Increasing *Medicago* resistance to soil residues of ALS-inhibiting herbicides

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Thesis submitted for the degree of

Doctor of Philosophy

in

The University of Adelaide

Faculty of Agricultural and Natural Resource Sciences,

Department of Applied and Molecular Ecology

November, 2000

A stand of *Medicago littoralis* regenerating in late autumn in a wheat stubble (South Australian Mallee).

ACKNOWLEDGMENTS

To my loving and supportive wife, Lesley, and to my children Angela and Geoffrey – thank you for so graciously allowing me to take this time from you. I dedicate this thesis to you. I would also like to thank my father and late mother for their unfailing encouragement and assistance towards my tertiary studies.

Thankyou to my supervisor, Chris Preston, who was always enthusiastic, supportive, encouraging – and always there. I could not have wished for a better supervisor. I am grateful to the Grains Research and Development Corporation (GRDC) for funding this research, and to the South Australian Research and Development Institute (SARDI), Primary Industries and Resources South Australia (PIRSA), the University of Adelaide, and the CRC for Weed Management for making this project possible.

In particular I would like to thank Rick Roush and Steve Powles for encouragement and support to begin this study; Mary Rieger for her patient tuition and assistance with techniques in molecular biology; Chris Preston, my brothers Ian Heap and Mark Heap, and Steve Powles, for reviewing the draft manuscript of this thesis; Noel Pederson, John Cooper, Glynn Scmidt and Robert Riemann for generous provision of field sites; Jake Howie, Steve Hughes and Ricki Drewry (SARDI) for provision of seed and technical advice; Ray Rose and Kim Nolan (University of Newcastle) for seed and advice on tissue culture techniques; and Bruce Burns for his cheerful and able assistance with field experiments.

I would also like to thank those friends and encouraging colleagues at Struan who gave compassionate support and friendship when it was most needed.

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ABSTRACT

Medics (annual *Medicago* spp.) are important and valuable leguminous pasture species in cereal-livestock farming systems of temperate southern Australia. Soil residues of acetolactate synthase (ALS) inhibiting herbicides (ALSIH) applied to cereals severely restrict medic growth in the following season, especially in regions with low rainfall and alkaline sandy soils. This study aimed to identify or develop medics with increased resistance to soil residues of ALSIH, particularly chlorsulfuron and triasulfuron.

In field experiments soil residues of chlorsulfuron and triasulfuron applied in the previous year reduced early medic production by 91% and 79% respectively. Response to chlorsulfuron in glasshouse soil bioassays was determined for 49 lines from the Australian Medicago Genetic Resource Centre. Cultivars of *M. rugosa* and *M. scutellata* were least affected by chlorsulfuron but root growth was severely inhibited by 1 ppb, a rate commonly encountered in the year following chlorsulfuron application. Approximately 32 million seedlings from four elite cultivars, and 723 plants which had survived ALSIH field applications, were screened for resistant mutants but none were found. Soils with residues of chlorsulfuron and triasulfuron clearly restricted the growth of *Medicago*, but there were no extant genotypes with sufficient resistance to deploy in these soils.

In the absence of resistant genotypes, cells were grown and mutated with EMS and selected with chlorsulfuron in tissue culture. Many lines of *M. truncatula* cv. Jemalong 2HA with high levels of chlorsulfuron resistance were selected, but calli lost competence for somatic embryogenesis and no resistant plantlets were regenerated.

Seeds of *M. littoralis* cv. Herald and *M. truncatula* cv. Mogul were mutated with EMS and selected with chlorsulfuron. One cv. Herald and four cv. Mogul lines with putative chlorsulfuron resistance were selected. None of the cv. Mogul lines had resistance, but the *M. littoralis* cv. Herald selection "FEH-1" was shown to have increased resistance to a range of ALSIH. *In vitro* ALS enzyme assays confirmed that FEH-1 has a mutated ALS gene which confers increased resistance to chlorsulfuron, flumetsulam, imazethapyr, metsulfuron-methyl, triasulfuron and sulfometuron-methyl. There was little or no cross-resistance to imazapyr or metosulam. Soil and foliar dose-response experiments concurred with these results at the whole plant level. Sections of the ALS genes for FEH-1 and cv. Herald were sequenced and it was found that the nucleotide sequence for the region spanning the highly conserved Domains A and D were identical. The sequences for Domains B, C, and E, and hence the location and nature of the nucleotide substitution underlying the change in amino acid sequence in the ALS enzyme, have not yet been determined.

FEH-1 growth in the field was far superior to cv. Herald in soils with residues of chlorsulfuron and triasulfuron, and the genotype has the potential to significantly increase pasture productivity in cereal-livestock farming systems in the presence of these residues. There is also potential for strategic weed control in FEH-1 pastures using post-emergence ALSIH applications, however this practice will be generally discouraged because it will increase development of herbicide resistance in weeds. Commercial development of the genotype has begun and may include transfer of the herbicide resistance in FEH-1 to other *M. littoralis* and *M. truncatula* cultivars using conventional crossing.

DECLARATION

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

I give my consent that this work may be photocopied or loaned from the university library.

Signed: John Heap: **Date**: May 3rd, 2001

CHAPTER 1

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1.1 ANNUAL MEDICS IN SOUTHERN AUSTRALIAN FARMING SYSTEMS

The Mediterranean climate in the southern Australian cropping zone is characterised by hot, dry summers, with rainfall concentrated in the cooler winter-spring growing season between May and October. Short season crops and winter-spring growing pastures are most suited to this climate. Following the summer drought, rains may begin any time between April and June. Wheat and barley are grown in areas with between 250 and 640 mm annual rainfall (Puckridge and French, 1983). Pulse and oilseed crops are grown in areas with rainfall greater than 350 mm. Cropping systems are based on continuous crop rotations, or ley-rotations incorporating an annual pasture phase. Ley cropping rotations involving grazed pastures are typical in cropping areas with less than 350 mm annual rainfall, however, in areas with 350-500 mm continuous cropping is becoming dominant. Key annual pasture legumes in these systems are subterranean clover (*Trifolium subterranean*), in wetter areas with acid to neutral soils, and medics (annual *Medicago* spp.) in drier areas with neutral to alkaline soils (Puckridge and French, 1983; Fig. 1.1).

Southern Australian soils are typically low in organic matter, nitrogen and phosphorus. Falling wheat yields from 1870 to 1900 were temporarily reversed between 1900 and 1920 using long bare fallowing, but declining organic matter levels resulted in soil structure decline, widespread soil erosion, and a return of declining wheat yields by 1930. Self-regenerating annual legume pastures, including medic pastures, were first introduced into Southern Australian cereal farming systems in the mid 1930's, leading to increases in soil fertility and stability, cereal yields, livestock productivity and farm income stability.

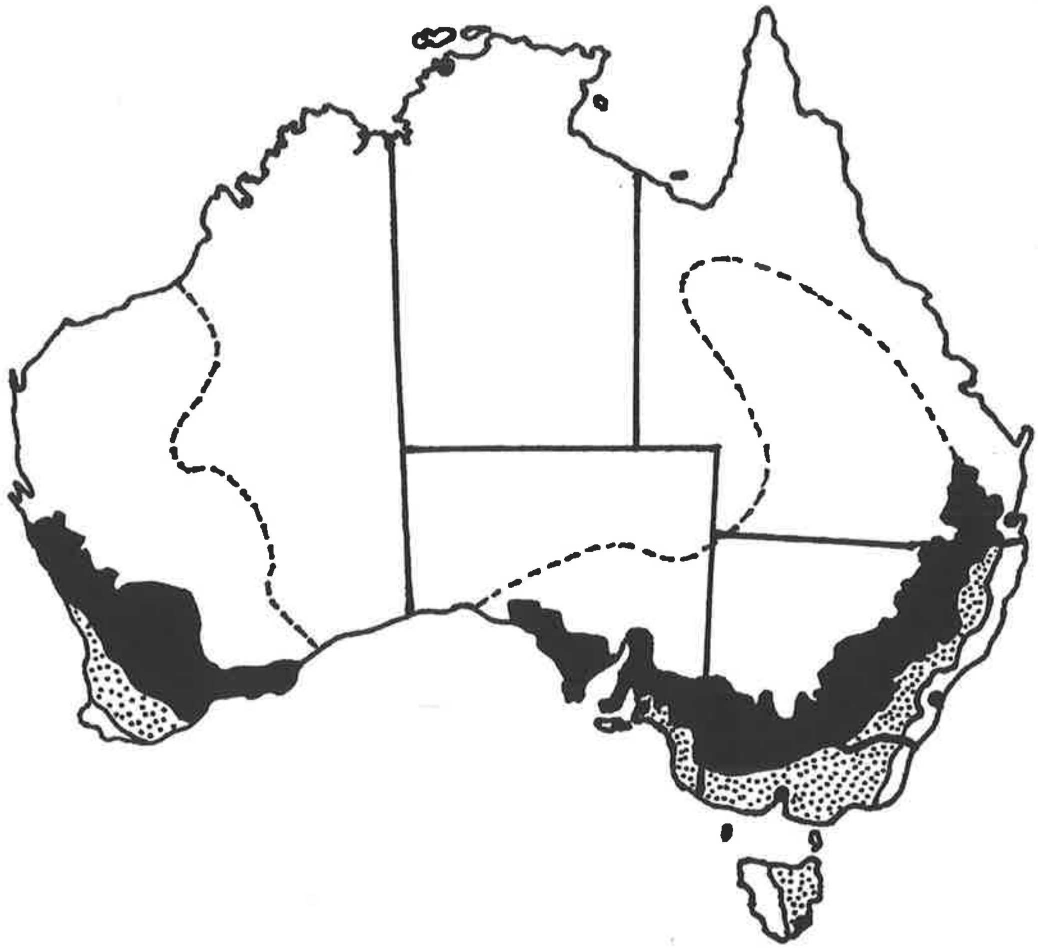


Figure 1.1. Location of the cereal-sheep zone in Australia where medics are most widely adapted and grown. The zone is marked in black and is between the high rainfall pasture zone (dotted) and the arid pastoral zone (bordered by dashed line) (After Puckridge and French, 1983).

The application of super phosphate alleviated the phosphorus deficiency, and in the 1950's very high wool prices allowed widespread sowing of annual legume pastures as growers concentrated more on wool production than crops. This pioneering development in world agriculture was suited to local conditions because the shallow, often fragile soils did not allow deep cultivation, and hence legume pasture seeds were not buried too deeply for reliable regeneration. Legume pasture ley rotation technology was subsequently adopted in many other countries with a Mediterranean climate (Puckridge and French, 1983).

Annual medics are self-regenerating autogamous pasture species which grow in autumn to spring in areas with at least 250 mm annual rainfall. Medics are native to the Mediterranean region and have been accidentally and intentionally introduced to Australia. They grow initially as a rosette of leaves, later producing leaf-bearing runners from a central crown (Fig. 1.2). Runners are either prostrate or semi-erect, depending on growing conditions and crowding. Hard seed coats, which are impervious to water and resist degradation, allow them to re-establish well after one or two years of cropping. Species with registered cultivars in Australia include *Medicago littoralis* (strand medics), *M. murex* (murex medics), *M. polymorpha* (burr medics), *M. rugosa* (gama medics), *M. scutellata* (snail medics), *M. sphaerocarpus, M. tornata* (disc medics), and *M. truncatula* (barrel medics) (CSIRO, 1990). *M. truncatula* contributes most of the commercially-grown cultivars. It is especially suited to drier regions of the wheat belt and is the major medic species in the Victorian and South Australian mallee, growing on light textured, alkaline soils. *M. littoralis* is suited to sandy soils in areas with short growing seasons between 4 and 5 months. *M. rugosa* is particularly adapted to alkaline soils. Volunteer burr medics (*M. polymorpha, M. minima* and *M. laciniata*) and volunteer *M. truncatula* are common and have been naturalised since early farming years. *M. polymorpha* has good forage qualities, but wool contamination from its spiny burrs has led to replacement with other species with less prominent burrs.

Medics are usually more suited to drier cropping zones with alkaline soils than subterranean clovers. Factors which facilitate persistence of medics in cropping rotations are high seed yield, a large soil seed bank, physiological dormancy, and hard-seededness. They are usually grown as a grazed pasture, but are also used for hay, green manure crops, and fodder crops. Medics can also be used to manage herbicide-resistant grass weeds, and are sometimes sown with cereal crops ("under-sown") so that seed is set for the basis of a pasture in the following

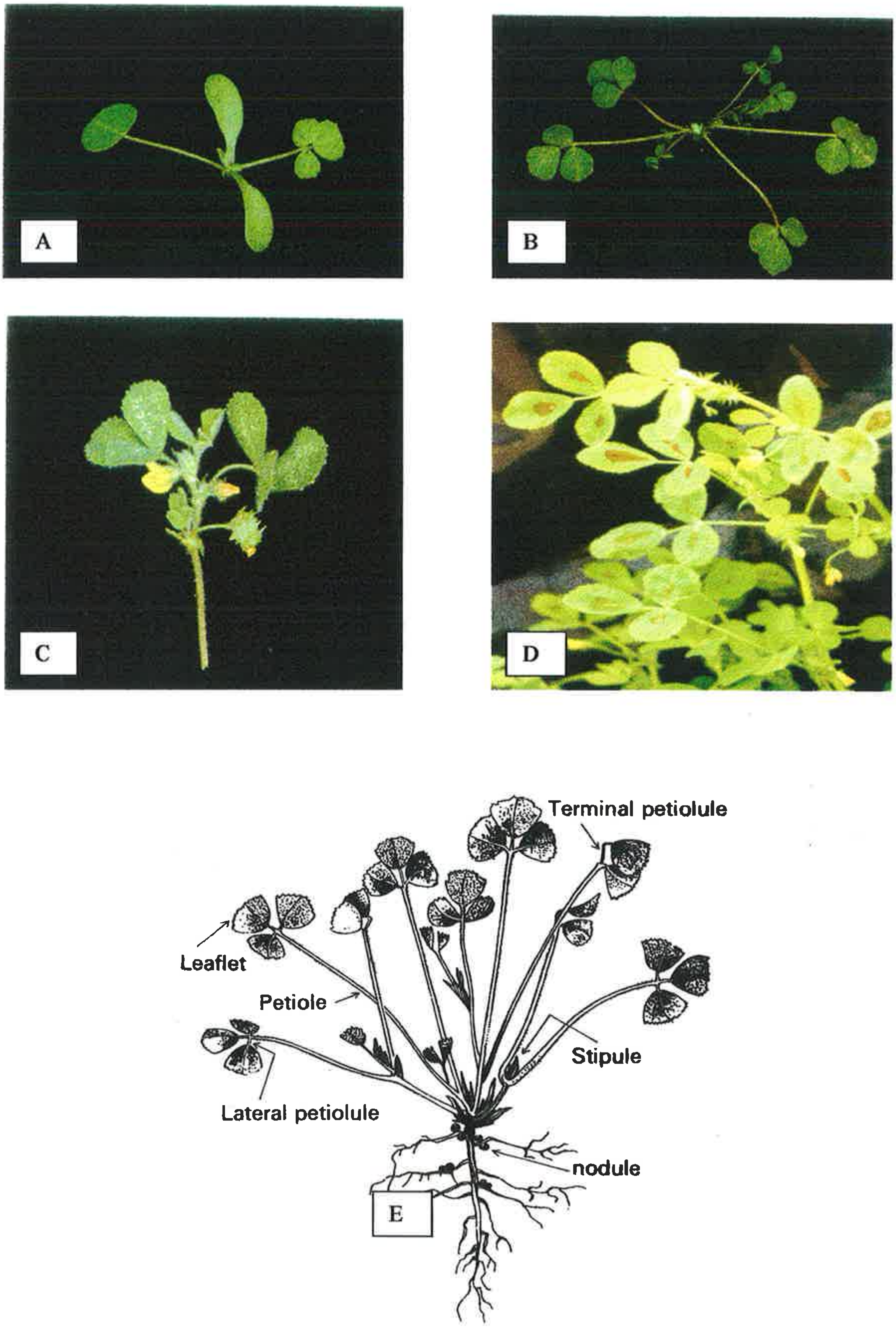


Figure 1.2. *Medicago truncatula*: (A) seedling at first trifoliate leaf stage; (B) seedling; (C) flowering stem; (D) *Medicago littoralis* flowering stem, and (E) *Medicago truncatula* showing roots and nodules (A, B, C: Wilding *et al*., 1986; E: Anon, 1977).

year. Krause (1995) examined the value of pastures in medium-rainfall areas of South Australia, in the light of a swing towards continuous cropping in these areas. This study found that pastures, if included in the rotation, should be managed as intensively as a crop. Returns for each dollar invested ranged from $0.87 to $2.67. Pastures were found to increase income stability, although continuous cropping returned higher short-term returns. High cropping intensity with cereals decreases soil fertility and degrades soil structure. In higher rainfall areas pulses, such as peas, beans, lupins and vetches, in addition to pasture legumes, can help to maintain fertility and structure but in drier areas medics are the only plants available to do this.

Productive medic pastures increase soil N and soil organic matter, reduce cereal root diseases, provide high quality green and dry forage, increase soil water-holding capacity, and reduce the potential for soil erosion. They also improve porosity and drainage, reducing erosion by surface water run-off. Dry seed pods also provide valuable summer feed for sheep (Crawford, 1987a; Puckridge and French, 1983). Medic-based pastures can produce up to 8 t ha^{-1} dry matter under optimum conditions (Puckridge and French, 1983) and they can produce between 50 to 200 kg ha^{-1} of N (Crawford, 1987b). Only about 10 to 15% of fixed N is available to the following crop, and the rest is stored in soil organic matter and slowly released in subsequent years. Medics do not provide an immediate large boost to available N, but ensure a maintenance and steady increase in fertility (Crawford, 1987b).

1.2 ANNUAL MEDIC DECLINE

The benefits of annual medics to cropping rotations in southern Australia have been well documented (Carter *et al.*, 1982; Crawford, 1987a; Krause, 1995; Puckridge and French, 1983). Agriculturalists observed the decline of medic performance and persistence during the 1970's with concern. Establishment density and production declined and self-regenerating medic pastures were characterised by increasing levels of weeds, or bare ground. This trend has continued over the past two decades. The decline of annual medics is the result of a complex interaction of many factors. In 1982, just prior to the widespread adoption of sulfonylurea herbicides in cropping rotations, many factors were thought to be contributing to medic decline. These included damage from red-legged earthmite (*Halotydeus destructor*), lucerne flea (*Sminthurus viridis*), sitona weevil (*Sitona humeralis*) and aphids (*Therioaphis trifolii* and *Acyrthosiphon kondoi*); reduced application of phosphorus; increased cropping intensity and grazing pressure; poor grazing management and fodder conservation practices; increased use of herbicides in cereal crops; decreased under-sowing of medics in cereal crops; spray-topping annual grasses with glyphosate and paraquat; and an increase in application of nitrogenous fertilisers (Carter *et al.*, 1982; Ewing, 1996). Recent surveys and assessments of the problem (Bellotti, 1996; Bretag, 1985; Mebalds, 1987; Neal *et al.*, 1997) have attributed the decline to inadequate soil nutrition (especially P and Zn); root diseases (*Rhizoctonia solani, Pratylenchus* spp., and *Pythium* spp.); sulfonylurea herbicide residues; unfavourable conditions for annual medic rhizobia; and poor management.

Economic and technological changes have favoured more intensive cropping over pastures. Pulses and oilseed crops have been substituted into many continuous cropping rotations, providing the benefits of disease suppression, complementary weed control and nitrogen fixation which were previously associated with pasture/cereal rotations. Continuous cropping is fundamentally incompatible with self-regenerating annual legume species because seed reserves become exhausted (Ewing, 1996).

1.3 ANNUAL MEDIC TOLERANCE TO HERBICIDES

Research on herbicide tolerance in annual *Medicago* species is limited, resulting in conservative registration of a small range of herbicides, which usually refer to medics generically. In general, medics have a poor tolerance to herbicides used for broadleaved weed control, and density, herbage growth, and seed yield can be reduced. There is a wider array of options available for subterranean clover. A greater range of herbicide tolerance or resistance would greatly improve weed control, and hence productivity, in medics (Dear *et al.*, 1995; Madin, 1993; Young *et al.*, 1992).

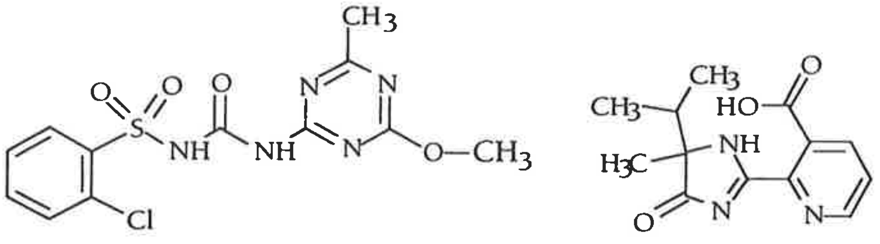
The small range of herbicides registered in Australia for broadleaved weed control in medics includes trifluralin, 2,4-DB amine, 2,4-DB salts, flumetsulam, and MCPA amine. Imazethapyr and metolachlor are also used in medics. When under-sown with a cereal crop, medics are thought to be significantly damaged by 2,4-D amine, 2,4-D ester, bromoxynil/diflufenican, bromoxynil/MCPA, chlorsulfuron, clopyralid, dicamba, diuron, fluroxypyr, MCPA ester, MCPA/diflufenican, metsulfuron-methyl, metosulam, triasulfuron and terbutryn (Heap, 1997; Primary Industries S.A., 1997a&b). Medic tolerance to acetolactate synthase (ALS) inhibiting herbicides (ALSIH) varies widely. They are very susceptible to the sulfonylurea herbicides chlorsulfuron, metsulfuron-methyl, sulfometuron-methyl, and triasulfuron. Amongst the imidazolinones, medics are sensitive to imazapyr (a non-selective herbicide), but have useful field tolerance to imazethapyr. Medics are also tolerant to one triazolopyrimidine, flumetsulam, but not to metosulam. Tolerance to pyrimidinyl thiobenzoates is unknown (Heap, 1997; Primary Industries S.A., 1997a&b).

1.4 ALS INHIBITING HERBICIDES

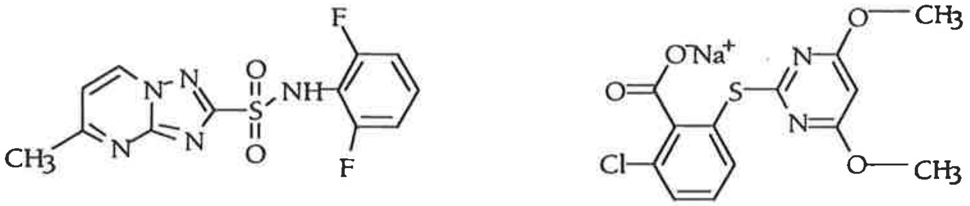
1.4.1 Range and characteristics

Five classes of ALSIH have been commercialised or are under commercial development world-wide; imidazolinones, pyrimidinyl thiobenzoates, sulfonylureas, sulfonylamino-carbonyltriazolinones and triazolopyrimidines (Heap, 2000; Lovell *et al.*, 1996; Fig. 1.3).

Chlorsulfuron (a sulfonylurea) was the first ALSIH commercialised in Australia and was released in 1982 for weed control in cereals.



CHLORSULFURON **IMAZAPYR**



FLUMETSULAM **PYRITHIOBAC-SODIUM**

Figure 1.3. Example herbicide structures representing four ALS inhibitor classes sulfonylureas (chlorsulfuron), imidazolinones (imazapyr), triazolopyrimidines (flumetsulam), and pyrimidinyl thiobenzoates (pyrithiobac-sodium) (Saari *et al.*, 1994).

The sulfonylurea herbicides give broad-spectrum weed control at very low application rates (approximately 2 to 75 g ha^{-1}), have good crop selectivity, sound environmental properties, and very low acute and chronic mammalian toxicity. The extremely high herbicidal potency allows use at 100 to 1000 times lower use rates than some other herbicides, thus improving handling, application and container disposal activities. Commercial ALSIH worldwide are comprised of 54 active ingredients, registered for selective weed control in at least 17 different crops. Of these, 34 are sulfonylureas, 8 are imidazolinones, 5 are triazolopyrimidines, 5 are pyrimidinyl thiobenzoates, and 2 are sulfonylamino-carbonyltriazolinones (Heap, 2000). There have also been ten other chemical classes that inhibit ALS described, of unknown commercial potential (Saari *et al.,* 1994).

Sulfonylureas are readily taken up by roots and foliage, and translocated via the xylem and phloem (Beyer *et al.*, 1988). Naturally-occurring crop tolerance for all ALSIH is based on rapid metabolic inactivation. Metabolic half-lives of ALSIH within tolerant plants is 1 to 5 h under growth room conditions, however in sensitive plants it is typically > 20 h (Brown, 1990). Chlorsulfuron is metabolised in two different ways by tolerant grasses and broadleaved plants, resulting in conjugated metabolites which are non-toxic to plants (Beyer *et al.*, 1988). Sulfonylureas are potent inhibitors of plant root and shoot growth, with visual symptoms including vein reddening, leaf chlorosis, terminal bud death, and tissue necrosis developing within several days of application. Inhibition of plant growth, specifically cell division, has been detected within 1 to 2 h of treatment with chlorsulfuron. Studies on peas confirmed the site of action in plants as ALS, and demonstrated that sulfonylureas have no other adverse affects on plants, even at very high rates (Ray, 1984). The specific activity on ALS confers the very low mammalian toxicity of the sulfonylureas, as mammals do not possess ALS, or the ability to synthesise branched-chain amino acids (Brown, 1990). Early research (La Rossa *et al.*, 1987) suggested that accumulation of α-ketobutyrate, due to ALS inhibition, may be phytotoxic. Later, Shaner and Singh (1993) demonstrated that high concentrations of 2-ketobutyrate (2-KB) and its transaminated product 2-aminobutyrate (2-AB) did not inhibit maize plants, and proposed that branched-chain amino acid starvation was the primary mode of action. The exact mode of action and the connecting links between metabolic changes and subsequent reductions in plant growth remain unknown. Assimilate transport disruption, probably due to ultrastructural cell damage which inhibits sucrose loading into phloem, may be a secondary effect related to amino acid pool imbalances (Kim and Vanden Born, 1997a&b).

1.4.2 Acetolactate synthase (ALS) – the target enzyme

Acetolactate synthase (ALS; EC.4.1.3.18; acetohydroxylated synthase (AHAS)) is the first enzyme common to the biosynthesis of the branch-chained amino acids valine, leucine, and isoleucine (Saari *et al.*, 1994; Fig. 1.4). These amino acids are essential for plant growth processes such as cell division. Disruption of ALS function is lethal to many susceptible plant species (Holt *et al.,* 1993). The sulfonylurea, imidazolinone, triazolopyrimidine, and pyrimidinyl thiobenzoate herbicides act to inhibit this enzyme (Shaner, 1991). ALS occurs in plants, bacteria, archaebacteria, algae and fungi, but not animals (Mazur and Falco, 1989). The ALS gene codes for an enzyme of 670 amino acids, with a molecular weight of about 73 kDa, and is highly conserved in various organisms (Holt *et al.*, 1993). Plants may possess more than one structural form of ALS, and *Brassica napus* cv. Topas was found to possess 5 distinct ALS genes (Rutledge *et al.*, 1991).

Mutations which confer resistance to some, but not all, classes of ALSIH suggest that there may be different binding sites on ALS for different ALSIH. A change in one region of ALS structure may prevent binding by one class of ALSIH, but allow binding of other classes at unchanged regions. There is evidence for examples of both overlapping and non-overlapping binding domains of ALSIH on ALS. Therefore, it is possible for different single mutations to confer resistance to one, some, or all ALSIH, and this is reflected in plant cross-resistance patterns (Saari *et al.*, 1994). At least 24 different amino acid substitutions at 10 different sites conferring sulfonylurea resistance have been patented, and herbicide resistant transgenic plants have been generated incorporating some of these substitutions (Stidham, 1991).

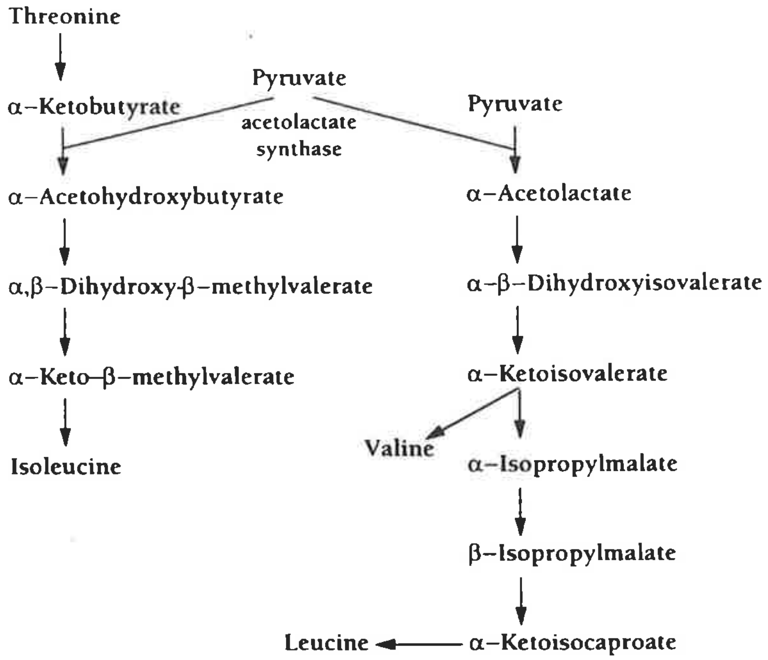


Figure 1.4. Biosynthetic pathway for branched-chain amino acids (Saari *et al.*, 1994).

1.4.3 Role and re-cropping constraints in southern Australia

Low use rates, broad weed control spectrum, crop safety, reliability, low acute and chronic mammalian toxicity and relatively low cost of most products have promoted widespread adoption of selective ALSIH in southern Australian farming systems. They are used in almost every major crop, including wheat, barley, oats, triticale, chickpeas, faba beans, lentils, lupins, field peas, linseed, safflower and pastures. Since 1982 there have been 4 sulfonylurea, 1 imidazolinone and 2 triazolopyrimidine herbicides successfully commercialised for southern Australian field crops.

Chlorsulfuron was the first ALSIH commercialised in Australia, in 1982. It is used pre-sowing or early post-emergence for grass and broadleaved weed control in cereals, and has persistent residues in alkaline soils. There was a widespread drought in southern Australia in 1982, leading to reduced soil residue breakdown, and many farmers were introduced to the issue of rotational crop injury from ALSIH soil residues in 1983. Despite this, chlorsulfuron was adopted widely and continues to be a major herbicide, although concerns over soil residues and recropping restrictions have resulted in reduced use in the 1990's. Metsulfuron-methyl was commercialised in 1987 for post-emergence broadleaved weed control in cereals. Breakdown in soil is relatively rapid, allowing more flexible crop rotation, and the wide array of companion herbicides for tank mixes provides a very broad spectrum of weed control. Metsulfuron-methyl is still a significant herbicide in cereals, and is almost always applied in mixture with other herbicides. Triasulfuron was commercialised in 1989 for pre-sowing control of grass and broadleaved weeds in cereals. While there are recropping restrictions, farmers have perceived the residual effects to be lower than those of chlorsulfuron, and in many cases have replaced chlorsulfuron with triasulfuron. Sulfosulfuron was commercialised in 1999 for control of several grasses and broadleaved weeds in wheat. Imazethapyr (imidazolinone) was commercialised in 1991 for post-sowing, pre-emergence and early post-emergence control of broadleaved weeds in pulse crops and pastures. It also has significant recropping constraints, and degrades more rapidly in alkaline soils than in acid soils. Flumetsulam (triazolopyrimidine) and metosulam (triazolopyrimidine) were commercialised in 1994 for post-emergence broadleaved weed control in cereals, pulses, pastures, and other crops. Flumetsulam recropping intervals are longer than those of metosulam. In addition, bensulfuron-methyl (sulfonylurea) has been developed for rice, sulfometuron-methyl (sulfonylurea) and imazapyr (imidazolinone) have been used for non-selective residual weed control, and a mixture of thifensulfuron-methyl and metsulfuron-methyl (sulfonylureas) is registered for post-emergence broadleaved weed control in cereals in NSW and Qld.

1.4.4 Persistence and phytotoxicity of soil residues

One of the major considerations for users of ALSIH are the recropping restraints imposed by soil residues. In particular, sulfonylureas can cause injury to sensitive crops at very low concentrations, and this is exacerbated by slower degradation in alkaline soils. Annual medics are particularly sensitive to soil residues of sulfonylureas and reports of field injury are common. Field use of sulfonylurea herbicides in Australia is prescribed by specific information on registered product labels. Chlorsulfuron recropping intervals are longer for higher pH soils. If soil pH is greater than 7.5, a total of 700 mm of rainfall is required between application and sowing of sensitive crops, and test plots of sensitive crops (including medics) are required to be grown through to maturity in the previous season. For soil pH of 6.5 or less, many sensitive crops can be grown within 12 to 18 months of application. Chlorsulfuron is not recommended for use on soils with pH > 8.5. These restrictions have important consequences for medics in South Australia, because they are typically grown in rotation with cereals on soils with pH between 7.5 and 8.5. Pragmatism and a lack of flexibility with crop-pasture rotations results in medics regenerating or being sown under conditions which do not satisfy chlorsulfuron label recropping requirements, and this frequently leads to plant damage from herbicide residues. Triasulfuron recropping intervals are also dependent on soil pH. Soils below pH 6.5 allow recropping with most sensitive crops 12 months after application, provided that ≥ 300 mm rain has fallen. In soils with pH > 7.5, typically 24 months with ≥ 500 mm rainfall is required for medics and other sensitive crops. Metsulfuron-methyl is not as persistent as chlorsulfuron or triasulfuron, and many sensitive crops can be grown within 9 to 14 months of application. If soil pH is > 8.5, test plots of sensitive crops are required to be grown through to maturity in the previous season. In soils with pH < 8.6 medics can be sown after 9 months.

The persistent nature of some ALSIH in alkaline soils, in particular chlorsulfuron and triasulfuron, has important implications for crops and pastures grown in the years after application. Annual medics are grown in predominantly alkaline soils (Puckridge and French, 1983) and are particularly sensitive to very low residues of chlorsulfuron and triasulfuron (Evans *et al.*, 1993; Gillett and Holloway, 1996; Noy, 1996). There is widespread concern in southern Australia that sulfonylurea herbicides may leach into subsoils, which are typically very alkaline, and accumulate because rates of hydrolysis and microbial breakdown are very low (Sarmah *et al.*, 1998; Wilhelm, 1997).

Degradation and movement

Sulfonylurea herbicides degrade and dissipate primarily through hydrolysis, microbial breakdown and leaching. Relative to these, photolysis and volatilisation are minor processes. The rates of hydrolysis and microbial breakdown are influenced by soil temperature, pH, moisture, and organic matter content (Beyer *et al.*, 1988; Sarmah *et al.*, 1998). Degradation is bi-exponential (non-first order) with an initial fast rate, as hydrolysis and microbial breakdown are additive. Later there is a herbicide fraction which is sequestered into molecular-sized spaces in soil particle surfaces and is unavailable to microbes, and this fraction is only slowly degraded by hydrolysis (Brown, 1990). Chemical hydrolysis of sulfonylurea herbicides is faster under warm, acid conditions. For example, the hydrolysis half-life of chlorsulfuron at 35°C increases from 6 days at pH 5 to 208 days at pH 8. Residue half-life is longer in drier soils than soils approaching field capacity, and breakdown is faster in light-textured soils than heavy soils (Beyer *et al.*, 1988).

Although sulfonylurea herbicide degradation is relatively rapid, residues are a problem because some species are sensitive to very low soil concentrations (Frederickson and Shea, 1986). For example, sugar beet can be damaged by soil residues of chlorsulfuron of between 0.1 and 1 ppb (Beyer *et al.*, 1988), and 99.5% of applied chlorsulfuron must degrade before sugar beet can be grown without injury (Brown, 1990).

Mobility of sulfonylurea herbicides through the soil profile is controlled by organic matter content, soil pH, soil texture, and porosity. Sulfonylureas are more mobile through soil profiles with high pH and low organic matter content. Chlorsulfuron and metsulfuron-methyl have similar mobility over a range of soil types. Field studies have shown that sulfonylureas move rapidly through alkaline soil profiles to below 60 cm, even in dry years (Wilhelm, 1997). Triasulfuron is less mobile than chlorsulfuron or metsulfuron-methyl (Walker and Welch, 1989). Models have been developed to predict behaviour and degradation of sulfonylurea herbicides, but so far they have not sufficiently integrated the interaction of the complex factors to give reliable predictions (Sarmah *et al.*, 1998).

Residue measurement

Although sulfonylureas usually have a relatively short half-life in soil, very low residue concentrations can significantly damage sensitive crops. If 0-10 cm soil samples were taken immediately after spraying commercial rates, metsulfuron-methyl (3 g a.i. ha^{-1}), chlorsulfuron (11 g a.i. ha^{-1}), and triasulfuron (25 g a.i. ha^{-1}) residues would be 2, 8 and 17 ppb respectively. The NOEL (No Observable Effect Level) for chlorsulfuron and metsulfuron-methyl on two very sensitive crops, sugar beet and lettuce, is between 0.05 to 0.1 ppb and the NOEL for field peas is estimated at 0.2 to 0.6 ppb. Thus, for chlorsulfuron the potentially damaging concentration of 0.05 ppb represents only 0.6 % of the initial dose (McQuinn, 1997).

Three main methods are used to determine sulfonylurea residues; bioassays, HPLC-MS (High Pressure Liquid Chromatography - Mass Spectroscopy), and ELISA (Enzyme Linked Immunosorbent Assays) techniques. Bioassays are the most sensitive, but all give comparable results (Sarmah *et al.*, 1998). Herbicide bio-availability is thought to be more important than herbicide concentration, *per se*, and this is most accurately measured by bioassays (Stork and Hannah, 1996). Bioassays using plants are very sensitive, do not require expensive equipment, measure only biologically active compounds, and do not require pre-extraction techniques. However, they are usually only semi-quantitative, have a limited response range for any one species, require separate calibration curves for each species and herbicide combination, and are relatively slow, requiring from several days to several weeks. Species very sensitive to chlorsulfuron residues include lentils, sugar beets, and onions (Beyer, *et al.*, 1988). Soil bioassays and ELISA assays are sensitive down to levels around 0.05 to 0.1 ppb, and HPLC-MS techniques can currently detect 0.1 ppb (McQuinn, 1997).

Bioassays involve growing sensitive species in soils with unknown herbicide concentration, and then comparing reduction in growth to dose-response curves derived from soil treated with known concentrations. Stork and Hannah (1996) used peas and lupins as test species to determine soil concentrations of chlorsulfuron, triasulfuron, metsulfuron-methyl, flumetsulam and metosulam. Peas and lupins were equally sensitive and reliable estimates were obtained in the range of 0.75 to 6 ppb in clay, and 0.125 to 8 ppb in sand.

Residue effects on rotational crops

Sulfonylurea herbicides are active at low rates, and often soil residues contribute to extended effective weed control. However, even at low concentrations herbicides can have a sub-clinical impact on crop productivity (Evans *et al.*, 1996), and the susceptibility of crops to a range of diseases and nutritional deficiencies can be increased (Ferris *et al.*, 1992; K. Hollaway, pers comm.).

Injury to rotational crops varies greatly and is influenced by many factors, including soil pH, initial applied dose, hydrolysis and microbial breakdown rate, time and rainfall since application, plant species, and stress caused by, for example, drought, frost, disease, or poor nutrition. In alkaline soils in southern Australia, annual medics, pulses and oilseed crops are particularly sensitive to low levels of chlorsulfuron soil residues (Wilhelm, 1997). A Canadian study measured crop growth up to 7 years after chlorsulfuron was applied at 0, 10, 20, and 40 g a.i. ha^{-1} to soils with pH 7.4-8.0. Safe recropping times for 40 g a.i. ha^{-1} were barley, 2 yr; canola, 3 yr; peas and beans, 4 yr; flax and potatoes, 5 yr; lucerne and sugar beets, 6 yr; and lentils, >7 yr (Moyer *et al.*, 1990).

Effect of residues on annual medics

In southern Australia, annual medics are typically grown on alkaline soils with low rates of hydrolysis and microbial breakdown of sulfonylurea herbicides. Alkaline sodic soils comprise the major proportion of soils in the Australian wheat belt, often exhibiting a gradient from pH 8 at the surface to pH 10 in the subsoil (Ferris *et al.*, 1992). In these areas herbicide residues are suspected as a prime cause of poor medic re-establishment (McCarthy, 1995). Damage to medics is restricted to alkaline soils and is normally the result of low rainfall or insufficient plant-back time (McQuinn, 1997). Symptoms include yellowing and stunting of shoot tips and restriction of root growth. An example of field damage to *Medicago littoralis* from chlorsulfuron residues is shown in Plate 1.1. Chlorsulfuron and triasulfuron persistence and leaching was studied in four alkaline sodic soils in the Australian wheat-belt. Chlorsulfuron caused damage to medics, chickpeas and lentils in the field up to 29 months after application. A persistence and leaching model predicted that application of chlorsulfuron every 4 years in an alkaline Mallee sand would potentially injure legumes (Ferris *et al.*, 1992).

The residual effects of chlorsulfuron (11 g a.i. ha^{-1}), metsulfuron-methyl (4 g a.i. ha^{-1}) and triasulfuron (21 g a.i. ha^{-1}) on annual *Medicago* was investigated on a sandy clay loam, with a surface pH of 8.5-9.0, and a subsoil (>10 cm) pH of 9.5. In the year following treatment, metsulfuron-methyl had no effect on annual medic survival, herbage yield or seed yield. However, chlorsulfuron reduced the three parameters by 31%, 77%, and 92% respectively,

Plate 1.1. The effect of chlorsulfuron soil residues (top picture, right hand side) on winter growth of *Medicago littoralis*. Close-up pictures are of affected plants (middle picture) and adjacent plants in soil without residues (bottom picture).

and triasulfuron had no effect on density, but reduced herbage yield by 63%, and seed yield by 55%. Two years after application, chlorsulfuron reduced density of the medic stand by 71%. It was concluded that chlorsulfuron damage was obvious, but triasulfuron damage was less evident, and may not be noticed by farmers unless careful comparison is made with untreated plants (Evans *et al.*, 1993). In a study on an alkaline soil in the Victorian Wimmera, three sulfonylureas were applied at maximum field rates to plots one and two years prior to sowing *Medicago truncatula* cv Mogul. One year after application, medic growth was reduced by 89% and 60% by chlorsulfuron (15 g a.i. ha^{-1}) and triasulfuron (25 g a.i. ha^{-1}), and by 75% and 33% two years after application. Metsulfuron-methyl (4.2 g a.i. ha^{-1}) residues may have slightly stimulated medic growth. Emergence was not effected by the residues (Noy, 1996).

Simulated soil residues 1% and 4% of triasulfuron field application rates, approximating levels typically found 12 months after application, produced no visual damage to shoots of *Medicago truncatula* cv. Caliph, however shoot dry weight was reduced. The growth reduction in the absence of obvious shoot damage raises concerns with farmer assessments of low potential for annual medic damage from triasulfuron residues, and indicates that root and shoot growth may be restricted in the field, significantly contributing to medic decline (Gillett and Holloway, 1996).

Interference with N-fixing symbiotic *Rhizobia*, found with *Medicago sativa* (Koopman *et al.*, 1995; Martensson, 1992; Martensson and Nilsson, 1989), also occurs with *Medicago truncatula*. Rovira *et al.* (1993) investigated the effect of chlorsulfuron residues equivalent to 0.02 - 0.16 g ha^{-1} on *Medicago truncatula* cv. Cyprus growth and nodulation in a sandy loam soil with pH 8.6. Nodulation was significantly reduced at 0.02 g ha^{-1} (c. 0.125% of field rate), and 0.16 g ha^{-1} (c. 1% of field rate) reduced nodule numbers to less than 10% of untreated.

After 40 days 0.16 g ha^{-1} had reduced root dry weight by 74%, and reduced taproot length by 53%.

1.5 RESISTANCE TO ALS INHIBITING HERBICIDES

1.5.1 Resistance mechanisms and natural tolerance

There are at least five mechanisms for herbicide resistance; morphological barriers, differential uptake/translocation, altered receptor sites, amplification of target enzymes and metabolic detoxification (Froud-Williams, 1991). The term "resistance" is usually applied to a population which was once predominantly susceptible to a herbicide, and "tolerant" is used to describe a naturally-occurring, pre-existing ability to survive a herbicide application (Holt and LeBaron, 1990). Multiple-resistance is the phenomenon of populations or individual plants possessing more than one resistance mechanism. Cross-resistance is where a population, following exposure to one herbicide, evolves resistance to herbicides from different classes to which it has not been exposed. It is possible for multiple- and cross-resistance to co-evolve within a population (Holt *et al.*, 1993).

A range of plant species have natural tolerance to ALSIH, and in all cases studied the mechanism has been determined to be metabolic inactivation. In naturally tolerant species ALSIH are broken down to non-phytotoxic metabolites rapidly enough to prevent lethal amounts from reaching the target-site ALS (Saari *et al.*, 1994). For example, the tolerance of wheat to triasulfuron appears to be due partly to reduced translocation, and mostly due to rapid metabolic inactivation (Meyer and Muller, 1989). There are at least nine known metabolic inactivation pathways for sulfonylureas, and cytochrome P450 monooxygenase systems have been implicated as a catalyst in some hydroxylation reactions (Brown *et al.*, 1991). Susceptible species cannot sufficiently metabolise ALSIH, thus exposing sensitive

ALS to lethal concentrations of herbicides. Hydroxylated derivatives may have some reduced activity on ALS, and species with intermediate susceptibility may metabolise, but not then conjugate the herbicide derivatives. Glucose conjugation often follows aryl and aliphatic hydroxylation and contributes to tolerance (Saari *et al.*, 1994).

Mechanisms for ALSIH resistance have been reviewed by Saari *et al.*(1994), and Devine and Eberlein (1997). In most cases in which weeds have developed resistance to ALS-inhibiting herbicides it is conferred by a mutation in a gene coding for the ALS enzyme (Devine and Eberlein, 1997), but there are also cases in which resistance is based on gene amplification or metabolic inactivation. Gene amplification is usually induced when cultured cells are subjected to step-wise selection, but this resistance is not always stable and heritable (Caretto *et al.,* 1994). Populations of *Lolium rigidum* have evolved resistance to ALSIH due to metabolic inactivation, likely conferred by high cytochrome P450 activity, however many cases of resistance in this species are based on mutated ALS (Christopher *et al.*, 1992; Christopher *et al.,* 1994). There are also populations of *Kochia scoparia* (Kwon and Penner, 1995) and *Sorghum halepense* which are thought to be resistant because of enhanced herbicide metabolism (Devine and Eberlein, 1997).

1.5.2 Resistant ALS enzyme target sites

Target-site resistance to ALSIH was first discovered in weeds (e.g. Mallory-Smith *et al.,* 1990), and was later utilised in crops. There are now more documented weed species (64) with resistance to ALSIH than to any other herbicide mode of action (Heap, 2000). Development of resistance is influenced by the selection pressure exerted by herbicides, absolute and relative fitness of resistant biotypes, initial frequency of resistant genes, soil seed bank dynamics, and gene flow. Selection intensity for ALS inhibitors is often high, especially where high rates are applied to control the least sensitive weed species present. More sensitive species often receive a dose at least twice that required to kill susceptible individuals. The long soil residual activity of many ALS inhibitors also increases the selection intensity. Herbicide resistance has usually developed most rapidly in rotations or mono-cultures dependent on herbicides with the same mode of action. These systems may become more prone to herbicide resistance as new ALS herbicides and ALS-resistant crops are introduced, thus increasing the frequency of use of ALSIH at the expense of herbicides with different modes of action.

Target-site resistance dominates in weeds that are very sensitive to ALSIH, suggesting that other mechanisms (e.g. metabolic inactivation) cannot overcome relatively large doses as effectively as target-site mutations. Studies have not yet identified any reduction in fitness associated with ALSIH resistance in the absence of herbicide. There are usually no large differences found between ALSIH resistant and susceptible biotypes for a range of parameters including seed production, seed germination, seed longevity, competitiveness, and ALS catalytic competency (enzyme performance). This suggests that where resistance has developed in the field, resistant gene frequencies may remain high even if ALSIH use is discontinued (Saari *et al.*, 1994).

In fungi, bacteria and plants, often a single base substitution in an ALS gene confers resistance to ALSIH. Laboratory-derived mutants and resistant weeds usually have a single base substitution in one of five highly conserved domains of the ALS gene (Fig. 1.5; Table 1.1; Devine and Preston, 2000). In two of the earliest cases studied, ALS mutations in weeds conferring ALSIH resistance involved changes from Pro_{173} to His (*Lactuca serriola*) and Pro_{173} to Thr (*Kochia scoparia*). It may be possible to elucidate the underlying substitution position and type indirectly by comparing enzyme cross-insensitivity patterns and whole plant cross-resistance patterns to those of biotypes with a known substitution (Mazur and Falco, 1989). ALSIH resistance in weeds usually involves a single major gene which may be dominant or semi-dominant (Anderson and Georgeson, 1989; Chaleff and Ray, 1984; Saari *et al.*, 1994). Resistant phenotypes are qualitatively described using herbicide rates required to reduce survival (LD_{50}), biomass (GR_{50} or ED_{50}), or specific enzyme activity (I_{50}) by 50% compared to untreated plants. The resistance factor is a ratio of the response to herbicides of resistant and susceptible isolates, at the whole plant or enzyme level. For whole plants it is the fold increase in herbicide rates required to inhibit some growth parameter (eg LD_{50} or GR_{50}) (Saari *et al.*, 1994).

ALSIH resistant weeds often have some degree of cross-resistance across ALS inhibitors. In weeds selected with sulfonylureas, cross-resistance has been found to virtually all other sulfonylureas. Most of these biotypes were also cross-resistant to imazapyr (imidazolinone), at a lower level, and there were varying patterns of cross-resistance to herbicides from other classes of ALS inhibitors. It is clear that many different ALS mutations confer resistance, and that these are reflected in different spectra of resistance and cross-resistance to a range of ALS inhibitors. Some laboratory-selected biotypes of plants, selected with a particular ALS inhibitor, are more sensitive to certain other ALS inhibitors, but this phenomenon has not been observed in the field. If weeds resistant to one class of ALSIH (e.g. sulfonylureas) are treated with an ALSIH from another class (e.g. imidazolinones) there is a possibility that a double mutant may be selected, thus conferring higher levels of resistance to a wider range of ALSIH.

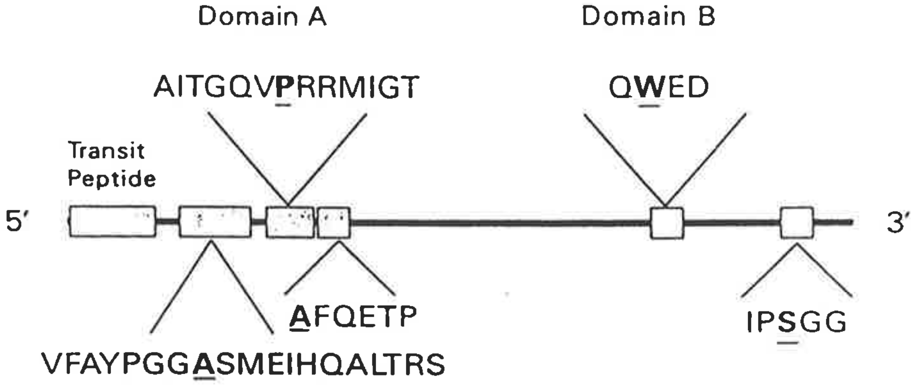


Figure 1.5. Conserved amino acid sequences in the ALS protein implicated in resistance to ALS-inhibitor herbicides. Within each conserved sequence, the specific amino acid substitution site implicated in resistance is shown in bold (Devine and Eberlein, 1997).

Table 1.1. Mutations conferring resistance to ALS-inhibiting herbicides, with generalised levels of resistance conferred by each mutation to three classes of ALS inhibitor (SU, sulfonylureas; TP, triazolopyrimidines; IM, imidazolinones). Amino acid position numbers refer to the functionally equivalent position in *Arabidopsis*. Amino acids shown in bold type are replaced in the resistant biotypes (Devine and Preston, 2000).

Domain	Mutation	Level of resistance to		
		SU	TP	IM
(A) AITGQV**P**RRMIGT	Pro_{197} to Ala	High	Mod-low	Zero
	Pro_{197} to Thr	High	?	Low-zero
	Pro_{197} to His	High	Low	Mod
	Pro_{197} to Lue	High	High	Mod-low
	Pro_{197} to Arg	High	?	?
	Pro_{197} to Ile[a]	High	Mod-low	Mod-low
	Pro_{197} to Gln	High	?	?
	Pro_{197} to Ser	High	High	Zero
(B) Q**W**ED	Trp_{591} to Lue	High	High	High
(C) VFAYPGG**A**SMEIHQALTRS	Ala_{122} to Thr	Low-zero	Low-zero	High
(D) **A**FQETP	Ala_{205} to Asp	High	?	?
(E) IP**S**GG	Ser_{670} to Asp	Low	Zero	High

[a] Double mutation, CCT to ATT (Boutsalis *et al.*, 1999).

Mazur and Falco (1989) identified, isolated and sequenced 41 spontaneous mutations on the *ILV2* gene of yeast which resulted in sulfonylurea-resistant ALS. There were 24 different amino acid substitution mutations which occurred over 10 positions. Site-directed mutagenesis produced many more different mutations at the 10 positions. Different amino acid substitutions, at the same or different positions, produced different resistance and enzyme activity properties. Analogous amino acid substitutions were found in ALS in resistant weeds, and these were transferred to crops using transformation. At some sites (e.g. Ala_{117}, Pro_{192} or Trp_{586}) almost all nucleotide substitutions induced resulted in less sensitive ALS, while at other sites only a few substitutions conferred resistance. The level of reduced sensitivity to chlorsulfuron ranged from 5 to 1000 times that of wild type ALS.

ALSIH are thought to bind primarily to a site distal to the catalytic site (Schloss *et al.*, 1988), however in a minority of mutants catalytic function and affinity for pyruvate are decreased (Rathinasabapathi and King, 1991; Subramanian *et al.*, 1990). Saari *et al.* (1994) have proposed both overlapping and non-overlapping binding domains for ALSIH, based on competitive binding studies and cross-resistance patterns. ALS is regulated by feedback inhibition from the three amino acids synthesised by the enzyme, and sometimes mutations conferring resistance to ALSIH can also alter the regulation mechanism. This can result in over-production of some amino acids and an imbalance in the free amino acid pool. Resistance to ALS inhibition by valine, a branched chain amino acid product of the ALS enzyme and an allosteric regulator of ALS, was found to be conferred by a single amino acid change to ALS in tobacco (Hervieu and Vaucheret, 1996).

Chaleff and Ray (1984) first reported on deliberate *in vitro* selection of plants resistant to ALSIH, selecting tobacco mutants with resistance to chlorsulfuron and sulfometuron-methyl. Since then there have been ALSIH resistant mutants selected in numerous species, including *Arabidopsis thaliana,* birdsfoot trefoil, canola, carrot, chicory, cotton, *Datura innoxia,* flax, lucerne, maize, rice, soybeans, sugarbeet, tobacco and wheat (Saari *et al.*, 1994). Sulfonylurea resistance has also been transferred to tomato, sugarbeet, oilseed rape, lucerne, lettuce and melon, using genetic transformation. These transformations resulted in resistance at the cellular level, and in some cases, the whole plant level (Mazur and Falco, 1989). Selection of mutants with altered ALS is relatively easy, and there are no reports of yield losses in crops selected with mutated ALS (Gressel, 1992). Most of the resistance is apparently based on target site insensitivity to ALSIH, but it is also possible to transfer genes for metabolic inactivation of ALSIH from fungi and bacteria to plants. While ALS-inhibiting herbicide-resistant crops have a potentially important role in agriculture, their use may increase the use of ALSIH, thus increasing the risk of ALSIH resistant weeds evolving (Saari *et al.*, 1994). There have been no reported attempts to select ALSIH resistance in annual *Medicago* species, however Stannard (1987) screened 20 million germinated lucerne (*Medicago sativa*) seeds with chlorsulfuron, and found 2 genotypes with increased resistance.

1.5.3 Resistance gene frequency

The likely success of identifying a mutant genotype with altered ALS is greatly influenced by the mutation frequency and survival of mutants. Mutation frequency can be increased by mutagenic treatment of seeds or cell cultures, or by the somatic variation generated during tissue culture regeneration. The relationship between gene frequency and population size screened to detect a resistant individual is broadly logarithmic, and an estimate of the likely gene frequency is useful to determine the appropriate plant or cell population size to screen to ensure a reasonable likelihood of success (Maxwell and Mortimer, 1994).

In plant populations, the mutation rate, herbicide selection pressure, seedbank dynamics and fitness interact to determine the frequency of genes conferring resistance. Density, competition and gene flow also have an influence (Holt and Thill, 1994). While intensity of selection pressure, and population size are relatively easily determined, there have been few published estimates of initial mutant gene frequency (Maxwell and Mortimer, 1994). Initial frequency of resistant phenotypes is influenced by the mutation rate, the number of genes involved, the dominance of the alleles, and the ploidy. Typically monogene-dominant resistant phenotypes are found at 10^{-5} to 10^{-6}, and monogene-recessive phenotypes at 10^{-9} to 10^{-11} (Gressel and Segel, 1982). A gene for resistance may confer a lower fitness than the wild type gene in the absence of herbicide, thus restricting the frequency of the gene to a very low level in unsprayed populations, however plants with altered ALS have been found to suffer no fitness disadvantage (Holt and Thill, 1994).

The initial frequency is a function of mutation rate (μ) and the selection co-efficient against the resistant plant with the allele (s). The frequency of the resistant alleles at equilibrium (q_e) is estimated by:

$$q_e = \mu/hs$$

where h is the degree of dominance of the allele (dominant = 1). If the fitness of the resistant plants is considerably reduced (eg 25% as fit as wild type) then the equilibrium frequency may only be 1 to 5 times higher than the mutation rate, however if there is no or little loss of fitness (e.g. ALS mutants) the resistant allelles can occur at equilibrium at 100 to 400 times the mutation rate. Assuming a mutation rate (μ) of 1×10^{-6}, a completely dominant allele ($h=1$) and 99% fitness ($s = 0.01$) the equilibrium frequency for a self-fertilised species is estimated at 1.09×10^{-4}. There are some limitations on the use of the model, including insufficient data for μ and s, and the number of generations (e.g. 500) required to attain equilibrium in some scenarios (Jasieniuk *et al.*, 1996).

For resistance to develop, there must first be genetic variation for resistance within the susceptible population. The source of this variation is likely to be mutation, which generally occurs spontaneously (Jasieniuk *et al.*, 1996). New mutations are continuously generated within natural plant populations, typically at 10^{-8} to 10^{-9} gametes per locus per generation. These values are often used when modelling single, nuclear gene inheritance of herbicide resistance (Jasieniuk *et al.*, 1996). Even with a low resistant gene frequency, the probability of at least one herbicide resistant mutant plant in a 30 ha field can be high, especially if the plant density is high, the mutant gene is dominant, and the species is outcrossing. Resistance is less likely to occur in 30 ha in species with low density ($<5m^{-2}$) and low mutation rates ($<1x10^{-8}$). For outcrossing species, there is a very low probability of resistance arising based on a recessive allele. By contrast, the probability of occurrence of a recessive resistant mutant plant in highly selfing species is similar to that of a plant with a dominant allele (Jasieniuk *et al.*, 1996).

Modelling studies suggest that typical frequencies for resistant individuals with ALSIH resistance in unsprayed populations are around 10^{-6} or less. The rapid and common occurrence of ALSIH resistance in weeds is probably not due to an unusually high resistant gene frequency, but rather the high selection intensity, high seed production of weeds involved, rapid and frequent seed germination, efficient seed and pollen distribution systems, and high levels of fitness (Saari *et al.*, 1994). As an example, if in *Kochia scoparia* the ALSIH resistant gene frequency is 10^{-7} and there are 1 million seeds ha^{-1} produced, one would expect to find 10 resistant individuals in a 100 ha field (Maxwell and Mortimer, 1994). *Arabidopsis thaliana* with mutated ALS conferring resistance to chlorsulfuron was selected following mutagenesis with EMS (ethyl methane sulphonate). EMS increases the general mutation rate in this species by over 1000-fold and it was estimated that spontaneous mutations conferring chlorsulfuron resistance occur at about 1×10^{-9} (Haughn and

Sommerville, 1987). Initial resistance frequency is apparently very high for annual ryegrass (*Lolium rigidum*), and in field and laboratory screening experiments Preston and Powles (2000) estimated the initial mutant ALS gene frequency to be between 4.6 x 10^{-5} to 1.2 x 10^{-4}. There have been no attempts to estimate the initial resistance frequency for ALSIH in annual *Medicago* species, however Stannard (1987) screened 20 million germinated lucerne (*Medicago sativa*) seeds for chlorsulfuron resistance, and found 2 genotypes with increased tolerance, suggesting a frequency of around 1 x 10^{-7}.

For predominantly self-fertilising plants, such as annual *Medicago* species, the probability of a resistant individual occurring in a 30 ha field increases from 0.28 at 1 plant m^{-2} to 1.0 at 500 plants m^{-2}, with a resistant gene frequency of 10^{-6}. There is a similar probability range (0.003 to 0.81) at a frequency of 10^{-8}, but if the frequency is 10^{-10} the range is only 0.00003 at 1 plant m^{-2} to 0.2 at 500 plants m^{-2}. If the frequency of resistant plants is 10^{-6} then at least 3 x 10^{6} plants must be screened to be 95% confident of there being at least one resistant plant in the sample. Due to the uncertain validity of underlying assumptions, model-estimates of initial gene frequency are highly approximate (Jasieniuk *et al.*, 1996).

There have been several estimates, between 0.5 x 10^{-6} and 2.7 x 10^{-8}, made for gene frequency conferring ALSIH resistance in cell cultures. Selections made from tobacco cells using primisulfuron, and treatment with the mutagen *N*-ethyl-*N*-nitroso-urea prior to selection resulted in a 25-fold increase in resistant lines. The mutation rate per cell generation was estimated to be 2.7 x 10^{-8} for non-mutagenized cells, and 30 times higher for mutagenised cells (Harms and DiMaio, 1991). Cotton cell lines resistant to the sulfonylurea primsulfuron had an estimated spontaneous resistant gene frequency of 0.5 x 10^{-6} (Rajasekaran *et al.*, 1996). *Datura innoxia* cells mutated with EMS produced sulfonylurea-resistant variants at about 1 to 3 x 10^{-7} (Saxena and King, 1988).

1.6 SELECTION FOR HERBICIDE RESISTANCE IN CROPS

1.6.1 Rationale for herbicide resistant crops

The impetus to develop herbicide resistant crops arose from the evolution of resistant weeds, and the underlying molecular mechanisms selected in resistant crops are often similar or identical to those which have spontaneously evolved in weeds (Saari *et al.*, 1994). There are four major perceived needs and opportunities leading to development of resistant crops. The first is poor weed control options in relatively minor crops (e.g. Pofelis *et al.*, 1992). The expensive development costs for new herbicides for use in minor crops has lead to a paucity of options in some cases, and selection of ALSIH herbicide-resistant crops can be a relatively inexpensive way of developing a new, effective herbicide option (Newhouse *et al.*, 1991). Development costs for new herbicides have risen sharply, and the proportion of compounds screened which are ultimately commercialised had decreased from 1 in 2,000 in the 1950's to about 1 in 20,000 by the 1980's (Mazur and Falco, 1989).

Secondly, many crop and pasture species grown in rotation are damaged by soil residues of some ALSIH (see 1.4.4). Development of plants with resistance to soil residues allows a wider diversity and flexibility in rotations, thus leading to increased finacial stability and agronomic sustainability. Sugarbeet (Saunders *et al.*, 1992), flax (Jordan and McHughen, 1987) and soybeans (Sebastian and Chaleff, 1987) are among crops sensitive to sulfonylurea soil residues, and attempts have been made to identify resistant lines to allow them to be grown in close rotation with crops in which residual sulfonylureas are applied. This relates to the major focus of this study; to identify a mutant line of an annual medic which can tolerate soil residues of sulfonylurea herbicides.

A third situation where herbicide-resistant crops are useful is where the crop is closely related to major weeds. For example, the likelihood of discovery of selective herbicides to control a range of brassica weeds in canola is very low (Newhouse *et al.*, 1991). The development of herbicide resistant canola cultivars with resistance to glyphosate, triazines, glufosinate, and imidizolinone herbicides now makes control of related weed species possible. The fourth impetus to develop herbicide resistant crops is the commercial advantage gained from providing cheaper and better broad spectrum weed control.

1.6.2 Selection techniques

Classical crop breeding has been improved and accelerated by a number of new sophisticated genetic approaches, and these techniques were first applied to selection for herbicide resistance in crops. Herbicide resistant genes have been developed for agronomic use, and also for use as selectable markers for transformations linked to other traits (Mazur and Falco, 1989).

Techniques used for development of herbicide resistant crops include classical breeding, somatic hybridisation, *in vitro* selection, and transgenic engineering. Classical breeding is time consuming, laborious, and often uses large spaces. Limited crosses can be made each year, and many species are incompatible when crossed with other species. Somatic hybridisation overcomes some interspecies incompatibility, and allows protoplasts from plants of closely related genera to be fused to produce resistant hybrids. *In vitro* mutant selection, sometimes using mutagens to generate genetic variability, is relatively cheap and allows many genotypes to be screened rapidly using little space. Not all species are amenable to regeneration from callus, and there is usually a need for further breeding to ensure that the cultivar is agronomically suitable. There has been rapid and successful progress transferring genes for herbicide resistance into crops, usually using the T_i plasmid of *Agrobacterium* *tumefaciens* as a vector (Froud-Williams, 1991). Transgenic engineering techniques were considered to be beyond the limited time scope of this study and so the topic is not further reviewed.

There is an array of techniques used to generate resistant mutants, using many culture methods. Selection has been applied as an initial high herbicide concentration, or sometimes as a series of step-wise increases (Caretto *et al.*; 1994), and in some cases haploid cells have been used for selection (Chaleff and Ray, 1984; Saxena and King, 1988). While resistance is commonly based on altered ALS structure, conferred by a mutation of a gene coding for ALS, there are also examples of gene amplification, especially where step-wise selection is used (Caretto *et al.*, 1994).

The success of these methods has been varied, but there are numerous examples of selection of stable mutants exhibiting high levels of ALSIH resistance at the whole plant level (e.g. Pofelis *et al.*, 1992). Some of these methods are reviewed in more detail below.

1.6.3 Mutagenesis

Mutations are genetic changes which occur naturally, or are induced by physical or chemical agents. They can be inherited, and form the basis for evolution and selection. Spontaneous mutations occur continuously in natural populations, but usually at a low rate. New mutations rarely confer an evolutionary advantage to the carrier. Plant breeding involves generation of genetic variability, selection of desirable genotypes and demonstration of the superiority of selected genotypes (Brock, 1970). Mutagenesis is a plant breeding technique used to artificially generate increased frequency and range of mutations, and variability. In the late 1920s and 1930s, researchers in Europe demonstrated that mutation frequency could be increased by irradiating live material with X-rays, and induced mutations are now regarded as a useful addition to conventional plant breeding methods. Variation generated by induced mutation is essentially no different to spontaneous mutations in nature, and laboratory mutagenesis may be considered as an evolutionary shortcut to that which occurs more slowly in nature (Darmency, 1994). Induced mutations have resulted in hundreds of released cultivars in cereals, legumes, fruit trees and other crops. Improved characters have included yield, lodging resistance, disease resistance, maturity time and quality (Halloran *et al.*, 1979).

It is very difficult to predict the size of populations which need to be treated and screened to obtain a given probability of generating a desirable genotype. Where seeds are used, experience has shown that large numbers (thousands to millions) and an efficient screening process are usually necessary (Brock, 1970). The untreated seed is called M_0, the treated seed generation is M_1, and the first generation from the treated material is M_2. Mutagenesis is more suited to autogamous species than allogamous species (Maliga *et al.*, 1981).

Mutation induction

There are three groups of mutatgenic agents: ionising radiation (e.g. X-rays, gamma rays and beta rays); non-ionising radiation (e.g. ultra-violet light) and chemical mutagens (Brock, 1970). UV-irradiation is sometimes used as an alternative to X-rays and gamma-rays, where the later are not available, and is an extremely efficient physical mutagen (Dix, 1986). Chemical mutagens were developed extensively from the 1960s. Physical and chemical mutagens result in three types of damage to plants: physiological (primary damage); factor mutations (point mutations, "gene" mutations); and chromosomal mutations (chromosomal aberrations). Successful treatment aims to maximise factor and chromosomal aberrations, while minimising physiological effects, which set an upper limit on dose intensity. For a given mutagenic treatment there is a correlation between seedling height and survival, and mutation frequency (Gaul, 1970). Different mutagens produce different spectra of mutations, which can be modified by the species, organ or even the chemical or physical state of the organ treated. Thus, when dealing with the uncertainty of effect, the best that can be hoped for is to maximise mutation rate, while minimising undesirable damage. Mutation rate increases with dose, but so does lethal damage. Chromosomal aberrations include translocations, inversions, deletions and duplication. Many of these may lead to sterility, often due to infertile pollen (Halloran *et al.*, 1979).

Dix (1986) suggests that use of mutagens prior to cell selection *in vitro* may not always be necessary, and often is not demonstrably superior to the mutation rate from somaclonal variation alone. For example, it is argued that if the desired mutation occurs at 10^{-7} to 10^{-8} per cell division, and that typical cell densities *in vitro* are 10^{4} to 10^{7} ml^{-1}, then desired variants should be present without recourse to mutagens. There is, however, sufficient evidence to suggest that use of mutagenesis is beneficial in many cases, and mutagens have been used to increase mutation frequency to around 10^{-3} (Maliga *et al.*, 1981).

Gamma rays were used to irradiate *Medicago polymorpha* var. *polymorpha* in an attempt to generate mutants. Selections were made which flowered 2 to 3 weeks earlier than controls, and had the same vigour. Mutation was recommended for use with self-fertilising annual species where the primary selection criteria are clear cut and simply measured (Brock *et al.*, 1971).

Alkylating agents and sodium azide

The most widely used and potent chemical mutagens are alkylating agents, including ethyl methane sulphonate (EMS), diethyl sulphate (dES), ethylene imine (EI), nitroso ethyl urethane (NEU) and nitroso ethyl urea (NEH). Some base analogues, antibiotics, and various miscellaneous compounds have also been found to be mutagenic (Heslot, 1970; Legator and

Flamm, 1973). Alkylating agents act by altering the pairing capacity of single bases in the DNA sequence, and can cause both DNA backbone breakages as well as point base sequence changes. Although DNA appears to be the main target, repair enzymes may also be inactivated, preventing repair of DNA modifications. Since mutations occur at the single cell level, resultant growth often results in a chimera, where only certain sectors of the tissue or organ carry the mutation. Due to this phenomenon, not all seeds of a plant carrying a mutated sector can be expected to carry the mutation, and single inflorescences are normally the unit of selection, rather than single plants (Halloran *et al.*, 1979). Dose depends on properties of the agent, solvent medium and biological system, and must take into account concentration, duration of treatment, and temperature (Kamra and Brunner, 1970).

EMS, diethyl sulfate and certain base-substitution nitroso compounds induce higher proportions of point mutations than chromosomal aberrations. EMS is the mutagen most frequently used in plant cell cultures, with mixed success (Dix, 1986). Due to the specific nucleotide substitutions caused by EMS, not all mutations may be represented in EMS-mutagenised populations, and in some cases additional mutagens may need to be used to induce particular mutations (Haughn and Sommerville, 1987). EMS is one of the least toxic chemical mutagens for humans, but most agents are also carcinogens, and hence inhalation and skin contact must be avoided (Kamra and Brunner, 1970). Sodium azide is not an alkylating agent, but causes either base substitutions or very small deletions within a specific locus (Nilan, 1981).

1.6.4 Cell and tissue culture

Cell and tissue culture techniques are important plant breeding tools and advances have been rapid because the techniques are crucial for gene transfer to create transgenic plants (Larkin, 1986). Somatic embryogenesis, a precursor to regeneration of plantlets, is complex and incompletely understood (reviewed by Dodeman *et al.*, 1997). In general terms, pieces (explants) from donor plants are incubated on media containing auxin and cytokinin compounds to initiate a callus of dedifferentiated cells. Explants can be derived from virtually any plant organ or cell type, including embryos, microspores, roots, leaves and protoplasts. Callus is then transferred to different media to promote formation of somatic embryos; "somatic embryogenesis". Embryos are transferred to another media to promote shoot and root development and then plantlets are acclimatised in media or soil. The ease and efficiency of the process varies greatly between species and between genotypes within species (Larkin and Scowcroft, 1981). Hybridisation, using somatic protoplast fusion, may allow regeneration of cell lines which cannot be regenerated by routine techniques (Maliga, 1984).

Mutation and direct selection in cell culture is well suited to selection for herbicide resistance expressed at the cellular level. Calli are placed on selective media and putative resistance is indicated by sectorial growth amongst predominantly dead or inhibited cells. These sectors can be isolated, and multiplied prior to regeneration of variant plantlets via somatic embryogenesis (Dix, 1986). Many types of cell and tissue culture have been developed, including suspended cell cultures, suspended protoplast cultures, cell cultures on solid media, and callus culture (Meredith, 1984). Liquid cell suspensions can be spread or sprayed onto agar surfaces, or mixed with molten agar to provide a selection matrix. Plated cell cultures produce growths from small aggregates of cells with relatively uniform genetic and physiological uniformity, and selected variant lines are likely to arise from single cells. Many plant species have a critical inoculation density below which cells will not divide. This effect appears, in practice, to be lessened by the delayed death of surrounding susceptible cells, which support resistant cell colonies until they reach critical size. Also, if there is a lag phase between mutation treatments and application of selective pressure, resistant variant colonies may grow to reach critical mass prior to selection. Small cell clusters have been successfully grown using conditioned media or nurse cells (Dix, 1986).

Somaclonal variation

Plant cell culture cycles generate a valuable and novel source of genetic variability - somaclonal variation (Larkin, 1986; Larkin and Scowcroft, 1981). Somaclonal variation may involve gross chromosomal changes, or changes to individual genes. The term "mutant" is applied to a stable and heritable genotypic change, and "variant" describes a novel phenotype which has not yet been shown to possess a genotypic change (Meredith, 1984). Somaclonal variants are often the result of chromosome doubling, or partial doubling, but may also be caused by aneuploidy, structural changes in chromosomes, qualitative genetic changes, quantitative or complex genetic changes, or the unstable expression of a single allele (Bingham *et al.,* 1988). Gene amplification occurs when the number of copies of a gene increases, thus increasing the expression of the gene function. Such amplification can result from sequential selection with increasing concentrations of selection agents. The reverse effect can occur with gene depletion (Larkin and Scowcroft, 1981).

Up to 30% of regenerants from tissue culture may exhibit somaclonal variation, even in the absence of selective pressure, and many variants have been characterised as mutants with heritable changes (Maliga, 1984). Genetic instability often increases with age of cultures, and additional variability can be induced by exposure of cells to mutagens, such as EMS (Bingham *et al.,* 1988). Prolonged culture, leading to genetic instability, can reduce the ease with which shoot regeneration or embryogenesis can be induced, or effect flowering and fertility of regenerated plants. It is often most practical to select desirable traits from genotypes which are known to regenerate easily under *in vitro* conditions, and then transfer the traits to target cultivars using traditional breeding techniques (Dix, 1986).

1.6.5 Tissue culture and regeneration in Medicago

Research in *Medicago* has focused on *M. sativa*, due to its worldwide agronomic importance. *M. sativa* was first regenerated in 1972 following pioneering work on tobacco and carrots (Saunders and Bingham, 1972). The three most important factors contributing to somatic embryogenesis are: 1) the structural quality and quantity of the exogenous auxin source; 2) the concentration of exogenous cytokinin; and 3) the level and nature of reduced N in the regeneration medium (Bingham *et al.,* 1988). Other *Medicago* species have since been regenerated, including *M. arborea, M. coerulea, M. difalcata, M. falcata, M. glutinosa, M. hemicycla, M. littoralis, M. orbicularis, M. polymorpha, M. rigidula, M. suffruticosa, M. truncatula,* and *M. varia* (Ibragimova and Smolenskaya, 1997; Li and Demarly, 1996; Nolan *et al.,* 1989; Scarpa *et al.*, 1993; Zafar *et al.*, 1995).

M. truncatula (barrel medic) has recently become a model plant for combined molecular and genetic studies on *Rhizobium*-legume symbiosis because, in contrast to *M. sativa*, it is diploid and autogamous (Chabaud *et al.,* 1996). *M. truncatula* is the most important annual medic in South-Eastern Australian pastures. Nolan *et al.* (1989) were the first to regenerate it from tissue culture, using somatic embryogenesis. Explants from leaves of *M. truncatula* cv. Jemalong were cultured through a four-step media cycle, based on NAA and BAP. Somatic embryogenesis frequency was greatly increased when explants from regenerated plants were used, leading to selection of a highly regenerable line (2HA3-9-10-3). Thomas *et al.* (1992) were the first to produce a transgenic annual *Medicago* species, transforming *M. truncatula*, using *Agrobacterium tumefaciens* to introduce a gene conferring kanamycin resistance. Chabaud *et al.* (1996) introduced a nodulin promoter gene and reported improved techniques which increased the frequency and speed of embryogenesis in line 2HA3-9-10-3, and a rapid transformation protocol using direct shoot organogenesis was reported by Trieu and Harrison (1996). Nolan and Rose (1998) published a revised and improved regeneration procedure for their *M. truncatula* cv. Jemalong 2HA3-9-10-3 selection. They found a requirement for both NAA and BAP for callus formation and somatic embryogenesis, and no requirement for a drop in auxin concentration to induce embryos. ABA, when used with NAA and BAP, increased the number of somatic embryos in light and dark, but did not improve plantlet conversion efficiency. When embryos formed under light, conversion to plantlets was markedly inhibited. The protocol is:

P4 + 10 μM NAA + 4 μM BAP
3 weeks for callus formation in dark
↓
P4 + 1 μm ABA + 10 μM NAA + 4 μM BAP
embryo formation in dark
↓
P40 + 1 μm NAA
embryos 4-8 weeks in dark
↓
P40 + 1 μm NAA
shoot formation in light
↓
P40 + 1 μm NAA
plantlet formation in liquid medium in light

where P4 and P40 are modified Gambourg B5 (Gamborg *et al.,* 1968) growth media.

Hoffmann *et al.* (1997) selected *M. truncatula* line R108-1 for its high somatic embryogenesis capacity and ease of transformation. Trinh *et al.* (1998) reported a rapid and efficient transformation protocol for specific lines of *M. truncatula* and *M. sativa* ssp. *falcata* lines using improved somatic embryogenesis, based on 2,4-D and BAP. Direct somatic embryogenesis was attained from *M. truncatula, M. littoralis, M. murex* and *M. polymorpha* using thidiazuron and BAP in MS media (Iantcheva *et al.*, 1999). A two-step protocol using foliole explants with MS media supplemented with 2,4-D (4.45 μM) and zeatin (9.3 μM) produced repetitive somatic embryogenesis in *M. truncatula* (das Neves *et al.*, 1999). Zafar *et al.* (1995) successfully regenerated *M. littoralis* cv Harbinger, an agronomically important medic in southern Australia. Calli derived from hypocotyls and incubated in 2,4-D/BAP based media grew most rapidly. The unselected line (cv Harbinger 1886) was found to have a high capacity for embryogenesis, compared to unselected *M. truncatula* and *M. polymorpha*.

1.6.6 Selection using seed mutation

Seeds of some species have been treated with chemical mutagens prior to selection for herbicide resistance. Soybean (*Glycine max*) seeds were mutagenised with ENU, sequentially selected with chlorsulfuron, and crossed to produce four mutants in the M_5 generation. There were three distinct single, recessive gene mutations, none of which conferred an altered ALS (Sebastian and Chaleff, 1987). Sebastian *et al.* (1989) selected a sulfonylurea-resistant soybean ALS mutant following treatment of seeds with EMS and N-nitroso-N-methylurea. Mutagenesis was used because, at the time, there was no totipotent cell regeneration methodology for soybeans. The selected resistance was monogenic, and semi-dominant. Single, nuclear, dominant mutations of *Arabidopsis thaliana* with high levels of resistance to sulfonylureas were also selected from EMS-treated M_2 seeds grown on agar treated with 75 ppb chlorsulfuron (Haughn and Sommerville, 1987).

There are many variables involved with seed mutation which can affect the nature and number of mutations. Large numbers of seeds (>10,000) are usually treated to maximise the frequency of desired mutations. Hydration and aeration of seeds in cold water (c. 0°C) for 11 to 24 hours prior to exposure to mutagens reduces the variability of the mutagen effect (Konzak *et al.*, 1970; Sebastian *et al.*, 1989; Van den Bulk *et al.*, 1990) and allows the use of lower concentrations at lower temperatures, producing good mutation frequencies and high M_1 survival (Kamra and Brunner, 1970). Hydration causes rapid leaching of germination inhibitors and free metabolites, while minimising metabolic activity and cell division (Konzak *et al.*, 1970). The optimum concentration, temperature and soaking time varies between mutagens and species. The rate of EMS uptake is influenced by the degree of hydration of embryonic tissue, but is not influenced by temperature to the same extent as hydration rate (Konzak *et al.*, 1970). EMS is usually used for about 0.5 to 2 hrs at about 25°C, using hydrated seeds, and it has typically been used at 0.1 to 1.5% (Kamra and Brunner, 1970; Rao *et al.,* 1993). Hydrolysis of alkylating agents in solution leads to acidification, which can cause significant physiological damage, as well as increasing the occurrence of chromosomal aberrations. To minimise this, solutions are usually buffered with phosphate (<0.1 M), however short duration (0.5 to 2 hr), high temperature treatments may not require buffering or periodic renewal of the solution. Post-treatment washing is important to remove excess mutagens (Kamra and Brunner, 1970), but some residues remain dissolved in lipids and continue to have an effect. Seeds can be dried after post-treatment washing, but the temperature should not exceed 35°C (Konzak *et al.*, 1970).

1.6.7 Selection from cell and tissue culture

Plant breeders rapidly recognised the potential for using selection agents in cell and tissue culture to identify desirable traits (Larkin, 1986). Cell culture selection techniques have produced crop plants with tolerance to bacterial, fungal and viral diseases, salt and herbicide tolerance, and tolerance to soil nutritional deficiencies (Bingham *et al.,* 1988; Chaleff and Ray, 1984; Pofelis *et al.,* 1992). Swanson and Tomes (1980) identified three critical factors for successful selection of herbicide-resistant variants: ability to isolate rapidly growing friable calli; a genotype which is readily regenerated; and a clear correlation between *in vitro* and *in vivo* systems for the selected resistance. Herbicides which interfere with basic metabolic activities can be expected to inhibit growth of cultured cells as well as whole plants (Chaleff and Ray, 1984). Cell culture techniques are indicated (Chaleff, 1983; Larkin, 1986; Meredith, 1984; Swanson and Tomes, 1980) for selection of a *Medicago truncatula* genotype resistant to ALSIH because the desired genotype is the result of a simple single nucleotide substitution (e.g. Boutsalis, 1996: Guttieri *et al.*, 1992), the trait is expressed and selectable at the cellular level (e.g. Pofelis *et al.*, 1992; Chaleff and Ray, 1984), and cell culture and regeneration techniques have been established for the species (Chabaud *et al.,*1996; Nolan *et al.*, 1989; Nolan and Rose, 1998).

Selection for traits at the cellular level has limitations which must be recognised, and many studies are tedious and often progress slowly (Larkin, 1986). Cell culture selection is not well suited to whole-plant characteristics involving growth habit, anatomy, or complex tissue-membrane interactions (Bingham *et al.,* 1988). Survival of cells in selection medium does not necessarily confer resistance in regenerated plants (MacLean and Grant, 1987), and often selected plants have poor agronomic adaptation (Bingham *et al.,* 1988). Calli typically grow slowly, and often only a small proportion of the cells are in direct contact with the selective toxin in the media. Translocation of toxins into calli may be variable, leading to shielding of susceptible cells. Plasmodesmatal connections between cells may also allow movement of essential supplies to susceptible cells, from resistant cells, thus allowing them to survive. Resistant sectors may also contain susceptible cells which may compete vigorously with resistant cells in the absence of the selection agent, producing susceptible or chimeric plantlets (Dix, 1986). Plated cell cultures overcome some of the problems of callus cultures, but require more labour. It may initially be necessary to select a large number of variants to obtain one stable mutant trait that is expressed in the appropriate tissue, and at the right time, in the regenerated plant (Meredith, 1984).

The first ALS-inhibiting herbicide resistance was selected in haploid tobacco callus, using chlorsulfuron and sulfometuron as selection agents, and ENU as a mutagen in some treatments. A 100-fold increase in resistance was obtained, and the mutation was dominant or semi-dominant (Chaleff and Ray, 1984). Since then resistant lines have been selected in many species, including birdsfoot trefoil (Pofelis *et al.*, 1992), carrots (Caretto *et al.,*1994), chicory (Dewaele *et al.*, 1997), corn (Anderson and Georgeson, 1989), cotton (Rajasekaran *et al.*, 1996), *Datura innoxia* (Saxena and King, 1988), flax (Jordan and McHughen, 1987), sugarbeet (Saunders *et al.*, 1992; Wright *et al.*, 1998), and tobacco (Caretto *et al.*, 1993; Harms *et al.*, 1991; Harms and DiMaio, 1991). A number of different selection techniques have been used, including suspended cell culture (e.g. Caretto *et al.*, 1993; MacLean and Grant, 1987), calli culture (e.g. Anderson and Georgeson, 1989; Pofelis *et al.*, 1992), plated dispersed cell culture (e.g. Saunders *et al.*, 1992), and direct shoot induction (e.g. Harms *et al.*, 1991). Where mutant ALS is selected, gene expression is usually dominant or semi-dominant (e.g. Anderson and Georgeson, 1989; Chaleff and Ray, 1984). Levels of resistance are typically 100 to 1,000-fold higher than the wild type ALS, but have been as high as 4,300-fold (Wright *et al.*, 1998), and in almost every case there is expression of cross-resistance to other ALSIH.

1.7 CONCLUSIONS AND RESEARCH DIRECTIONS

Annual medics are an important component of cereal-livestock farming systems in temperate southern Australia. It is clear that productivity from medic pastures is currently severely restricted by soil residues of chlorsulfuron and triasulfuron. Medic genotypes with increased resistance to soil residues of these herbicides would be very valuable. They would produce vigorous growth in the pasture phase, thus allowing continued use of cheap and efficient ALSIH for weed control in cereals. There have been a number of crop and pasture species with increased resistance to ALSIH successfully developed to overcome ALSIH soil residues, using an array of well-documented selection techniques.

Research in this study began by considering the agronomic ramifications of deploying an ALSIH resistant medic in southern Australian farming systems. Potential advantages and disadvantages were investigated and, on balance, it was concluded that it would be desirable to deploy a resistant medic (Chapter 2). The results from experiments on the interactive effects of chlorsulfuron and triasulfuron residues and summer weeds on medic growth then confirmed the severity of damage under field conditions (Chapter 3). The search for resistant genotypes began with screening of a selection of known lines of *Medicago* from the Australian Medicago Genetic Resource Centre, but none had sufficient resistance to be useful (Chapter 4). Large numbers of medic seedlings of elite cultivars were then screened in attempt to identify existing resistant mutant individuals. None were found in this commercial seed, prompting screening of field-selected plants from farmers' paddocks which had been sprayed with ALSIH, and which had a history of selection pressure from ALSIH. These selections also failed to identify any resistant individuals (Chapter 5), leading to the conclusion that resistant genotypes would need to be selected from induced mutations, or from somaclonal variants in cell culture.

A line of *M. truncatula* cv. Jemalong (2HA), selected for its capacity to regenerate from somatic embryos, was established in cell culture and mutated with EMS. Many cell lines with resistance to chlorsulfuron were selected, but in the process the calli lost embryogenic competence, thus preventing regeneration of plantlets with putative resistance (Chapter 6). Concurrent experiments with EMS-treated seed produced one *M. littoralis* and four *M. truncatula* lines with putative resistance to chlorsulfuron. One of these lines, *M. littoralis* cv. Herald selection "FEH-1", was confirmed as having a mutant ALS gene which coded for an altered ALS enzyme which had resistance to a range of ALSIH. Research then concentrated on determining the mechanism of resistance, characterising the cross-resistance pattern of FEH-1, attempting to identify the nature and position of the mutation in nucleotide sequence of the FEH-1 ALS gene, and comparing the response of FEH-1 and its parent to ALSIH soil residues and foliar applications (Chapters 7 and 8). Finally, the merits and mechanisms of developing and deploying the specific selection FEH-1 were explored (Chapter 9).

CHAPTER 2

AGRONOMIC IMPLICATIONS OF HERBICIDE RESISTANT MEDICS

2.1 INTRODUCTION

A major aim of this work is to identify or select a genotype of annual medic with resistance to field levels of sulfonylurea soil residues. Such a plant could be expected to have both advantages and disadvantages when compared to current annual medics. The following analysis of the likely behaviour and utilisation of a selected resistant medic is valuable in determining the merit of pursuing this aim.

Crop rotations can be made more flexible and sustainable if crops which are susceptible to soil herbicide residues are selected for resistance (Conner and Field, 1995; Dear *et al.*, 1995). Before herbicide-resistant crops are introduced the impact on the total agricultural system should be carefully considered, including environmental implications (Huppatz *et al.*, 1995). Gressel (1997) proposes that "rational case by case risk/cost/benefit analysis must replace irrational fear-striking generalisations, as agriculture can benefit from many herbicide-resistant crops".

Herbicide-resistant crops have been developed for a number of reasons (reviewed in section 1.6.1), including poor weed control options in relatively minor crops (e.g. Pofelis *et al.*, 1992), damage to crop and pasture species by soil residues of some ALS-inhibiting herbicides when grown in rotations (e.g. Jordan and McHughen, 1987; Saunders *et al.*, 1992), and where a crop is closely related to major weeds so that naturally-occurring herbicide selectivity is unlikely (Newhouse *et al.*, 1991). There are other areas with potential such as selective control of parasitic weeds (e.g. *Striga* and *Orabanche*), and new resistance for crops such as wheat and maize to alleviate weed resistance problems and broaden herbicide choice (Gressel, 1992).

The primary motivation for this study is the severe restriction in growth of the agronomically-important, ALSIH-sensitive annual medics by soil residues of sulfonylurea herbicides applied in previous phases of the cropping rotation (Evans *et al.*, 1993; Gillet and Holloway, 1996; Noy, 1996). The concept of developing a resistant crop or pasture cultivar in response to soil residues of sulfonylurea herbicides is not new, and has been attempted overseas for species including flax (Jordan and McHughen, 1987), bird's foot trefoil (Pofelis *et al.*, 1992), cotton (Rajasekaran *et al.*, 1996) and tobacco (Chaleff and Ray, 1984).

There have been a number of crops with herbicide resistance released in Australia. In general the crops have been readily adopted by Australian farmers, however persistence of volunteers and over-use of specific herbicide groups has caused concern. Experience following the release and adoption of these crops may only have limited relevance to this research, as it aims to be the first to produce a plant for which direct herbicide application is not intended.

The following analysis aims to identify and discuss potential areas of advantage and disadvantage, to arrive at a balanced opinion on the desirability of a resistant medic. The discussion assumes that soil residual ALSIH are used in rotational crops within the system.

2.2 POTENTIAL BENEFITS FROM AN ALSIH RESISTANT MEDIC

2.2.1 Increased productivity and quality of medic-based pastures

Annual medics are a valuable component of ley farming systems in southern Australia. They increase soil nitrogen and organic matter, reduce cereal root diseases, provide high quality green and dry forage, increase soil water-holding capacity and drainage, reduce potential for soil erosion and provide an opportunity to reduce soil seed reserves of annual grass weeds in cropping rotations (Crawford, 1987a; Puckridge and French, 1983). Well managed medic pastures also increase income stability (Krause, 1995). Residues from sulfonylurea herbicides, in particular chlorsulfuron and triasulfuron, have severe effects on annual medic growth and persistence. Herbage yields are typically reduced by 60-90% in the year following chlorsulfuron or triasulfuron application, and seed yields are also severely reduced (Carter *et al.,* 1982; Evans *et al.*, 1993; Gillet and Holloway, 1996; Noy, 1996). Resistance to soil residues of sulfonylureas would allow more vigorous growth of medic plants, leading to an increase in the many other beneficial contributions they make to the rotation. Improved winter growth through reduced herbicide damage would increase the quality and quantity of livestock feed at a critical time during the year and provide increased competition for weeds not controlled by herbicides. Increased winter/spring production would increase nitrogen fixation, with benefits to rotational crops. Improved nitrogen fixation would reduce reliance on continuous cropping and thus improve stability and sustainability of the system (Dear *et al.*, 1995). It is also possible that interactive nutritional (eg Zn and P; W.D. Bellotti, pers. comm.) and disease (e.g. *Rhizoctonia*; Streeter *et al.*, 1997) stress may be lowered if sulfonylurea damage is reduced. In addition medic seed production would be increased, improving the persistence from one pasture phase to the next.

2.2.2 Increased use of medic-based pastures in cropping rotations

The use of annual medic pastures has declined in the cereal-sheep belt of southern Australia over the last 20 years, primarily due to the relatively high returns from cropping compared to wool, and the poor performance of medic-based pastures (Carter *et al.*,1982, Ewing, 1996, Krause, 1995). The substitution of pulse and oilseed crops for medic pastures has been most pronounced in regions with medium to high annual rainfall. In regions with low rainfall there are no suitable crops to replace medic pastures and they have remained as part of the ley rotation. There is an intermediate transition zone of medium to low rainfall (275 to 350 mm) which is most elastic for substitution. An increase in wool prices, or greater medic productivity, would result in a significant increase in medic pastures in this zone. These areas are predominantly characterised by alkaline soils, and medics are therefore particularly at risk to damage from soil residues of sulfonylureas. Overriding the factors of wool price and medic performance is the increasing problem of herbicide resistant weeds. Many farms which have adopted continuous cropping practices, at the expense of annual pasture phases, now face a very difficult problem of managing resistant weed populations. It has long been recognised that annual pastures are a valuable tool for management of resistant weeds, and many of these farms may be forced to re-introduce pastures in order to continue cropping.

2.2.3 Greater competition for weeds of pastures

Vigorous annual medic growth, in the presence of sulfonylurea residues, would provide increased competition for broadleaved and grass weeds in the pasture. This would increase the beneficial contribution of the medics, as well as reducing the seed set and consequent soil seed bank of weedy species (Dear *et al.*, 1995). This would in turn reduce the weed pressure in following crops, and would lead to a decline in weed density throughout all phases of the rotation.

2.2.4 Option of under-sowing medics in crops treated with sulfonylurea herbicides

Traditionally, many pastures are resown as an under-sown crop in the final cereal crop before the pasture. This practice allows cheaper pasture reseeding costs because the seedbed preparation for the crop is utilised for pasture seeds at no extra cost. There is some crop yield loss due to competition from under-sown pasture plants in the crop, but the technique allows soil pasture seed reserves to increase over the spring/summer prior to the pasture phase. The practice has declined as the use of selective herbicides in cereals has increased, many of which kill under-sown legumes (Dear *et al.*, 1995). An annual medic with ALS-inhibiting herbicide resistance could be under-sown in cereals which are treated with these herbicides, set adequate seed reserves, and then establish and grow vigorously in the pasture phase. This attribute would increase the adoption of the under-sowing technique, and generally help to increase the use of medic pastures.

2.2.5 Management of herbicide resistance in weeds

Annual pastures are a recognised tool for management of herbicide resistant weeds in cropping rotations, especially grass weeds such as *Lolium rigidum.* More vigorous medic stands would compete directly with weeds to reduce seed set, and would also better withstand applications of glyphosate and paraquat applied in spring to reduce seed set of weeds. Another major benefit of a medic with resistance to ALSIH would be that farmers would probably need to use a herbicide with a different mode of action to control medics in rotational cereal crops (section 2.3.2). This would reduce the selection intensity of ALSIH within the rotation and thus delay or prevent the development of weeds with resistance to these herbicides.

2.3 POTENTIAL PROBLEMS WITH AN ALSIH RESISTANT MEDIC

2.3.1 Possible direct application of ALSIH to medic-based pastures

The introduction of a medic with a sufficiently high level of ALSIH resistance would create the obvious temptation for farmers to apply these herbicides directly to pastures to control weeds. Even though the intention of this project is to alleviate damage from soil residues, many farmers would also see a perceived benefit from direct ALSIH application. This would give an unprecedented level of control of many weeds during the pasture phase. However, the practice would need to be actively and strongly discouraged in most circumstances because it would increase herbicide resistance in weeds through over-reliance on ALSIH, and would also contribute to elevated levels of soil residues throughout the rotation. It should be recognised that it is likely that a small percentage of farmers would pursue this option through lack of understanding, or for short-term financial considerations, despite the best efforts of advisers to educate against the practice.

2.3.2 Increased weediness of annual medics in rotational crops

Herbicide-resistant crop plants may also become weeds, and may necessitate a change of herbicide choice to control them in following crops (Swain, 1996), and the problem may be serious if there is no effective way to control them (Conner and Field, 1995). However, in most foreseeable cases, herbicide resistant plants growing as volunteers within a subsequent rotational crop could be controlled by treatment with a herbicide from an alternative chemical family (Huppatz *et al.*, 1995). In a broader sense, apart from resistance to herbicides, a single foreign gene is unlikely to cause a crop to become weedy. There is no reason that herbicide-resistance, *per se,* should increase the competitiveness or invasiveness of crops or pastures (Dear *et al.*, 1995).

Annual medics are very useful pasture plants, but are sometimes also weeds of crops in the rotation. They are only moderately competitive, and usually only cause concerns to farmers at high densities. Some farmers who rely on medic pastures as part of the rotation are prepared to accept a certain level of medic infestation within crops to ensure adequate medic seed reserves for the pasture phase. A medic with resistance to ALSIH would be able to grow unaffected in crops which rely on these herbicides for weed control. This would be most significant for cereal crops treated with chlorsulfuron, triasulfuron and metsulfuron-methyl. Farmers would be forced to use a herbicide with an alternative mode of action if control of medics was required. The major crops in the cereal-sheep zone of southern Australia are cereals, pulses and canola. Medics are listed as weeds of pulse crops, but not cereals, in weed control guides in South Australia, and medics are not normally a major weed of canola crops.

Cereals. The approximate tolerance of under-sown annual medics to cereal herbicides is given in the cereal weed spraying chart, published by Primary Industries South Australia (1997a).

The following herbicides are not tolerated by medics and so would be suitable for controlling or suppressing them in cereal crops: 2,4-D, bromoxynil/dicamba/MCPA, bromoxynil/diflufenican, clopyralid, chlorsulfuron, dicamba, dicamba/MCPA, fluroxypyr, metosulam, metsulfuron-methyl, and triasulfuron. Of these, chlorsulfuron, metosulam, metsulfuron-methyl, and triasulfuron are ALSIH and may be ineffective against a resistant medic. This leaves seven potential alternative options for control, in addition to six other herbicides which are not recommended for use on under sown medics due to unacceptable damage to medics. Where medics are a weed in cereals the ineffectiveness of ALSIH would be inconvenient, but there are a range of alternative options which carry the benefit of reducing the risk of developing herbicide resistant weeds.

Pulses and canola. The ALSIH used in pulses are flumetsulam, imazethapyr, and metosulam. Of these, only metosulam would be considered as an option for controlling medics, and then only in lupins. Simazine, which kills medics, is widely used in lupins and so medics are unlikely to be a weed in this crop. There are no ALSIH registered for use in canola. Clopyralid, simazine and atrazine are widely used in canola (triazines in triazine tolerant cultivars only) and these herbicides control medics very effectively. Therefore the impact of medics as weeds in pulses and canola is unlikely to be increased by resistance to ALSIH (Heap, 1997; Primary Industries and Resources South Australia, 1997 a and b).

2.3.3 Increase in sulfonylurea use in rotational crops prior to medic-based pastures

There are an increasing number of weed species and populations developing resistance to ALSIH in southern Australia (Boutsalis, 1996), and these herbicides are well known as being a high risk class (Saari *et al.*, 1994). As a consequence it is prudent to minimise reliance on this class, and encourage use of herbicides with alternative modes of action. The release of a medic with resistance to ALSIH would probably increase the use and therefore selection intensity of these herbicides, leading to an increased risk of developing weeds with ALSIH resistance. Farmers would be likely to use ALS-inhibiting herbicides in crops more often, and perhaps at higher rates, if productive medic pastures could be grown in the following year. There will probably also be a minority who will apply ALSIH directly to medic pastures, despite strong advice to the contrary. The principles of herbicide resistance in weeds are now well understood by advisers and most farmers, and so a release of a resistant medic would need to be accompanied by strong advice against increasing reliance on ALSIH.

2.3.4 Natural spread to all properties

Annual medics are selected for persistence in farming systems, and readily become naturalised in disturbed areas. Once released, a medic with resistance to ALSIH could potentially spread to any property within the naturalised range of annual medics. As a consequence, the choice to adopt the technology will not be an individual choice, but will have to be made collectively by farmers. It is therefore essential that farming representative organisations and the farming sector at large be fully briefed on any proposal to release a resistant medic. Although there appears to be a very good case for release of a resistant medic, it is essential that a collective decision to do so is made after thorough consideration of the implications.

2.4 EVALUATING HERBICIDE RESISTANT PASTURE LEGUMES

Dear *et al.* (1995) suggest the following agronomically-orientated checklist for potential releases of herbicide resistant pasture legume cultivars. The discussion relates to an annual medic with a single mutant gene conferring ALSIH resistance in a rotational cropping-grazing rotation in temperate southern Australia:

1) Cultivar should be agronomically sound so that it will not be superseded rapidly.

There are three cultivars which will be the main focus of selection: *M. truncatula* cv. Jemalong; *M. truncatula* cv. Mogul; and *M. littoralis* cv. Herald. Mogul and Herald are elite current cultivars, while Jemalong is a widely-accepted older cultivar for which there is a selected line with proven high regeneration competency in tissue culture (Nolan and Rose, 1998).

2) *There should be a suitable array of herbicides available to adequately control the herbicide-resistant legume within the rotation.*

There are adequate alternatives available. This point is discussed in detail in section 2.3.2.

3) *The potential for gene transfer to other cultivars or wild relatives should be low; herbicides with a high risk for herbicide resistant weed development should be avoided.*

Even though the ALSIH are a high risk class for resistance in weeds, the rate of outcrossing in annual medics is extremely low and has never been observed in the field in Australia. The risk of gene transfer to naturalised *Medicago* is therefore considered negligible (Dear *et al.*, 1995).

4) *Herbicide resistance should not be used as a selectable marker for other transformed traits (this wastes future opportunities to use the herbicide resistant, per se, in future cultivars).*

Not relevant to this project.

5) *Herbicides should have low environmental impact.*

ALSIH have a low mammalian toxicity, are used at very low application rates, and are only known to affect plants and other lower organisms with ALS enzymes. They are, however, active at very low concentrations and so soil residues can damage non-target species (Saari *et al.*, 1994).

6) *Herbicide resistant pasture legumes should form the basis of an IWM strategy, which should be widely promoted.*

A resistant medic would grow vigorously in the presence of ALSIH, thus restricting the growth and seed production of pasture weeds. Farmers would be strongly advised not to routinely apply ALSIH directly to the pasture, or increase reliance on these herbicides in crops. Ignoring this advice would almost certainly accelerate development of weeds with resistance to ALSIH.

7) *Herbicide should differ from those groups commonly used in other phases of the rotation, to avoid over-reliance on one mode of action and possible development of herbicide resistant weeds.*

The proposed aim of this study appears to be in clear contravention of this guideline, but the aim is to produce a pasture legume which can withstand residues which are already present, and direct application or increase in reliance of ALSIH will be discouraged.

8) *Use of broad spectrum, non-selective herbicides should be avoided, to encourage species diversity in the sward.*

ALSIH used in cropping rotations are selective and would be expected to maintain species diversity. In any case, direct application to the pasture would be discouraged. There may, however, be a reduction in species diversity over time as seed production of susceptible weeds is restricted by soil residues of ALSIH and increased competition from medics.

2.5 CONCLUSIONS

Some very clear potential benefits from release of a medic with ALSIH resistance have been identified. Pasture productivity and quality would be increased, thus increasing income stability, increasing soil nitrogen and organic matter, reducing cereal root diseases, providing high quality green and dry forage, increasing soil water-holding capacity and drainage, reducing potential for soil erosion and providing an opportunity to reduce soil seed reserves of annual grass weeds in cropping rotations. The use of medic pastures would be increased and under sowing of medics could become more widespread.

The main disadvantage appears to be the potential for increased reliance on ALSIH, thus increasing the risk of weeds developing resistance. In addition, release of a resistant medic could force growers to use alternative herbicides in cereal crops, however, this will help to prevent herbicide resistance developing in weeds. Another major concern is the inability to prevent natural spread of a resistant medic to anywhere within the naturalised range of medics. In this sense farmers will not be able choose whether or not to adopt the technology onto their farm. Sooner or later it could become naturalised on all properties whether it is wanted or not.

On balance there appears to be a strong case for development of a medic with resistance to ALSIH, provided that the farming sector and other stakeholders are given the chance to make an informed collective decision to do so.

CHAPTER 3

EFFECT OF SULFONYLUREA RESIDUES AND SUMMER WEEDS ON MEDIC GROWTH

3.1 INTRODUCTION

Annual medic decline is a syndrome caused by a complex and interactive array of factors. Sulfonylurea soil residues contribute significantly to the syndrome, but little is known about the interactive effects of these residues with other factors (Bellotti, 1996; Carter *et al.*, 1982; Ewing, 1996; Neal *et al.*, 1997). This study aims primarily to develop medic genotypes with tolerance to soil residues of ALSIH, but literature research and discussions also identified questions about field interactions. Information about other factors that can be manipulated could lead to improved medic production in pastures.

Chlorsulfuron and triasulfuron are important herbicides for wheat, although chlorsulfuron use has declined due to effects of soil residues on following crops and pastures. Farmers in the low rainfall wheat areas of South Australia (e.g. G. Schmidt, pers comm., 1997) perceive that medic growth, although usually poor following chlorsulfuron application, is often enhanced after triasulfuron is used in the previous season. These observations have been made in areas which typically use a two year wheat-volunteer medic pasture rotation on alkaline (pH 8-8.5) sands or sandy loams. Growers attribute this phenomenon to suppression of summer weeds by triasulfuron residues which persist long enough to suppress weeds such as *Heliotropium europaeum* (heliotrope),*Citrullus lanatus* and *Cucumis myriocarpus* (Afghan and prickly paddy melons), *Asphodelus fistulosis* (onion weed) and *Tribulus terrestris* (caltrop), but degrade sufficiently by the break of the winter growing season so as not to suppress medic establishment and growth.

Farmers, however, were unsure of the relative counter-acting effects of summer weed competition and medic suppression from triasulfuron residues. It is important to separate these two effects experimentally because if triasulfuron residues suppress medics significantly there may be benefits from growing an ALSIH-resistant medic, or from controlling summer weeds with non-residual herbicides while reducing or avoiding triasulfuron soil residues. The field experiments described here were designed to determine the relative effects of summer weed control and chlorsulfuron and triasulfuron residues on medic growth.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

In autumn, 1998, chlorsulfuron (CSF: 5.6, 11.3 and 22.5 g a.i. ha^{-1}) and triasulfuron (TSF: 7.5, 15 and 30 g a.i. ha^{-1}) were applied to plots sown to wheat (cv. Frame). Plots were 3 x 16 m and treatments were replicated three times. During the summer of 1998/99 the main herbicide plots were split into two sub-plots (3 x 8 m). One was sprayed, as required, with the non-residual herbicides paraquat/diquat and 2,4-D to control summer weeds. The experiments were fenced to exclude stock after the 1998 wheat harvest, and annual medics (*M. littoralis* cv. Harbinger) and other volunteer pasture species were allowed to regenerate in autumn 1999. In mid-May, 1999, approximately one year after chlorsulfuron and triasulfuron application, medic establishment and growth (spade leaf to 6 cm diameter stage) and summer weed growth were measured using 3 x 0.25 m^2 quadrats per sub-plot. Medic density and growth were measured again in August, 1999. Data were analysed using a two way analysis of variance.

Sites were established at Lowbank and Wunkar, in the Riverland area of South Australia, in paddocks with a history of a two year wheat-volunteer annual medic rotation. The Lowbank site was on an alkaline sand (pH = 8.4), and the Wunkar site was on an alkaline (pH = 8.5) sandy-loam. Annual rainfall for both sites is approximately 200 mm. Prior to the first sampling in May, 1999 Wunkar received 13 mm for February, 28 mm for March, 13 mm for April, and 1 mm for May. Lowbank received 5.5 mm for January, 42 mm for February, 34.5 mm for March, and no rain for April or May.

3.2.2 Herbicide application and summer weed control

Chlorsulfuron and triasulfuron were applied pre-sowing with a 2 m wide plot sprayer (147 ha^{-1}; 200 kPa) on 29/4/98 and incorporated by sowing within one week. On 3/8/98 the non-residual herbicides MCPA ester and 2,4-D ester were applied uniformly to the sites to control winter weeds in the wheat crops. The summer weed control sub-plots at Wunkar were sprayed on 20/1/99 (paraquat/diquat + 2,4-D amine) to control a light infestation of *Heliotropium europaeum* and on 26/2/99 a light infestation of *Citrullus lanatus* was removed by hand. The sub-plots at Lowbank were hand-weeded to remove a light infestation of *Citrullus lanatus* on 21/1/99, and on 26/2/99 paraquat/diquat + 2,4-D amine was applied to control a moderate infestation comprising seedlings of annual grasses, *Asphodelus fistulosis*, volunteer wheat, and *Tribulus terrestris*. The sites were checked on 22/3/99, and there was almost no green growth in the summer weed control sub-plots. Summer weed density and growth in unsprayed plots were light at Wunkar and moderate at Lowbank.

3.3 RESULTS

3.3.1 Summer weed control

Relatively low January to late May rainfall (82 mm at Lowbank; 55 mm at Wunkar) resulted in only low to moderate summer weed growth. At Lowbank, where the rainfall was highest, there were 73 g shoot drywt m^{-2} in untreated plots, compared to only 10 g at Wunkar (Figs. 3.1 and 3.2). The non-residual herbicides and hand-weeding in the summer weed control sub-plots allowed almost no weed growth at Wunkar, and 33 g shoot drywt m^{-2} at Lowbank. In the sub-plots where summer weeds were allowed to grow at Wunkar, residues remaining from the lowest rate of triasulfuron (7.5 g ha^{-1}) suppressed weed growth more than the lowest rate of chlorsulfuron (5.6 g ha^{-1}) and growth declined to very low levels at the highest rates for both herbicides. At Lowbank, volunteer wheat, which is relatively unaffected by either herbicide, comprised a high proportion of growth and so treatment effects for other species were masked.

3.3.2 Early establishment and growth of medics

Establishment of medics in untreated plots with uncontrolled summer weeds, measured in late May, varied from 40 seedlings m^{-2} at Lowbank to 100 m^{-2} at Wunkar (Figs 3.3 and 3.4). This difference was probably due to greater summer weed growth at Lowbank (Figs 3.1 and 3.2). When summer weeds were suppressed in plots without residual herbicides at Lowbank, establishment rose from 40 seedlings m^{-2} to 200 m^{-2} (Fig 3.4). This result demonstrates the potential for summer weeds to significantly reduce medic performance. A similar increase was not apparent at Wunkar (Fig 3.3) because summer weed growth was low. Control of summer weeds increased medic establishment for most chlorsulfuron and triasulfuron treatments. Both chlorsulfuron and triasulfuron residues reduced medic establishment (Figs 3.3 and 3.4), but chlorsulfuron was much more damaging than triasulfuron. Even at the lowest rate, chlorsulfuron reduced establishment by 70 to 90% when summer weeds were controlled (Fig 3.3 and 3.4). At Lowbank, triasulfuron also caused a 40% reduction at the lowest rate (Fig 3.4). The effect of chlorsulfuron was particularly severe at Lowbank (Fig 3.4), where there was greatest competition from summer weeds for moisture.

Shoot dry weight production (Figs 3.5 and 3.6) closely reflected the data for establishment (Figs 3.3 and 3.4). Summer weeds suppressed medic growth severely, even at Wunkar (Fig 3.5) where summer weed growth was relatively low. In sub-plots where summer weeds were controlled, medic production was significantly reduced by all residual treatments, and chlorsulfuron was more damaging than triasulfuron. In the most severe case, chlorsulfuron at 22.5 g ha^{-1} combined with competition from summer weeds reduced production by 99.6% (Fig 3.6). In this experiment, triasulfuron at the lowest rate (7.5 g ha^{-1}) reduced production by 30 to 50% (Figs 3.5 and 3.6), contradicting the perception by some farmers that triasulfuron has little residual effect on medic growth.

An important feature of the results in Figs 3.3 to 3.6 is the effect of summer weed growth on the apparent damage caused by triasulfuron in May. Where summer weeds were not controlled, the various rates of triasulfuron caused proportionally less damage (relative to appropriate untreated control) than corresponding triasulfuron treatments in which summer weeds were controlled.

3.3.3 Density and growth of medics in late winter

By August, medics in many treatments had made a striking recovery (Fig 3.7 and 3.8) from the severe damage evident in May. There was an increase in density for most treatments, suggesting the emergence of multiple cohorts. The strong effect of summer weed control on density evident in May had declined markedly by August. At Wunkar, density was reduced by 30% or less by triasulfuron, and by 30 to 60% for chlorsulfuron, for treatments with no summer weed control. These reductions were slightly less when summer weeds were controlled (Fig 3.7). Medic production for chlorsulfuron treatments at Wunkar (Fig 3.9) was equal to or higher than for control plots. However, higher rates of triasulfuron reduced medic growth. This suggests that the higher concentrations of residual chlorsulfuron were important in the mechanism leading to the recovery of medic growth in August at Wunkar, probably through control of winter weeds. At Lowbank, chlorsulfuron reduced production more than triasulfuron (Fig 3.10) but the benefits of summer weed control persisted to a greater extent than at Wunkar, probably due to greater control of volunteer wheat. Density was also reduced more by residual treatments at Lowbank, but summer weed control had little influence (Fig 3.8).

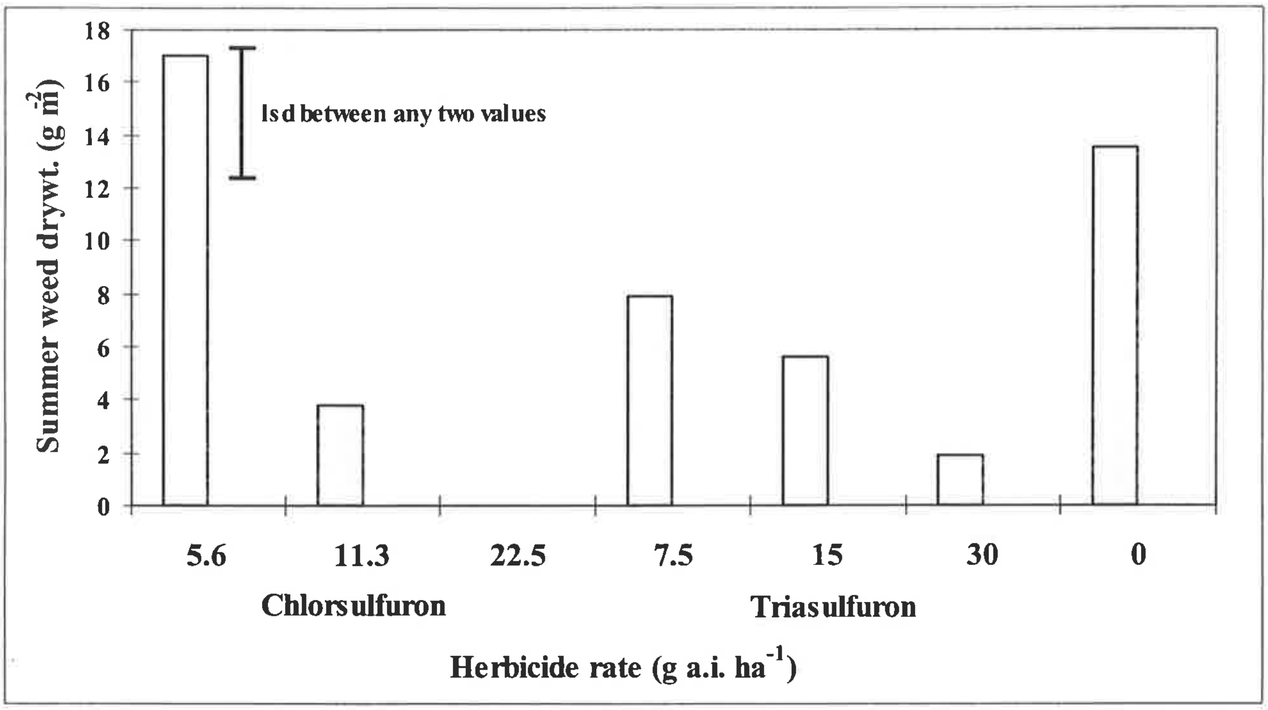


Figure 3.1. Summer weed shoot growth (g drywt. m^{-2}) in sulfonylurea soil residues with (dark shade: all zero values) and without (light shade) summer weed control at Wunkar.

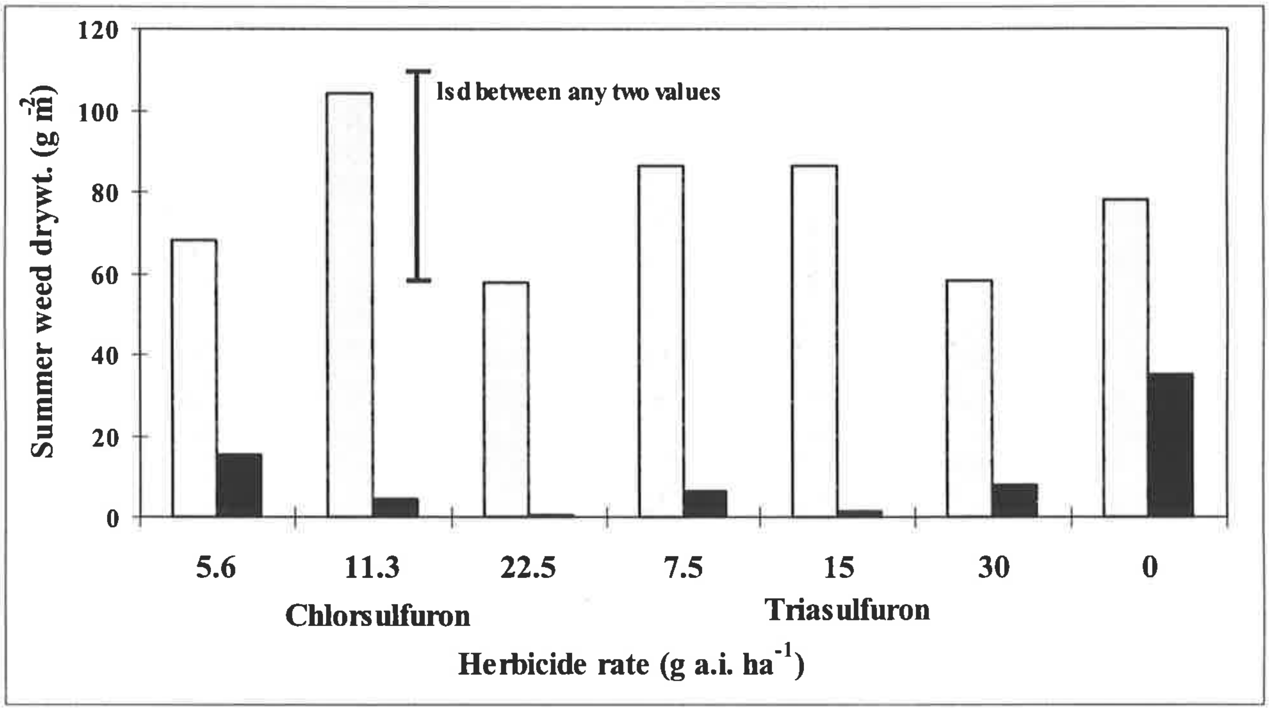


Figure 3.2. Summer weed shoot growth (g drywt. m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Lowbank.

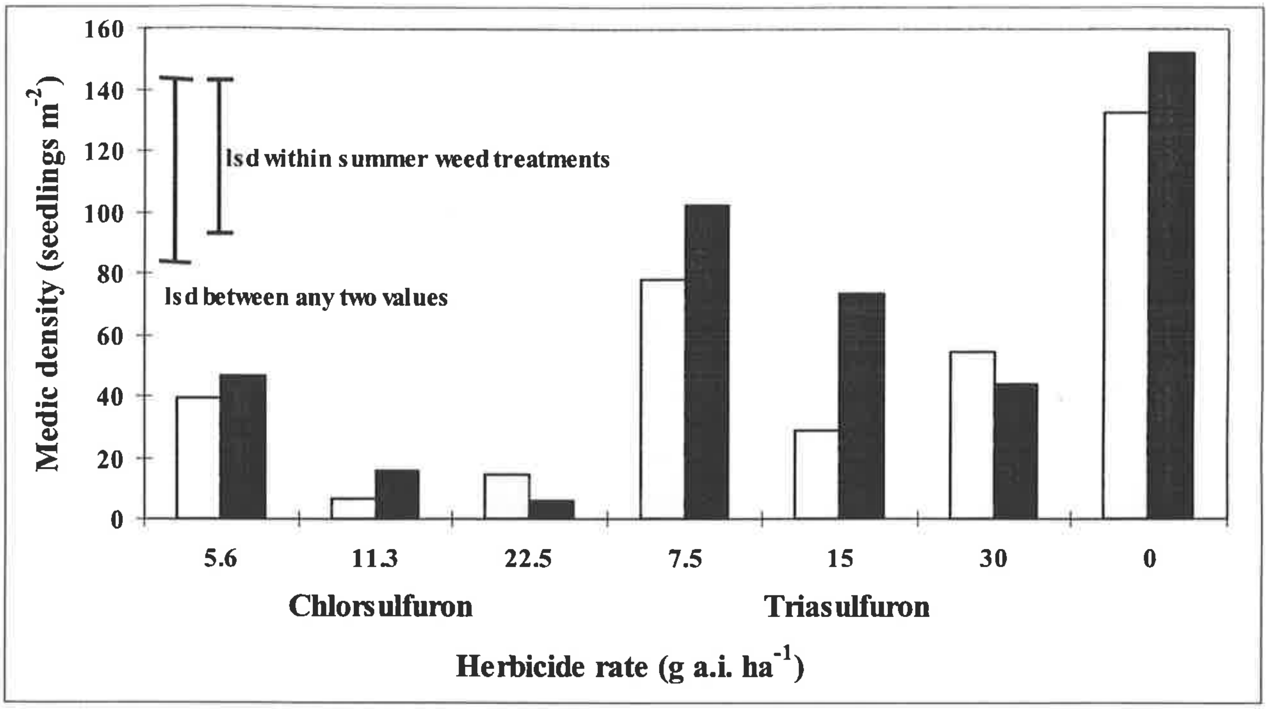


Figure 3.3. Medic density (seedlings m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Wunkar in May.

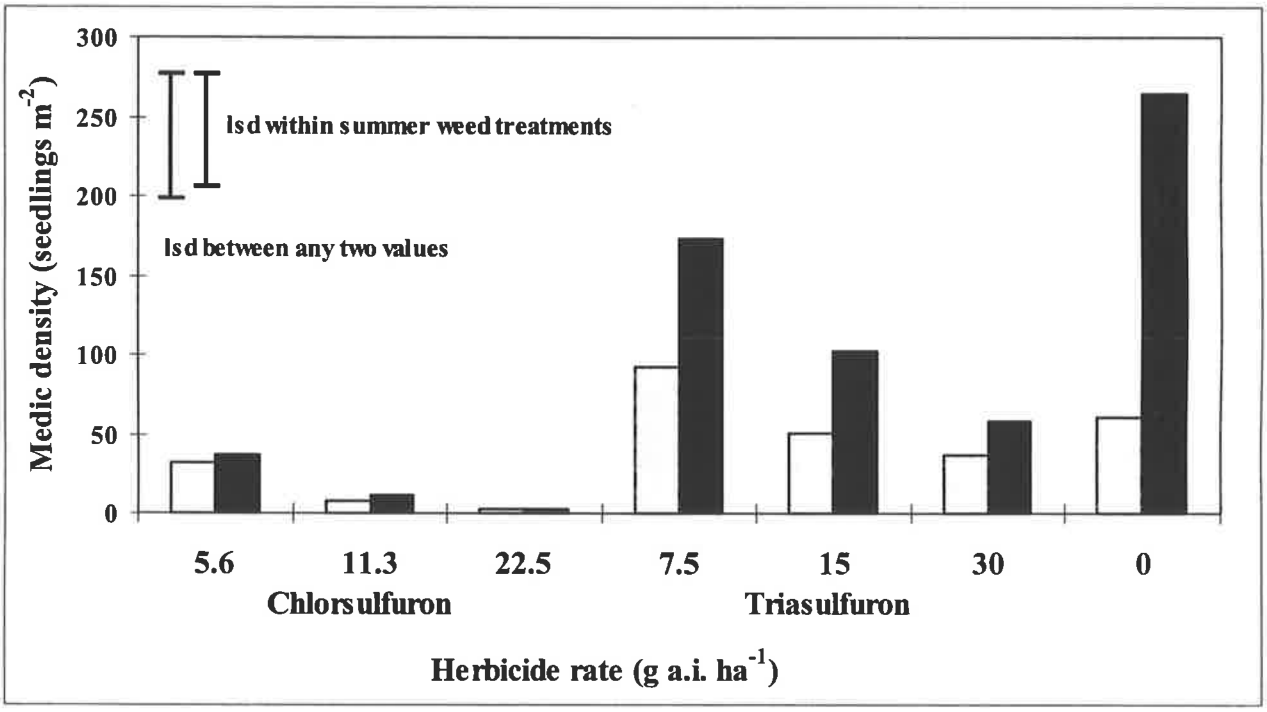


Figure 3.4. Medic density (seedlings m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Lowbank in May.

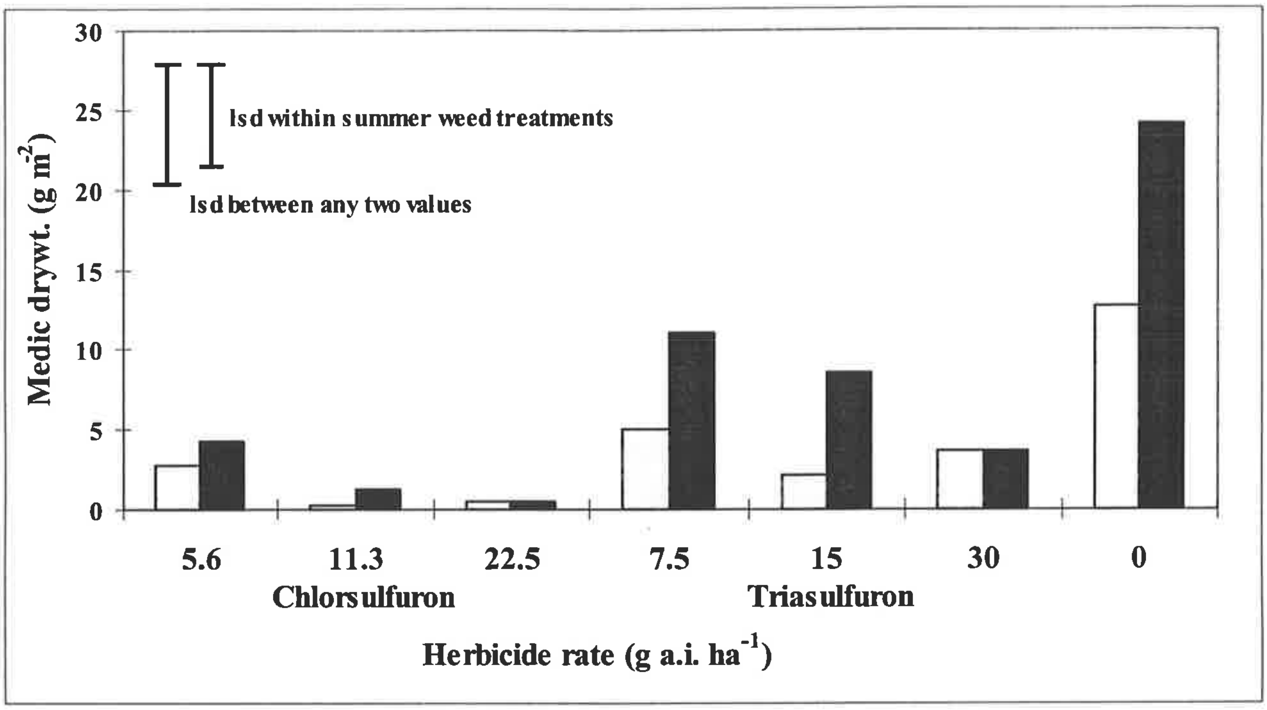


Figure 3.5. Medic shoot drywt. (g m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Wunkar in May.

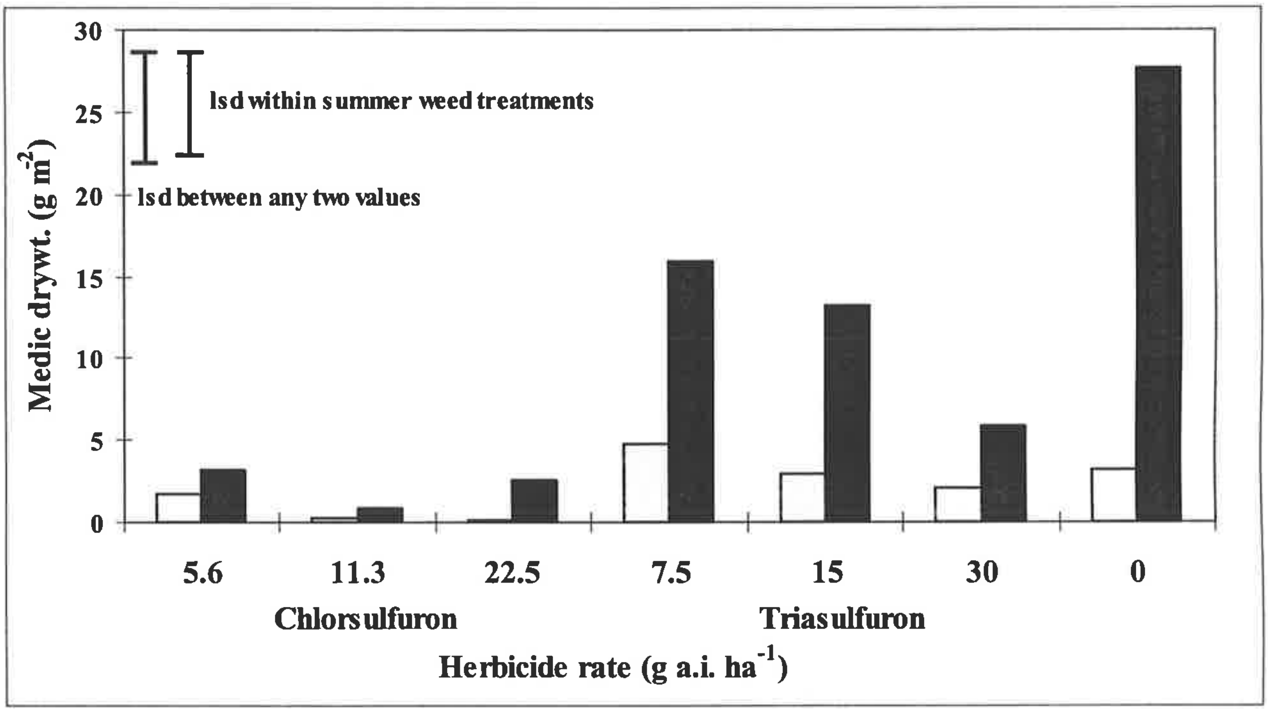


Figure 3.6. Medic shoot drywt. (g m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Lowbank in May.

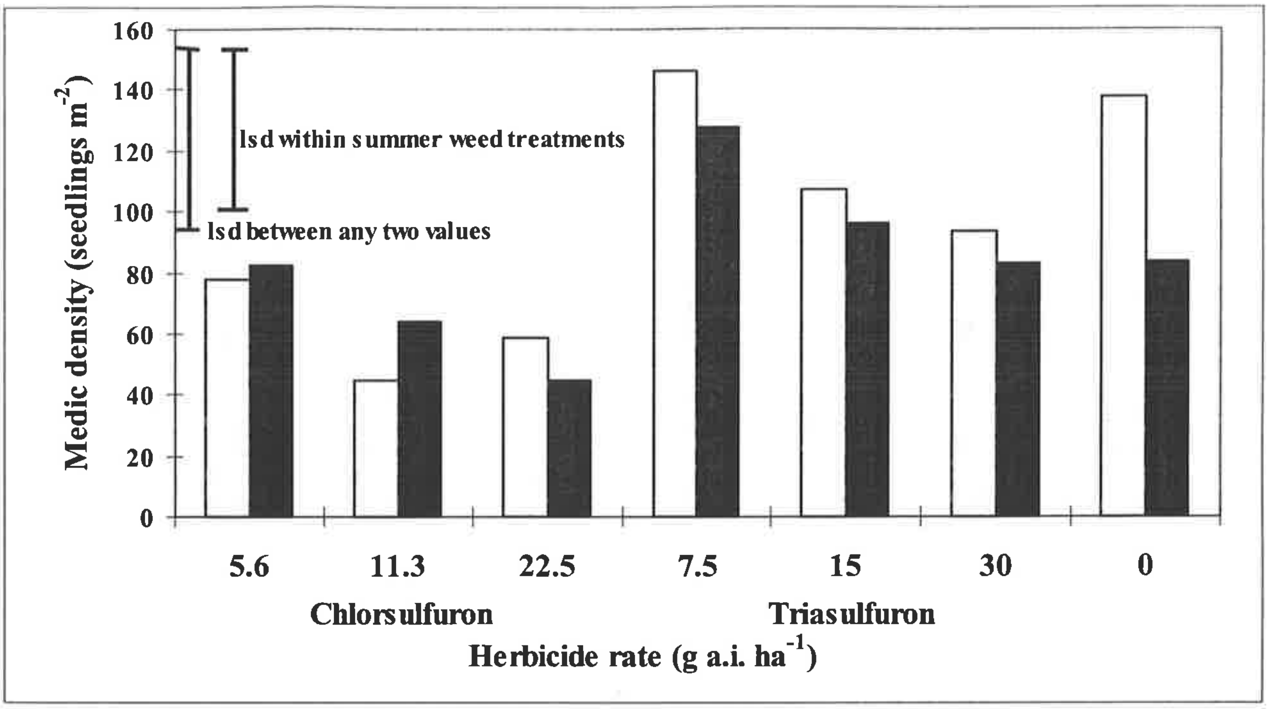


Figure 3.7. Medic density (seedlings m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Wunkar in August.

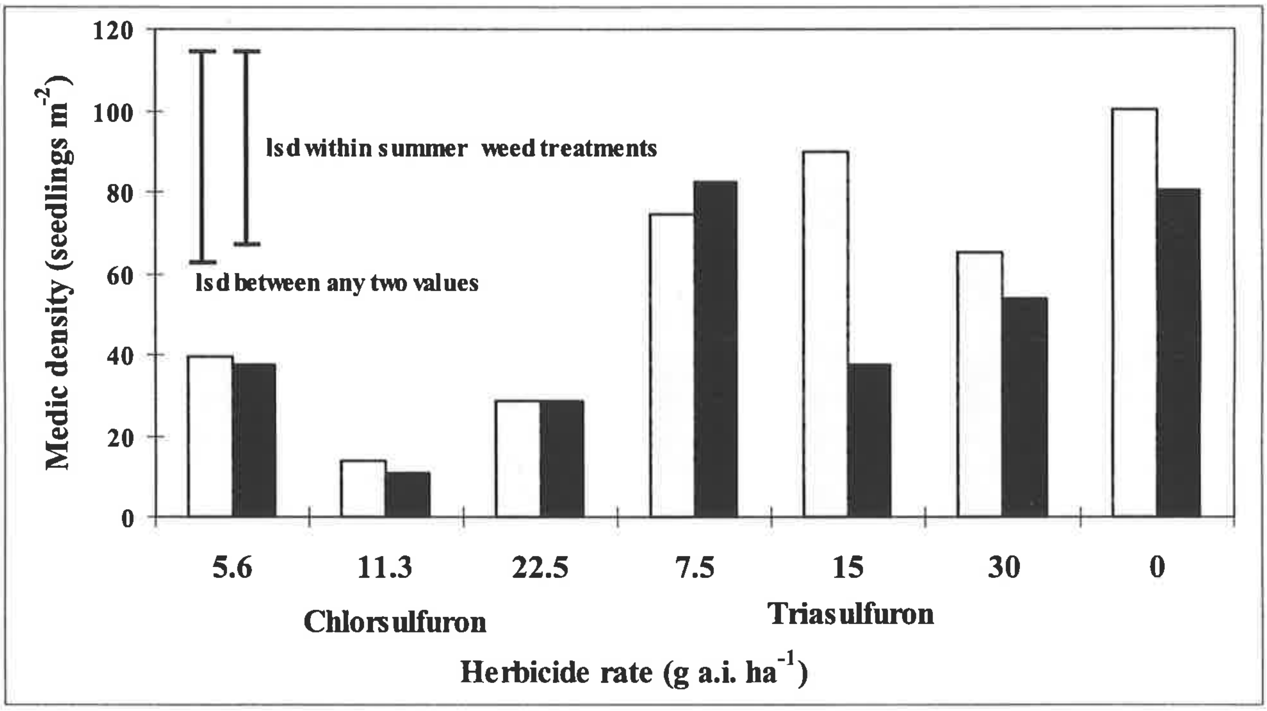


Figure 3.8. Medic density (seedlings m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Lowbank in August.

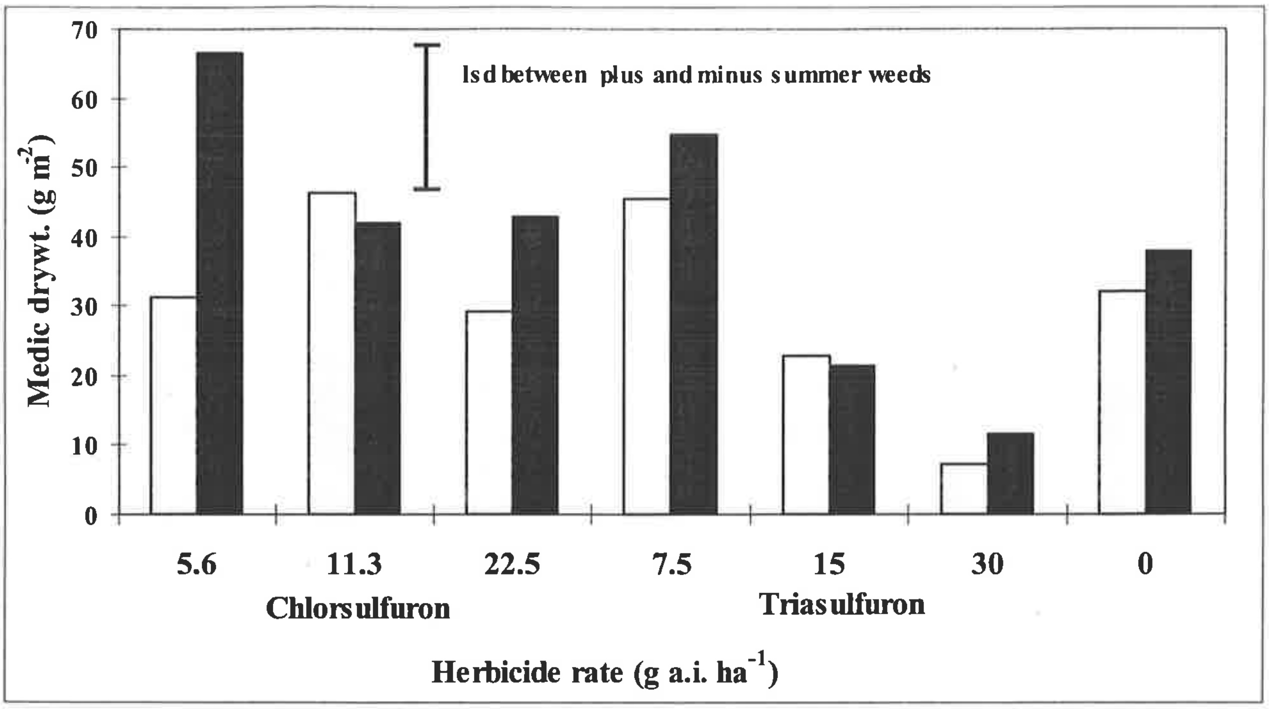


Figure 3.9. Medic shoot drywt. (g m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Wunkar in August.

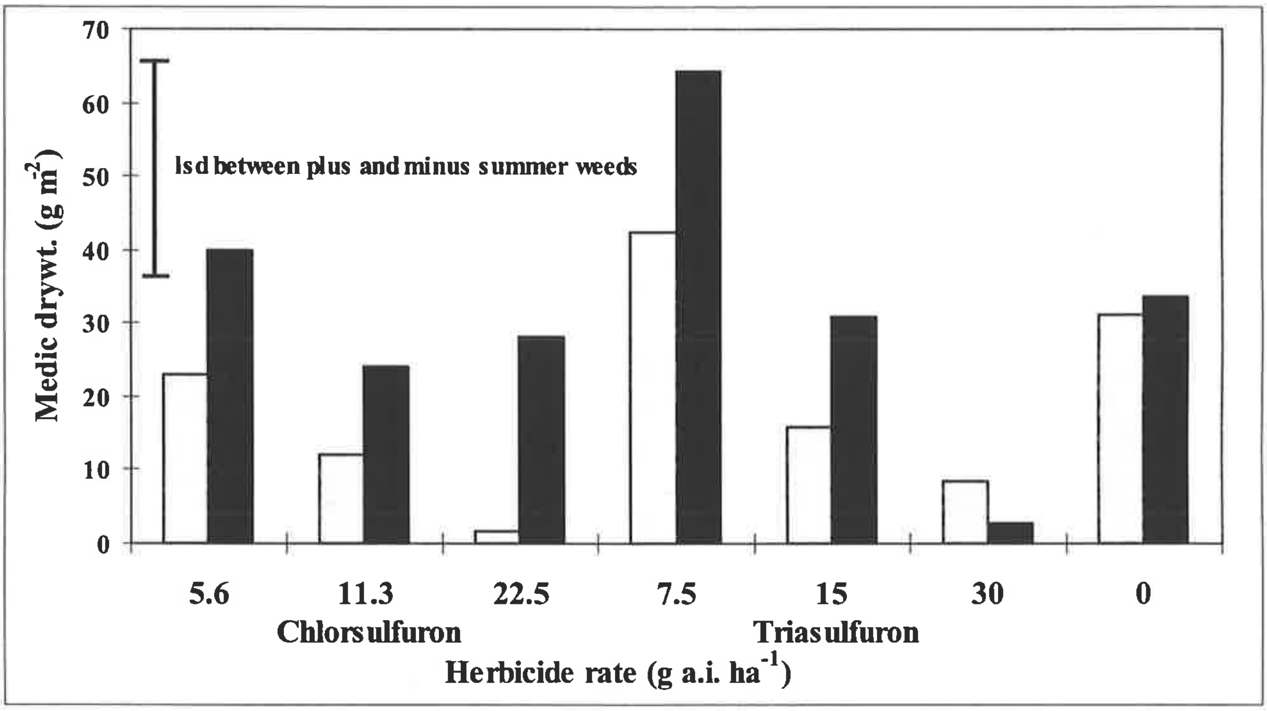


Figure 3.10. Medic shoot drywt. (g m^{-2}) in sulfonylurea soil residues with (dark shade) and without (light shade) summer weed control at Lowbank in August.

3.4 DISCUSSION

Summer weeds clearly have a large influence on medic establishment and growth in low rainfall environments. At Lowbank, when moderate summer weed growth was controlled, there was a five-fold increase in medic establishment (Fig 3.4) and six-fold increase in early production (Fig 3.6). Higher levels of damage could be expected in weedier paddocks in seasons with heavy summer rainfall. These experiments were established in low rainfall regions and most of the damage probably occurred through moisture stress, but nutrient depletion and shading would also have an effect. The results highlight the potential increase in early pasture production from control of summer weeds. The effects of summer weeds also masked the effects of triasulfuron and chlorsulfuron residues on medic growth, possibly explaining farmer observations that triasulfuron residues enhanced medic growth.

When competition from summer weeds was low, both chlorsulfuron and triasulfuron severely reduced early medic establishment and growth. As in previous studies (e.g. Evans *et al.*, 1993; Gillet and Holloway, 1996; Noy, 1996), chlorsulfuron was more damaging than triasulfuron, and damage increased with rate for both herbicides. Damage to medics from triasulfuron residues demonstrates that good early medic stands established after triasulfuron application are substantially compensated by reduced competition from summer weeds. Reduction in autumn and winter pasture production is highly undesirable because often there is a forage shortage due to slow growth caused by short days and cold temperatures.

The recovery of medic stands in August, after application of triasulfuron in particular, illustrates how farmers come to perceive that triasulfuron benefits following pastures. In addition, triasulfuron effects in May were proportionally less when summer weeds were present, thus contributing to the perception that residual effects of triasulfuron on medic growth are not important. Even though there was obvious medic suppression early in the season where summer weeds had been controlled, the visual appearance of the stand in spring was good compared to stands suffering the effects of earlier weed competition. It seems likely that triasulfuron soil residues cause a net reduction in medic production when summer weed growth is low. However, where summer weed growth is higher, the benefits of control by sulfonylurea residues outweighs the damage to medics caused by the residues. The results from sub-plots in which summer weeds were controlled (Figs 3.9 and 3.10) suggest that low rates of triasulfuron may suppress winter weeds, thus allowing later-emerging medic cohorts to grow strongly in late winter when residues have declined. Thus, the farmer's perception which prompted this research appears to be correct under high summer weed pressure.

Suppression of summer weeds by soil residues of chlorsulfuron or triasulfuron is a bonus to winter weed control in wheat, for no extra cost. These experiments suggest that maximum medic production would involve using alternatives to these herbicides in wheat, coupled with control of summer weeds without using residual herbicides. Sulfonylurea herbicides are used so widely because alternative treatments are either more expensive, or do not give sufficient residual control during winter and early spring. In addition, control of summer weeds by other means would involve the expense of cultivation or at least one, and possibly two, spraying operations. The decision to change or not could only be made by individual farmers, by comparing the value of extra pasture production to the additional cost of using alternatives to sulfonylureas and undertaking active summer weed control. Deployment of an ALSIH-resistant medic would also capture the benefits of residual control of summer weeds, without the current associated medic growth penalties.

CHAPTER 4

TOLERANCE OF MEDIC GENOTYPES TO SULFONYLUREAS

4.1 INTRODUCTION

Leguminous pasture species are very sensitive to soil residues of sulfonylureas and their growth is restricted by them in many pastures. While this is widely recognised there is little information available on the relative tolerance of species and cultivars. This information would be valuable when pasture legumes are to be sown into known sulfonylurea soil residues. Pasture production would be maximised if the most tolerant species and cultivars were sown. Identification and development of *Medicago* genotypes with tolerance to ALSIH was a major aim of this study. Identification of such genotypes began with testing the tolerance of a range of known genotypes with the aims of determining the range of variation present, ranking major species, and identifying genotypes with outstanding and useful tolerance. Before this could be done, a rapid, sensitive and reliable bioassay was required to quantitatively discern differences in medic tolerance to ALS-inhibiting herbicides. Most bioassays are based on root growth in soil in pots (e.g. Stork and Hannah, 1996), but are relatively labour-intensive and time consuming (Sarmah *et al.*, 1998). This chapter describes the development of bioassay protocols, and the use of a bioassay to rank the tolerance of a wide range of species and cultivars to chlorsulfuron and triasulfuron, the two most commonly encountered soil herbicide residues in Southern Australia. The experiments also sought to determine the variation in tolerance present, and to identify any outstanding genotypes with useful tolerance.

4.2 MATERIALS AND METHODS

4.2.1 Bioassay development

Assays with increasing requirements for labour and incubation time were evaluated until a suitable assay was identified. Initially seedlings were grown on aqueous and agar-solidified solutions of herbicides, then seeds were soaked in aqueous herbicide solutions, then roots were grown in treated soil between glass plates, and finally seedlings were grown in soil in pots. Preliminary experiments with a range of assays showed that a soil assay in pots was the most sensitive and reliable. Root length of seedlings grown in pots of soil was reduced by as little as 0.25 ppb chlorsulfuron after 7 days, and so a soil-based assay method was chosen, despite the relatively high labour requirements. The bioassay was based on that developed by Stork and Hannah (1996), used routinely by researchers at the Victorian Institute for Dryland Agriculture (VIDA). Experiment one identified the sensitive range of *Medicago truncatula* cv. Mogul for chlorsulfuron, and experiment two provided data for the effect of triasulfuron on *Medicago truncatula* cv. Mogul and *M. littoralis* cv. Herald.

The methods of Stork and Hannah (1996) were modified to measure root elongation of medics in herbicide-supplemented soil. Plastic pots, 90 mm diameter and 100 mm high, were filled with 110 ml water, then approximately 650 g of sieved (3.2 mm) sandy loam soil (pH 6.6 - water). The soil had chlorsulfuron added at 0, 0.25, 0.5, 1, 2, or 4 ppb in experiment one (cv. Mogul), and in experiment two triasulfuron was added at 0, 0.63, 0.125, 0.25, 0.5, 1, 2, and 4 ppb (cvs. Mogul and Herald). Seeds were incubated for 20 hr at 25°C in the dark and germinated seeds with 1 to 2 mm long radicles were placed on top of the soil and covered with 5 mm of untreated soil, watered with a further 15 mls, and then 5 mm of dry vermiculite was spread onto the surface as a mulch. In experiment one there were 15 seedlings per replicate and 3 replicates, and in experiment two there were 20 seedlings per replicate and 3 replicates. Pots were placed in a randomised complete block design in a glasshouse at 25 to 30°C for 7 days without further watering. After 7 days seedlings were washed from the soil in a sieve and maximum root length was measured by hand.

4.2.2 Genotypes from the Australian Medicago Genetic Resource Centre

Seeds of a range of leguminous species (see tables 4.1a and 4.1b) were obtained from the Australian Medicago Genetic Resource Centre, Adelaide, South Australia (courtesy of Mr Steven Hughes and Mr Jake Howie). Genotypes were selected to represent a range of leguminous species, with an emphasis on current and potential use in southern Australia. The seeds were germinated in 9 cm petri dishes with three filter papers and 8 ml of water at 25 ° C in the dark. Germinated seeds were planted, over a three day period, into small plastic pots containing herbicide-supplemented soil, using modified methods of Stork and Hannah (1996), described in section 4.2.1. The soil was supplemented with chlorsulfuron at 1 ppb or triasulfuron at 1 ppb. Pots were placed in a randomised complete block design in a glasshouse at 25 to 30°C for 7 days without further watering. After 7 days seedlings were washed from the soil in a sieve and root length was measured. There were 10 seeds per replicate, and three replicates. Data were analysed using a two-way analysis of variance. Germination of some lines was insufficient to allow testing, including *Medicago scutellata* cvs. Sava and Sair, *Scorpiurus vermiculatus* lines 8487 and 5067, *Trigonella balansae* line 19767, *Astragalus hamosus* lines 12734 and 16046, and *Medicago rugosa* cv. Paraponto.

4.2.3 Genotypes from ALSIH-treated breeding plots

Seed from 15 genotypes of *Medicago* were collected from a site at the Northfield Research Laboratories in Adelaide which was used for irrigated multiplication of cereal cultivars from breeding programs. The site had previously been used to evaluate a wide range of imported *Medicago* species and cultivars, and had a relatively intense history of chlorsulfuron application between 1988 and 1995 while used as a cereal breeding area. The coincidence of many different *Medicago* species and cultivars with heavy selection intensity from chlorsulfuron drew attention to a number of genotypes which were growing in chlorsulfuron soil residues which would normally be expected to severely stunt them. Mr Peter Schutz (SARDI) made 16 limited line collections, and the author made a separate collection of one prolific *Medicago scutella* population in June, 1997. In this experiment, seven 15 lines were grown with *M. truncatula* cv. Mogul and *M. littoralis* cv. Herald in soil containing 1 ppb chlorsulfuron (using the assay described in 4.2.1) to detect any lines with unusually high tolerance to chlorsulfuron. The 1 ppb rate corresponds to about 10% of a typical initial field dose, and any plants able to grow well in this concentration may possess useful field tolerance. After root measurement seedlings were transplanted into polystyrene boxes filled with sandy loam and grown for 6 weeks until they were at the 2-4 trifoliate leaf stage. Chlorsulfuron at 11.3 g a.i. ha^{-1} plus 0.2% BS1000 wetting agent was then applied with a 2 m wide plot sprayer (147 l ha^{-1}; 200 kPa) and survival was assessed 7 weeks after treatment. Data were analysed using a two-way analysis of variance.

4.3 RESULTS

4.3.1 Bioassay development

Seedling emergence in experiment one began after 3 days and by 7 days 97% of seedlings had emerged. Seedling emergence rate was not influenced by herbicide concentration and seedling roots attained a certain minimum length even at high concentrations. Experiment one showed that the sensitive range for cv. Mogul was between 0 and 0.5 ppb chlorsulfuron (Fig. 4.1) and that root growth was reduced to 39% between 0 and 0.25 ppb. In experiment two, triasulfuron inhibited both cvs. Mogul and Herald root growth in a similar way, and the I_{50} was about 1 ppb (Fig. 4.2).

4.3.2 Genotypes from the Australian Medicago Genetic Resource Centre

Tolerance to chlorsulfuron and triasulfuron varied widely between the species tested. Mean root growth was reduced to between 14 to 52% of untreated and, in general, genotype tolerance to chlorsulfuron and triasulfuron were similar (Tables 4.1a and 4.1b; Fig 4.3). *M. rugosa* was the most tolerant species tested, with the two representative cultivars having the highest (52%) and second highest (44%) level of tolerance. *M. sphaerocarpus* cv. Orion was the third most tolerant (39%). Most cultivars of *M. truncatula* and *M. littoralis*, which are currently the most agronomically significant group, had a higher level of tolerance than most other genotypes tested. Generally, *M. polymorpha* and *M. sativa* were the least tolerant species. Of the species other than *Medicago*, *Lotus maroccanus* (19681) and *Scorpiurus vermiculatus* (25698) were slightly more tolerant than *M. truncatula* and *M. littoralis* (Table 4.1b; Fig 4.4).

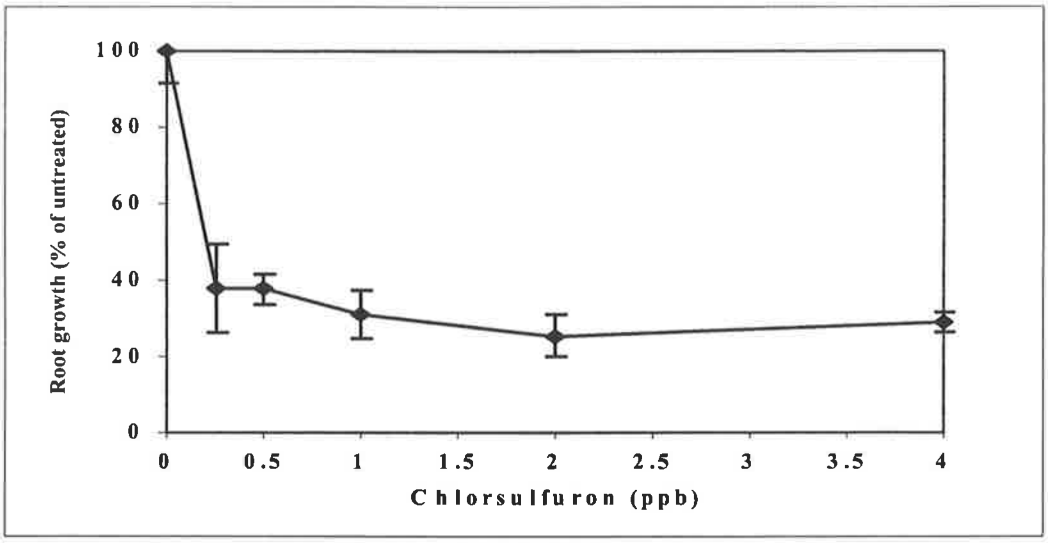


Fig. 4.1. *M. truncatula* cv. Mogul maximum root length (% of untreated) in soil supplemented with chlorsulfuron in pots after 7 days in experiment one.

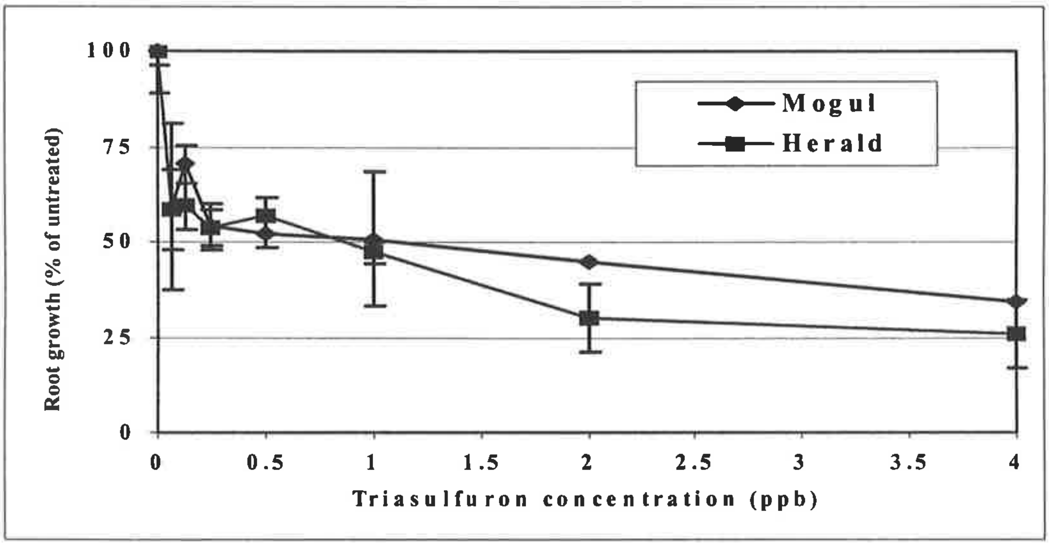


Fig. 4.2. *M. truncatula* cv. Mogul and *M. littoralis* cv. Herald maximum root length (% of untreated) in soil supplemented with triasulfuron in pots after 7 days in experiment two.

Table 4.1a. Root growth (% of maximum length in untreated soil) of a range of *Medicago* species and cultivars in soil containing 1 ppb chlorsulfuron (CSF) and triasulfuron (TSF) residues.

Species	Cultivar (SA collection #)	Root length (mm)	Root growth (% of untreated)		
			CSF	TSF	Mean
Medicago littoralis	Harbinger	52.6	26.9	ID*	26.9
Medicago littoralis	Harbinger AR	76.9	31.4	ID*	31.4
Medicago littoralis	Herald	66.6	27.0	35.3	31.2
Medicago littoralis	Pildapa	71.9	23.2	23.4	23.3
Medicago murex	Zodiac	90.2	20.7	21.1	20.9
Medicago polymorpha	Circle Valley	90.4	18.8	ID*	18.8
Medicago polymorpha	Serena	88.8	17.1	15.4	16.3
Medicago polymorpha	Santiago	95.8	13.7	13.5	13.6
Medicago rugosa	Paragosa	85.9	50.5	54.3	52.4
Medicago rugosa	Sapo	93.5	43.7	45.8	44.8
Medicago sativa	Aquarius	79.3	17.8	16.9	17.4
Medicago sativa	Aurora	84.6	17.0	14.5	15.8
Medicago sativa	Eureka	83.0	21.9	24.4	23.2
Medicago sativa	Hunterfield	89.9	16.8	19.9	18.4
Medicago sativa	Trifecta	78.3	17.2	15.6	16.4
Medicago scutellata	Kelson	98.5	16.4	29.8	23.1
Medicago sphaerocarpus	Orion	97.6	39.8	38.3	39.1
Medicago tornata	Tornafield	86.7	17.5	24.1	20.8
Medicago tornata	Murraylands	98.8	18.6	29.1	23.9
Medicago tornata	Rivoli	80.9	21.4	18.7	20.1
Medicago truncatula	Jemalong	83.5	20.5	ID*	20.5
Medicago truncatula	Mogul	83.2	26.7	32.2	29.5
Medicago truncatula	Paraggio	78.7	30.3	25.9	28.1
Medicago truncatula	Hannaford	71.4	28.2	22.3	25.3
Medicago truncatula	Parabinga	90.3	31.8	31.0	31.4
lsd (P=0.05)			8.5	11.1	

ID* = Insufficient data due to poor germination or growth of line.

Table 4.1b. Root growth (% of maximum length in untreated soil) of a range of leguminous species and cultivars in soil containing 1 ppb chlorsulfuron (CSF) and triasulfuron (TSF) residues.

Species	Cultivar (SA collection #)	Root length (mm)	Root growth (% of untreated)		
			CSF	TSF	Mean
Hymenocarpus circinnatus	(9759)	92.7	15.9	15.4	15.7
Hymenocarpus circinnatus	(13615)	105.6	22.8	21.0	21.9
Lotus maroccanus	(729)	51.6	19.2	25.0	22.1
Lotus maroccanus	(19681)	25.9	43.8	28.0	35.9
Scorpiurus vermiculatus	(25698)	94.9	26.6	42.2	34.4
Trigonella arabica	(919)	68.4	34.7	29.5	32.1
Trigonella arabica	(5042)	71.1	29.3	ID*	29.3
Trigonella arabica	(8583)	64.3	31.5	ID*	31.5
Trigonella balansae	(6136)	48.3	24.2	32.7	28.5
lsd (P=0.05)			8.5	11.1	

ID* = Insufficient data due to poor germination or growth of line.

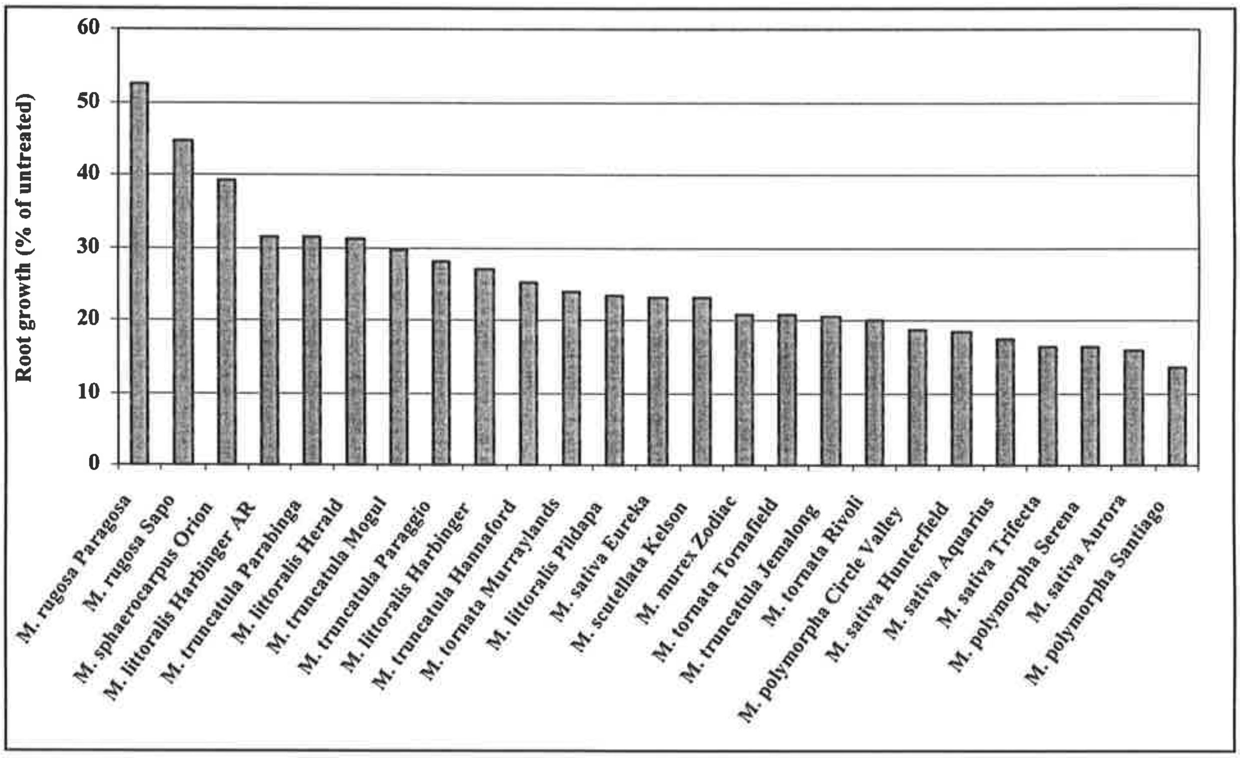


Fig. 4.3. Root growth (% of maximum length in untreated soil) of a range of *Medicago* species and cultivars – mean of 1 ppb chlorsulfuron and 1 ppb triasulfuron.

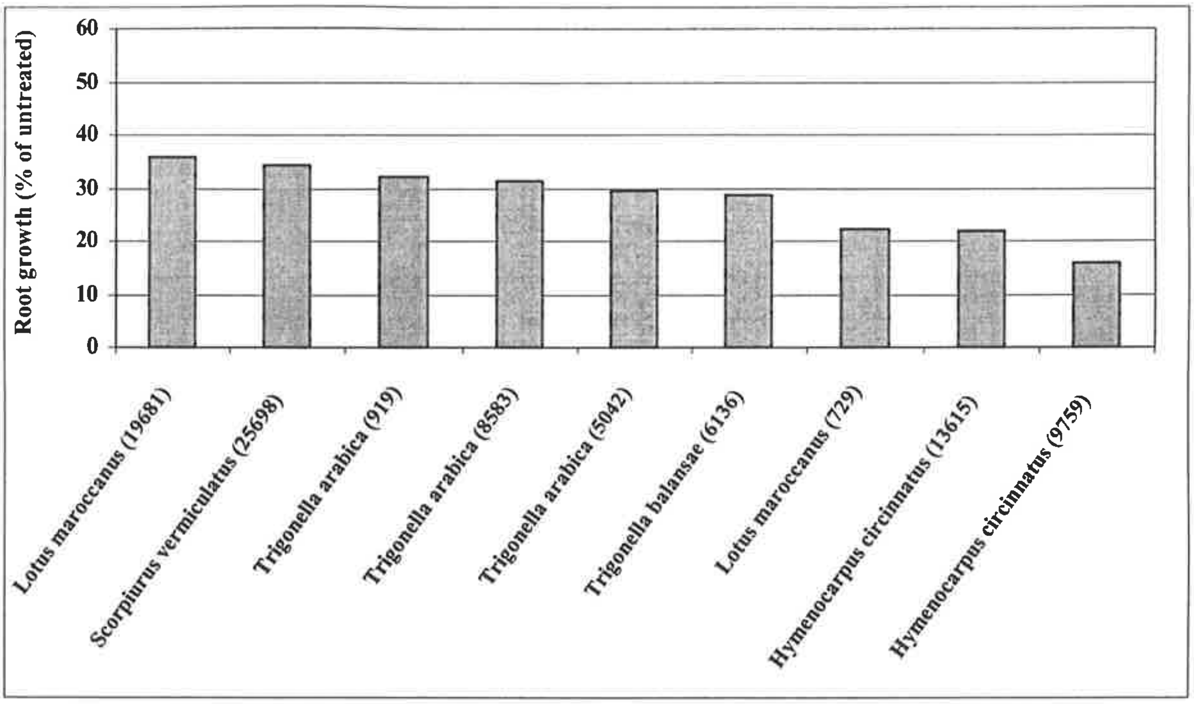


Fig. 4.4. Root growth (% of maximum length in untreated soil) of a range of leguminous species and cultivars – mean of 1 ppb chlorsulfuron and 1 ppb triasulfuron.

4.3.3 Genotypes from ALSIH-treated breeding plots

Root growth in 1 ppb chlorsulfuron ranged from 28 to 84% of untreated root length (Fig 4.5). Under the conditions of this experiment root growth of cvs. Mogul and Herald was greater than in the previous experiment (section 4.3.2). There were five genotypes which had significantly higher tolerance than cvs. Mogul and Herald, including one *M. scutellata*, one *M. rugosa*, one *M. polymorpha* and two *M. intertexta*. The *M. scutellata* genotype had the highest apparent tolerance of any existing genotype tested during these studies. None of the seedlings transplanted and later sprayed with chlorsulfuron at 11.3 g a.i. ha^{-1} survived, confirming that there was no high level resistance present.

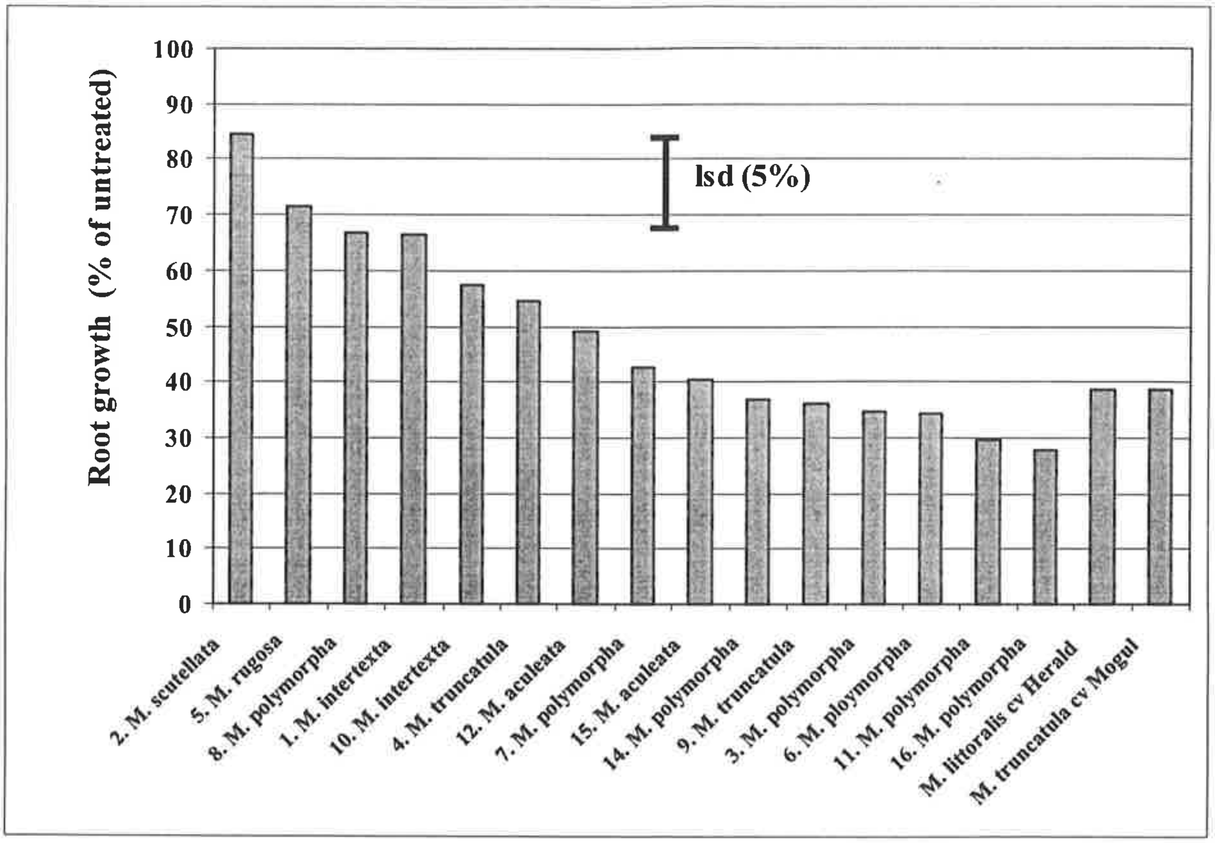


Fig. 4.5. Root growth (% of maximum length in untreated soil) of 15 genotypes from Northfield in 1 ppb chlorsulfuron (numbers preceding species name are collection identification).

4.4 DISCUSSION

A range of assays was evaluated, requiring increasing levels of labour and incubation time. Finally, root growth in pots of soil was identified as the most sensitive and reliable bioassay. The failure of the other bioassay methods was remarkable for the apparent ability of medic roots to elongate in the presence of very high concentrations of sulfonylurea herbicides in some media. The sensitive and reliable results from the soil bioassay in pots led to adoption and refinement of this bioassay as a standard for determination of tolerance. Soil bioassay experiments confirmed the results of K. Hollaway and D. Noy (pers. comm., 1997) that annual medic root growth in soil over 7 days is reduced to less than 50% of untreated by concentrations of chlorsulfuron below 0.5 ppb. These experiments aimed to identify a concentration which could be used to discern the level of tolerance of medics to sulfonylurea herbicides. A concentration was required which inhibited root growth, while allowing expression of useful levels of tolerance. A concentration of 1 ppb was chosen for both chlorsulfuron and triasulfuron because this concentration did not completely inhibit root growth. It also approximates typical field residues one year after application, and so genotypes identified with tolerance to 1 ppb may have useful field tolerance.

All of the genotypes tested from the Australian Medicago Genetic Resource Centre showed significant susceptibility to 1 ppb chlorsulfuron, and many were reduced to 10 to 15% of untreated root length. In dose-response experiments it was observed that, even at very high herbicide concentrations, seeds were able to germinate and attain a minimum shoot and root size before seed reserves of amino acids were exhausted. It is likely that some of the species with lower ranked tolerance did not grow at all beyond this point. The herbicide concentrations used to test for tolerance were not high. Immediately after application and incorporation (to 100 mm) chlorsulfuron and triasulfuron concentrations are about 8 and 17 ppb, respectively (McQuinn, 1997). The rates used in these experiments represent about 13% and 6% of the applied dose, simulating 87 to 94% breakdown between application in a cereal crop and pasture regeneration.

Although none of the genotypes displayed outstanding tolerance, *M. rugosa* cvs. Paragosa and Sapo were the most tolerant, and any further research should evaluate other cultivars within this species. *M. rugosa* is particularly adapted to alkaline soils (Puckridge and French, 1983), where soil residue problems are greatest. *M. littoralis* and *M. truncatula* are the most widely grown medic species in southern Australia, and they were found to be more tolerant than most genotypes tested. *M. sativa* was very sensitive, confirming the sensitivity of lucerne seedlings observed in the field.

Several of the genotypes from the Northfield breeding plots showed promise, however, none survived a post-emergence application of 11.3 g a.i. ha^{-1} chlorsulfuron. Limited seed supplies prevented further evaluation of the lines, but the *M. scutellata* and *M. rugosa* genotypes, in particular, warrant more detailed characterisation. The relatively high tolerance of two *M. intertexta* genotypes suggests that this species may also warrant more detailed testing.

Most members of the Leguminosae are sensitive to sulfonylurea soil residues, due to both low rates of herbicide metabolism and sensitive ALS. It is likely that they share similar ALS structures which are descended from one or several common sensitive ancestral ALS. It therefore seems likely that a medic with a high degree of resistance would only result from selection of a rare spontaneous mutation, mutation breeding, or gene transfer. The former two possibilities were explored in the following chapters.

CHAPTER 5

FIELD SELECTION AND MASS SCREENING FOR SULFONYLUREA RESISTANCE

5.1 INTRODUCTION

The results from section 4.3 suggested that useful tolerance was unlikely to be found in known *Medicago* genotypes, and that alternative approaches were needed. There are four methods for producing herbicide resistant crops: classical breeding, somatic hybridisation, *in vitro* selection, and transgenic engineering (Froud-Williams, 1991). Although classical breeding is time consuming, laborious, and often uses large spaces, it was chosen as the most desirable method for identifying a herbicide-resistant genotype for a number of reasons. A resistant genotype selected directly from an elite commercial cultivar is likely to retain its desirable agronomic traits without carrying detrimental traits which may be introduced using some other techniques. The mass-screening method is also technologically simple, requiring few specialised resources or techniques. Classical breeding relies on the generation or identification of genetic variation, in this case a genotype resistant to ALSIH. Genes for herbicide resistance are usually present in plant populations at low frequencies (Gressel and

Segel, 1982) but the chances for finding such a mutant in a large population were thought to be good, considering the measurements and estimates of other researchers for similar traits in other species. For example, in field and laboratory screening experiments Preston and Powles (2000) estimated the initial frequency for genes conferring ALSIH resistance in *Lolium rigidum* to be between 4.6 x 10^{-5} to 1.2 x 10^{-4}. Also, Stannard (1987) screened 20 million germinated lucerne (*Medicago sativa*) seeds for chlorsulfuron resistance, and found 2 lines with increased tolerance, suggesting a frequency of around 2 x 10^{-7}.

To maximise the probability of identifying a resistant annual medic plant it was desirable to challenge billions of plants to field residues over many years. This situation has been occurring throughout the cropping zone of southern Australia since 1982, as chlorsulfuron and triasulfuron have been applied in dry regions with high soil pH. Therefore, volunteer medic plants were selected from paddocks which had been subjected to intensive sulfonylurea herbicide use, with the aim of identifying possible resistant mutants which may be persisting in soils subject to a high intensity of herbicide selection pressure.

In a second experiment large numbers of four elite cultivars from two major medic species were established in the field and screened for resistance to metsulfuron-methyl. The aim of this experiment was to characterise survivors and attempt to transfer any resistance to other elite cultivars through conventional crossing.

5.2 MATERIALS AND METHODS

5.2.1 *Field selection screening*

<u>Region and paddock selection.</u> Paddocks were sought in the Riverland and Lower North of South Australia where annual medic pastures are grown in rotation with cereals on alkaline

soils. Rotations involving volunteer pastures in these areas are typically cereal-pasture, or wheat-barley-pasture. Initially, paddocks which had been treated with sulfonylurea herbicides in the current or previous year were selected, but most searches were conducted in wheat paddocks which had been treated with triasulfuron at sowing, to minimise the number of medic plants present and maximise the herbicide selection intensity. Collections were made from 2/9/97 to 12/9/97 and records of ALSIH use were obtained from the land owners (Table 5.1).

Plant collection. Paddocks were searched on foot, walking at about 3 km hr^{-1} in a zig-zag pattern at 45° to drill rows to maximise visibility along the rows and minimise the chances of collecting disproportionately in strips which had been accidentally missed by the spray boom. Collection time in paddocks ranged from 20-70 minutes and a maximum of 60 plants were collected from an individual paddock. Where there were significant clusters of plants discovered a GPS reading was taken to facilitate return to the point. Selected plants were dug up and kept moist prior to transplanting into foam boxes filled with a sandy-loam soil. There was a range of species collected, including *M. littoralis, M. polymorpha* and *M. truncatula*. Medic density was generally very low in cereal crops. Details of paddocks searched are given in Table 5.1.

Screening. Transplanted medics were allowed to establish and resume growth for 6 weeks in a glasshouse and then metsulfuron-methyl was applied (as Ally®) on 22/10/97 at 8.4 g a.i. ha^{-1}, with 0.2%v/v BS 1000® non-ionic wetting agent, using a 2m bicycle plot sprayer applying 147 l ha^{-1} at 200 kPa. Medics were 2 to 20 cm high at the mid-flowering stage and survival was assessed 4 weeks after application.

Table 5.1. Collection details for field selections of annual *Medicago* with putative sulfonylurea resistance in 1997.

Location	Crop type 1997	Soil texture	ALS-inhibiting herbicide applied (g a.i. ha^{-1})		Area searched (ha)*	Number of medics collected
			1996	1997		
Balaklava	-	S-loam	Tria 19	Tria 19	0.2	45
Balaklava	-	C-loam	Tria 19	Tria 19	0.2	60
Balaklava	-	C-loam	-	Tria 19	0.2	120
Bute	B	S-loam	Imaz 48	None	0.5	60
Bute	B	Loam	Tria 23	None	0.3	60
Bute	B	Loam	Tria 23	None	0.2	60
Bute	VP	S-loam	Tria 14	None	0.2	60
Kulpara	W	Loam	Tria 23	Mets 2.5	0.5	50
Lowbank	W	L-sand	None	Tria 8	0.3	20
Lowbank	W	L-sand	None	Tria 8	0.3	20
Lowbank	W	L-sand	None	Tria 11	0.2	46
Paringa	W	S-loam	-	Tria 14	0.3	30
Paringa	W	S-loam	None	Tria 14	0.3	20
Paskeville	B	S-loam	None	None	0.2	2
Paskeville	B	S-loam	None	None	0.2	3
Wunkar	W	S-loam	None	Tria 11	0.2	7
Wunkar	W	L-sand	None	Tria 11	0.2	8
Wunkar	W	L-sand	None	Tria 11	0.2	6
Wunkar	W	L-sand	None	Tria 11	0.3	43
Wunkar	W	L-sand	None	Tria 11	0.4	3
Total collected						723

Crop type: B = barley; VP = Volunteer pasture, W = Wheat
Soil texture: C-loam = clay-loam; L-sand = loamy sand; S-loam = sandy-loam.
ALS-inhibiting herbicide: Mets = metsulfuron-methyl; Tria = triasulfuron; Imaz = imazethapyr.
* Based on walking at 3 km hr^{-1} with a 1.5 m wide search width.

5.2.2 *Mass screening experiment*

Site, sowing and plant establishment. *Medicago truncatula* cvs. Caliph, Mogul, Parragio and *Medicago littoralis* cv. Herald were sown in a randomised complete block design with four replicates within an area 105m x 120m. Each of the 16 plots was 7.5m x 105m. The site was near Bool Lagoon (approximately 20 km SW of Naracoorte, SA) on a loamy-clay soil with a pH (water) of 7.1. The experiment was sown on 26/6/97 using a tractor-drawn small plot cone seeder. Seeds were obtained from commercial suppliers (cvs. Caliph, Herald, and Mogul from Revell Seeds at Dimboola, Vic., and Paraggio from David Verner at Mallala, SA) and inoculated with "Nitrogerm Group AM or AL" medic *Rhizobium meliloti* inoculant. Bitenthrin (as Talstar® 100 EC) insecticide was applied at 10 g ai ha^{-1} immediately after sowing for control of red-legged earthmite (*Halotydeus destructor*). Fluazifop-methyl (as Fusilade®) + 0.2% v/v BS1000® non-ionic wetting agent was applied at 318 g a.i. ha^{-1} on 17/9/97 to control *Lolium rigidum*. Very high seeding rates were used to maximise the number of seedlings established within the 1.3 ha area (Table 5.2). Seedling numbers were estimated on 22/8/97 by sampling 15 cm lengths of drill rows at 8 places within each plot. Seeding rates, and numbers of established seedlings 8 weeks after sowing are shown in Table 5.2.

Herbicide application. The first application of metsulfuron-methyl (as Ally®) was applied on 26/8/97 at 8.4 g a.i. ha^{-1}, with 0.2%v/v BS 1000® non-ionic wetting agent, using a 10m boom mounted on a 4-wheel drive vehicle applying 92 L ha^{-1} at 210 kPa and the dose was applied as two half rate applications, the second at 90 degrees to the first. Medics were at the 1-2 trifoliate leaf stage. A second application of metsulfuron-methyl at 8.4 g ha^{-1}, using the same methods as above, was made 8 weeks later on 22/10/97.

Survival assessment. Seedlings which were still green on 30/9/97, 5 weeks after the first metsulfuron-methyl application, were identified and marked. Green plants were easily seen against a background of brown-white dead seedlings. Surviving plants from the first application of metsulfuron-methyl were assessed on 6/11/97, 2 weeks after the second application.

5.3 RESULTS

5.3.1 *Field selection screening*

Plants collected from paddocks recovered from transplanting and were growing actively at the time of metsulfuron-methyl application. This treatment killed all of the plants, and an assessment 4 weeks after spraying confirmed 100% mortality.

5.3.2 *Mass screening experiment*

Seedlings established well and by the time of the first metsulfuron-methyl application there was a very dense stand in each plot (Table 5.2). Conditions were favourable for herbicide activity, and a careful foot search of each plot 5 weeks after spraying identified only two small surviving patches of *M. truncatula* cv Mogul. The colonies consisted of 6 and 4 plants, and were not obviously shaded from spraying. The clumping of the survivors indicated that they were unlikely to be resistant. All of these survivors were subsequently killed by a second application of metsulfuron-methyl.

Table 5.2. Estimated seedling number and seeding rate for four medic cultivars in the mass field screening experiment.

Cultivar	Caliph	Herald	Mogul	Parragio
Sowing rate (kg ha^{-1})	160	80	170	160
Seedlings 1.2 m^{-1} drill row	667 ±119	381 ±32	759 ±91	482 ±43
Estimated total plant number	9.34 x 10^6	5.33 x 10^6	10.63 x 10^6	6.80 x 10^6

5.4 DISCUSSION

Screening of around 32 million medics grown from commercial seed (Table 5.2), and 723 selected plants with a putatively high likelihood of being resistant (Table 5.1), failed to identify any individuals with resistance to twice the field dose of metsulfuron-methyl. The apparent absence of resistance amongst the four cultivars in the mass-screening experiment suggests that the frequency of ALSIH resistant individuals in medics is less than $0.5\text{-}1.1 \times 10^{-7}$ (Table 5.2). Failure to detect resistance in plants collected from treated paddocks was probably the result of low population density in cereal paddocks, and low resistant gene frequency. The dramatic decline in chlorsulfuron use, due mainly to grower's concerns over soil residues, was illustrated in Table 5.1. Paddocks treated with chlorsulfuron were uncommon and none of the 20 paddocks surveyed had been treated with chlorsulfuron, even though its use was widespread in the 1980's. Thus, resistance to chlorsulfuron was sought in remnant chlorsulfuron resistant plants with cross-resistance to triasulfuron, or through chlorsulfuron cross-resistance in triasulfuron resistant plants.

Given the low frequency of genes for resistance, it is likely that the population sizes for both the mass screening and field selections were insufficient. The elite cultivars which are growing in farmers' paddocks, and which were used in the mass screening experiment, have been through relatively few generations since selection from a very narrow genetic base. Consequently, there has been little opportunity for new ALS mutations to occur or become established in the genomes. It is possible that there were medics killed which were resistant to ALSIH other than metsulfuron-methyl. However, in most cases of ALSIH resistance there is wide cross-resistance amongst the sulfonylureas (Saari *et al.*, 1994).

In many paddocks medic density is low due to medic decline (section 1.2). Even if medic density is relatively high in the pasture preceding the crop, the early germination and emergence of medics soon after the opening autumn rains subjects the population to high mortality rates during crop preparation. The cumulative mortality due to pre-seeding, non-selective herbicide application, cultivation, and the sowing operation is later compounded by competition from the crop. As a result a relatively low proportion of the population survives to be subjected to ALSIH, which are usually applied around the time of sowing. The susceptibility of surviving plants collected from cereal crops suggests that most plants emerged after the crop was sown, in soil which for some reason (e.g. missed strip or shielding) did not contain a lethal concentration of herbicide. The long soil bank life of medics would also provide a high proportion of susceptible plants to compete with any resistant mutants, thus restricting an increase in resistant gene frequency.

In a population of *Kochia scoparia* with an ALSIH-resistant gene frequency of 10^{-7} producing 1 million seeds ha^{-1}, only 10 resistant individuals would be expected in a 100 ha paddock (Maxwell and Mortimer, 1994). The area searched for each paddock in this study was typically 0.2 to 0.5 ha (Table 5.1). Assuming a resistant gene frequency for medics of 10^{-7}, and a population density of 20 m^{-2}, using Maxwell and Mortimer's (1994) estimates only two resistant medics are expected in a 100 ha field. This equates to only 0.01 medics per each 0.5 ha searched in each paddock, or on average one resistant medic in every 100 areas searched. This suggests that the resistant gene frequency would have needed to be around 10^{-5} or higher to expect a reasonable likelihood of success.

The gene frequency for new medic cultivars is probably less than 0.5 to 1.1×10^{-7} (Table 5.2), suggesting that many more plants need to be screened to increase the probability of identifying a resistant individual. Jasieniuk *et al.* (1996) estimated that if the frequency of resistant plants is 1×10^{-6} then at least 3×10^{6} plants must be screened to be 95% confident of there being at least one resistant plant in the sample. The mass screening experiment used 5 to 10 x 10^6 medic plants in each population, covered 1.3 ha and used 175 kg of seed. The area could only practically be increased to 2 or 3 times without access to greater resources. If the resistance frequency was 10^{-9}, for example, then a similar screening experiment would need to cover at least 100 ha, and use 17.5 tonnes of seed, to be confident of finding a resistant individual.

It seems likely from the failure of both the mass screening and field selection techniques used that a much higher population size would need to be screened to improve the likelihood of success. The resources needed to achieve this are beyond those of the current study. For these reasons, research involving cell selection techniques and mutagenesis was initiated as a more practical way to screen higher population (cell) sizes and increase resistance gene frequency in seeds.

CHAPTER 6

MUTATION, SELECTION AND CHARACTERISATION OF RESISTANT CELL LINES

6.1 INTRODUCTION

The results from the experiments described in Chapters 4 and 5 suggested that it was unlikely that useful tolerance to soil residues of ALSIH would be found within existing medic genotypes, selected from ALSIH-treated paddocks, or identified using mass screening techniques. This led to the conclusion that resistance would have to be generated using mutations induced by somaclonal variation in cell culture, or by mutagenic treatment of either cell cultures or intact seeds.

Cell culture techniques were chosen for selection of a *Medicago truncatula* genotype resistant to ALSIH because the desired genotype is the result of a simple single nucleotide substitution (e.g. Boutsalis, 1996: Guttieri *et al.*, 1992), the trait is expressed and selectable at the cellular level (e.g. Chaleff and Ray, 1984; Pofelis *et al.*, 1992), and successful cell and tissue culture techniques have been established for the species (Nolan *et al.*, 1989; Nolan and Rose, 1998; Chabaud *et al.,* 1996).

Herbicide resistant crop plants have been developed using tissue culture and cell selection techniques to regenerate resistant plantlets via somatic embryogenesis (e.g. Chaleff and Ray, 1984; Jordan and McHughen, 1987; Pofelis *et al.*, 1992). The general methodology employed is to initiate cell callus from an explant, sub-culture the callus, generate mutations through somatic variation or specific mutagenic treatment, and then select resistant cell lines with herbicides. Somatic embryogenesis is then induced and embryos are grown into plantlets and tested for stable genetic changes conferring resistance at the whole plant level.

This chapter describes the development of techniques needed to attempt this plant breeding in annual *Medicago* spp. The aim was to mutate cell callus cultures, then disperse mutated cell clumps onto growth media containing herbicides, identify and then multiply any tolerant cell colonies, induce formation of somatic embryos and regenerate tolerant plantlets. Reliable methods were required for callus initiation and sub-culture, somatic embryogenesis, regeneration, cell dispersal, cell mutation and selection with herbicides. *M. littoralis* cv. Herald and *M. truncatula* cv. Mogul were used as test genotypes because they are elite cultivars from the two most important species in southern Australia, and *M. truncatula* cv. Jemalong selection 2HA was used because it is highly regenerable (Nolan and Rose, 1998). Chlorsulfuron was used as the selection herbicide because it has been the most important ALSIH involved in field residue problems in medic growing areas, and cross-resistance to other ALSIH is frequently conferred by chlorsulfuron resistance.

6.2 MATERIALS AND METHODS

6.2.1 Callus initiation and cell culture, somatic embryogenesis and regeneration

Media. Tissue culture and regeneration media were based on those developed by Nolan and Rose (1998) for their highly regenerable *M. truncatula* cv. Jemalong line 2HA. Regeneration involves sequential culture on three media: Callus Induction Media (CIM); Embryo Development Media (EDM); and Plantlet Development Media (PDM). All media were prepared using distilled and de-ionised Milli-Q water. CIM was prepared using Gamborg's B5 basal major and minor salts (Gamborg *et al.,* 1968) as a pre-mixed powder (Sigma Chemical Company). Added to the basal salt solution were *myo*-inositol (100 mg l^{-1}), thiamine-HCl (10 mg l^{-1}), pyridoxine-HCl (1 mg l^{-1}), nicotinamide (1 mg l^{-1}), NAA (10 μM), BAP (4 μM), casein enzymatic hydrolysate (casamino acids; 250 mg l^{-1}), sucrose (30 g l^{-1}), and agar (8 g l^{-1}). The solution was adjusted to pH 5.8 using 1 M KOH, then autoclaved at 121°C for 20 min. Approximately 20 mls of each media were poured into 9 cm plastic petri dishes. EDM was the same as CIM, except that 1 μM ABA was added through a 0.2μM filter after autoclaving. PDM was the same as CIM, except that it contained no *myo*-inositol or BAP, and the NAA concentration was lowered to 1μM.

Callus initiation and cell culture of *M. littoralis* and *M. truncatula*. Callus initiation and cell culture was attempted by two methods. Firstly, the method of Nolan and Rose (1998) was used. Seeds of *M. littoralis* cv. Herald and *M. truncatula* cv. Mogul were sown in pots and grown in a glasshouse at 25±5°C for two (Herald) or six (Mogul) weeks. The spade leaf (Herald) or the youngest fully-expanded leaf (Mogul) was removed and surface sterilised for 20 min in a 1% (v/v) available chlorine NaOCl solution with 0.1% Triton detergent, then double rinsed in de-ionised water cold-filtered through a 0.2μm filter. Full-width strips, approximately 2 mm wide, were cut from the widest part of the leaflet, perpendicular to the mid-vein, and placed abaxial side down in 9 cm petri dishes containing CIM agar media. Plates were sealed with parafilm and placed in a growth cabinet at 27°C constant in the dark. Calli were cut from explants after three weeks and subcultured to fresh CIM. After 3 weeks on CIM explants were sub-cultured to EDM. Explants were taken from 28 (Mogul) or 10 (Herald) seedlings. There were five (Herald) or ten (Mogul) explants per plate, and two (Herald) or three (Mogul) replicates per explant donor. Development of cultures was recorded weekly.

The method of Zafar *et al.* (1995) relating specifically to regeneration of *M. littoralis* was adapted and applied to *M. littoralis* cvs. Herald and Harbinger. Explants were taken from aseptically grown seedlings, and plants grown in the glasshouse. Seedlings were grown from seeds sterilised for 20 min in 1.2% (v/v) NaOCl + 0.1% Triton detergent, double rinsed in sterile de-ionised water, then incubated at 20°C with 10 hr light on quarter strength Gamborg's B5 basal major and minor salts (Gamborg *et al.,* 1968) in polycarbonate growth jars. Hypocotyl (8-10 mm long) explants were taken from 10 day old seedlings, and leaf explants were taken from 11 week old plants, using the sterilisation methods described above. All explants were placed on B5DB callus initiation media (Gamborg's B5 + 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP) and incubated at 25°C with 16 hr light. There were 10 explants per plate and four replicates per explant donor. Callus was excised from explants after three weeks and sub-cultured to B5DB. After a further four weeks calli were transferred to MSN1 embryo development media (Murashige and Skoog, 1962; MS salts + 1 mg l^{-1} NAA + 1 mg l^{-1} BAP). Development was assessed weekly.

Somatic embryogenesis and regeneration of *M. truncatula* cv. Jemalong 2HA. The methods of Nolan and Rose (1998), described in detail above, were applied to their highly regenerable *M. truncatula* cv. Jemalong 2HA selection. Leaf explants were taken from three week old seedlings grown in a glasshouse. Calli were excised from explants after three weeks and sub-cultured to CIM. Four weeks later embryos were transferred to PDM in polycarbonate growth jars and transferred to 20°C with 10 hr light. After 8 weeks on PDM a plantlet was selected with 5 trifoliate leaves and well-developed roots, and was transferred to a sand/vermiculite/perlite mixture (2:1:1) in a polycarbonate jar. The plantlet was watered with quarter strength Gamborg's B5 mixture and grown at 20°C with 10 hr light. Over a two week period the jar lid was removed for increasing periods to acclimatise the plantlet and then at the 8 trifoliate leaf stage it was potted in soil in the glasshouse.

6.2.2 Cell dispersal and mutation

Suspension cultures. Calli derived from *M. truncatula* cv Jemalong 2HA leaf explants were crushed through a sterile metal sieve (1.5 mm), then 2.5 ml of the pulp was mixed with 5 ml of sterile water. A pipette was used to apply 1 ml of the suspended cell solution as drops to the surface of solidified CIM in 9 cm petri dishes, which were swirled to smear the drops.

Mutagenesis. Calli were initiated on CIM from 14 week-old *M. truncatula* cv. Jemalong 2HA leaf explants, were subcultured 4 times over 14 weeks and then used to produce surface dispersed cell cultures on CIM, as described above. During preparation of dispersed cell cultures a range of concentrations of sodium azide or ethyl methane sulphonate (EMS) were included in the suspension solution. Plates were sealed with parafilm and incubated for 3 weeks in the dark at 27 °C prior to weighing cell growth. There were three replicates per treatment.

6.2.3 Dose response of Jemalong 2HA and Herald callus to chlorsulfuron

Five c. 20 mg calli pieces of *M. truncatula* cv. Jemalong (2HA) and *M. littoralis* cv. Herald calli were placed on 9 cm petri dishes of CIM. CIM was prepared (section 6.2.1) without added casamino acids and allowed to cool to 48 °C, then 0.2μm-filtered solutions of technical grade chlorsulfuron were added prior to pouring. The final concentrations of chlorsulfuron were 0, 0.25, 0.5, 1, 2, 4 and 8 nM. There were 3 replicates per treatment and calli were harvested and weighed 3 after weeks. Casamino acids were omitted from the CIM because preliminary experiments demonstrated that *M. truncatula* cv Jemalong 2HA cells grew on 1,000 nM chlorsulfuron when the media included casamino acids (results not presented). It is likely that the casamino acids supplied isoluecine, luecine and valine to cells so that inactivation of ALS by chlorsulfuron was not critical to growth (Ray, 1984).

6.2.4 Selecting chlorsulfuron resistant cells

Production of EMS treated and non-EMS stock cell cultures. Leaf explants from *M. truncatula* cv. Jemalong 2HA plants were placed on CIM and callus was subcultured on CIM five times at 3 to 4 week intervals, following the methods in section 6.2.1. Calli were then crushed through a sieve and suspended in water or 1% EMS, as described in section 6.2.2. One ml of the cell suspension was added to each petri dish of CIM and swirled to spread the cell clumps over the agar surface. Cultures were allowed to grow for 6 weeks to allow individual mutated resistant cells to establish larger resistant cell clumps prior to selection. This was done because single resistant cells or small resistant clumps may not have grown to attain critical mass for survival (Dix, 1986; Meredith, 1984), if selection were applied immediately and supporting susceptible cells were killed.

Selection for putative resistance (2HA ne lines). Calli from the stock cell cultures were harvested and used to prepare suspensions in water, which were dispersed (section 6.2.2) onto selective CIM media with chlorsulfuron added at 5 nM (section 6.2.3), and then dishes were incubated at 27 ° C for 8 weeks. There were 50 dishes prepared using EMS-treated cell cultures, 100 dishes of untreated cell cultures, and two control dishes without herbicide for each cell culture type. After incubation, calli (2-8 mm diameter) which appeared to be growing actively on 5 nM chlorsulfuron, along with calli from control plates without herbicides, were transferred to CIM supplemented with 20 nM chlorsulfuron. Calli which continued to grow on 20 nM chlorsulfuron were subcultured to herbicide-free CIM without casamino acids for multiplication.

6.2.5 Chlorsulfuron resistance of 8 selected cell lines

Calli pieces (c. 20 mg) from eight resistant (2HA ne#1-ne#8) and a susceptible (2HA susc) line (selected as described in section 6.2.4) were placed on EIM media without added casamino acids in 9 cm petri dishes. Chlorsulfuron was filter-sterilised (0.2 µm) into the media after autoclaving at 48°C to make concentrations of 0, 8, 16, 32, 63, 125, and 250 nM. There were six calli pieces per replicate, and three replicates. The dishes were incubated in the dark at 27 ° C and fresh weight was recorded after three weeks.

6.2.6 Mechanism of chlorsulfuron resistance of 8 selected lines

Resistance to sulfometuron. Calli pieces (c. 20 mg) from eight resistant (2HA ne#1-ne#8) and a susceptible (2HA susc) line were placed on EIM media without added casamino acids in 9 cm petri dishes. Sulfometuron-methyl was filter-sterilised (0.2 µm) into the media after autoclaving at 48°C to make concentrations of 0, 2, 4, 8, 16, 32, 64 and 128 nM. There were five calli pieces per replicate, and three replicates. The dishes were incubated in the dark at 27 ° C and fresh weight was recorded after three weeks.

Malathion dose response experiment. The function of P450 cytochromes conferring metabolic resistance to chlorsulfuron is disrupted by the insecticide malathion (Christopher *et al.*, 1994). Thus, in the presence of malathion, chlorsulfuron resistance breaks down. Before this test for metabolism-based resistance could be applied to the selected resistant cell lines, a dose-response curve was needed so that an appropriate concentration of malathion could be chosen. Calli from the lines 2HA ne#2, 2HA ne#8, and the susceptible line 2HA susc were cut into small pieces (c. 20 mg) and placed on CIM media without added casamino acids in 9 cm petri dishes. Malathion was filter-sterilised (0.2 *µ*m) into the media after autoclaving at 48°C to make concentrations of 0, 12.5, 25, 50, 100, and 200 µM. There were six calli pieces per replicate, and three replicates. The dishes were incubated in the dark at 27 ° C and fresh weight was recorded after three weeks.

Effect of malathion on chlorsulfuron resistance. Calli from the 2HA ne resistant lines #1, #3, #5, #6, #7, and #8, and a susceptible line (2HA susc) were cut into small pieces (c. 20 mg) and placed on EIM media without added casamino acids in 9 cm petri dishes. Chlorsulfuron was filter-sterilised (0.2 *µ*m) into the media after autoclaving at 48°C to make concentrations of 0, 8, 63 and 125 nM. Malathion was added to one set of chlorsulfuron treatments at 50 *µ*M. There were five calli pieces per replicate, and three replicates. The dishes were incubated in the dark at 27 ° C and fresh weight was recorded after three weeks. Prior to analysis 100 mg (approximate starting weight of the six calli) was subtracted from each value so that the data reflected actual growth.

6.2.7 ALS enzyme resistance to chlorsulfuron

The method of Singh *et al.* (1988), as adapted and modified by Boutsalis (1996), was used. Callus from a susceptible line with a similar subculture history was compared to the putatively chlorsulfuron resistant *M. truncatula* cv. Jemalong 2HA lines ne #1,2,3, and 8. Three grams of fresh callus was ground with 0.1 g sand and 6 ml of pH 7.0 KH_2PO_4/ K_2HPO_4 grinding buffer comprising 100 mM KPO_4, 0.5 mM $MgCl_2$, 0.5 mM TPP, 10% glycerol, 10 mM sodium pyruvate, 10 μM FAD, 1 mM DTT, 1 mM PMSF, and 0.5% PVP, then centrifuged at 4 °C at 15,000 rpm for 10 min. The supernatent was diluted with an equal volume of saturated $(NH_4)_2SO_4$, stirred for 30 min, centrifuged at 4 °C at 15,000 rpm for 30 min. The pellet was resuspended in 0.5 ml of grinding buffer (without DTT, PMSF and PVP) and eluted through a Sephadex column with a pH 7.0 KPO_4 buffer comprising 100 mM KPO_4 , 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM TPP, and 20 μM FAD. Alliquots (50μL) of chlorsulfuron solutions, 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M were added to 50μL of the eluted solution containing ALS in plastic 96 well plates. There was also a nil chlorsulfuron treatment in which the protein was deactivated with 20μL of 6N H_2SO_4 prior to incubation. The wells were then incubated for 30 min at 37 °C in a water bath, after which time reactions were terminated with 20μL of 6N H_2SO_4, then incubated at 60 °C for 15 min to decarboxylate acetolactate to acetoin. Creatine (95μL of 0.55% w/v in water) then α-naphthol (95μL 5.5% w/v in 5N NaOH) were added to each well, incubated for a further 15 min at 60 °C to develop colour, and read with an automatic plate-reading spectrometer at 530nm. Wherever practical, materials and solutions were cooled on ice throughout the assay. ALS activity (μmol acetolactate mg protein^{-1} h^{-1}) was determined from an acetoin standard curve. Total protein was determined using the method of Bradford (1976).

6.3 RESULTS

6.3.1 Callus initiation and cell culture of M. littoralis and M. truncatula

Using the method of Zafar *et al.* (1995), cv. Herald and cv. Harbinger hypocotyl explants had produced healthy callus growth after 14 days. Leaf explants also produced calli after three weeks, however embryo and shoot formation on all explants was poor. Using the method of Nolan and Rose (1998), Mogul and Herald explants readily formed callus on CIM. Leaf explants from three week old *M. truncatula* cv. Jemalong 2HA seedlings placed on CIM produced embryos within five weeks of plating and by six weeks there were many advanced embryos and shoots up to 6 mm long. Embryos transferred to jars on PDM in the light continued to develop and by 12 weeks there were shoots with up to four trifoliate leaves and some well developed root systems. After 16 weeks from explant plating one of these shoots with roots was successfully transferred to a sand/vermiculite/pearlite substrate and continued to grow. The seedling was then transferred to soil in a pot and grew to flower and set seed. Leaf explants, calli, somatic embryos and plantlets of *M. truncatula* cv. Jemalong 2HA are shown in Plates 6.1 and 6.3.

6.3.2 Mutagenesis of cells

Cell clump size, determined by sieve size, and cell dilution were probably the most important variables in successful dispersion of cells. Suspension of cells in too much water led to over-wetting of the agar surface, thus reducing growth rate through lack of aeration. Both sodium azide and EMS greatly reduced cell growth within the concentration range tested. Sodium azide was more toxic than EMS at lower concentrations, causing 50% suppression of cell growth at less than 0.125%. EMS suppressed growth by 50% at around 1% (Figure 6.1). EMS at 1% v/v was chosen as a concentration likely to suppress growth by around 50%. At this concentration the mutation frequency was expected to be high, while still allowing a useful rate of cell growth.

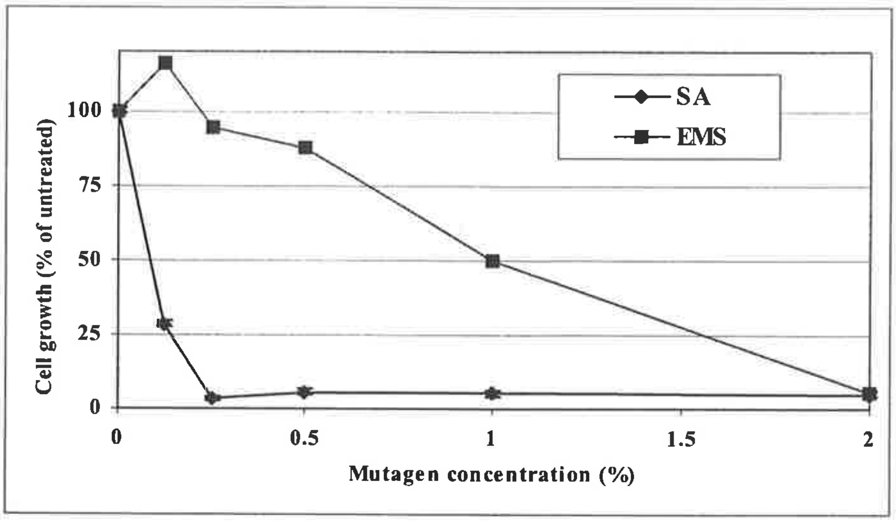


Fig. 6.1. Effect of sodium azide (SA) and EMS on *M. truncatula* cv. Jemalong 2HA dispersed cell clump growth (total fresh calli weight).

6.3.3 Dose response of Jemalong 2HA and Herald callus to chlorsulfuron

Sensitivity of *M. truncatula* and *M. littoralis* cell cultures to chlorsulfuron were very similar (Fig. 6.2). Cell growth was completely inhibited at 1 nM and the I_{50} was c. 0.25 nM The callus weight (15-25% of untreated) recorded at concentrations of >1 nM represents the weight of the original starting callus pieces, not growth. A concentration of 5 nM chlorsulfuron was chosen as a standard selection pressure for subsequent selection experiments. This concentration killed susceptible cells, but allowed relatively prolonged death of affected susceptible cells so that they could provide a suitable environment for division and growth of single mutant cells or small cell clumps.

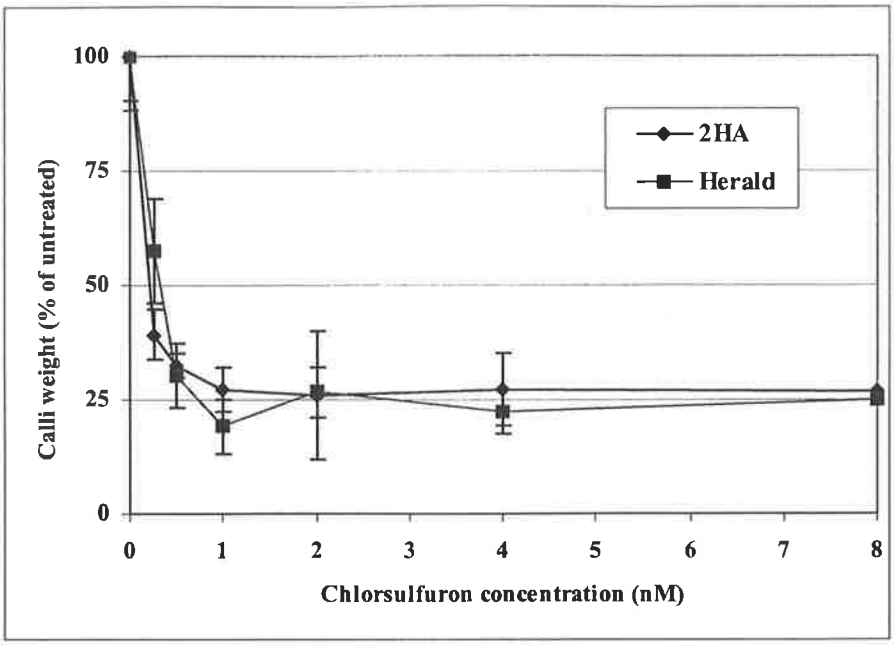


Fig 6.2. Effect of chlorsulfuron on *M. truncatula* cv. Jemalong 2HA and *M. littoralis* cv. Herald cell growth (total fresh calli weight) in experiment two.

6.3.4 Selecting chlorsulfuron resistant cells

Media supplemented with 5 nM chlorsulfuron severely restricted the growth of dispersed cell cultures of *M. truncatula* cv. Jemalong 2HA. Non-destructive visual estimates of growth for non-EMS treated cultures were 40% surface coverage on herbicide-free media, and 2% coverage on media with 5 nM chlorsulfuron. Corresponding estimated coverage of EMS-treated cultures was 30% and 4%. Calli selection from 5 nM chlorsulfuron dishes and subsequent survival on 20 nM chlorsulfuron is summarised in Table 6.1. None of the 99 putatively resistant colonies from non-EMS stock survived on 20 nM chlorsulfuron, whereas 8 of the 117 from EMS-treated stock continued to grow. This is strong evidence that EMS caused mutations conferring resistance to chlorsulfuron. None of the 37 colonies grown on herbicide-free media and selected at random from each cell type survived on 20 nM chlorsulfuron. None of the calli grown from selected lines produced embryos. Calli were subsequently placed on a wide range of agar-based plant growth regulator treatments (results not presented) but no embryos were produced, thus preventing regeneration of resistant plantlets.

Table 6.1. Number of *M. truncatula* cv. Jemalong 2HA calli selected from plates supplemented with 5 nM chlorsulfuron (CS), and subsequent growth on 20 nM chlorsulfuron.

Cell culture	Calli selected from		Survivors on 20 nM CS from	
	5 nM CS	Control	5 nM CS	Control
Non-EMS	99	37	0	0
EMS-treated	117	37	8	0

Cell colonies with putative resistance to 5 nM chlorsulfuron are shown in Plate 6.2.

6.3.5 Chlorsulfuron resistance of 8 selected cell lines

Cell lines with high levels of chlorsulfuron resistance were produced by mutation with EMS. The results confirmed that the resistance was stable through repeated sub-culturing, and that growth of the susceptible line was inhibited at a low concentration (Figs. 6.3 and 6.4). There appeared to be three distinct groupings for resistance (Figs. 6.3 and 6.4). The susceptible control line and lines #2 and #3 had no or very little resistance, while lines #1, #3, #6, and #7 had intermediate resistance. Lines #5 and #8 had high levels of resistance, with #8 maintaining 70% growth (cf. untreated) at 250 nM. The I_{50} concentrations ranged from 0.2 to >250 nM, with resistance ratios from 1 to >1,250 (Table 6.2).

Plate 6.1. Fresh leaflet explants (right) and dedifferentiating leaflet explants (left: 2 weeks old) from *Medicago truncatula* cv. Jemalong line 2HA.

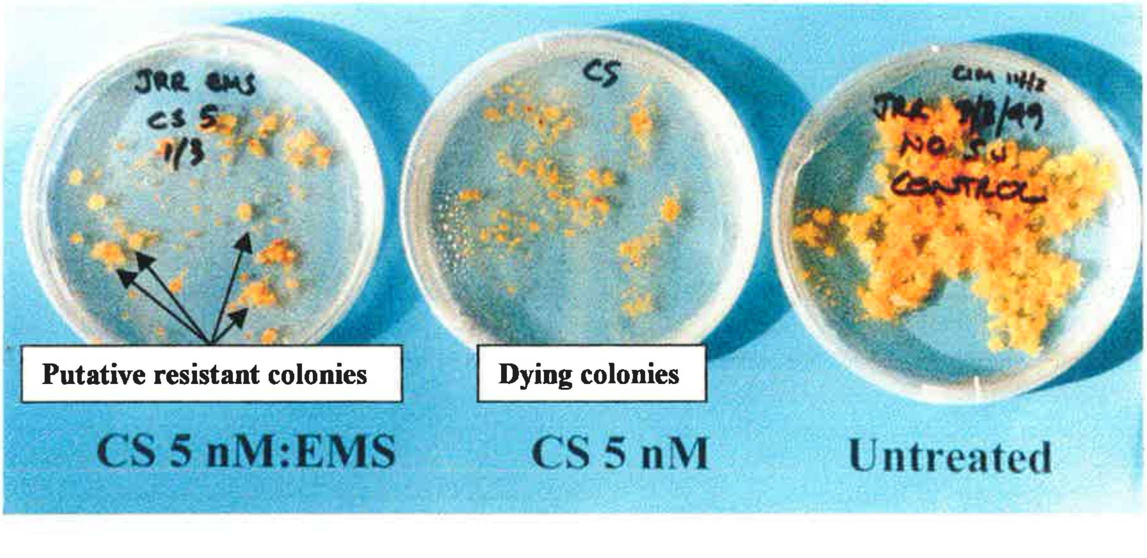
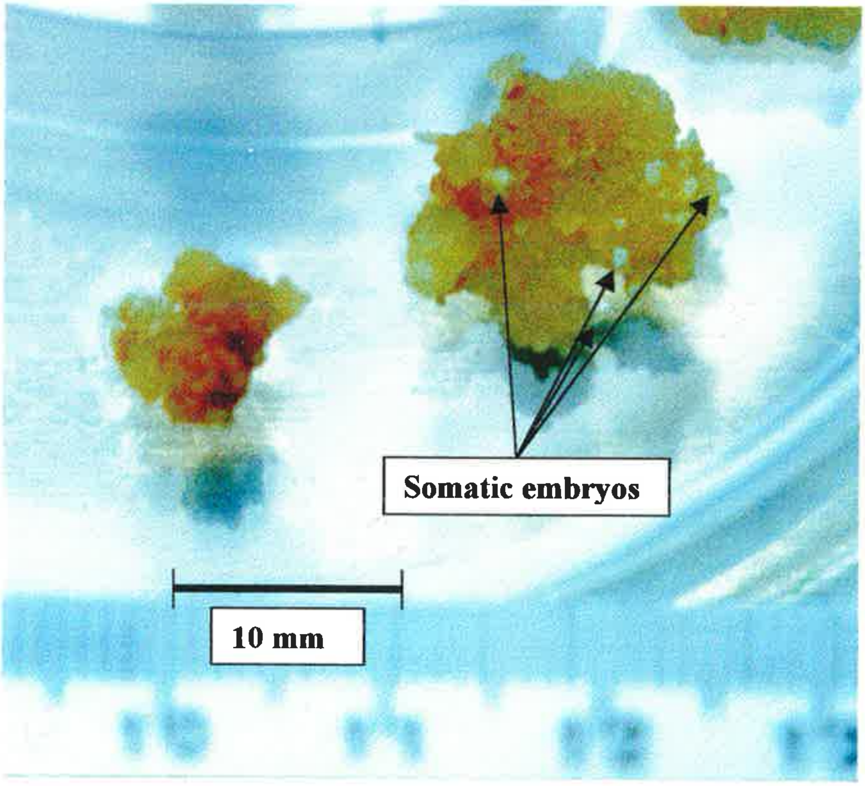


Plate 6.2. Dispersed cell cultures derived from *Medicago truncatula* cv. Jemalong line 2HA calli grown with EMS (left) or without EMS (centre) and placed on selective media containing 5 nM chlorsulfuron. The cells on the right are from non-EMS calli and are growing on herbicide-free media.



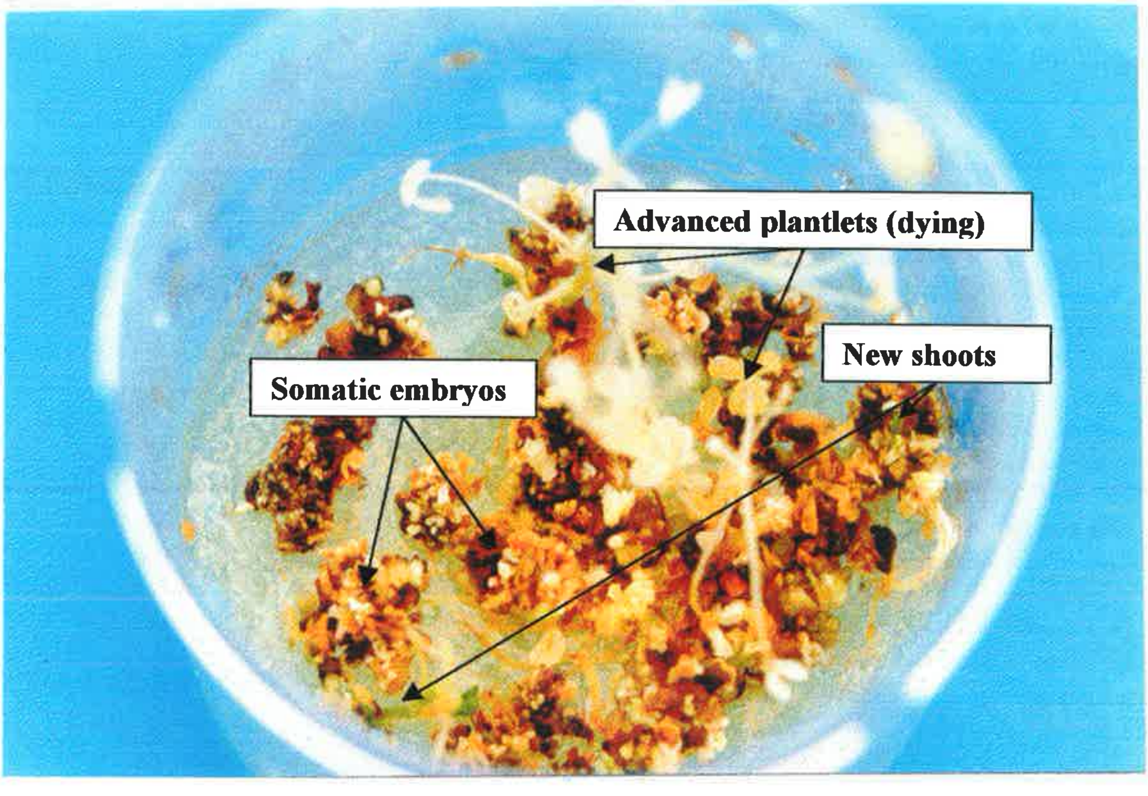


Plate 6.3. *Medicago truncatula* cv. Jemalong line 2HA: Somatic embryos growing from callus derived from leaflet explants (top) and shoots developing from somatic embryos on exhausted calli.

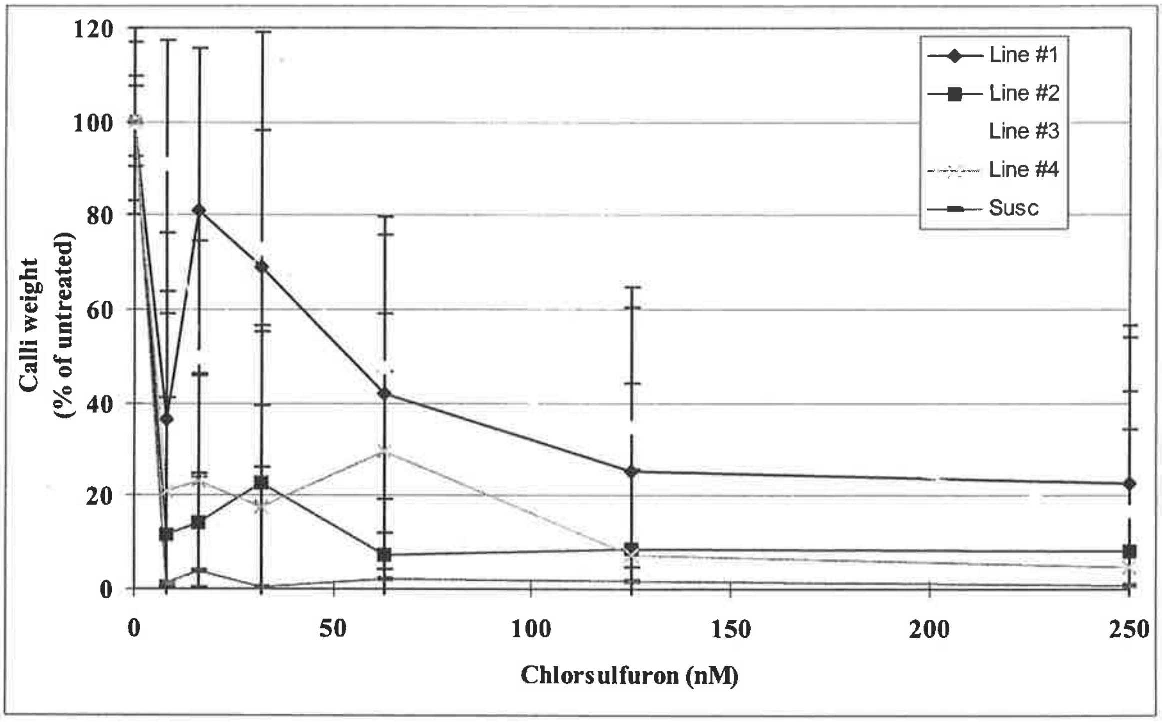


Figure 6.3. Calli weight (% of untreated) for chlorsulfuron resistant cv. Jemalong lines #1 to #4 and susceptible when grown with chlorsulfuron.

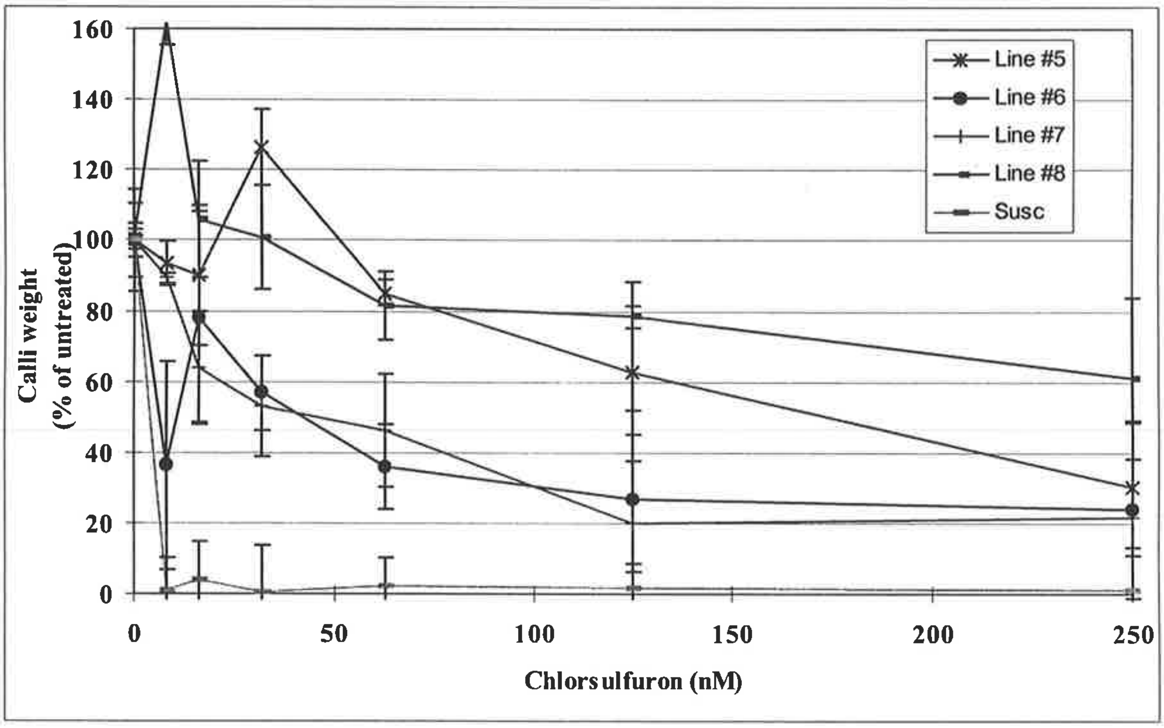


Figure 6.4. Calli weight (% of untreated) for chlorsulfuron resistant cv. Jemalong lines #5 to #8 and susceptible when grown with chlorsulfuron.

6.3.6 Mechanism of chlorsulfuron resistance of 8 selected lines of 8 selected lines

Resistance to sulfometuron-methyl. In the few cases of metabolic resistance to chlorsulfuron described (e.g. Christopher *et al.*, 1992) it has been found that plants with the P450 cytochrome conferring the resistance are susceptible to sulfometuron-methyl. In contrast, many of the mutated target site based resistant lines have mutated ALS, often with cross-resistance to sulfometuron-methyl (Devine and Eberlein, 1997). Thus, with lines resistant to chlorsulfuron, cross-resistance to sulfometuron-methyl is evidence in support of a mutated ALS target site, while susceptibility to sulfometuron-methyl suggests that metabolic resistance may be involved. Sulfometuron-methyl concentrations for I_{50} ranged from 1 to 100 nM (Table 6.2). All lines resistant to chlorsulfuron were also cross-resistant to sulfometuron-methyl, providing evidence against the mechanism being enhanced metabolism. The level of sulfometuron-methyl resistance (Figs. 6.5 and 6.6) was generally lower than for chlorsulfuron (Table 6.2). Lines #2 and #4 had little or no resistance to either chlorsulfuron or sulfometuron-methyl. Lines #5 and #8 were most resistant to sulfometuron-methyl. These lines were also the most resistant to chlorsulfuron.

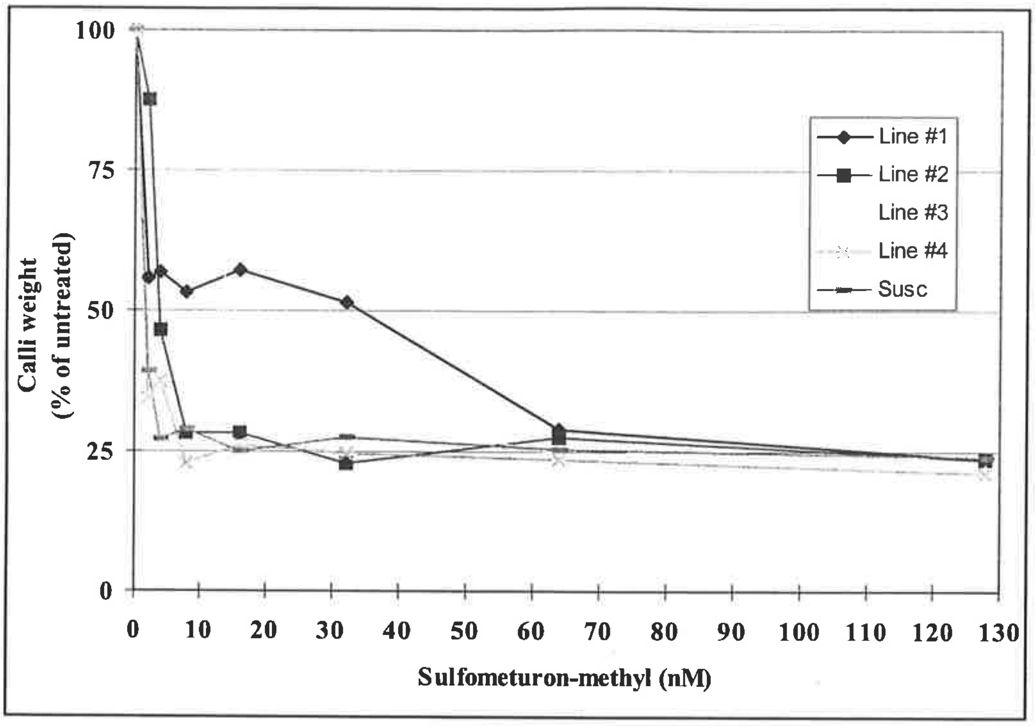


Figure 6.5. Calli weight (% of untreated) for chlorsulfuron resistant cv. Jemalong lines #1 to #4 and susceptible when grown with sulfometuron-methyl.

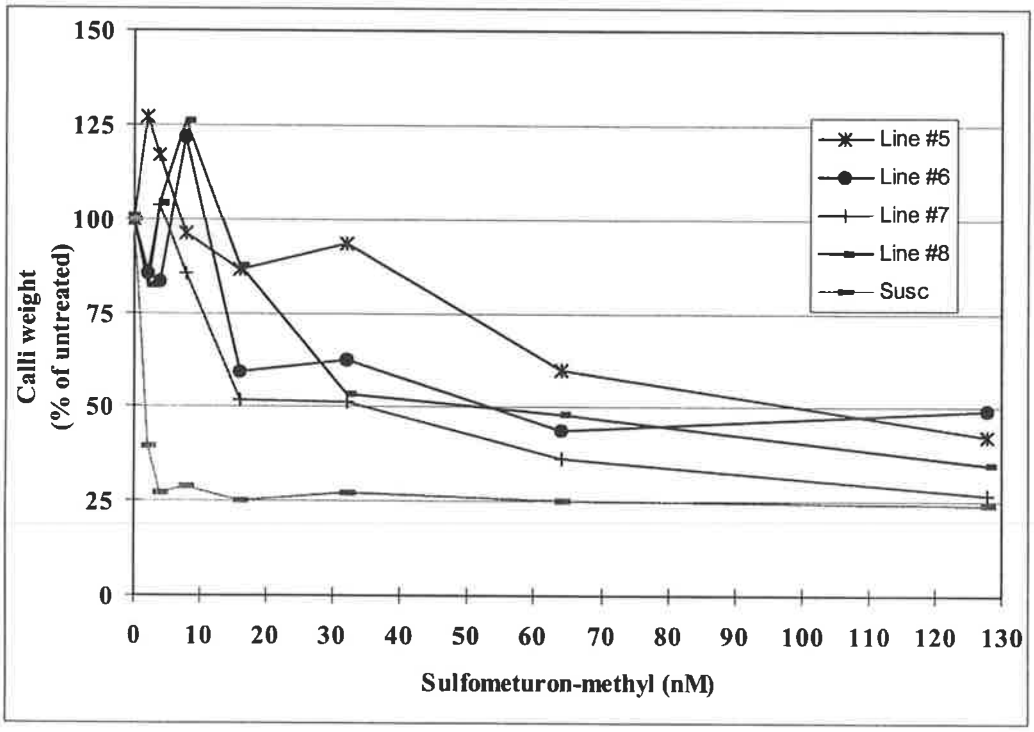


Figure 6.6. Calli weight (% of untreated) for chlorsulfuron resistant cv. Jemalong lines #5 to #8 and susceptible when grown with sulfometuron-methyl.

Table 6.2. Approximate concentrations of the herbicides chlorsulfuron (CSF) and sulfometuron-methyl (SMT) inhibiting chlorsulfuron resistant selections of *M. truncatula* cv. Jemalong 2HA calli growth by 50% (I_{50}), and resistance ratios (RR).

Line #	I_{50}		RR*	
	CSF	SMT	CSF	SMT
1	65	35	325	35
2	<<5	3	<<25	3
3	80	15	400	15
4	<<5	1	<<25	1
5	210	100	1050	100
6	60	55	300	55
7	80	35	400	35
8	>250	55	1250	55
Susceptible	0.2	1	1	1

* Resistance ratio based on I_{50} of 0.2 nM for chlorsulfuron (Fig 6.2) and 1 nM for sulfometuron-methyl for the susceptible line (Figs 6.5 and 6.6).

Malathion dose response experiment. Malathion inhibited both cell lines equally (Fig.6.7), and the I_{50} was c. 115 to 165μM. The 50μM concentration reduced growth by 15-20%, and was chosen for use with chlorsulfuron dose-response experiments because it allowed relatively strong calli growth.

Effect of malathion on chlorsulfuron resistance. Cell growth was completely inhibited in the susceptible control line between 0 and 8 nM chlorsulfuron (Fig. 6.14), and lines which had chlorsulfuron resistance based on enhanced metabolism would be expected to have a curve similar to this when malathion was present. Generally, growth of lines in the presence of both chlorsulfuron and malathion was similar to growth in the presence of chlorsulfuron alone (Figs. 6.8 to 6.13). The similar dose-response curve shapes for the six lines with and without malathion is further evidence against resistance being based on enhanced metabolism.

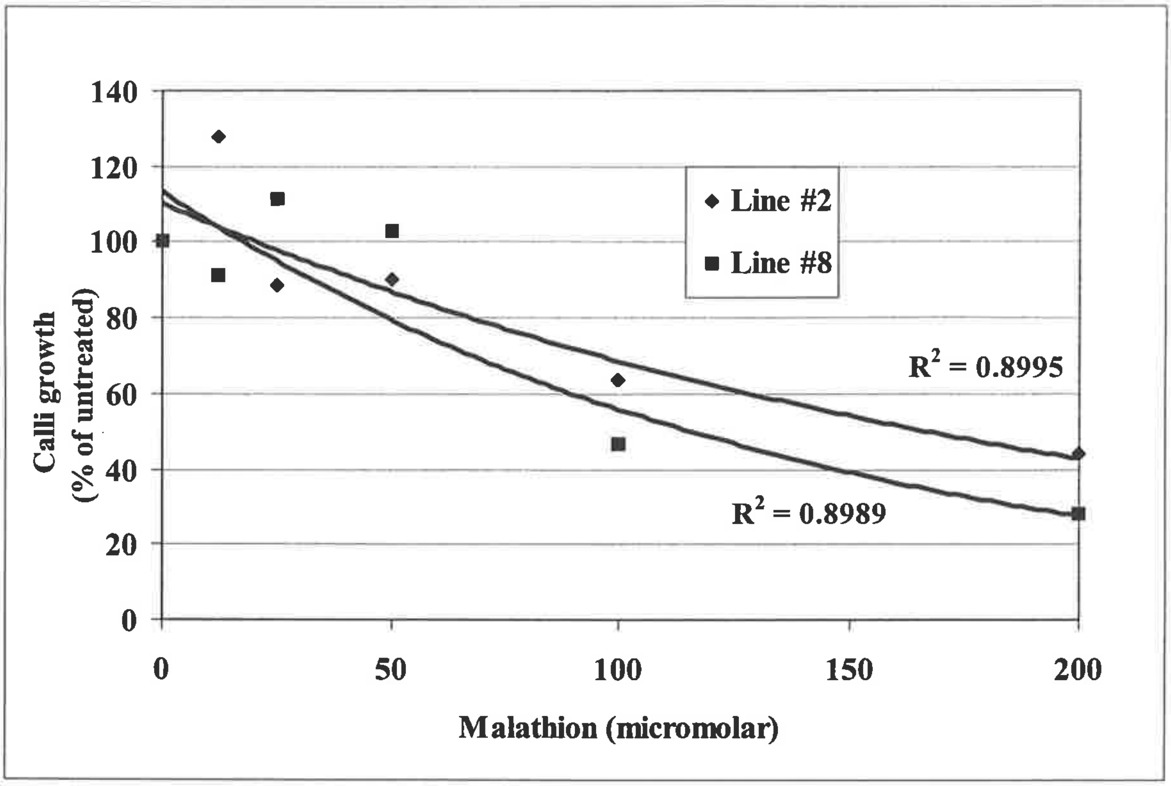
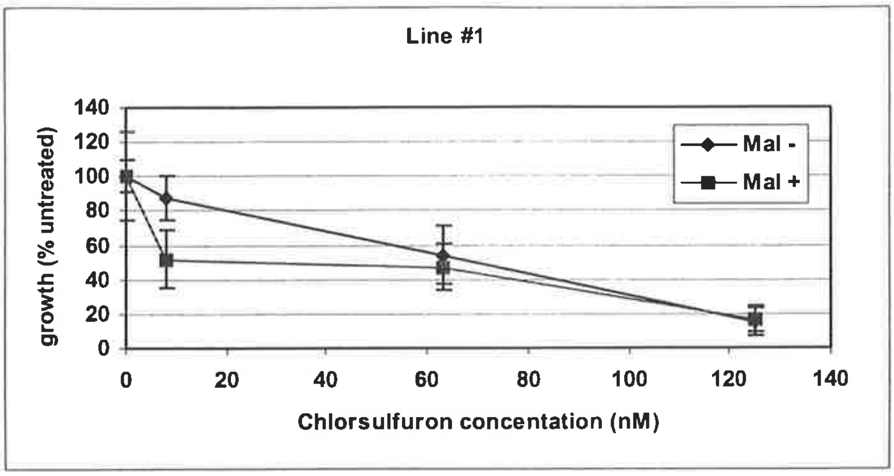
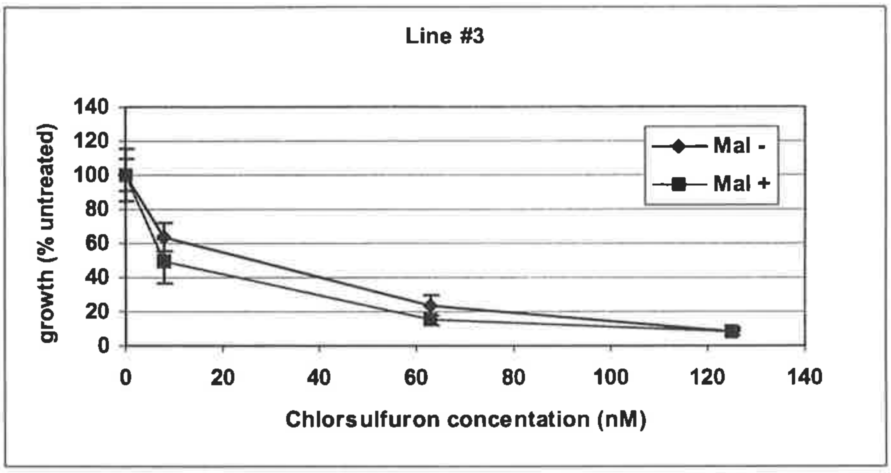
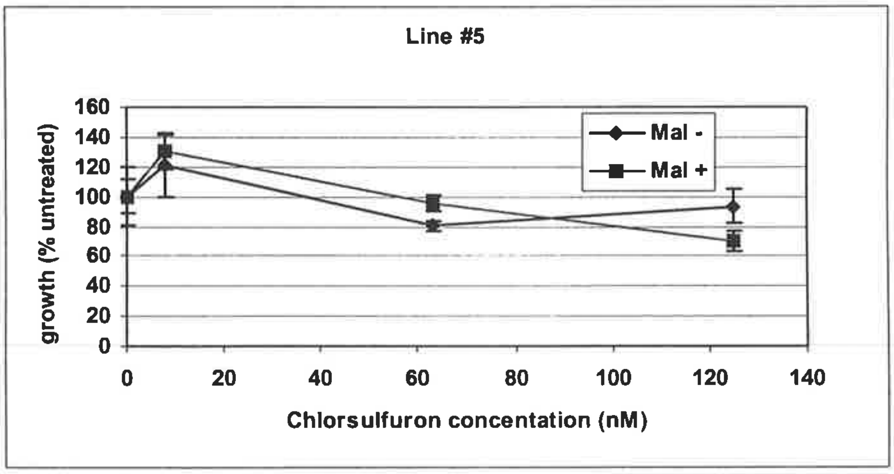


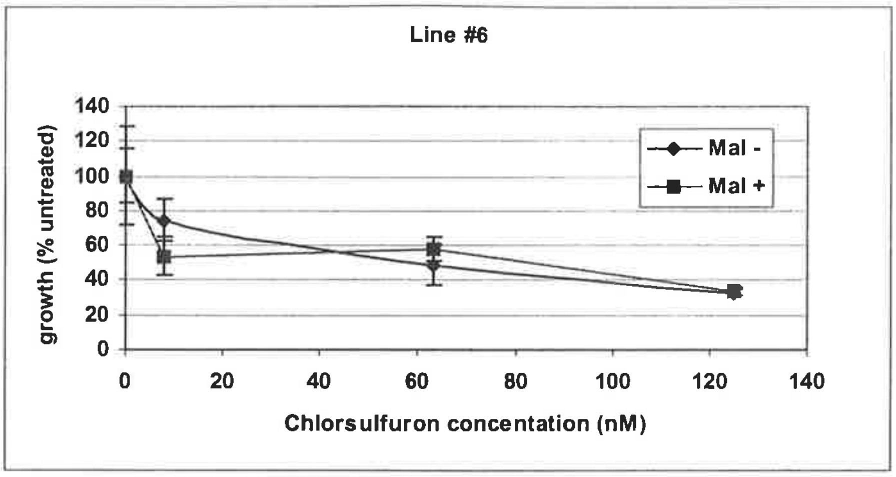
Figure 6.7. Effect of malathion concentration on callus growth (% of untreated calli weight) of lines Jemalong 2HA lines ne #2 and #8.

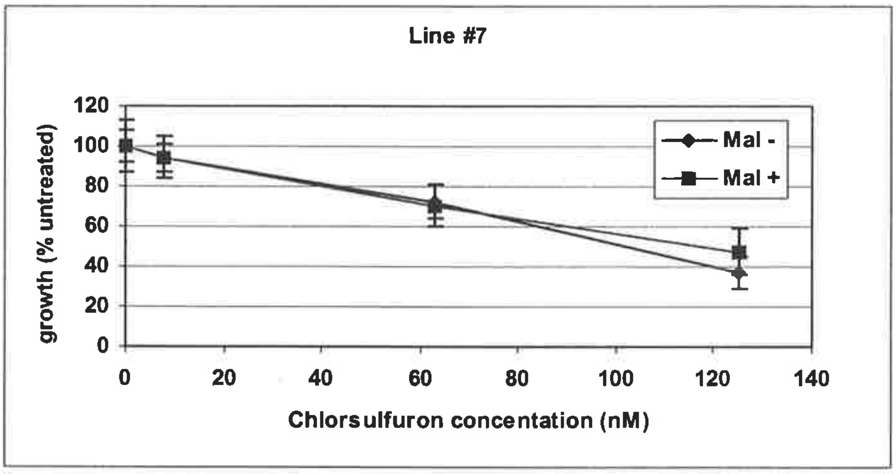


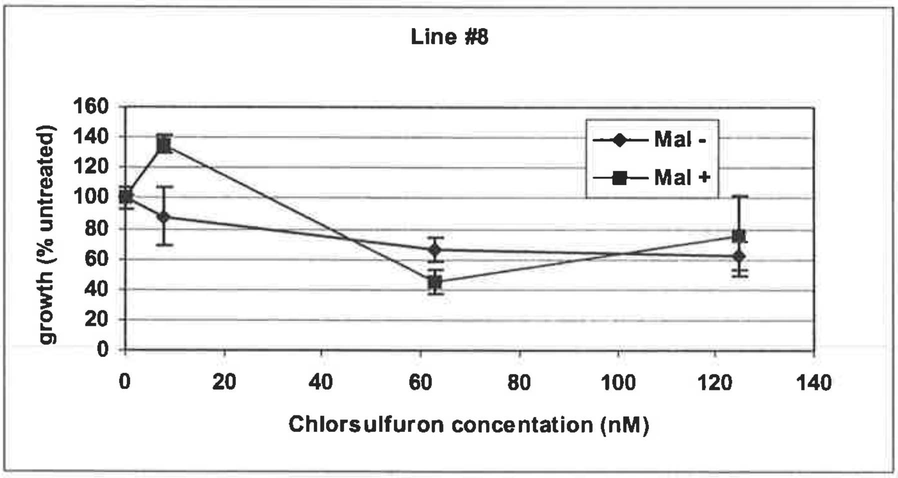




Figs. 6.8 to 6.10. Callus growth (% of untreated calli weight) of *M. truncatula*.cv. Jemalong 2HA ne lines #1, #3 and #5 with and without 50µM malathion after three weeks.







Figs. 6.11 to 6.13. Callus growth (% of untreated calli weight) of *M. truncatula*.cv. Jemalong 2HA ne lines #6, #7 and #8 with and without 50μM malathion after three weeks.

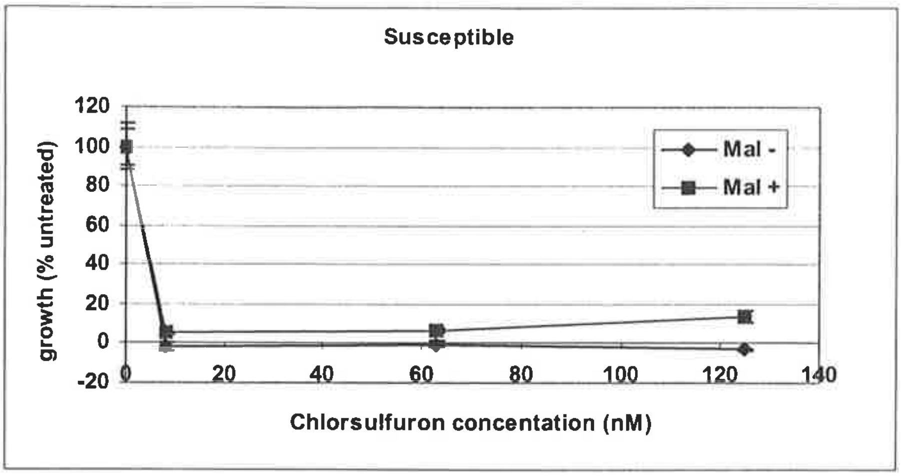


Fig 6.14. Callus growth (% of untreated calli weight) of *M. truncatula* cv. Jemalong 2HA ne susceptible line with and without 50μM malathion after three weeks.

6.3.7 ALS enzyme resistance to chlorsulfuron

Activity of ALS, extracted from calli and assayed *in vitro,* declined with increasing chlorsulfuron concentration (Fig. 6.15). Line #8 was more resistant at the enzyme level than the susceptible control line, but lines #1, #2, and #3 were more susceptible. Line #8 was highly resistant (1,250-fold) at the cellular level (Fig. 6.4), and appears to have a mutated ALS. Line #2 was susceptible at both the cellular (Fig. 6.3) and enzyme level (Fig. 6.15). Lines #1 and #3 were moderately resistant at the cellular level (Fig. 6.3), but very susceptible at the enzyme level (Fig. 6.15), suggesting resistance based on a mechanism other than mutated ALS. The ALS activity, relative to the susceptible control (R:S ratio), was between 0.75 and 1.09 (Table 6.3). This is strong evidence that none of the lines had been selected on the basis of gene amplification.

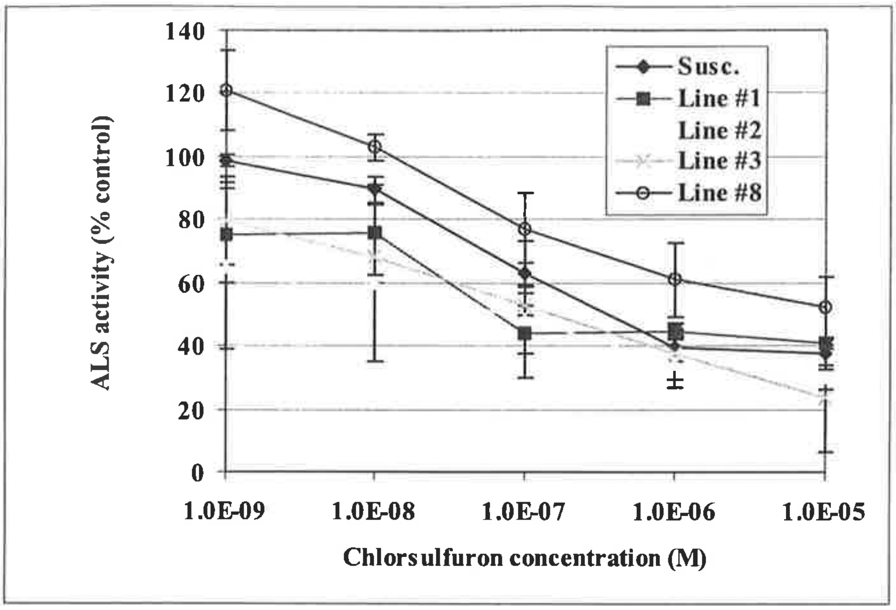


Fig 6.15. Effect of chlorsulfuron on ALS activity (% of untreated) from selected cell calli lines of *M. truncatula* cv. Jemalong 2HA.

Table 6.3. ALS relative activity (nmol acetolactate mg protein^{-1} h^{-1}: ratio of R:S) in Jemalong 2HA ne lines #1, #2, #3, #8.

Line	#1	#2	#3	#8
Mean	1.09	1.17	0.75	0.95
(s.e.)	(0.02)	(0.32)	(0.27)	(0.09)

6.4 DISCUSSION

These experiments demonstrate that *Medicago* cell lines with resistance to ALSIH can be readily generated using mutagens and tissue culture techniques. Some were highly resistant, but loss of embryonic competence prevented regeneration of resistant plantlets. Cell mutation and selection have great potential for breeding non-transgenic herbicide resistant crops, which are not subject to GMO regulations, provided that somatic embryogenesis and regeneration can be achieved. Successful development of a surface dispersal technique for suspended cell clumps was an important step towards mutation of cells and selection of resistant colonies. The technique developed was relatively simple and rapid, and allowed research to proceed to evaluation and selection of mutation treatments. EMS was chosen as the preferred mutagen because it had the least sensitive dose-response curve (Fig. 6.1), and was judged to be the least hazardous of the two exceptionally dangerous compounds (Kamra and Brunner, 1970). EMS is also the most frequently-used chemical mutagen (Dix, 1986), and produces higher proportions of base substitutions or deletions than chromosomal aberrations (Haughn and Sommerville, 1987).

M. truncatula and *M. littoralis* cells were sensitive to very low concentrations of chlorsulfuron (< 1nM), reflecting the similar sensitivity of whole plants to low concentrations of chlorsulfuron soil residues. Chaleff and Ray (1984) selected tobacco cells with 5.6 nM chlorsulfuron and sulfometuron-methyl, and Jordan and McHughen (1987) used 100 to 200 nM chlorsulfuron for flax cells. An experiment with selection concentration (results not presented) showed that the number of putatively resistant *Medicago* colonies selected declined with increasing concentration of chlorsulfuron from 5 to 1,000 nM.

It is significant that resistant variants were only recovered from EMS-treated cells. Growth was more vigorous in non-EMS cells and allowed a number of colonies to persist on 5 nM chlorsulfuron and to be selected as putative variants, but all 116 of these colonies were subsequently killed by 20 nM chlorsulfuron. Although the overall vigour of EMS-treated cells was lower, there were 39 colonies selected as putative variants, of which 37 were resistant to 20 nM chlorsulfuron. This suggests that it is unlikely that chlorsulfuron resistant variants could be selected relying solely on somaclonal variation in *M. truncatula,* and that routine production of variants with chlorsulfuron resistance is dependent on induced mutation. Harms and DiMaio (1991) reported a 30-fold increase in mutations conferring primsulfuron resistance in tobacco cells following mutation with *N*-ethyl-*N*-nitroso-urea. The absence of resistant variants amongst the array of somaclonal variants almost certainly present (Larkin and Scowcroft, 1981) is further evidence that spontaneous mutations conferring ALS resistance in annual medics are rare. Attempts to identify spontaneous mutations also failed in field selection and mass screening experiments (Chapter 5).

Apparent resistance in selected cell lines can be unstable and does not always produce resistant plantlets following regeneration of embryos (MacLean and Grant, 1987). Susceptible cells can survive on selective media by cross-feeding from surrounding resistant cells through plasmodesmatal connections or incomplete translocation of herbicides to all cells. When the selective pressure is removed it is possible for susceptible cells to grow into large sectors in the putatively resistant callus (Dix, 1986). This may have occurred in lines 2HA ne #2 and #4, which survived on 20 nM chlorsulfuron shortly after selection, but later appeared to have little or no resistance (Table 6.2). Resistance in the other six selected and tested lines remained stable through many sub-cultures, suggesting that remnant susceptible cells had been eliminated from these cultures.

The known mechanisms for resistance to chlorsulfuron are an altered ALS target site (Saari, *et al.,* 1994), gene amplification (Caretto *et al.*, 1994), and enhanced metabolism (Devine and Eberlein, 1997). The range of I_{50} chlorsulfuron concentrations found in the six resistant lines tested (Table 6.2) suggests that there were a number of different mutations induced by EMS. EMS most commonly produces point mutations through single base substitution or deletion, rather than chromosomal aberrations (Nilan, 1981), and so it was thought most likely that the selected lines had resistance based on single base mutations coding for a mutant ALS. Experiments on the mechanism of resistance provided very strong evidence against resistance based on enhanced metabolism, because the six tested lines had cross-resistance to sulfometuron-methyl (Table 6.2; Figs 6.5 and 6.6). In the few cases of chlorsulfuron resistance based on enhanced metabolism, so far all have been susceptible to sulfometuron-methyl (Christopher *et al.*, 1992), whereas in the many cases of chlorsulfuron based on altered ALS, cross-resistance to sulfometuron-methyl is common (Devine and Eberlein, 1997). In addition, metabolic resistance to chlorsulfuron is usually greatly reduced in the presence of malathion (Christopher *et al.*, 1994), but this was not the case for any of the six resistant lines tested (Figs. 6.8 to 6.13). The ALS activity in the presence of a range of chlorsulfuron concentrations was assayed in four selected lines to test for target site mutation. Relative ALS activity in the absence of chlorsulfuron was similar for all lines (Table 6.3), providing strong evidence against gene amplification. Line 2HA ne #2 was susceptible at both the enzyme and cellular level. Line 2HA ne #8 was only c. 20-fold resistant at the enzyme level, but > 1,250-fold resistant at the cellular level. Similarly, lines 2HA ne # 1 and #3 were moderately resistant at the cellular level (65-fold and 80-fold, respectively), but more sensitive than the susceptible line at the enzyme level. This suggests that either the ALS assay technique was inaccurate, or that two mechanisms of resistance were present in line 2HA ne #8, and resistance in lines 2HA ne #1 and #3 is based on a mechanism other than mutated ALS. Given the low probability for selecting two independent mutations in the same cell line, it is more probable that the ALS assay methods, developed for use with leaf tissue, are not reliable for cell callus material. The resistance mechanism is expected to be an altered target site because there is indirect evidence against gene amplification and enhanced metabolism, the only other two known alternatives to an altered target site. Given the ambiguity of the ALS assay results, the mechanism for resistance in the six selected lines tested remains unresolved.

Mutation and selection techniques produced 31 variant lines with resistance to chlorsulfuron, however none of these produced somatic embryos. This was a critical limitation to production of a resistant plant line because somatic embryogenesis is required as a first step for regeneration of plantlets. Induction of somatic embryogenesis is a very complex science (Dodeman *et al.*, 1997) and different species, and sometimes genotypes within species, require different and very specific protocols. Optimum protocols for annual *Medicago* spp. vary widely, and sometimes appear contradictory. Somatic embryogenesis for *M. truncatula* in particular is highly genotype-specific (Chabaud *et* al, 1996; Hoffmann *et al.*, 1997; Nolan *et al.*, 1989). Embryonic competence was reported to decline rapidly in *Medicago sativa* during repeated sub-culturing (Piccioni *et al.*, 1996), and was influenced by exogenous auxins and cytokinins, and the concentration and nature of reduced N in the regeneration medium (Bingham *et al.*, 1988). In this study, sieved and dispersed calli of *M. truncatula* ceased embryo production 5.5 months after callus initiation, whereas undispersed calli produced embryos for 10 months through successive sub-cultures (results not presented). This suggests that the protocol, rather than the age of the culture, was responsible for loss of embryogenic competence.

CHAPTER 7

FIELD SELECTION OF A RESISTANT GENOTYPE USING SEED MUTATION

7.1 INTRODUCTION

Screening of existing genotypes, field selections, and mass screening (Chapters 4 and 5) failed to identify any useful tolerance to sulfonylurea herbicides. Experiments were therefore undertaken to use somaclonal variation and mutagenesis to select resistant genotypes in tissue culture (Chapter 6). At the same time parallel experiments began using EMS to mutate seeds in an attempt to select for resistance at the whole plant level. This chapter describes EMS mutation of *Medicago* seed, selection of a *M. littoralis* genotype with chlorsulfuron resistance, determination of the pattern of cross-resistance, and evaluation of growth of the selected line in soil residues of chlorsulfuron and triasulfuron.

Mutation of seed has been used in numerous plant breeding projects (e.g. Chaleff and Ray, 1984; Harms and DiMaio, 1991) to induce mutations which could not be identified in existing genotypes. EMS was chosen as the mutation agent for *Medicago* spp. in this study because it often produces single base changes, rather than gross chromosomal changes (Nilan, 1981), and because it is relatively safe and convenient to use compared to irradiation or some other chemical mutatgens. Mutagenised seed must be grown through one generation of selfing (M_1) prior to selection to avoid the complication of chimeric variation (Halloran *et al.*, 1979), and to expose recessive mutations in the M_1. Seeds of *M. truncatula* cv. Mogul and *M. littoralis* cv. Herald were mutagenised, and then grown for one season in the field. Seed from these field-grown plants was then sown in the following season and selected for resistance to chlorsulfuron.

Following selection of cv. Herald and cv. Mogul plants with putative resistance to chlorsulfuron it was important to collect seed and test for resistance to determine whether the survivors were resistant or had just escaped exposure to chlorsulfuron. Initially two soil bioassays were conducted using a range of chlorsulfuron and triasulfuron concentrations. When sufficient seeds were available, detailed dose-response experiments using ALSIH were conducted. A field site was also established to compare the growth of putative resistant lines and the susceptible parent in soil containing residues of chlorsulfuron and triasulfuron.

7.2. MATERIALS AND METHODS

7.2.1 Seed mutagenesis and selection for resistance

EMS mutation of *Medicago* seed. Literature on EMS mutation of seeds of other species (e.g. Konzak, 1970; Rao *et al.*, 1993) was used to design a preliminary experiment to determine an appropriate concentration and duration of exposure for *Medicago truncatula* cv. Mogul. This experiment (data not presented) suggested that exposure to 0.75% EMS for 30 min at 20°C would cause damage to seeds, but still allow an adequate level of seedling survival. One kg each of scarified *M. truncatula* cv. Mogul and *M. littoralis* cv. Herald seed was soaked for 4 hr in 3L of de-ionised water at 10°C, and stirred for 15 s every 30 min. Seeds were then drained, rinsed, and soaked in 3 L of 0.75% EMS (v/v) for 30 min at 20°C. Seeds were then rinsed for one min in running tap water, soaked for 20 min in tap water, rinsed again in running tap water for one min then dried at 20°C for 20 hr. *Rhizobium* inoculatant (100 g kg^{-1}) was mixed with the seed prior to drying.

Production of M_2 seed. Mutated seed (M_1) was hand-scattered over field plots on 7/7/98, then incorporated into the soil with light harrows. Each cultivar was sown into 30 x 15 m plots of loam soil (pH 5.2:water) at Struan, South Australia. Based on thousand seed weights, 471,000 cv. Herald and 279,000 cv. Mogul seeds were sown. Haloxyfop at 42 g a.i. ha^{-1} was applied over all plots for grass control on 18/8/98. Emergence was measured for both cultivars at the first trifoliate leaf stage. Seed pods were harvested from the field in February, 1999.

EMS-treated and untreated seeds of both cultivars were also sown into a sandy-loam soil in 20 cm plastic pots in a glasshouse at 15°C to test the effect of EMS on establishment. There were 50 seeds per treatment, with two replicates. Plants with a trifoliate leaf were counted four weeks after sowing.

Selection of plants grown from M_2 seed. A site at Struan with a loam soil (pH 5.2: water) was cultivated and harrowed, then on 27/6/99 chlorsulfuron was applied PSI at 15 g a.i.ha^{-1}. Two half doses were applied at 90°, and were incorporated by harrows in two directions to ensure even herbicide distribution. Eight untreated control areas (90 x 40 cm) for each cultivar were masked to exclude herbicide prior to spraying. Scarified M_2 seed produced from M_1 plants in 1998 (12 kg Mogul; 22 kg Herald) was spread by hand onto 28 x 36 m plots on 28/6/99.

There were about 3,438,000 cv. Mogul seeds and 11,526,000 cv. Herald seeds sown. By the end of August there were more surviving plants than could be evaluated. Many of these appeared to be escapes, indicated by a clustered pattern of survival. On 31/8/99 a second application of chlorsulfuron was made POST at 15 g a.i. ha^{-1} (0.2% v/v BS1000 non-ionic wetting agent) when the seedlings were 20 to 50 mm in diameter. Five weeks after the second chlorsulfuron application surviving M_2 plants were dug from the field and transferred to pots of soil in a glasshouse. These transplants were grown to maturity and seed pods (M_3 seed) were collected from each plant and stored in the dark at 37°C for 3 weeks to break dormancy.

7.2.2 FEH-1 herbicide resistance

Resistance of selected plants to chlorsulfuron in tissue culture. Shortly after transfer to the glasshouse, surviving selected and unsprayed plants were used as donors for leaf explants to initiate tissue culture of cell calli, using the techniques described in section 6.2.1. This was done so that resistance could be tested without waiting several months for mature seed to form on selected plants. Callus growth from cv. Mogul selections was very poor, so only a cv. Herald putative chlorsulfuron resistant selection, FEH-1, was able to be tested. Petri dishes of EIM media without casamino acids was prepared with chlorsulfuron added at 0, 5 and 20 nM as described in section 6.2.3. Five calli pieces (c. 20 mg) from FEH-1 and a cv. Herald control plant (FEH-C) were used per treatment, with three replicates. The dishes were incubated in the dark at 27 °C and fresh weight of calli was recorded after three weeks.

Resistance to chlorsulfuron and triasulfuron in soil. Once seed of the putative chlorsulfuron resistant selection FEH-1 was available, germinated seeds of *M. littoralis* cv. Herald and FEH-1 were sown as described for the pot bioassay (section 4.2.1). Soil used in the bioassay contained 0, 0.25, 0.5, 1, 2, 4, 8 or 16 ppb chlorsulfuron and 0, 0.063, 0.125, 0.25, 0.5, 1, 2 and 4 ppb triasulfuron. Seedlings were allowed to grow for four weeks in the glasshouse before fresh weight (including roots) was recorded. There were 5 seeds per pot, and three replicates per treatment. Seedlings which survived were replanted for seed multiplication, as supplies of FEH-1 seed were low.

Resistance to foliar applied herbicides. Germinated Herald and FEH-1 seeds were sown into 10 cm diameter pots and grown in a glasshouse for 4-5 weeks until the 4-6 trifoliate leaf stage. Commercial formulations of six ALSIH were then applied at a range of rates (Table 7.1) with a 2 m wide plot sprayer delivering 100 l ha^{-1} at 150 kPa. BS 1000® non-ionic wetting agent was applied at 0.2% v/v with each treatment, including water control treatments. Plants were harvested and weighed 3 to 4 weeks after application. A subjective estimate of the level of probable field tolerance was also made, based on plant vigour and elongation and development of reproductive stems.

Table 7.1. ALS-inhibiting herbicides* and rates applied to seedlings of *M. littoralis* cv. Herald and FEH-1 at the 4 to 6 trifoliate leaf stage.

Herbicide	Rate (g a.i. ha^{-1})								
Chlorsulfuron	0	0.75	1.5	3	6	12	24		
Flumetsulam	0	10	20	40	80				
Imazethapyr	0	24	48	96	192	384	768	1536	3072
Metsulfuron-methyl	0	0.6	1.2	2.4	4.8	9.6	19.2	38.4	76.8
Triasulfuron	0	1.5	3	6	12	24	48		
Sulfometuron-methyl	0	1.5	3	6	12	24	48	96	

* BS 1000® non-ionic wetting agent was applied at 0.2%v/v with each treatment.

Comparative growth in field residues of chlorsulfuron and triasulfuron. A field site on a sandy loam soil (pH 5.2; water) was established at Struan Research Centre, in the south-east of South Australia. Chlorsulfuron (5.6, 11.3 and 22.5 g a.i. ha^{-1}) and triasulfuron (7.5, 15 and 30 g a.i. ha^{-1}) were applied on 18/5/00 using a wheel-mounted plot-sprayer delivering 147 L ha^{-1} at 200 kPa. There were 3 replicates and plots were 6 x 2 m. The site was sprayed with glyphosate on 18/5/00, and then cultivated 5 days later with three passes of a scarifier with trailing harrows to thoroughly incorporate the sulfonylurea herbicides into the soil. Each plot was planted with 36 germinated *M. littoralis* cv. Herald and FEH-1 seeds on 26/6/00. Establishment was recorded 4 weeks after sowing, and seedling dry weight was measured (10 plants plot^{-1}) 7 and 12 weeks after sowing. Flowering stage for untreated plants was also measured 12 weeks after sowing. Surplus FEH-1 plants were destroyed to prevent seedset in the field. Soil samples were taken from plots treated with the highest rates of chlorsulfuron and triasulfuron and untreated plots two weeks after sowing to determine residual concentrations of chlorsulfuron and triasulfuron, using soil bioassay methods described in section 4.2.1. Growth of *M. truncatula* cv. Mogul roots in soil from treated plots was compared to growth in soil from untreated plots which was supplemented with a range of known chlorsulfuron and triasulfuron concentrations. There were 10 seeds per replicate, three replicates, and root length was measured after 9 days.

7.3 RESULTS

7.3.1 Seed mutagenesis and selection for resistance

EMS mutation of *Medicago* seed and production of M_2 seed from M_1 plants. Seedling emergence and survival (to the first trifoliate leaf stage) of EMS treated *Medicago* seeds was estimated to be 11,325 ± 1407 for cv. Mogul and 226,500 ± 14,450 for cv. Herald, in 1998. This equates to 4.1 and 48.1% of seeds sown respectively. Emergence in the glasshouse (Table 7.2) strongly suggests that the EMS treatment caused more damage to cv. Mogul than to cv. Herald. There were 15 kg of cv. Mogul and 25 kg of cv. Herald harvested from the field.

Table 7.2. Effect of EMS treatment on Herald and Mogul seeds in a glasshouse (% of seeds producing a seedling with a trifoliate leaf) four weeks after sowing.

Cultivar	Untreated	EMS-treated
Herald	43.0 (± 17.0)	38.0 (± 4.0)
Mogul	50.0 (± 8.0)	3.0 (± 1.0)

Selection of plants grown from M_2 seed. Total seedling emergence for the 1,008 m^2 plots four weeks after sowing in 1999 was 1,614,000 ± 155,000 for Mogul and 6,415,000 ± 471,000 for Herald. Seedling emergence (as % of seed sown) was 55.7% for Herald and 46.9% for Mogul.

The two chlorsulfuron applications (PSI and POST) killed almost all seedlings. There were 3 cv. Herald and 27 cv. Mogul single plant M_2 selections made and transplanted to the glasshouse (Plate 7.1), along with unsprayed plants from the masked areas. Of the putatively resistant plants transplanted to pots in the glasshouse all but one cv. Herald (FEH-1) and 4 cv. Mogul plants (FEM-1, FEM-3, FEM-10 and FEM-14) died within one week, presumably from the cumulative effects of transplanting stress and chlorsulfuron.

7.3.2 FEH-1 herbicide resistance

Resistance of selected plants to chlorsulfuron in tissue culture. Leaf explants from *M. littoralis* cv. Herald and FEH-1 produced viable callus which showed clear differences in susceptibility to chlorsulfuron (Fig. 7.1). Herald growth was completely inhibited below 5 nM chlorsulfuron, while FEH-1 growth was reduced to 55% of untreated at 5 nM. This was the first clear quantitative evidence that the FEH-1 selection had greater resistancc to chlorsulfuron than the parent Herald cultivar. Callus growth for the four cv. Mogul selections was very poor and was not suitable for use in dose-response experiments.

Plate 7.1. Examples of field selections of *Medicago littoralis* cv. Herald (top) and *Medicago truncatula* cv. Mogul (centre and bottom) plants grown from M_2 seed (treated with EMS in M_1) and selected with chlorsulfuron.

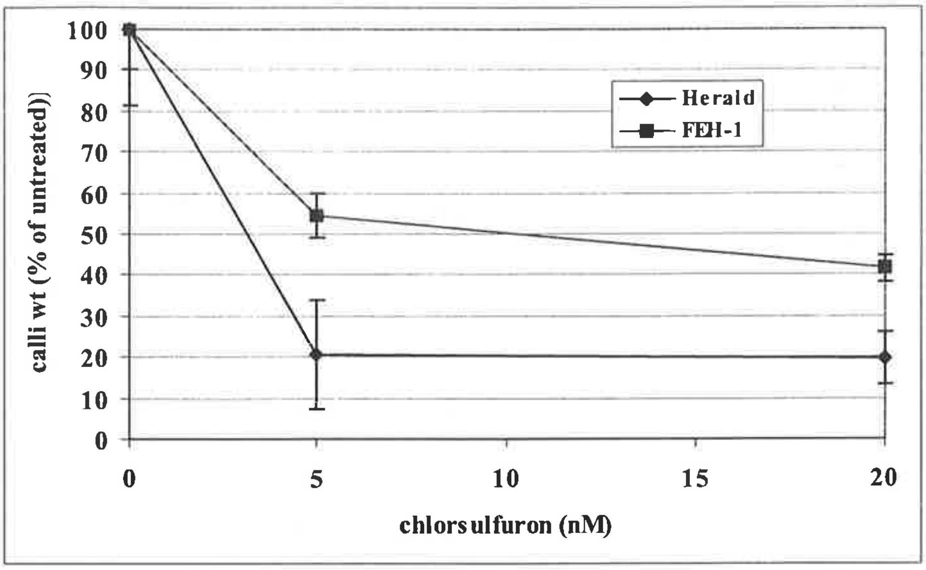


Figure 7.1. Growth (% of untreated calli) of cv. Herald and FEH-1 cell callus with chlorsulfuron in tissue culture after three weeks.

Resistance to chlorsulfuron and triasulfuron in soil. Seedling growth of *M. littoralis* FEH-1 was greater than for cv. Herald in soil treated with chlorsulfuron (Fig. 7.2) and triasulfuron (Fig. 7.3). Herald seedling growth was almost totally inhibited below 0.25 ppb for chlorsulfuron, while FEH-1 seedling growth appeared to be totally inhibited at around 4 ppb (Fig. 7.2; Plate 7.2). Both Herald and FEH-1 seedling growth were almost totally inhibited at 1 ppb triasulfuron, however FEH-1 growth was much stronger at lower rates. These results demonstrate that FEH-1 has greater resistance than Herald to soil residues of chlorsulfuron and triasulfuron, but the level of resistance is not large. None of the FEM cv. Mogul lines had greater resistance to chlorsulfuron than cv. Mogul (results not presented) and no further research was undertaken on these lines.

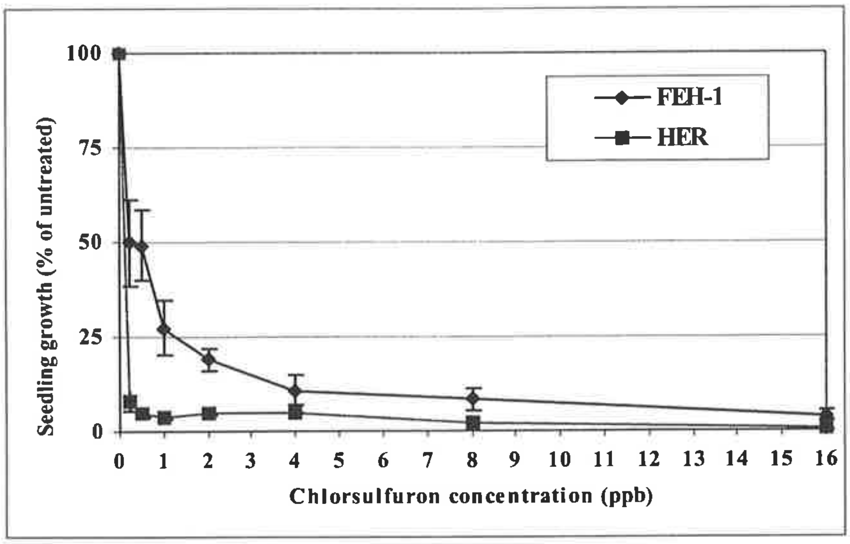


Figure 7.2. FEH-1 and Herald seedling growth (% of untreated) in chlorsulfuron-supplemented soil after 4 weeks.

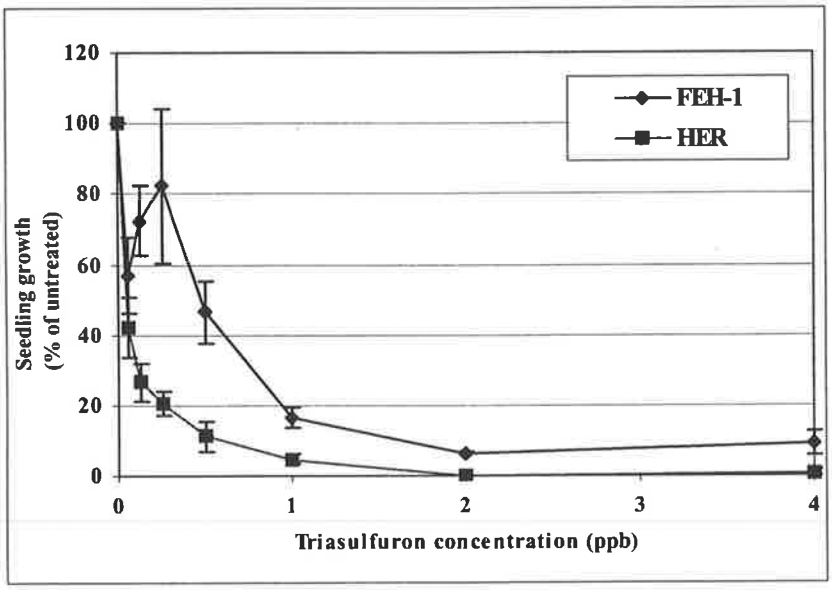
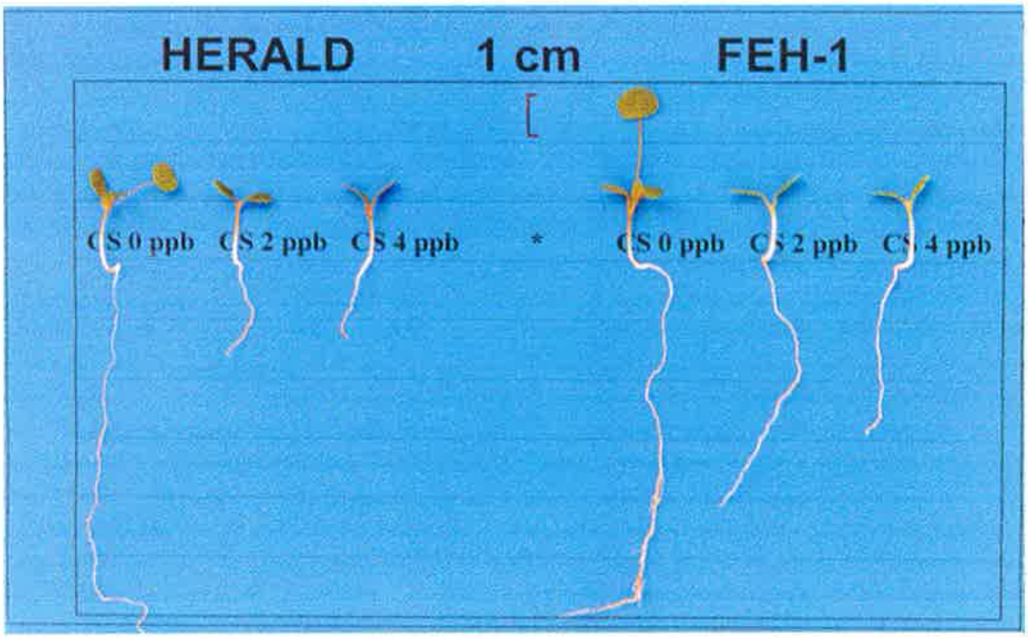


Figure 7.3. FEH-1 and Herald seedling growth (% of untreated) in triasulfuron-supplemented soil after 4 weeks.



Medicago littoralis cv. Herald (left) and FEH-1 root growth in soil treated with 0, 2 or 4 ppb chlorsulfuron, after 9 days.

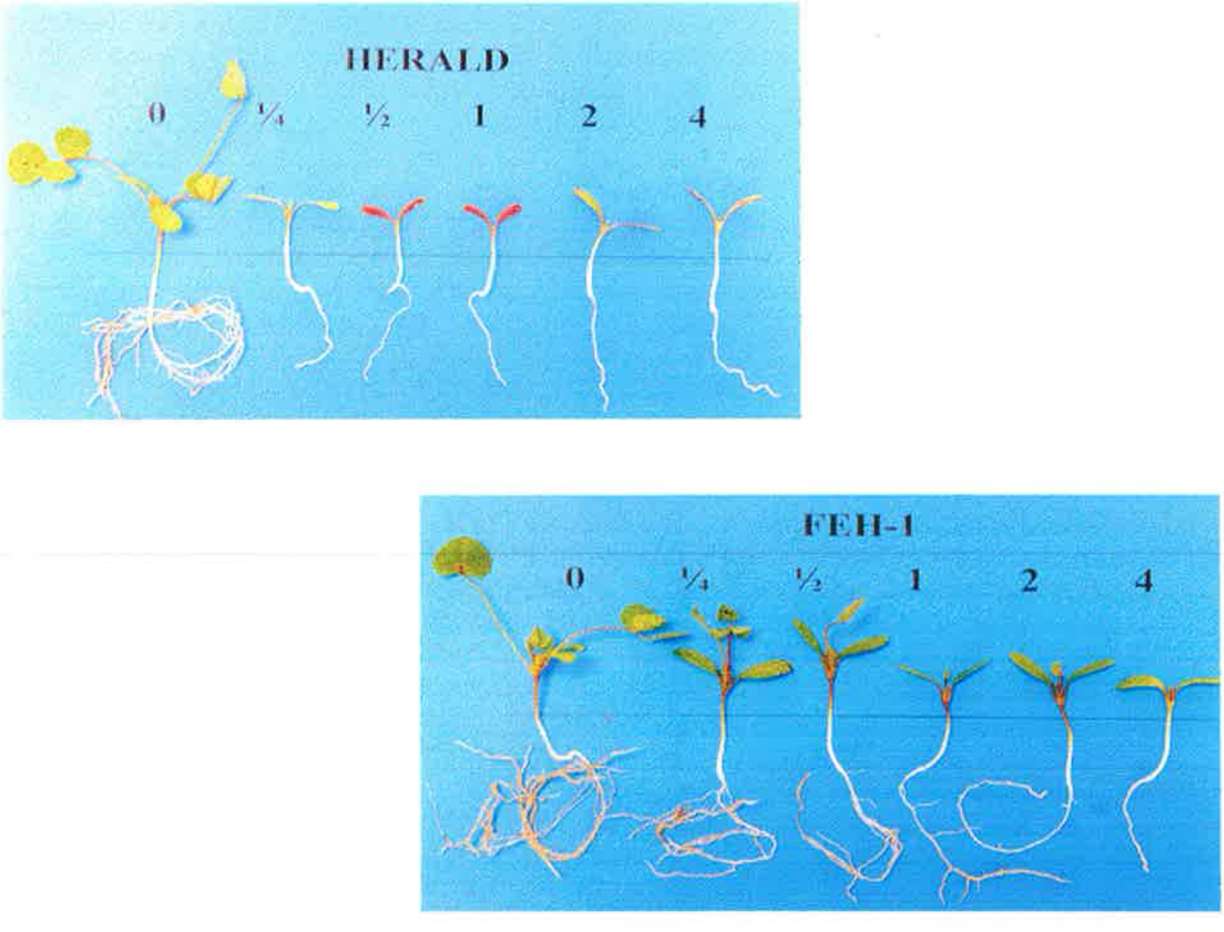


Plate 7.2. *Medicago littoralis* cv. Herald (top plate) and FEH-1 (bottom plate) root growth in soil treated with 0, 0.25, 0.5, 1, 2 or 4 ppb chlorsulfuron, after four weeks.

Resistance to foliar applied herbicides. Results from the post-emergence dose-response experiments demonstrated that *M. littoralis* FEH-1 had greater resistance than cv. Herald to imazethapyr (Fig. 7.4), flumetsulam (Fig. 7.5), metsulfuron-methyl (Fig. 7.6), sulfometuron-methyl (Fig. 7.7), chlorsulfuron (Fig. 7.8), and triasulfuron (Fig. 7.9). Photographs of these experiments are shown in Plates 7.3 to 7.8. Herald was resistant to surprisingly high rates of imazethapyr (Fig 7.4; Plate 7.3), suggesting that it has a high rate of imazethapyr metabolism, and FEH-1 was only clearly more resistance at very high rates (c. 15 to 60 x field rate). Both lines were resistant to high rates of flumetsulam, but FEH-1 was more resistant at the highest rate (4 x field rate; Fig. 7.5; Plate 7.4). Herald was very sensitive to metsulfuron-methyl and growth was totally inhibited below 0.6 g a.i. ha^{-1} (Fig. 7.6, Plate 7.5). FEH-1 was more resistant but growth was reduced at all rates, and probable field tolerance is only 1.2 g a.i. ha^{-1}. FEH-1 showed potentially useful resistance to sulfometuron-methyl (Fig. 7.7; Plate 7.6), with probable field tolerance to 12 g a.i. ha^{-1}. Resistance to soil residues of chlorsulfuron and triasulfuron in FEH-1 (Figs. 7.2 and 7.3) was reflected in similar levels of resistance to foliar treatments (Figs 7.8 and 7.9; Plates 7.7 and 7.8). Herald was very sensitive to these herbicides, with growth almost totally inhibited at around 1 g a.i. ha^{-1}. Although FEH-1 resistance was not large, it is probable that it has field tolerance to around 3 g a.i. ha^{-1} chlorsulfuron and 24 g a.i. ha^{-1} triasulfuron.

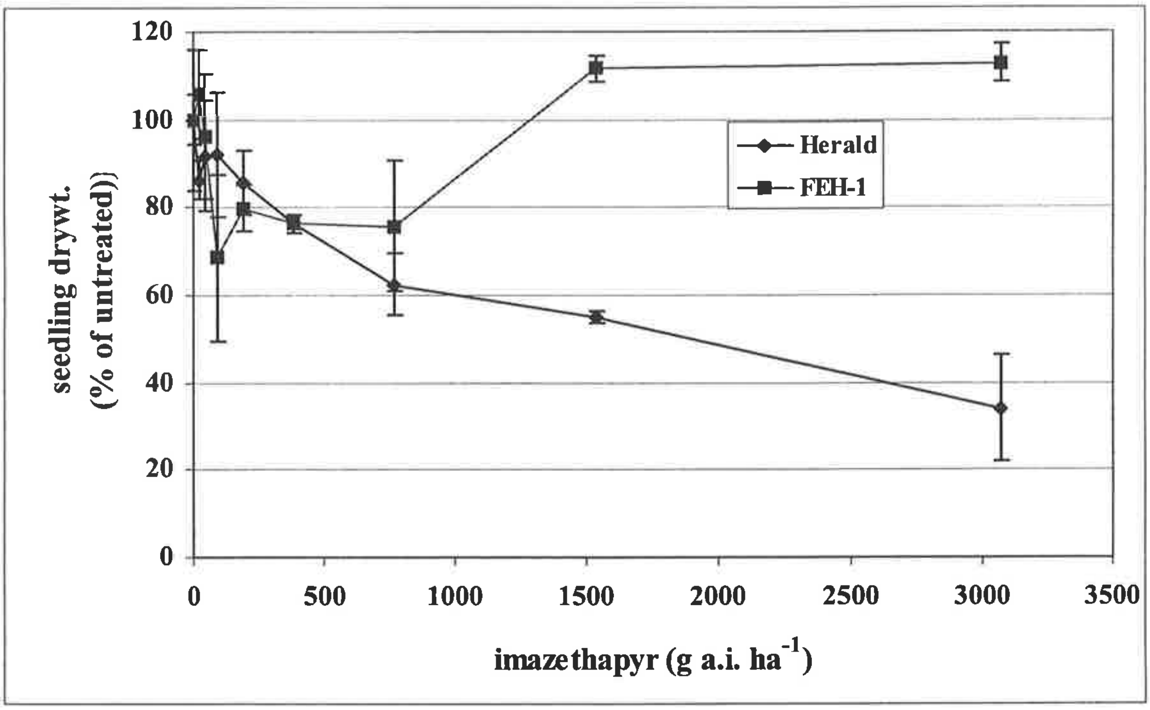


Figure 7.4. Effect of imazethapyr on seedling growth of *M. littoralis* cv. Herald and FEH-1 four weeks after foliar application.

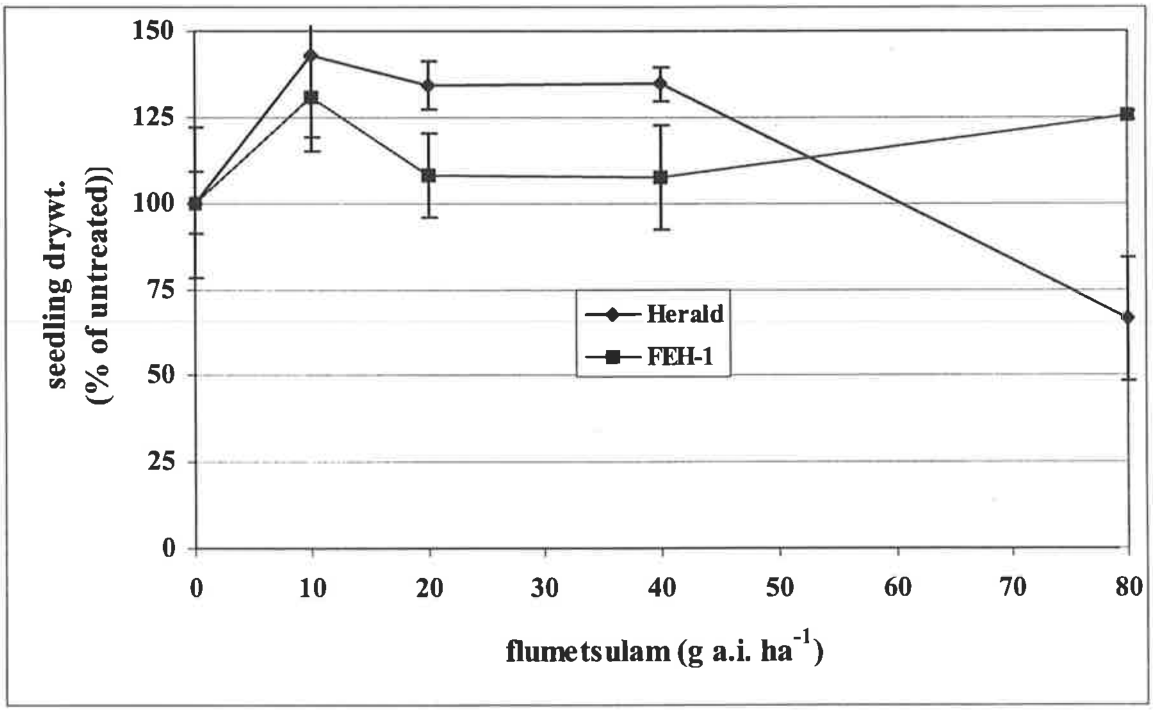


Figure 7.5. Effect of flumetsulam on seedling growth of *M. littoralis* cv. Herald and FEH-1 four weeks after foliar application.

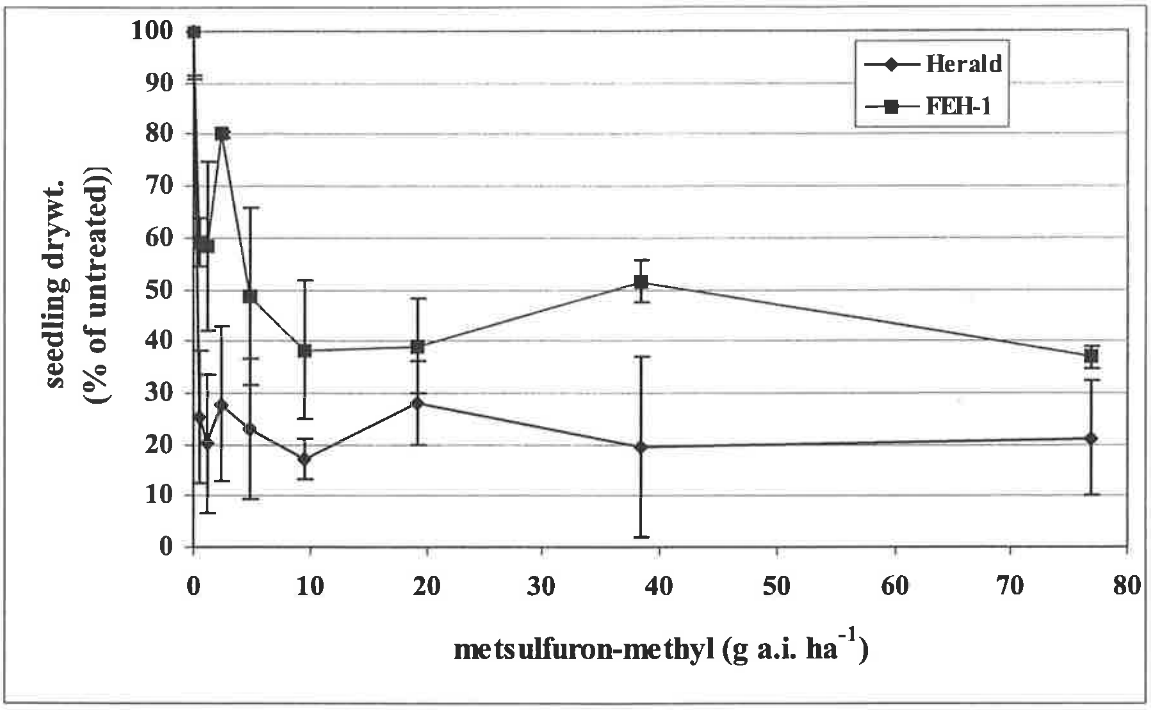


Figure 7.6. Effect of metsulfuron-methyl on seedling growth of *M. littoralis* cv. Herald and FEH-1 four weeks after foliar application.

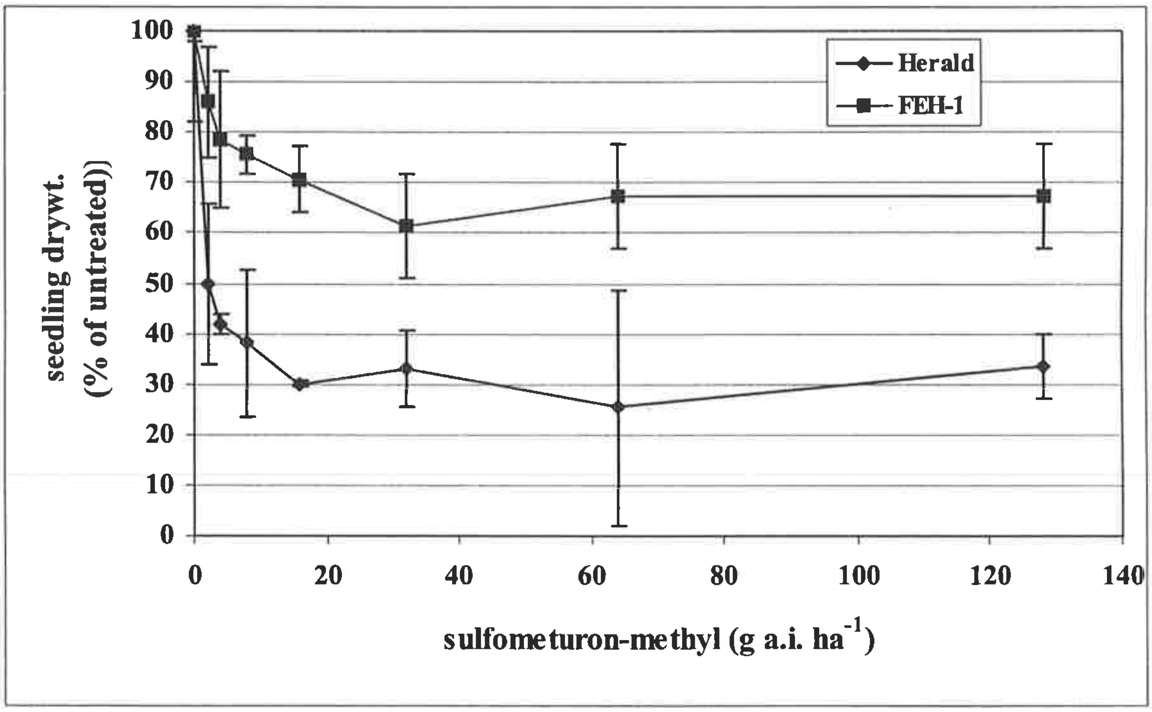


Figure 7.7. Effect of sulfometuron-methyl on seedling growth of *M. littoralis* cv. Herald and FEH-1 four weeks after foliar application.

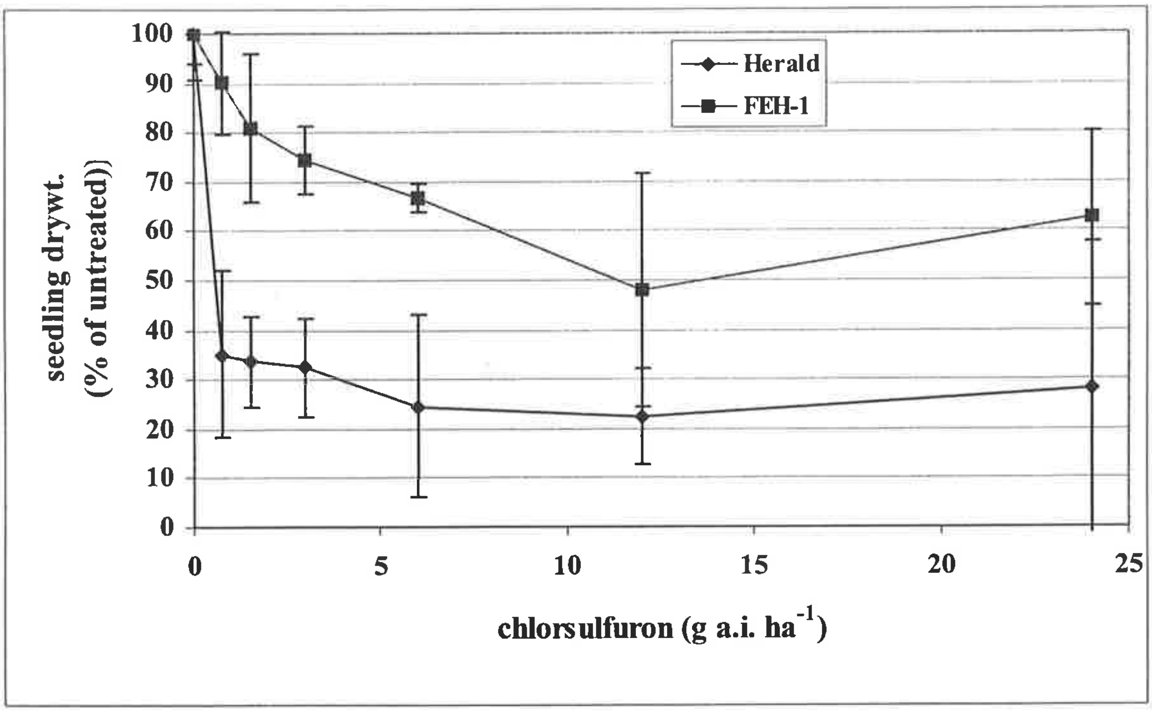


Figure 7.8. Effect of chlorsulfuron on seedling growth of *M. littoralis* cv. Herald and FEH-1 four weeks after foliar application.

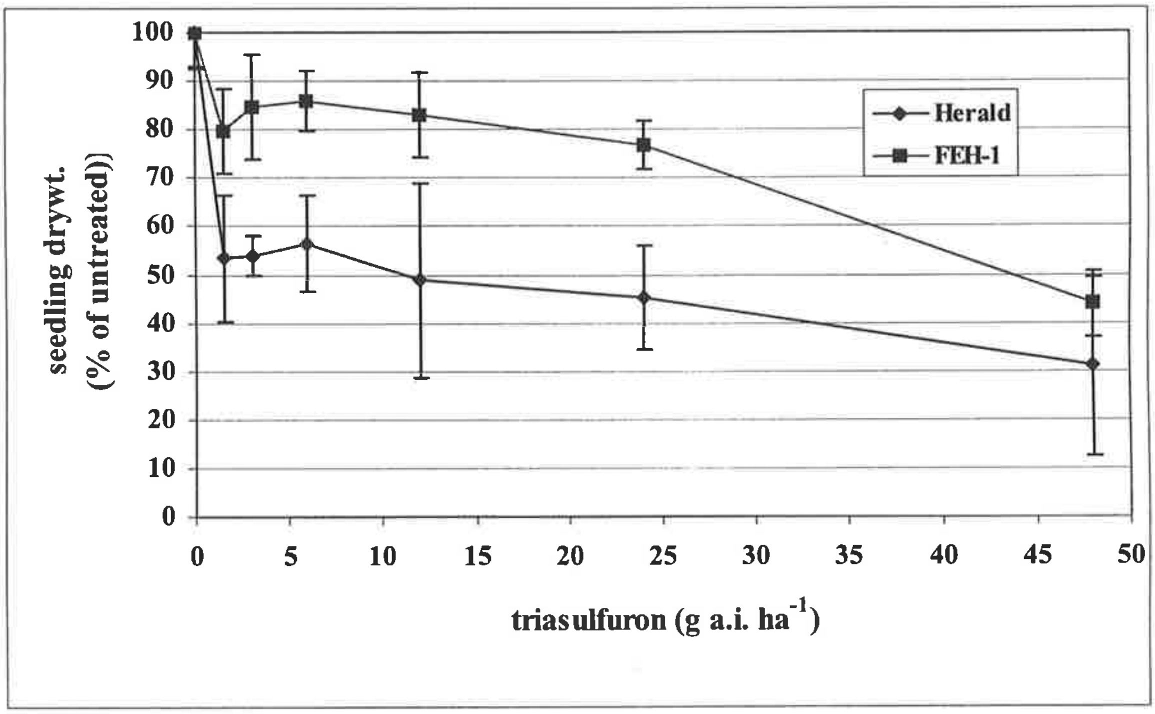


Figure 7.9. Effect of triasulfuron on seedling growth of *M. littoralis* cv. Herald and FEH-1 four weeks after foliar application.

Plate 7.3. Growth of *M. littoralis* FEH-1 (top row) and Herald (bottom row) four weeks after foliar application of imazethapyr at (L to R) 0, 24, 48, 96, 192, 384, 786, 1536, and 3072 g. a.i. ha^{-1}.

Plate 7.4. Growth of *M. littoralis* FEH-1 (top row) and Herald (bottom row) four weeks after foliar application of flumetsulam at (L to R) 0, 10, 20, 40, and 80 g. a.i. ha^{-1}.

Plate 7.5. Growth of *M. littoralis* FEH-1 (top row) and Herald (bottom row) four weeks after foliar application of metsulfuron-methyl at (L to R) 0, 0.6, 1.2, 2.4, 4.8, 9.6, 19.2, 38.4, and 76.8 g. a.i. ha^{-1}.

Plate 7.6. Growth of *M. littoralis* FEH-1 (top row) and Herald (bottom row) four weeks after foliar application of sulfometuron-methyl at (L to R) 0, 1.5, 3, and 6 g. a.i. ha^{-1}.

Plate 7.7. Growth of *M. littoralis* FEH-1 (top row) and Herald (bottom row) four weeks after foliar application of chlorsulfuron at (L to R) 0, 0.75, 1.5, 3, 6, 12, and 24 g. a.i. ha^{-1}.

Plate 7.8. Growth of *M. littoralis* FEH-1 (top row) and Herald (bottom row) four weeks after foliar application of triasulfuron at (L to R) 0, 1.5, 3, 6, 12, 24, and 48 g. a.i. ha^{-1}.

Comparative growth in field residues of chlorsulfuron and triasulfuron. FEH-1 growth was far greater than Herald in field residues of chlorsulfuron and triasulfuron. Seedlings of the lines established equally well (results not presented), but grew slowly under the cold and wet conditions. Bioassay dose-response experiments (as described in 4.2.1) using soil from plots treated with the highest rates of chlorsulfuron and triasulfuron suggested that the highest rates of chlorsulfuron and triasulfuron had resulted in residues of approximately 1 ppb by two weeks after sowing (Table 7.3).

Table 7.3. Root length (% of untreated) of *M. truncatula* cv. Mogul seedlings grown in soil supplemented with chlorsulfuron (CSF) or triasulfuron (TSF) after 9 days.

Herbicide Concentration (ppb)	CSF	(± se)	TSF	(± se)
0	100.0	(22.1)	100.0	(8.1)
0.063	87.6	(5.4)	91.0	(0.7)
0.125	73.6	(3.9)	76.6	(2.5)
0.25	75.5	(7.9)	68.4	(2.8)
0.5	65.1	(4.5)	67.5	(2.0)
1.0	60.9	(4.8)	62.4	(7.6)
Unknown field sample	52.6	(3.2)	62.1	(4.5)

Plant weight measurements taken 7 weeks after sowing showed significant stunting of Herald (Fig. 7.10) which increased with herbicide application rate. FEH-1 growth was reduced slightly by the residues, but was commercially acceptable for all treatments. Herald seedlings were severely stunted by the highest rates of chlorsulfuron and triasulfuron, and many plants ceased growth at the spade leaf stage.

There were very large differences in growth between FEH-1 and Herald 12 weeks after sowing (Fig. 7.11, Plate 7.9). Untreated plants were 30 to 40 cm in diameter, with 5 to 6 reproductive stems. None of the plants were flowering on 15/9/00, however on 25/9/00 both Herald and FEH-1 were in early flower, with no difference in the flowering stage evident in untreated plots (results not presented). Herald growth in residues from application of 11.3 and

22.5 g a.i. ha^{-1} chlorsulfuron and 30 g a.i. ha^{-1} triasulfuron was very poor, with most plants ceasing growth at the spade leaf to several trifoliate leaf stage. In contrast to Herald, FEH-1 growth was vigorous and apparently unaffected even at the highest rates, suggesting that it had overcome any early suppression.

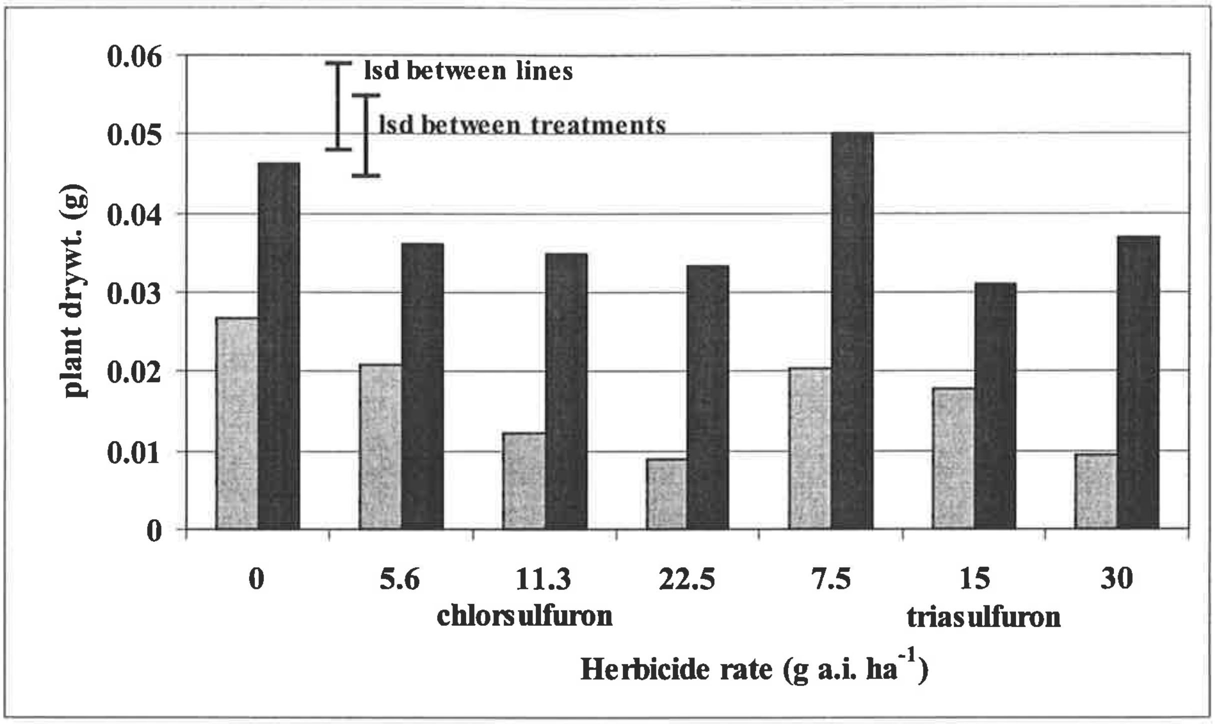


Figure 7.10. Field growth of *M. littoralis* cv. Herald (light shade) and FEH-1 (dark shade) after 7 weeks of growing in soil residues of chlorsulfuron and triasulfuron.

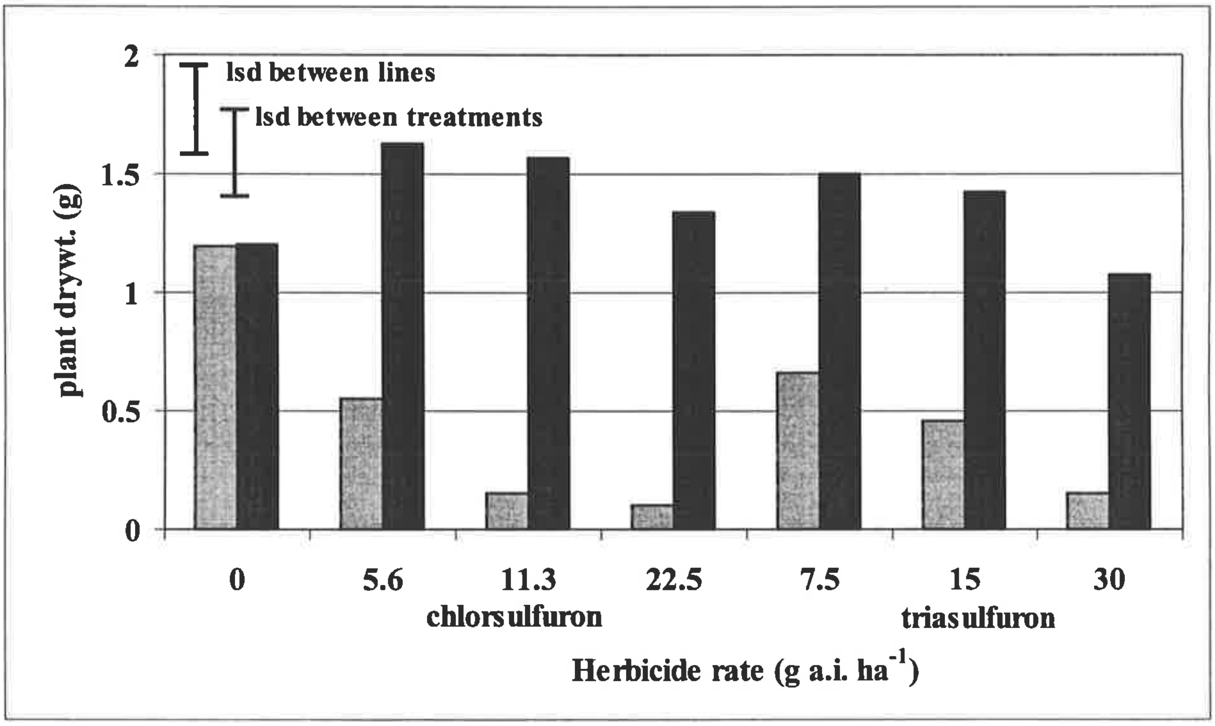


Figure 7.11. Field growth of *M. littoralis* cv. Herald (light shade) and FEH-1 (dark shade) after 12 weeks of growing in soil residues of chlorsulfuron and triasulfuron.

Plate 7.9. Field growth of *M. littoralis* cv. Herald (left) and FEH-1 (right) after 12 weeks of growing in untreated soil (top picture) or residues from application of 22.5 g. a.i. ha^{-1} chlorsulfuron (bottom picture).

Plate 7.10. *Medicago littoralis* cv. Herald (left) and FEH-1 (right) seedlings, flowering stems and seed pods.

7.4 DISCUSSION

These experiments have confirmed the increased resistance of the selected *M. littoralis* cv. Herald line FEH-1 to a range of herbicides including chlorsulfuron, flumetsulam, imazethapyr, metsulfuron-methyl, triasulfuron, and sulfometuron-methyl. It was also demonstrated that a herbicide resistant line with apparently sound agronomic traits can be generated relatively quickly using seed mutation instead of genetic engineering techniques.

Seedling establishment was sufficient to allow selection from large populations of both cultivars, but relatively few survivors were successfully transplanted to the glasshouse. Most survivors were probably suffering moderate to severe growth retardation from chlorsulfuron, and the added stress of transplantation resulted in death for all but one cv. Herald and four cv. Mogul plants. Recovery of putative resistant individuals was 1.56×10^{-7} for cv. Herald and 2.48×10^{-7} for cv. Mogul. Maliga (1984) estimated that mutagenic agents such as EMS typically increase mutations from 10^{-5}–10^{-8} to around 10^{-3}. This suggests that for *M. truncatula* and *M. littoralis*, the natural frequency for ALS mutants with resistance to chlorsulfuron may be in the range 10^{-9}–10^{-12}, implying that a very large population would need to be screened to detect a resistant individual from seed which had not been mutated. For example, the area required to screen c. 4×10^{7} seedlings for naturally-occurring mutants (section 5.2.2) was 1.26 ha. To use similar methodology to screen 10^{-9} to10^{-12} seedlings would require 31.5 to 31,500 ha, clearly an impractical approach without the use of mutagens to increase the frequency of resistant mutants. Both *M. truncatula* cv. Mogul and *M. littoralis* cv. Herald are relatively new cultivars and have passed through few generations following selection from a narrow genetic base. Consequently, there has been little time for spontaneous mutations conferring ALSIH resistance to occur and become established in populations. In contrast, Preston and Powles (2000) estimated that the frequency of resistant mutants in

Lolium rigidum was 4.6 x 10^{-5} to 1.2 x 10^{-4}, and Saari *et al.* (1994) estimated that it was typically in the range of 10^{-6} or less for most species.

Tissue culture, using cell calli initiated from leaves of FEH-1 shortly after selection, was a valuable approach for assessing putative resistance well before seed was available. Levels of chlorsulfuron resistance from this first experiment were later supported by experiments with whole plants using soil residues and foliar application. In future studies it may be possible to screen all survivors using cell culture, so that subsequent efforts can be concentrated on individual selections with resistance confirmed by tissue culture.

FEH-1 was developed primarily to overcome the effects of chlorsulfuron and triasulfuron soil residues in cropping-livestock rotations, and results from Figs. 7.2, 7.3, 7.10, and 7.11 confirm that there is a moderate, but agronomically-useful, increase in resistance over Herald. The results suggest that FEH-1 has resistance to soil residues of chlorsulfuron and triasulfuron in the range 0 to 2 ppb. Carry-over residues are typically around 0.5 to 1 ppb, and very sensitive species are affected at >0.1 ppb (McQuinn, 1997). FEH-1 grew strongly in the field five weeks after application of high rates of chlorsulfuron and triasulfuron, but a rapid breakdown of the residues to around 1 ppb within 7 weeks was promoted by an acid soil and high soil moisture (Beyer *et al.*, 1988). It is likely that the apparently unaffected growth of FEH-1 in residues 12 weeks after sowing (Fig. 7.11) reflects a continuation of the rapid residue breakdown. In contrast, Herald plants which were severely affected by residues in the first seven weeks (Fig. 7.10), were too damaged to exploit the lower residue levels present later in the season, and remained stunted (Fig. 7.11). It was not determined how FEH-1 growth would be affected by an extended duration of elevated residue levels, such as might be encountered in a Mallee paddock, but the results from this experiment give cause for cautious optimism that it may withstand soil residues of chlorsulfuron and triasulfuron under alkaline, low-rainfall conditions. However, further field experiments are needed to demonstrate this over a range of soil types, residue levels and growing conditions.

Foliar applications of six ALSIH suggested that FEH-1 has greater resistance to these herbicides than does Herald. The unexpectedly high resistance of Herald to imazethapyr suggests that it has an efficient imazethapyr metabolism mechanism, which has been augmented by a resistant ALS in FEH-1 to produce an extremely high level of resistance in FEH-1. Growth of FEH-1 was apparently unaffected at 3,000 g a.i. ha^{-1} (Fig. 7.4), a rate which is approximately 60 times the field rate. This resistance may be useful in specific situations such as controlling the important parasitic weed branched broomrape (*Orabanche ramosa*) in the South Australian Mallee. Branched broomrape is susceptible to imazethapyr and FEH-1 is agronomically suited to the region. Annual *Medicago* are known to be resistant to flumetsulam, and the increased resistance at four times the field rate (Fig. 7.5) is of little practical significance. It appears that FEH-1 may tolerate foliar applications of useful rates of sulfometuron-methyl (12 g a.i. ha^{-1}) and triasulfuron (24 g a.i. ha^{-1}). These rates would be expected to control most weeds which are normally controlled by these herbicides, and FEH-1 may offer opportunities for strategic weed control using them. If this were to be done, careful consideration of the implications for development of herbicide resistance in weeds would be needed (discussed in 2.3.3). Triasulfuron is a selective herbicide already used widely in cropping-livestock rotations, but sulfometuron-methyl is a non-selective herbicide not normally used in arable areas. Application of sulfometuron-methyl to FEH-1 pastures may be particularly attractive to farmers, because selective control of all weeds may be possible. Although FEH-1 was more resistant to foliar applications of metsulfuron-methyl and chlorsulfuron than Herald, the level of resistance appears to be low (Figs. 7.6 and 7.8) and it is likely that selective control of only very sensitive weeds would be possible.

Observations on FEH-1 in glasshouse and field experiments suggest that it is visually indistinguishable from Herald (Plate 7.10), and that growth rates and flowering times are very similar. Although field research is required to further compare FEH-1 to Herald, it is likely that FEH-1 is identical to Herald except for the trait of ALSIH resistance. If this is the case, then commercial development and deployment of FEH-1 should be possible.

CHAPTER 8

MOLECULAR BASIS FOR FEH-1 RESISTANCE

8.1 INTRODUCTION

Results from section 7.3.2 confirmed that FEH-1 has greater resistance to chlorsulfuron and five other ALSIH than its parent cultivar Herald. This section describes experiments conducted to determine the resistance mechanism of FEH-1. When the experiments began mature seeds were not available from the single selected FEH-1 plant, and so cell callus in tissue culture was used to determine the effect of malathion on chlorsulfuron resistance, as well as cross-resistance to sulfometuron-methyl. As discussed in chapter 6, the results from these experiments can be used to infer whether resistance is likely to be based on enhanced metabolism of chlorsulfuron, or an altered ALS target site. Limited seed from FEH-1 was later used for seed multiplication, and leaf material from these seedlings was used for *in vitro* ALS enzyme assays to characterise the herbicide cross-resistance spectrum.

These experiments were conducted to examine if FEH-1 has a mutated ALS enzyme, conferring resistance to at least six ALSIH, and an attempt was made to determine the ALS gene base sequence for both FEH-1 and cv. Herald in order to identify the mutation (s) conferring resistance. There have been a large number of mutant ALS genes described from bacteria, yeast, fungi and higher plants (reviewed in Saari *et al.*, 1994). Mutants usually have one, or rarely two, base substitutions which encode for a different amino acid in the enzyme. These substitutions typically occur in one of five highly conserved areas of the ALS gene, in Domains A-E (Fig. 1.5). Substitutions in the same position on the gene have been found in independent cases of weed resistance, and it is possible to see correlations between the position and nature of the substitution and herbicide cross-resistance patterns (Devine and Preston, 2000).

8.2 MATERIALS AND METHODS

8.2.1 Effect of malathion on chlorsulfuron resistance

The results from section 6.3.6 suggested that 50 μM malathion suppressed the growth of callus by around 15 to 20%. This concentration was chosen for combination with a range of chlorsulfuron concentrations to determine whether the selected resistance in FEH-1 was likely to be based on enhanced metabolism. Calli derived from FEH-1 and cv. Herald (section 7.2.2) were cut into small pieces (c. 20 mg) and placed on EIM media in 9 cm petri dishes without added casamino acids. Chlorsulfuron was filter-sterilised (0.2 μm) into the media after autoclaving at 48°C to final concentrations of 0, 8, 16, 32, 64, 128, and 256 nM. One treatment group had malathion added at 50μM. There were 5 calli pieces per replicate and three replicates. The dishes were incubated in the dark at 27 ° C and fresh weight was recorded after four weeks. Prior to analysis, 80 mg (approximate starting weight of calli) was subtracted from each weight so that the data reflected actual growth.

8.2.2 Cross-resistance to sulfometuron-methyl

Calli from FEH-1 and cv. Herald (section 7.2.2) were cut into small pieces (c. 20 mg) and placed on EIM media in 9 cm petri dishes without added casamino acids. Sulfometuron-methyl was filter-sterilised (0.2 μm) into the media after autoclaving at 48°C to make concentrations of 0, 2, 4, 8, 16, 32, 64 and 128 nM. There were 5 calli pieces per replicate, and three replicates. The dishes were incubated in the dark at 27 ° C and fresh weight was recorded after three weeks.

8.2.3 Effect of ALS-inhibiting herbicides on FEH-1 ALS

These experiments measured the effect of chlorsulfuron, flumetsulam, imazapyr, imazethapyr, metosulam, metsulfuron-methyl, sulfometuron-methyl and triasulfuron on ALS activity of FEH-1 and cv. Herald, using a method described in 6.2.7 which is based on that of Singh *et al.* (1988), and adapted and modified by Boutsalis (1996). ALS extractions were from 1g of young expanding leaf material from plants grown in the glasshouse. Eppendorf tubes (1.5 ml) were used instead of 96 well plates and reaction volumes were doubled to allow for reading absorbance in a cuvette. Herbicide concentrations were 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M for chlorsulfuron, metosulam, metsulfuron-methyl, sulfometuron-methyl and triasulfuron; 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M for flumetsulam and imazapyr; and 0, 10^{-7}, 10^{-6}, and 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M for imazethapyr. Herbicide concentrations were replicated three times.

Resistance ratios were calculated based on the herbicide concentration required to inhibit ALS activity by 50% (I_{50}). The ratio is expressed as FEH-1 I_{50}/Herald I_{50}. Activity (µmol acetolactate mg protein^{-1} h^{-1}) of the FEH-1 and cv. Herald ALS enzymes was measured in the absence of herbicide and are the mean of 12 replicates over four experiments.

8.2.4 Mutation in FEH-1 ALS gene

Genomic DNA extraction. FEH-1 and *M. littoralis* cv. Herald plants were grown in a glasshouse for three months and young expanding leaves were harvested and stored in liquid nitrogen. The method used for DNA extraction was based on that for isolation of DNA from fresh tissue of *Banksia* spp. (Doyle and Doyle, 1998). One gram of frozen leaves from each medic line was ground to powder in liquid N and added to a solution at 60°C containing 5 ml of CTAB isolation buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl (pH 8.0)) in a 10 ml plastic conical centrifuge tube. The solutions were incubated at 60°C for 30 min and gently inverted 2 to 3 times. Samples were then extracted with an equal volume of chloroform:isoamyl alcohol (24:1; v:v), mixed gently on a rotary rack for 30 min, then centrifuged at 10,000 rpm for 10 min at 20°C. The aqueous phase was collected and the nucleic acids were precipitated by addition of 2.5 volumes of cold isopropanol, followed by freezing at -20°C for 20 min. The nucleic acids were pelleted by centrifuging at 15,000 rpm for 10 min at 4°C, washed in 5 ml wash buffer for 20 min, centrifuged at 10,000 rpm for 5 min, then air dried for 60 min at 20°C. The pellet was then resuspended in 400 µl TE buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA) overnight at 4°C. Gel electrophoresis of the nucleic acids confirmed the presence of high molecular weight DNA. RNA was degraded in the samples by adding 800 µL of TE buffer and 8 µL (1:10 dilution of 10 mg ml^{-1} RNAase “A”) RNAase, then heating at 37°C for 40 min. Samples were then diluted in two volumes of TE buffer, one volume of 7.5 M ammonium acetate, and two volumes of cold absolute ethanol, precipitated at 4°C for 1 hr, and centrifuged at 10,000 rpm for 10 min. The pellet was washed in 70% ethanol, centrifuged at 10,000 rpm for 10 min, dried for 30 min at 20°C, dissolved in 200 µL of TE, and stored at 4°C awaiting polymerase chain reaction (PCR).

Oligonucleotide primers. A region of the ALS gene was amplified using specific oligonucleotide primers. Two sets of primers previously used to amplify ALS genes in *Brassicaceae* (Boutsalis, 1996) were used to amplify the *Medicago* ALS genes (Table 8.1). The primers were prepared by the Centre for Basic and Applied Plant Molecular Biology at the University of Adelaide. The first set, designated Primers 1 and 2, were designed to amplify Domains A and D (Fig. 1.5 and Table 1.1), and the second set, Primers 4 and 5, were designed to amplify most of the gene.

Table 8.1 Nucleotide sequences of the four oligonucleotide primers selected for PCR amplification and sequencing of ALS genes in *Medicago littoralis* cv. Herald and FEH-1.

Primer*	Target	Orientation	Sequence
1	Domains A&D	5'→ 3'	5' GCATGTCTAGAACGTCCTTCC(T/C)CGTCACGAACA 3'
2	Domains A&D	3'→ 5'	5' CGTGGATCCT(A/C)GTTACCTCAACAA 3'
4	Most of gene	3'→ 5'	5' GAA(G/A)GTGCC(G/A)CCACT(A/T)GGGAT 3'
5	Most of gene	5'→ 3'	5' ATCCT(C/G)GT(C/G)GAAGCCCT(C/G)GAGCGTCA 3'

* Boutsalis, 1996

Polymerase chain reaction. Polymerase chain reactions were conducted in a programmable thermal controller (MJ Research Inc., PTC-100) using oligonucleotide primers to amplify a specific region of the ALS gene for *M. littoralis* cv. Herald and FEH-1. Prior to the PCR 49.8 µL solutions were made comprising 1 µL isolated DNA (see above), 5 µL 10x thermophylic buffer solution (670 mM Tris-HCl, pH 8.8; 166 mM $(NH_4)_2SO_4$; 2 mg ml^{-1} gelatin; 4.5% v/v Triton X-100), 7 µL 25mM $MgCl_2$, 4 µL oligonucleotide primer, 1 µL dNTPs, and 31.8µL double autoclaved water. A control solution was also prepared using water instead of DNA solution. There were five samples for FEH-1 and cv. Herald, and two control samples.

Solutions were placed in 0.5 ml eppendorf tubes, microfuged for 20 sec, then loaded into the programmable thermal controller. The solutions were pre-heated ("hot-start") at 94°C for 8.5 min prior to the addition of 0.2μL Taq polymerase (Bresatec; 10 U μl^{-1}). After the Taq polymerase was added the cycle program was: denaturation at 94°C for 1.5 min; anneal at 55°C for 2 min; elongate at 72°C for 2 min. This cycle was repeated 34 times then the solution held at 72°C for 10 min before cooling to 25°C.

PCR product purification using gel electrophoresis and DNA extraction. The products of the PCR were separated electrophoretically on a TBE buffer 1.0 % agarose gel. The gel was stained with ethidium bromide and stained DNA bands at approximately 300 bp (primers 1 and 2) and 1400 (primers 4 and 5) excised. The 1400 bp band was smaller than expected, but it was decided to extract the DNA and attempt to sequence it. DNA was extracted from agarose using a "Bio 101 Geneclean 2" kit, then frozen at -20°C.

DNA cycle sequencing. DNA purified from ethidium bromide stained agarose (see above) was made into 20μL DNA cycle sequencing reaction solutions containing 8.0 μL terminator ready reaction mix (supplied by NAPCU, W.A.R.I.), 5 μL DNA PCR product, 3.0μL primer, and 4μL water in 0.5 ml eppendorf tubes. The tubes were then subject to a PCR program consisting of a rapid thermal ramp to 96°C (held for 10 s), a rapid thermal ramp to 50°C (held for 5 s), a rapid thermal ramp to 60°C (held for 4 min), repeated through 25 cycles, then a rapid thermal ramp to 4°C and held. Following the PCR, the 20μL PCR product was added to 20μL 3M sodium acetate (pH 4.6) and 50μL 95% ethanol in a 1.5 ml eppendorf tube, vortexed, placed on ice for 10 min, then centrifuged at 14,000 rpm for 30 min. The ethanol solution was then aspirated and the pellet rinsed with 250μL 70% ethanol. The ethanol was then aspirated and the tubes dried at 95°C for 10 min. The samples were then submitted to

NAPCU (W.A.R.I.) for automated sequencing in a terminal sequencing machine (Applied Biosystems 373 DNA Sequencer Stretch, Foster City, CA, USA).

8.3 RESULTS

8.3.1 Effect of malathion on chlorsulfuron resistance

The lowest concentration of chlorsulfuron used (8 nM) greatly reduced growth of cv. Herald and the addition of malathion completely prevented growth (Fig. 8.1). FEH-1 exhibited resistance to chlorsulfuron, and there was still slow growth at 250 nM. Malathion generally reduced FEH-1 callus growth at all chlorsulfuron concentrations, but did not overcome expression of resistance, suggesting that the mechanism of resistance was not enhanced metabolism.

8.3.2 Cross-resistance to sulfometuron-methyl

FEH-1 was found to be highly cross-resistant to sulfometuron-methyl (Fig. 8.2). Herald callus growth was prevented below 8 nM sulfometuron-methyl, while FEH-1 maintained greater than 50% of untreated growth at 128 nM. Low levels of sulfometuron-methyl (2 to 8 nM) appeared to stimulate growth in FEH-1, but it was not determined whether this was an experimental artefact. The strong cross-resistance to sulfometuron-methyl was further evidence against resistance based on enhanced metabolism.

8.3.3 Effect of ALS-inhibiting herbicides on FEH-1 ALS

ALS from FEH-1 was clearly more resistant to some ALSIH in *in vitro* enzyme assays than cv. Herald (Table 8.2; Figs. 8.3 to 8.6). The curve equations derived from data used for figures 8.3 to 8.6 were used to calculate the I_{50} values (herbicide concentration which inhibited ALS activity by 50%). The resistance ratio (FEH-1 I_{50}/Herald I_{50}) was then calculated for each herbicide (Table 8.2). The most striking result was the apparent >73,000-fold resistance to imazethapyr (Table 8.2; Fig 8.6). There were also high levels of resistance to metsulfuron-methyl (>141-fold), sulfometuron-methyl (70-fold), and flumetsulam (>54-fold). Resistance to the two herbicides of most practical interest was only low to moderate, with 9-fold resistance to chlorsulfuron, and 11-fold resistance to triasulfuron. ALS from FEH-1 showed little or no resistance to imazapyr or metosulam.

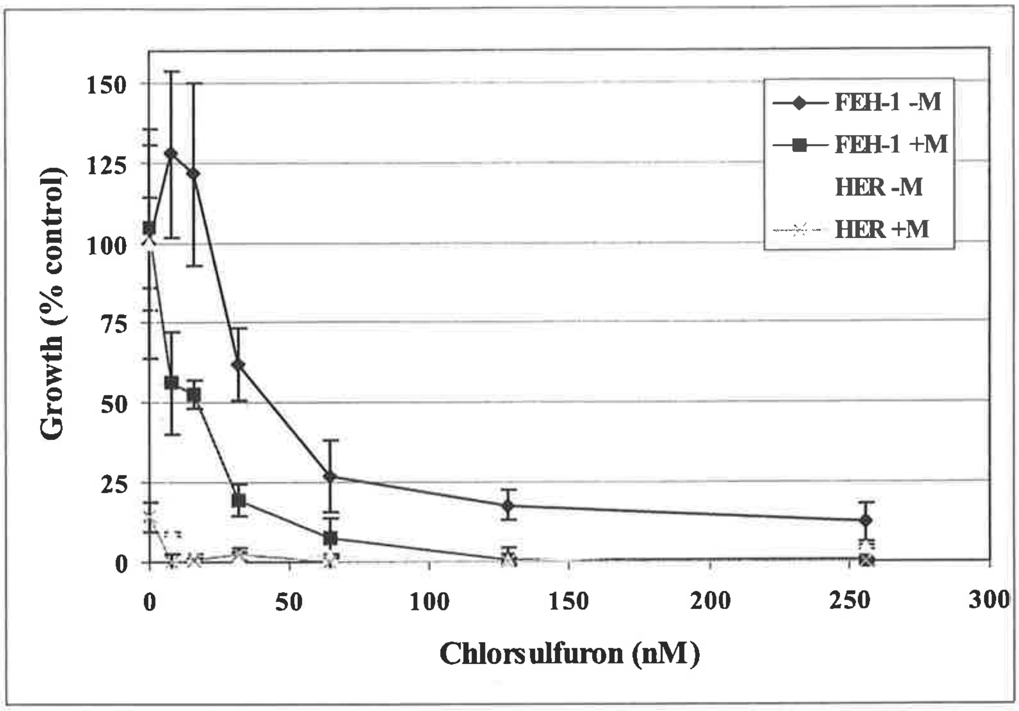


Figure 8.1. Effect of malathion (50μM) on growth (callus weight) of FEH-1 and cv. Herald callus with chlorsulfuron.

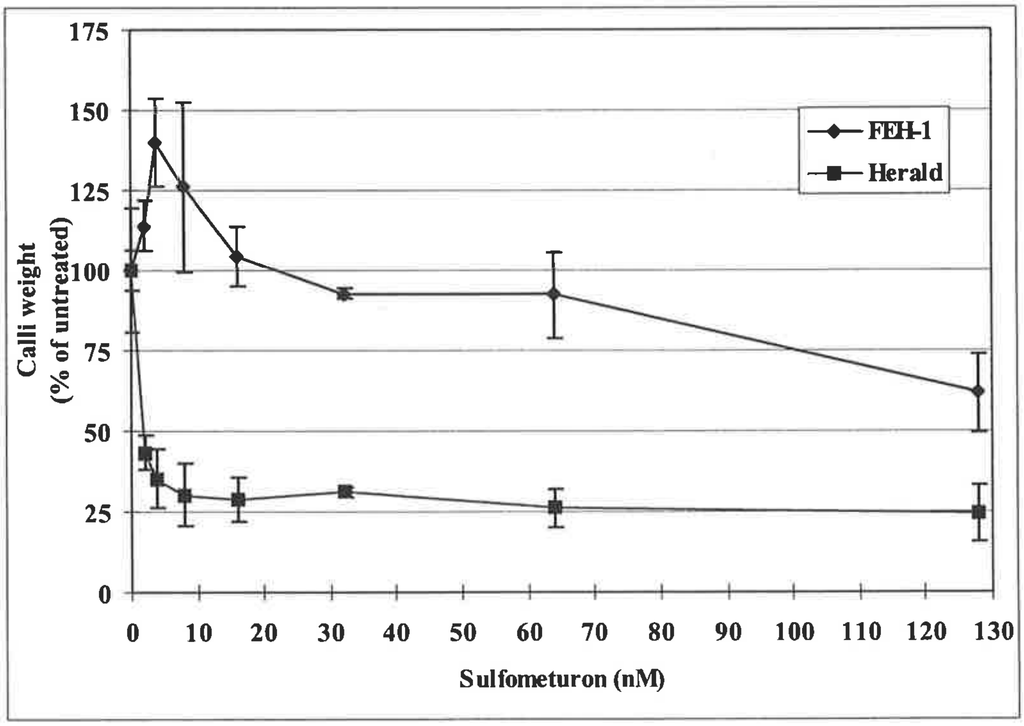


Figure 8.2. Calli weight (% of untreated) for FEH-1 and cv. Herald grown with sulfometuron-methyl.

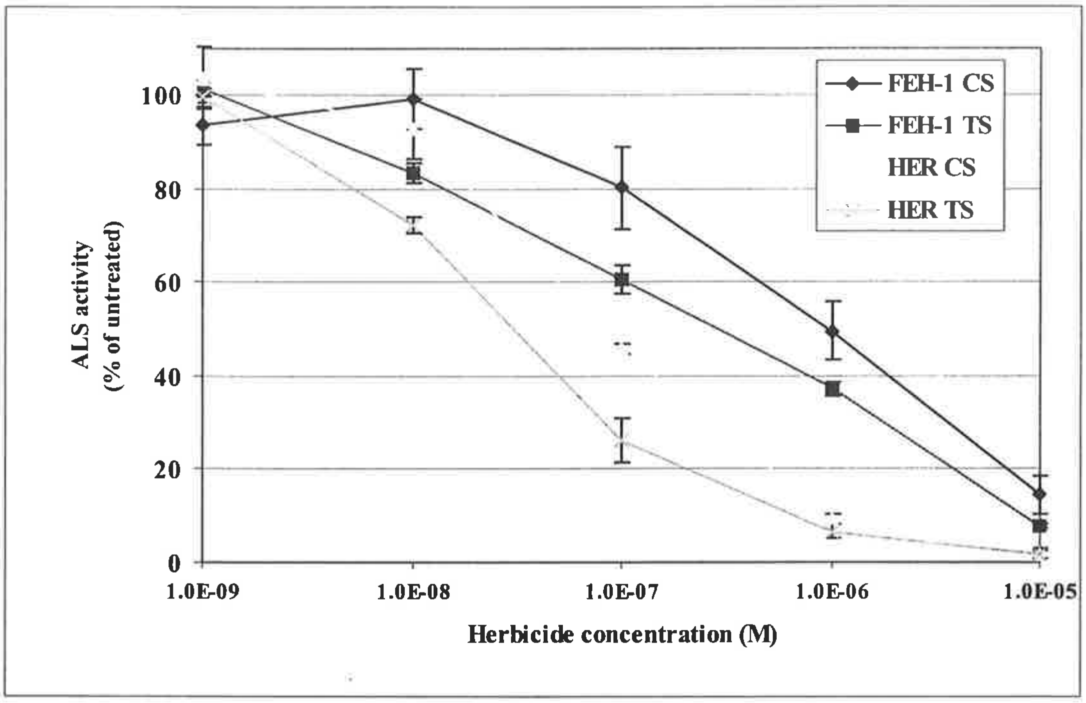


Fig. 8.3. Effect of chlorsulfuron (CS) and triasulfuron (TS) on activity of ALS from FEH-1 and cv. Herald.

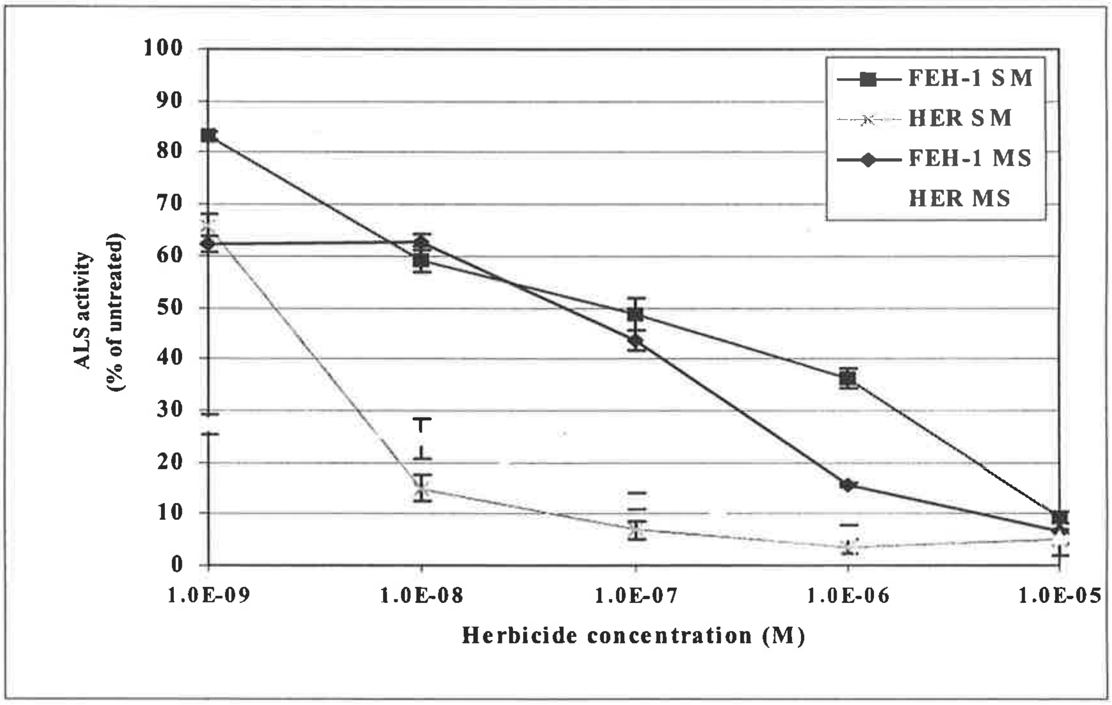


Fig. 8.4. Effect of metsulfuron-methyl (MS) and sulfometuron-methyl (SM) on activity of ALS from FEH-1 and cv. Herald.

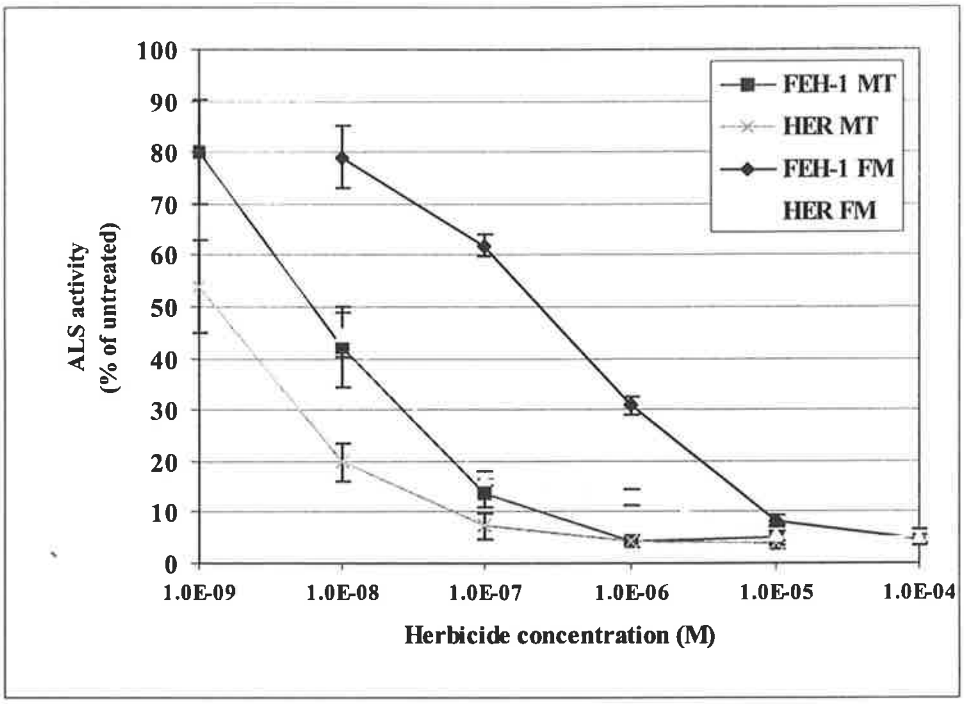


Fig. 8.5. Effect of flumetsulam (FM) and metosulam (MT) on activity of ALS from FEH-1 and cv. Herald.

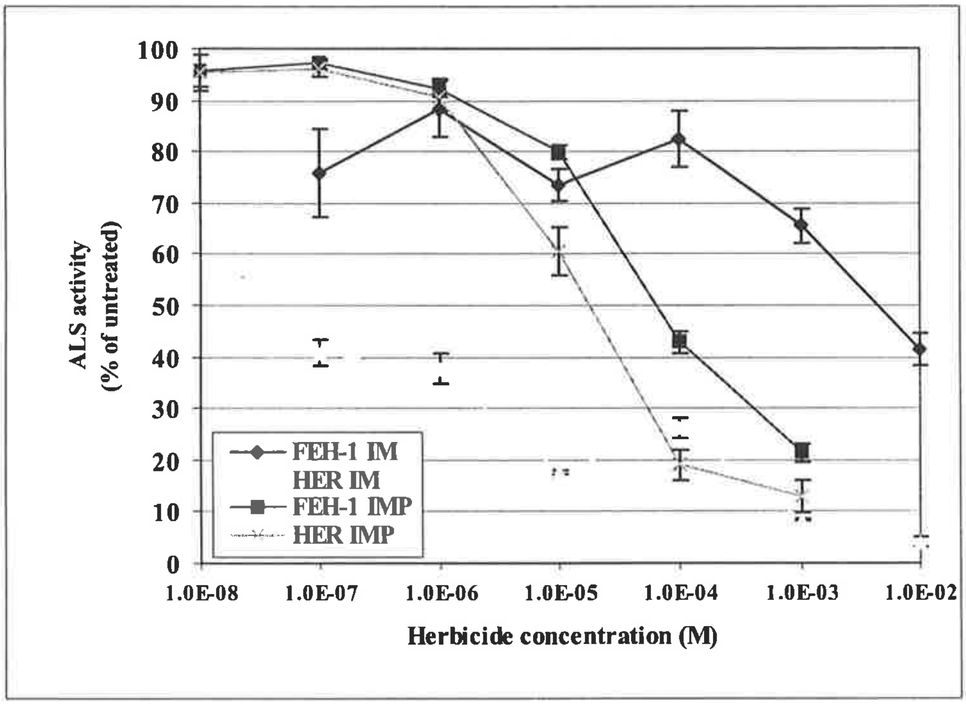


Fig. 8.6. Effect of imazethapyr (IM) and imazapyr (IMP) on activity of ALS from FEH-1 and cv. Herald.

Table 8.2. ALS enzyme response to three classes of ALS-inhibitors and relative activity of ALS in *M. littoralis* cv. Herald and FEH-1.

Herbicide	Class	Herbicide concentration (M) causing 50% ALS inhibition (I_{50})		Resistance ratio
		cv. Herald	FEH-1	(FEH-1 I_{50}/Herald I_{50})
Chlorsulfuron	SU	3.61×10^{-8}	3.34×10^{-7}	9
Metsulfuron-methyl	SU	$<10^{-9}$	1.14×10^{-7}	>141
Sulfometuron-methyl	SU	3.81×10^{-9}	2.67×10^{-7}	70
Triasulfuron	SU	5.98×10^{-8}	6.40×10^{-7}	11
Imazapyr	IMI	4.80×10^{-5}	8.37×10^{-5}	2
Imazethapyr	IMI	$<10^{-7}$	7.39×10^{-3}	>73,870
Flumetsulam	TP	$<10^{-8}$	5.38×10^{-7}	>54
Metosulam	TP	2.04×10^{-9}	8.15×10^{-9}	4
ALS activity*		452 (±114)	426 (±19)	1.061(±0.25)

* μmol acetolactate mg protein^{-1} h^{-1}

8.3.4 Mutation in FEH-1 ALS gene

DNA was successfully extracted from cv. Herald and FEH-1 leaf material. A PCR amplification, using primers 1 and 2, produced bands at approximately 300 bp, and primer 4 produced a band at c. 1400 bp, which was smaller than that expected (c. 1665 bp). Both bands were extracted, amplified by the PCR with fluorescent terminators, and then sequenced. No differences in ALS gene nucleotide sequence were found between Herald and FEH-1. However, a portion of the gene was sequenced for both cv. Herald and FEH-1, allowing Domains A and D to be eliminated as potential sites for mutation. Primer 1 was successful and provided sequence data for cv. Herald (two replicates) and FEH-1 (two replicates). Primer 2 provided data for FEH-1 only (one replicate), Primer 4 failed, and time constraints prevented use of Primer 5. The sequence obtained for the region sequenced spanning Domains A and D (Figure 8.7) showed no differences in base sequence between cv. Herald and FEH-1.

Figure 8.7. Base sequences for part of Region 1 of genomic DNA coding for ALS from *M. littoralis* cv. Herald (two replicates (R1 and R2) forward with primer 1(P1)) and FEH-1 (two replicates forward with primer 1 and one replicate reverse with primer 2 (P2)), and deduced consensus amino acid (AA) sequence compared to homologous region in ALS from *Brassica napus***. Amino acid (peptide) sequences for Domains A and D (in that order) are shaded in rows 8 and 9.

* Consensus base and amino acid sequence from the five *Medicago littoralis* cv. Herald and FEH-1 sequences.

** *Brassica napus* ALS sequence published by Bekkaoui *et al.* (1991).

```
POSITION          250 - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + 30
HER P1 R1           1                                                                 G G T T A C G C T C G T T C C T C T
HER P1 R2           2                                                                 G G T T A C G C T C G T T C C T C T
FEH-1 P1 R1         3                                                                 G G T T A C G C T C G T T C C T C T
FEH-1 P1 R2         4                                                                 G N T T A C G C T C G T T C C T C T
FEH-1 P2 R1         5                                                                 G G T T A C G C T N G T T C C T C T
M. litt. Bases*     6   >PRIMER1>>                                                    G G T T A C G C T C G T T C C T C T
B. nap.Bases**      7   T C A C G A A C A A G G A G G A G T C T T C G C C G C C G A G G G T T A C G C T C G T T C C T C C
M. litt. AAs*       8                                                                   G     Y     A     R     S     S
B. nap.AAs**        9                                                                   G     Y     A     R     S     S

POSITION          300 - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + 35
HER P1 R1           1 G G T C T A C C T G G T G T C T G C A T C G C C A C T T C C G G C C C T G G T G C C A C T A A T C T
HER P1 R2           2 G G T C T A C C T G G T G T C T G C A T C G C C A C T T C C G G C C C T G G T G C C A C T A A T C T
FEH-1 P1 R1         3 G G T C T A C C T G G T G T C T G C A T C G C C A C T T C C G G C C C T G G T G C C A C T A A T C T
FEH-1 P1 R2         4 G G T C T A C C T G G T G T C T G C A T C G C C A C T T C C G G C C C T G G T G C C A C T A A T C T
FEH-1 P2 R1         5 G G T C T A C C T G G T G T G T G C A T C G C C A C T T C C G G C C C T G G T G C C A C T A A T C T
M. litt. Bases*     6 G G T C T A C C T G G T G T c T G C A T C G C C A C T T C C G G C C C T G G T G C C A C T A A T C T
B. nap.Bases**      7 G G C A A A C C G G G A A T C T G C A T A G C C A C T T C G G G T C C C G G A G C T A C C A A C C T
M. litt. AAs*       8   G     L     P     G     V     C     I     A     T     S     G     P     G     A     T     N     L
B. nap.AAs**        9   G     K     P     G     I     C     I     A     T     S     G     P     G     A     T     N     L

POSITION          350 - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + 40
HER P1 R1           1 T G T C A G T G G A C T T G C T G A T G C T A T G C T T G A C A G T G T T C C G C T T A T T G C T A
HER P1 R2           2 T G T C A G T G N A C T T G C T G A T G C T A T G C T T G A C A G T G T T C C G C T T A T T G C T A
FEH-1 P1 R1         3 T G T C A G T G G A C T T G C T G A T G C T A T G C T T G A C A G T G T T C C G C T T A T T G C T A
FEH-1 P1 R2         4 T G T C A G T G G A C T T G C T G A T G C T A T G C T T G A C A G T G T T C C G C T T A T T G C T A
FEH-1 P2 R1         5 T G T C A G T G N A C T T G C T G A T G C T A T G C T T G A C A G T G T T C C G N T T A T T G C T A
M. litt. Bases*     6 T G T C A G T G G A C T T G C T G A T G C T A T G C T T G A C A G T G T T C C G C T T A T T G C T A
B. nap.Bases**      7 C G T C A G C G G G T T A G C A G A C G C G A T G C T T G A C A G T G T T C C T C T T G T C G C C A
M. litt. AAs*       8     V     S     G     L     A     D     A     M     L     D     S     V     P     L     I     A
B. nap.AAs**        9     V     S     G     L     A     D     A     M     L     D     S     V     P     L     V     A

POSITION          400 - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + 45
HER P1 R1           1 T C A C C G G T C A G G T T C C C C G G A N A A T G A T T G G T A C T G A T G C T T T T C A A G A A
HER P1 R2           2 T C A C C G G T C A G G T T C C C C G G A G A A T G A T T G G T A C T G A T G C T T T T C A A G A A
FEH-1 P1 R1         3 T C A C C G G T C A G G T T C C C C G G A N A A T G A T T G G T A C T G A T G C T T T T C A A G A A
FEH-1 P1 R2         4 T C A C C G G T C A G G T T C C C C G G A G A A T G A T T G G T A C T G A T G C T T T T C A A G A A
FEH-1 P2 R1         5 T C A C C G G T C A G G T T C C C C G G A G A A T G A T T G G T A A T G A N G C T N T N N A A G A A
M. litt. Bases*     6 T C A C C G G T C A G G T T C C C C G G A G A A T G A T T G G T A c T G A T G C T T T T C A A G A A
B. nap.Bases**      7 T T A C A G G A C A G G T C C C T C G C C G G A T G A T C G G T A C T G A C G C C T T C C A A G A G
M. litt. AAs*       8 I     T     G     Q     V     P     R     R     M     I     G     T     D     A     F     Q     E
B. nap.AAs**        9 I     T     G     Q     V     P     R     R     M     I     G     T     D     A     F     Q     E

POSITION          450 - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + 50
HER P1 R1           1 A C T C C G A T T G T T G A G G T A A C T A G G A T
HER P1 R2           2 A C T C C G A T T G T T G A G G T A A C T A G G A T
FEH-1 P1 R1         3 A C T C C G A T T G T T G A G G T A A C T A G G A T
FEH-1 P1 R2         4 A C T C C G A T T
FEH-1 P2 R1         5 A A N C C G A A A
M. litt. Bases*     6 A c T C C G A t t G T T G A G G T A A C T A G G A T
B. nap.Bases**      7 A C A C C A A T C G T T G A G G T A A C G A G G T C T A T T A C G A A A C A T A A C T A T T T G G T
M. litt. AAs*       8   T     P     I     V     E     V     T     R     ?
B. nap.AAs**        9   T     P     I     V     E     V     T     R     S     I     T     K     M     M     Y     L     V

POSITION          500 - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + - - - - - - - - - + 55
HER P1 R1           1
HER P1 R2           2
FEH-1 P1 R1         3
FEH-1 P1 R2         4
FEH-1 P2 R1         5
M. litt. Bases*     6                           <<<<<<<<<<<PRIMER2<<<<<<<<<<<
B. nap.Bases**      7 G A T G G A T G T T G A T G A C A T A C C T A G G A T C G T T C A A G A A G C T T T C T T T
M. litt. AAs*       8
B. nap.AAs**        9     M     D     V     D     D     I     P     R     I     V     Q     E     A     F     F
```

8.4 DISCUSSION

The results from the ALS *in vitro* assays clearly show that FEH-1 has altered ALS enzyme activity. The large differences in effect of some ALSIH on ALS between FEH-1 and cv. Herald (Table 8.2) may be the result of a changed enzyme structure, reflecting a change in the amino acid sequence caused by a nucleotide substitution on the ALS gene. There is also strong evidence against metabolism-based resistance. As discussed in 6.3.6, in all reported cases of metabolism-based resistance to chlorsulfuron there has been no cross-resistance to sulfometuron-methyl. FEH-1 is resistant to chlorsulfuron and is strongly cross-resistant to sulfometuron-methyl at the enzyme (Fig. 8.4), cellular (Fig. 8.2) and whole plant (Fig. 7.7) levels. It has also been shown (Christopher *et al.*, 1994) that malathion can disrupt metabolism of chlorsulfuron, but FEH-1 maintained resistance to chlorsulfuron in the presence of malathion (Fig. 8.1), further discounting resistance based on enhanced metabolism. Resistance was expected to be based on a mutated ALS gene because there have only been a few cases reported which are based on enhanced metabolism, and many documented cases based on mutated ALS genes (Devine and Preston, 2000; Saari *et al.*, 1994).

FEH-1 has an unusual pattern of cross-resistance. Results from 7.3.2 showed that, at the whole plant level, it has high resistance to imazethapyr, low resistance to flumetsulam, sulfometuron-methyl and triasulfuron, and slight resistance to chlorsulfuron and metsulfuron-methyl (Figs. 7.2 to 7.9; Plates 7.2 to 7.6). Results from the ALS assays (Table 8.2) generally concur with results from 7.3.2, except that resistance to metsulfuron-methyl appeared to be higher in the enzyme assay than with whole plants. ALS assay experiments (Table 8.2) also showed that FEH-1 had little or no resistance to imazapyr, but unusually there was high cross-resistance to imazethapyr, another imidazolinone. Similarly, FEH-1 had little or no resistance to metosulam, but exhibits low resistance to flumetsulam, another triazolopyrimidine herbicide. There was no data collected for the pyrimidinyl thiobenzoates. These results demonstrate that, assuming resistance is based on a mutated ALS gene, the level of resistance expressed can be measured at the enzyme, cellular or whole plant level.

Having established the mechanism of resistance and broadly defining the pattern of cross-resistance, it was of interest to determine the underlying molecular change present in the FEH-1 ALS gene. It was hoped that this could be achieved directly by sequencing the cv. Herald and FEH-1 ALS genes, and identifying any changes in the nucleotide sequence. As discussed in 8.3.4, a sequence for only part of the genes was obtained. It is useful to compare unknown ALS mutations to known and documented cases, because mutations frequently occur at one of a number of identified positions, and specific mutations are known to confer specific cross-resistance patterns (Devine and Preston, 2000).

Devine and Preston (2000) have summarised the position and cross-resistance patterns of a number of known ALS mutations over the five highly conserved Domains A, B, C, D and E (Table 1.1). The eight Pro_{197} (Domain A) and one Ala_{205} (Domain D) mutations can be excluded because the sequence for these domains was obtained and found to be identical for cv. Herald and FEH-1 (Fig 8.7). The Trp_{591} mutation in Domain B confers high SU, TP and IM resistance. FEH-1 is unlikely to have this mutation, because it has only low resistance to SU. The Ala_{122} mutation in Domain C confers low-zero resistance to chlorsulfuron and flumetsulam, and 7-fold resistance to imazethapyr (Bernasconi *et al.*, 1995 a; b). This matches the cross-resistance pattern for FEH-1, except that resistance to imazethapyr in FEH-1 is c. 10,000-fold higher. The final mutation in Table 1.1 is Ser_{670} in Domain E, conferring low resistance to SU, zero resistance to TP, and high resistance to imazapyr (Haughan and Somerville, 1990; Sathasivan *et al.,* 1991). This also partially matches FEH-1, except for the anomaly that FEH-1 resistance to imazapyr is zero-low. It is therefore not possible to clearly align the mutation in FEH-1 to any previously-described mutations, and there is the likelihood that it has a mutation which has not been described in detail. Attempts are continuing to obtain nucleotide sequences for the entire cv. Herald and FEH-1 ALS genes, and until this data is available the exact position and type of nucleotide substitution will remain unknown.

CHAPTER 9

CONCLUSIONS

9.1 EFFECT OF ALSIH SOIL RESIDUES ON ANNUAL *MEDICAGO*

9.2 NATURAL OCCURRENCE AND GENERATION OF ALSIH RESISTANCE IN ANNUAL *MEDICAGO*

9.3 CHARACTERISTICS AND POTENTIAL DEPLOYMENT OF FEH-1

9.1 EFFECT OF ALSIH SOIL RESIDUES ON ANNUAL *MEDICAGO*

Sulfonylurea herbicide soil residues have been a major factor in the decline of annual *Medicago* pastures in southern Australia. Chlorsulfuron residues are particularly damaging, but it is clear that triasulfuron also causes significant damage. In many areas with low annual rainfall (<350 mm) and alkaline soils there are no alternatives to cereal/pasture rotations, and vigorous annual medic production is crucial to the sustainability and profitability of these systems. Unfortunately, the combination of low rainfall and alkaline soil in these systems favours the persistence of sulfonylurea soil residues and, with little rotational flexibility, medics are often damaged. *M. littoralis* has the potential to be the dominant pasture legume over 10 million ha in New South Wales, South Australia, Victoria, and Western Australia, contributing an estimated $450 million dollars annually (J Howie, pers. comm., Dale Manson, pers. comm.). This potential contribution is severely reduced by widespread damage from soil residues of ALSIH, and previous studies have found that herbage production is typically reduced by 60-90% following application of chlorsulfuron (Evans *et al.,* 1993; Gillet and Holloway, 1996; Noy, 1996).

The results from this study confirmed that chlorsulfuron residues can cause severe damage to *Medicago littoralis*, with a 99% dry weight reduction recorded at one field site following application of chlorsulfuron at 22.5 g a.i. ha^{-1} in the previous year. At the same site, the low rate of 7.5 g a.i. $ha^{-1}$of triasulfuron applied in the previous season caused 50% dry weight reduction, thus highlighting the potential damage from this herbicide as well (section 3.3.2). Competition from summer weeds was also found to be very damaging, reducing medic establishment from 200 m^{-2} to 40 m^{-2} at one site (section 3.3.2). These results demonstrate that damage from triasulfuron residues is to some extent ameliorated by residual control of summer weeds, and provides some insight into farmer's perception that triasulfuron residues promote medic growth.

The data collected during paddock surveys (Table 5.1) confirms that chlorsulfuron use has declined markedly since its rapid and widespread adoption in the 1980s. Chlorsulfuron is a cheap and effective herbicide which controls a broad spectrum of weeds, and its use has declined primarily due to concerns about damage to sensitive species in cropping rotations, particularly annual medics. In areas with low rainfall and alkaline soils deployment of the ALSIH resistant line FEH-1, derived in this study from *M. littoralis* cv. Herald, may facilitate renewed use of chlorsulfuron without the associated medic production losses.

9.2 NATURAL OCCURRENCE AND GENERATION OF ALSIH RESISTANCE IN ANNUAL *MEDICAGO*

At the beginning of this study it was believed that resistant *Medicago* plants might be detectable, in paddocks with a substantial history of sulfonylurea use, or as selectable mutants within commercial seed. This belief was subsequently found to be optimistic, as screening of 723 selections with putative resistance, and around 32 million plants grown from commercial seed, did not identify any resistance (section 5.3). Analysis of the data and methods suggested that the scale of the screening was probably inadequate, due to the low frequency of resistant genes. These experiments suggested that the resistant gene frequency may be less than 10^{-7}, and also that it may be logistically impractical to increase the scale of screening to a level likely to succeed (section 5.4). The apparent low frequency of resistant genes is probably due primarily to the relatively few generations of these new elite cultivars since selection from a very narrow genetic base. Screening of known recognised genotypes (Chapter 4) also failed to identify lines with outstanding resistance, suggesting that the genus has very weak sulfonylurea metabolic detoxification mechanisms.

When no useful resistance could be identified from extant genotypes, it was necessary to attempt to create a new mutation in cells in tissue culture, or in seed. Tissue culture techniques are very suitable for selection of ALSIH resistance in cells, because there are published methods for callus initiation and culture, the desired trait is often based on a single nucleotide substitution in other species (Saari *et al.*, 1994), and the resistance is expressed at the cellular level. Once appropriate methods were refined, using EMS as a mutagen, cell lines with resistance to chlorsulfuron were generated routinely in a relatively short time (section 6.2.4). There appears to be no reason why similar results could not be obtained with any species for which methods for callus culture are known. The major constraint to this system is loss of the capacity for somatic embryogenesis, and in this study this prevented recovery of resistant plantlets from highly resistant cell lines. Future research on the *M. truncatula* cv. Jemalong 2HA line used in this research may discover a method to preserve or induce embryogenic competence following selection of resistant cell lines. If this could be achieved, the system would be more efficient, and probably more productive, than mutation of seed.

Although seed mutation yielded a resistant line, the process took almost two years (section 7.2.1).

The use of EMS to mutate medic seed resulted in the selection of the ALSIH resistant medic genotype FEH-1, and has provided exciting opportunities for commercial development and deployment of the genotype. The resistance could be easily transferred to other *M. littoralis* cultivars using artificial cross-pollination, and it is possible to cross *M.littoralis* with *M. truncatula* cultivars (J. Howie, pers. comm, 2000), thus providing resistant cultivars suited to most medic areas in southern Australia. In addition, due to the apparently novel pattern of cross-resistance and extremely high level of imazethapyr resistance, there may be commercial interest in transferring the mutated ALS gene to other species using transgenic technology.

9.3 CHARACTERISTICS AND POTENTIAL DEPLOYMENT OF FEH-1

Experiments (Chapters 7 and 8) have established that FEH-1 has altered ALS activity which confers resistance to a range of ALSIH, and herbicide experiments with whole plants have provided information about the likely level of field resistance (section 7.3.2). FEH-1 appears to tolerate field residues of chlorsulfuron and triasulfuron of around 1 ppb, which should allow it to grow in the year following herbicide application in most cases. It also appears to grow following direct foliar applications of 770 g.a.i ha^{-1} of imazethapyr, 80 g.a.i ha^{-1} of flumetsulam, 12 g.a.i ha^{-1} of sulfometuron-methyl, 3 g.a.i ha^{-1} of chlorsulfuron, 1.2 g.a.i ha^{-1} of metsulfuron-methyl, and 24 g.a.i ha^{-1} of triasulfuron (section 7.3.2). Further research is required to more accurately define resistance under field conditions.

Chapter 2 critically assessed the likely advantages and disadvantages of deployment of a resistant medic in a general sense, and concluded that on balance there was a strong case for development and release of a medic with resistance to ALS inhibiting herbicides. Having induced a mutation and selected a biotype of *M. littoralis* cv. Herald with resistance to ALSIH, it is very important to consider the ramifications of developing it as a cultivar and releasing it into southern Australian farming systems. In the following section the merits of commercial development and deployment of the selected line FEH-1 are specifically assessed, using current criteria developed for evaluating release of genetically modified oganisms GMOs.

The framework chosen for evaluation of the FEH-1 line is based on a Standing Committee on Agriculture and Resource Management (SCARM) document titled "Good agricultural practice – Guidelines for the use of genetically modified plants" (March, 1999). These guidelines were followed by a SCARM-commissioned report by a task force in the Plant Industry Commission (Agricultural and Resources Management Council of Australia and New Zealand) titled "A strategy to integrate herbicide tolerant crops and pastures into Australian farming systems" (November, 1999). This strategy discusses assessment of HR crops and pastures in general terms, and proposes a structure of roles and responsibilities for prudent introduction and monitoring of their deployment. The strategy proposes that "although the regulation of transgenic and non-transgenic HR crops and pastures may differ, both should be treated similarly when integrating into farming systems".

The SCARM guidelines (March, 1999) are intended to be the definitive directions for the development and release of GMOs in Australia, and incorporate consideration of the activities and responsibilities of the Genetic Manipulation Advisory Committee (GMAC), Australian Quarantine and Inspection Service (AQIS), National Registration Authority (NRA), and the Australia New Zealand Food Authority (ANZFA). GMAC are currently (2000) the body responsible for GMOs in Australia, but their role is soon to be taken by the Office of the Gene Technology Regulator (OGTR). A key feature of the guidelines is the requirement to convene two workshops; one during the Deliberate Release (open field trial) proposal stage, and a second during the General Release (commercial approval) proposal stage. The guidelines provide a checklist of questions for the workshops designed to ensure that all relevant issues related to the development and release of a GMO are adequately addressed. The checklist for workshop one is used below as the basis for this analysis. The checklist for workshop two is designed for use once extensive field experiments have been completed. The nature and scope of questions posed in the checklist from workshop one are similar to earlier checklists proposed by Millis (1995) and Dear *et al.* (1995).

It must be emphasised that the FEH-1 line produced by the mutation breeding and selection used in this study is not a GMO and does not possess transgenes. Although the methodology used in this study does not legally constitute "genetic transformation", there appears to be little difference between crops with herbicide resistance developed through plant breeding or tissue culture selection, and those produced using genetic engineering (Huppatz *et al.,* 1995; Gressel, 1997). Rigid statutory regulations in most countries require stringent evaluation of GMOs produced by transgenic methods. This creates an anomaly because virtually identical genotypes can be developed using traditional genetic manipulation methods (e.g. mutagenesis and/or cell selection). This has made non-transgenic methods more attractive, but in Canada the products of these technologies are now subject to increasing scrutiny (Conner and Field, 1995). Given that, potentially, medics selected using non-transgenic methods may be genotypically identical to a medic transformed with a known mutant ALS gene, it seems prudent to examine the likely attributes of a selected medic as if it were transformed.

The checklist from workshop one is reproduced in italics *verbatim* below, and answers are given in plain font. Where large sections of text are not applicable to the analysis only the major headings are reproduced.

1. POINTS TO BE CONSIDERED BEFORE FIELD TRIALS OF GENETICALLY MODIFIED PLANTS

Section A: Why use a GMO?

A1. What is the size and value to Australia of the crop into which the transgene has been introduced? In which region(s) is this industry most important?

Medicago littoralis is potentially the dominant pasture legume species for an estimated 10 million ha, comprising low rainfall areas (250 to 400 mm annual rainfall) with sandy to loamy alkaline soils (e.g. Mallee regions) in New South Wales, South Australia, Victoria and Western Australia. Agricultural systems in these areas are typically a two-year rotation of cereal and grazed pasture, so that an estimated 5 million ha of *M. littoralis* are grown each year. The value from annual medics is derived mainly from the value of produce from the grazed pasture and fixed nitrogen for the following crop. Sheep meat and wool production from volunteer pastures in these areas are typically \$70 $ha^{-1}yr^{-1}$ (\$350 million) and the value of nitrogen is estimated at \$20 $ha^{-1}yr^{-1}$ (\$100 million) giving a total estimate of \$450 million per annum (J. Howie, pers. comm.; D. Manson, pers. comm.).

A2. What problem is the GMO designed to overcome, or what benefits will be provided? Is the problem local, or Australia-wide.

M. littoralis with resistance to ALSIH is designed to overcome the problem of poor pasture growth due to carryover soil residues of sulfonylurea herbicides applied in the previous cereal crop. The problem is widespread over New South Wales, South Australia, Victoria and Western Australia in areas with low rainfall, and sandy alkaline soils. Where ALSIH soil residues are present, a resistant medic will provide increased productivity, utilisation and quality of medic-based pastures; greater competition for weeds of pastures; a more flexible under-sowing option; and assist with management of resistant weeds. More vigorous annual medic growth would increase soil nitrogen and organic matter, reduce cereal root diseases, provide high quality green and dry forage, increase soil water-holding capacity and drainage, reduce potential for soil erosion and provide an opportunity to reduce soil seed reserves of annual grass weeds in cropping rotations (Crawford, 1987a; Puckridge and French, 1983).

A3. Is the use of the GMO the most agronomically appropriate use of the gene?

Deployment of a gene for an ALS enzyme resistant to sulfonylurea herbicides is an agronomically appropriate use because it allows increased pasture production (with the benefits stated in A2) without promoting additional application of herbicides.

A4. What farming systems are likely to incorporate the GMO?

The resistant *M. littoralis* would be deployed in two-year cereal-volunteer medic pasture rotations. These rotations are constrained by low rainfall and poor soils. There are currently no economic pasture legume alternatives to *M. littoralis* in these areas.

A5. Will the introduction of the gene into the GMO prejudice the use of the gene in other plants or agricultural GMOs?

Introduction of this medic has potential to interact with future GMOs with similar genes in two ways; (i) contributing to the frequency of ALSIH use within the rotation being too high; or (ii) becoming a weed in a future GMO crop. Given the rainfall and soil type constraints on future GMOs with ALSIH resistance, in the short to medium term the crop would be expected to be a cereal or pasture legume. The introduction of this medic does not require additional application of ALSIH, therefore it is unlikely that its introduction would increase the frequency of use. Most cereals already grown have tolerance to ALSIH, so that the weediness problem would be similar to the current system. If, however, a more desirable GM pasture legume with ALSIH resistance is introduced, the resistant medic may become a significant weed of that pasture.

Section B: Plant biotechnology and plant breeding

Plant properties

B1. Are the genetics and population dynamics of the host plant well understood?

Yes. Medics have been the subject of ongoing breeding for decades in southern Australia. The ease of hybridisation between species is known for most of the cultivated species, and factors influencing persistence and vigour are well documented.

B2. Is the host plant self-fertile or outcrossing or both?

Annual *Medicago* species are self-pollinated (>99%). Spread of resistant genes in the field would be expected to very slow, and almost certainly restricted to other cultivars of *M. littoralis*.

B3. Does the plant produce numerous seeds? Does it have seed that persists in the soil (hard seed)? If so, what are the management options? Is the seed transported by wind, animals or any other natural means?

M. littoralis is an annual species with non-persistent roots and moderate seed production. Seeds survive in the soil for 5 to 8 years. The hard seeds of this species, needed for adequate persistence in cropping rotations, would contribute to the difficulty of eradicating any population. Unwanted resistant medics can be controlled using cultivation, or a wide range of non-ALSIH (see 2.3.2).

Seeds are held within a tough pod which is not readily transported by wind. Pods may be transported by animals as a contaminant of hair or wool, or seeds may survive passage through the gastro-intestinal tract of grazing animals.

B4. Does the plant naturalise in the environment, form a volunteer population in subsequent crops, or grow as a roadside weed?

Most annual medics, including *M. littoralis*, naturalise readily and grow in crops and disturbed waste places. They are already widely naturalised over most of temperate Australia.

B5. Does the plant have any wild or weedy relatives in Australia? If so, does the plant cross with any of them?

The rate of outcrossing in annual medics is extremely low and has not been observed in the field in Australia. The risk of gene transfer to naturalised *Medicago* is therefore considered negligible (Dear *et al.*, 1995). The greatest risk would be development of a herbicide resistant spiny species such as *M. polymorpha,* which can be weedy due to spiny pod contamination of wool, but the likelihood of such an inter-specific cross with *M. littoralis* is negligible (J. Howie, pers. comm., 2000).

Introduced DNA

B6. What property is to be introduced? Is the DNA fully characterised?

Resistance to a range of ALSIH has been introduced. It is very likely that the only change from the parent line (*M. littoralis* cv. Herald) is that of a single base mutation on the ALS gene (Saari *et al.*, 1994). Sequencing has commenced on the mutated gene and results suggest that the mutation is in Domains B, C or E (section 8.4).

B7. Does the transgene express in all parts of the plant or only in specific organs or at particular stages of the plant's growth?

Resistance is expressed at the enzyme, cellular and whole plant level. Evidence from similar mutations in other crops and weeds suggest that the trait will be expressed in all tissues.

B8. Does the introduced gene improve the plant's fitness?

The fitness of the resistant medic is likely to be the same as the parent line in the absence of ALSIH. It has significantly increased fitness in the presence of ALSIH. Enzyme activity measurements and comparative whole plant growth studies suggest that growth rate and fecundity are similar. This has been the case for most crop and weed studies with resistant ALS.

B9. Will a marker gene be used in construction of the GMO? If so, will it be removed after the wanted gene(s) is introduced, or remain in the GMO after general release?

There is no marker gene in this plant.

B10. If the marker gene remains after release, what property will be expressed and will this, or its coding DNA, in any way pose a hazard to the public, livestock, or the biota in the environment? Could it affect acceptance of the GMO or its products in the market?

Not applicable.

Section C: Farm systems

General

C1 What is the geographic distribution of the GMO? Will the GMO be grown in all areas? Is its management well understood?

M. littoralis is grown or naturalised throughout the cereal-sheep zone of southern Australia. If FEH-1 is commercially developed and released it is likely that it will be sown in arable areas of southern Australia with neutral to alkaline soils with an annual rainfall between 250 and 400 mm. Given the propensity of annual *Medicago* to spread and become naturalised in agricultural systems, it is likely that it will eventually occur throughout this range. Management of *M. littoralis* is already well understood, and management of FEH-1 will need modification to accommodate its herbicide resistance.

C2. Are there cultivars of the plant already in existence which carry other novel genes which could impact upon the use of the GMO?

No.

Management of the GMO in the farm system

C3. Thinking across time and space (e.g. paddocks, years, rotations), are there threats to farming systems or the environment arising from deployment of the technology?

The most likely threats to farming systems are the increased risk of herbicide resistant weeds developing due to increased ALSIH use intensity (discussed in 2.3.1), and the management constraints which might apply if FEH-1 needed to be controlled as a weed of cereal crops using non-ALSIH (discussed in 2.3.2). There is no reason to believe that FEH-1 will be any more invasive of the environment than *M. littoralis* because it will only have increased fitness in the presence of ALSIH, which are not normally applied outside of arable fields.

C4. What changes do you foresee to present management practices as a result of incorporation of the GMO into farming systems?

There are a number of probable changes to management practices:

(i) Application of chlorsulfuron and triasulfuron in cereal crops prior to volunteer medic pastures will increase in frequency and perhaps in rate, due to soil residues being less damaging.

(ii) The practice of under-sowing medics in cereal crops may increase if ALSIH are able to be applied in the cereal crop without killing the medics.

(iii) Non-ALSIH may be used more frequently in cereal crops if FEH-1 is dense enough to warrant control. The use of herbicides from another group will assist in a general weed resistance management strategy.

(iv) More productive pastures will provide the opportunity for increased livestock carrying capacity. However, livestock numbers will continue to be primarily influenced by the relative commodity prices for livestock and crops.

(v) Experimental results suggest that FEH-1 may be able to survive post-emergence applications of chlorsulfuron, metsulfuron-methyl, sulfometuron-methyl and triasulfuron. Despite sound agronomic advice to the contrary, it is likely that some managers will apply these herbicides directly to the FEH-1 pasture in a non-strategic way. This will almost certainly lead to accelerated development of weed resistance to ALSIH, necessitating more fundamental management changes.

C5: Will the presence of this GMO interfere with other crops or pastures, or other GMOs on the farm?

As discussed in C4 (iii) above, FEH-1 may require control in cereal crops with non-ALSIH to minimise yield loss through competition.

C6. What infrastructure is established in the industry to devise, implement and monitor compliance with the industry strategy for management of the transgene?

FEH-1 is not a GMO and will not be the subject of formal strategic management.

C7. Is it envisaged that the technology will be packaged such that producers will receive an information package or guidelines with seed and be tied to a contract?

It would be prudent to provide written information about the agronomic significance and best practice management of FEH-1 at the point of sale. This information would relate predominantly to sound management to minimise herbicide resistance in weeds. It is unlikely that the seed would be grown under contract.

Section D: Environmental issues

Whole plants

D1. Can the GMO be retrieved from the environment?

Medics have been selected for their ability to persist indefinitely in farming systems, often without re-sowing. Once whole paddocks have been sown to FEH-1 it will be practically impossible to retrieve it from the environment. The hard-seededness of medics, combined with their propensity to become naturalised would prevent retrieval.

D2. Will the GMO pose any threat as a weed in the environment, for example to remnant vegetation or to national parks? If it does, will it be a greater threat than normally bred or current varieties?

FEH-1 is expected to have the same invasive potential as its parent line *M. littoralis* cv. Herald, which is already widely naturalised. FEH-1 would only be more invasive in areas to which ALSIH are applied.

D3. Is there any threat from transfer of the GMO to other regions or farming systems?

Over time FEH-1 will almost certainly become naturalised in other regions and farming systems through passive spread.

GMO residues

D4. Will any GMO residues, including genetic material from the GMO in soil pose any hazard to the environment?

FEH-1 residues will be almost identical to *M. littoralis* cv. Herald residues. It is likely that increased vigour will result in higher levels of dry shoot material in early summer, but this would be utilised as valuable forage prior to seedbed preparation in the following year. Residues in soil will pose no threat to the environment.

Section E: Use of the produce from the GMO

Chemical residues

E1. Will the technology package cause any problems with chemical residues in produce for domestic or export markets (for example, as a result of increased or altered use of a herbicide or pesticide on the crop)?

The proposed deployment of FEH-1 will not increase residues in crops or pasture plants above existing levels. There are currently no known chemical residue issues in produce related to ALSIH use in the farming system.

E2. Will the technology package cause any problems with chemical residues in (i) grain, (ii) stubble, (iii) fodder fed to animals on farms?

No (see E1 above).

Section F: Herbicide resistance genes

Weed problem to be addressed

F1. What is the magnitude of the target weeds in crops?

ALSIH are currently applied to cereal crops in the farming system to control a wide range of broadleaved and grass weeds. FEH-1 will not typically be deployed to aid in control of any specific weed species, but rather will minimise the damage caused to following pastures as a result of controlling a broad spectrum of weeds with ALSIH.

F2. Is the herbicide to which the plant has been made resistant currently used to control volunteers of the crop?

Yes. Chlorsulfuron, metsulfuron-methyl and triasulfuron are currently applied to cereals to control a spectrum of weeds which sometimes includes annual medics. These herbicides are rarely applied primarily to control medics.

Introduced gene

F3. Does the transgenic plant build on existing tolerance found in the plant, or does it create a new use for the herbicide?

Experiments have demonstrated that FEH-1 has enhanced resistance to chlorsulfuron, flumetsulam, imazethapyr, metsulfuron-methyl, sulfometuron-methyl, and triasulfuron. Of these, the parent line *M. littoralis* cv. Herald has some existing natural tolerance (probably metabolic) to flumetsulam and imazethapyr, but is very sensitive to the other herbicides. The proposed deployment of FEH-1 does not create a new use for any of the herbicides.

Weed management strategy, including resistance management

F4. How does incorporation of the transgene fit in the context of integrated weed management on farm? Will the technology lead to sequential use in the crop rotation of like-herbicides, or of herbicides in groups known to give rise to cross-resistance problems?

The proposed deployment of FEH-1 does not seek to alter herbicide use patterns or alter weed management practices. It will primarily increase production in volunteer medic pastures. If deployment guidelines are followed, it is likely that the net impact on development of herbicide resistance will be neutral. Use of ALSIH in cereals may increase due to the amelioration of residual effects on medics in the following pasture, but this will be balanced by the requirement to rotate to non-ALSIH when control of FEH-1 is required in the cereal crops.

F5. Are the weeds, given their history and biology, likely to develop resistance to the herbicide used in the transgene-herbicide package, or cross-resistance to other herbicides? If so, describe the elements of a resistance management strategy which might be used for the transgene-herbicide package.

Sequential applications of an ALSIH readily leads to weeds with resistance to that herbicide, and often cross-resistance to others in the group. There is already a suite of weeds which have become resistant, and it is almost inevitable that there will be other species in the future. The proposed deployment of FEH-1 does not seek to alter current ALSIH use, and so it is not expected to significantly increase the rate of development of resistance. If, however, managers chose to apply ALSIH directly to FEH-1 pastures, then the selection pressure will increase and resistance will almost certainly increase. For this reason managers will be discouraged from applying ALSIH directly to FEH-1 pastures unless this is done as part of a sound strategic management plan.

F6. Are the weeds likely to be mobile to the point where a regional strategy for deployment of the technology will be required or desirable?

Resistant weeds, some highly mobile, have already developed and will continue to do so irrespective of whether FEH-1 is deployed. Deployment of FEH-1 will not influence the level of coordinated control already devised for existing herbicide resistant weeds.

Herbicide use in the management strategy

F7. Will the technology lead to greater use of herbicides (i) per hectare, or (ii) per region?

No, deployment of FEH-1 does not seek to increase herbicide use.

F8. Will the technology lead to use of more or less benign herbicides?

FEH-1 will contribute to the commercial life of ALSIH by reducing their negative impact on volunteer pastures. As a group these herbicides are thought to have very low mammalian toxicity, and are considered to be relatively environmentally benign. There are concerns about the persistence of some under specific conditions, but these relate to the effects of soil residues on following crops and pastures rather than human or environmental well being.

F9. Will the technology change the range of herbicides which can be used?

FEH-1 will present the new option of using a range of ALSIH in the pasture phase of the farming system. This will be generally discouraged to avoid accelerating the development of herbicide resistance in weeds. Most of these herbicides are already used in the cereal phase of the system, with the exception of imazethapyr and sulfometuron-methyl.

F10. Is the herbicide commonly applied in mixtures? Does this affect deployment of the technology?

ALSIH applied in cereal crops are sometimes applied in mixtures, but the partner herbicides have substantially shorter residual half lives in the soil. Although partner herbicides applied in crops will have negligible effects on medics in the following pasture phase, choice will be restricted if FEH-1 is under-sown in the cereal crop.

Section G: Insecticidal or nematicidal genes *(Not applicable)*

Section H: Viral resistance genes *(Not applicable)*

Section I: Fungal or bacterial resistance genes *(Not applicable)*

Section J: Genes altering quality *(Not applicable)*

This analysis has confirmed the general conclusion reached in chapter two that deployment of an annual medic with resistance to ALSIH is desirable, but there are several points of concern which need to be considered. The specific analysis of FEH-1 using the SCARM GMO checklist did not expose any new potential problems which were not previously addressed in Chapter 2.

There are clear benefits from growing a medic such as FEH-1 which can maintain vigorous production in the presence of ALSIH soil residues. Where these residues are present, deployment of FEH-1 would lead to increased productivity, utilisation and quality of medic-based pastures; greater competition for weeds of pastures; a more flexible under-sowing option; and assist with management of resistant weeds. More vigorous annual medic growth would increase soil nitrogen and organic matter, reduce cereal root diseases, provide high quality green and dry forage, increase soil water-holding capacity and drainage, reduce potential for soil erosion and provide an opportunity to reduce soil seed reserves of annual grass weeds in cropping rotations (Crawford, 1987a; Puckridge and French, 1983).

M. littoralis is used in a farming system which is currently constrained, by low rainfall and poor soils, to growing cereals and regenerating medic-based pastures. Sulfonylurea herbicides (e.g. chlorsulfuron and triasulfuron) are widely used in this system because they control a broad spectrum of weeds, are cheap, and are relatively benign for the user and environment. Deployment of FEH-1 will extend the commercial life and enhance the use of these herbicides, but will not seek to increase herbicide use. Given the basis for resistance, experience with many other crops and weeds suggests that FEH-1 will almost certainly be as productive as cv. Herald in the absence of herbicide residues. In the presence of herbicide residues FEH-1 would also suppress weeds more than cv. Herald through increased competition, with negligible risk of introgression of the gene into weeds.

The potential problems of most concern if FEH-1 is deployed are the inability to control the spread of the plant; the possibility that managers will choose to apply ALSIH directly to the pasture in a non-strategic way; and that FEH-1 may become a weed of cereal crops where ALSIH are used alone. FEH-1 will spread within and between regions and eventually become naturalised throughout the current range of *M. littoralis* cultivars. Once established, it would be almost impossible to retrieve from the environment. While it is no greater threat than *M.*

littoralis cv. Herald to natural areas, it is of concern that individual farmers will not be able to choose whether or not to use the technology. As a consequence, the choice to adopt the technology will not be an individual choice, but will have to be made collectively by farmers. This may be of particular significance to organic growers, many of whom are opposed to GMOs, who may not readily accept the distinction between FEH-1 and GMOs. It is therefore essential that farming representative organisations and the farming sector at large be fully briefed on any proposal to release a resistant medic and that a collective decision to do so is made only after thorough consideration of the discussion presented in this analysis. This stakeholder consultation will require significant time and resources.

Some managers will choose to apply ALSIH directly to the pasture in a non-strategic way. FEH-1 would be deployed primarily to overcome the growth reduction caused by residues of chlorsulfuron and triasulfuron. However, growers will inevitably discover that it is also possible to apply chlorsulfuron, metsulfuron-methyl, sulfometuron-methyl and triasulfuron directly to FEH-1 at sufficient rates to attain selective weed control. This may be a particularly attractive option in the case of sulfometuron-methyl because it is normally a non-selective herbicide. This practice would be actively discouraged in most cases to limit development of resistance in weeds, especially when ALSIH are also used in the cereal phase. Despite this, experience with herbicide resistance management in *Lolium rigidum* L. suggests that some farmers are likely to do this for short-term benefit, particularly if they are under acute financial pressure. There may, however, be some circumstances where strategic use of the cross-resistance may be acceptable.

FEH-1 may become a weed of cereal crops where ALSIH are used alone. Annual medics are occasionally weeds of cereal crops and are easily controlled by a range of herbicides, including most ALSIH. In situations where the density of FEH-1 in a cereal would warrant control there are a range of non-ALSIH which could be used. This rotation of herbicide mode of action would also delay development of herbicide resistant weeds.

There is a very small chance that FEH-1 also carries an unknown and inconspicuous mutation which might pose some threat to animals or humans. This also applies to new cultivars produced from spontaneous mutations or by crossing. In general, most of the public risk associated with herbicide-resistant crops or pastures comes not from herbicide use patterns, but from the biochemistry of transformed plants. Crop plants may either produce toxins, or produce novel and toxic herbicide breakdown products (Duke, 1995). Despite this, Millis (1995) observes that literally tens of thousands of analyses have been done on a range transgenic crops, including transgenic canola, cotton, potato, soybeans and tomato, with no significant unintended changes to plants found. She concluded that there now seems little need for such exhaustive testing, except where the host plant, or near relatives, contain undesirable chemistry (e.g. alkaloids in *Solanum* species).

A typical deployment of FEH-1 would begin with application of chlorsulfuron or triasulfuron in a cereal crop to control weeds and susceptible *Medicago* cultivars, to reduce the soil seed bank surviving into the following pasture phase. FEH-1 would then be sown with adequate fertiliser and arthropod control, then grazed in a manner which allowed adequate seed set to establish a soil seed reserve for subsequent regeneration every second year. This may also involve grass control if grass densities are high. Alternatively, FEH-1 might be under-sown in the cereal crop, and triasulfuron applied strategically at a low rate to control some weeds, but allow FEH-1 growth and seedset. Once a soil seed bank is established FEH-1 could be managed as any other *M. littoralis* cultivar. In special circumstances there may be merit in one or two strategic foliar applications of chlorsulfuron, flumetsulam, imazethapyr, metsulfuron-methyl, or triasulfuron to contribute towards an integrated control strategy for a weed which is difficult to control with available herbicides in cereals.

On balance there appears to be a strong case for further development and release of FEH-1. Pasture legume breeders have been consulted and have verified the potential commercial value of FEH-1. Further research is required on important characteristics (e.g. palatability, hard-seededness, relative productivity, persistence, insect and disease susceptibility, and field resistance to ALSIH) which should be determined before release. Before further research is undertaken organisations representing farmers, agronomy and livestock researchers and advisers, and a number of individual farmers should be consulted to ensure that there is no significant initial opposition to the principle of release. These people should be consulted again if results from further field research encourage commercial development of FEH-1.

This research has successfully provided a potential practical solution to a significant field production problem, combining and applying a range of eclectic techniques from related studies. The techniques used in this study are not as sophisticated or precise as transgenic techniques. However, the results have shown that it is possible to generate and deploy resistant plants more rapidly than is currently the case for transgenic plants, because plants derived by the method used to select FEH-1 are not currently deemed to be genetically modified organisms (GMOs), and as such are not subject to rigorous and lengthy legislated testing protocols. The techniques can also be applied to elite lines, allowing rapid deployment of selected traits in agronomically desirable genotypes. It may be possible to apply similar techniques to crops of local significance which do not warrant large research and development investment from commercial organisations. For example, lentils are extremely sensitive to residues of sulfonylurea herbicides, and production in southern Australia is currently constrained by these residues. There are published methods for callus culture and regeneration of lentils, and there is an opportunity to increase the area available to lentil production through the generation of sulfonylurea-resistant cultivars.

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