	IP**S**GG
Laboratory					
A. thaliana	-	S_{197}	-	-	N_{653}
B. napus	-	S_{173}	-	L_{557}	-
N. tabacum	-	Q/S_{196}	D_{199}	L_{557}	N_{653}
Z. mays	T_{57}	-	-	L_{557}	-
Field					
K. scoparia	-	T/R/L/Q/ A/S_{173}	-	-	-
L. serriola	-	H_{173}	-	-	-
S. iberica	-	L_{173}	-	-	-
X. strumarium	T_{133}	-	-	L_{552}	-

Figure 1.3. Schematic representation of the ALS gene showing five conserved domains (A to E) each containing a variable site known to confer ALS herbicide resistance in plants. The peptide sequence encoded by susceptible ALS for each domain is presented below the figure with the variable site endowing ALS herbicide resistance in bold and underlined. Tabulated below are species for which susceptible and resistant ALS genes have been sequenced. Numbered amino acids below each conserved domain list mutations conferring ALS resistance in different biotypes of laboratory and field selected plants. Information for this figure was obtained from Tables 1.7 and 1.8. Amino acids are in single letter code. A dash '-' indicates this mutation has not been identified in this species (Devine and Eberlein, 1996).

Table 1.8. Mutations identified conferring ALS resistance in field selected ALS herbicide resistant weeds in North America (after Devine and Eberlein, 1996).

Species	Biotype	Selective agent	Amino acid changes	Mutation site (Domain)	Resistance	Reference
K. scoparia	KS-R	SU	Pro_{173} to Thr	A	SU 119/ IM 2	(1)
	ND-R	SU	Pro_{173} to Arg	A	NA	(1)
	MAN-R	SU	Pro_{173} to Leu	A	NA	(1)
	MT-R	SU	Pro_{173} to Gln	A	NA	(1)
	ID#5-R	SU	Pro_{173} to Ala	A	NA	(1)
	TX-R	SU	Pro_{173} to Ala	A	NA	(1)
	SLV-R	SU	Pro_{173} to Ser	A	NA	(1)
	CO-R	SU	Pro	not A	SU 111/ IM 11	(1)
	SD-R	SU	Pro	not A	SU 31/IM 3	(1)
	ID#2	SU	Pro	not A	SU 138/ IM 3	(1)
L. serriola		SU	Pro_{173} to His	A	NA	(2)
S. iberica		SU	Pro_{173} to Leu	A	NA	(3)
X. strumarium	MO-XANST	IM	Trp_{552} to Leu	B	SU 6250/ IM >7.2/ TP 1000/ POB 2200	(4)
	MS-XANST	IM	Ala_{133} to Thr	C	SU 2.4/ IM >7.2/ POB 3.5	(4)

Resistance column indicates what herbicides the ALS is resistant to.

Numbers in this column indicate I_{50} ratios. NA: not available

References: (1) Guttieri et al., 1995; (2) Guttieri et al., 1992; (3) Guttieri and Eberlein, pers. comm.; (4) Bernasconi et al., 1995

1.6 AIM OF THIS STUDY

Numerous reports of dicot weeds resistant to SU herbicides in North America have been documented (reviewed in Saari et al., 1994). In contrast, ALS resistance has only been identified in one species in Australia, the grass weed, *L. rigidum*, after selection with SU herbicides (Christopher et al., 1992). However, the strong selection pressure imposed by SU herbicides has now led to failure to control three weed species in three widely separated regions of the Australian cropping zone. Sulfonylurea herbicide failure was reported for three biotypes of *Sis. orientale*, two from the Yorke Peninsula of South Australia in 1991 and one from northern New South Wales in 1992 (Boutsalis and Powles, 1995). Also in 1991, a biotype of *Son. oleraceus* from southern Queensland was not controlled with repeated applications of chlorsulfuron (Boutsalis and Powles, 1995). Finally in 1992, a report from Western Australia identified a biotype of *B. tournefortii* that had survived pre-emergent chlorsulfuron application (G. Gill, pers. comm.).

An investigation on the three aforementioned purported resistant weed species was commenced in July 1992 to confirm their resistance status by herbicide screening of outdoor pot grown plants. In addition, field experiments were commenced to investigate changes in dormancy and seedbank life of the resistant populations over a three year period. After determining the herbicide resistance spectrum of the resistant biotypes, genetic crosses between resistant and susceptible plants were performed to follow the mode of inheritance of ALS resistance. Information gathered from the herbicide screening of pot grown plants indicated that these biotypes exhibit resistance only to the ALS herbicides, indicative of target site ALS resistance. Thus, *in vitro* ALS enzyme assays in the presence of various herbicides were carried out revealing a modified ALS enzyme as the main mechanism of resistance in all cases. This finding prompted a molecular investigation of the ALS gene to identify mutations responsible for endowing a resistant enzyme.

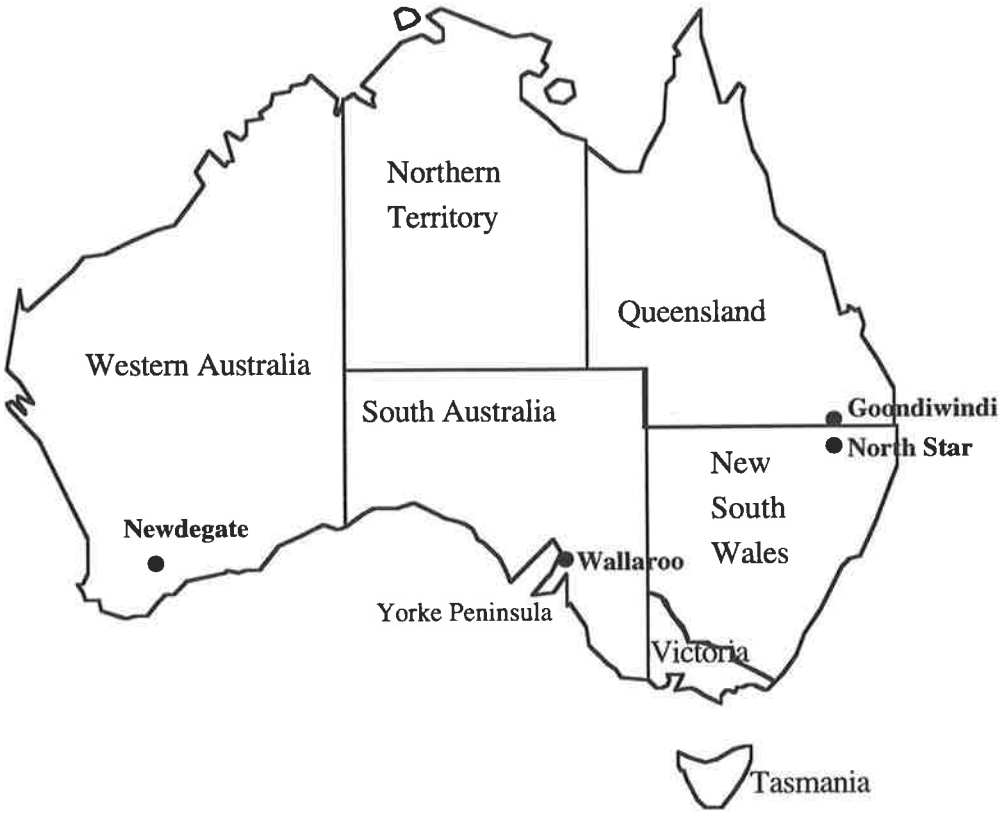
CHAPTER 2

2.0. WHOLE PLANT RESPONSE TO ALS HERBICIDES

2.1 INTRODUCTION

Resistance to ALS herbicides has occurred in *L. rigidum* in Australia (Christopher et al., 1992), and in at least eight dicot and two monocot weed species in North America (reviewed in Saari et al., 1994), following selection with ALS herbicides. In Australia, SU herbicides are the most widely and frequently used ALS herbicides. These herbicides, in particular chlorsulfuron and triasulfuron, are used to control *L. rigidum* and many dicot weed species in cereals. As will be subsequently documented, resistance to ALS herbicides in dicot weeds is now a reality in Australia. Over 150 cases of SU herbicide failure to control dicot weeds, particularly *Sis. orientale* and *Lactuca serriola* in the Yorke Peninsula (Fig. 2.1a), have occurred in the 1995 season (C. Clarke, pers. comm.). Ten percent of these fields have been re-tested on site by spraying with SU herbicides and the results indicate failure due to herbicide resistance in all cases. Although resistance cannot be assumed until pot

(a) Australia



(b) South Australia

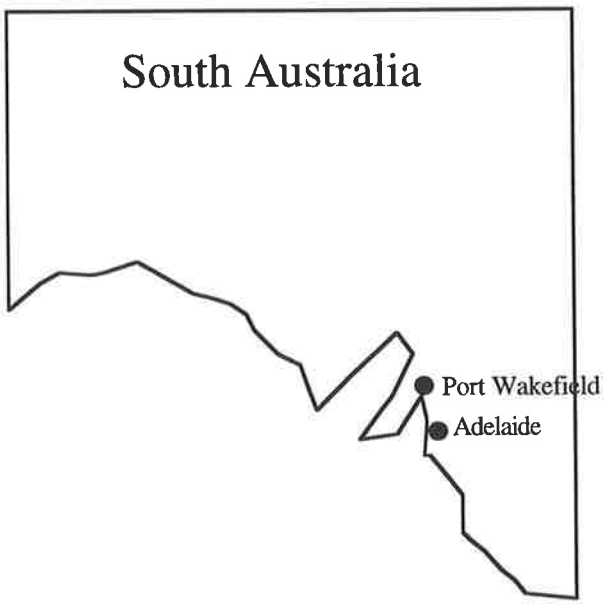


Figure 2.1. (a) Location of ALS resistant weed biotypes of *Sis. orientale* (North Star and Wallaroo), *Son. oleraceus* (Goondiwindi) and *B. tournefortii* (Newdegate) and (b) location of susceptible weed biotypes of *Son. oleraceus* (Adelaide) and *B. tournefortii* (Port Wakefield).

experiments are conducted, these findings indicate that widespread resistance is imminent.

The first indications of dicot weeds resistant to field applied SU herbicides in Australia appeared in the early 1990's. The first report was a chlorsulfuron failure on a population of *Son. oleraceus* which had a long history of chlorsulfuron use. Similarly, there were reports of triasulfuron failure on populations of *Sis. orientale* from the Yorke Peninsula in South Australia (Fig. 2.1a) after only two previous applications of SU herbicides. A failure was also reported with a *Sis. orientale* population from northern New South Wales which survived field rates of triasulfuron after a long history of exposure to chlorsulfuron (Fig. 2.1a). Finally, failure with a third weed species, *B. tournefortii* was reported after repeated use of chlorsulfuron. All these species are introduced annual weeds common to most Australian cropping zones. In Australia, *Son. oleraceus* is a weed of summer crops and pastures, whereas the other two species are weeds of winter crops and pastures. This chapter establishes the herbicide resistance spectrum of these populations. Pot experiments were conducted in which plants were treated with ALS herbicides or herbicides with other modes of action to test for cross-resistance. All experiments were conducted during the normal growing season for the three species, autumn-winter for *Sis. orientale* and *B. tournefortii* and spring for *Son. oleraceus*.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Son. oleraceus: In March 1991, *Son. oleraceus* seeds were collected from a field near Goondiwindi, Queensland (Fig 2.1a). In this field, chlorsulfuron had been applied between 11 to 13 g ha^{-1}, either pre- or post-emergent, for eight consecutive years. Hereafter this population is referred to as the resistant *Son. oleraceus* biotype. Susceptible seeds (hereafter referred to as the susceptible *Son. oleraceus* biotype) were collected in Adelaide, South Australia (Fig. 2.1b), from a field with no prior herbicide application. To multiply seed, plants of the resistant and susceptible biotypes were grown in the glasshouse in the summer

of 1991. Resistant plants used to generate new seed were survivors of 15 g ha^{-1} chlorsulfuron.

Sis. orientale: Seeds of a biotype (NSO1) were collected in 1992 from a wheat field near North Star, New South Wales which had been treated with 27 g ha^{-1} triasulfuron (Fig. 2.1a). This biotype had been exposed to an annual chlorsulfuron application for seven years, with triasulfuron used in the eighth year. Seeds of a second biotype (SSO3) were collected in 1991 from a wheat field near Wallaroo, South Australia (Fig. 2.1a). This biotype had survived a pre-emergent application of 29 g ha^{-1} triasulfuron after previous triasulfuron and metsulfuron-methyl application over a four year period. Susceptible seeds (biotype SSO2) were collected from an untreated area 10 km south east from the location of biotype SSO3.

B. tournefortii: Seeds were collected from *B. tournefortii* plants that survived 11.25 g ha^{-1} pre-emergent chlorsulfuron in a wheat field near Newdegate, Western Australian in 1992 (Fig. 2.1a). The herbicide history of this field included six applications of chlorsulfuron before failure was evident following the seventh application. Susceptible *B. tournefortii* seeds were collected from a non-cropping area near Port Wakefield, South Australia (Fig. 2.1b).

2.2.2 Treatment with herbicides

Son. oleraceus: Seeds were germinated on 0.6% (w/v) agar in a seed incubator with a 12 h photoperiod and a light intensity of 19 $\mu E\ m^{-2}\ s^{-1}$. Temperature was maintained at 20°C during the light period and 16°C during the dark period. Seedlings were transplanted into 17 cm pots (5 plants per pot) containing pasteurised potting soil and grown outdoors. Plants were sprayed at the 3-4 leaf stage using a precision laboratory herbicide sprayer delivering 97 l ha^{-1} (at a pressure of 250 kPa) through two flat-fan hydraulic nozzles (Tee-Jet, 001). Both biotypes were tested with six post-emergent ALS herbicides and three other herbicides with different modes of action (five replications per rate). The pot experiments were

repeated twice, once per growing season. Surfactant (0.2% v/v Agral 600) was applied with each herbicide.

Chlorsulfuron was applied at nine rates, while eight rates of sulfometuron-methyl, seven rates of metsulfuron-methyl, eight rates of imazapyr, five rates of imazethapyr and seven rates of flumetsulam were used. The non-ALS herbicides diuron, MCPA and diflufenican were applied at five rates each.

Sis. orientale: Seeds were sprinkled on sterile potting soil in 17 cm pots and grown outdoors. After emergence, the seedlings were thinned to 10 per pot and grown as described for *Son. oleraceus*. Spraying was performed when seedlings were at the 3-4 leaf stage. The three biotypes were tested with five ALS and three non-ALS post-emergent herbicides with three replications at each herbicide rate. Nine rates of chlorsulfuron, sulfometuron-methyl, metsulfuron-methyl, imazethapyr and flumetsulam were applied and seven rates per non-ALS herbicide (diuron, MCPA and diflufenican). The pot experiments were repeated twice, once per growing season.

B. tournefortii: Dormancy in this species was broken by immersing the seeds in concentrated sulfuric acid for 6 min and then rinsing for 5 min under running water. Seeds were germinated on agar, transplanted into pots, grown and sprayed as described for *Son. oleraceus*. Both biotypes were treated with six rates of chlorsulfuron.

After herbicide application, the treated plants were returned outdoors and the pots positioned in a randomised design. The plants were watered as required. Thirty days after treatment, plant survival for the three species was recorded. Dry weight was obtained from plants harvested at ground level and dried at 60^{o}C for 48 h. The experiments were repeated and the data combined for statistical analysis for each species. Percentage survival and percentage dry weight reduction were recorded with the standard error of the mean calculated for each rate. LD_{50} and GR_{50} ratios were computed using regression analysis.

2.3 RESULTS

2.3.1 *Son. oleraceus*

These experiments revealed that the *Son. oleraceus* biotype from Goondiwindi is highly resistant to chlorsulfuron (Plate 2.1a; Fig. 2.2a, 2.3a; Table 2.1). Susceptible plants were all killed with 2.5 g ha^{-1} whereas only 20% of resistant plants were killed at the highest chlorsulfuron rate, 90 g ha^{-1} (Fig. 2.2a). Analysis of dry weights indicated that chlorsulfuron reduced dry matter accumulation in both biotypes with a greater reduction in the susceptible; 24-fold more chlorsulfuron was required to reduce dry weight of the resistant biotype by 50% when compared with the susceptible biotype (Fig. 2.3a, Table 2.1). The differential response, measured in mortality and dry weight is reflected in the high LD_{50} and GR_{50} ratios, >86 and >24, respectively (Table 2.1). Resistance to two other SU herbicides, metsulfuron-methyl and sulfometuron-methyl was observed (Fig. 2.2b,c, Fig. 2.3b,c, Table 2.1). As only chlorsulfuron had been used on this population, resistance to metsulfuron-methyl and sulfometuron-methyl had occurred without prior exposure to these chemicals.

In North America, several SU resistant weed species show cross-resistance to IM herbicides (Primiani et al., 1990; Saari et al., 1992; Hall and Devine, 1990). Imazethapyr, the only selective ALS herbicide from the IM group available in Australia, is marketed for the pre-emergent control of several dicot crop weeds. Resistant *Son. oleraceus* is 10-fold less resistant to imazethapyr and imazapyr than to SU herbicides as indicated by LD_{50} ratios (Fig. 2.4a,b, 2.5a; Table 2.1).

The TP compounds are the most recent class of ALS herbicides to become available to Australian agriculture. Flumetsulam was released in 1994 for dicot weed control in various crops (Table 1.2). Neither *Son. oleraceus* biotype was affected by flumetsulam at rates up to eight times the recommended field rate for control of other dicot weed species (Fig. 2.4c, 2.5b). This is consistent with technical information supplied by Dow Elanco regarding the inactivity of this herbicide on this species.

Plate 2.1. Response of resistant and susceptible biotypes of (a) *Son. oleraceus* and (b) *Sis. orientale* to chlorsulfuron.

(a) *Son. oleraceus*

(b) *Sis. orientale*

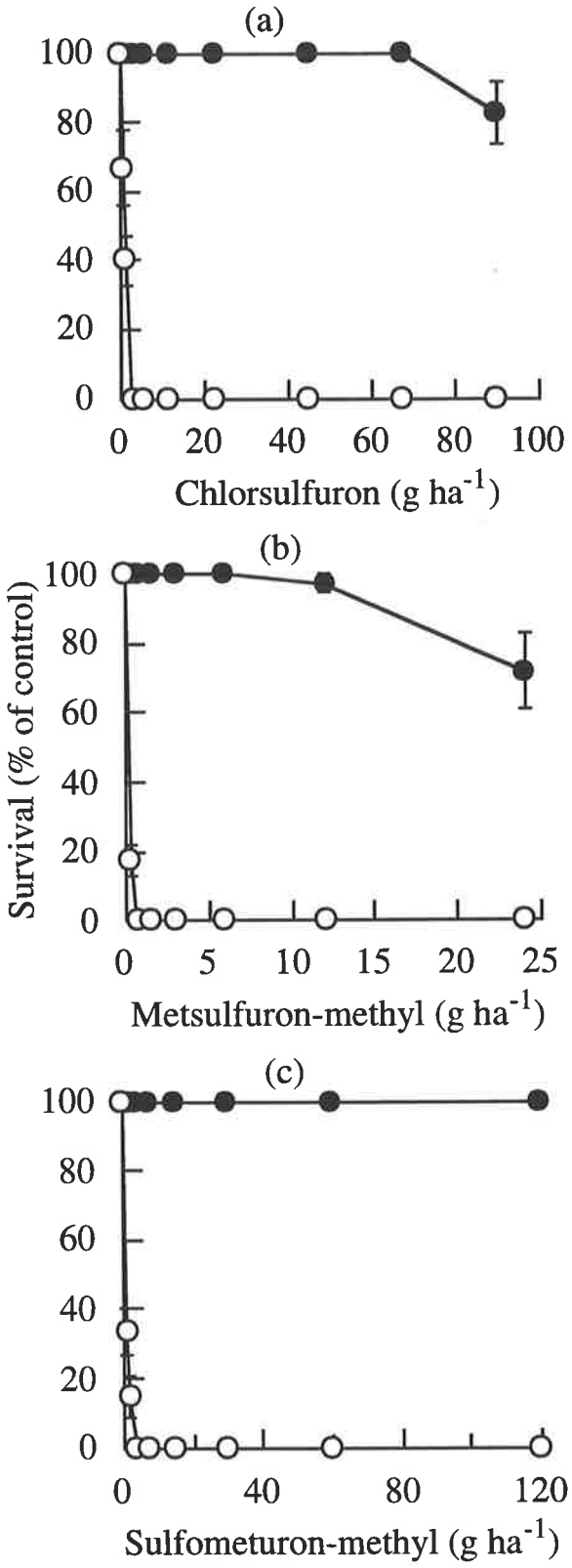


Figure 2.2. Survival of susceptible (O) and resistant (●) *Son. oleraceus* biotypes 30 days after treatment with the ALS herbicides (a) chlorsulfuron, (b) metsulfuron-methyl and (c) sulfometuron-methyl. Points are means per pot of survival as a percentage of the control. Bars indicate ± SE of the means.

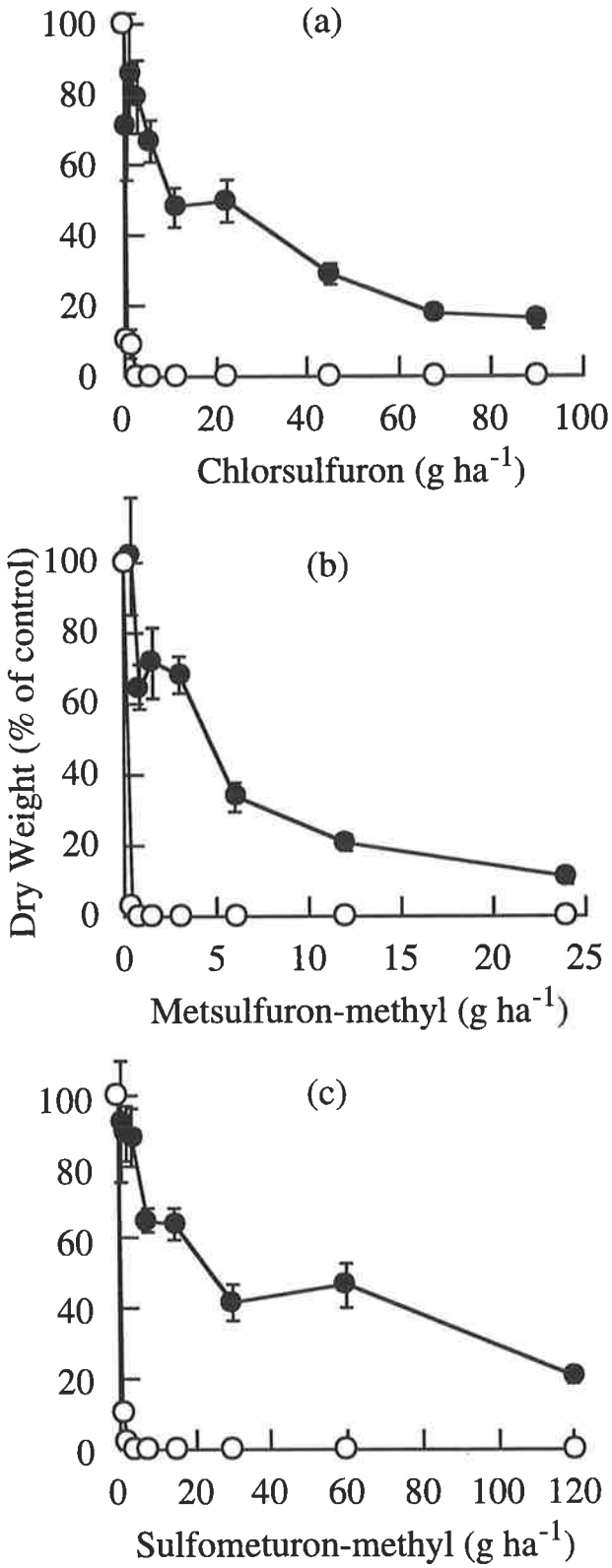


Figure 2.3. Dry weight of susceptible (O) and resistant (●) *Son. oleraceus* biotypes 30 days after treatment with the ALS herbicides (a) chlorsulfuron, (b) metsulfuron-methyl and (c) sulfometuron-methyl. Points are means per pot of dry weight as a percentage of the control. Bars indicate ± SE of the means.

Table 2.1. LD_{50} ratios (the herbicide dose required to kill 50% of resistant plants divided by the dose required to kill 50% of susceptible plants) and GR_{50} ratios (the dose of herbicide required to inhibit resistant plant growth by 50% divided by the dose required to inhibit susceptible plant growth by 50%) for the resistant *Son. oleraceus* biotype.

Herbicide Class	Herbicide	LD_{50} Ratio	GR_{50} Ratio
Sulfonylurea	Chlorsulfuron	>86	>24
	Sulfometuron-methyl	>128	>28
	Metsulfuron-methyl	>64	>9
Imidazolinone	Imazapyr	8.4	7.4
	Imazethapyr	4.5	NA
Triazolopyrimidine	Flumetsulam	NR	NR
Phenoxyacetic acid	MCPA	1.1	0.9
Substituted urea	Diuron	0.7	0.5
Nicotinamide	Diflufenican	1.1	0.7

NA: no dry weight measurement taken

NR: Not recorded because no mortality of plants from the resistant or susceptible biotypes were observed to flumetsulam.

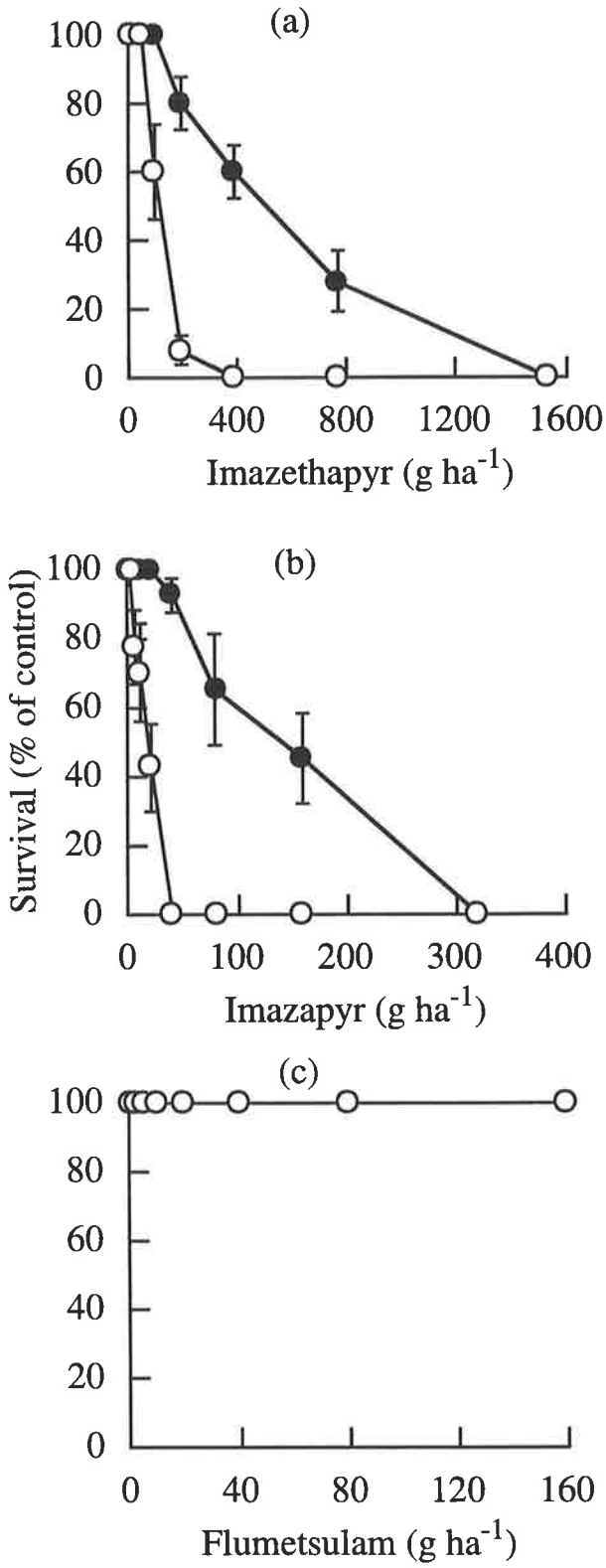


Figure 2.4. Survival of susceptible (O) and resistant (●) *Son. oleraceus* biotypes 30 days after treatment with the ALS herbicides (a) imazethapyr, (b) imazapyr and (c) flumetsulam (for flumetsulam, the resistant and susceptible biotype symbols are superimposed). Points are means per pot of survival as a percentage of the control. Bars indicate ± SE of the means.

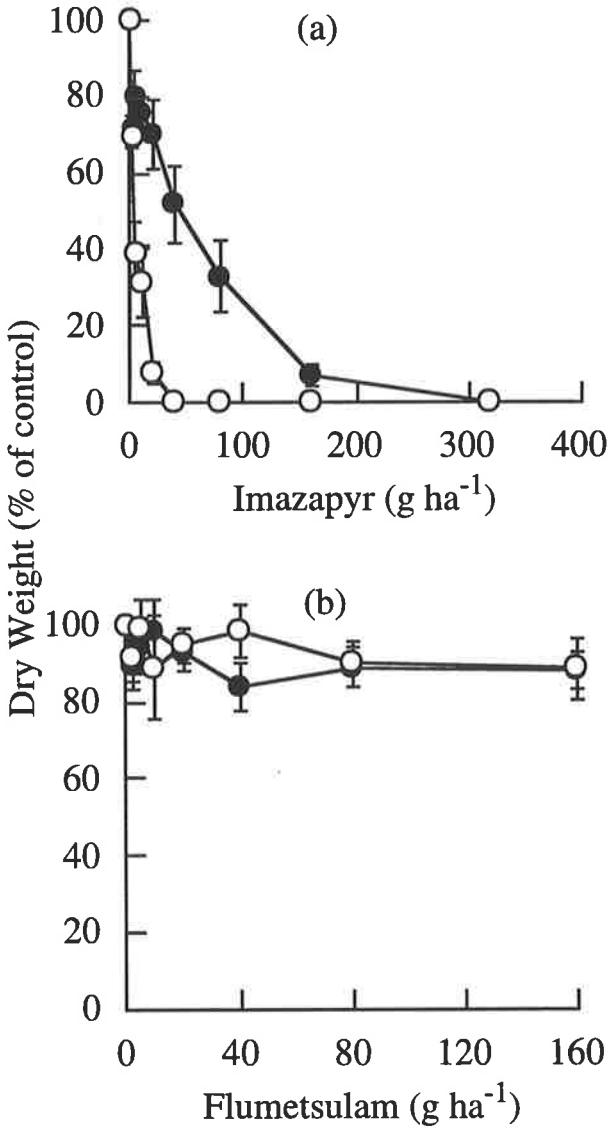


Figure 2.5. Dry weight of susceptible (O) and resistant (●) *Son. oleraceus* biotypes 30 days after treatment with the ALS herbicides (a) imazapyr and (b) flumetsulam. Points are means per pot of dry weight as a percentage of the control. Bars indicate ± SE of the means.

There are several non-ALS herbicides recommended for *Son. oleraceus* control. This study used the non-ALS herbicides diuron, diflufenican and MCPA. Each of these herbicides are from a different chemical group with a different mode of action. No resistance to these non-ALS herbicides was observed for either biotype (Fig. 2.6, 2.7). This response was confirmed by both LD_{50} and GR_{50} ratios less than 1.1 (Table 2.1). Both biotypes were killed at rates below the recommended field rates of 450 g ha^{-1} diuron, 250 g ha^{-1} MCPA or 100 g ha^{-1} diflufenican (Fig. 2.6, 2.7). Based on these observations, this resistant *Son. oleraceus* biotype exhibits cross-resistance to the SU and IM herbicides only.

2.3.2 *Sis. orientale*

ALS herbicides normally provide effective control of *Sis. orientale*. This is evident in the response of a susceptible biotype of *Sis. orientale* treated with ALS herbicides (Figs. 2.8-2.11). However, two *Sis. orientale* biotypes, NSO1 and SSO3, which chlorsulfuron had failed to control in the field were confirmed resistant to ALS herbicides by pot experiments (Table 2.2). Neither biotype showed mortality at the highest chlorsulfuron rate of 45 g ha^{-1}, four times the recommended field rate for susceptible *Sis. orientale* control (Plate 2.1b; Fig. 2.8a). Similarly, these two biotypes were found to be resistant to metsulfuron-methyl, with biotype NSO1 showing a higher level of resistance than biotype SSO3 (Fig. 2.8b, 2.9b); the LD_{50} ratio of the former was 54 compared to 7 for the latter (Table 2.2). The response to metsulfuron-methyl, between resistant biotypes to sulfometuron-methyl also differed (Fig. 2.8c, 2.9c, Table 2.2). No mortality was recorded for biotype NSO1, whereas only 10% of biotype SSO3 survived above 23 g ha^{-1} sulfometuron-methyl (Fig. 2.8c).

The IM and TP herbicides are effective in controlling susceptible but not resistant *Sis. orientale* (Fig. 2.10, 2.11). However, the SU resistant biotype NSO1 showed higher levels of resistance to imazethapyr and flumetsulam than biotype SSO3. At the highest imazethapyr (192 g ha^{-1}) and flumetsulam (160 g ha^{-1}) rates there was only 12% and 20% survival, respectively, of biotype SSO3, whereas 100% of biotype NSO1 plants survived both

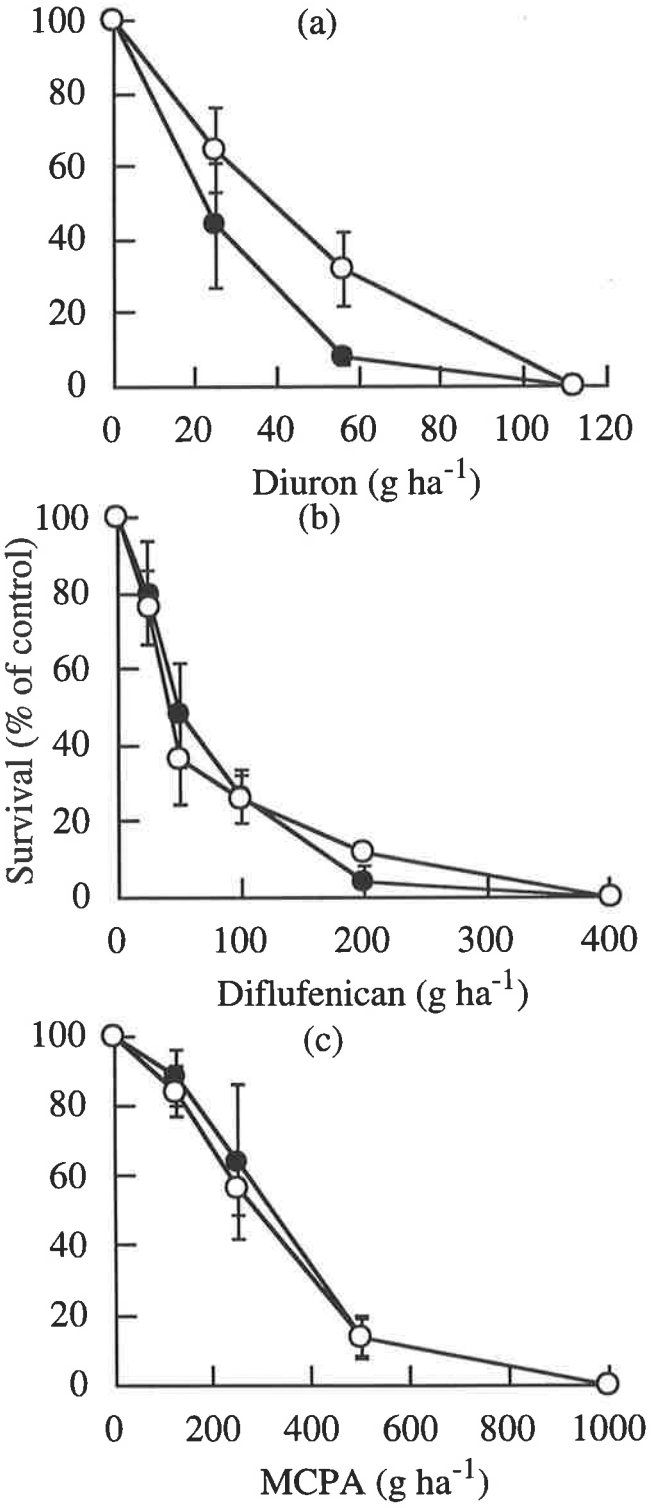


Figure 2.6. Survival of susceptible (O) and resistant (●) *Son. oleraceus* biotypes 30 days after treatment with the non-ALS herbicides (a) diuron, (b) diflufenican, and (c) MCPA. Points are means per pot of survival as a percentage of the control. Bars indicate ± SE of the means. The highest rates for diuron and MCPA are not shown because 100% mortality was obtained at lower rates.

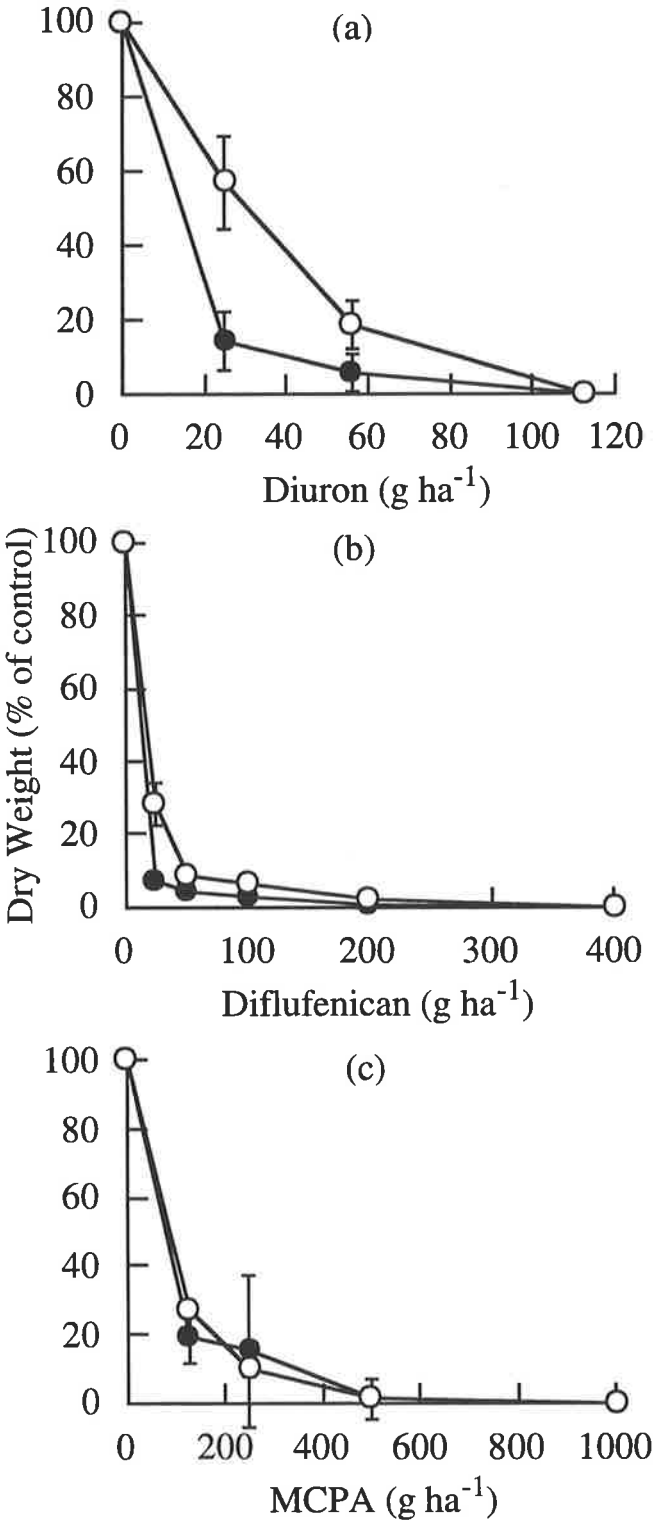


Figure 2.7. Dry weight of susceptible (O) and resistant (●) *Son. oleraceus* biotypes 30 days after treatment with the non-ALS herbicides (a) diuron, (b) diflufenican, and (c) MCPA. Points are means per pot of dry weight as a percentage of the control. Bars indicate ± SE of the means. The highest rates for diuron and MCPA are not shown because 100% mortality was obtained at lower rates.

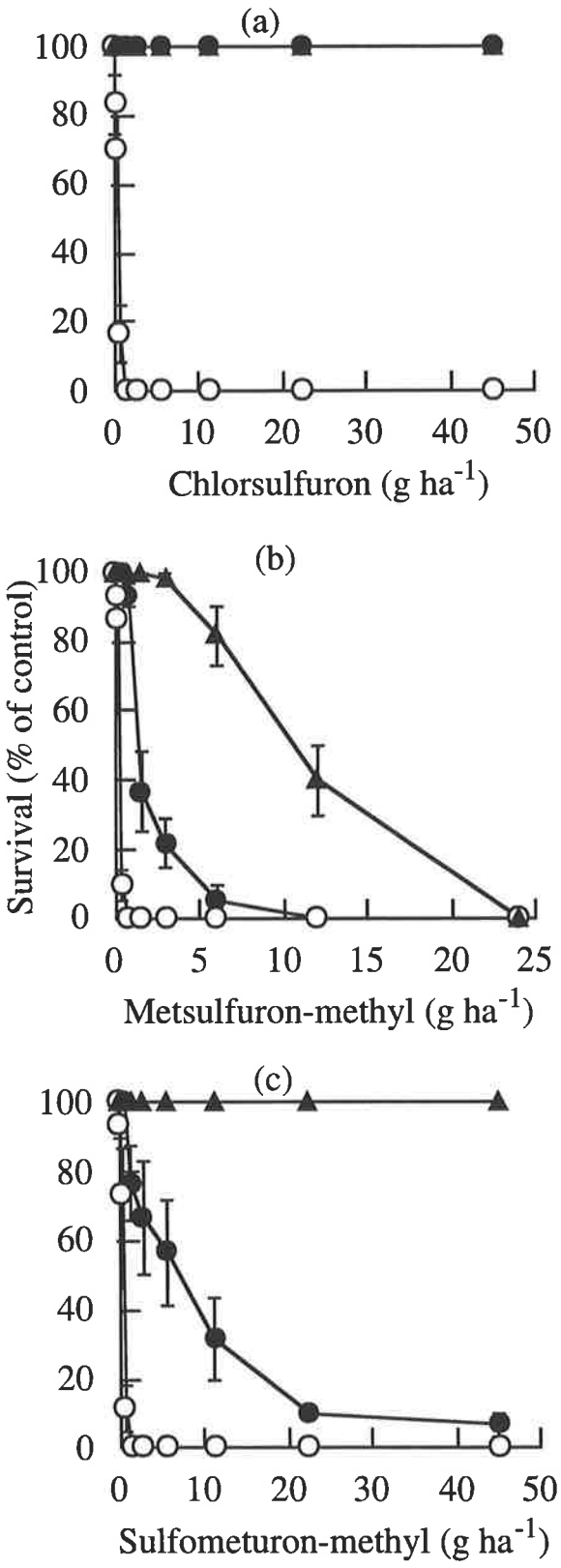


Figure 2.8. Survival of *Sis. orientale* biotypes SSO2 (○), SSO3 (●) and NSO1 (▲) 30 days after treatment with (a) chlorsulfuron (symbols for biotypes SSO3 and NSO1 are superimposed), (b) metsulfuron-methyl and (c) sulfometuron-methyl. Points are means per pot of survival as a percentage of the control. Bars indicate ± SE of the means.

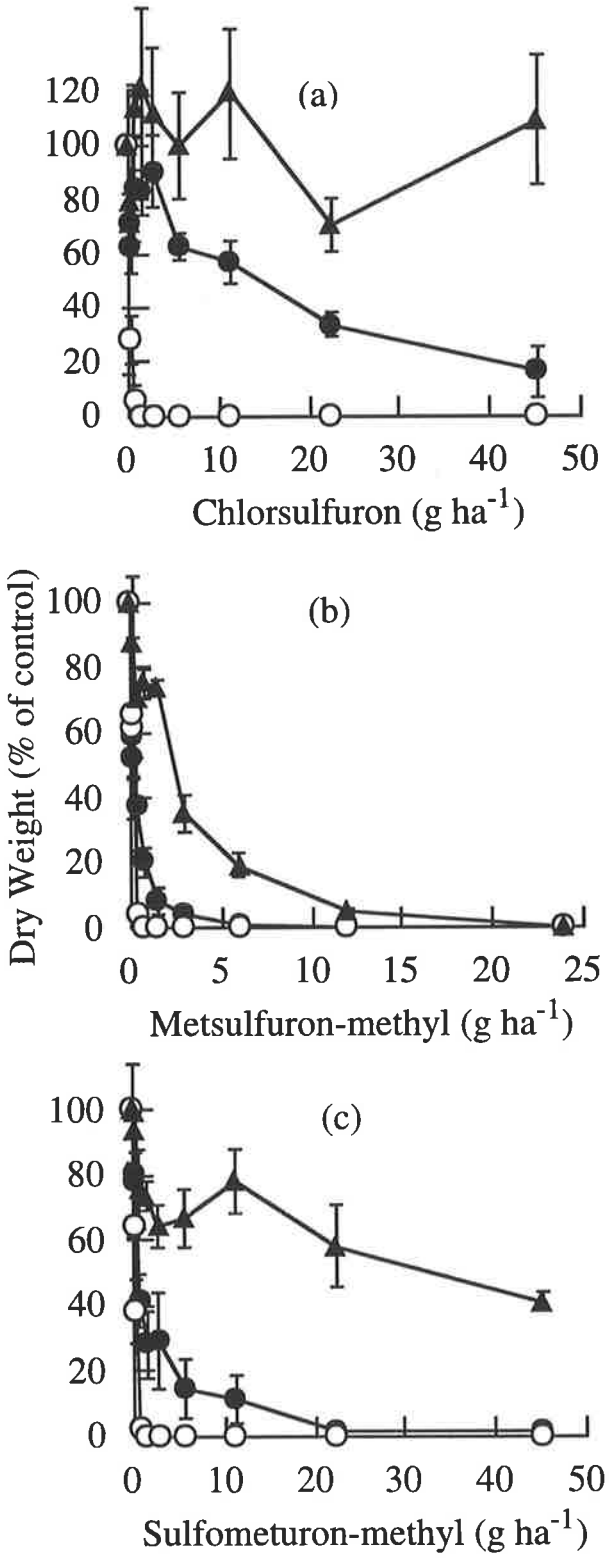


Figure 2.9. Dry weight of *Sis. orientale* biotypes SSO2 (O), SSO3 (●) and NSO1 (▲) 30 days after treatment with (a) chlorsulfuron, (b) metsulfuron-methyl and (c) sulfometuron-methyl. Points are means per pot of dry weight as a percentage of the control. Bars indicate ± SE of the means.

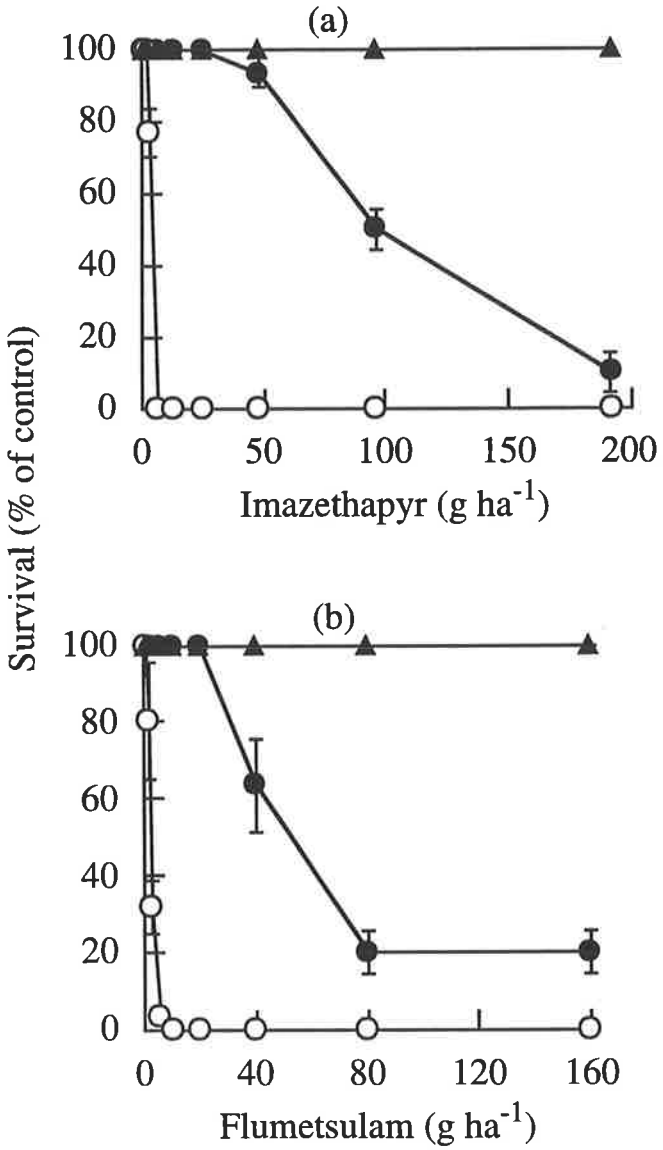


Figure 2.10. Survival of *Sis. orientale* biotypes SSO2 (O), SSO3 (●) and NSO1 (▲) 30 days after treatment with the ALS herbicides (a) imazethapyr, (b) flumetsulam. Points are means per pot of survival as a percentage of the control. Bars indicate ± SE of the means.

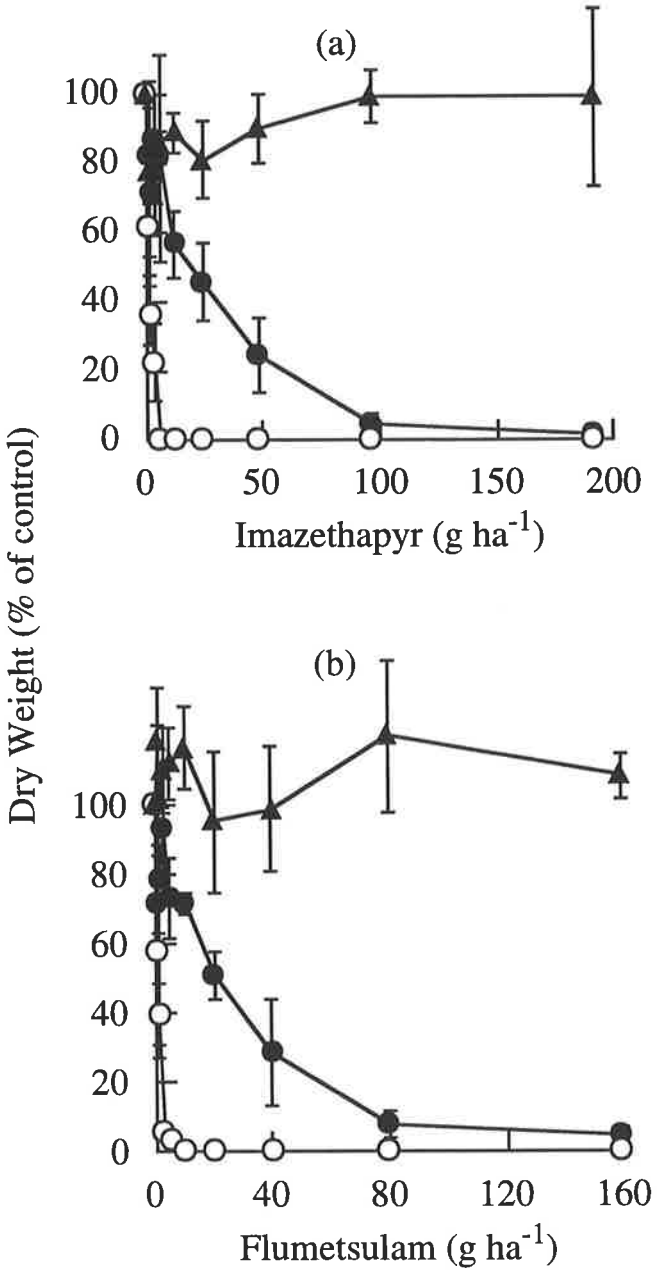


Figure 2.11. Dry weight of *Sis. orientale* biotypes SSO2 (O), SSO3 (●) and NSO1 (▲) 30 days after treatment with the ALS herbicides (a) imazethapyr, (b) flumetsulam. Points are means per pot of dry weight as a percentage of the control. Bars indicate ± SE of the means.

Table 2.2. LD_{50} ratios and GR_{50} ratios for the *Sis. orientale*, biotypes SSO3 from Wallaroo, SA and NSO1, from North Star, NSW.

Herbicide Class	Herbicide	LD_{50} Ratio		GR_{50} Ratio	
		SSO3	NSO1	SSO3	NSO1
Sulfonylurea	Chlorsulfuron	>110	>110	129	>502
	Sulfometuron-methyl	>15	102	5	126
	Metsulfuron-methyl	7	54	1.3	12.8
Triazolopyrimidine	Flumetsulam	24	>79	24	>203
Imidazolinone	Imazethapyr	29	>57	17	>185
Phenoxyacetic acid	MCPA	1	0.9	0.6	0.7
Substituted urea	Diuron	0.8	0.8	1	1
Nicotinamide	Diflufenican	1.3	1.3	0.7	0.7

herbicides (Fig. 2.10, 2.11; Table 2.2). Susceptible *Sis. orientale* plants were all killed by 10 g ha^{-1} flumetsulam or 6 g ha^{-1} imazethapyr (well below recommended field rates).

Diuron, diflufenican and MCPA are non-ALS herbicides which normally control *Son. oleraceus* and *Sis. orientale*. Survival and dry weight reduction of susceptible *Sis. orientale* and the two resistant biotypes to these herbicides was not significantly different for any of the non-ALS herbicides tested (Fig. 2.12, 2.13; Table 2.2). As 100% control of both resistant biotypes was achieved at rates below the recommended field rates for *Sis. orientale* control, these biotypes are not resistant to any of these alternative mode of action herbicides. Thus these resistant *Sis. orientale* biotypes are resistant to ALS herbicides only.

2.3.3 *B. tournefortii*

Excellent control of susceptible biotypes of *B. tournefortii* is achieved by the recommended field rate to chlorsulfuron (11.25 g ha^{-1}). However, no mortality of the resistant *B. tournefortii* biotype was observed at rates up to 45 g ha^{-1} chlorsulfuron, the highest rate used (Figure 2.14). In contrast, 100% mortality of the susceptible biotype was obtained at 4 g ha^{-1} chlorsulfuron. Therefore, the Western Australian biotype of *B. tournefortii* is resistant to chlorsulfuron. *B. tournefortii* was not treated with other herbicides.

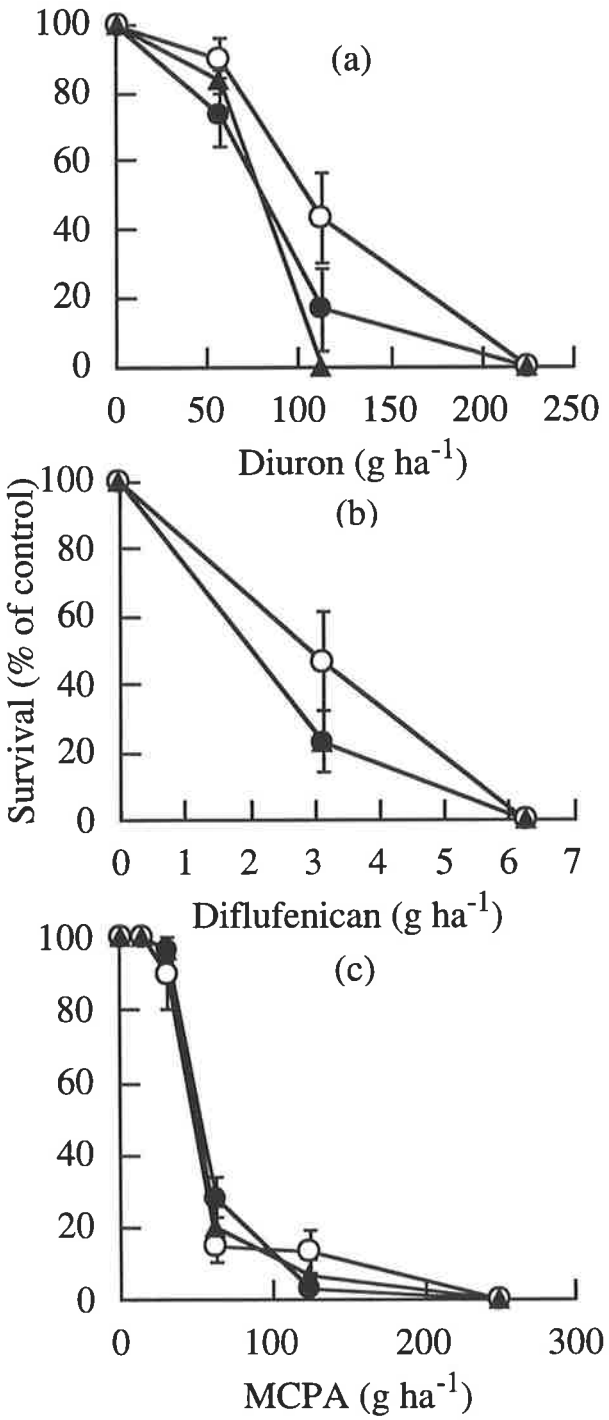


Figure 2.12. Survival of *Sis. orientale* biotypes SSO2 (O), SSO3 (●) and NSO1 (▲) 30 days after treatment with non-ALS herbicides (a) diuron, (b) diflufenican and (c) MCPA. Some symbols are superimposed. Points are means per pot of survival as a percentage of the control. Bars indicate ± SE of the means. The highest rates are not shown because 100% mortality was obtained at lower rates.

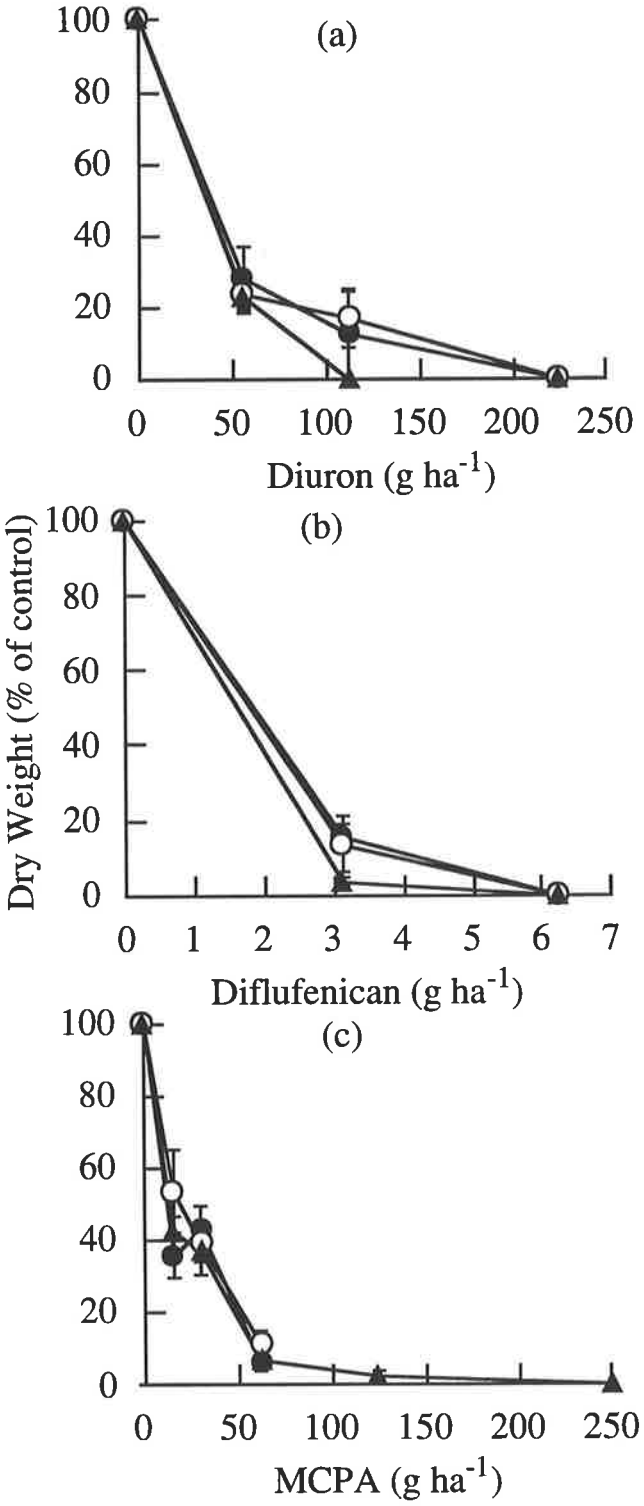


Figure 2.13. Dry weight of *Sis. orientale* biotypes SSO2 (O), SSO3 (●) and NSO1 (▲) 30 days after treatment with non-ALS herbicides (a) diuron, (b) diflufenican and (c) MCPA. Some symbols are superimposed. Points are means per pot of dry weight as a percentage of the control. Bars indicate ± SE of the means. The highest rates are not shown because 100% mortality was obtained at lower rates.

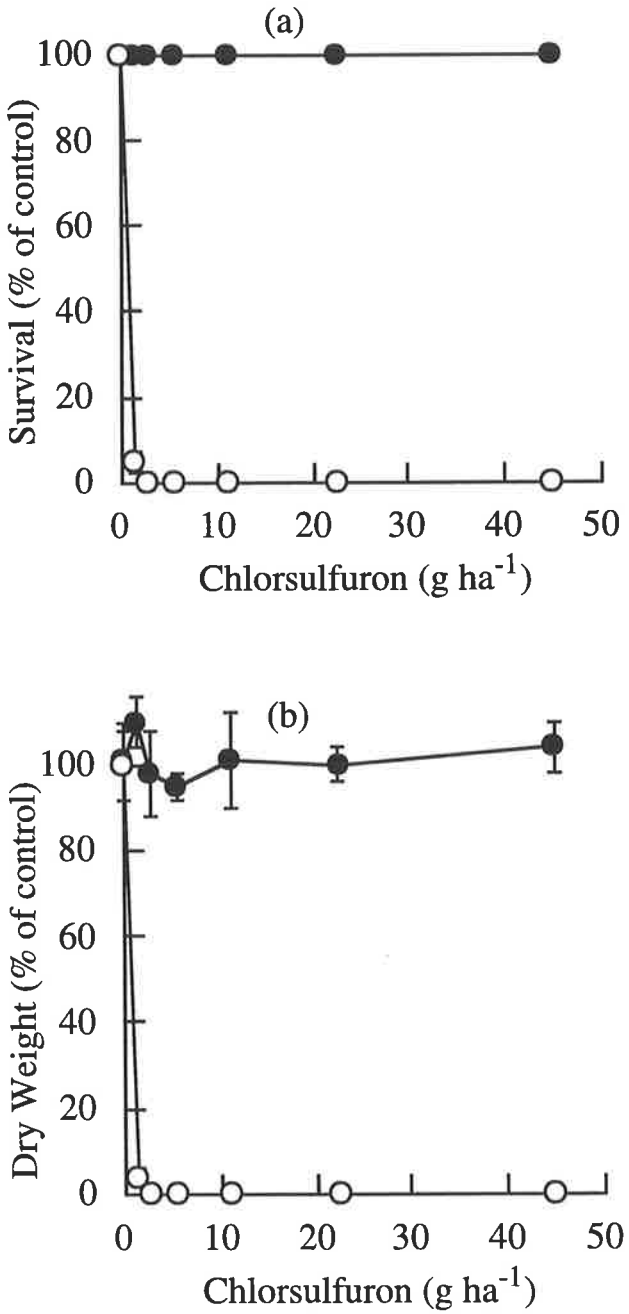


Figure 2.14. Survival (a) and dry weight (b) of susceptible (O) and resistant (●) *B. tournefortii* biotypes 30 days after treatment with chlorsulfuron. Points are means per pot of survival or dry weight as a percentage of the control. Bars indicate ± SE of the means.

2.4 DISCUSSION

The rapid and widespread adoption of ALS herbicides by Australian farmers for control of a wide range of dicot weed species imposes strong selection pressure for resistance. Prior to the start of this study, the only weed species in Australia documented to have evolved resistance following selection with ALS herbicides was the grass weed *L. rigidum*. Resistance in *L. rigidum* can be conferred by at least two mechanisms, increased herbicide metabolism or a resistant ALS enzyme (Christopher et al., 1992).

The results reported herein confirm that ALS resistance has occurred in three dicot weed species, until now controlled by ALS herbicides (Figures 2.2-2.14). The three dicot weed species studied here (*Son. oleraceus*, *Sis. orientale* and *B. tournefortii*) have biotypes with a similar spectra of resistance to ALS herbicides as those reported for resistant biotypes of dicot weeds in North America. *Son. oleraceus* GR_{50} ratios to chlorsulfuron, metsulfuron-methyl, sulfometuron-methyl and imazethapyr are similar to those recorded for resistant *Kochia scoparia* (Primiani et al., 1990). However, *Stellaria media*, *Lolium perenne* and *Salsola iberica* resistant biotypes have higher GR_{50} ratios (Saari et al., 1992), suggesting plant growth reduction was not as sensitive to these SU herbicides as in resistant *Son. oleraceus*. GR_{50} ratios for resistant *Sis. orientale* biotype NSO1 to chlorsulfuron, metsulfuron-methyl and sulfometuron-methyl are similar to *S. media* (Saari et al., 1992; Hall and Devine, 1990). Both resistant *S. media* and *Sis. orientale* biotype NSO1 were quite insensitive to growth reduction by chlorsulfuron.

The identification of resistant dicot weed biotypes and the large difference in LD_{50} and GR_{50} ratios between the two resistant *Sis. orientale* biotypes to metsulfuron-methyl, sulfometuron-methyl, imazethapyr and flumetsulam (Table 2.2) has prompted further work (Chapter 4) to determine their precise resistance mechanisms. Biotype NSO1 has strong resistance to all tested ALS herbicides, whereas biotype SSO3 exhibits strong chlorsulfuron resistance but lower resistance to other ALS herbicides (Fig. 2.10-2.11). If, as is the case for ALS-resistant dicot weed species in North America, resistance is endowed by a resistant ALS enzyme, the differing levels of resistance to ALS herbicides between these biotypes may be

due to distinct mutations in the ALS gene (Guttieri et al., 1992, 1995; Bernasconi et al., 1995). In addition to intra-species differences, inter-species differences to ALS herbicides was evident. Resistance to the IM herbicide, imazethapyr was higher in the resistant *Sis. orientale* biotypes than in resistant *Son. oleraceus*. These inter-species differences in sensitivity to IM herbicides may, as for the inter-biotypic differences, be due to different mutations in ALS genes as well as species specific characteristics such as uptake and translocation rates or metabolism rates (see later chapters). Guttieri et al. (1992) analysed resistant *K. scoparia* samples from different sites and concluded that resistance to ALS herbicides was due to several mutations causing different amino acid substitutions in ALS. Thus genetic variability with respect to herbicide response also exists between biotypes of the same species with the same mechanism.

Whole plant responses to some herbicides can sometimes indicate the likely resistance mechanism. For example, the non-selective SU herbicide, sulfometuron-methyl is slowly metabolised by plants and thus is toxic even to plant species that can rapidly metabolise selective SU herbicides (Sweetser, 1985; Anderson & Swain, 1992). For example, *L. rigidum* biotype, SLR 31, which is resistant to chlorsulfuron due to enhanced metabolism is killed by sulfometuron-methyl which it cannot metabolise rapidly (Christopher et al., 1991). Resistant *Son. oleraceus* and *Sis. orientale* biotypes are resistant to sulfometuron-methyl (Figs. 2.2, 2.3, 2.8, 2.9) indicating (but not proving) that resistance is unlikely to be due to enhanced metabolism. Furthermore, the resistant weed biotypes investigated exhibit cross-resistance to IM and TP herbicides after field selection with SU herbicides. It follows that the mechanism of resistance is most likely a resistant enzyme making each biotype resistant only to ALS herbicides.

The SU selected Australian weed biotypes discussed here are resistant to ALS herbicides but not resistant to the non-ALS herbicides diuron, diflufenican and MCPA (Figs. 2.6, 2.7, 2.12, 2.13). Similarly ALS resistant *K. scoparia* (Primiani et al., 1990) and *L. serriola* (Mallory-Smith et al., 1990a) from North America were not resistant to MCPA and diuron. Thus, neither North American ALS-resistant weed biotypes, nor resistant *Son. oleraceus* and *Sis. orientale* biotypes show cross-resistance to herbicides with different modes of action.

The practical implications of this study suggest that control of the ALS-resistant biotypes can be achieved by alternative herbicides which do not inhibit ALS.

It is clear that persistent selection pressure by ALS herbicides in Australian agriculture has resulted in the first three confirmed resistant Australian dicot species, *Son. oleraceus*, *Sis. orientale* and *B. tournefortii*. For several reasons there are likely to be many more cases of ALS resistance in Australia. Firstly, minimum tillage practices, currently widespread in Australian broadacre agriculture, rely heavily on selective herbicides such as SU for weed control. Following the expiry of patent protection for several SU herbicides, other manufacturers have begun marketing these compounds. This has resulted in the marketing of chlorsulfuron, triasulfuron and metsulfuron-methyl under several trade names (Table 1.3). A lack of awareness by many farmers often results in annual applications of the same or chemically-related active ingredient. In addition, the availability of ALS herbicides which are not only selective in cereals, but also in dicot crops has resulted in their persistent use. To date, however, no resistant biotypes selected with IM or TP herbicides have been confirmed in Australia. These modern selective herbicides include imazethapyr in field peas (*Pisum sativum* L.) and faba beans (*Vicia faba* L.), flumetsulam in some *Medicago* and *Trifolium* pasture species, wheat and field peas and metosulam in winter cereals and lupins. The likely outcome of increased ALS herbicide use will be increasing incidence of resistance.

CHAPTER 3

3.0. INHERITANCE OF RESISTANCE TO HERBICIDES INHIBITING ALS

3.1 INTRODUCTION

To date, in laboratory generated plant strains where resistance to ALS herbicides is endowed by a mutated ALS enzyme, the resistance trait is inherited as a single gene with various degrees of dominance (Falco and Dumas, 1985; Haughn and Somerville, 1986; Harnett et al., 1987; LaRossa et al., 1987; Mourad et al., 1993). However, where the mechanism of resistance to ALS herbicides is not a resistant ALS, such as for a strain of soybean, resistance is inherited as a recessive trait (Sebastian and Chaleff, 1987). These patterns of inheritance of resistance were also noted in IM resistant maize where a recessive trait was identified for herbicide susceptible ALS (Anderson and Georgeson, 1989) and a semi-dominant trait for resistant ALS from three strains of maize (Newhouse et al., 1991).

In contrast to the numerous studies on the inheritance of ALS resistance in laboratory selected resistant plants, there are few reports on weed populations. Studies on ALS resistant *Lactuca serriola* (Mallory-Smith et al., 1990b) and *Kochia scoparia* (Thompson et al., 1994) have shown that resistance to ALS herbicides in these biotypes is conferred by a single nuclear gene with incomplete or complete dominance. Thus, the mode of inheritance of ALS resistance is the same whether the result of laboratory or field selection.

Resistance to ALS herbicides in the dicot weed species, *B. tournefortii, Son. oleraceus* and *Sis. orientale* has been confirmed (Chapter 2). Here I report the mode of inheritance of resistance in one biotype of *Son. oleraceus* and three biotypes of *Sis. orientale* resistant to ALS herbicides.

3.2 MATERIALS AND METHODS

3.2.1 Selection of parents

Seeds of susceptible and resistant *Son. oleraceus* biotypes were germinated on 0.6% (w/v) agar in a germination incubator set at 12 h, 20°C, 20 μE m^{-2} s^{-1} light/ 12 h, 18°C dark cycle. After five days, seedlings were transplanted into two plastic trays (40 cm x 30 cm x 12 cm) and transferred to a glasshouse.

Sis. orientale biotype SSO1 seed used in this study was collected from a barley field in 1991, 2 km from the location of susceptible biotype SSO2, after surviving 26 g ha^{-1} triasulfuron. In order to confirm that biotype SSO1 was resistant an outdoor pot trial was conducted with plants at the 3 leaf stage treated with 22.5 g ha^{-1} chlorsulfuron. At this rate all plants from susceptible biotype SSO2 died whereas all plants from biotype SSO1 survived (data not shown). Seeds of three resistant *Sis. orientale* biotypes SSO1, SSO3 and NSO1 and a susceptible biotype (SSO2) were sown in 20 cm pots containing potting soil, watered, and transferred to the glasshouse.

At the 2 to 3 leaf stage the plants were sprayed with 23 g ha^{-1} chlorsulfuron plus 0.2% (v/v) non-ionic surfactant in a laboratory sprayer delivering 97 L ha^{-1}. Three weeks after

treatment, three unsprayed susceptible plants and three surviving plants from each resistant biotype were transplanted into 25 cm diameter pots and transferred to the glasshouse. These plants were used for the inheritance study.

3.2.2 Generation of *Son. oleraceus* F_1, F_2 and F_3 populations

Son. oleraceus is self-fertile (Lewin, 1948). The capitulum inflorescence of *Son. oleraceus* commonly contains 140 florets (Salisbury, 1942). Two distinct floret types are evident; inner and outer florets, the latter having a purple tinge on the dorsal side (Lewin, 1948). Studies involving removal of inner or outer florets with subsequent bagging of each capitulum (to prevent unwanted pollen contamination) revealed that only inner florets produced seed (data not shown). Also, Tsun-Shih et al. (1972) report that the flowers of *Son. oleraceus* are partially sterile. This information was used to devise a method for artificially crossing *Son. oleraceus* without the need for emasculation. One day prior to flower opening the sheath tissue of capitula was cut, exposing the closed florets. Except for 4-6 purple tinged outer florets the remaining florets were removed and each capitulum immediately bagged. The following morning the selected florets had their ripened stigma protruding from the corolla. Crossing was performed by brushing anthers of the resistant or susceptible biotype against the exposed stigma of the other biotype. Control florets which were not pollinated did not produce seed (data not shown). Mature F_1 seeds from florets cross-pollinated in this manner were harvested two weeks after crossing. Seeds from parents and the F_1 crosses were germinated, transplanted into trays and transferred to the glasshouse, as described above. At the 2-3 leaf stage the seedlings were sprayed with 4, 23 or 45 g ha^{-1} chlorsulfuron. Phenotypes were scored 28 d after treatment as either resistant, susceptible or intermediate. Plants were designated as resistant if they showed no herbicide damage, susceptible if they all died and intermediate if they displayed severe stunting with narrow and short new leaves produced from the meristem.

Three R♀ x S♂ F_1 and three S♀ x R♂ F_1 herbicide survivors were transplanted into 25 cm diameter pots and transferred to the glasshouse. Just prior to anthesis each plant was covered with a plastic sleeve to ensure self-pollination. F_2 seed collected from these plants

together with seed collected from the susceptible and resistant parents was germinated and the seedlings treated with 23 g ha^{-1} chlorsulfuron. Plant symptoms were scored as above, 30 d after treatment.

To study segregation in the F_3 generation, 18 F_2 herbicide survivors classed intermediate and 18 classed as resistant were transplanted into 25 cm diameter pots and allowed to self-pollinate as described for the F_2 seed production. Seeds were collected, germinated and F_3 seedlings treated with herbicide as described above.

3.2.3 Generation of *Sis. orientale* F_1 and F_2 populations

Sis. orientale flowers are bisexual and self-compatible (Jessop, 1986b; pers. observation). Using this information a method was devised to follow the inheritance of ALS resistance in three resistant *Sis. orientale* biotypes. One day prior to anthesis, florets were emasculated. Crossing was performed as described for *Son. oleraceus*. Control florets which were not pollinated did not produce seed (data not shown). Mature F_1 seeds from florets cross-pollinated in this manner were harvested four months after crossing. Seeds from parents and the F_1 crosses were germinated, transplanted into trays and transferred to the glasshouse, as described above. At the 1-2 leaf stage the seedlings were sprayed with 1.4, 23 or 90 g ha^{-1} chlorsulfuron. Phenotypes were scored 14 d after treatment as either resistant, intermediate or susceptible. Plants were designated as resistant if new shoots were evident, intermediate if they were smaller than the resistant parents but still alive at the time of scoring and susceptible if they were dead.

F_2 seed was generated from three F_1 individuals from each reciprocal cross for each resistant biotype as described for *Son. oleraceus*. F_2 plants were screened with 15 g ha^{-1} chlorsulfuron. Plant symptoms were scored 10 d after treatment.

3.2.4 Statistical analysis

Chi-square analysis of the segregation of ALS herbicide resistance in F_2 and F_3 plants was performed and a Chi-square homogeneity test conducted to compare the segregation ratios between families of the same generation (Fisher, 1970).

3.3. RESULTS

3.3.1 *Son. oleraceus*

There was no mortality in the resistant *Son. oleraceus* parents treated with 23 g ha^{-1} chlorsulfuron, whereas as expected, all treated susceptible plants were killed (data not shown). All F_1 hybrids survived 4, 23 and 45 g ha^{-1} chlorsulfuron (Table 3.1). At the higher herbicide rates, F_1 hybrid plant growth was reduced relative to the resistant parents, suggesting that the gene for ALS herbicide resistance is incompletely dominant. This differential growth reduction was used as the basis for scoring the intermediate phenotypes in the F_2 and F_3 generations.

Seeds from six self-pollinated F_1 parents were collected to generate six F_2 families. Treatment of the F_2 seedlings with 23 g ha^{-1} chlorsulfuron produced three distinct phenotypes: a resistant phenotype with slight injury, an intermediate phenotype with severe stunting but no leaf necrosis or chlorosis (similar to the F_1 parents) and a susceptible phenotype that was killed (Plate 3.1). ALS herbicide resistance in F_2 plants, from each reciprocal cross, segregated in a 1:2:1 (R:I:S) ratio (Table 3.2). Observed segregation ratios for each individual family (χ^2_5=8.59, P=0.13) supports the hypothesis that each F_2 family segregated in a 1:2:1 ratio (Table 3.2). Furthermore, the total 804 F_2 plants screened (χ^2_2=0.49, P=0.78) were not significantly different from the predicted 1:2:1 segregation ratios (Table 3.2). Hence, these studies establish that ALS resistance in this biotype of *Son. oleraceus* is endowed by a single, incompletely dominant gene.

To further confirm single gene Mendelian inheritance, inheritance of ALS resistance was followed into the F_3 generation. Eighteen F_2 intermediate and 18 F_2 resistant plants were

Table 3.1 Effect of chlorsulfuron on survival of resistant, susceptible, R♀ x S♂ F_1 and S♀ x R♂ F_1 *Son. oleraceus* plants 30 days after treatment. Data is survival out of 20 treated plants.

	Chlorsulfuron (g ha^{-1})			
Population	0	4	23	45
R♀ x S♂ F_1	20	20	20	20
S♀ x R♂ F_1	20	20	20	20
Resistant	20	20	20	20
Susceptible	20	0	0	0

Plate 3.1. The phenotypic response of F_2 *Son. oleraceus* derived from selfed F_1 plants whose phenotype was intermediate to resistant and susceptible parents, 28 d after treatment with 23 g ha^{-1} chlorsulfuron. Resistant, susceptible and intermediate F_2 phenotypes were scored 30 d after plants were treated with 23 g ha^{-1} chlorsulfuron.

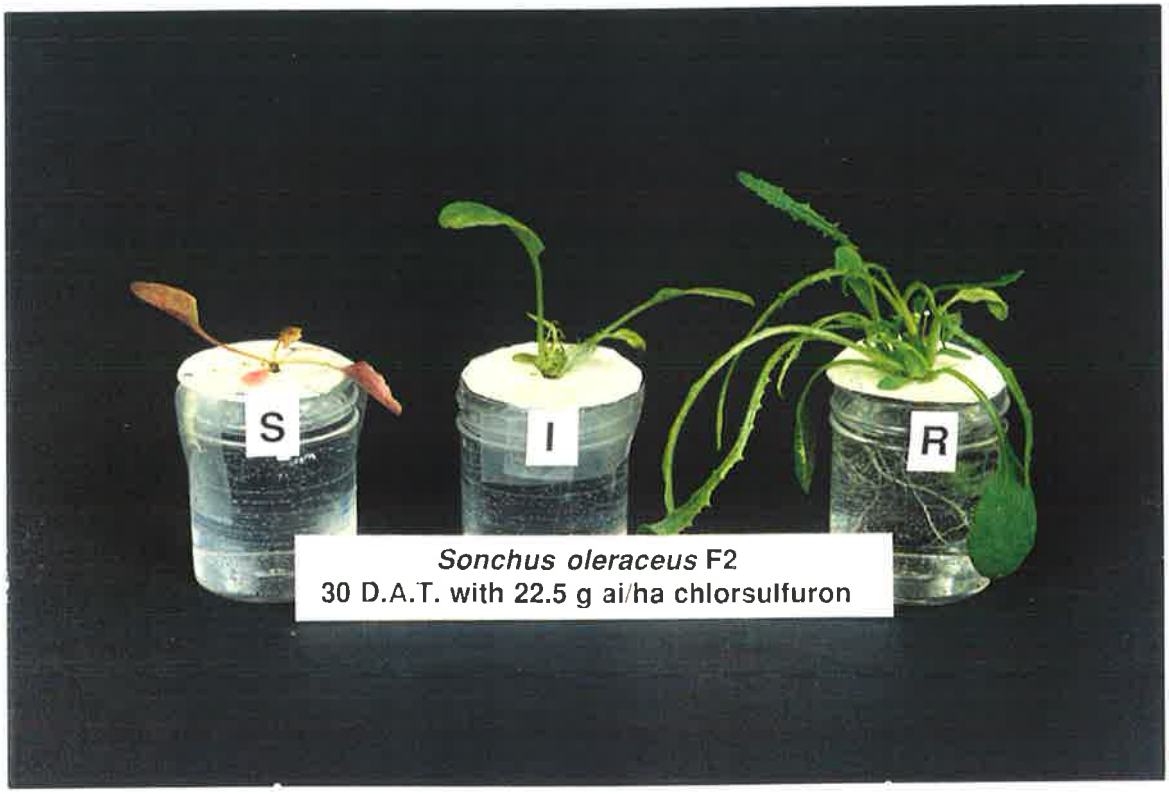


Table 3.2. χ^2 analysis of the segregation for chlorsulfuron resistance in F_2 families generated from self-pollination of three R♀ x S♂ and three S♀ x R♂ *Son. oleraceus* F_1 plants, 28 days after treatment with 23 g ha^{-1} chlorsulfuron. Phenotype classes are resistant (as resistant parent), intermediate (intermediate to both parents) and susceptible (as susceptible parent).

	Segregation by phenotype				χ^2(1:2:1)	df	Prob.
FAMILY	R	I	S	Total			
F_2 (R♀ x S♂)							
1	38	73	36	147	0.06	2	0.97
2	37	72	45	154	1.48	2	0.48
3	24	47	29	100	0.86	2	0.65
F_2 (S♀ x R♂)							
1	32	86	29	147	4.37	2	0.11
2	38	73	46	157	1.59	2	0.45
3	25	51	23	99	0.72	2	0.92
Observed	194	402	208	804	0.49	2	0.78
Expected	201	402	201				
Test of homogeneity among F_2 families					8.59	5	0.13

Table 3.3. χ^2 analysis of the segregation for chlorsulfuron resistance in F_3 families generated from self-pollination of 18 intermediate F_2 *Son. oleraceus* survivors of 23 g ha^{-1} chlorsulfuron 30 days after application.

	Segregation by phenotype				χ^2(1:2:1)	df	Prob.
FAMILY	R	I	S	Total			
1	10	22	14	46	0.78	2	0.68
2	13	24	14	51	1.16	2	0.56
3	14	30	13	57	0.19	2	0.91
4	16	24	10	50	1.52	2	0.47
5	10	30	9	49	2.51	2	0.29
6	16	34	12	62	1.10	2	0.58
7	18	23	15	56	2.11	2	0.35
8	16	25	9	50	1.96	2	0.38
9	12	30	14	56	0.43	2	0.81
10	14	21	11	46	0.74	2	0.69
11	10	29	13	52	1.04	2	0.60
12	9	24	8	41	1.24	2	0.54
13	14	26	15	55	0.20	2	0.90
14	14	22	11	47	1.60	2	0.45
15	14	23	12	49	0.35	2	0.84
16	13	24	13	50	0.08	2	0.96
17	15	28	17	60	0.40	2	0.82
18	13	30	10	53	1.26	2	0.53
Observed	241	469	220	930	1.02	2	0.60
Expected	232.5	465	232.5				
Test of homogeneity among F_3 families					17.65	17	0.41

self-pollinated, producing 18 F_3 intermediate and 18 F_3 resistant families. F_3 plants were herbicide treated as described for F_2 plants. The 18 intermediate F_3 families segregated in a 1:2:1 (R:I:S) ratio (Table 3.3). Chi-square analysis of a 1:2:1 segregation ratio of the F_3 generation (χ^2_2=1.02, P=0.60) and of each individual family (χ^2_{17}=17.65, P=0.41) supports the hypothesis that ALS resistance in this biotype of *Son. oleraceus* is endowed by a single, incompletely dominant, nuclear gene. No segregation for ALS herbicide resistance was observed for the 18 resistant F_3 families as all plants displayed the resistant phenotype.

3.3.2 *Sis. orientale*

The resistant *Sis. orientale* plants used as parents for the F_1 cross all survived 23 g ha^{-1} chlorsulfuron, a rate at which all treated susceptible plants were killed. Survival and dry weight response of the F_1 hybrids to chlorsulfuron was intermediate between those of susceptible an resistant parents (Figs. 3.1-3.3). At both 23 and 90 g ha^{-1} chlorsulfuron, growth of the F_1 hybrids was reduced to a greater extent than that of resistant parents, suggesting that the gene endowing ALS herbicide resistance is incompletely dominant, as was observed for the *Son. oleraceus* F_1 hybrids (Plate 3.2).

Seeds from the six reciprocal F_1 crosses were collected to generate six F_2 *Sis. orientale* families. Treatment of the F_2 seedlings with 15 g ha^{-1} chlorsulfuron produced a susceptible phenotype that was killed 10 days after treatment, and survivors with symptoms ranging from no visual herbicidal effects to severe stunting and chlorosis. For this reason all survivors were classed as resistant. Thus, on the basis of this classification ALS herbicide resistance in F_2 plants from each reciprocal cross segregated in a 3:1 (R:S) ratio (Table 3.4). Observed segregation ratios for each individual family were not significantly different from the predicted 3:1 segregation ratio (Table 3.4). Thus, ALS resistance in the resistant *Sis. orientale* biotypes is endowed by a single, dominant gene showing incomplete dominance, as evidenced by the range of symptoms in the F_2 survivors.

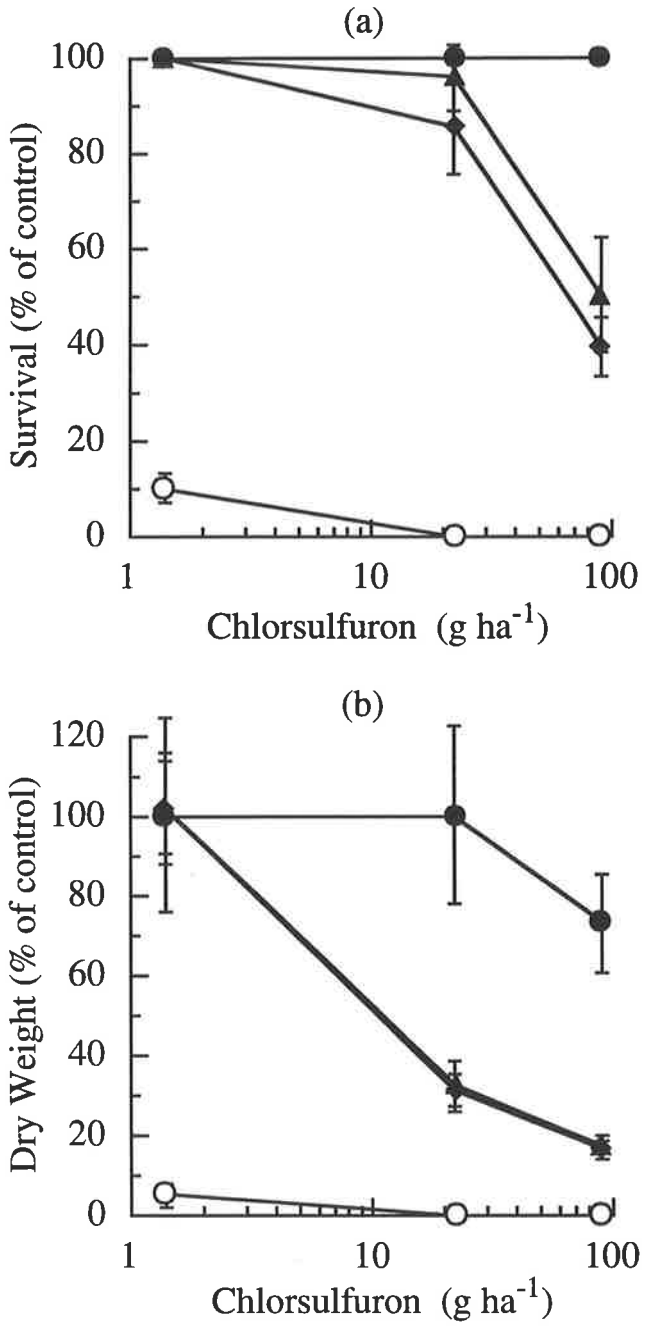


Figure 3.1. Survival (a) and dry weight (b) of F_1 reciprocal hybrids (R♀ x S♂ F_1 (◆) and S♀ x R♂ F_1 (▲)) treated with chlorsulfuron. Hybrids were produced by artificially crossing plants from a susceptible biotype (O) with resistant biotype (●) NSO1. Phenotypes were scored as either resistant, susceptible or intermediate 21 days after herbicide treatment. Points are means per pot of survival and dry weight as a percentage of the control. Vertical bars represent the standard errors of the means.

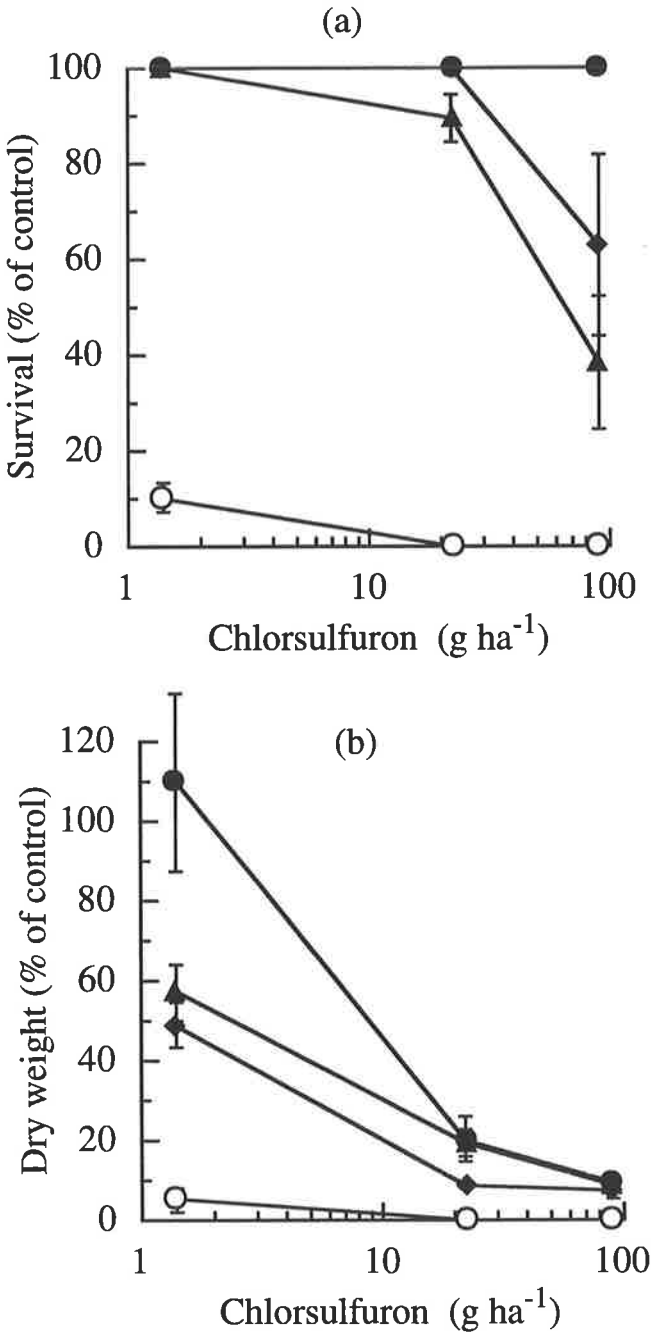


Figure 3.2. Survival (a) and dry weight (b) of F_1 reciprocal hybrids (R♀ x S♂ F_1 (◆) and S♀ x R♂ F_1 (▲)) treated with chlorsulfuron. Hybrids were produced by artificially crossing plants from a susceptible biotype (O) with resistant biotype (●) SSO3. Phenotypes were scored as either resistant, susceptible or intermediate 21 days after herbicide treatment. Points are means per pot of survival and dry weight as a percentage of the control. Vertical bars represent the standard errors of the means.

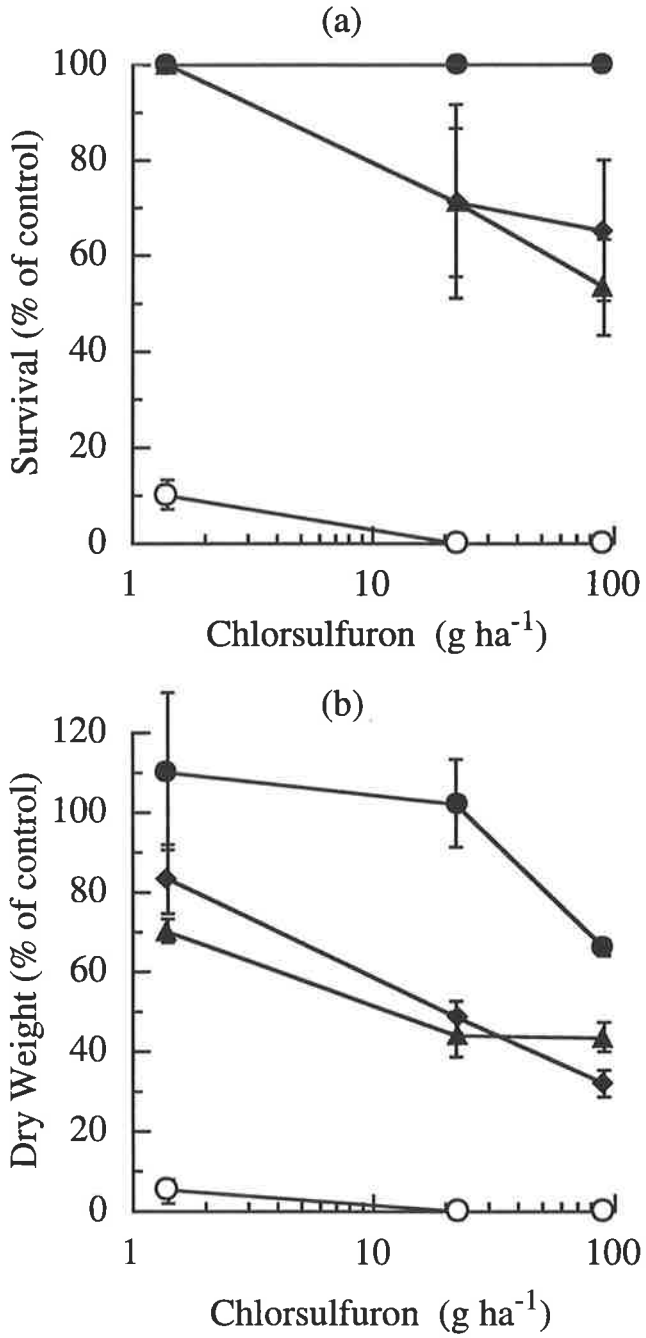


Figure 3.3. Survival (a) and dry weight (b) of F_1 reciprocal hybrids (R♀ x S♂ F_1 (◆) and S♀ x R♂ F_1 (▲)) treated with chlorsulfuron. Hybrids were produced by artificially crossing plants from a susceptible biotype (O) with resistant biotype (●) SSO1. Phenotypes were scored as either resistant, susceptible or intermediate 21 days after herbicide treatment. Points are means per pot of survival and dry weight as a percentage of the control. Vertical bars represent the standard errors of the means.

Plate 3.2. The phenotypic response of F_1 *Sis. orientale* hybrids generated by reciprocal crosses between an ALS susceptible biotype with resistant biotype NSO1. F_1 hybrids were scored 14 d after plants were treated with 23 g ha^{-1} chlorsulfuron and had intermediate growth relative to susceptible and resistant parents.

Table 3.4. χ^2 analysis of the segregation for chlorsulfuron resistance in F_2 families generated from self-pollination of three reciprocal F_1 crosses (R♀ x S♂ and S♀ x R♂) of resistant *Sis. orientale* biotypes, NSO1, SSO3 and SSO1 with a susceptible biotype. Plants were scored 10 days after treatment with 15 g ha^{-1} chlorsulfuron. Phenotype classes are resistant (survived) and susceptible (died).

	Phenotype			χ^2 (3:1)	df	Prob.
FAMILY	R	S	Total			
NSO1						
F_2 (NSO1♀ x S♂)						
1	20	9	29	0.60	1	0.44
2	41	18	59	0.99	1	0.32
3	74	26	100	0.05	1	0.82
F_2 (S♀ x NSO1♂)						
1	33	9	42	0.29	1	0.60
2	38	19	57	2.16	1	0.14
3	72	16	88	2.18	1	0.14
Observed	278	97	375	0.15	1	0.70
Expected	281.25	93.75				
Test of homogeneity among F_2 families.				6.12	5	0.29
SSO3						
F_2 (SSO3♀ x S♂)						
1	50	22	72	1.19	1	0.28
2	61	12	73	2.81	1	0.09
3	53	17	70	0.02	1	0.89
F_2 (S♀ x SSO3♂)						
1	59	16	75	0.52	1	0.47
2	59	22	81	0.21	1	0.64
3	59	18	77	0.10	1	0.75
Observed	341	107	448	0.30	1	0.59
Expected	336	112				
Test of homogeneity among F_2 families				4.55	5	0.47
SSO1						
F_2 (SSO1♀ x S♂)						
1	52	19	71	0.13	1	0.72
2	56	13	69	1.37	1	0.24
3	31	11	42	0.03	1	0.86
F_2 (S♀ x SSO1♂)						
1	62	17	79	0.49	1	0.48
2	48	22	70	1.54	1	0.21
3	48	17	65	0.05	1	0.82
Observed	297	99	396	0	1	1.00
Expected	297	99				
Test of homogeneity among F_2 families				3.61	5	0.61

3.4 DISCUSSION

A single major gene determines herbicide resistance in most weed biotypes examined to date which have developed herbicide resistance (reviewed in Darmency, 1994). The predominance of major gene inheritance can be attributed to two factors. Firstly, modern herbicides are highly target site specific, interfering with a single enzyme in major metabolic pathways. Mutation of the gene encoding the enzyme may decrease the plant's sensitivity to the herbicide and result in resistance. Secondly, some weed species, including those in the Brassicaceae family, are very sensitive to ALS herbicides. Therefore repeated applications of ALS herbicides impose strong selection pressure on susceptible populations and if resistant weeds are present, even at very low frequencies rapid selection for resistance can occur.

The present studies on the inheritance of resistance to ALS herbicides in *Son. oleraceus* and *Sis. orientale* established that resistance was maintained through successive generations. Segregation of ALS resistance exhibited Mendelian single gene genetics. The lack of significant differences between herbicide treated F_1 plants from the two reciprocal crosses demonstrates that genetic control of ALS resistance is nuclear and not cytoplasmic. The intermediate phenotype observed for F_1, F_2 and F_3 heterozygous *Son. oleraceus* plants, distinguishable from the parental phenotypes, establishes that an incompletely dominant allele confers resistance to ALS herbicides. Plants with an intermediate response to herbicide were also observed in the *Sis. orientale* F_2 generation, but unlike *Son. oleraceus* there was no clear distinction between resistant and intermediate plants. For this reason, although all surviving F_2 plants were classed as resistant for the statistical analysis, ALS resistance is likely to be inherited as an incompletely dominant trait. This is highlighted by the dose response of the F_1 populations to chlorsulfuron which clearly demonstrate that the F_1 has an intermediate level of resistance. The findings from these analyses are strong evidence that ALS resistance in the resistant *Sis. orientale* biotypes is endowed by a single, incompletely dominant nuclear gene.

Adaptive evolution in plant and animal populations is mostly achieved by selection of phenotypes encoded by many genes with small additive effects (Lande, 1983). Herbicide resistance is an exception to this generalisation as most cases are determined by single genes. Single gene inheritance of ALS resistance found in the resistant dicot weed biotypes studied here has also been demonstrated for other ALS resistant species. Studies of ALS resistant biotypes of *L. serriola* and *K. scoparia* in North America have shown that resistance segregated in a 1:2:1 (R:I:S) ratio in the former and in a 3:1 (R:S) ratio for the latter biotype. Identification of an intermediate phenotype in resistant *L. serriola* indicates that ALS resistance is controlled by a single nuclear gene with incomplete dominance (Mallory-Smith et al., 1990b) while complete dominance is indicated for *K. scoparia* since no intermediate phenotype was distinguished (Thompson et al., 1994). Studies on the inheritance of ALS resistant mutants of *N. tabacum*, *A. thaliana*, algae, yeast and bacteria also report a dominant or incompletely dominant single gene endowing resistance in each case (reviewed in Saari et al., 1994). Thus, the inheritance of resistance reported for various organisms resistant to ALS herbicides due to a resistant ALS is conferred by a single nuclear gene with varying degrees of dominance.

Is there a fitness differential between ALS resistant and susceptible biotypes? Two studies have not revealed any large differences in the competitive ability (fitness) between resistant and susceptible biotypes (Alcocer-Ruthling et al., 1992; O'Donovan et al., 1994). Evidence to date suggests that the high selection pressure imposed by ALS herbicides in unselected populations favour the selection of ALS resistance endowed by a dominant gene with no reported fitness penalty. This indicates that there is probably no strong selection pressure against the evolution of dominant ALS resistance genes within unselected weed populations. The fact that ALS resistance genes occur in low frequencies in unselected weed populations suggests some slight deleterious effects. Moreover, once resistance is established there is likely to be no strong pressure driving the loss of resistance genes in the absence of selection pressure in resistant field populations.

There is always difficulty in determining the homozygosity of field collected plants for a particular trait. However, the general lack of variability in response to herbicides of the

resistant populations (see Chapter 2) suggests that these biotypes are homozygous for this trait. Additionally as no segregation for ALS resistance was observed in the F_1 generations, this is further evidence for homozygosity of the resistant parents.

Gene flow by pollen dispersal can be an important factor in the spread of herbicide resistance among outcrossing weed species (reviewed by Maxwell and Mortimer, 1994). One study has documented the movement of resistance genes within sulfonylurea resistant *Kochia scoparia* populations, suggesting that outcrossing of resistant pollen to susceptible plants can be significant (Stallings et al., 1993). Additionally, another study has reported movement of *K. scoparia* pollen to distances up to 50 m indicating long distance spread of resistance genes is possible (Mulugeta et al., 1992). There is little published information on pollen dispersal for *Son. oleraceus* and *Sis. orientale*. Lewin (1948) reported that bees and various flies, especially syrphids, visit *Son. oleraceus* flowers. It follows that because the ALS gene is nuclearly encoded the potential for cross-pollination of susceptible plants with resistant pollen exists. However, because *Sis. orientale* is self-pollinated with dehiscence occurring before the flowers opens, it is unlikely that pollen contamination will be a problem (Salisbury, 1991). Gene flow between populations is also possible by movement of weed seed by poor hygiene practices such as contamination of seed crops or agricultural machinery by resistant seeds. Such careless practices can rapidly lead to infestations with resistant weeds in the presence of selection.

In section 3.1, reference is given to studies on soybeans and maize resistant to ALS herbicides. These studies established that in lines where ALS resistance is inherited by a single, incompletely dominant gene, the mechanism of resistance has been found to be target site resistance (Sebastian et al., 1989; Newhouse et al., 1991). However, in resistant soybean and maize lines with non-target site based resistance, the inheritance of ALS resistance was found to be recessive (Sebastian and Challeff, 1987; Anderson and Georgeson, 1989). Based on these findings, the incompletely dominant nature of the inheritance of ALS resistance in *Son. oleraceus* and *Sis. orientale* resistant biotypes suggests that the mechanism of resistance may be target site resistance. This is examined in the next chapter.

CHAPTER 4

4.0. PROPERTIES OF ALS FROM RESISTANT DICOT WEED BIOTYPES.

4.1 INTRODUCTION

The site of action of the commercially important SU, IM and TP herbicide classes is the plastidic enzyme ALS (reviewed in section 1.4). Over the past decade, ALS herbicides have become extensively used worldwide, particularly for selective weed control in a variety of crops. As a result, and discussed in Chapter 1, ALS resistance has occurred in at least twenty species. Weeds selected with ALS herbicides from one chemical class are frequently resistant to ALS herbicides from other chemical classes (target site cross-resistance). One exception is an imazaquin resistant *X. strumarium* biotype which is not cross resistant to chlorimuron or flumetsulam, herbicides from the SU and TP classes (Schmitzer et al., 1993). Where investigated, the biochemical mechanism of target site cross-resistance to ALS herbicides in field populations has often been found to be due to a herbicide resistant form of ALS (Hall and Devine, 1990; Devine et al., 1991; Saari et al., 1990, 1992). However, several resistant *L. rigidum* biotypes possess a susceptible ALS

(Christopher et al., 1991; 1992; Burnet, et al., 1994) but can metabolise chlorsulfuron to non-phytotoxic conjugates (Christopher et al., 1991; Cotterman and Saari, 1992), probably by enhanced cytochrome P_{450} activity (Christopher et al., 1994). This type of resistance in *L. rigidum* is non-target site resistance.

Over-expression of the ALS target site is a potential mechanism by which ALS resistance could develop. Such a resistance mechanism has been established in the laboratory with a resistant *Daucus carota* line (SC1000) exhibiting a 10-fold amplification of the ALS gene following incremental selection with chlorsulfuron (Caretto et al., 1994). In this line, a 6-fold increase in extractable ALS activity results in 80-fold resistance to chlorsulfuron. Resistance to chlorsulfuron was solely attributed to over-expression of ALS, as no difference in sensitivity of the enzyme to the herbicide was measured between this line and the parental line from which it was selected. Additionally, tobacco line SU-27D5 with a resistant ALS and increased ALS activity was selected by stepwise selection with primisulfuron after an initial selection with a sublethal concentration of cinosulfuron (Harms et al., 1992). This tobacco line is resistant to SU and IM herbicides due to the possession of a mutant ALS enzyme, and a 20-fold amplification of the ALS gene resulting in a 6-fold increase in ALS specific activity (Harms et al., 1992). No such resistance due to over-expression of ALS has been observed in field populations.

Differences in the affinity of ALS for the substrate pyruvate have not been reported for field selected biotypes with a resistant ALS. However, SU-resistant *Datura innoxia* cell lines CSR2, CSR6, and CSR10 had higher K_m values than susceptible strains indicating that the mutation endowing resistance altered pyruvate binding to ALS (Rathinasabapathi and King, 1991). A higher K_m for pyruvate may have physiological implications such as less efficient synthesis of branched-chain amino acids *in vivo*, as identified in line CSR6. ALS mutants with increased K_m are likely to be less competitive or even lethal and as such are unlikely to survive in the field.

The studies reported in Chapter 2 reveal that Australian biotypes from three weed species are resistant to ALS herbicides following repeated applications of SU herbicides. These biotypes are not resistant to herbicides with other modes of action, suggesting that the

mechanism of resistance is related to the target site of the ALS herbicides (Chapter 2). Furthermore, these resistant biotypes survived foliar treatment with the non-selective SU herbicide, sulfometuron. As discussed in section 2.4, resistance to sulfometuron indicates that the mechanism of resistance is unlikely to be metabolism based. Therefore, all studies performed indicate that resistance is due to a resistant ALS in the weed biotypes studied in this thesis. Hence, in this chapter an *in vitro* assay was used to investigate the effect of SU, IM and TP herbicides on ALS from resistant *Son. oleraceus*, *Sis. orientale* and *B. tournefortii*. The kinetic properties of the enzyme were also examined.

4.2 MATERIALS AND METHODS

4.2.1 Herbicides

Technical grade SU, IM and TP herbicides used for enzyme assays were supplied by DuPont Agricultural Products (Newark, DE) and American Cyanamid Co. (Princeton, NJ).

4.2.2 Plant material and growth conditions

Seeds of a susceptible *Sis. orientale* biotype, three resistant *Sis. orientale* biotypes (NSO1, SSO3 and SSO1), and susceptible and resistant *Son. oleraceus* and *B. tournefortii* were germinated in plastic trays (40 cm x 30 cm x 12 cm) containing pasteurised potting soil in a growth cabinet with a 14 h, 18.5^{o}C, 490 μE m^{-2} s^{-1} light/ 10 h, 14^{o}C dark cycle. Plants were grown to the 2 to 3 leaf stage and then subjected to experiment.

4.2.3 ALS extraction and assay

The ALS extraction and assay protocols were modified from procedures described by Ray (1984) and Singh et al. (1988b). Green leaf tissue from 3 week old plants was ground in liquid nitrogen with 0.5 g polyvinyl polypyrrolidone. After grinding, extraction buffer

(buffer:plant material, 2:1 v/w) containing 1 M potassium phosphate buffer (pH 7.5), 0.62% (w/v) dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyruvate, 5 mM $MgCl_2$, 5 mM thiamine pyrophosphate (TPP), 100 µM flavine adenine dinucleotide (FAD) and 10% (v/v) glycerol were then added and the tissue homogenised for 30 seconds with an Ultra-Turrax homogeniser (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 30, 000 g for 20 min at 4°C and the supernatant filtered through one layer of miracloth (Behring Diagnostics, La Jolla, CA). The supernatant was brought to 60% (v/v) saturation by the dropwise addition of saturated ammonium sulfate and stirred for 30 min at 4°C . The crude enzyme pellet was collected by centrifugation for 30 min and then resuspended in 500 µl elution buffer (1 M potassium phosphate buffer [pH 7.5], 500 mM sodium pyruvate, 100 mM $MgCl_2$). The resuspended pellet was loaded on a Sephadex G-25 column (Pharmacia PD-10) equilibrated with elution buffer, and eluted with 1.2 ml of elution buffer. Enzyme assays were performed in 400 µl well microassay plates (Labsystems).

4.2.4 Determination of ALS kinetic parameters

The assays were initiated by the addition of 20 µl crude enzyme fraction to 20 µl of a series of concentrations of sodium pyruvate and 60 µl of assay buffer (1 M potassium phosphate buffer (pH 7.0), 100 mM $MgCl_2$, 50 mM TPP and 100 µM FAD). Enzyme activity for the pyruvate assays was expressed as nmol acetolactate produced mg^{-1} protein h^{-1} after determining acetoin content (Westerfield, 1945) and protein concentration (Bradford, 1976). The enzyme kinetic parameters, K_m and V_{max} were estimated using Eadie-Hofstee plots.

4.2.5 ALS inhibition by herbicides

The assays were incubated at 35°C for 30 min after the addition of 20 µl of herbicide solution (prepared in 20 mM KH_2PO_4, pH 7.0). The enzymic reaction was terminated by

the addition of 20 µl of 6N H_2SO_4 to each well, followed by a 15 min incubation at 60°C. Finally, 95 µl of 0.55% (w/v) creatine and 95 µl of 5.5% (w/v) naphthol (in 5N NaOH) were added. Following a second 15 min incubation at 60°C, the absorbance was measured at 530 nm by a microassay plate reader (Titertek Multiskan MCC/340). Background optical densities (determined by adding 20 µl 6N H_2SO_4 before the enzyme) were subtracted from the mean of 4 replicates. Activity was expressed as percentage of activity in the samples where no herbicide was added. I_{50} values were determined from logarithmic or linear regression analysis (depending on r^2) of ALS activity against herbicide concentration. Both ALS kinetic and herbicide experiments were repeated in triplicate with five and four replications, respectively. The data were pooled with standard error of the means calculated for each concentration.

4.3 RESULTS

To establish the mechanism of ALS herbicide resistance in biotypes of three weed species resistant to ALS herbicides, the target enzyme, ALS, was partially purified. Affinity of ALS for the substrate pyruvate, measured as the K_m (the concentration of pyruvate required for 50% ALS activity) was found to be similar between resistant and susceptible biotypes of all three species (Table 4.1, Figs. 4.1-4.3). Thus, resistance to ALS herbicides in the resistant biotypes is not due to a change in the affinity of the enzyme for pyruvate. Furthermore, enzyme specific activity of ALS (V_{max}) from susceptible and resistant *Son. oleraceus* and *B. tournefortii* biotypes were found to be similar within each species (Table 4.1, Figs. 4.1, 4.3). This suggests that ALS over-expression in resistant biotypes of *Son. oleraceus* and *B. tournefortii* is not contributing to resistance. However, the V_{max} for *Sis. orientale* biotypes SSO3 and NSO1 was 1.6- and 2.3-fold higher, respectively than for the susceptible biotype. Therefore, over-expression of ALS may be contributing slightly to resistance in these resistant biotypes.

Potentially, any mutations of ALS which could alter the binding site of ALS herbicides to the enzyme could result in resistance. To test this possibility, *in vitro* assays were

Table 4.1. The catalytic properties K_m (pyruvate) and V_{max} of ALS isolated from resistant and susceptible biotypes of *Son. oleraceus*, *Sis. orientale* and *B. tournefortii*. The catalytic properties were determined by regression analysis of Eadie-Hofstee plots from data obtained from three replicate experiments. Data is presented as mean K_m and Vmax with standard error of the means.

Biotype	K_m (μM)	V_{max} (nmol acetolactate mg^{-1} protein h^{-1})
Son. oleraceus		
R	13 ± 5	449 ± 123
S	16 ± 3	541 ± 85
Sis. orientale		
NSO1	16 ± 1	1246 ± 123
SSO3	14 ± 2	972 ± 98
S	17 ± 2	546 ± 176
B. tournefortii		
R	13 ± 3	153 ± 86
S	11 ± 3	259 ± 42

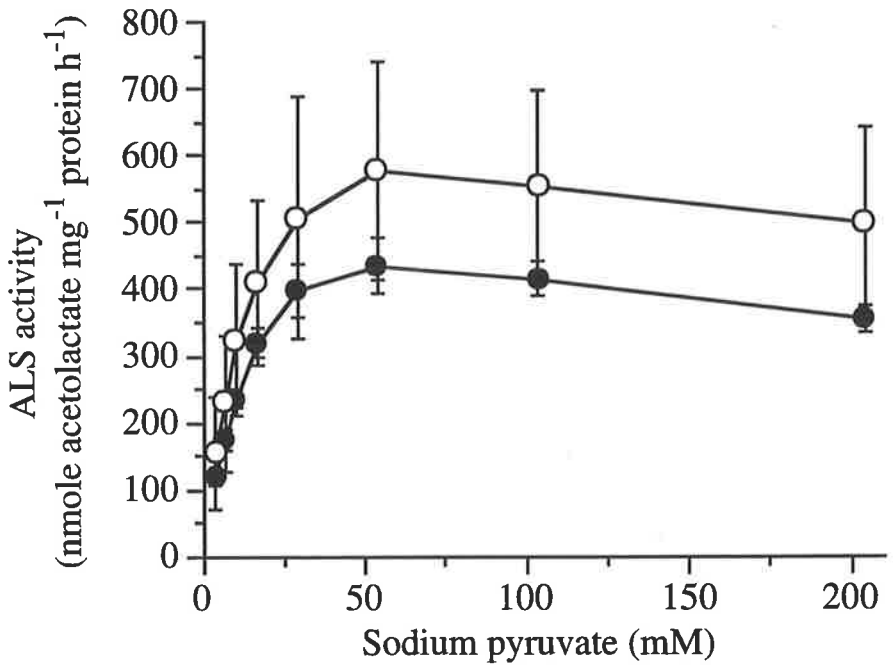


Figure 4.1. Pyruvate dependence of ALS activity in crude extracts from ALS herbicide resistant (●) and susceptible (O) *Son. oleraceus*. Each point is the mean of three experiments with five replicates conducted. Vertical bars represent the standard errors of the means.

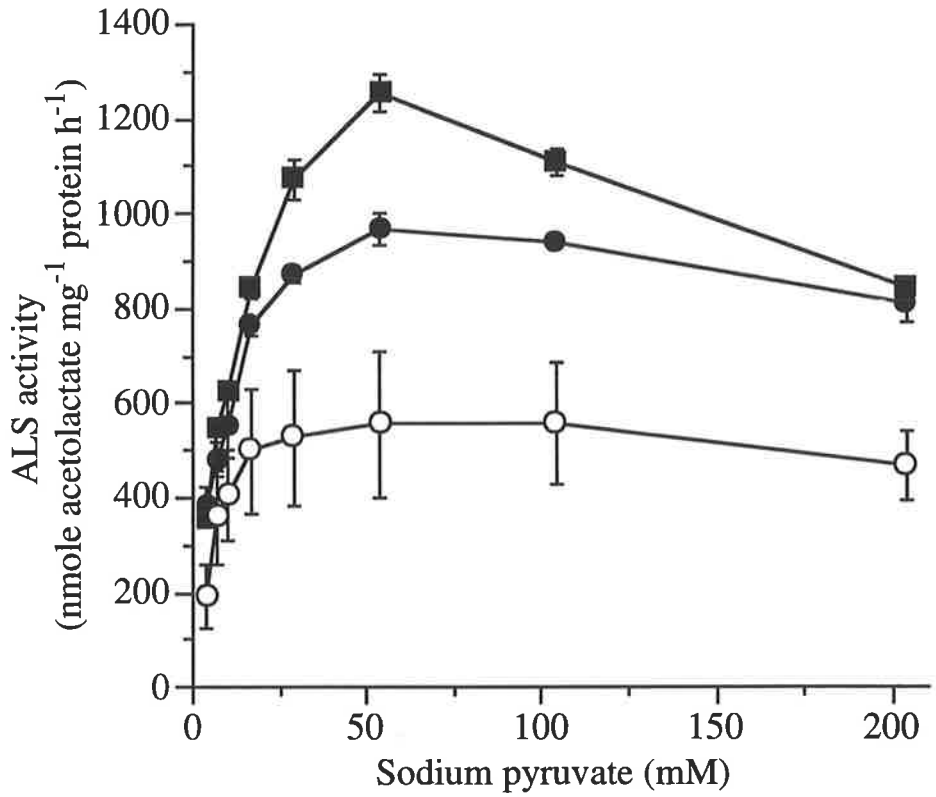


Figure 4.2. Pyruvate dependence of ALS activity in crude extracts from two ALS herbicide resistant *Sis. orientale* biotypes NSO1 (■), SSO3 (●) and a susceptible (○) biotype. Each point is the mean of three experiments with five replicates conducted. Vertical bars represent the standard errors of the means.

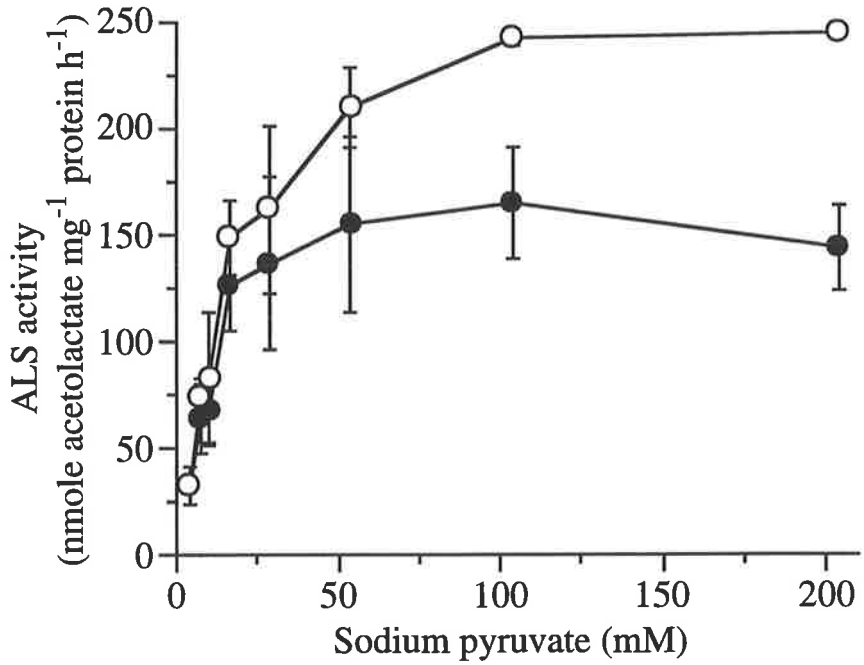


Figure 4.3. Pyruvate dependence of ALS activity in crude extracts from ALS herbicide resistant (●) and susceptible (○) *B. tournefortii*. Each point is the mean of three experiments with five replicates conducted. Vertical bars represent the standard errors of the means.

performed with various ALS herbicides using the optimum pyruvate concentration (104 μM) (Figs. 4.1-4.3). ALS from the resistant *Son. oleraceus* biotype was found to be highly resistant to the SU herbicide chlorsulfuron (Fig. 4.4a). The concentration of chlorsulfuron required to inhibit *in vitro* ALS activity from resistant biotypes by 50% (I_{50}) was found to be 14-fold that for the susceptible biotype (Tables 4.2). Furthermore, ALS from resistant *Son. oleraceus* was found to exhibit 20-fold resistance to the SU herbicide sulfometuron (Fig. 4.4b), 3.3-fold resistance to the triazolopyrimidine herbicide flumetsulam (Fig. 4.5c) and to be only slightly resistant to the imidazolinone herbicides imazethapyr and imazapyr (Table 4.2, Fig. 4.5a,b).

ALS from the three resistant *Sis. orientale* biotypes (Fig. 4.6) not only showed higher levels of resistance to SU, IM and TP herbicides than that from resistant *Son. oleraceus*, but also exhibited a different pattern of resistance to the three herbicide classes (Tables 4.2-4.3). There was also variation in the level of resistance between the *Sis. orientale* biotypes, with biotype NSO1 the most resistant to all three herbicide classes. This variation is unlikely to be due to dilution of resistant enzyme with susceptible in the other two biotypes, because all three were homogeneous for resistance, evident from the low variation in both survival and dry weight response of whole plants to ALS herbicides (Figs. 2.8-2.11).

ALS from the resistant *B. tournefortii* exhibited a higher level of resistance to chlorsulfuron than to flumetsulam, and showed no resistance to imazethapyr (Fig. 4.7, Table 4.4). This pattern is different to that observed with the resistant *Son. oleraceus* and *Sis. orientale* biotypes. Thus, variation in the pattern of resistance between the resistant species indicates two possibilities; the involvement of more than one mechanism, or the presence of different mutations within the herbicide binding domain of ALS.

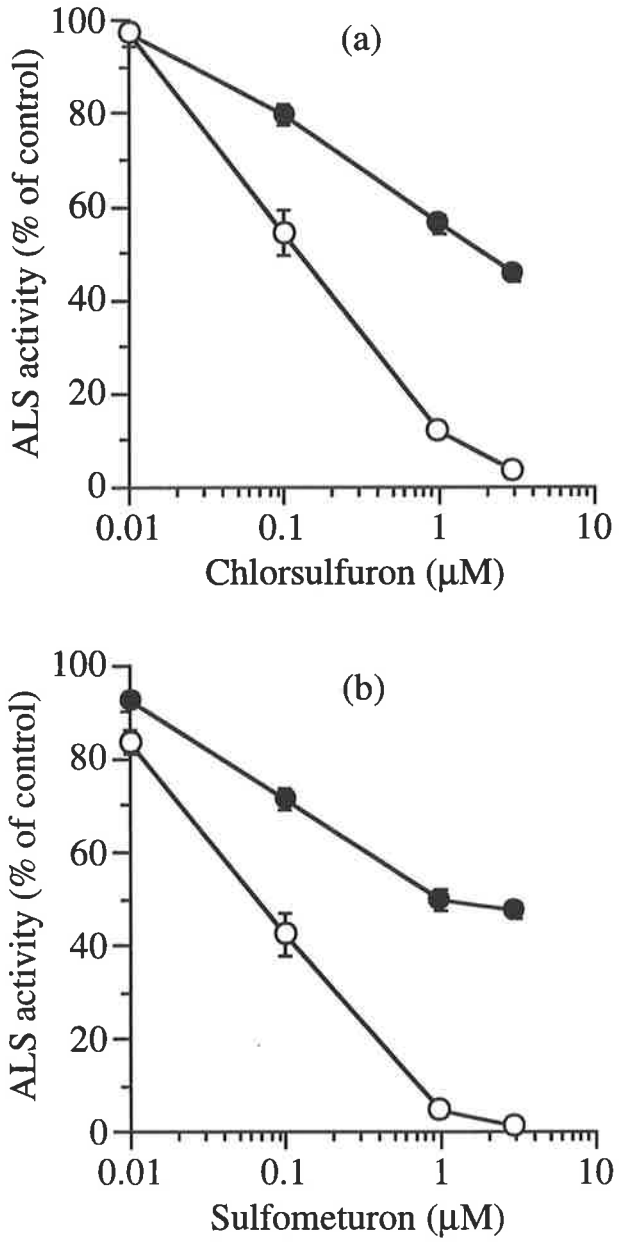


Figure 4.4. Inhibition of ALS isolated from resistant (●) and susceptible (○) *Son. oleraceus* biotypes by (a) chlorsulfuron and (b) sulfometuron. ALS activity is expressed as a percentage of activity in the absence of herbicide. Each point is the mean of three experiments, each containing four replicates. 100% activity was 337 ± 95 and 421 ± 88 nmol acetolactate mg^{-1} protein h^{-1} for resistant and susceptible, respectively. Vertical bars represent the standard errors of the means.

Table 4.2. I_{50} values for three classes of ALS herbicides: the SU herbicides chlorsulfuron and sulfometuron, the IM herbicides imazethapyr and imazapyr and the TP herbicide flumetsulam determined using crude extracts from resistant and susceptible *Son. oleraceus*. I_{50} values were estimated by regression analysis of linear or logarithmic plots depending of r^2 values.

Herbicide	R	S	R/S ratio[2]
	I_{50}[1] (μM)		
Chlorsulfuron	2.0	0.14	14
Sulfometuron	1.5	0.08	20
Imazapyr	75	53	1.4
Imazethapyr	50	43	1.2
Flumetsulam	1.0	0.31	3.3

(1) I_{50} is defined as the concentration of herbicide required to inhibit enzyme activity by 50 %.

(2) I_{50} ratio calculated by dividing the I_{50} of resistant ALS by the I_{50} of susceptible ALS.

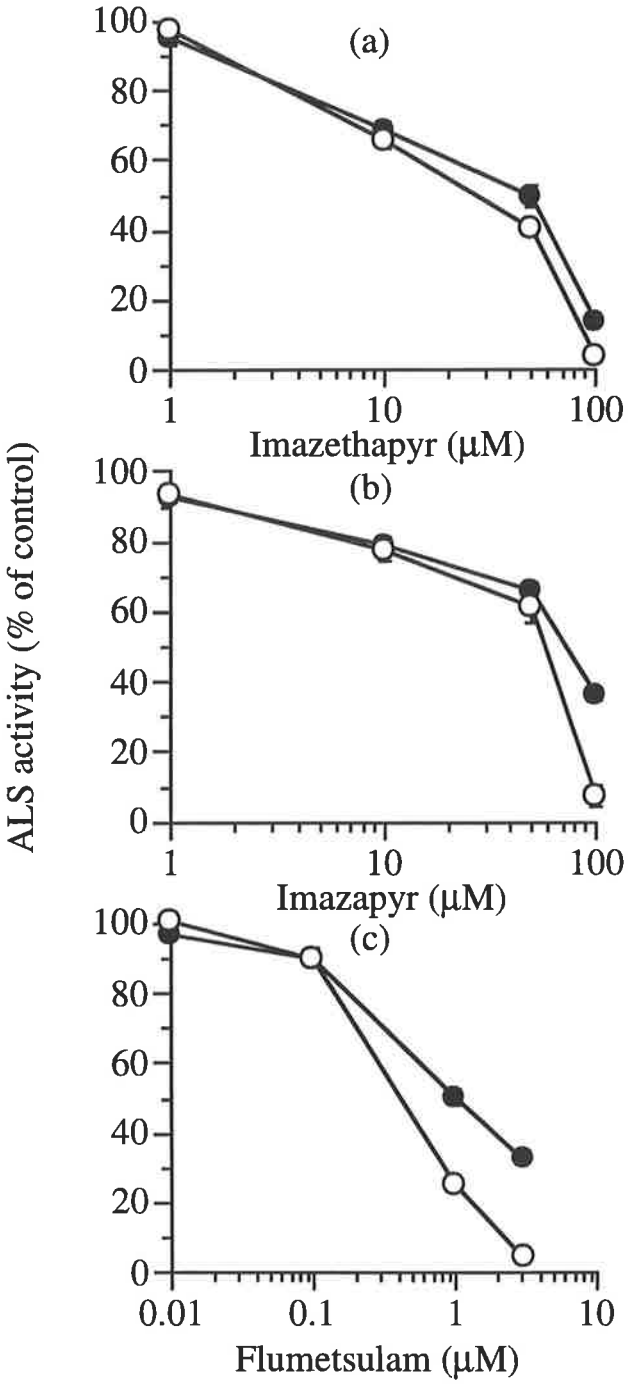


Figure 4.5. Inhibition of ALS isolated from resistant (●) and susceptible (○) *Son. oleraceus* biotypes by (a) imazethapyr, (b) imazapyr and (c) flumetsulam. ALS activity is expressed as a percentage of activity in the absence of herbicide. Each point is the mean of three experiments, each containing four replicates. 100% activity was 337 ± 95 and 421 ± 88 nmol acetolactate mg^{-1} protein h^{-1} for resistant and susceptible, respectively. Vertical bars represent the standard errors of the means.

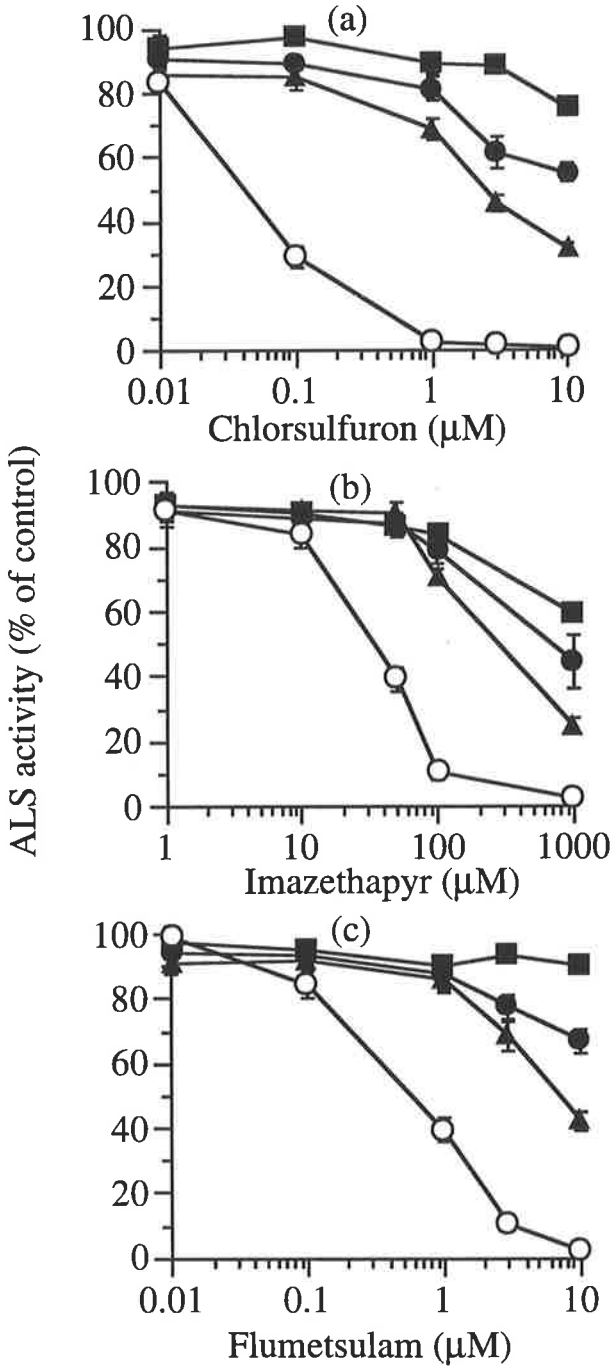


Figure 4.6. Inhibition of ALS isolated from *Sis. orientale* resistant biotypes NSO1 (■), SSO3 (●), SSO1 (▲) and a susceptible (O) biotype by (a) chlorsulfuron, (b) imazethapyr and (c) flumetsulam. ALS activity is expressed as a percentage of activity in the absence of herbicide. Each point is the mean of three experiments, each containing four replicates. 100% activity was 1369 ± 261, 900 ± 98, 620 ± 123 and 605 ± 99 nmol acetolactate mg^{-1} protein h^{-1} for biotypes NSO1, SSO3, SSO1 and susceptible, respectively. Vertical bars represent the standard errors of the means.

Table 4.3. I_{50} values for the ALS inhibiting herbicides chlorsulfuron, imazethapyr and flumetsulam determined with crude extracts of resistant biotypes NSO1, SSO3 and SSO1 and susceptible *Sis. orientale*.

Herbicide	NSO1	SSO3	SSO1	S	NSO1/S ratio	SSO3/S ratio	SSO1/S ratio
		(μM)					
Chlorsulfuron	>10	>10	5.4	0.06	>167	>167	90
Imazethapyr	>1000	864	304	47	>21.3	18.4	6.5
Flumetsulam	>10	>10	8.1	0.7	>14.3	>14.3	11.6

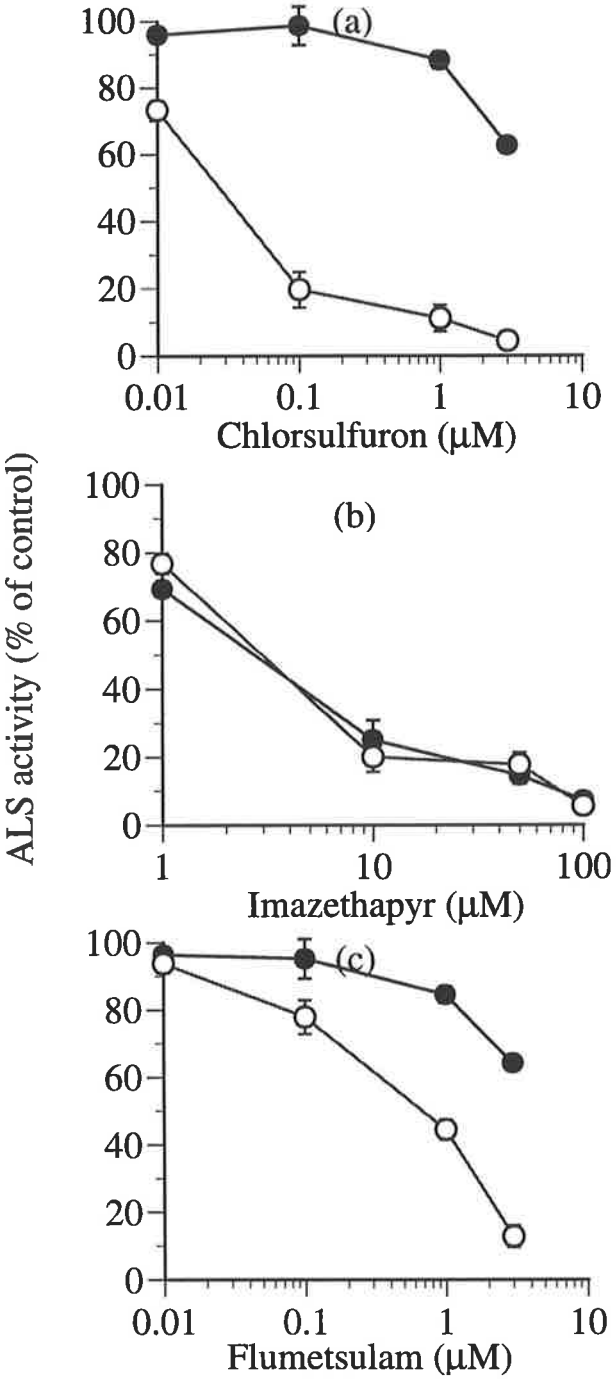

ALS activity (% of control)
100
80
60
40
20
0
(a)
0.01
0.1
1
10
Chlorsulfuron (μM)
(b)
1
10
100
Imazethapyr (μM)
(c)
Flumetsulam (μM)

Table 4.4. I_{50} values for the ALS inhibiting herbicides chlorsulfuron, imazethapyr and flumetsulam determined with crude extracts of resistant and susceptible *B. tournefortii*.

Herbicide	R	S (μM)	R/S ratio
Chlorsulfuron	>3	0.03	>100
Imazethapyr	3.1	3.8	0.8
Flumetsulam	>3	1.3	>2.3

4.4 DISCUSSION

Possible biochemical mechanisms endowing herbicide resistance in plants include increased herbicide detoxification, reduced uptake and/or translocation, sequestration, and modification of the herbicide target site (Powles and Holtum, 1994). Both target site and non-target site resistance mechanisms have been reported for the SU, IM and TP herbicides (reviewed in Saari et al., 1994). The present study investigated whether resistance could be endowed by changes in ALS, the target site for ALS herbicides, in resistant *Sis. orientale*, *Son. oleraceus* and *B. tournefortii* biotypes. It also examined whether increased levels of the ALS enzyme could endow resistance to herbicides.

Increased catalytic efficiency or increased levels of an enzyme can be measured as increased specific activity. Any increase in the specific activity of ALS could cause resistance to ALS herbicides. However, this is not the mechanism of resistance in *Son. oleraceus* and *B. tournefortii* resistant biotypes as similar ALS activities were measured between susceptible and resistant biotypes from each species (Table 4.1). Similarly, other studies of different weed species resistant to ALS herbicides have shown no differences in ALS activity between susceptible and resistant biotypes (Christopher et al., 1992;

Matthews et al., 1990; Saari et al., 1990, 1992). A 2.3 and 1.6-fold increase in ALS activity over susceptible levels was measured for *Sis. orientale* biotypes NSO1 and SSO3, respectively. Whilst ALS resistant field selected weeds with increased enzyme activity have not been reported, resistant plant cell lines of *Daucus carota* with ALS gene amplification giving rise to increased ALS activity (Caretto et al., 1994) and tobacco mutants, resistant with two mechanisms (a gene amplification and a resistant ALS gene) (Harms et al., 1992) have been selected with SU herbicides. What level of resistance these two examples of increased ALS activity would give at the whole plant level is unknown.

Reduced vigour is often observed with plant biotypes resistant to triazine herbicides due to a modified target site (reviewed in Holt and Thill, 1994). This is due to reduced binding of Q_B to the D1 protein of Photosystem II and consequently reduced electron transport (reviewed in Gronwald, 1994). It follows that reduced fitness of field selected ALS resistant plants by possession of a herbicide resistant ALS might reduce the affinity of ALS for the substrate, pyruvate. For example, ALS resistant *D. innoxia* cell lines with higher K_m values have lower branched-chain amino acid levels (Rathinasabapathi and King, 1991). However, there was no significant difference in K_m for pyruvate between ALS isolated from resistant and susceptible biotypes of *Son. oleraceus*, *Sis. orientale* or *B. tournefortii* (Table 4.1). Similarly, ALS extracted from susceptible and resistant *K. scoparia* selected with field applications of chlorsulfuron displayed no difference in affinity for pyruvate (Saari et al., 1990). Therefore the mutations conferring resistance to ALS in the above mentioned field selected material do not affect binding of the substrate.

The results summarised in Tables 4.2-4.4 establish that the resistant biotypes of *Son. oleraceus*, *Sis. orientale* and *B. tournefortii* examined exhibit target site cross-resistance. Although the resistant biotypes were selected in the field with SU herbicides, their spectrum of resistance varies across three classes of ALS herbicides (Tables 4.2-4.4, Figs. 4.4-4.7). This is probably due to their possessing different amino acid substitutions that alter binding of herbicides to the enzyme. For example, the mutation(s) conferring resistance in *Son. oleraceus* give(s) high resistance to SU herbicides, intermediate resistance to flumetsulam and low resistance to IM herbicides (Table 4.2). This pattern has

also been reported for other resistant weed species (Devine et al., 1991; Christopher et al., 1992; Saari et al., 1992). In contrast, the mutation(s) in all three resistant *Sis. orientale* biotypes endows high resistance to SU, IM and TP herbicides (Table 4.3). ALS from the resistant *B. tournefortii* has high SU and TP resistance with susceptibility to IM herbicides which is a different pattern again (Table 4.4).

A strong relationship was observed between whole plant response to ALS herbicides and the effect of these herbicides on ALS assayed *in vitro*. For example, both whole plants and ALS isolated from resistant *Son. oleraceus* were resistant to chlorsulfuron and sulfometuron with low resistance to IM herbicides (Tables 2.1, 4.2). Furthermore, the correlations between whole plant and ALS responses to chlorsulfuron, imazethapyr and flumetsulam from *Sis. orientale* biotypes NSO1 and SSO3 were similar (Tables 2.2, 4.3). For example, biotype NSO1 was more resistant to SU, IM and TP herbicides at both the whole plant and ALS levels than biotype SSO3 (Fig 4.3). This suggests that the modified ALS is the major mechanism of resistance in all of these biotypes and that other possible mechanisms contribute little or not at all.

CHAPTER 5

5.0. MOLECULAR BASIS OF RESISTANCE TO HERBICIDES INHIBITING ALS

5.1 INTRODUCTION

The molecular basis for resistance to ALS herbicides based on target site enzyme changes has been thoroughly investigated in bacteria, yeast and higher plants selected for resistance in the laboratory (reviewed in Saari et al., 1994). ALS genes from several plant species have now been cloned and sequenced from herbicide susceptible and resistant biotypes. In all cases reported, the mutations identified in the ALS protein conferring resistance are each encoded by a single nucleotide change.

As discussed in Section 1.5.5 above, it appears that *A. thaliana* (a diploid species) possesses only one form of the ALS enzyme and one copy of the ALS gene (Mazur et al., 1987). In contrast, most other diploid and tetraploid species examined to date appear to have multiple

copies of the ALS gene, although not all copies are active (Lee et al., 1988; Mazur and Falco, 1989; Rutledge et al., 1991; Grula et al., 1995). Certain *Brassica* species, both diploid (*B. campestris*) and allotetraploid (*B. napus*) have been shown to encode and express at least two distinct ALS isoforms (Rutledge et al., 1991). Of three active ALS genes in *B. napus*, one (ALS II) is only expressed in floral tissues and shares only 85% amino acid identity with ALS I and III (Ouellet et al., 1992). The latter two (ALS I and ALS III) share 98% sequence similarity, are both constitutively expressed and are thought to be homologues of the targets for herbicide selection leading to resistance in field isolates of weed species (Rutledge et al., 1991).

Investigations of the molecular basis of ALS herbicide resistance in higher plants have centred around five highly conserved regions (Domains A-E) ranging in size from 16 to 39 bp (Fig. 1.3; Table 1.7). Single nucleotide substitutions encoding amino acid changes at the protein level within each of the five domains have been shown to independently confer resistance to ALS herbicides in laboratory strains (Section 1.5.5, Table 1.7). Three of these five sites have been observed to vary in field selected ALS resistant weeds, Pro in Domain A (Guttieri et al., 1992, 1995), Trp in Domain B and Ala in Domain C (Bernasconi et al., 1995; Table 1.8).

Six different amino acid substitutions, each resulting from single base change at Pro_{173} (CCG) have been identified in resistant *K. scoparia* (Table 1.8; Guttieri et al., 1995). Gutierri et al. (1992) have also reported a single base change at Pro_{173} for *L. serriola* causing substitution of Pro for His (Table 1.8). Recently, Guttieri and Eberlein (pers. comm.) have reported a Pro_{173} to Leu change in Domain A of the ALS gene of an ALS resistant *S. iberica* biotype. Unfortunately, ALS inhibition studies have been done only with sulfonylurea herbicides for *K. scoparia*, *L. serriola* and *S. iberica* so their spectrum of herbicide resistance is unknown.

Single nucleotide changes conferring mutations at two other sites were identified in two ALS herbicide resistant cocklebur (*X. strumarium*) biotypes (Table 1.8; Bernasconi et al., 1995). ALS inhibition studies on one biotype with a Trp_{552} to Leu change in Domain B showed this substitution endows an ALS with high level resistance to all four classes of ALS herbicides

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(Bernasconi et al., 1995), identical to the mutation engineered in corn line 3180 IR resistant to all ALS herbicide classes (Siehl et al., 1996). A second resistant cocklebur biotype had an Ala_{133} to Thr mutation in Domain C (Bernasconi et al., 1995), identical to the mutation in ALS resistant corn line ICI 8532 IT, conferring resistance to IM herbicides only (Siehl et al., 1996; Table 1.8). These findings suggest that mutations within the different domains of ALS confer different resistance patterns. Mutations in Domains D and E conferring ALS resistance have not yet been identified in field selected weeds but have been selected in the laboratory.

In Chapter 4 it was shown that three biotypes of *Sis. orientale* and one biotype of *B. tournefortii* are resistant to ALS herbicides as a result of modifications of ALS. Here the molecular basis for herbicide resistance of these biotypes is determined.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Seeds of resistant *Sis. orientale* biotypes NSO1, SSO3 and SSO1, the resistant *B. tournefortii* biotype and the standard susceptible biotype from each species were germinated and grown in a growth room as described previously (section 4.2.2). At the five leaf stage the youngest emerged leaf was removed from plants and frozen at -70^{o}C. To ensure that the plants from which leaves were taken were truly susceptible or resistant, the plants were then sprayed with 23 g ha^{-1} sulfometuron plus 0.2% surfactant in a laboratory sprayer. Twenty one days after spraying only plants from the susceptible biotypes were dead (data not shown).

5.2.2 Genomic DNA extraction

Individual leaves, collected from resistant and susceptible plants as described above, were frozen in liquid nitrogen and ground to a fine powder. The powder was homogenised in

DNA extraction buffer (1% sarkosyl, 100 mM Tris-HCl, pH 8.5, 100 mM NaCl and 10 mM EDTA). Samples were then extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), genomic DNA precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 4.8 and two volumes isopropanol, and centrifuged for 5 min to pellet the DNA. After washing for 5 min with 70% ethanol, the DNA was resuspended in 50 µl of TE (10 mM Tris HCl, pH 8.4, 1 mM EDTA) containing 40 µg ml^{-1} RNase (Boehringer Mannheim).

5.2.3 Oligonucleotide primers

Six oligonucleotide primers (Table 5.1) were synthesised by the Centre for Basic and Applied Plant Molecular Biology of the University of Adelaide for sequencing of two regions of genomic DNA (regions 1 and 2) of the ALS gene (Fig. 5.1). These two regions contain sites that have previously been shown to encode amino acid substitutions conferring ALS resistance (Guttieri et al., 1992, 1995; Hattori et al., 1995; Bernasconi et al., 1995; Hartnett et al., 1990). Primers 5 and 4 were used to PCR amplify a 1665 bp fragment of ALS from resistant and susceptible *B. tournefortii* and *Sis. orientale* genomic DNA (Fig. 5.1). This fragment represents all but 72bp of the nucleotide sequence encoding the mature ALS peptide in higher plants (Fig. 5.1). Primers 1 to 6 were then used for sequencing regions 1 and 2 of these PCR products. Primers 1 and 2 are identical to primers 1 and 4, respectively from Guttieri et al. (1992). Primers 3 to 6 were designed by the author, primers 3 to 5 from regions of high similarity among the published sequences of *B. napus*, *N. tabacum* and *A. thaliana* (EMBL Nucleotide Sequence Database). Primer 6 was designed to specifically bind to region 1 of the 1665 bp *B. tournefortii* PCR product (Fig 5.1).

Table 5.1. Nucleotide sequences of the six oligonucleotide primers used for PCR amplification and sequencing of regions 1 and 2 (Fig. 5.1) of *Sis. orientale* and *B. tournefortii* ALS genes.

Primer	Orientation	Sequence
1.	5' --> 3'	5' GCATGTCTAGAACGTCCTTCC(T/C)CGTCACGAACA 3'
2.	3' --> 5'	5' CGTGGATCCT(A/C)GTTACCTCAACAA 3'
3.	5' --> 3'	5' GTTGTTGACATTGA(C/T)GG(C/T)GATGG 3'
4.	3' --> 5'	5' GAA(G/A)GTGCC(G/A)CCACT(A/T)GGGAT 3'
5.	5' --> 3'	5' ATCCT(C/G)GT(C/G)GAAGCCCT(C/G)GAGCGTCA 3'
6.	3' --> 5'	5' TGGTGTCTCCTGGAACGCGTC 3'

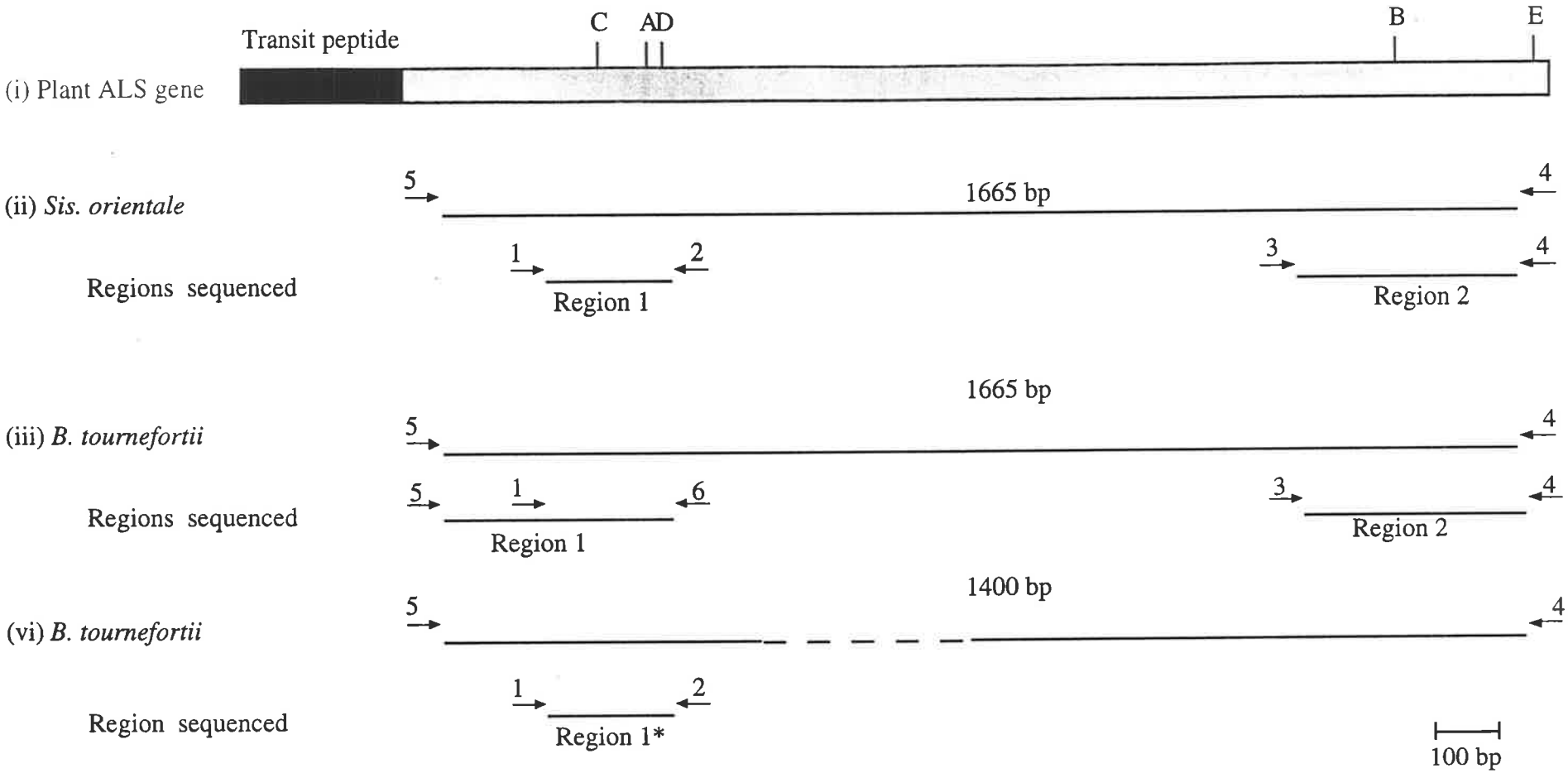


Figure 5.1. (i) Schematic representation of a plant ALS gene showing the relative positions of the five conserved domains (C, A, D, B, E) and transit peptide. Diagrams below represent the 1665 bp PCR fragments amplified from (ii) *Sis. orientale* and (iii) *B. tournefortii* genomic DNA using primers 5 and 4 (vi). The dashed line in the shorter 1400 bp *B. tournefortii* product also amplified with primers 5 and 4 indicates nucleotide deletions in one or more unidentified regions. Regions 1, 1* and 2 were subsequently sequenced using combinations of primers 1 to 6 (presented as labelled arrows).

5.2.4 Polymerase chain reaction

PCR amplifications were conducted in 50 µl reactions containing a 1:10 dilution of the genomic DNA extract, 0.2 µM of each primer combination, 200 µM of each deoxynucleoside-5'-triphosphate (dNTP), 3.5 mM $MgCl_2$, 5 µl of 10x thermophilic buffer (Promega, WI, USA), 2.5 units of Promega Taq polymerase and covered with mineral oil. The amplification cycle used was denaturation at 94°C for 1.5 min, ramp to 60°C at 2 s^oC^{-1}, anneal at 60°C for 2.0 min, ramp to 72°C at 3 s^oC^{-1}, elongate at 72°C for 2.0 min, ramp to 94°C at 2 s^oC^{-1}, for 34 cycles. The PCR products were separated electrophoretically on 1.5% SeaKem LE agarose gel (FMC BioProducts, ME, USA) and the relevant ethidium bromide stained band excised. Purification of the excised DNA for sequencing was performed with a BANDPURE™ DNA purification kit (Progen Industries Ltd., Australia).

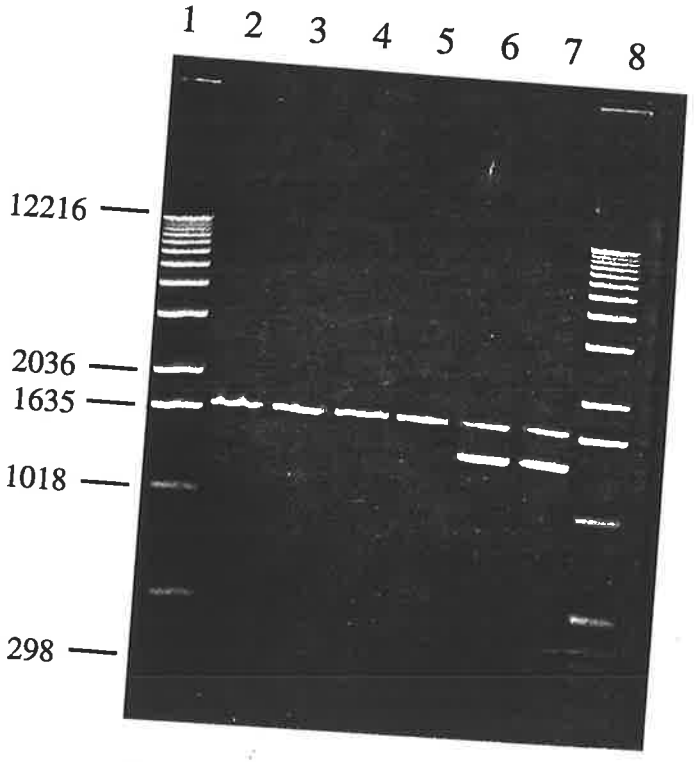
5.2.5 DNA sequencing

The double stranded purified PCR product was sequenced with an Applied Biosystems Model 373A DNA sequencer using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems, Australia Pty. Ltd.). The DNA regions were sequenced in both directions in duplicate.

5.3 RESULTS

PCR amplification of *Sis. orientale* and *B. tournefortii* genomic DNA with primers 5 and 4 (Fig. 5.1) produced one ethidium bromide stained band of the expected size (1665 bp) for each *Sis. orientale* biotype but two bands (1665 bp and 1400 bp) for each *B. tournefortii* biotype (Plate 5.1). When compared to the only other published sequences available for the Brassicacea (ALS I, ALS II and ALS III of the allotetraploid *B. napus,* Rutledge et al., 1991) it is clear that the inferred amino acid sequences in regions 1 and 2 of the 1665 bp

Plate 5.1. Ethidium bromide stained PCR products amplified from genomic DNA of *Sis. orientale* biotypes SSO2 (lane 2), SSO1 (lane 3), SSO3 (lane 4) and NSO1 (lane 5), and the susceptible (lane 6) and resistant (lane 7) *B. tournefortii* biotypes using primers 5 and 4. Lanes 1 and 8 were loaded with 216 ng of a DNA marker (1 kb ladder, BRL). Marker units are base pairs.



fragments from *Sis. orientale* and *B. tournefortii* most closely resemble the constitutively expressed *B. napus* ALS I and ALS III genes (Table 5.2, Fig. 5.2) and are more distantly related to the flower-specific ALS II. In contrast, the region 1 sequence from the 1400 bp PCR product obtained from both *B. tournefortii* biotypes appears to be more closely related to the ALS II gene of *B. napus* (Table 5.2, Fig. 5.2). Thus, it appears that there are at least two genes with similarity to ALS in *B. tournefortii*. However, the fact that the 1400 bp fragment is around 265 bp shorter than the 1665 bp from all active ALS genes sequenced to date implies that the smaller fragment is either from an inactive gene or has a different function. For this reason investigation of mutations conferring herbicide resistance has been concentrated on the 1665 bp fragments from both species.

At the amino acid level, region 1 from both *B. tournefortii* and *Sis. orientale* are identical to the *B. napus* ALS I and ALS III sequences, but the four clearly differ at the nucleotide sequence level. Figures 5.3 and 5.4 compare the nucleotide sequences of regions 1 and 2 of the 1665 bp PCR products amplified from susceptible biotypes of *Sis. orientale* and *B. tournefortii* and region 1* of the 1400 bp product from *B. tournefortii* to the corresponding sequences from the three *B. napus* ALS genes. Ignoring the ALS II and 1* sequences, both regions show that as expected *B. tournefortii* is more closely related to *B. napus* than either is to *Sis. orientale*, which reflects the classical systematics of these species.

Four (Domains A to D) of the five conserved domains shown to endow, as a result of a single change in their nucleotide sequence, resistance to ALS herbicides in higher plants, have been sequenced for each biotype of *Sis. orientale* and *B. tournefortii*. Domain E could not be sequenced in this study due to its proximity to the 3' end of the coding region, its nucleotide sequence forming part of primer 4. Domains A, C and D are encoded by sequenced region 1, Domain B by sequenced region 2 (Fig. 5.1).

Table 5.2. Percentage identity among inferred ALS amino acid sequences from susceptible biotypes of higher plants. Figures above the diagonal compare 1: a 64 residue segment (corresponding to residues 127-191 in the *B. napus* ALS III sequence) of sequenced region 1 from the 1665 bp fragments of *B. tournefortii* (*B. tou*) and *Sis. orientale* (*S. ori*), the 1400 bp fragment of *B. tournefortii* (*B. tou* *) and 2: a 109 residue segment (corresponding to residues 522-630 in the *B. napus* ALS III sequence) of sequenced region 1 from the 1665 bp fragments of *B. tournefortii* (*B. tou*) and *Sis. orientale* (*S. ori*) to corresponding regions of the three *B. napus* (*B. nap* III, I and II), *A. thaliana* (*A. thal*), *N. tabacum* (*N. tab*) and *Z. mays* ALS sequences (Genbank accession numbers Z11526, Z11524, Z11525, X51514, X07644 and X63553, respectively). Figures below the diagonal show similarities for the sequences of the entire mature ALS peptides (residues 79 to 652 in the *B. napus* ALS III sequence) from the latter four species. Though in general similarities in region 1 are higher but those in region 2 are lower than for the entire mature peptide, the relationships between the various loci remain the same.

	B. tou	*B. tou* *	*S. ori*	*B. nap* I	*B. nap* II	*B. nap* III	*A. thal*	*N. tab*	*Z. mays*
B. tou		1: 86 2: na	1: 100 2: 95	1: 100 2: 98	1: 89 2: 79	1: 100 2: 98	1: 97 2: 94	1: 88 2: 85	1: 84 2: 73
B. tou *	na		1: 86 2: na	1: 86 2: na	1: 91 2: na	1: 86 2: na	1: 89 2: na	1: 80 2: na	1: 75 2: na
S. ori	na	na		1: 100 2: 96	1: 89 2: 80	1: 100 2: 96	1: 98 2: 95	1: 91 2: 84	1: 86 2: 71
B. nap I	na	na	na		1: 89 2: 78	1: 100 2: 100	1: 98 2: 93	1: 91 2: 84	1: 91 2: 73
B. nap II	na	na	na	85		1: 89 2: 78	1: 92 2: 82	1: 84 2: 73	1: 86 2: 61
*B. nap*III	na	na	na	99	85		1: 98 2: 93	1: 91 2: 84	1: 91 2: 73
A. thal	na	na	na	94	85	94		1: 92 2: 84	1: 88 2: 72
N. tab	na	na	na	84	78	84	83		1: 86 2: 73
Z. mays	na	na	na	77	73	77	76	76	

na: not available

```
Region 1
                                                                  Dom C
B. n III  ILVEALERQGVETVFAYPGGASMEIHQALTRSSTIRNVLPRHEQGGVFAAEGYARSSGKP 143
B. n I    ............................................................
B. n II   ...........DV.................N............I.............
B. tou        ........................................................
B. tou *                                                 .........T......
S. ori                                                   ..............

                                                Dom A      Dom D
B. n III  GICIATSGPGATNLVSGLADAMLDSVPLVAITGQVPRRMIGTDAFQETPIVEVTRSITKH 203
B. n I    ............................................................
B. n II   ...........M.........LF.....I.............M......V.....T....
B. tou    ............................................................
B. tou *  ...M.......M.........LF.....I.T......G....M.....
S. ori    ..................................................

B. n III  NYLVMDVDDIPRIVQEAFFLATSGRPGPVLVDVPKDIQQQLAIPNWDQPMRLPGYMSRLP 263
B. n I    ............................................................
B. n II   .....E........R........V......I.....V...F.....E......L...TM.
B. tou    ........
                                      .
                                      .
                                      .
Region 2
                                                                  Dom B
B. n III  IGASVANPDAIVVDIDGDGSFIMNVQELATIRVENLPVKILLLNNQHLGMVMQWEDRFYK 562
B. n I    ............................................................
B. n II   M..AI...G.V.............I..............V..I........L....H..A
B. tou                ..................................................
S. ori                      ..........................................

B. n III  ANRAHTYLGDPARENEIFPNMLQFAGACGIPAARVTKKEELREAIQTMLDTPGPYLLDVI 622
B. n I    ............................................................
B. n II   ....DSF.....NPEAV..D..L..AS.........RR.D..............F....V
B. tou    ..............S.........................D...................
S. ori    ......F.....K............A.............N...................

                     Dom E
B. n III  CPHQEHVLPMIPSGGTF                                          639
B. n I    .................
B. n II   ....D....L.......
B. tou    ........
S. ori    ..........
```

Figure 5.2. Comparison of inferred amino acid sequences (single letter code) of regions 1 and 2 of the 1665 bp fragment from susceptible biotypes of *Sis. orientale* (*S. ori*) and *B. tournefortii* (*B. tou*) and from region 1 of the 1400 bp fragment of *B. tournefortii* (*B. tou* *) to the three published ALS sequences (*B. n* III, *B. n* I, *B. n* II) from susceptible *B. napus* (Rutledge et al., 1991). Dots in the lower five sequences indicate matches to the reference *B. napus* ALS III sequence (numbered from Met1), differences indicated by single amino acid code of the altered residue. The peptide sequences of Domains A to E are underlined in the reference sequence.

Figure 5.3. Comparison of nucleotide sequences of region 1 of the 1665 bp fragment from susceptible biotypes of *Sis. orientale* (*S. ori*) and *B. tournefortii* (*B. tou*), and from the 1400 bp fragment of *B. tournefortii* (*B. tou* *) to the three published ALS genes (*B. n* III, *B. n* I, *B. n* II) from susceptible *B. napus* (Rutledge et al., 1991; Genbank accession numbers Z11526, Z11524 and Z11525, respectively). Dots in the lower five sequences indicate matches to the reference *B. napus* ALS III nucleotide sequence (numbered as in Rutledge et al., 1991), differences indicated by A, C, G or T. Positions of the four primers used to amplify and sequence this region shown as underlined regions in the reference sequence, each is labelled above and its orientation indicated by an arrow. The peptide sequences of Domains A, C, and D are boxed in the inferred amino acid sequence (single-letter code, numbered from Met1) of the reference sequence included above.

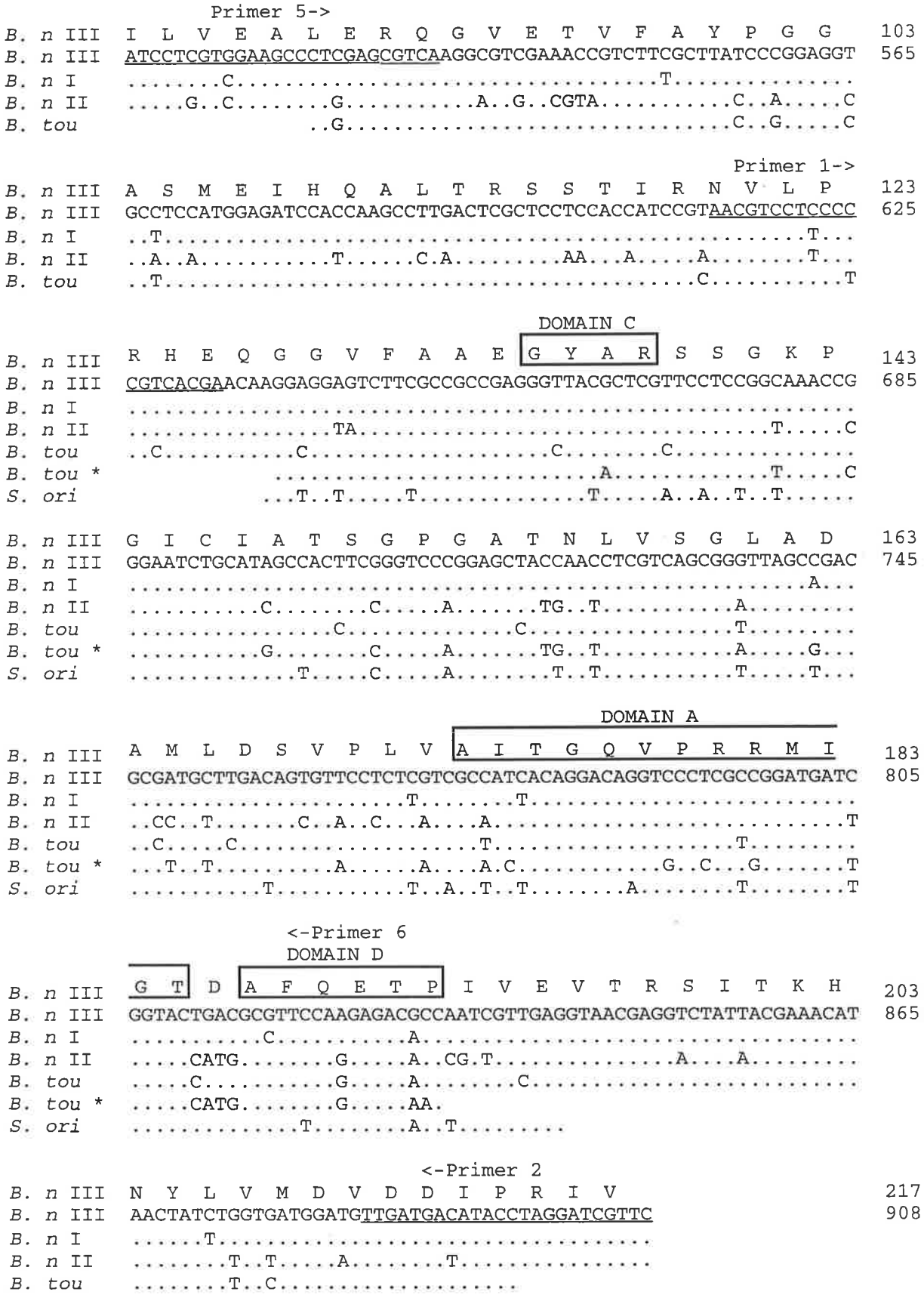

Primer 5->
B. n III I L V E A L E R Q G V E T V F A Y P G G 103
B. n III ATCCTCGTGGAAGCCCTCGAGCGTCAAGGCGTCGAAACCGTCTTCGCTTATCCCGGAGGT 565
B. n I .....C.....T.....
B. n II .....G..C.....G.....A..G..CGTA.....C..A.....C
B. tou ..G.....C..G.....C
Primer 1->
B. n III A S M E I H Q A L T R S S T I R N V L P 123
B. n III GCCTCCATGGAGATCCACCAAGCCTTGACTCGCTCCTCCACCATCCGTAACGTCCTCCCC 625
B. n I ..T.....T...
B. n II ..A..A.....T.....C.A.....AA...A.....A.....T...
B. tou ..T.....C.....T
DOMAIN C
B. n III R H E Q G G V F A A E G Y A R S S G K P 143
B. n III CGTCACGAACAAGGAGGAGTCTTCGCCGCCGAGGGTTACGCTCGTTCCTCCGGCAAACCG 685
B. n I .....
B. n II .....TA.....T.....C
B. tou ..C.....C.....C.....C.....
B. tou * .....A.....T.....C
S. ori ...T..T.....T.....T.....A..A..T..T.....
B. n III G I C I A T S G P G A T N L V S G L A D 163
B. n III GGAATCTGCATAGCCACTTCGGGTCCCGGAGCTACCAACCTCGTCAGCGGGTTAGCCGAC 745
B. n I .....A...
B. n II .....C.....C.....A.....TG..T.....A.....
B. tou .....C.....C.....T.....
B. tou * .....G.....C.....A.....TG..T.....A.....G...
S. ori .....T.....C.....A.....T..T.....T.....T...
DOMAIN A
B. n III A M L D S V P L V A I T G Q V P R R M I 183
B. n III GCGATGCTTGACAGTGTTCCTCTCGTCGCCATCACAGGACAGGTCCCTCGCCGGATGATC 805
B. n I .....T.....T.....
B. n II ..CC..T.....C..A..C...A....A.....T
B. tou ..C.....C.....T.....T.....
B. tou * ...T..T.....A.....A....A.C.....G..C...G.....T
S. ori .....T.....T..A..T..T.....A.....T.....T
<-Primer 6
DOMAIN D
G T D A F Q E T P I V E V T R S I T K H 203
B. n III GGTACTGACGCGTTCCAAGAGACGCCAATCGTTGAGGTAACGAGGTCTATTACGAAACAT 865
B. n I .....C.....A.....
B. n II .....CATG.....G.....A..CG.T.....A....A.....
B. tou .....C.....G.....A.....C.....
B. tou * .....CATG.....G.....AA.
S. ori .....T.....A..T.....
<-Primer 2
B. n III N Y L V M D V D D I P R I V 217
B. n III AACTATCTGGTGATGGATGTTGATGACATACCTAGGATCGTTC 908
B. n I .....T.....
B. n II .....T..T.....A.....T.....
B. tou .....T..C.....

Figure 5.4. Comparison of nucleotide sequences of region 2 of the 1665 bp fragment from susceptible biotypes of *Sis. orientale* (*S. ori*) and *B. tournefortii* (*B. tou*) to the three published ALS genes (*B. n* III, *B. n* I, *B. n* II) from susceptible *B. napus* (Rutledge et al., 1991). Dots in the lower four sequences indicate matches to the reference *B. napus* ALS III nucleotide sequence (numbered as in Rutledge et al., 1991), differences indicated by A, C, G or T. Positions of the two primers used to amplify and sequence this region shown as underlined regions in the reference sequence, each is labelled above and its orientation indicated by an arrow. The peptide sequences of Domains B and E are boxed in the inferred amino acid sequence (single-letter code, numbered from Met1) of the reference sequence included above.

```
                Primer 3->
B.n III V  V  D  I  D  G  D  G  S  F  I  M  N  V  Q  E  L  A  T  I     533
B.n III GTTGTGGACATTGACGGTGATGGAAGCTTCATAATGAACGTTCAAGAGCTGGCCACAATC  1856
B.n I   ........T.....................................................
B.n II  .....C........T..G.....T........C......A.......A.....A..C...
B.tou   ..C.................C.....................................
S.ori                        ..................G........C.........

B.n III R  V  E  N  L  P  V  K  I  L  L  L  N  N  Q  H  L  G  M  V     553
B.n III CGTGTAGAGAATCTTCCTGTGAAGATACTCTTGTTAAACAACCAGCATCTTGGGATGGTC  1916
B.n I   .....................................................
B.n II  A.G..T..........A..C...G.TT.GC..A.T..T..T.....C..C..A......
B.tou   .....G.....................................................
S.ori   ................G.....................................C.....T

                DOMAIN B
B. n III M  Q  W  E  D  R  F  Y  K  A  N  R  A  H  T  Y  L  G  D  P     573
B. n III ATGCAATGGGAAGATCGGTTCTACAAAGCTAACAGAGCTCACACTTATCTCGGGGACCCG  1976
B. n I   .....................................................
B. n II  C.T..G........C.AC......GC............CG.TT...T...G..A......
B. tou   ..................................G...........T........A
S. ori   .....G.....................................G.T...........T

B. n III A  R  E  N  E  I  F  P  N  M  L  Q  F  A  G  A  C  G  I  P     593
B. n III GCAAGGGAGAACGAGATCTTCCCTAACATGCTGCAGTTTGCAGGAGCTTGCGGGATTCCA  2036
B. n I   .....................................................
B. n II  ..G.ACCCTG.G.C.G.A.....GG.T......TT...C..C.C.T.G.....T..A...
B. tou   ..T.......G.....................................................
S. ori   ....A...........A.....C........T.........C.....................

B. n III A  A  R  V  T  K  K  E  E  L  R  E  A  I  Q  T  M  L  D  T     613
B. n III GCTGCGAGAGTGACGAAGAAAGAAGAACTCCGAGAAGCTATTCAGACAATGCTGGATACA  2096
B. n I   .....................................................
B. n II  ..C..C..G..C..C.GA.GG..G..C........G..A..C..............C...
B. tou   ...................G.....T.............................C...
S. ori   ..G...........A.........A.C.....................................


B. n III P  G  P  Y  L  L  D  V  I  C  P  H  Q  E  H  V  L  P  M  I     633
B. n III CCTGGACCGTACCTGTTGGATGTCATCTGTCCGCACCAAGAACATGTGTTACCGATGATC  2156
B. n I   ..A.....A.............G..A.....................................
B. n II  ........A.T.T.........GG.......T.....G..C...........AC.C...
B. tou   ..G..G..A.............G..A.....................................
S. ori   ..A.....A...T.........G..T.............................T......

                <-Primer 4
           DOMAIN E
B. n III P  S  G  G  T  F                                              639
B. n III CCAAGTGGTGGCACTTTC                                            2174
B. n I   ..................
B. n II  ..T.....C.....C...
```

5.3.1 Region 1

Region 1 of the 1665 bp fragments amplified from *Sis. orientale* and *B. tournefortii* contained three of the conserved domains: the 39 bp Domain A, the 12 bp Domain C and an 18 bp Domain D (Fig. 5.3). The nucleotide sequences of Domain A for resistant *Sis. orientale* biotypes NSO1 and SSO3 do not differ from that of susceptible biotype SSO2 (Fig. 5.5). However, two adjacent nucleotide substitutions within Domain A (CCT in SSO2 to ATT in SSO1 at positions 155 and 156, respectively) predict a Pro in the susceptible, but an Ile in the resistant sequence (Fig. 5.5). Only one other nucleotide difference was detected in region 1 from *Sis. orientale*. Biotypes SSO2 and NSO1 have a T at position 40 whereas SSO3 and SSO1 have a C (Fig. 5.5). However, this difference does not result in any change to the primary amino acid sequence.

When sequences of region 1 from susceptible and resistant *B. tournefortii* were compared, they differed by a single nucleotide substitution at the variable Pro codon of Domain A (Fig. 5.6). This C (in susceptible) to G (in resistant) difference at position 280 predicted a Pro in the susceptible but an Ala in the resistant biotype (Fig. 5.6). A Pro to Ala substitution at this position in ALS has previously been shown to encode a resistant ALS in tobacco (Lee et al., 1988), and in *K. scoparia* (Guttieri et al., 1995). The nucleotide sequences of Domains C and D within region 1 were identical between resistant and susceptible biotypes of both species (Figs. 5.5, 5.6).

However, the sequence of region 1* from the 1400 bp fragment of *B. tournefortii* revealed a total of 33 nucleotide substitutions when compared to region 1 from both susceptible and resistant *B. tournefortii* (Fig 5.3). One of the changes predicts for an Ala to Thr change in the amino acid sequence of region 1* from both susceptible and resistant biotypes (Fig. 5.2). This change was not observed in Domain C within the 1665 bp fragment for any of the resistant biotypes. Two other amino acid substitutions are seen in Domain A of the region 1* sequence relative to all other Domain A sequences published here or elsewhere, I to T and R to G (Fig. 5.2). In addition, there were 9.5 replacement substitutions causing a further six amino acid differences outside the conserved domains and 21.5 non-coding nucleotide

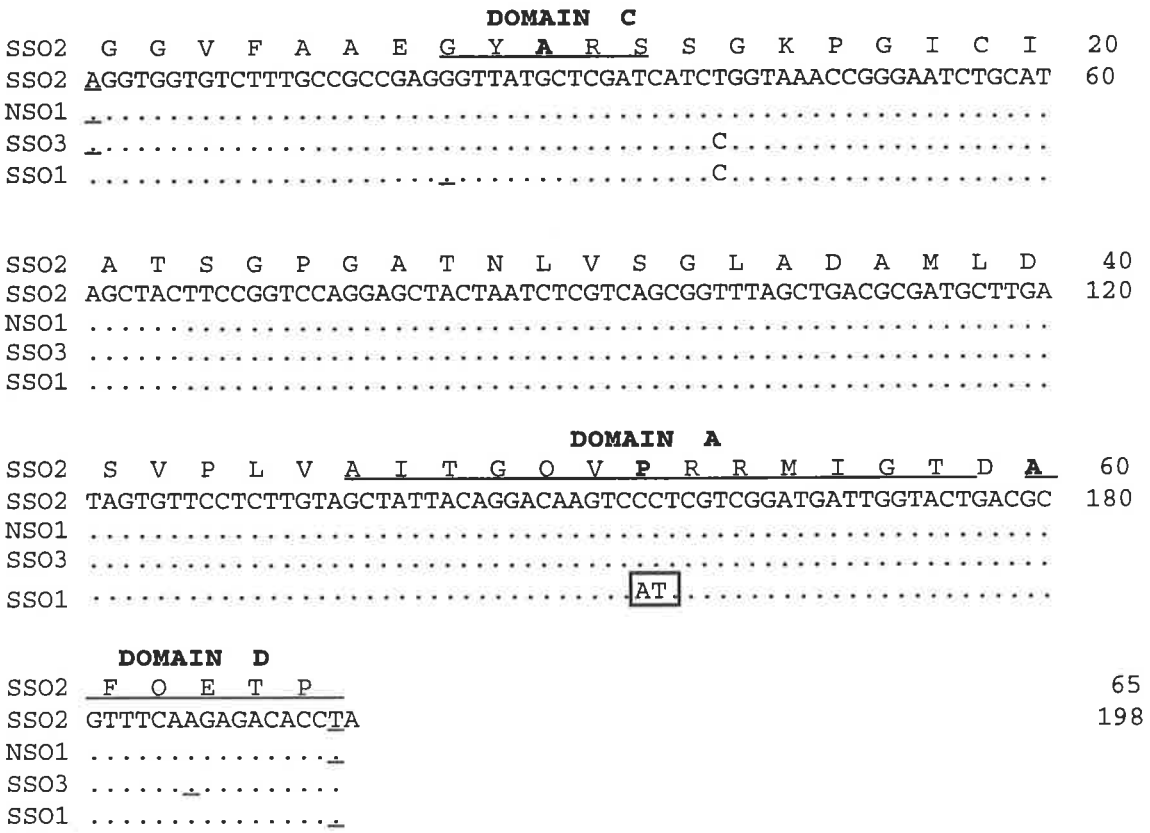


Figure 5.5. Comparison of nucleotide sequences of region 1 of the 1665 bp fragment from susceptible (SS02) and resistant (NS01, SS03, SS01) biotypes of *Sis. orientale* using SS02 as a reference. Dots in the lower three sequences indicate matches to the reference nucleotide sequence, differences indicated by A, C, G or T. Regions between underlined individual nucleotides (or the dots representing them) represent double stranded sequence and outside represent single stranded sequence. The peptide sequences of Domains A, C, and D are underlined in the inferred amino acid sequence (single-letter code) of the reference sequence included above. Bold print in the amino acid sequence indicates sites where mutations confer ALS resistance. The boxed codon (ATT) in the resistant SS01 sequence encodes Ile, and is the only amino acid difference predicted between the four sequences.

```
B.tou S1 L  E  R  Q  G  V  E  T  V  F  A  Y  P  G  G  A  S  M  E  I    20
B.tou S1 CTGGAGCGTCAAGGCGTCGAAACCGTCTTCGCTTACCCGGGAGGCGCTTCCATGGAGATC  60
B.tou R1 .........A.....................................................

B.tou S1 H  Q  A  L  T  R  S  S  T  I  R  N  V  L  P  R  H  E  Q  G    40
B.tou S1 CACCAAGCCTTGACTCGCTCCTCCACCATCCGCAACGTCCTCCCTCGCCACGAACAAGGC 120
B.tou R1 .....................................................

                              DOMAIN  C
B.tou S1 G  V  F  A  A  E  G  Y  A  R  S  S  G  K  P  G  I  C  I  A    60
B.tou S1 GGAGTCTTCGCCGCCGAGGGCTACGCTCGCTCCTCCGGCAAACCGGGAATCTGCATAGCC 180
B.tou R1 .....................................................

B.tou S1 T  S  G  P  G  A  T  N  L  V  S  G  L  A  D  A  M  L  D  S    80
B.tou S1 ACCTCGGGTCCCGGAGCCACCAACCTCGTCAGCGGTTTAGCCGACGCCATGCTCGACAGT 240
B.tou R1 .....................................................

                           DOMAIN  A
B.tou S1 V  P  L  V  A  I  T  G  Q  V  P  R  R  M  I  G  T  D  A  F   100
B.tou S1 GTTCCTCTCGTCGCTATCACAGGACAGGTCCCTCGTCGGATGATCGGTACCGACGCGTTC 309
B.tou R1 ..............................G..............................

         DOMAIN D
B.tou S1 Q  E  T  P  I  V  E  V  T  R                                110
B.tou S1 CAGGAGACACCAATCGTCGAGGTAACGAGG                               330
B.tou R1 ............................A
```

Figure 5.6. Comparison of nucleotide sequences of region 1 of the 1665 bp fragment from susceptible (S1) and resistant (R1) biotypes of *B. tournefortii* using S1 as a reference. Dots in the R1 sequence indicate matches to the reference nucleotide sequence, differences indicated by A, C, G or T. Regions between underlined individual nucleotides (or the dots representing them) represent double stranded sequence and outside represent single stranded sequence. The peptide sequences of Domains A, C and D are underlined in the inferred amino acid sequence (single-letter code) of the reference sequence included above. Bold print in the amino acid sequence indicates sites where mutations confer ALS resistance. The boxed codon (GCT) in the resistant R1 sequence encodes Ala, and is the only amino acid difference predicted between the two sequences.

substitutions in region 1 of the 1400 bp fragment for both biotypes, relative to that of the 1665 bp fragment.

5.3.2 Region 2

Region 2 from *Sis. orientale* biotypes SSO1 and SSO2 was identical over the 333 nucleotides sequenced (Fig. 5.7). A single nucleotide change was observed at position 104 for sequences of *Sis. orientale* biotypes SSO3 and NSO1. This change in the Trp codon within the susceptible Domain B sequence (TGG to TTG) predicts an amino acid change from Trp to Leu in the resistant enzyme. The nucleotide sequences of region 2 from resistant and susceptible biotypes of *B. tournefortii* were identical (Fig. 5.8).

```
SSO2 S  F  I  M  N  V  Q  E  L  A  T  I  R  V  E  N  L  P  V  K      20
SSO2 AGCTTCATAATGAACGTGCAAGAGCTCGCCACAATCCGTGTAGAGAATCTTCCGGTGAAG    60
NSO1 ..................A.....................................
SSO3 ...A.....................................................
SSO1 .........A...............................................

                                                DOMAIN  B
SSO2 I  L  L  L  N  N  Q  H  L  G  M  V  M  Q  W  E  D  R  F  Y      40
SSO2 ATACTCTTGTTAAACAACCAGCATCTTGGCATGGTTATGCAGTGGGAAGATCGGTTCTAC    120
NSO1 ..........................................[.T.].............
SSO3 ..........................................[.T.].............
SSO1 .....................................................

SSO2 K  A  N  R  A  H  T  F  L  G  D  P  A  K  E  N  E  I  F  P      60
SSO2 AAAGCTAACAGAGCTCACACGTTTCTCGGGGACCCTGCAAAGGAGAACGAGATATTCCCC   180
NSO1 .....................................................
SSO3 .....................................................
SSO1 .....................................................

SSO2 N  M  L  Q  F  A  A  A  C  G  I  P  A  A  R  V  T  K  K  E      80
SSO2 AACATGCTTCAGTTTGCAGCAGCTTGCGGGATTCCAGCGGCGAGAGTGACAAAGAAAGAA   240
NSO1 .....................................................
SSO3 .....................................................
SSO1 .....................................................

SSO2 N  L  R  E  A  I  Q  T  M  L  D  T  P  G  P  Y  L  L  D  V     100
SSO2 AACCTCCGAGAAGCTATTCAGACAATGCTGGATACACCAGGACCATACTTGTTGGATGTG   300
NSO1 .....................................................
SSO3 .....................................................
SSO1 .....................................................

SSO2 I  C  P  H  Q  E  H  V  L  P  M                            111
SSO2 ATTTGTCCGCACCAAGAACATGTGTTACCTATG                           333
NSO1 ...........A.....................
SSO3 ..............................A...
SSO1 ........................A........
```

Figure 5.7. Comparison of nucleotide sequences of region 2 of the 1665 bp fragment from susceptible (SS02) and resistant (NS01, SS03, SS01) biotypes of *Sis. orientale* using SS02 as a reference. Dots in the lower three sequences indicate matches to the reference nucleotide sequence, differences indicated by A, C, G or T. Regions between underlined individual nucleotides (or the dots representing them) represent double stranded sequence and outside represent single stranded sequence. The peptide sequences of Domain B is underlined in the inferred amino acid sequence (single-letter code) of the reference sequence included above. Bold print in the amino acid sequence indicates the site where mutations confer ALS resistance. The boxed codon (TTG) in the two resistant NS01 and SS03 sequence encodes Leu, and is the only amino acid difference predicted between the four sequences.

```
B.tou S1 V  V  D  I  D  G  D  G  S  F  I  M  N  V  Q  E  L  A  T  I    20
B.tou S1 GTCGTGGACATTGACGGTGACGGAAGCTTCATAATGAACGTTCAAGAGCTGGCCACAATC  60
B.tou R1 ..................................................... 60

B.tou S1 R  V  E  N  L  P  V  K  I  L  L  L  N  N  Q  H  L  G  M  V    40
B.tou S1 CGTGTGGAGAATCTTCCTGTGAAGATACTCTTGTTAAACAACCAGCATCTTGGGATGGTC  120
B.tou R1 ..................................................... 120

            DOMAIN  B
B.tou S1 M  Q  W  E  D  R  F  Y  K  A  N  R  A  H  T  Y  L  G  D  P    60
B.tou S1 ATGCAATGGGAAGATCGGTTCTACAAAGCTAACAGAGCGCACACTTATCTTGGGGACCCA  180
B.tou R1 ..................................................... 180

B.tou S1 A  R  E  S  E  I  F  P  N  M  L  Q  F  A  G  A  C  G  I  P    80
B.tou S1 GCTAGGGAGAGCGAGATCTTCCCTAACATGCTGCAGTTTGCAGGAGCTTGCGGGATTCCA  240
B.tou R1 ..................................................... 240

B.tou S1 A  A  R  V  T  K  K  E  D  L  R  E  A  I  Q  T  M  L  D  T   100
B.tou S1 GCTGCGAGAGTGACGAAGAAGGAAGATCTCCGAGAAGCTATTCAGACAATGCTGGACACA  300
B.tou R1 ..................................................... 300

B.tou S1 P  G  P  Y  L  L  D  V  I  C  P  H  Q  E  H  V  L          117
B.tou S1 CCGGGGCCATACCTGTTGGATGTGATATGTCCGCACCAAGAACATGTGTTA           351
B.tou R1 ..................................................         351
```

Figure 5.8. Comparison of nucleotide sequences of region 2 of the 1665 bp fragment from susceptible (S1) and resistant (R1) biotypes of *B. tournefortii* using S1 as a reference. Dots in the RI sequence indicate matches to the reference nucleotide sequence, differences indicated by A, C, G or T. Regions between underlined individual nucleotides (or the dots representing them) represent double stranded sequence and outside represent single stranded sequence. The peptide sequences of Domains B is underlined in the inferred amino acid sequence (single-letter code) of the reference sequence included above. Bold print in the amino acid sequence indicates the site where mutations confer ALS resistance. Both nucleotide and amino acid sequences are identical between S1 and R1 biotypes for the entire region.

5.4 DISCUSSION

The isolation of a single amplified DNA product (using primers 5 and 4) of the expected size (1665 bp) and sequence of all four biotypes of *Sis. orientale* is consistent with the findings of the genetic study in Chapter 3, that a single ALS gene confers ALS resistance in this species. However, PCR amplification of genomic DNA from both biotypes of *B. tournefortii* using primers 5 and 4 resulted in two distinct amplified DNA products, both with similarity to ALS. These are the expected 1665 bp product and a smaller 1400 bp product, implying the existence of at least two ALS gene copies in *B. tournefortii*. As discussed in Section 1.5.5, multiple copies of the ALS gene are evident for several other *Brassica* species (Rutledge et al., 1991) but have to date only been cloned and sequenced for *B. napus* (Wiersma et al., 1989; Rutledge et al., 1991; Bekkaoui et al., 1991).

Although there was no genetic study to map the inheritance of ALS resistance in *B. tournefortii*, there is indirect evidence to suggest that at least one of the two amplified products is from a gene which is either inactive or producing a different ALS product that is not involved in resistance. Firstly, one or more deletion events have evidently occurred within the smaller *B. tournefortii* gene leading to loss of 265 bp between the positions of primers 4 and 5. This lost sequence may contain domain(s) vital to ALS enzyme function. Furthermore, the mature peptides predicted for all active ALS genes sequenced to date differ in length by only a few amino acid residues and are not interrupted by introns.

While DNA hybridisation studies indicate that homologues of the distinct flower-specific ALS II gene sequenced from *B. napus* are also present in the genomes of some but not all diploid *Brassica* species (Rutledge et al., 1991; Ouellet et al., 1992) and both diploid and tetraploid cotton species (Grula et al., 1995), this is the first report of DNA sequence of a ALS II-like gene from outside *B. napus*, albeit from a truncated and most likely inactive gene copy. Transcriptional analysis and heterologous expression of these sequences from *B. tournefortii* were not in the scope of this study but should shed light on whether the gene is transcribed, translated and in the unlikely event that it is, whether it shares the developmental and tissue specific expression patterns of the ALS II gene from *B. napus*. In addition, Southern hybridisation of ALS probes to *Sis. orientale* and *B. tournefortii* at

various stringencies should determine ALS copy number. Although the latter attempted in this study, the results were inconclusive (data not shown).

Interestingly, sequencing of the 193 bp region 1* from the 1400 bp product (encompassing Domains C, A and D) revealed a nucleotide change relative to the susceptible region 1 sequence predicting substitution of Thr_{10} in Domain C for Ala in region 1* of both susceptible and resistant biotypes (Figs. 5.2, 5.3). This mutation has been reported to confer high level IM resistance in *X. strumarium* biotype MS-XANST (Bernasconi et al., 1995) and has been used to develop IM resistant corn line ICI 8532 (Siehl et al., 1996). However, ALS enzyme extracted from the leaves of both susceptible and resistant *B. tournefortii* biotypes was sensitive to imazethapyr. This evidence alone cannot discount the activity of the second gene: if it was active and shared the same pattern of expression as the *B. napus* ALS II gene (flower), it would not have been expressed in the tissues assayed for ALS activity. Wiersma et al. (1989) showed that if mutated *in vitro* (Pro to Ser in Domain A), *B. napus* ALS II was capable of conferring herbicide resistance on transgenic tobacco although this was presumably under the control of a constitutive heterologous promoter. However, as ALS herbicides are applied either pre-emergence or early post-emergence, it is unlikely that a gene not active until flowering could confer resistance in the field.

Mutations within five conserved domains of ALS have been reported to confer resistance in higher plants to herbicides which inhibit ALS (Haughn et al., 1988; Lee et al., 1988; Sathasivan et al., 1991; Bernasconi et al., 1995; Hartnett et al., 1990). In this study mutations at two sites that have previously been shown to confer ALS resistance were identified, the Pro residue in the centre of Domain A in both species (Figs. 5.5, 5.6), and the Trp residue of Domain B in *Sis. orientale* (Fig. 5.7 and summarised in Table 5.3). No other amino acid substitutions in the ALS protein were identified in regions 1 and 2 of the 1665 bp PCR products from either species.

Table 5.3. Summary of mutations conferring resistance to ALS herbicides in four weed biotypes. S, susceptible; R, resistant.

Species	Biotype	Domain A Pro site		Domain B Trp site	
		Codon	Residue	Codon	Residue
Sis. orientale	SSO2 (S)	CCT	Pro	TGG	Trp
	NSO1 (R)	CCT	Pro	TTG	**Leu**
	SSO3 (R)	CCT	Pro	TTG	**Leu**
	SSO1 (R)	ATT	**Ile**	TGG	Trp
B. tournefortii	Susceptible	CCT	Pro	TGG	Trp
	Resistant	GCT	**Ala**	TGG	Trp

All substitutions at the Pro residue of Domain A in higher plants reported to date have been due to a single nucleotide change. All six possible amino acid substitutions that could result from such a change (S, H, Q, A, T, and L) confer in higher plants an ALS which is highly resistant to SU and TP herbicides, but in most cases susceptible or only slightly tolerant to IM herbicides (Haughn et al., 1988; Mourad and King, 1992; Harms et al., 1992; Guttieri et al., 1992; 1995). The ALS enzyme from *B. tournefortii* displays this very pattern of resistance, consistent with the Pro to Ala change in its Domain A (Fig. 5.6, Table 5.3). In contrast, for *Sis. orientale* biotype SSO1, two adjacent nucleotide substitutions within the Pro codon of Domain A have resulted in a Ile residue at that position in the resistant enzyme (Fig. 5.5, Table 5.3). This enzyme is highly resistant to all three classes of ALS herbicides. Therefore, it appears that an Ile substitution for Pro within Domain A results in an ALS enzyme with a different resistance pattern compared with a resistant enzyme with an Ala substitution. It follows that the presence of Leu versus Ala probably alters the herbicide binding site in distinct ways causing differences in the herbicide binding capacity of the enzyme.

The substitution of Trp (susceptible) for Leu in Domain B of resistant plants has been shown to endow ALS with high level resistance to all ALS herbicide classes in *X. strumarium*, *Z.*

mays and *B. napus* (Bernasconi et al., 1995 and Hattori et al., 1995). *Sis. orientale* biotypes NSO1 and SSO3 which possess this mutation were resistant to SU, IM and TP herbicides as expected (Fig. 5.7). However, ALS from biotype NSO1 is more resistant to these herbicides than is that from SSO3 (Table 4.3). This raises two possibilities. Firstly, a substitution elsewhere in the ALS gene could be modifying the level of ALS resistance in either biotype, or secondly the higher extractable ALS activity from NSO1 (see Section 4.3) might result in increased resistance in this biotype. Extractable ALS activity of NSO1 is 2.3 times higher than that of the susceptible *Sis. orientale* biotype, whilst ALS activity of SSO3 was 1.6 times greater. As indicated in Chapter 4, this increased activity might contribute in part to resistance.

In conclusion, a mutation in one of two regions (Domains A or B) has been identified in each of the ALS resistant biotypes of *Sis. orientale* and *B. tournefortii*. The sites where these mutations were identified have been previously shown to encode a resistant ALS enzyme in other field selected resistant weeds (Guttieri et al., 1992, 1995; Bernasconi et al., 1995). However, one of the three substitutions identified in this study (Pro to Ile) has never been identified in either laboratory or field selected resistant plants. It is highly likely that the mutations identified herein are responsible for conferring resistance to ALS herbicides in the resistant biotypes of *Sis. orientale* and *B. tournefortii*.

CHAPTER 6

6.0. SEEDBANK STUDIES OF ALS RESISTANT *SIS. ORIENTALE* AND *SON. OLERACEUS*

6.1 INTRODUCTION

Weed seedbanks have been studied longer and more intensely than other seedbanks because of their agricultural significance. In agriculture, weeds are responsible for yield losses by competing with crop plants for nutrients, light and moisture. Some weed species can also act as hosts for pests and pathogens. One strategy to reduce weed infestations is to limit the number of weed seeds that enter the seedbank, which is often more practical than total seed eradication.

Dormancy, defined as a process by which viable seeds do not germinate under any set of environmental conditions (Baskin and Baskin, 1989), is one of the most important factors influencing seedbank dynamics. Seeds that are innately dormant following maturation possess physical or physiological dormancy (Baskin and Baskin, 1989). Seeds with

physical dormancy will not germinate because some barrier prevents the entry of water, which is necessary for germination (Baskin and Baskin, 1989). A more common dormancy mechanism of seedbanks in temperate regions is physiological dormancy (Baskin and Baskin, 1985) in which a physiological mechanism inhibits germination of the embryo. Once seeds with physiological dormancy become non-dormant they can germinate under favourable conditions or can enter secondary or enforced dormancy under unfavourable environmental conditions such as darkness, adverse temperature or lack of moisture.

Little is known about seed dormancy and germination of crop weeds in Australia compared to the information available on weeds of European and North American crops. In southern Australia, studies have shown that successful weed species are suited to a Mediterranean type climate with effective rainfall occurring from April to October. Most of the weeds infesting crops in southern Australia are annual crucifers (for example *Sis. orientale* and *B. tournefortii*), composites (for example *Son. oleraceus*) and grasses (Moore, 1960). Seeds of many winter growing annual weeds that ripen in late spring have summer/autumn dormancy (innate dormancy) and germinate in the following autumn-winter in response to rain (Cheam, 1987). Winter growing annual dicot weeds in general have longer seed dormancy than most annual grass weeds (Cheam, 1987).

Considerable effort has gone into investigation of techniques that could alleviate weed seed dormancy in the field and therefore potentially aid in weed control. Tillage is a technique that has been shown to relieve dormancy and stimulate germination of weed seeds (Evans and Young, 1972) by exposing them to favourable temperatures, oxygen, release from chemical inhibitors (including ethylene and carbon dioxide), and light regimes, for example, photoperiod, spectral quality and intensity (Baskin and Baskin, 1989). However, tillage has been reported to decrease germination in some species, for example in *Sonchus* spp. and *Taraxacum* spp. (Cheam, 1987).

The introduction of modern selective herbicides has resulted in a major shift from more traditional practices (such as mechanical weed control, burning, cutting crops for hay) to, in many cases, the sole reliance on herbicides for weed control. However, herbicide resistance has necessitated a change from herbicide monoculture to making herbicides only one of a

number of alternative weed control strategies. Furthermore, the development of non-target site cross-resistance to chemically dissimilar herbicide classes has reduced the effectiveness of selective herbicides as a component of weed control strategies. To date in Australia cross-resistance has only been identified in *L. rigidum* (Christopher et al., 1991; Burnet et al., 1994). In such cases the remaining options to reduce seedset are often not profitable in the short term resulting in lower rates of adoption by the farming community. Thus, an understanding of weed seed ecology is essential if weed control is to be successful, particularly where herbicide resistance complicates weed control.

The following illustrates how knowledge of the seedbank is imperative in devising successful control programs to combat herbicide resistant weeds. Herbicide resistance in *L. rigidum* is a well recognised problem in Australia (Hall et al., 1994). Studies on the biology of this species (Monaghan, 1980) and interactions of various agronomic practices on the seedbank life (Medd et al., 1985) have aided in devising successful control strategies. Investigation of the longevity of the seedbank life of *L. rigidum* indicates that three years of seedset prevention are required to greatly reduce seedbank numbers of both resistant and susceptible biotypes (Heap, 1988). Such reductions of weed seedbank numbers in combination with careful planning have been used successfully to keep weed numbers to manageable levels whilst ensuring maximum returns.

The appearance of dicot weed biotypes resistant to ALS herbicides has been confirmed for *Son. oleraceus*, *Sis. orientale*, and *B. tournefortii* (Chapter 2). Here I report the findings of experiments designed to quantify seedbank longevity and seed dormancy characteristics of resistant and susceptible biotypes of *Son. oleraceus* and *Sis. orientale* by investigating germination and emergence.

6.2 MATERIALS AND METHODS

6.2.1 Seed dormancy

Son. oleraceus achenes (hereafter referred to as 'seeds') from the resistant and susceptible biotypes were picked over a 10 day period from several plants grown under uniform glasshouse conditions. This procedure provided sufficient experimental seed of resistant and susceptible biotypes that had matured under identical conditions. Similarly, mature *Sis. orientale* seeds from the susceptible biotype and from resistant biotype SSO3 were collected on the same day from the sites of the original collections (see section 2.2.1), and processed the following day. Therefore, in all the experiments reported in this chapter the seed from resistant and susceptible plants were grown, harvested and processed under identical conditions so as to avoid any environmental variation.

Five hundred freshly harvested seeds from resistant and susceptible biotypes from both species were placed immediately onto 0.6% agar in Petri dishes in a growth cabinet (5 replicates of 100 seeds) to test their germinability. Several thousand freshly harvested seeds from both biotypes of each species were placed separately into black porous polypropylene bags (5 replications per biotype) and buried outdoors at a depth of 2 cm in November 1992. Over the following 13 months the bags were exhumed at monthly intervals and thereafter at 6 monthly intervals. The bags were opened outdoors, approximately 100 to 150 ungerminated seeds were collected from each replicate at each exhumation and the bags immediately re-buried. Therefore, seeds were briefly exposed to light. The excavated seeds were counted and germinated on 0.6% agar in Petri dishes in a growth cabinet (see section 4.2.2). Seeds that had germinated in the bags while still buried were not counted. Germination of seeds was scored five days later.

6.2.2 Seedling emergence

The following experiments were established in November 1992 and completed in July 1995.

Sonchus oleraceus

Open-ended plastic cylinders (25 cm diameter, 30 cm length) were sunk in the ground in an agricultural field at the Waite Campus so that their rims were about 5 cm above ground level. Pasteurised potting soil was added so that after settling, the soil surface was 7 cm below ground level. The seeds (same source as from 6.2.1) were then mixed with one litre volume of potting soil, which was placed in the cylinders, thereby occupying the top 5 cm. There were eight replicated cylinders from each biotype, each containing 1000 seeds. Half of the cylinders containing seeds from each biotype were covered with a transparent roof 30 cm above the ground level to protect against rainfall. The other half were exposed to natural rainfall.

The soil surface between the cylinders was covered by a porous black polypropylene sheet to prevent weed growth, and the cylinders netted to exclude birds. Seedlings were counted and removed as they appeared and monthly totals derived. The cylinders were maintained in the field for the duration of the experiment (2.5 years). After 2.5 years, the top 7 cm of soil was removed from each cylinder and distributed within plastic trays. The trays were placed in randomised positions on the bench of an air conditioned glasshouse (22^{o}C), kept moist and the soil periodically disturbed over a further 2 months to induce germination of viable seeds. It was not possible to directly quantify the seedbank by physically identifying the remaining seed because the small seed of *Son. oleraceus* could not be separated from other soil particles on the basis of size.

Sisymbrium orientale

A field site within a cropping field (lat. 34^{o}, long. 137^{o} 30', alkaline sandy loam) with a high infestation of *Sis. orientale* in the field where resistant biotype SSO3 was collected was

chosen in November 1992. This field has been in a pea/ wheat/ barley cropping rotation since 1988. Standing mature *Sis. orientale* plants were thrashed to hasten seed-fall. Ten 5 m x 3 m plots were marked in the corner of the field over which there was a uniform infestation of standing *Sis. orientale*. Seed from these plants therefore entered the soil seedbank at the normal time in November 1992. The experimental area was exposed to the hot, dry summer conditions that prevail throughout southern Australia. Following the arrival of the season-opening autumn rains, a cultivation treatment (the normal agricultural practice in this area) was imposed on some plots. This involved three light cultivations with a rake (with 4 cm teeth spaced 5 cm apart) in late autumn (over a 30 day period) to mimic shallow cultivation. At each cultivation treatment the plots were cultivated twice (second cultivation perpendicular to the first) to a maximum depth of 1 cm. At either end of the experiment, control plots (where no weed control was practiced) were established, separated by a 2 m strip from the treatment plots to reduce seed contamination. On these control plots, normal seedset and seedfall of the natural infestation of *Sis. orientale* occurred. It is emphasised that on all plots other than the control plots after November 1992, there was zero seedset allowed to occur over the duration of the experiment and therefore the seedling emergence than occurred was from seed that entered the seedbank in November 1992, or from residual seed from previous years. Over the 1993 to 1995 seasons, seedling emergence was counted and then the seedlings were killed (by treatment with 750 g ha^{-1} glyphosate) and annual total seedling emergence estimated.

6.2.3 Statistical analysis

Seedlings per replicate were scored for each treatment and the standard error of the means calculated. The data is presented as emerged seedling for the *Sis. orientale* emergence experiment and as percentage germination for the remaining experiments.

6.3 RESULTS

6.3.1 Seed dormancy

Sonchus oleraceus

Freshly harvested mature seed from both resistant and susceptible biotypes harvested over a 10 day period showed no dormancy. As there was 100% germination of this seed in November 1992 (Fig. 6.1) it is clear that seed from neither resistant nor susceptible biotypes is initially dormant. When seed was buried at 2 cm depth in the soil and then periodically exhumed to check germination capacity it was found that the percentage germination of seed of both the resistant and susceptible biotypes remained above 90% over the 36 month duration of the experiment (Fig. 6.1).

Sisymbrium orientale

In contrast to *Son. oleraceus* (Fig. 6.1), freshly harvested seed of both resistant and susceptible *Sis. orientale* biotypes was strongly dormant (Fig. 6.2). However, when the seed was placed at 2 cm depth in the soil and then exhumed at monthly intervals it was evident that within one month after burial, 50 to 60 percent of the seeds from both biotypes germinated (Fig. 6.2). Two months after maturation 85% of the seed germinated. This result indicated that freshly matured seed of both resistant and susceptible *Sis. orientale* has a short period of seed dormancy but that when subsequently placed under optimum conditions the majority will germinate within two months of seed maturation. Over the following 11 months the percentage of exhumed seed that would germinate fluctuated from 30% to 70% (Fig. 6.2). Thirteen months after burial, exhumations were conducted at 6 month intervals for the next 18 months with germination declining uniformly in both biotypes (Fig. 6.2). The reduction in germination of seed buried 19 months or more was attributed to seed dormancy or seed death. No large differences in germination of seed from the resistant and susceptible biotypes was observed at any time point indicating that there were no fundamental differences between the two biotypes.

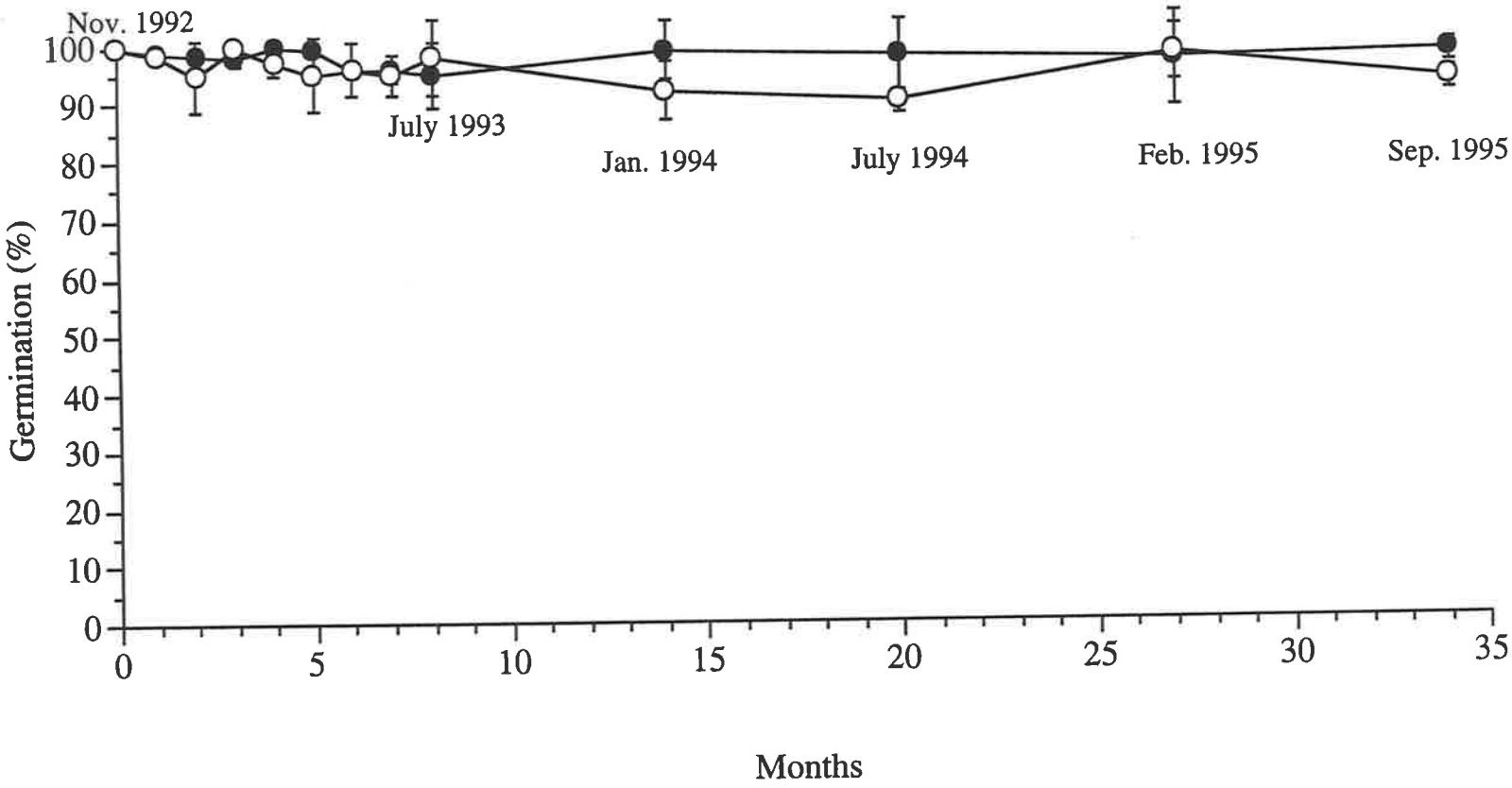


Fig. 6.1. Germination of seeds of the resistant (●) and susceptible (O) biotypes of *Son. oleraceus* exhumed from the soil following burial of fresh seed. Bars indicate the standard error of the means.

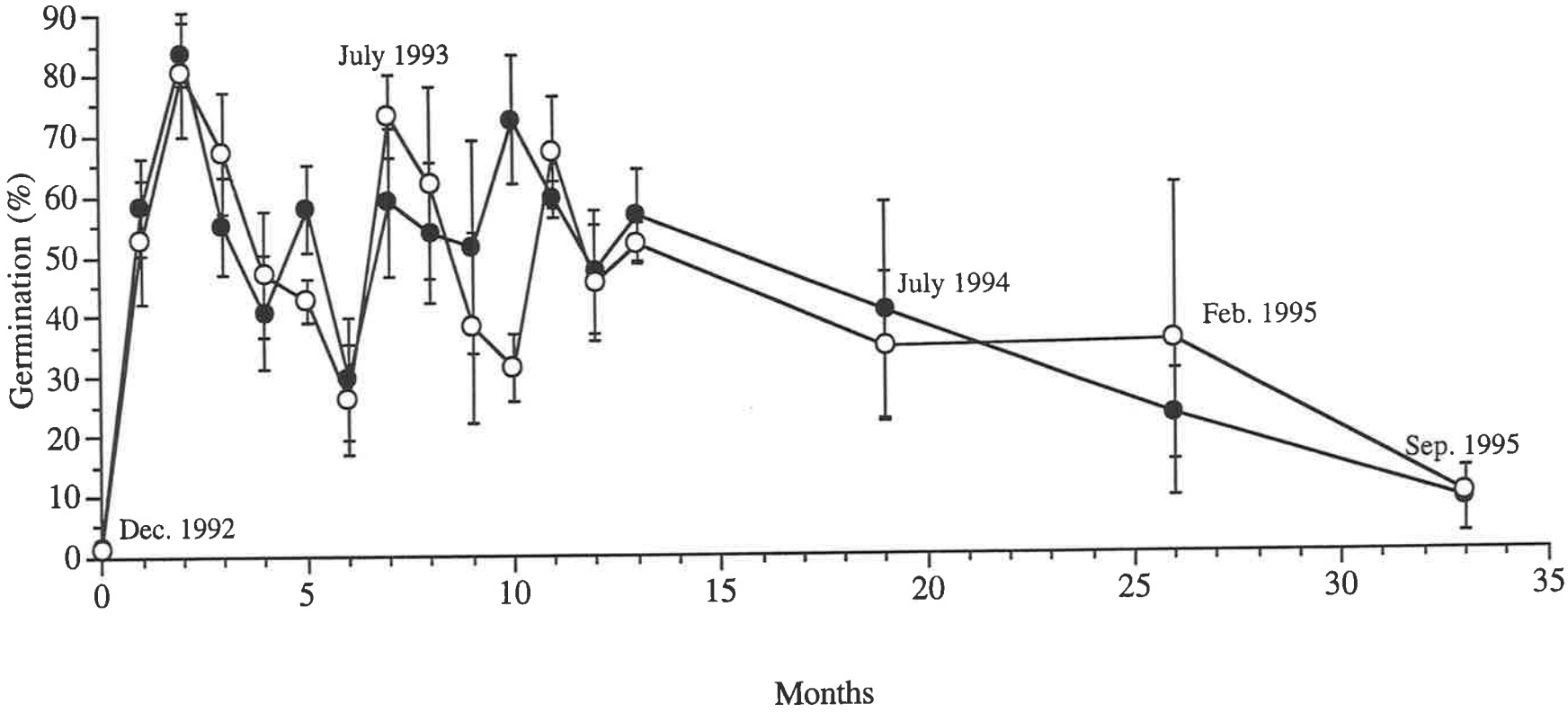


Figure 6.2. Germination of seeds of resistant (●) and susceptible (O) biotypes of *Sis. orientale* exhumed from the soil following burial of fresh seed. Bars indicate the standard error of the means.

6.3.3 Seedling emergence

Sonchus oleraceus

Emergence of seed from the soil for both resistant and susceptible *Son. oleraceus* biotypes in cores exposed to natural rainfall was high during the first month after burial (10 to 14%), followed by a dramatic reduction over the summer months (Fig. 6.3) which in southern Australia is the driest season. No emergence was observed over the 30 month duration of the experiment for seeds from either biotype that were not subjected to rainfall (data not shown). In the cores exposed to rainfall, flushes of emergence followed rainfall during the winter months, whereas there was no response to spring or summer rainfall (Fig. 6.3) over the course of the experiment. After the final measurement in June 1995, 69.2% ± 2.3% of the total seeds from the resistant biotype and 69.7% ± 1.2% from the susceptible biotype had emerged. Approximately 308 seeds from the resistant biotype and 303 seeds from the susceptible biotype did not emerge over the course of the experiment. Unfortunately, the remaining seed could not be separated from the soil to allow quantification of viable seeds. Thus, a germination test of recovered soil was established to test for germination of seeds. Seedlings were observed (data not shown) indicating some of the remaining seeds were still viable.

Emergence (data not shown) was observed for seeds from both biotypes that had not been exposed to rainfall but were placed in the glasshouse and watered as above. This suggests that seeds were still viable in the dry soil some 34 months after initial placement of freshly matured seed in the soil.

Sisymbrium orientale

The purpose of this field experiment was to establish the seedbank longevity of *Sis. orientale* under realistic agronomic conditions by observing for three years seedling emergence from the seedbank in the absence of seed replenishment, and furthermore to determine what effect cultivation had on the emergence pattern of *Sis. orientale*. In this field experiment there was

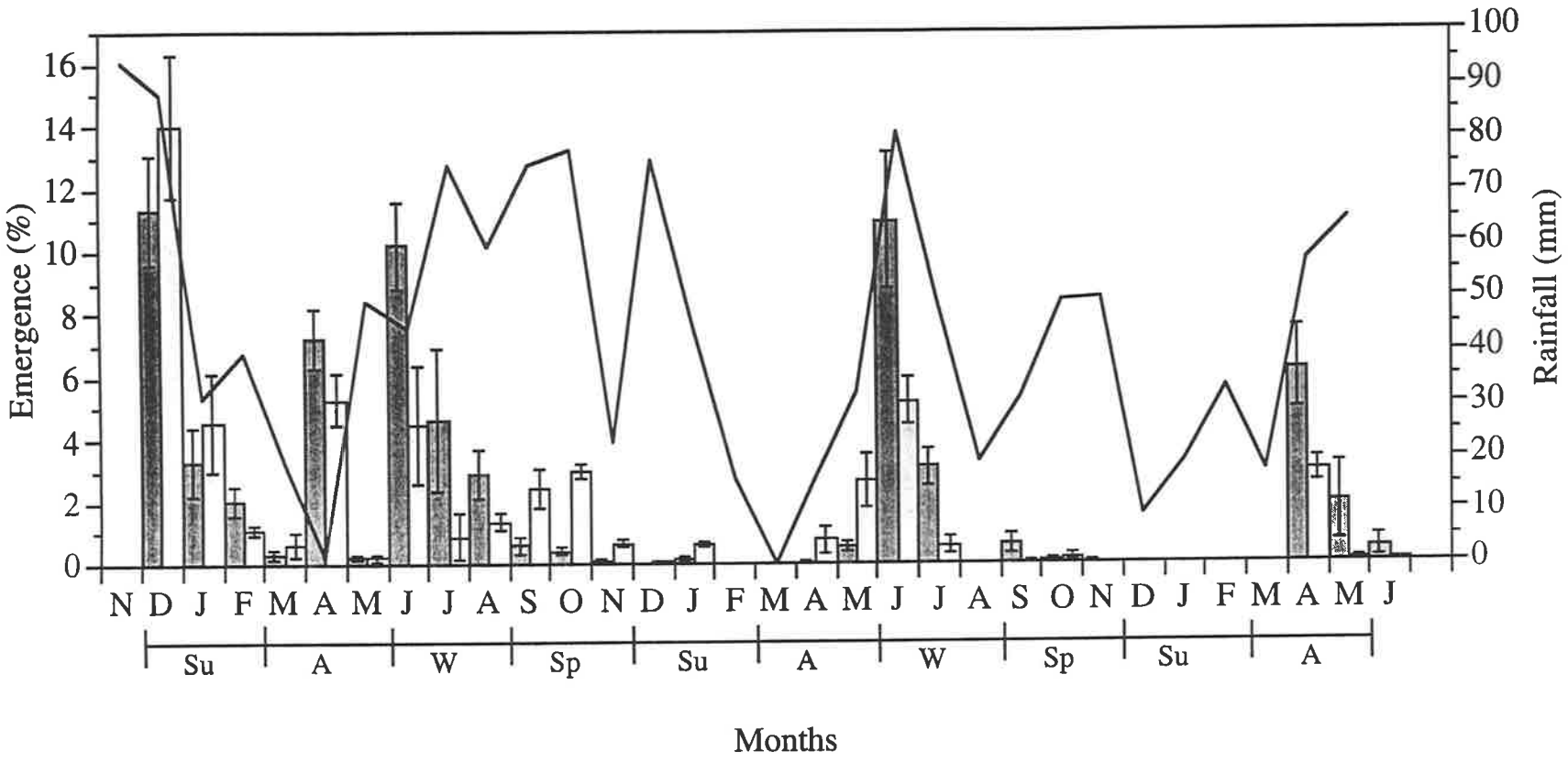


Figure 6.3. Monthly emergence of *Son. oleraceus* seeds from resistant (■) and susceptible (□) biotypes buried in open ended cylinders outdoors and exposed to natural rainfall. Bars indicate the standard error of the means. Line plot represents monthly rainfall. Seasons indicated are summer (Su), autumn (A), winter (W) and spring (Sp).

zero new seed production on any plots other than control plots in which natural seed production was allowed to occur. Seedling emergence from the plots was recorded on a regular basis throughout the course of this experiment and then these seedlings were killed so as to prevent seed production. The absence of seed set for three years resulted in a 7-fold reduction in seedling emergence from both cultivated and non-cultivated plots (Fig. 6.4). Quantification of the *Sis. orientale* seedbank was only possible by this method of counting seedling emergence. It was not possible to directly sample the soil seedbank because the small sized seed (1 mm long) made it difficult to separate seed from soil particles. The experiment has shown a major reduction in seedling numbers, and thus a reduction in the seedbank of *Sis. orientale* in the absence of seed replenishment. Furthermore, the experiment highlights the effect cultivation has in stimulating germination. Germination was found to be dependent on rainfall since 1900 seedlings m^{-2} were counted in the control plots in 1995 (which had double the rainfall in the March to July period than in 1994) compared to 300 seedlings m^{-2} in 1994 (Fig. 6.4).

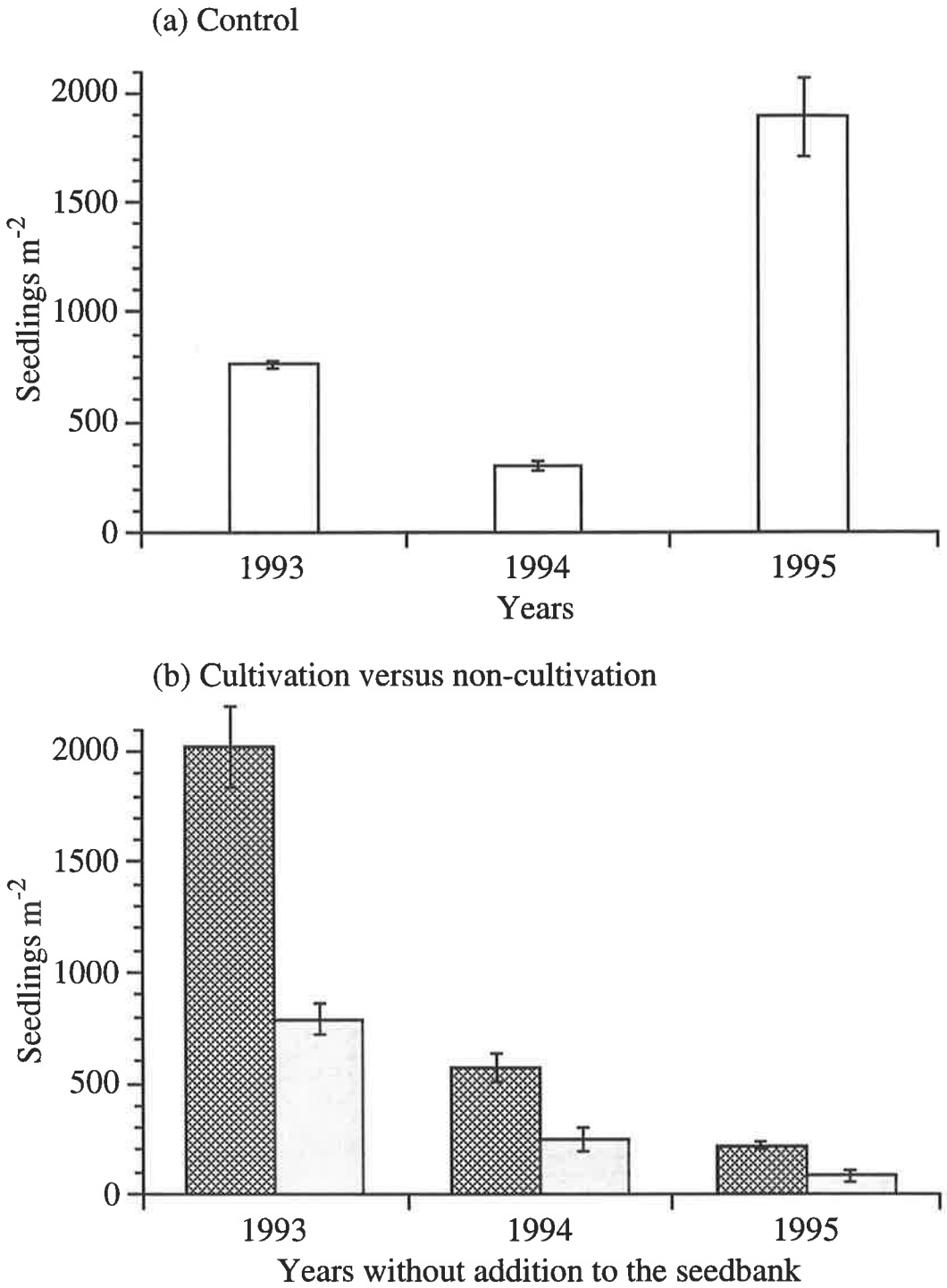


Figure 6.4. Emergence of *Sis. orientale* over a three year period in the absence of seedset. Seedlings were counted, removed at regular intervals as they appeared and annual totals derived. In control plots (a) no seed set limitation or plant removal was performed. Treatments imposed (b) were plots with three annual cultivations (▩) and plots with no cultivation (□). Bars represent standard errors of the means.

6.4 DISCUSSION

Freshly ripened seeds of *Son. oleraceus* immediately placed in favourable germinating conditions all germinate indicating that freshly matured seed does not exhibit innate dormancy. However, not all seed germinated upon subsequent burial indicating that the seed had entered enforced dormancy which was relieved when the seed was put in favourable conditions (Fig. 6.1). These findings support the results of the emergence experiment (Fig. 6.3) which indicates that burial of *Son. oleraceus* seed places them in conditions which result in enforced dormancy, because not all the 1000 buried seeds germinated at the beginning of the experiment. The dormancy experiment also revealed that a high proportion of seeds from both biotypes germinated while still buried, indicating that enforced dormancy was periodically relieved in some seeds.

Emergence of both biotypes of *Son. oleraceus* followed a cyclical pattern, with the highest emergence corresponding to periods of high rainfall between April to July (Fig. 6.3). However, rainfall during the warmer months did not stimulate emergence indicating that high temperature inhibits germination. One contributing factor could be that after high rainfall during warm conditions soil moisture falls rapidly, a condition that does not seem to favour *Son. oleraceus* seed germination. This indicates that enforced dormancy in *Son. oleraceus* seeds may be imposed by low soil moisture. A similar experiment investigating emergence of *Son. oleraceus* seeds in the United Kingdom (Roberts and Neilson, 1981) showed emergence peaking in late spring, periods of high soil moisture and warmer soil temperatures resembling Australian autumn-winter conditions.

In the present study, soil containing seed of *Son. oleraceus* (Fig. 6.3) removed from cores 2.5 years later and placed in a glasshouse with frequent watering, seedling emergence was observed from soil both exposed and not exposed to rainfall. This finding suggests that the seedbank life of *Son. oleraceus* can be greater than 2.5 years. Furthermore, periods of low soil moisture may enforce dormancy, and periods of increasing moisture relieve dormancy.

Freshly matured *Sis. orientale* seed exhibited innate dormancy with zero initial germination (Fig. 6.2). However, after only one month of burial, 50% of seeds germinated indicating

innate dormancy had been partially relieved. It therefore seems that one month of summer conditions is sufficient to relieve innate dormancy by 50%. Several annual weeds of southern Australian agriculture also exhibit summer/ autumn innate dormancy as a survival mechanism against the hot dry summers and from abortive early rains (McGowan, 1970). McGowan (1970) suggested that for these weeds increasing soil moisture stimulated germination.

Germination of *Sis. orientale* seed of both the resistant and susceptible biotypes was reduced to 10% after 33 months of burial (Fig. 6.2). This indicates that *Sis. orientale* seed has at least a three year life which supports the field results (Fig. 6.4). Other studies have reported that in the fourth year of burial of a known number of *Sis. orientale* seeds (in an experiment similar to that performed here for *Son. oleraceus*, see section 6.2.2), only 1.5% germinated (Wilson, 1985). Such a reduction in a herbicide resistant biotype would be manageable in agricultural systems. In this experiment however, 70% of the seeds had not emerged after the fourth year and no reference is made to their viability (Wilson, 1985). In the present experiment the reduction in the *Sis. orientale* seedbank could not be estimated, because initial and final seed counts (Fig. 6.4) were not made. Thus, the seedbank life of *Sis. orientale* was not elucidated from this experiment. In order to determine the seedbank life, it would have been necessary to continue the experiment until no further seedling emergence was observed, and then to verify by artificial irrigation. However, the data shows that preventing *Sis. orientale* seedset for three years reduces seedling numbers 7-fold from the original emerged densities (not original seed numbers). This experiment does not reveal if the ungerminated seeds in the non-cultivated plots (represented by the difference between the cultivated and non-cultivated plots) are still viable. These seeds are likely to have enforced dormancy because cultivation stimulated germination. If the seeds in the non-cultivated plots are viable, then the seedbank life of *Sis. orientale* under non-disturbed situations will be longer. Thus, this experiment has revealed that cultivation can reduce the seedbank of *Sis. orientale*, probably through exposure of weed seeds to light which breaks dormancy, particularly in small-seeded weeds (Dyer, 1995).

In fields where dicot weeds with resistance to ALS herbicides has become a problem, reducing the soil seedbank of the population to manageable levels should be a priority. One strategy is to stimulate germination by cultivation with subsequent re-cultivations to kill emerged seedlings. Following up with a herbicide with a mode of action different to ALS (e.g. an auxin analogue), will ensure escapes of cultivation are controlled. Such a strategy should drastically reduce the dicot weed population. For ALS resistant Sis. orientale and Son. olerceus, implementation of such a strategy for three years will reduce the soil seedbank to low levels. However, non-till or minimum-till techniques are unlikely to disturb the soil sufficiently in order to achieve adequate seed germination. Therefore, a more intensive cultivation regime is probably necessary. The above strategy involving cultivation and herbicides for dicot weed control could only be used in crops such as cereals that are not damaged by auxin analogue herbicides, or in a fallow system where non-selective herbicides can be used. If strategies to reduce fresh seed input into the soil seedbank are practised, then weed numbers can be maintained at levels low enough to be manageable.

CHAPTER 7
7.0. GENERAL DISCUSSION

7.1 Selection pressure

This thesis has demonstrated that biotypes of the dicot weed species *Son. oleraceus*, *Sis. orientale* and *B. tournefortii* with target site resistance to ALS herbicides have been selected following 2 to 8 applications of these herbicides. Compared to the photosystem I-inhibiting bipyridyl herbicides (reviewed in Preston 1994) and the auxin analog herbicides such as 2,4-D (reviewed in Coupland, 1994) which have been widely used for 30 to 50 years with few instances of resistance, resistance to the ALS herbicides has occurred rapidly. This raises the question as to why resistance to the ALS herbicides has developed so quickly? Two major factors affecting the onset of herbicide resistance in weeds are the initial frequency of resistant individuals in a population and the intensity of the selection pressure imposed. It is clear from the data presented in Figs. 2.2-2.5, 2.8-2.11 and 2.14 that plants from the susceptible *Son. oleraceus*, *Sis. orientale* and *B. tournefortii* biotypes were killed at ALS herbicide rates much lower than field recommendations. ALS resistant biotypes of *Lolium rigidum* (Christopher et al., 1992; Burnet et al., 1994), *Lactuca serriola* (Mallory-Smith et al., 1990a), *Kochia scoparia*, *Salsola iberica* and *Stellaria media* (Thill et al., 1991) *Ixophorus unisetus*, *Eleucine indica* (Valverde et al., 1993) and *Xanthium strumarium* (Schmitzer et al., 1993) have been selected with three to six field applications of ALS

herbicides. This highlights the intense selection pressure that ALS herbicides impose on susceptible weed populations.

The level of selection pressure is determined by the frequency and intensity of herbicide use. Some weed species are very sensitive to ALS herbicides, particularly species in the Brassicaceae family (which includes *Sis. orientale* and *B. tournefortii*). By applying a strong selection pressure such as an ALS herbicide, not only are susceptible individuals removed from the initial population, but any individuals with low levels of resistance are also killed. Thus, only individuals with high levels of resistance remain to enrich the seedbank for following generations. Selection pressure is intensified if herbicides remain active in the soil for considerable time. As many ALS herbicides are active in the soil, herbicide soil residues persisting into the next season can impose a selection pressure on future generations.

It has been well established that degradation of SU herbicide is slow in alkaline soils (Joshi et al., 1985; Walker and Welch, 1989). *Sis. orientale* biotypes SSO1 and SSO3 originate from a region with soils that are very alkaline (pH 8.9) due to the presence of lime (French et al., 1968). It is therefore very likely that degradation of SU herbicides, such as triasulfuron which is used in this region, in these high pH soils is slow and that herbicide persistence from one season to the following season may impose selection pressure on subsequent weed germinations. Furthermore, higher rates than is necessary for the control of most dicot weeds are used because one of the target weeds is *L. rigidum*, which is not as sensitive to ALS herbicides. Thus, a combination of relatively high herbicide rates, slow herbicide degradation because of high soil pH and weeds very sensitive to ALS herbicides result in a very strong selection pressure.

What is the explanation for the rapid selection for resistance to ALS herbicides? Is the frequency of ALS resistance genes higher that the frequency of resistance genes to other herbicide classes such as the bipyridyl and auxin analog herbicides mentioned above (reviewed in Coupland, 1994), or glyphosate for which no cases of field resistance have been reported (reviewed in Dyer, 1994). There is little information on the initial frequency of ALS resistance in the field. However, laboratory studies screening *Arabidopsis thaliana*

with chlorsulfuron (Haughn and Somerville, 1987) and imazapyr (Haughn and Somerville, 1990), chlorsulfuron screening of *Medicago sativa* seedlings (Stannard, 1987) and primisulfuron screening of *Nicotiana tabacum* (Harms and DiMaio, 1991) have suggested that the frequency of ALS resistance is less than 10^{-6}. These findings suggest that the rapid appearance of ALS resistance in agricultural fields is probably not a function of an unusually high frequency of ALS resistance genes but rather due to the high selection pressure these herbicides impose combined with other characteristics, such as high seed production, rapid and frequent seed germination, efficient seed and pollen distribution systems (reviewed in Saari et al., 1994) and slow ALS herbicide degradation due, for example, to high soil pH.

7.2 Resistance mechanisms

So far this discussion has dealt with issues of selection pressure imposed by ALS herbicides, but has not addressed what mechanisms of resistance are selected by ALS herbicides. Resistant *Sis. orientale*, *Son. oleraceus* and *B. tournefortii* all possess a herbicide resistant ALS enzyme (Chapter 4). To date, this is the sole mechanism of resistance that has been identified in dicot weed species worldwide (reviewed in Saari et al., 1994). However, biotypes of the grass weed *L. rigidum* have developed resistance to ALS herbicides through enhanced herbicide metabolism as well as a resistant ALS target enzyme (Christopher et al., 1991, 1992; Cotterman and Saari, 1992). Why has enhanced ALS herbicide metabolism not been found as a resistance mechanism in dicot weeds? One possible explanation is that, in general, susceptible dicot weeds are much more sensitive to ALS herbicides than susceptible *L. rigidum* because they have lower rates of ALS herbicide metabolism than *L. rigidum* (reviewed in Saari et al., 1994). As ALS herbicide use rates in Australia are set at the higher levels that control or suppress susceptible *L. rigidum*, only mechanisms which endow high levels of resistance (such as a resistant ALS enzyme) will be selected in many dicot weed species. Most individuals of dicot weed species with mechanisms endowing low levels of resistance will probably be killed by the field rates of ALS herbicides (reviewed in Saari et al., 1994). Thus, a small increase in metabolism which endows resistance to ALS herbicides for resistant *L. rigidum* (Christopher et al., 1991) may

be less likely to endow resistance to a highly susceptible dicot weed. Since target site resistance endows higher levels of resistance than enhanced metabolism (Christopher et al., 1992) this may suggest why only target site resistance has been identified in dicot weeds.

7.3 Spread of resistance

The widespread, persistent and increasing use of ALS herbicides in Australia is of concern. In 1995, over 150 cases of ALS herbicide failure (all SU herbicides) to control *Sis. orientale* and *L. serriola* were reported in one small region of South Australia (C. Clarke, pers. comm.). This is a dramatic increase since the first confirmed case of resistant *Sis. orientale* in 1991 (Boutsalis and Powles, 1995). It is inevitable that with the current range of selective ALS herbicides available, the incidence of resistance will escalate. ALS herbicides are available for control of dicot weeds not only in cereal crops but herbicides such as imazethapyr, metosulam and flumetsulam also selectively control dicot weeds in dicot crops and pastures (Table 1.2). Resistance is therefore likely to increase, particularly in fields that already have an SU herbicide history. It is inevitable that such practices are going to select for widespread resistance and possibly make ALS herbicides ineffective in some situations. At present farmers are often unable to distinguish between herbicides with a similar mode of action. For this reason mandatory labelling of all herbicide containers with a clearly visible letter according to the mode of action has been implemented in 1996 to make farmers aware of overuse of one herbicide mode of action.

Little emphasis has been placed on the spread of resistance by pollen. Since ALS herbicide resistance in at least four weed species is inherited by a nuclearly encoded single gene (Chapter 3; Mallory-Smith et al., 1990b; Thompson et al., 1994) this matter should be further investigated. In self-pollinating species such as *Sis. orientale*, *B. tournefortii* (Salisbury, 1991) and *L. serriola* (Mallory-Smith et al., 1990b) there is little concern of resistance spreading via pollen. In cross-pollinating species such as *Raphanus raphanistrum* (Salisbury, 1991), regarded as one of the more serious weeds of cropping in Australia, the spread of resistance by pollen should be of concern. Resistance in *R. raphanistrum* to ALS

herbicides has recently been reported in Western Australia (G. Gill, pers. comm.). Moreover, cross-pollination between ALS resistant and susceptible individuals has been documented for the weed species, *K. scoparia* (Stallings et al., 1993; Mulugeta et al., 1992).

The potential for the transfer of resistant seed by poor hygiene should also not be overlooked. This transfer may be in the form of contamination of farm machinery, clothing or animals. Contamination of seed crops is of major concern because seeds for future sowing are spread over much larger areas and can therefore spread resistance much faster than by any other method. For example, in Australia, herbicide resistant *L. rigidum* has been identified as contaminating perennial grass seed used for pasture regeneration and is responsible for the introduction of resistance to some fields (J. Matthews, pers. comm.).

In order to investigate the mechanism by which resistance has developed at any particular site, be it a new selection or by contaminating pollen or seed, a sensitive detection system is required. One such technique is DNA sequencing to identify mutations in ALS gene, assuming the mechanism of resistance is a resistant ALS enzyme. The present study was able to identify three different ALS mutations in four resistant biotypes (Chapter 5). Interestingly, *Sis. orientale* resistant biotypes SSO1 and SSO3 possess different mutations endowing herbicide resistance. However, each biotype was represented by the DNA sequence from a single plant. These two biotypes were collected in fields only 10 kilometres apart and farmed by the same individual. Given the sampling method, the obvious question is whether resistance developed independently in the two fields or was there contamination from one field to another. To answer this question one must know how homogeneous resistance mutations are within each field, by screening several plants from each for mutations at the DNA level. If all resistant plants from the same field had the same mutation, then resistance probably occurred independently in both fields. On the other hand, if resistance in each field was endowed by several ALS mutations then it would be difficult to predict if ALS resistance had occurred independently, particularly if individual mutations were present in both populations.

7.4 Preventing and combating ALS herbicide resistance in dicot weeds

An important factor in preventing or slowing the development of herbicide resistance is reducing the selection pressure imposed. The introduction of herbicide mode of action labelling, where all herbicide products carry an alphabetical label according to mode of action, is an attempt to educate farmers about reducing the selection pressure. Hopefully, by keeping a simple record of the herbicide history of each field, herbicide selection pressure can be monitored. In agriculture however, decisions are usually made according to economic opportunities often with the hidden costs, such as the development of herbicide resistance, sacrificed.

In order to address the issue of combating ALS herbicide resistance once it has developed, knowledge of the herbicide resistance spectrum is important. The whole-plant herbicide studies in Chapter 2 show that these ALS resistant *Sis. orientale* and *Son. oleraceus* biotypes possess target site ALS resistance, ie. they are resistant to ALS herbicides only. Non-ALS herbicides, however, should still remain effective in controlling these ALS resistant biotypes.

Knowledge of the seedbank life of weed seeds and how to break dormancy is also important if herbicide resistance is to be effectively combated. This study has revealed that the seedbank life of *Sis. orientale* and *Son. oleraceus* is greater than three years (Chapter 6). Furthermore, *Son. oleraceus* exhibits only enforced dormancy whereas *Sis. orientale* exhibits both enforced and innate dormancy (Figs. 6.3, 6.4). This information suggests that it would be easier to stimulate germination of *Son. oleraceus* than *Sis. orientale* in the field and thus more rapidly reduce the seedbank of the former species.

7.5 Future work

Seldom is a Ph.D. study completed with no further work required. This study is no exception. One area requiring further investigation is sequencing the entire ALS gene to determine if there are any other mutations conferring resistance such as in Domain E

(Chapter 5). Southern hybridisation of specific ALS sequences to genomic DNA of the *Sis. orientale* and *B. tournefortii* biotypes should reveal how many ALS genes (silent and active) each species possesses. Time constraints prevented me from conducting RNA hybridisation analysis on the *Sis. orientale* biotypes to determine if increased mRNA levels could be measured for resistant biotypes NSO1 and SSO3, for which increased ALS activities were observed. More time was required to complete the *Son. oleraceus* seedbank experiment (Fig. 6.3) because all the seed buried was not accounted for as seedlings. The *Sis. orientale* seedbank experiment also required more time because seedling emergence was still observed in the final year. Finally, the increased incidence of ALS herbicide failures against dicot weeds reported in 1995 indicates that considerable more work is required in the area of management of ALS resistant weed populations.

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