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Resistance to ALS inhibiting herbicides in UK populations of the grass weed *Alopecurus myosuroides*

Doctor of Philosophy

School of Plant Sciences

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BBSRC CASE Studentship based at Rothamsted Research with support from DuPont (U.K.) Limited

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Abstract

The objective of this project was to characterise resistance to sulfonylurea herbicides in UK biotypes of the grass-weed *Alopecurus myosuroides*. Investigations included field studies, glasshouse bioassays, biochemical and molecular analyses. Responses to the selective sulfonylureas mesosulfuron-methyl + iodosulfuron-methyl sodium mixture and flupyrsulfuron-methyl were investigated, while extensive use was made of the non-selective sulfonylurea herbicide sulfometuron-methyl as a screen for possible ALS target site resistance in *A. myosuroides*.

Glasshouse tests identified three populations from the UK where resistance to sulfonylurea herbicides was present. DNA sequencing of the ALS gene confirmed that a single point mutation segregated with resistance in these populations. All highly resistant individuals showed a single point mutation in the first position of the Pro197 codon of an *A. myosuroides* ALS gene, conferring a predicted proline to threonine target site change. Enzyme assays confirmed that resistance was due to an altered form of the ALS enzyme less susceptible to inhibition by sulfonylureas, making this the first fully characterised case of ALS target site resistance in a European grass-weed.

Results from segregation of sulfonylurea resistant and susceptible phenotypes in crossing experiments indicated that ALS target site resistance in *A. myosuroides* is conferred by a single, dominant nuclear allele but that additional effects are also present. An association was found between parental resistance levels and the degree of resistance in progeny.

Field work at one site with ALS target site resistance showed that distribution of resistant plants was uneven. The highest proportions of resistant plants were concentrated in a single area with lower levels present across the whole field. No evidence of the resistant trait was found in neighbouring fields. ALS target site resistant *A. myosuroides* is predicted to increase with more widespread use of mesosulfuron+iodosulfuron in coming years.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed:

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Acknowledgements

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1. Introduction

1.1 Weed control in arable cropping systems

1.1.1 Motivation for weed control

Weeds are plants that occur in managed ecosystems such as farmland, forestry, gardens, public areas and industrial land where they can be considered pests, often reducing crop yields or otherwise damaging the potential of the system for human use. Weeds provide a recurrent threat to agricultural production across all arable systems and so agricultural weed control is a constant priority. Successful weeds tend to have certain characteristics associated with their biology, often referred to collectively as 'weediness', which amount to the ability of a plant to successfully colonise a particular managed ecosystem, often out-competing or displacing other species in the process. The weediness potential of any particular species can be summed up according to several characteristics associated with a successful weed (Baker, 1965). These include: discontinuous germination and persistence in the seed-bank, rapid seedling growth upon germination, rapid attainment of reproductive growth stage, a long seed production period and high seed output, special adaptations for seed dispersal, germination and seed production under a wide range of different conditions, self-compatibility but not obligatory self pollination, use of the wind or an unspecialised pollinator in the case of outcrossing, good competitiveness, and the ability to regenerate via vegetative methods, if perennial. In general these characteristics favour flexibility over specialisation and allow the successful weed to thrive across a range of different environmental situations.

Weeds have the potential to interfere with agricultural productivity and so require management to keep numbers under control. Specifically weeds can cause problems for farmers by reducing crop yields, decreasing crop quality, and interfering with harvesting machinery. Weeds reduce crop yield in several ways. Firstly, weeds use up nutrients that would otherwise be available for crop plants, resulting in poorer crop growth. Secondly,

weeds use up water, reducing the amount available to crop plants. Thirdly, weeds may shade crop plants. In addition to these economically motivated reasons, farmers also attempt to control weeds for aesthetic reasons. Many people regard weed control as a 'good thing' and weed-free fields are associated with farming skill, regardless of crop yield or profitability. This attitude has the potential to lead to weed control beyond what makes sense economically and in turn can lead to increased water pollution and herbicide resistance development. For example, some weed species do not cause heavy yield loss at low plant densities while also providing food and/or habitat for beneficial fauna. Efforts to control such species can be counter productive. Finding a balance between yield loss and excessive management is a major challenge for farmers, and research carried out in this area has the potential to provide benefits in terms of more cost effective weed control, while maintaining biodiversity and decreasing herbicide usage.

Most research on crop-weed interactions has examined the effect of a single weed species on a particular crop. Yield reduction is used in combination with data on the costs of weed removal to produce economic weed threshold levels which are defined as the level of weed infestation above which the net return of the best herbicide option exceeds the no-herbicide option (Munier-Jolain *et al.*, 2002). This approach takes account of the fact that below certain weed density levels, crop productivity is not seriously affected and therefore removing weeds is not economically justified. Weed threshold values depend on many factors, with crop competitiveness being very important. Winter cereal crops are relatively strong competitors with weeds and typical fixed thresholds are 20 to 30 plants m^{-2} for grass weeds and 40 to 50 plants m^{-2} for most broad leaved weeds (Gerowitt & Heitefuss, 1990; Zanin *et al.*, 1993). Weed threshold values currently have potential as a useful tool in arable systems with low weed pressure or in high weed pressure systems, after application of weed control measures, to assess the potential of weed escapes in seed return. Thresholds are more applicable in competitive cereal crops for example than in high value vegetable crops in systems with highly competitive weeds, where tolerable weed numbers may be near zero (Swanton *et al.*, 1999).

1.1.2 The integrated weed management approach

Basic weed economic threshold values have some drawbacks in that they only consider the risk of yield loss from a single crop weed interaction in a single cropping year and fail to take account of seed return and long term trends (Munier-Jolain *et al.*, 2002; Zanin *et*

al., 1993). Attempts have been made to address this problem through the formulation of more detailed models. These include estimation of multiple weed species effects (Swinton *et al.*, 1994), timing of weed emergence (Cousens *et al.*, 1987), and 'economic optimum thresholds' taking account of seed return and associated net benefits over the long term (Cousens *et al.*, 1986). The idea of weed thresholds, and in particular detailed mechanistic threshold modelling approaches over empirical weed crop interaction data, are important elements in the goal of integrated weed management systems that are reliable and usable across a wide range of farming systems (Swanton & Murphy, 1996). Integrated weed management (IWM) incorporating crop breeding, fertilisation, rotation, chemical and mechanical weed control, competition, seed return, and soil management has the goal of reducing the negative effects of weed species through a logical integrated approach, while maintaining acceptable crop yields and minimising adverse environmental effects. While agricultural producers have been slow to accept weed thresholds as a management tool, research in this area has shed new light on the complex problem of economically and environmentally viable and sustainable weed management, thus increasing understanding of crop-weed interactions. In future it is hoped that this research will provide the basis for more robust and workable IWM systems.

1.1.3 Current trends in European cereal cropping systems

Since the introduction of selective herbicides, cereal cropping systems in Europe and the UK have moved away from traditional weed control methods such as rotations, spring cropping, and ploughing for reasons of cost and effectiveness (Moss, 1987a). A recent survey of the European agrochemical market showed that spending on herbicides was €2.8 billion which amounted to 41% of the total agrochemical spend, with fungicides making up 35% and insecticides and other pesticides making up the remainder. This is in line with global figures where herbicides represent over 45% of total agrochemical sales (AGROW World Crop Protection News, 2005). AGROW (2005) also reported a continued rise in overall pesticide use coupled with a trend towards ever increasing farm size and overall efficiency, emphasising the continued reliance on agrochemicals, especially herbicides, in modern European farming. Wheat growing in the UK is currently high input/output based with typically around 200kg N ha^{-1} applied annually, high herbicide and fungicide inputs, and yields averaging around 8t ha^{-1}. While successful integrated management systems remain a goal of much research effort in weed science, and there are signs of limited implementation, (Anderson, 2005; Blackshaw *et al.*, 2006),

the present trends are towards intensification and higher agrochemical inputs in general. Currently cereal crops are the most widely grown arable crops in the UK; figures from 2005 show a total of around 2.93 million hectares of cereals with 1.87 million hectares of wheat, 0.94 million of barley and the remainder as a mixture of oats, rye, corn and triticale, with the total arable area for the UK in 2005 being 4.44 million hectares (DEFRA, 2005). The importance of cereal cropping systems in UK farming means that an understanding of effective weed management in cereals is especially important in order to maintain yields and quality in this important arable sector. Weeds infesting UK winter cereal crops include a wide range of grass-weeds and also many broadleaved species presenting a complex management problem for farmers. Several grass-weed species currently provide the greatest problem with regards to control by herbicides and these include *Alopecurus myosuroides* Huds. (black-grass), *Anisantha sterilis* (L.) Nevski (barren brome), *Avena* ssp. (wild oats), *Bromus commutatus* Schrad. (meadow brome), *Lolium multiflorum* Lam. (Italian rye-grass) and *Poa annua* L. (annual meadow grass) (Clarke *et al.*, 2000).

1.2 *Alopecurus myosuroides* biology, control, and the problem of herbicide resistance

1.2.1 *Alopecurus myosuroides biology*

Alopecurus myosuroides (black-grass) is an annual allogamous species possessing a self incompatibility system, meaning that obligate out-breeding can be assumed for most practical purposes, although self pollination does take place to a limited extent. Cytological studies reveal *A. myosuroides* to be diploid (2n=14 chromosomes). The species exhibits a high level of genetic polymorphism, with around 60 per cent of loci being polymorphic, as well as a particularly low proportion of total genetic diversity being caused by differences between populations from different geographical locations in contrast to the proportion due to within population diversity (Cavan *et al.*, 2000b; Chauvel & Gasquez, 1994; Sieber & Murray, 1979). Genetic diversity in this case is defined as the expected heterozygosity under Hardy-Weinberg equilibrium for each population, and overall diversity within the species is partitioned into between- and within-population diversity (Cavan *et al.*, 2000b). High within-population diversity coupled with low levels of inter-population divergence is typical of out-crossing species

and has potential consequences for the spread of herbicide resistance (see section 1.2.2). *Alopecurus myosuroides* is a common weed of winter cereals in the UK with an economic threshold of around 12 plants m^{-2} in winter wheat. Studies show that the competitiveness of *A. myosuroides* with wheat can vary depending on location and year and this is attributed to factors such as soil type, nitrogen level, and weather with rainfall being particularly important (Clarke *et al.*, 2000). The variability of *A. myosuroides* competitive impact depending on local conditions serves to highlight the difficulty of implementing an integrated weed management approach for this weed and so herbicides are a very important part of many management strategies.

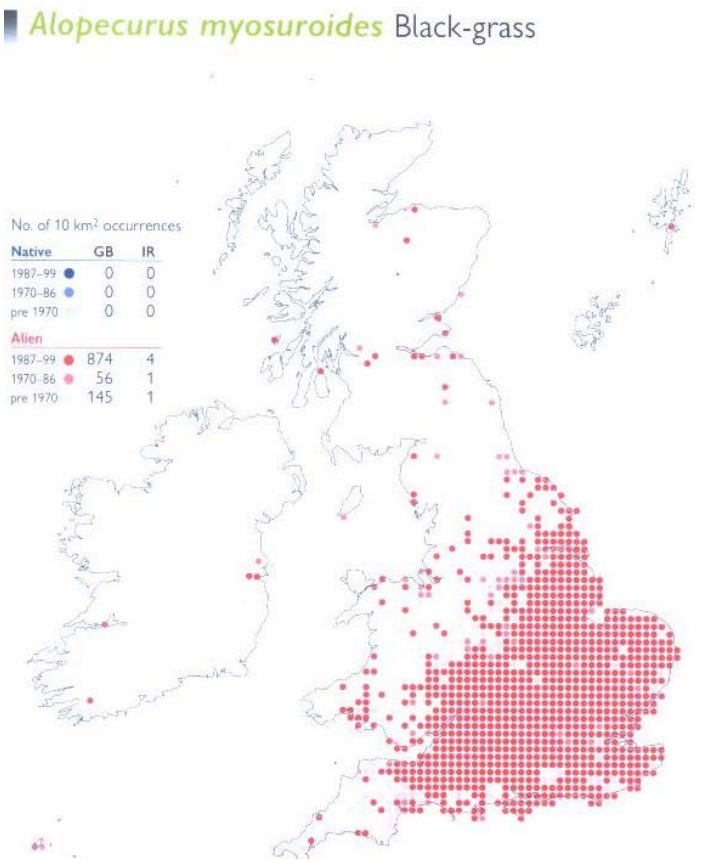

Figure 1.1 The distribution of *Alopecurus myosuroides* in the British Isles. Each dot represents at least one record in a 10 km square of the National Grid. Taken from "New Atlas of British and Irish Flora" (Preston *et al.*, 2002)

Lowland arable areas in central, southern and eastern parts of England are most likely to suffer from heavy infestations of *A. myosuroides* (see Figure 1.1), and high densities are

most common in winter cereal monoculture where cultural control measures such as rotations, ploughing and late drilling are not practised. Management of *A. myosuroides* populations in winter cereal cultivation is made more difficult by its germination pattern, with plants emerging from late autumn, into spring (Chapman, 1996; Moss, 2002a; Naylor, 1972). *A. myosuroides* is sensitive to nitrogen levels and crop density (Colbach & Sache, 2001) with seed quality and quantity varying accordingly. As many as 7614 seeds have been observed per plant with lower limits of around 100 depending on conditions and timing of germination (Naylor, 1972). In terms of the weed seed bank, *A. myosuroides* seeds are relatively short lived in the soil (Moss & Cussans, 1985) and this is why ploughing is such a useful cultural control method for this particular weed (Cavan *et al.*, 2000a; Chauvel *et al.*, 2001).

1.2.2 Potential for the development of resistance in *Alopecurus myosuroides*

Herbicide resistance can be defined as the inherited ability of a weed to survive a rate of herbicide which would normally result in effective control (Moss, 2002a). Resistance builds up in a weed population as the proportion of resistant individuals increases over time due to repeated selection by herbicide. The build up of resistance in any weed population depends on a number of factors and these can potentially be used to slow down its development through careful weed management strategies. Most cases of resistance to herbicides occur in weed populations where the same herbicide, or herbicide mode of action, has been used frequently over a number of years. Using this information it has been shown that using more than one herbicide mode of action in successive years slows the development of resistance quite considerably in a model *A. myosuroides* population (Cavan *et al.*, 2000a).

Development of resistance in a weed population also depends on the particular resistance mechanisms involved: for example the initial frequency of the resistant trait in weed populations, single gene versus polygenic resistant traits, the mating system of the weed species in question, the dominance of the resistant trait, possible fitness penalties for resistant individuals and differences in selection pressure all have to be taken into account (Maxwell & Mortimer, 1994). The spread of a dominant resistant trait in an out-crossing species like *A. myosuroides* for example will be very different from that observed in a primarily self-pollinating species, with gene flow through dispersal of pollen leading to more rapid and widespread development of other resistant populations nearby compared

to spread through seed movement. Understanding these phenomena better should allow the implementation of cultural control methods best suited to particular resistance problems.

Moss (1985) listed several characteristics of *A. myosuroides* that favour the development of herbicide resistance. These include high reproductive capacity allowing populations to increase very rapidly; the absence of a large dormant seed-bank which, coupled with minimum tillage cultivation systems, means that changes in the population genetic makeup in response to herbicides are subject to minimal dilution by older seeds; and the frequent absence of crop rotations in arable systems susceptible to infestation with *A. myosuroides*. Reliance on herbicides, along with a reluctance to implement routine cultural control methods such as ploughing, spring cropping, and late drilling, compound the problem and means that the pressure on herbicides as the only method of control is very great. A ban on stubble burning as a means of cultural control since 1992 has also favoured the heavy reliance on herbicides for *A. myosuroides* control in winter cereal crops. Encouraging farmers to adopt cultural control where available as a matter of course would greatly relieve the selection pressure from herbicides and delay the development of resistance (Cavan *et al.*, 2000a; Chauvel *et al.*, 2001).

The terminology used in studies of herbicide resistance weeds like *A. myosuroides* which are resistant to several different herbicides is often ambiguous. In particular there are no universally accepted definitions of the terms cross resistance and multiple resistance, and this can cause confusion when talking about different resistant *A. myosuroides* populations. Hall *et al.,* (1994) define cross resistance as: "Expression of a mechanism that endows the ability to withstand herbicides from different chemical classes. Cross resistance may be conferred either by a single gene or, in the case of quantitative inheritance, by two or more genes influencing a single mechanism". Multiple resistance is defined by the same authors as: "Expression (within individuals or populations) of more than one resistance mechanism, endowing the ability to withstand herbicides from different chemical classes". In this thesis the term cross resistance has been used exclusively, with the details of mechanism explained in the text. The reason for this is that separating multiple and cross resistance can cause confusion and is not strictly necessary. For example a single population could be said to show 'cross resistance' to different herbicides based on an enhanced metabolism mechanism or 'multiple resistance' to the

same herbicides based on defining two different types of enhanced metabolism (eg P450 vs GST). It was felt that use of the term cross resistance to cover both meanings was less confusing provided mechanistic explanations accompanied each specific case.

1.2.3 The current situation: herbicide resistant *Alopecurus myosuroides*

Herbicide resistant *A. myosuroides* is a particular management problem in winter cereals across the low-lying arable areas of southern and eastern England and also on a wider scale in France, Germany, Belgium, Israel, Switzerland, Spain, Netherlands, and Turkey (Heap, 2006). A large scale survey on the weeds of winter cereal crops in the UK from 1989 showed *A. myosuroides* infesting 38% of fields in total with levels reaching 70% of fields in the arable areas of Eastern England making it the third most common grass weed in Britain overall and the most prevalent grass weed of lowland eastern areas at the time (Whitehead & Wright, 1989). The first case of herbicide resistant *A. myosuroides* in the UK was from a site near Faringdon in Oxfordshire in 1982, where plants exhibited partial resistance to the substituted phenyl-urea herbicide chlorotoluron, an inhibitor of photosynthesis at photosystem II. This was followed in 1984 by the Peldon population from Essex which exhibited higher levels of chlorotoluron resistance (Moss & Cussans, 1985). Since that time resistance to inhibitors of acetyl-CoA carboxylase has also been confirmed at Faringdon (Cocker *et al.*, 1999), and the Peldon population has emerged as one of the most highly resistant in the country with cross resistance to a wide range of different herbicides and herbicide modes of action (Kemp *et al.*, 1990; Moss & Cussans, 1987). More recently *A. myosuroides* from Peldon has exhibited resistance to the acetolactate inhibitor flupyrsulfuron-methyl (Moss *et al.*, 2005b) with the first cases being picked up around 1999 (Steve Cranwell, 2006, personal communication). Overall Peldon has one of the most herbicide resistant and extensively studied populations of *A. myosuroides* in the country and provides a unique resource for work on the mechanisms of resistance in this weed species.

Herbicide resistant *A. myosuroides*, along with *Lolium* ssp. (rye-grass), is currently the major resistance problem within Europe and resistant biotypes have been confirmed across a range of different countries (Moss, 2003). Surveys conducted in order to assess the scale of resistance in any weed species have presented problems since they are often biased towards populations where control problems have already been reported. Fully random resistance surveys are rare due to the amount of work involved and often tend to

be small scale. The most recent survey of herbicide resistance in British weeds lists *A. myosuroides* as being present on 2085 farms across 31 counties in England, equivalent to around 10% of the total number of farms estimated to be using herbicides to control this weed (Moss *et al.*, 2005a), see Figure 1.2.

Resistance in *A. myosuroides* was shown to a wide range of different herbicides including the acetyl-CoA carboxylase inhibitors fenoxaprop, clodinafop, cycloxydim and sethoxydim; the acetolactate synthase inhibitors flupyrsulfuron and mesosulfuron/iodosulfuron; and the inhibitors of photosynthesis at photosystem II isoproturon and chlorotoluron. The proportion of samples showing resistance to one or more herbicides in the most recent tests was 87%, with resistance to the acetyl-CoA carboxylase inhibitor fenoxaprop being the most common, averaging 83%. Compared to the previous large scale compilation of data for herbicide resistant UK weeds from 1999, the figures for 2005 represented almost a three fold increase in the number of farms with herbicide resistant *A. myosuroides* (Moss *et al.*, 1999).

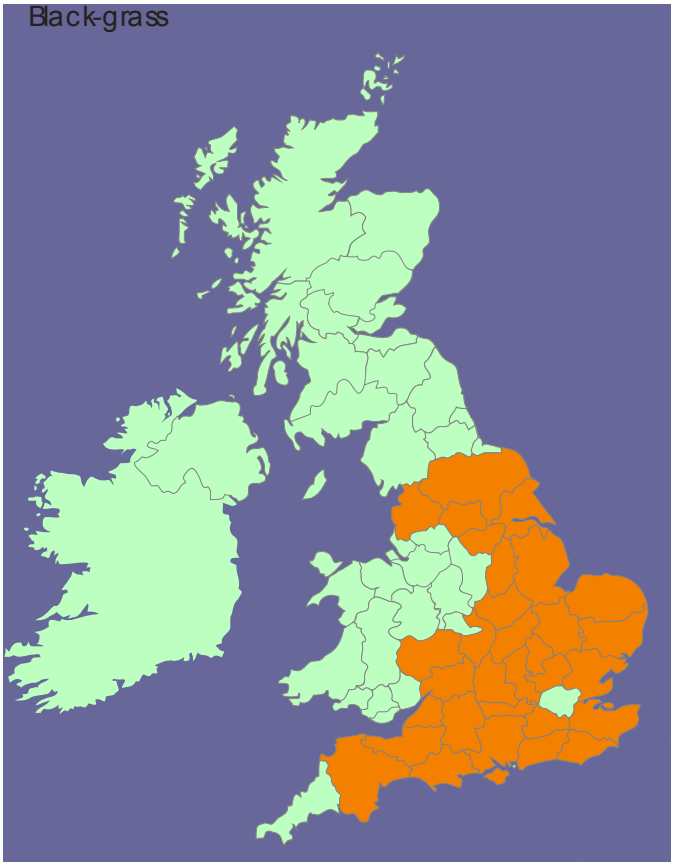

Figure 1.2 The distribution of herbicide-resistant *Alopecurus myosuroides* in the

British Isles (Moss *et al.*, 2005a). Counties where resistance is present are marked orange. One of the most comprehensive recent European surveys of resistant *A. myosuroides* came from the wheat growing areas of northern France in 2006 (Menchari *et al.*, 2006). This work, supported by the local growers' cooperative, went further than most large scale surveys in that it addressed the spatial distribution of seven different resistant alleles of the acetyl-CoA carboxylase (ACCase) gene on two different geographical scales. In total over 130000 plants were genotyped from 243 fields in the Cote d'Or district (random selection) and across the whole of France (resistant samples only). The results seemed to show a lack of spatial structure in terms of different resistant ACCase alleles. It was concluded that the most likely explanation for this distribution was multiple independent appearance of resistant ACCase alleles, with restricted propagation due to gene flow from pollen dispersal or transport of seeds through human activity. This survey is the only one of its type in *A. myosuroides* at the time of writing. As an approach it has an advantage over traditional resistance surveys in terms of the detailed information generated but was very time consuming and expensive and also focussed on a single herbicide mode of action. A smaller field scale experiment taking a genetic fingerprinting approach reached similar conclusions based on genetic differences between and within ACCase resistant and susceptible patches on a single farm (Cavan *et al.*, 1998). This study found no evidence that resistance had spread between patches, with genetic distances between resistant plants from different patches not related to geographical distance between patches. The French study went further in postulating likely dispersal mechanisms for resistant ACCase alleles but this was highly speculative. It is worth noting that much less detailed surveys can yield useful information, for example on the mechanisms of resistance, and provide usable information to farmers to form the basis of decision making. A similar survey with acetolactate synthase resistant alleles in *A. myosuroides* would be a very worthwhile goal for the future in the UK, especially since the introduction of the highly effective ALS inhibiting herbicide mixture mesosulfuron+iodosulfuron in 2003.

1.3 Resistance and resistance mechanisms

Herbicide resistance, and in particular resistance to acetolactate inhibiting herbicides, is a growing problem worldwide. Currently there are 185 confirmed resistant weed species and 310 unique herbicide resistant biotypes, of which 95 are resistant to ALS inhibitors,

65 are resistant to PSII inhibitors and 35 are resistant to ACCase inhibitors. The remainder are resistant to other herbicide modes of action such as inhibitors of microtubule assembly, and synthetic auxin mimics (Heap, 2006). Grasses make up a significant proportion of the known herbicide resistant species and in 2003 accounted for 33% of all resistant species and 40% of resistant biotypes (Moss, 2003). Of the 95 weed species resistant to ALS inhibitors, grass weeds are under represented with only 21 resistant species recorded to date (Heap, 2006).

1.3.1 The history of herbicide resistance

The first selective herbicides, 2,4-D and MCPA, were introduced in the late 1940's and provided agriculture with a powerful new weapon against weeds, allowing control of broad-leaved weeds in cereals. Prior to the introduction of selective herbicides weed control relied on cultural methods such as mechanical tillage, spring cropping, crop rotation and stubble burning. The introduction of selective chemical herbicides meant that land could be farmed with much reduced manpower and allowed more intensive production with reduced need for rotations and unprofitable crops. 2,4-D and MCPA belong to a group of herbicides known as synthetic auxins and work by mimicking the action of plant growth hormones. Resistance to synthetic auxin herbicides is relatively uncommon and only 36 unique resistant biotypes have been identified to date, despite the extended period of time these herbicides have been in use (Heap, 2006). Disadvantages of auxin mimics include their high application rates and their relatively poor selectivity with the potential to cause damage to non-target plants. Alternatives to the first generation of herbicides were soon developed to meet the growing need for selective weed control in a wide range of crops. The urea and carbamate herbicides, inhibitors of photosynthesis and cell division respectively, were first marketed in the early 1950s, closely followed by the triazines (also photosynthesis inhibitors), allowing control of grass weeds in broad-leaved crops for the first time. Most other modes of action in use today were released between the mid 1950s and late 1970s, including important groups such as the acetyl-CoA carboxylase inhibitors (inhibition of lipid synthesis), the dinitroanilines (inhibitors of microtubule assembly) and the thiocarbamates (inhibition of lipid synthesis, not ACCase). Only three new modes of action have been introduced since 1980 (Reade & Cobb, 2002), of which the ALS inhibitors are by far the most important commercially.

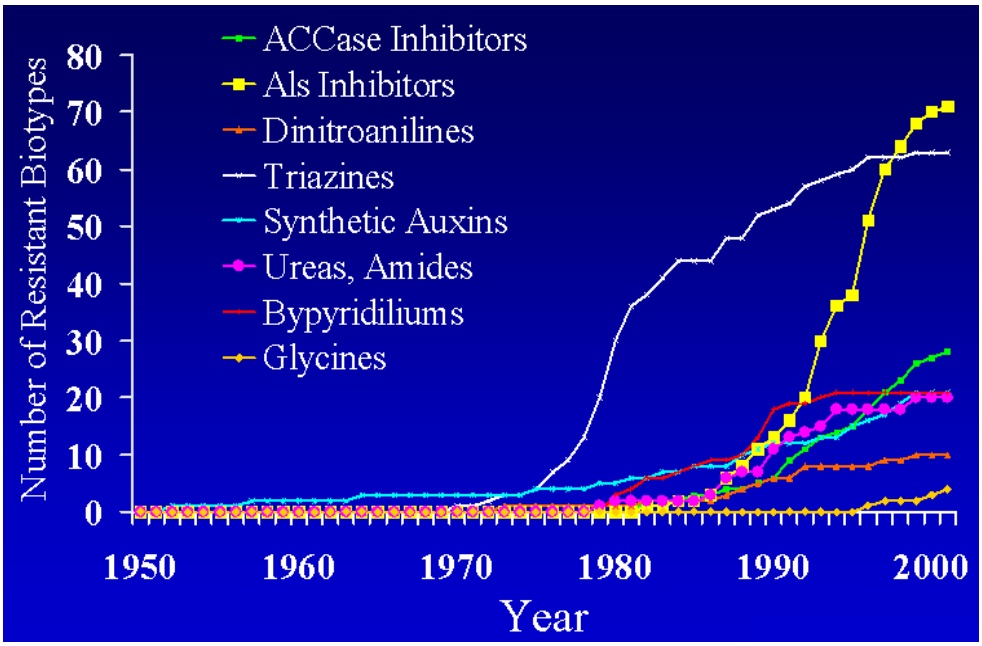

Figure 1.3 The chronological increase by mode of action in unique cases of herbicide-resistant weeds worldwide since 1950 (Heap, 2006)

A few cases of partial resistance to the synthetic auxins were recorded from the 1950's onwards, but the first documented case of high level resistance was simazine and atrazine resistant *Senecio vulgaris* (groundsel) in 1968 (Ryan, 1970). The problem started to increase significantly in the mid 1970s, and today the rate of emergence of new resistant biotypes shows no sign of slowing down (see Figure 1.3). Initially herbicide resistance was limited mainly to the United States, Australia and Europe, but this is now changing as farmers from Asia, Africa and South America increase herbicide inputs and intensive agricultural practices become more common.

1.3.2 The development of acetolactate synthase inhibiting herbicides

Inhibitors of the acetolactate synthase enzyme (ALS inhibitors) were first introduced in 1982 when DuPont launched the non-selective sulfonylurea herbicide sulfometuron-methyl and soon afterwards the first selective sulfonylurea, chlorsulfuron (Russell *et al.*, 2002). Currently there are 27 different active ingredients registered in the sulfonylurea class of herbicides and this makes them one of the largest single herbicide classes. Sulfonylureas are so called because of their basic chemical structure where the central portion of the molecule is made up of linked sulfonyl and urea groups. This chemical

backbone is common to all sulfonylurea herbicides and different herbicidal and crop selective properties are conferred by a wide range of possible substitutions on the terminal ring groups of the molecule (see Figure 1.4).

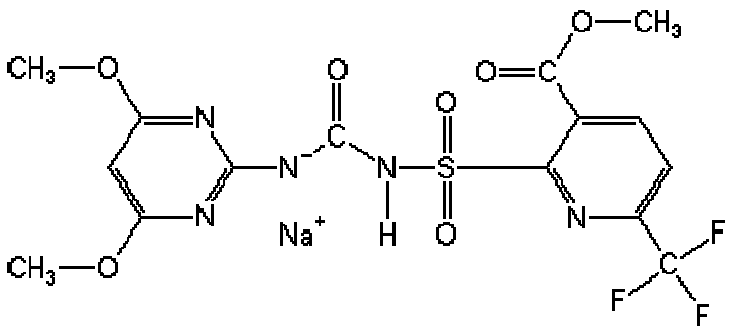

Figure 1.4 Chemical structure of the sulfonylurea herbicide flupyrsulfuron-methyl sodium which is active against *A. myosuroides* in cereals. Taken from http://www.alanwood.net/pesticides/

Acetolactate synthase is a key enzyme in the first common step of branched chain amino acid (BCAA) synthesis in plants (Ray, 1984). The primary mechanism of plant death upon treatment with acetolactate inhibiting herbicides is thought to be due to a lack of essential amino acids caused by inhibition of the enzyme, although alternative explanations include toxicity from the accumulation of precursor molecules such as aminobutyrate (Schloss, 1989; Shaner & Singh, 1993), and inhibition of the cell cycle (Spackman & Cobb, 1999). Cell cycle inhibition in particular is interesting because there is some evidence that depletion of the BCAA pool is not the sole reason for the observed effect on mitosis, and this fits in with the observation that ALS inhibitor treated plants stop growing immediately only to die much later. The conventional explanation for ALS mode of action (plant death due to lack of BCAA) is supported by several studies where supplementing ALS inhibitor treated plants with branched chain amino acids prevented growth inhibition and plant death (Ray, 1984; Shaner & Reider, 1986) but the possibility remains that there are secondary mechanisms at work.

In addition to the sulfonylureas, a number of other ALS inhibiting herbicides with different chemistry including the imidazolinone, triazolopyrimidine,

pyridinylthiobenzoate and sulfonylaminocarbonyltriazolinone groups are currently used as herbicides. Of these, after the sulfonylureas, the imidazolinones are the most commonly used. Imidazolinone chemistry was developed by researchers at American Cyanamid Company and the resulting herbicides commercialised in 1986 with the release of imazaquin as a selective herbicide in soya (Shaner & Singh, 1997). The mechanism of action in imidazolinones is the same as for sulfonylureas; they are potent inhibitors of ALS. Imidazolinones are unrelated structurally to sulfonylureas and the respective binding sites are different, although overlapping, with sulfonylureas binding more deeply into the ALS active site channel than do the imidazolinones.

Following their introduction the ALS inhibitors rapidly gained popularity due to their low use rates, good environmental profiles, low mammalian toxicity, residual activity, wide crop selectivity and high efficacy (Saari *et al.*, 1994). While these properties made ALS inhibitors very attractive to agrochemical companies and farmers, their high efficacy and tendency to leave residues in the soil imposed a heavy selection pressure on weed populations and resistance emerged very quickly; the first reported ALS target site resistant broadleaved weeds occurred only five years after the introduction of the sulfonylureas (Preston & Mallory-Smith, 2001; Tranel & Wright, 2002). The rate of development of resistance to ALS inhibitors was worsened by their popularity and subsequent heavy use, often on the same crop in successive years, and today resistance to ALS inhibitors is the most widespread type of herbicide resistance in the world, see Figure 1.3.

1.3.3 The acetolactate synthase enzyme

Ray demonstrated in 1984 that chlorsulfuron acts by inhibiting the acetolactate synthase (ALS) enzyme, preventing the first common step in the synthesis of the branched chain amino acids leucine, valine and isoleucine (Ray, 1984). Since then it has been shown that two parallel reactions are carried out by acetolactate synthase. These involve either the synthesis of 2-acetolactate from two molecules of pyruvate or the synthesis of 2-aceto-2-hydroxybutyrate from a molecule of pyruvate and a molecule of 2-ketobutyrate (Shaner & Singh, 1997). Acetolactate synthase (EC 2.2.1.6) is a member of a superfamily of enzymes dependent on the cofactor thiamine diphosphate (ThDP), members of which catalyse a variety of reactions including decarboxylation of 2-ketoacids. In addition to ThDP, ALS also requires a molecule of FAD, although this is not directly involved in the

principal reactions. The final requirement for the ALS enzyme is a divalent metal ion, for example Mg^{2+}, which coordinates to ThDP and two conserved residues of the enzyme (McCourt *et al.*, 2006).

At the present time, acetolactate synthases have been characterised from a number of different species including yeast (Pang *et al.*, 2002), bacteria (Hill *et al.*, 1997; Schloss *et al.*, 1985), and higher plants (Chang & Duggleby, 1997; McCourt *et al.*, 2006). Plants in particular have represented a challenge due to the very low abundance and high lability of the enzyme in plant tissues. Most commonly ALS enzymes have been found to consist of two subunits: a 60-65kDa catalytic unit and a 9-54kDa regulatory subunit which confers sensitivity to inhibition by the branched chain amino acids (Lee & Duggleby, 2001). Recent work has focussed on the crystal structure of the catalytic subunit in complex with cofactors and inhibitors (both imidazolinones and sulfonylureas). This work has the potential to show how plants from resistant populations are able to survive treatment with ALS inhibitors, explain patterns of cross resistance in resistant weed populations, and possibly allow the design of more effective ALS inhibiting herbicides.

In 2006 x-ray crystallography data giving the structure of *Arabidopsis thaliana* acetolactate synthase bound to various sulfonylurea and imidazolinone herbicides including sulfometuron, chlorsulfuron, and imazaquin became available (McCourt *et al.*, 2006). For the purposes of understanding resistance to ALS inhibitors, the plant enzyme is much more relevant than yeast or bacterial forms and so this work represents a step forward in the understanding of herbicide interaction with the ALS enzyme. Results showed that *A. thaliana* ALS is a tetramer and sulfonylurea and imidazloinone herbicides do not function by mimicking the enzyme substrate (i.e. they are not competitive inhibitors) but instead block a pathway through which access to the enzyme active site is gained, thus preventing substrate binding. It was shown that the binding sites for imadizolinones and sulfonylureas are different but overlapping, and that sulfonylureas bind more strongly due to more Van der Waals contacts and hydrogen bonding. The ALS enzyme tetramer is made up of four identical subunits with each subunit being composed of three domains, designated α, β and γ in *A. thaliana*, along with a C-terminal tail (McCourt *et al.*, 2006; Pang *et al.*, 2003). A more detailed discussion of ALS enzyme characteristics and diagrams of the enzyme in complex with inhibitors are included in Chapter 7.

1.3.4 Acetolactate synthase genes in higher plants

In higher plants acetolactate synthase is coded by a nuclear gene with the mature ALS enzyme being localised in the chloroplasts (Chipman *et al.*, 1998). Available sequence data shows a high degree of conservation across different plant species and almost all gene sequences from higher plants lack introns (Prado *et al.*, 2004; Uchino & Watanabe, 2002). ALS genes differ in length between species due to additions and deletions which are not conserved between species; in the Gramineae complete ALS gene sequences range from around 1950 to 2600 base pairs. Resistance studies have often adopted different numbering systems based on amino acid positions in the mature protein and the result of the differences in ALS length between species means that confusion can arise on the location of particular residues. Tranel and Wright (2002) suggest the use of *A. thaliana* numbering to help avoid such problems and theirs is the system used here.

Multiple ALS genes encoding multiple isoenzyme forms of acetolactate synthase have been confirmed in bacteria (LaRossa & Schloss, 1984; Milano *et al.*, 1992; Newman & Levinthal, 1980). These multiple ALS forms show different functions and in particular differing feedback inhibition characteristics in the presence of branched chain amino acids. Investigations in yeast have revealed only a single nuclear gene and corresponding mitochondrial form of the enzyme (Falco *et al.*, 1985; Yadav *et al.*, 1986) but with higher plants the situation is more complex and ALS genes are found in single copy (Dumas *et al.*, 1993; Mazur *et al.*, 1987; Scarabel *et al.*, 2004; Wright & Penner, 1998), and also multiple copy form (Fang *et al.*, 1992; Grula *et al.*, 1995; Keeler *et al.*, 1993; Ouellet *et al.*, 1992; White *et al.*, 2003). Plants with multiple forms of the gene have been shown in some cases to demonstrate divergent expression patterns and different functions (Grula *et al.*, 1995; Ouellet *et al.*, 1992) , while other species show constitutive expression with the different enzyme forms expressed at low levels in all tissues (Keeler *et al.*, 1993). The variation observed in higher plant ALS gene families and associated isoenzyme forms has invited much speculation as to why some plants contain more than one ALS. Possible reasons include the need for variable expression in different plant organs, reflecting different conditions within the plant, different developmental stages, or as a reflection of the evolutionary origins of different plant species (Keeler *et al.*, 1993). There seems to be some association between ALS gene number and ploidy, with diploids more likely to possess a single ALS gene and polyploid species more often showing diverse gene families. More details on ALS genes in different plant species are described in Chapter 7.

1.3.5 Mechanisms of resistance to ALS inhibitors- target site resistance

Inheritance of the ALS target site resistance trait is controlled by a nuclear gene with incomplete dominance in most cases (Saari *et al.*, 1994) and this has implications for the spread of ALS target site resistance depending on the crossing system of the weed species in question. Resistance to ALS inhibitors is most often due to target site mutations which prevent herbicide binding at the enzyme target site (Preston & Mallory-Smith, 2001). Target site mutations conferring resistance to ALS inhibitors are usually associated with a single amino acid substitution. In the weed biotypes investigated so far all amino acid substitutions resulting in resistance have been to one of five conserved residues encoded as part of five separate highly conserved domains of the acetolactate synthase gene (Boutsalis *et al.*, 1999). These are Ala_{122} (Dom C), Pro_{197} (Dom A) and Ala_{205} (Dom D) situated near the amino terminal end of ALS along with Trp_{574} (Dom B) and Ser_{653} (Dom E) near the carboxyl terminal end (Tranel & Wright, 2002). Conserved Domains A-E range in size from 12 to 57 base pairs and show very little variation between species, resulting in an equivalent conservation of encoded amino acid residues in mature ALS proteins. Crystallisation and visualisation of ALS enzyme allows explanation of how amino acid substitutions can result in resistance to ALS inhibiting herbicides.

The following discussion of target site amino acid substitutions and resistance is based on recent x-ray crystallography work where the structure of *A. thaliana* ALS was determined in complex with various inhibitors and co-factors (McCourt *et al.*, 2006). This section sums up the conclusions on cross resistance and structure in terms of different amino acid substitutions at different positions. It is worth noting that while knowledge of enzyme-inhibitor structural relationships can allow limited predictions of cross-resistance patterns, often the interactions between species sensitivity and possible amino acid substitutions at particular sites are so complex that broad predictions become difficult (Roux *et al.*, 2005).

- Ala_{122} is not a common amino acid substitution and only three examples are known where this substitution confers resistance in weed species (Heap, 2006). Substitutions at this position are limited to threonine and cross resistance tests show resistance to imidazolinones but not sulfonylureas. This can be explained by the fact that Ala_{122} makes hydrophobic contact to the substituents of the imidazolinone ring of the model imidazolinone, imazaquin, while interactions with sulfonylureas are either limited or non-existent. Threonine is a large polar amino acid and a substitution of threonine for

the smaller non-polar alanine at this position is likely to interfere with imidazolinone binding but not with that of sulfonylureas.

- Pro_{197} is the commonest position for amino acid substitution in ALS target site resistant weeds. Eight different amino acid substitutions have been observed at Pro_{197} with all conferring resistance to sulfonylureas, but only leucine segregating with significant resistance to imidazolinones. Pro_{197} is located at the entrance to the ALS active site access channel and makes contact with the aromatic ring of sulfonylureas but does not directly interact with imidazolinones, which bind at an overlapping but slightly different site, further from the catalytic centre. This explains why almost any substitution at this position interferes with sulfonylurea binding while only very bulky residues like leucine have the potential to interact with imidazolinones.
- Substitutions at the Ala_{205} position are the least commonly observed in ALS inhibitor resistant weed populations. Only two examples exist and both show substitution of valine for alanine at this position. No particular patterns of cross resistance are apparent and the valine substitution appears to confer low-level resistance to all ALS inhibiting herbicides.
- Mutations resulting in amino acid substitution at Trp_{574} are the second most common after Pro_{197} and always involve the substitution of tryptophan for leucine in resistant plants. This mutation typically results in strong cross resistance to sulfonylureas and imidazolinones, as well as other ALS inhibiting chemistry where tested. The Trp_{574} residue is involved in defining the active site channel shape of the ALS enzyme and in herbicide binding for both sulfonylureas and imadizolinones. Substitution with leucine alters the herbicide binding site and results in the loss of several interactions.
- The final common site where amino acid substitutions result in resistance to ALS inhibitors is Ser_{653} which is replaced with threonine or asparagine in imidazolinone resistant individuals. No cross resistance is observed to other ALS inhibiting herbicide groups. Substitution of the polar Ser_{653} for larger polar residues such as threonine and asparagine could conceivably impair imidazolinone binding by taking up space required for the aromatic ring of the herbicide molecule. Sulfonylurea binding is not obstructed by this substitution.

1.3.6 Mechanisms of resistance to ALS inhibitors- enhanced metabolism

In addition to target site resistance, there are some cases of ALS inhibitor resistance which are due to an enhanced ability to metabolise herbicides. Several different metabolic pathways exist in plants that have the capacity to detoxify ALS inhibitors including de-alkylation, hydroxylation, glucose conjugation, de-esterification, urea bridge cleavage, and ring cleavage (Cobb, 1992; Shaner & Singh, 1997). Differences in metabolic capacity between crops and weeds are often the basis for herbicide selectivity and sulfonylureas are no exception. In general, sulfonylurea tolerant crops rapidly transform the herbicide to inactive metabolites whereas susceptible weeds lack the ability to do this and are killed (Russell *et al.*, 2002). The most common metabolic pathway conferring herbicide selectivity in crop species is hydroxylation followed by glucose conjugation. Most susceptible weed species can metabolise selective herbicides to some degree but not sufficiently to avoid being killed. Weed biotypes which have evolved resistance to herbicides through enhanced metabolism generally detoxify herbicides at a greater rate than susceptible biotypes; the difference is usually in terms of up-regulation of existing metabolic pathways rather than the employment of completely different ones. Two notable examples exhibiting this type of ALS inhibitor resistance are *L. rigidum* in Australia (Burnet *et al.*, 1994; Christopher *et al.*, 1992; Preston *et al.*, 1996) and *A. myosuroides* from Peldon in the UK (Kemp *et al.*, 1990; Moss, 1987b; Moss & Cussans, 1985; Moss *et al.*, 2005b). Both of these grass weeds display cross-resistance, and multiple resistance mechanisms have been identified in *L. rigidum*. These populations are therefore able to tolerate applications of herbicides from various chemical classes. Less studied examples of grass weed populations resistant to ALS inhibitors by enhanced metabolism include *Bromus tectorum* (Park *et al.*, 2003) and *Setaria* faberi (Carey *et al.*, 1997). In general enhanced metabolism resistance to ALS inhibitors is more common in grass-weeds, although a few examples are known to exist in broad-leaved species (Veldhuis *et al.*, 2000; Warwick *et al.*, 2005).

1.3.7 Enhanced metabolism resistance in *Alopecurus myosuroides* from Peldon

One of the first cases of herbicide resistant *A. myosuroides* in the UK was from a farm in the Peldon area of Essex where resistance to chlorotoluron was detected in seed collected from the field in 1984. Good herbicide records exist from this farm dating back to 1977, see Chapter 5. These records show that the herbicides used for *A. myosuroides* control at the Peldon site up to 1984 included simazine, glyphosate, paraquat, chlorotoluron,

isoproturon, and triallate. Initial experiments led to the conclusion that chlorotoluron resistance at Peldon was a quantitatively inherited multi-gene trait (most likely enhanced metabolism), based on the patterns of resistance observed and the crossing system in *A. myosuroides* (Moss & Cussans, 1985). Following the first confirmed resistance to chlorotoluron in the Peldon population, and the implication of enhanced metabolism as a likely explanation, a large number of follow up studies were performed assessing the resistance characteristics of Peldon *A. myosuroides*. Cross resistance to several herbicides with different modes of action was soon demonstrated in the Peldon biotype and these included phenylureas and triazines (inhibitors of photosynthesis at photosystem II), dinitroanilines (inhibitors of microtubule assembly), aryloxyphenoxypropionates and cyclohexanediones (inhibitors of fatty acid synthesis by the ACCase enzyme), thiocarbamates (non-ACCase inhibitors of fatty acid synthesis) chloroacetamides (inhibitors of cell division) and the ALS inhibitor chlorsulfuron (Kemp *et al.*, 1990; Moss & Cussans, 1987). More recently, resistance to the sulfonylurea herbicide flupyrsulfuron-methyl has been observed at Peldon (Moss *et al.*, 2005b).

In addition to herbicide resistance studies using whole plants, the mechanisms of resistance in *A. myosuroides* from Peldon were also investigated in great detail. Several enzyme systems have been implicated in enhanced-metabolism based herbicide resistance in *A. myosuroides* from the Peldon population and these include cytochrome P450 monooxygenases (Hall *et al.*, 1995; Hyde *et al.*, 1996) and glutathione *S*-transferases (Cummins *et al.*, 1997; Reade & Cobb, 1999). In these studies the Peldon biotype typically showed elevated activities of cytochrome P450 and glutathione *S*-transferase enzyme systems compared to susceptible standard biotypes, and this was associated with the increased rate of metabolism and detoxification of applied herbicides. A number of additional enzyme systems have been investigated in the Peldon biotype including *O*-Glucosyltransferases (Brazier *et al.*, 2002) and esterases (Cummins *et al.*, 2001). In the case of *O*-Glucosyltransferase, the Peldon population showed higher intrinsic enzyme activities than a susceptible standard population but treatment with herbicides caused no increase in activity. In terms of esterase activities, no differences were detected between Peldon and the susceptible standard population. At the present time P450's and GST's are the only enzyme systems definitely linked to herbicide resistance in the Peldon population compared to susceptible standards. Overall Peldon *A. myosuroides* exhibits an enhanced ability to rapidly detoxify a range of different herbicides, including the sulfonylureas

flupyrsulfuron and possibly chlorsulfuron, based on higher enzyme activities compared to susceptible standards, and this translates to wide ranging cross resistance across herbicide groups with different modes of action.

An interesting result from some Australian studies with ALS inhibitor resistant *L. rigidum* biotypes is that resistance to the non-selective ALS inhibitor sulfometuron-methyl, which is normally used in forestry and amenity weed control, has been associated with altered ALS target site based resistance as opposed to enhanced metabolism (Christopher *et al.*, 1991; Gill, 1995). This unique characteristic of sulfometuron-methyl is thought to be due to its low susceptibility to metabolism in *L. rigidum*. Preston and Powles (2002) exploited this initial finding when they used sulfometuron-methyl as part of a selective germination medium for *L. rigidum* seeds and showed that a dose of 60 to 90 g a.i. ha^{-1} allowed only seedlings with an altered ALS enzyme to grow normally. Together these studies indicate that it might be possible to use sulfometuron-methyl as an indicator of target site resistance in *A. myosuroides* from the UK which displays a similar complicated resistance profile to Australian *L. rigidum*. If sulfometuron-methyl could provide such a discriminatory role it would enable the rapid screening of *A. myosuroides* biotypes and highlight any which showed possible target site resistance for work on the mechanisms involved. Using sulfometuron in this way might also eventually lead to rapid Petri dish screening tests similar to those for acetyl-CoA carboxylase target site resistance where the non-selective cyclohexanedione herbicide sethoxydim can act as an indicator (Moss *et al.*, 2003).

1.3.8 Resistance to ALS inhibitors in grass weeds: the current situation

A review of ALS target site resistance from around the world shows that confirmed cases of probable ALS target site resistance (indicated by reduced enzyme sensitivity) in grass weeds are limited to *Bromus tectorum* L. (downy brome) in the USA (Park & Mallory-Smith, 2004), *Lolium rigidum* Gaud. (rigid ryegrass) in Australia (Christopher *et al.*, 1992; Kaundun *et al.*, 2006), *Lolium perenne* L. (perennial ryegrass) in the USA (Saari *et al.*, 1992) *Apera spica-venti* (L.) P. Beauv. (wind bentgrass) in the Czech Republic (Novakova *et al.*, 2006), *Setaria viridis* (L.) P. Beauv. (green foxtail) in Canada (Laplante, 2006) and *Sorghum bicolour* (L.) Moench (shattercane) in the USA (Anderson *et al.*, 1998). The total number of ALS inhibitor resistant grass weed biotypes is currently 21 and the remainder of these cases are either due to enhanced metabolism or remain

uncharacterised at present (Heap, 2006). Target site resistance is therefore relatively less common in grasses than in broad leaved weeds where most cases of ALS inhibitor resistance are due to an insensitive enzyme and enhanced metabolism is rare.

Of the six resistant grass weed biotypes where enzyme insensitivity has been confirmed, the molecular basis of the trait has been characterised in only three: primisulfuron-resistant *Bromus tectorum* from Oregon in the USA (Park & Mallory-Smith, 2004), imazethapyr-resistant *Setaria viridis* from Ontario in Canada (Laplante, 2006) and sulfonylurea resistant *Lolium rigidum* biotypes from Australia (Kaundun *et al.*, 2006). All three cases were associated with single point mutations in the ALS gene conferring potential target site changes in resistant individuals compared to susceptibles. The precise mutations involved were Pro_{197} to Ser in *B. tectorum*, Ser_{653} to Thr, Asn or Ile in *S. viridus*, and either Trp_{574} to Leu or Pro_{197} to Ser or Thr in *L. rigidum*. The current lack of information on the extent of ALS target site resistance in grass weeds makes characterisation of resistance mechanisms in existing resistant biotypes very important. Knowledge of the herbicide treatment regimes which have led to the evolution ALS target site resistance rather than metabolism-based resistance in grass weeds may be useful in the development of treatments which minimise the risk and prolong the useful life of the ALS inhibiting herbicides currently available. ALS target site resistance has not been confirmed in *A. myosuroides* or any other grass weed in Europe so far, although it is the likely explanation for the resistance observed in *A. spica-venti* from the Czech Republic. If an altered ALS target site is confirmed in the Peldon biotype it will set an important precedent, having far reaching consequences for *A. myosuroides* management in the UK.

1.4 Thesis aims and objectives

The aim of this study was to combine field work and glasshouse bioassays with biochemical and molecular analyses to characterise the basis of resistance to the acetolactate synthase inhibiting sulfonylurea herbicides in *A. myosuroides* biotypes from the UK. The project focused on the following five main objectives:

1. *To study the potential of whole plant testing protocols utilising the non-selective sulfonylurea herbicide sulfometuron as an indicator of ALS target site resistance.*

Previous work by Christopher *et al*., (1992) demonstrated the low potential for resistance to the non-selective ALS inhibitor sulfometuron by enhanced metabolism in the grass-weed *L. rigidum*, while Burnet *et al*., (1994) showed that sulfometuron could be used as an indicator of ALS target site resistance in the same species. A testing protocol for the identification of possible target site resistant *A. myosuroides* populations using sulfometuron was an important goal following categorisation of the degree of resistance in standard populations through dose response assays. Whole plant tests using sulfometuron may provide a method to overcome the confounding effects of enhanced metabolism resistance in *A. myosuroides* which has the potential to complicate mechanistic studies where ALS target site resistance is also present.

2. *To investigate the mechanisms responsible for the high level sulfometuron resistance demonstrated by A. myosuroides plants from Peldon in Essex.*

The Peldon *A. myosuroides* biotype shows resistance to a wide variety of different herbicides through enhanced metabolism. Preliminary work at Rothamsted identified a proportion of plants able to survive sulfometuron in Peldon seed collected from the field in 1996. An investigation of the basis of the observed sulfometuron resistance and determination of the mechanisms involved was felt to be a priority in order to provide a cohesive overall picture of the mechanism behind sulfometuron resistance in the Peldon biotype. Thus it is appropriate to combine the results from whole plant studies of Peldon biotypes with investigations of ALS enzyme inhibition, herbicide metabolism and ALS gene sequence data from resistant and susceptible plants as required.

3. *To determine the heritability of the sulfometuron resistant trait.*

Crossing experiments using available lines of sulfometuron resistant Peldon *A. myosuroides* were carried out in an effort to clarify the inheritance of the resistant trait. Of particular interest is whether the trait is dominant or semi dominant and determined by a single nuclear gene suggesting ALS target site resistance, or whether it is a quantitatively inherited multi-gene trait more indicative of enhanced metabolism.

4. *To investigate the evolution of resistance to ALS inhibiting herbicides in terms of management practices and consequences for the development of resistance in future.*

Field and glasshouse experiments were carried out in order to investigate the evolution of resistance to ALS inhibiting herbicides in *A. myosuroides* from the Peldon area of Essex. The Peldon biotype presents an ideal opportunity for such work based on the accurate herbicide records from the site and the availability of viable seed samples dating back to the 1990's.

5. *To evaluate the presence and mechanisms of resistance to ALS inhibitors in a wider range of UK populations.*

Seed samples from a range of resistant populations were screened using sulfometuron in order to identify those displaying possible ALS target site resistance. Identification of mechanisms in any additional sulfometuron resistant populations would then be a priority. It is important to know whether ALS target site resistant *A. myosuroides* is an emerging threat in UK agriculture.

2. General Materials and Methods

2.1 Introduction

Several chapters in this thesis include results compiled from a number of separate experiments. The present chapter includes descriptions of general materials and methods that were used in more than one such experiment. Specific materials and methods are given where appropriate in the specific chapters to which they apply.

2.2 Collection and Storage of seeds

Seeds of *Alopecurus myosuroides* (black-grass) were collected from a number of sites for use in various indoor and outdoor pot, container and field experiments. Seed was also collected from survivors of glasshouse and outdoor container experiments.

2.2.1 Collection and storage of field samples

Ripe seeds (technically spikelets) were collected in the field from winter cereal crops in mid July by gently rubbing heads of individual plants over a tray or envelope. The use of more vigorous rubbing was avoided so as to minimise collection of unripe seeds. After collection all samples were labelled including geographical reference and collection date. Seeds were air dried in foil trays for 2 or 3 days and then weighed, cleaned using an air column seed cleaner (see Figure 2.1), and re-weighed. The seed cleaner ensured removal of chaff and debris from samples, as well as most non-viable seeds. Re-weighing after cleaning was done to give an indication of sample quality. High quality seed samples containing little in the way of chaff and non-viable seed typically lose only ten to twenty percent of weight with cleaning while very low quality samples can lose up to ninety-five percent of their original weight. After re-weighing seeds were stored in labelled envelopes in a designated seed store at 17°C, 65% relative humidity.

2.2.2 Collection and storage of glasshouse samples

To encourage panicle and seed production, glasshouse grown plants were subjected to a two week period of vernalisation at ambient temperatures which was achieved by moving

them to an unheated glasshouse compartment, or outdoors. Plants were crossed inside individual glasshouse compartments and pollinated by gently tapping the heads with a bamboo cane to promote release of pollen. Seeds were collected from mature heads in the same manner as described for field samples. Treatment and storage were also the same.

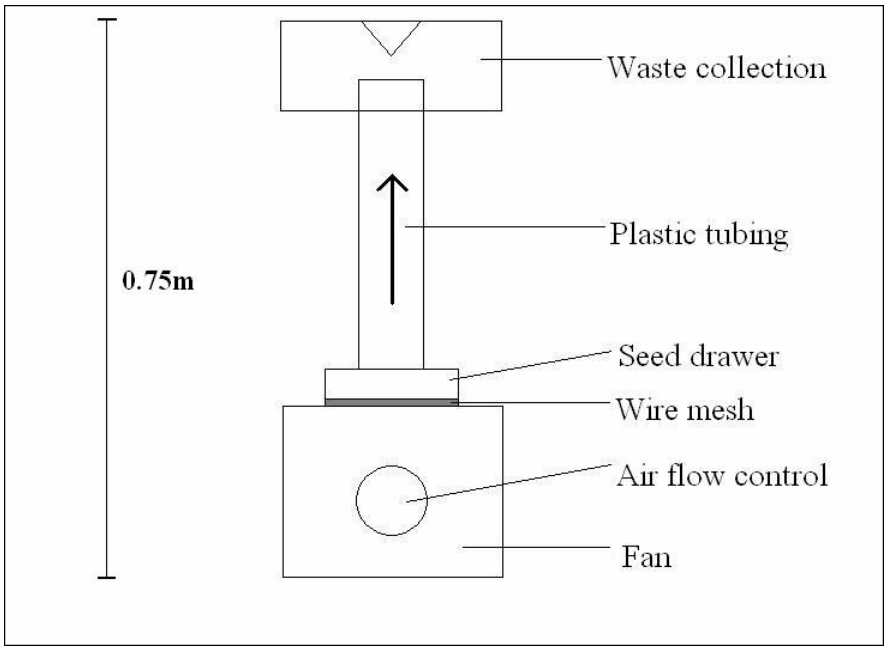

Figure 2.1 The seed cleaning machine

2.2.3 Seed dormancy

Freshly harvested *A. myosuroides* seeds typically have innate dormancy which lasts about four to six weeks. Beyond this period only ten to twenty percent of seeds remain dormant (Holm *et al.*, 1997). After collection, seeds were stored at 30 - 35°C for about four weeks to facilitate dormancy release prior to long term storage.

2.2.4 Seed viability testing

Seed viability tests were carried out with very low quality samples either before storage or just before use in experiments. This was done in order to give an indication of the number of seeds required for pre-germination. In all cases two samples of 100 seeds were dissected to determine whether a caryopsis was present or not. Seeds containing a caryopsis were classed as viable and the results used to determine percentage viability for each sample (Moss, 2002b).

2.2.5 Germination testing

In some cases where seeds had been stored for long periods it was necessary to perform germination tests. Fifty *A. myosuroides* seeds were placed in each of four replicate 9cm Petri dishes containing three Whatman cellulose filter papers covered by a single Whatman glass fibre paper (Moss, 2002b). Potassium nitrate solution (7ml, 2g L^{-1}) was applied to each dish immediately in order to help break dormancy (Beckie *et al.,* 2000) and the four dishes were then placed together in a polythene bag which was sealed to prevent moisture loss. The bag was placed in an incubator running at settings of approx 17^{o}C 14h day; 11^{o}C 10h night. After two weeks the dishes were removed and germination was assessed.

2.3 Whole Plants

2.3.1 Determination of plant growth stage

Plant growth stage was recorded using the BBCH (BASF, Bayer, Ciba-Geigy and Hoechst) system (Meier, 1997), which is widely used to define phenological phases of crop species. The BBCH coding system subdivides the entire developmental cycle of the plants into ten clearly distinguishable developmental stages and secondary growth stages are used to indicate precisely the points of time or steps in plant development. Specific information on the growth stages of grasses was obtained from a paper by Zadoks *et al*, (1974). Growth stage was used to ensure that plants received herbicide treatments at the correct time and that harvest times were coordinated.

2.3.2 Plant growth and management for glasshouse studies

Plant germination, growth and management were carried out as follows unless stated otherwise. Seeds were pre-germinated in 9cm Petri dishes as described in section 2.2.5 using roughly 50 to 80 seeds per dish. After one week in an incubator germinated seeds were transplanted into pre-wetted Kettering loam mix containing 80% w/w screened sterilised loam, 20% w/w 3-6mm screened lime free grit and 2.0kg m^{-3} Osmocote mini 5/6 month fertiliser. Kettering loam mix has a low organic matter content of around 4% organic matter and was supplied by Petersfield Products of Leicester. Low organic matter content was required because reduced efficacy of sulfonylurea herbicides has been observed at Rothamsted in growing media with high organic matter levels due to

adsorption. Seedlings were lightly covered with damp loam mix and watered thoroughly from above twice per day for the remainder of the growing period. More seedlings were planted than required and thinned out to leave the requisite number of healthy plants in each pot. All plants were maintained for the entire period of growth, according to the experiment, in a glasshouse with a 14h, 16°C day achieved with supplementary lighting providing 200-250μmol s^{-1} m^{-2}, and a 10h, 8°C night phase.

2.3.3 Plant growth and management for outdoor container experiments

After the initial glasshouse work, outdoor experiments using plastic containers were conducted in order to better simulate field conditions. The plastic containers (Kaiser and Kraft Ltd, 285mm L x 185mm W x 130mm D) had six 7cm drainage holes drilled in the base and were filled with 1.5L of 'Hydroleca' (Sinclair, UK) clay granular aggregate to assist drainage followed by 3L of Kettering loam mix. Approximately 200 of the required seeds were then mixed with a final 1.5L of loam and added as the top layer to each container. Seed numbers were determined by weight and seeds were not pre-germinated. After planting, containers were moved to an outdoor sand bed and then watered with an overhead sprinkler.

Following an initial setup period of one week, outdoor container experiments received only natural rainfall, hence mimicking field conditions. The sand bed used in these experiments was completely open to the air, being covered only by a net to keep out birds and animals.

2.4 Track Sprayer and Herbicide Preparation

All herbicide treatments were applied at BBCH growth stage 13-14 (three to four-leaf stage of the weed) using a track sprayer (see Figure 2.2) delivering spray solution through a single 'Teejet' spraying systems TP110015VK flat fan ceramic nozzle. The sprayer was calibrated to deliver between 230 and 250L spray solution ha^{-1} using pressurised CO_2 at 210kPa. Precise calibration was achieved by spraying water from the track sprayer onto pre-weighed Petri dishes with three placed along the central line of the room and one off to each side. These were then quickly capped and re-weighed and the results used to calculate output in L ha^{-1}. Two calibrations were performed and the mean output used for herbicide dose calculations. Commercial herbicide formulations and adjuvants were used

according to manufacturers' recommendations. Pots and containers were sprayed in an enclosure housing only the sprayer and fitted with extractor fans; all pots or containers were placed along the central line of the room directly under the path of the spray nozzle.

After spraying, plants were left to dry before being transferred back to the glasshouse or sand bed for randomisation. All plants were left without water for at least 12 but not more than 16h post spraying and were then watered as required.

Figure 2.2 The Rothamsted track sprayer

2.4.1 Herbicide formulation details

The following herbicide formulations were used in outdoor and glasshouse studies throughout the course of this project.

2.4.1.1 Sulfometuron-methyl ('sulfometuron', product name 'Oust XP')

Sulfometuron-methyl (2-(4,6-dimethylpyrimidin-2-ylcarbamoyl)sulfamoyl)benzoic acid), CAS number 74222-97-2, was applied as the Oust XP formulation unless stated otherwise

and was supplied by DuPont Agricultural Products (Stevenage UK). Oust XP is a water soluble granule containing 75% sulfometuron-methyl by weight. Sulfometuron-methyl is an ALS inhibitor in the sulfonylurea chemical group.

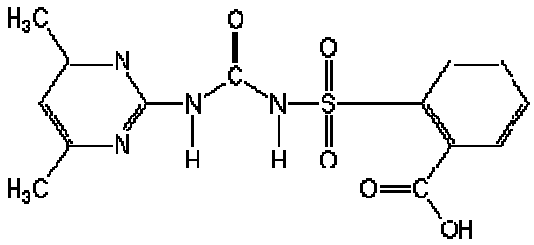

Figure 2.3 Chemical structure of sulfometuron-methyl. Taken from http://www.alanwood.net/pesticides/

2.4.1.2 Flupyrsulfuron-methyl sodium '(flupyrsulfuron', 'Lexus 50DF')

Flupyrsulfuron-methyl sodium (2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-6-(trifluoro methyl)nicotinic acid), CAS number 144740-54-5, was applied as the Lexus 50DF formulation unless stated otherwise and was supplied by DuPont Agricultural Products (Stevenage UK). Lexus 50DF is a water soluble granule containing 50% flupyrsulfuron-methyl by weight. Flupyrsulfuron-methyl is an ALS inhibitor in the sulfonylurea chemical group.

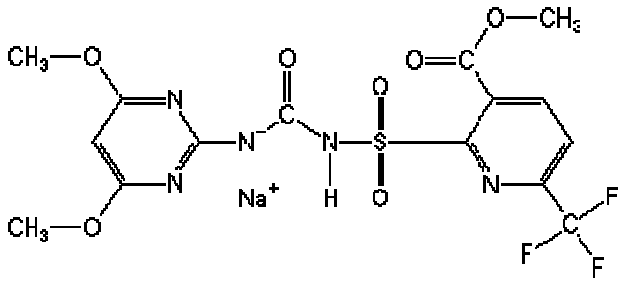

Figure 2.4 Chemical structure of flupyrsulfuron-methyl sodium. Taken from http://www.alanwood.net/pesticides/

2.4.1.3 Mesosulfuron-methyl + iodosulfuron-methyl-sodium mixture ('mesosulfuron+iodosulfuron', 'Atlantis WG')

Mesosulfuron-methyl (2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-α-

(methane-sulfonamido)-*p*-toluic acid), CAS number 208465-21-8, iodosulfuron-methyl-sodium (4-iodo-2-[3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)ureidosulfonyl]benzoic acid), CAS number 144550-36-7 and safener mafenpyr-diethyl ((RS)-1-(2,4-dichlorophenyl)-5-methyl-2-pyrazoline-3,5-dicarboxylic acid), CAS number 135590-91-9, were applied together as the Atlantis WG formulation. Atlantis WG is a water dispersible granule formulation containing 3% mesosulfuron-methyl and 0.6% iodosulfuron-methyl-sodium by weight along with 9% safener mefenpyr-diethyl. All applications of Atlantis WG were in mixture with the recommended adjuvant 'Biopower', a suspension concentrate formulation containing 6.7% w/w 3,6-dioxaeicosylsulphate sodium salt and 20.2% w/w 3,6-dioxaoctadecylsulphate sodium salt, at a rate equivalent to 1 L ha^{-1}. Atlantis WG and Biopower adjuvant were supplied by Bayer Crop Science (Cambridge UK). Mesosulfuron-methyl and iodosulfuron-methyl are ALS inhibitors in the sulfonylurea chemical group.

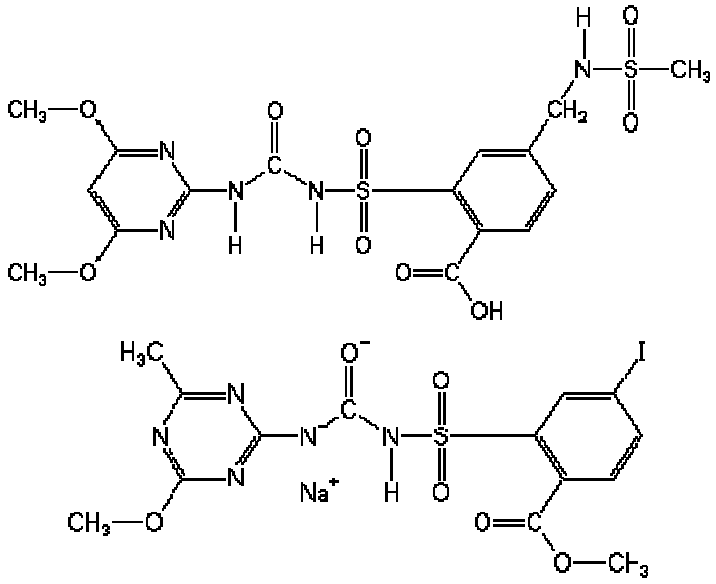

Figure 2.5 Chemical structures of mesosulfuron-methyl and iodosulfuron-methyl sodium. Taken from http://www.alanwood.net/pesticides/

2.4.1.4 Propoxycarbazone-sodium ('propoxycarbazone', 'Attribut WG70')

Propoxycarbazone-sodium (methyl 2-(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1*H*-1,2,4-triazol-1-yl)carboxamidosulfonylbenzoate), CAS number181274-15-7, was applied as the Attribut WG70 formulation and was supplied by Bayer Crop Science (Cambridge UK). Attribut WG70 is a water soluble granule containing 70% propoxycarbazone-sodium by

weight. All applications of Attribut WG70 were in mixture with recommended adjuvant 'Actipron', a formulation containing solvent refined mineral oil and additives, at a rate equivalent to 1L ha^{-1}. Propoxycarbazone-sodium is an ALS inhibitor in the sulfonylamino-carbonyl-triazolinone chemical group.

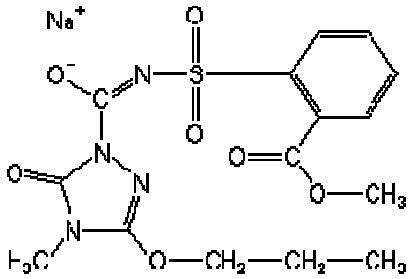

Figure 2.6 Chemical structure of propoxycarbazone-sodium. Taken from http://www.alanwood.net/pesticides/

2.4.1.5 Imazamethabenz-methyl ('imazamethabenz', 'Dagger')

Imazamethabenz-methyl (a reaction mixture of (*RS*)-6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*m*-toluic acid and (*RS*)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*p*-toluic acid), CAS number 81405-85-8, was applied as the Dagger formulation and supplied by Cyanamid of Great Britain (Gosport UK). Dagger is a suspension concentrate containing 28.9% imazamethabenz-methyl. All applications of Dagger were in mixture with recommended adjuvant 'Agral 90', a non-ionic surfactant containing nonylphenoxy polyethyoxyethanol, at a rate equivalent to 0.5L ha^{-1}. Imazamethabenz-methyl is an ALS inhibitor in the imidazolinone chemical group.

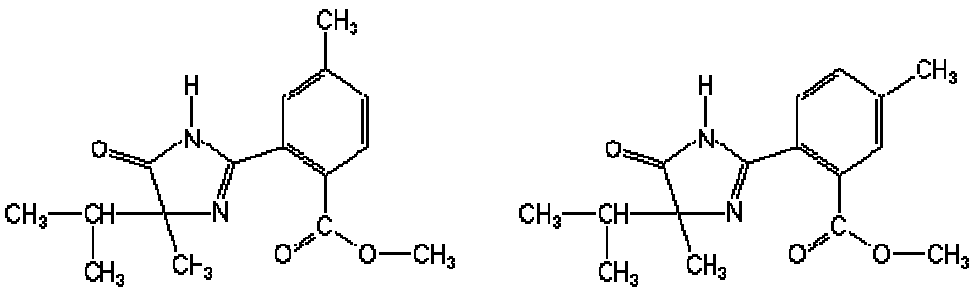

Figure 2.7 Chemical structures of imazamethabenz-methyl isomeric forms. Taken from http://www.alanwood.net/pesticides/

2.5 Harvest and Injury Scoring of Herbicide Treated Plants

A. myosuroides plants grown in the glasshouse were harvested 28-35 days after treatment with herbicide unless stated otherwise. The precise harvest date for each experiment was judged using susceptible standard *A. myosuroides* populations; harvest date corresponded with complete control of the susceptible standard (see Figure 2.3).

2.5.1 Injury Scoring

Prior to harvest all plants were visually scored for phenotypic response to herbicide treatment using five categories with untreated plants as a control. The categories were as follows (see Figure 2.8):

1. Plants showing no characteristic symptoms of ALS inhibitor damage compared to untreated plants.
2. Plants showing no serious symptoms of ALS inhibitor damage. Some stunting and reduced growth, but obvious new growth present.
3. Obvious symptoms of ALS inhibitor injury but plants still green and alive. Commonly show curled and damaged leaves, some discolouration, ground-hugging habit, damaged roots and reduced foliage mass. Some new growth present but plants quite stunted.
4. Serious symptoms including discoloration, leaf curl, severe stunting, swollen leaf base and extensive root damage. No new growth present but some green foliage still visible.
5. Plant completely dead, no green leaf material present at all.

In all experiments scoring was carried out using untreated plants of the same population as a reference along with treated plants from a susceptible standard population. Plants indistinguishable from the untreated control group were scored in category 1 while the treated susceptible standards were used as a visual reference for injury score 5. The inclusion of plants from a susceptible standard population also acted as a check of herbicide efficacy. In general percent fresh weight reduction for treated plants in each category showed good levels of consistency according to calculated coefficient of variation.

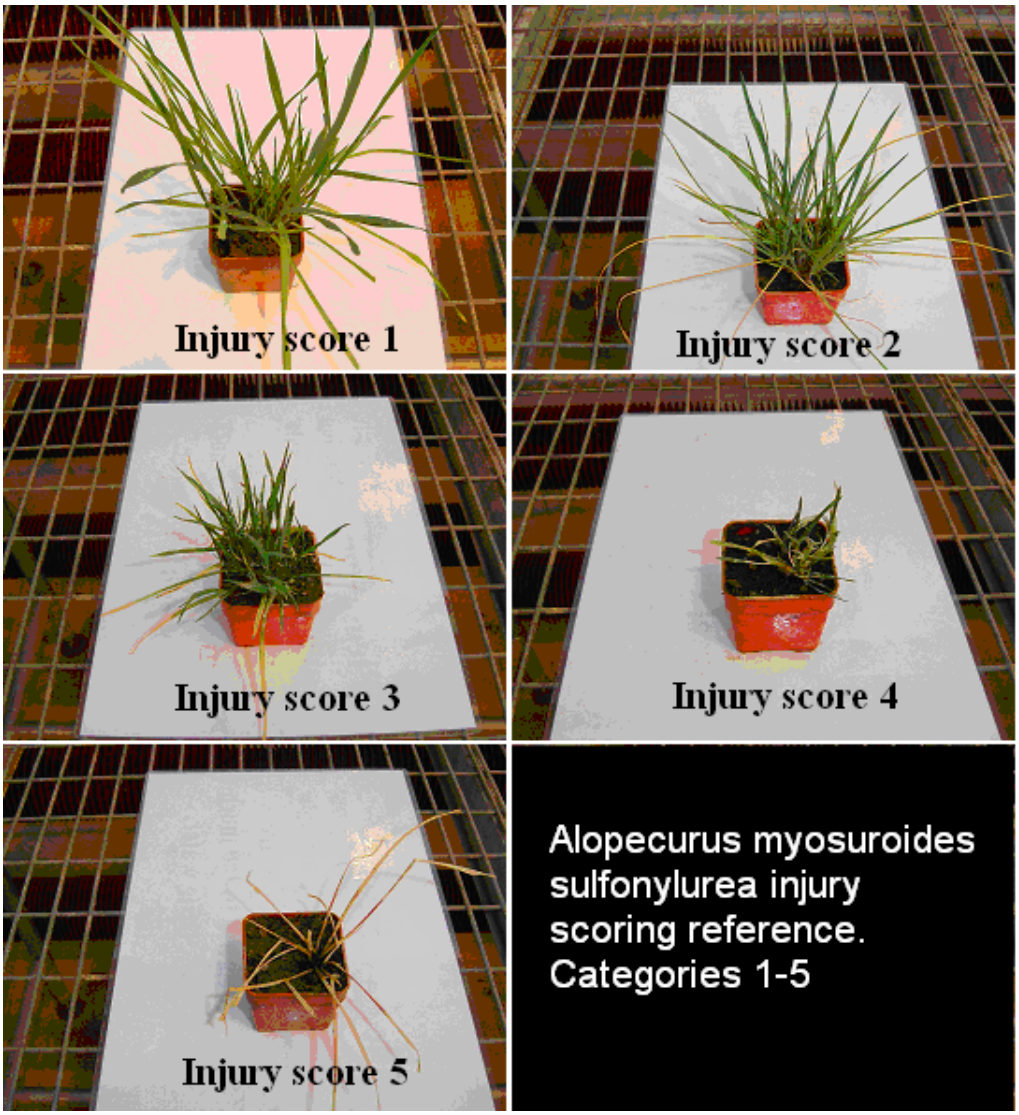

Figure 2.8 Injury scoring scale showing examples of *A. myosuroides* plants falling into each category

2.5.2 Plant harvest and fresh weight determination

After phenotypic scoring plants were harvested and fresh foliage weights taken unless stated otherwise. Fresh weight was always measured and samples were never subjected to dry weight determination. In general fresh weights give a good estimate of differences in total biomass are more convenient for large numbers of plant samples. Also after treatment with ALS inhibitors some plants stop growing but remain green and obviously alive. With dry weight determination the distinction between these plants and those that are completely dead tends to be lost. It was felt that use of fresh weights was preferable

due to the nature of studies with herbicides and the need to distinguish between dead plants and affected survivors.

Biomass assessments were carried out by cutting plants at soil level using scissors and weighing to three decimal places. If required for further analysis, foliage was transferred to thick polythene storage bags and labelled with population and unique identifying number. Plants required for later molecular work were harvested using scissors cleaned with 70% ethanol solution and dried to prevent cross contamination of samples. Disposable gloves were worn for all such harvests. After harvest leaf material was immediately frozen using liquid N_2 and stored at -80°C until required. Plants for enzyme extraction were harvested as bulk samples from the same population typically containing around 40 individuals and weighing approximately 50g. Enzyme assay material was not sprayed with herbicide and was harvested at BBCH growth stage 24-28.

2.6 Standard biotypes of *Alopecurus myosuroides*

Throughout the course of this project all work, particularly at the whole plant level, was based on the use of several standard *A. myosuroides* biotypes. These were developed and maintained at Rothamsted by Dr Stephen Moss over several years and provide a unique resource for the study of herbicide resistance in this particular weed species.

2.6.1 Susceptible standard biotype ('wild type')

The importance of a truly susceptible standard in studies of herbicide resistance cannot be over-emphasised. Without such a population there is no baseline against which resistance can be measured. The biotype selected for use as a susceptible standard in this project was termed 'Rothamsted' (Roth) and is grown on Broadbalk field on the Rothamsted Estate in Hertfordshire. Broadbalk *A. myosuroides* has proved its usefulness as a susceptible standard in many different studies, e.g. (Cocker *et al.*, 1999; Cummins *et al.*, 1997; Hall *et al.*, 1995; Hall *et al.*, 1997). The Roth biotype was collected from section 8 at Broadbalk which has received no herbicide input at any time in its over 160 year history and is part of the Broadbalk long-term experiment (Moss *et al.*, 2004). Broadbalk section 8 is one of the few arable sites in the UK where herbicides have never been applied. Seeds are collected from the site every year and stored according to the procedures detailed in section 2.2. This project used *A. myosuroides* seeds collected on Broadbalk in

the summer of 2001 or 2003 as a susceptible standard in all cases.

2.6.2 High metabolism standard biotype

Previous work on herbicide resistance in *A. myosuroides* from the UK identified the Peldon population from Essex as one of the first British populations to develop resistance to a wide range of herbicide modes of action. This population was first identified when it demonstrated resistance to the urea herbicides chlorotoluron and isoproturon in glasshouse tests (Moss & Cussans, 1985). Since then further work has identified that enhanced metabolism mechanisms (P450 and GST) play a role in the cross resistance patterns exhibited in the Peldon biotype (Cocker *et al.*, 1999; Hall *et al.*, 1997; Reade *et al.*, 1997). *A. myosuroides* seeds collected at Peldon in the summer of 1996 (Pel96) have been used throughout this project, both in the glasshouse and the lab, as a standard biotype with the ability to degrade herbicides through metabolic pathways and showing full or partial resistance to a wide spectrum of different products (Kemp *et al.*, 1990). In addition to being a population with the correct characteristics for use as a metabolism standard, the Peldon biotype had the advantage of being one of the most studied field populations of the weed anywhere in the world. Good herbicide records are available for the farm and a comprehensive collection of Peldon seeds (collected annually for 20 years) were available at Rothamsted when this project began.

2.6.3 Other *A. myosuroides* biotypes

Several other *A. myosuroides* biotypes were used throughout this project in specific sections. Some of these were Peldon collections made in different years; some were herbicide selected seed sets; and others were field samples from sites where herbicide resistance was observed. Descriptions of these other *A. myosuroides* biotypes are made in individual chapters as appropriate.

2.7 General laboratory materials

2.7.1 Chemicals

All chemicals were supplied by Sigma (Poole, UK), Fisher Scientific (Loughborough, UK) or Promega (Southampton, UK) unless stated otherwise.

2.7.2 Herbicides

Technical herbicides used in enzyme assays were supplied by DuPont (Stevenage UK).

2.7.3 Molecular biology standard solutions

10x TBE buffer 0.89 M Tris-borate, pH 8.3
0.025 M EDTA

Agarose gel loading buffer 45 mM Tris-borate
1 mM EDTA
5.3 % (v/v) glycerol
0.005 % (w/v) bromophenol blue
0.005 % (w/v) xylene cyanol

1x TE buffer 10 mM Tris-HCl, pH 8.0
1 mM EDTA

2.8 General laboratory methods

The laboratory methods used in enzyme and molecular sections of this project are described in detail in Chapters 6 and 7, respectively.

2.9 Statistical analysis of data

All ANOVA analyses were carried out using the GenStat 7.0 statistics programme (Lawes Agricultural Trust, Rothamsted). Curve fitting was performed using either Genstat 7.0, Sigmaplot 8.0 (SPSS Inc, Chicago, USA) or MLP v3.09 (Lawes Agricultural Trust, Rothamsted). Chi square analysis was done manually using Microsoft Excel. Refer to individual chapters for more information on statistical treatment of data and more detailed methodology. Experimental design for glasshouse work was formulated in consultation with the Rothamsted statistics department. All large experiments required approval by a statistician before any work was undertaken. All error bars on graphs in this thesis should be assumed to represent the standard error of the mean, unless stated otherwise.

3. Whole Plant Response to ALS Inhibitors

3.1 Introduction

Response to herbicides at the whole plant level is the most widely used method to characterise herbicide resistance. Whole plant experiments are useful because they simulate field conditions to an extent and provide comparative data on aspects such as dose response, cross resistance patterns, and percentage of resistant individuals within a population. Whole plant experiments also provide clues about the mechanisms of resistance, primarily through the degree of resistance detected and the extent of cross resistance to herbicides with different modes of action. In short, whole plant work is an essential component of any herbicide resistance study and serves to constantly validate differences detected at the molecular and enzyme levels through correlation with a phenotypic response.

This chapter covers work carried out at the whole plant level to investigate the use of the non-selective ALS inhibiting herbicide sulfometuron-methyl (sulfometuron) as an indicator of high level resistance, and also how populations resistant to this herbicide respond to other ALS inhibitors. Two herbicides were of particular interest in comparison with sulfometuron; flupyrsulfuron-methyl (flupyrsulfuron) and a formulated mixture of mesosulfuron-methyl and iodosulfuron-methyl-sodium (mesosulfuron+iodosulfuron, see Chapter 2 for details). Flupyrsulfuron was the main sulfonylurea herbicide approved for control of *A. myosuroides* in cereals in the UK in 2003. Work done at Rothamsted suggested that enhanced metabolism was the probable mechanism in at least some of the flupyrsulfuron resistant *A. myosuroides* populations (Moss *et al.*, 2005b). The mesosulfuron+iodosulfuron mixture 'Atlantis' was granted approval for use in the UK in November 2003 and so it was important to evaluate the response of resistant populations to this new herbicide. Resistance to sulfometuron has been associated with altered target site ALS enzyme in a survey of Australian ALS inhibitor-resistant weeds (Gill, 1995). Sulfometuron was also used in another Australian study as an indicator of possible ALS target site resistance in grass weeds, due to its low susceptibility to metabolism

(Christopher *et al.*, 1992). A third study used sulfometuron in a selective germination medium for grass weed seeds and showed that a dose of 60 to 90g a.i. ha^{-1} allowed only seedlings with an altered ALS enzyme to grow normally (Burnet *et al.*, 1994). Taken together, these studies indicated that sulfometuron might possibly be used at the whole plant level in *A. myosuroides* as an indicator of possible ALS target site resistance and provided the basis for the work described in this chapter. If this was to be successful in *A. myosuroides*, the first goal was to establish the correct dose rate of sulfometuron to use for selection of highly resistant plants, and then to establish whether it was possible to discriminate between plants with different resistance mechanisms using sulfometuron on whole plants.

3.1.1 Scope of work at the whole plant level

Work at the whole plant level was based on experiments designed to generate cross resistance and dose response data for the standard populations described in section 2.5. These were tested through a series of experiments in conjunction with several others which included some or all of the seed sets described below. Results from glasshouse tests were checked by performing an outdoor experiment more closely replicating field conditions with the same *A. myosuroides* populations.

In addition to dose response and cross resistance tests, screening experiments were set up to evaluate the extent of resistance in problem populations of UK *A. myosuroides* sent to Rothamsted for evaluation. This screening process was ongoing throughout the course of this project and was useful in the identification of field populations possessing high level resistance. Populations showing high levels of resistance were subjected to further investigations examining the mechanisms which are discussed in detail in later chapters. The initial screening tests described in this chapter used eleven seed samples collected from fields where flupyrsulfuron had given inadequate control of *A. myosuroides* in 2002. Later tests focused on samples that showed resistance to mesosulfuron+iodosulfuron in the field and were from 2003 onwards (see Chapter 7).

3.1.2 *A. myosuroides* biotypes used in whole plant work

In addition to the Rothamsted susceptible standard and the Peldon 1996 metabolism standard biotypes, the following seed sets were used extensively in glasshouse and outdoor experiments.

3.1.2.1 Peldon 2002 (Pel02)

A. myosuroides seed collected from the farm at Peldon in Essex where herbicide resistant plants were identified as long ago as 1984 (Moss, 1987b; Moss & Cussans, 1985). Seed collections had been made at this site every year since the 1980s and provided an unmatched resource for examining changing herbicide resistance characteristics over time. Seed from 2002 represented the most recent collection made at the Peldon site when the project was started and was included in some whole plant experiments as an example of a highly resistant field population. Importantly, the ALS inhibitor flupyrsulfuron had been used at Peldon since 2000 and so seed from 2002 was included for comparison with Pel96 seed. Information from the farmer at Peldon indicated some problems with resistance to flupyrsulfuron, which at the time was the main ALS inhibiting herbicide approved for use against *A. myosuroides* in the UK. Peldon *A. myosuroides* has shown resistance by enhanced metabolism to a number of different herbicides including fenoxaprop-p-ethyl (Cocker *et al.*, 1999), diclofop-methyl (Hall *et al.*, 1997), pendimethalin (James *et al.*, 1995), and chlorotoluron (Hall *et al.*, 1995; Hyde *et al.*, 1996).

3.1.2.2 Peldon sulfometuron selected line (PelRES02 and PelRES03)

A single Pel96 plant found to be highly resistant to sulfometuron was self pollinated to produce seed in 2002, giving rise to a suspected ALS target site resistant population, PelRES02. This work and the associated crossing experiments are discussed in detail in Chapter 4. Very little seed was obtained from the initial self crossed plant and most was of low viability. In order to obtain a larger quantity of highly resistant seed, twelve PelRES02 plants which survived treatment with sulfometuron at 100g a.i. ha^{-1} were bulk crossed giving rise to the PelRES03 seed set. PelRES seed represented the most highly resistant seed set available for testing at the start of this project.

3.1.2.3 Faringdon 1995 (Far95)

Faringdon in Oxfordshire is another UK site where *A. myosuroides* has been extensively studied (Moss & Cussans, 1985). Seed from Faringdon has shown partial resistance to inhibitors of acetyl-CoA carboxylase (disruption of fatty acid synthesis) and also substituted phenyl-urea herbicides (inhibition of photosynthesis at photosystem II). Resistance at Faringdon was shown to be due to an enhanced rate of herbicide metabolism (Cocker *et al.*, 1999). Seed collected at Faringdon has consistently exhibited

lower levels of resistance than the Peldon biotype and was used as a second metabolism standard biotype in this project (see section 3.2.3).

3.2 Materials and methods

Several different experimental approaches were adopted in the initial whole plant phase of this project:

- A dose response experiment was performed in order to assess the response of standard populations to the non-selective sulfonylurea herbicide sulfometuron and decide on a dose which would allow discrimination of high-level SU resistance from background enhanced metabolism in the Pel02 population.
- A cross resistance experiment using ALS inhibitors from different chemical classes was included in order to investigate possible mechanisms of resistance in the Peldon biotype.
- A sulfometuron screening test with eleven flupyrsulfuron resistant samples was performed to assess the spread of high level resistance to ALS inhibitors at UK locations other than Peldon.
- Outdoor testing was included to verify that the results from glasshouse tests were applicable to a situation better representing conditions in the field.

3.2.1 Dose response experiment

Seeds sets selected for use in the dose response experiment were the Roth susceptible standard, Pel96 enhanced metabolism standard, Pel02 resistant field population and PelRES03 sulfometuron selected line. Plants were germinated and planted into Kettering loam in 5cm square pots. Two seedlings were initially planted into each pot and these were later thinned to leave a single seedling. Each population was sprayed with seven doses of flupyrsulfuron and sulfometuron with 16 replicate pots per dose. Due to lack of plants only two doses of mesosulfuron+iodosulfuron were used; field rate (12 + 2.4g a.i. ha^{-1}) and 2x field rate (24 + 4.8g a.i. ha^{-1}) with the exception of Roth03 where only the field rate was applied. Plants were sprayed at the three-leaf stage with flupyrsulfuron at 2.5, 5, 10, 20, 40, 80, 160, and 320g a.i. ha^{-1}; mesosulfuron+iodosulfuron formulation at 12 + 2.4 and 24 + 4.8g a.i. ha^{-1} or sulfometuron at 3.125, 6.25, 12.5, 25, 50, 100, 200, 400g a.i. ha^{-1}. Thirty untreated pots per population were included in the experimental design. All pots were sprayed using a track sprayer at the three leaf stage and then

returned to the glasshouse and placed in a completely randomised design as described in Chapter 2.

A. myosuroides plants were harvested 28 days after treatment with herbicide. Harvest date corresponded with complete control of the susceptible standard. Each plant was rated using a 1-5 injury scale compared to control plants as described in Chapter 2. Plants were then harvested by cutting at soil level with scissors and the foliage fresh weight determined for each pot. Sulfometuron and flupyrsulfuron data were analysed by fitting a four parameter logistic curve to the foliage fresh weights using MLP v3.09; ED50 and ED80 values were determined and R/S ratios calculated where possible.

3.2.2 Cross resistance characteristics

The activities of ALS inhibiting herbicides from different chemical groups towards the *A. myosuroides* biotypes Roth99, Pel96, and PelRES02 were compared in a single dose cross resistance experiment. The doses chosen for the experiment were set fairly high in an effort to identify high level resistance. Each population was sprayed with a single dose of sulfometuron, flupyrsulfuron, imazamethabenz or propoxycarbazone-sodium with 16 replicate pots per dose containing either one or two plants per pot. Herbicide doses were sulfometuron at 100g a.i. ha^{-1}, flupyrsulfuron at 20g a.i. ha^{-1} (2x normal field rate, n-rate for control of metabolically resistant *A. myosuroides*), imazamethabenz at 1.2 L ha^{-1} (2x field rate) or propoxycarbazone-sodium at 140g a.i. ha^{-1} (2x field rate). Refer to Chapter 2 for further details of herbicide formulations and adjuvants. Sixteen untreated pots per population were included in the experimental design. All pots were sprayed using the track sprayer and then returned to the glasshouse and completely randomised.

A. myosuroides plants were harvested 35 days after treatment as described in section 3.2.1. Injury scores were determined for each individual plant while fresh foliage weights were determined on a per-pot basis and corrected to give mean fresh weight per plant in the case of pots containing two plants. Plant weight data were analysed using GenStat 7. A one-way ANOVA test was conducted for each herbicide treatment using percent reduction in fresh weights relative to mean weight of untreated plants as a measure of control. This was followed by a two-way ANOVA directly comparing fresh weights.

3.2.3 Initial screening test with flupyrsulfuron resistant seed samples

Plants grown from seed from eleven sites where spraying with flupyrsulfuron gave inadequate control in 2002 were compared with the Pel96 (metabolism standard) and Roth99 (susceptible standard) populations. These samples were collected in the field from surviving plants by farmers, industry consultants and agronomists. Details of the eleven field samples are given in Table 3.1.

Table 3.1 Flupyrsulfuron resistant seed samples from the field in 2002

Sample name	Details (site, rate of flupyrsulfuron applied in harvest year 2001/2002)
BoxA	ADAS Boxworth, Cambridgeshire, 1x field rate flupyrsulfuron
BoxB	ADAS Boxworth, Cambridgeshire, 1x field rate flupyrsulfuron
BoxC	ADAS Boxworth, Cambridgeshire, 1x field rate flupyrsulfuron
BoxD	ADAS Boxworth, Cambridgeshire, 1x field rate flupyrsulfuron
Berr	Farm near Oundle, Northamptonshire, 2x field rate flupyrsulfuron
Chan	Farm near Muston, Leicestershire , 2x field rate flupyrsulfuron
Flaw	Farm near Flawborough, Nottinghamshire, 2x field rate flupyrsulfuron
GBE-01-040	DuPont (Stevenage, Herts), unspecified test site, 2 x maximum field rate
GBB-01-101	DuPont (Stevenage, Herts), unspecified test site, 2 x maximum field rate
Plum	Farm near Oundle, Northamptonshire, 2x field rate flupyrsulfuron
Sand	Unspecified test site, mesosulfuron+iodosulfuron trial

Plants were established in 9cm pots and thinned to six plants per pot. A total of 35 pots were prepared for each population. Plants were sprayed with flupyrsulfuron at 10, 20 and 40g a.i. ha^{-1} (1x, 2x, 4x field rate), mesosulfuron+iodosulfuron mixture at 12 + 2.4 and 24 + 4.8g a.i. ha^{-1} (1x, 2x field rate) and sulfometuron at 100g a.i. ha^{-1}. Untreated controls were also included. Five reps (pots) were sprayed for each treatment with each population. Fresh foliage weights were measured 4 weeks after treatment and each plant given an injury score as described in Chapter 2. Data analysis was performed using GenStat 7. A two-way ANOVA was conducted for each herbicide using percentage reduction in fresh weights relative to untreated plants as a measure of control. For sulfometuron, a one-way ANOVA was used since only one rate was applied.

3.2.4 Outdoor container experiment

This experiment was aimed at identifying whether the results from glasshouse studies could be repeated in a scaled-up outdoor container experiment. Outdoor containers provide a more realistic simulation of field conditions than pots under glass. Populations selected for this experiment were; Roth03 (susceptible standard), Pel96 (a population which can metabolise a wide variety of herbicides including flupyrsulfuron), Berr02 (a representative population from 11 found to be flupyrsulfuron resistant in 2002), Pel02, Pel02SS (seed from Pel02 plants which survived sulfometuron treatment in 2003), and PelRES03 (sulfometuron selected suspected ALS target site resistant population).

Before planting into containers all seed samples were cleaned to the same level using a seed cleaner then subjected to viability assessments and germination tests. Refer to Chapter 2 for details of container preparation and plant growth and management. A total of 21 containers were prepared per population and placed outside on a sand-bed in October of 2003. Containers were watered as required for the first few days until the dry loam was sufficiently moistened. Plants were sprayed at the three-leaf stage in January 2004 with flupyrsulfuron at 10, 20 and 40g a.i. ha^{-1} (1x, 2x, 4x field rate), (mesosulfuron+iodosulfuron formulation at 12 + 2.4 and 24 + 4.8g a.i. ha^{-1} (1x and 2x field rate) or sulfometuron at 100g a.i. ha^{-1}. The experiment comprised a randomised block design with three replicates. There were two untreated containers per population per replicate. After spraying, pots were returned to the sand-bed and randomised in replicate blocks.

Plants were harvested on 08 April 2004. Prior to harvest the total number of surviving plants in each container was counted and each was assigned an injury score on a 1 to 3 scale as compared to control plants of the same population. The injury scoring used was a modification of the five category method described in section 2.5.1. Plants were harvested by cutting at soil level with scissors and the foliage fresh weight determined for each container. Data was analysed by ANOVA using GenStat 7. Analysis of variance was conducted using percentage reductions in fresh weights relative to untreated plants, foliage fresh weights and numbers of surviving plants in order to compare different approaches to analysis.

3.3 Results

3.3.1 Dose response experiment

MLP was used to fit four parameter logistic curves to the flupyrsulfuron and sulfometuron dose response data allowing calculation of ED_{50} and ED_{80} values and resistance indices compared to the susceptible standard, Roth03. For flupyrsulfuron ED_{50} the calculated R/S ratios were Pel96 (103), Pel02 (275) and PelRES03 (98) (inferred from Table 3.2). For ED_{80} the ratios were 88, 308 and 157, respectively.

Table 3.2 Summary of flupyrsulfuron ED_{50} and ED_{80} values calculated using MLP. R/S ratios are resistant ED_{50} value divided by susceptible value

Herbicide	Population	log_{10}ED50 (S.E.)	ED50 g a.i. ha^{-1}	log_{10}ED80 (S.E.)	ED80 g a.i. ha^{-1}
flupyrsulfuron	Roth03	0.0190 (0.1270)	<2.5	0.8879 (0.0541)	7.7
	Pel96	2.0316 (0.0705)	107.5	2.8330 (0.1384)	680.8
	Pel02	2.4581 (0.0914)	287.1	3.3770 (0.2436)	2382.3
	PelRES03	2.0113 (0.0992)	102.6	3.0837 (0.2444)	1212.6

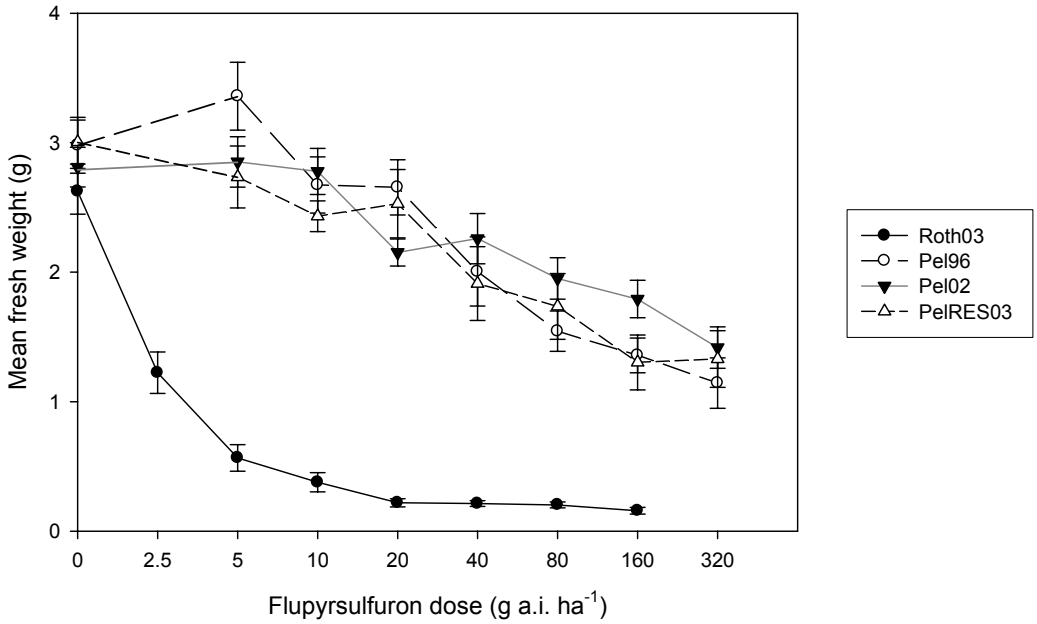

Figure 3.1 Mean fresh weight plotted against flupyrsulfuron dose for all populations. Error bars are S.E. of the means.

Curve fitting was not as successful for the sulfometuron as for the flupyrsulfuron data. This was due to selection of starting doses which were somewhat high. In retrospect it would perhaps have been more useful to cover a wider range of dose levels and use a lower starting dose. See Table 3.3 for a summary of results.

Table 3.3 Summary of sulfometuron ED_{50} and ED_{80} values calculated using MLP

Herbicide	Population	$log_{10}ED50$ (S.E.)	ED50 g a.i. ha^{-1}	$log_{10}ED80$ (S.E.)	ED80 g a.i. ha^{-1}
sulfometuron	Roth03	-3.5909 (1.4609)	<3.125	0.8742 (0.6461)	7.5
	Pel96	0.0706 (0.2320)	<6.25	1.4682 (0.0800)	29.4
	Pel02	0.6091 (0.2227)	<6.25	2.0987 (0.1339)	125.5
	PelRES03	Fitting failed	NC	Fitting failed	NC

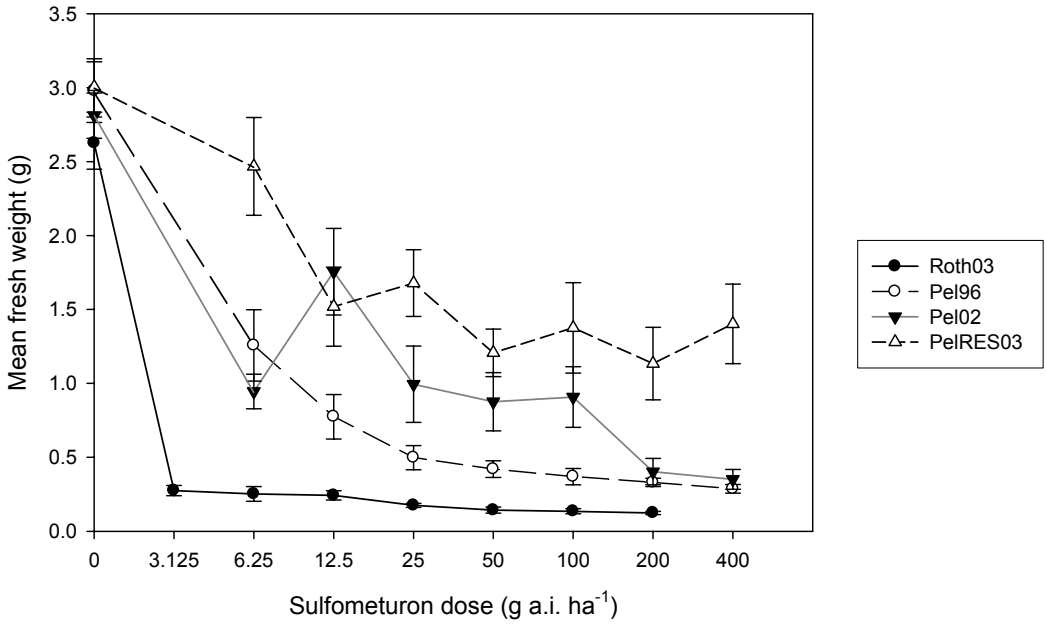

Figure 3.2 Mean fresh weight plotted against sulfometuron dose for all populations. Error bars are S.E. of the means.

Further work was performed where curves were fitted to the mean weights for each population at each sulfometuron dose rate. ED_{50} values were calculated as before using MLP. Results were as follows: Roth03 (<3.125g a.i. ha^{-1}), Pel96 (<6.25g a.i. ha^{-1}), Pel02

(<6.25g a.i. ha^{-1}), PelRES03 (47.5g a.i. ha^{-1}). Obtaining an ED50 value for the PelRES03 seed set was a useful result and showed that a screening dose of 100g a.i. ha^{-1} is likely to be sufficient for discrimination between target-site and non target-site resistant plants. This was further supported by counting the numbers of plants in each injury category at 100, 200 and 400g a.i. ha^{-1} sulfometuron in the suspected target site resistant PelRES03 seed set. The results are summarised in Table 3.4 and show that increasing sulfometuron dose rate from 100 to 400g a.i. ha^{-1} had no appreciable effect on the distribution of injury scores according to visual assessment. At 100g a.i. ha^{-1} sulfometuron, the proportion of plants falling into injury categories 1 and 2 was as follows: Roth03 (0), Pel 96 (0), Pel02 (12.5%) and PelRES03 (37.5%).

Table 3.4 Number of PelRES03 plants per injury category following treatment with sulfometuron. Each treatment consisted of 16 replicate plants.

Injury score	Number of plants in each injury category per sulfometuron dose rate		
	100g a.i. ha^{-1}	200g a.i. ha^{-1}	400g a.i. ha^{-1}
1	4	3	3
2	2	4	3
3	4	2	5
4	3	2	2
5	3	5	3

The proportion of plants surviving mesosulfuron+iodosulfuron with either no injury (category 1) or slight injury (category 2) was calculated for each population and these plants were categorised as 'resistant'. At the 12 + 2.4g a.i. ha^{-1} dose (field rate) the Pel02 population contained 38% resistant plants while the PelRES03 population consisted of 70% resistant plants. The Roth03 and Pel96 populations contained no resistant plants but 50% of Pel96 plants survived with moderate to severe damage. At the 24 + 4.8g a.i. ha^{-1} dose (2 x proposed field rate) Pel02 contained 13% resistant plants and PelRES03 was again the most resistant population with 50% of plants showing little damage at all. The Pel96 population showed 44% damaged survivors at the higher dose rate.

3.3.2 Cross resistance characteristics

Table 3.5 Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants within herbicide treatments

Seed set	Mean untreated weight (g)	Mean % reduction relative to mean weight of untreated plants			
		Flupyrsulfuron 20g a.i. ha^{-1} 2x field rate	Propoxycarb-azone 140g a.i. ha^{-1} 2x field rate	Imazametha-benz 1200g a.i. ha^{-1} 2x field rate	Sulfometuron 100g a.i. ha^{-1}
Roth99	5.6	97	97	97	97
Pel96	6.8	29	39	28	95
PelRES02	6.0	25	38	31	66
S.E.±	0.5	6.1	7.6	6.8	4.1
LSD,5%	1.5	17.6	22.0	19.5	11.7

3.3.2.1 General observations

The Roth99 susceptible standard was well controlled by all herbicide treatments with more than 98% of all plants killed overall and the remainder showing severe effects (See Figure 3.3 for representative Roth99 plants 35 days after treatment). Fresh weight reduction relative to untreated plants was 97% in all cases. With flupyrsulfuron, propoxycarbazone and imazamethabenz there were significant differences in control between the Rothamsted and Peldon seed sets (both Pel96 and PelRES02). This was supported by the scoring which showed that Pel96 and PelRES02 had more than 70% of plants showing either no effects or only slight effects with each of these herbicide treatments. There were no significant differences between control of the Pel96 and PelRES02 populations (see Figures 3.4 and 3.5 for pictures of representative plants from each seed set 35 days after herbicide treatment and Table 3.5 for a summary of results).

Sulfometuron gave good control of the Pel96 plants, with no significant difference in fresh weight reduction when compared to the Roth99 population ($p \leq 0.05$). Significantly poorer control (66% fresh weight reduction compared to untreated plants) was observed for the PelRES02 population (see Figure 3.5). In terms of the plant vigour assessment, 44% of PelRES02 plants showed no effects or only slight effects. None of the Pel96 plants fell into these two categories, with all showing at least moderate effects, and 84%

being killed or showing serious effects after sulfometuron treatment. The PelRES02 population was significantly less well controlled than the Pel96 population ($p \leq 0.05$).

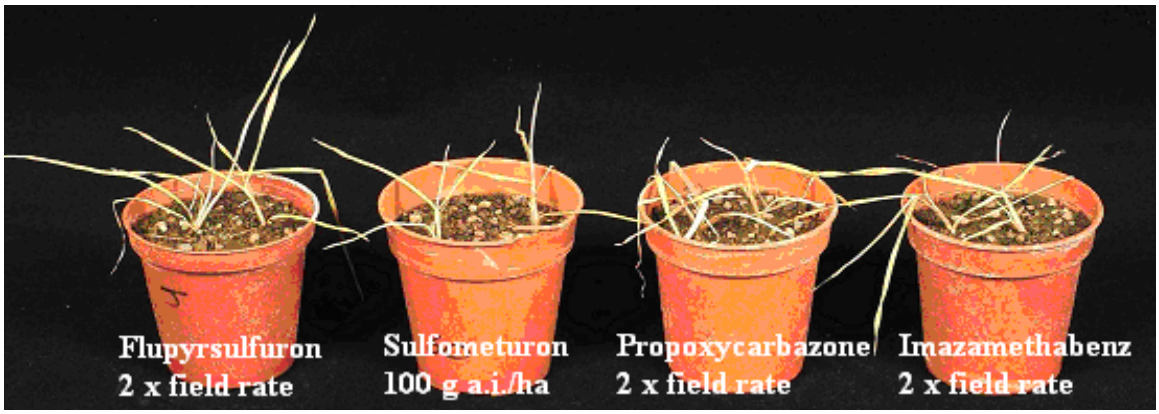

Figure 3.3 Roth99 susceptible standard plants 35 days after treatment with various ALS inhibiting herbicides

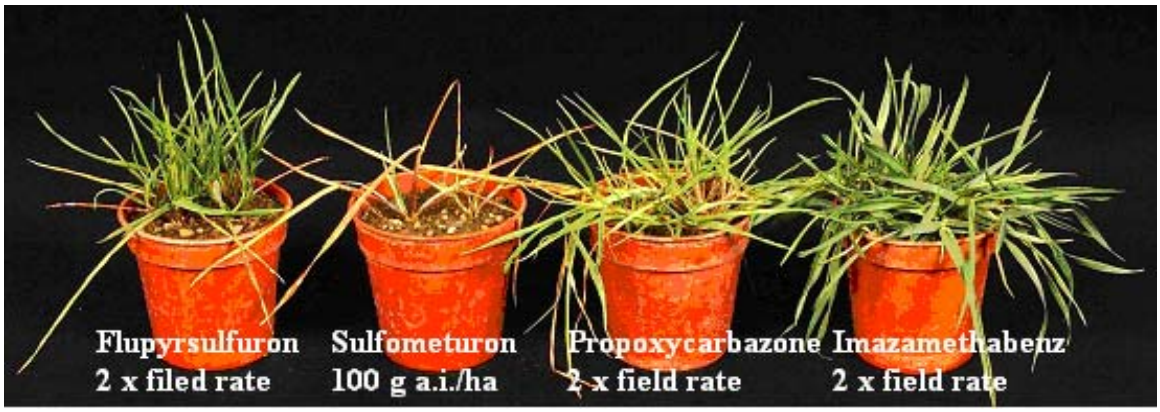

Figure 3.4 Pel96 metabolism standard plants 35 days after treatment with various ALS inhibiting herbicides

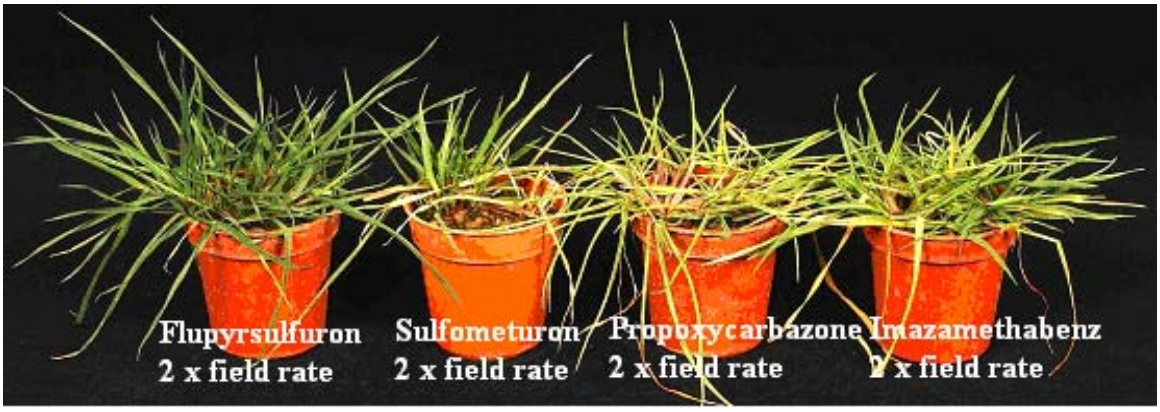

Figure 3.5 PelRES02 suspected target site resistant plants 35 days after treatment with various ALS inhibiting herbicides

3.3.2.2 Further analysis

Results were further analysed using direct foliage fresh weight comparison. A two way ANOVA test using GenStat 7 was performed with herbicide treatment (including nils) and seed set as the independent variables and mean fresh weight per plant as the dependent variable. Results are summarised in Table 3.6. Values marked ^ represent populations poorly controlled relative to the susceptible standard for that particular treatment.

Table 3.6 Analysis of variance comparing fresh foliage weights across treatments and populations

Seed set	Mean untreated weight (g)	Mean fresh weight per plant (g)			
		Flupyrsulfuron 20g a.i. ha^{-1} 2x field rate	**Propoxycarb-azone 140g a.i. ha^{-1} 2x field rate**	**Imazametha-benz 1200g a.i. ha^{-1} 2x field rate**	**Sulfometuron 100g a.i. ha^{-1}**
Roth99	5.6	0.2	0.2	0.2	0.2
Pel96	6.8	4.7^	4.2^	4.5^	0.4
PelRES02	6.0	4.4^	3.7^	4.0^	2.0^
S.E.±	0.5	0.4			
LSD,5%	1.5	1.2			

All treated weights for each population were significantly lighter than the corresponding untreated weights and all of the herbicides had a measurably significant effect upon plant weights compared to untreated plants regardless of seed set. There were no significant differences in herbicide activity between flupyrsulfuron, propoxycarbazone and imazamethabenz; however plants treated with sulfometuron showed significantly lower fresh foliage weights 5 weeks after treatment compared to all other herbicide treatments (Pel96 and PelRES02 seed sets). The PelRES02 plants showed significantly poorer control after sulfometuron treatment than either the susceptible Roth99 plants or those from the metabolism standard seed set Pel96. Overall the results were the same as for percentage reduction analysis and added little additional information other than highlighting the significantly better performance of sulfometuron against all populations. Residual plots were less skewed with direct fresh weight analysis than percentage reduction analysis.

3.3.3 Screening test with flupyrsulfuron resistant seed samples

3.3.3.1 General observations

Roth99, the susceptible standard, was well controlled by all herbicide treatments. One hundred percent control was achieved in all cases with the exception of flupyrsulfuron at 1x field rate, where 13% survived with severe effects. In the case of the susceptible standard, complete control corresponded to fresh weight reduction greater than or equal to 89% relative to untreated plants. A summary of percentage reduction values are presented in Table 3.7.

Figure 3.6 Flupyrsulfuron resistant seed sets after treatment with sulfometuron

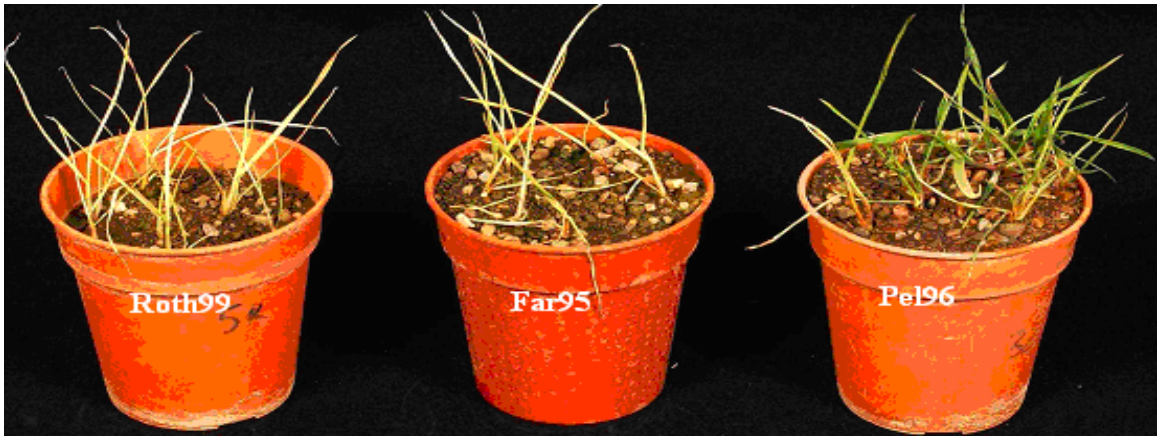

Figure 3.7 Plants from standard seed sets following treatment with mesosulfuron+ iodosulfuron mixture at field rate

3.3.3.2 Flupyrsulfuron

Flupyrsulfuron gave good control of the Roth99 susceptible standard at all three rates. The Pel96 enhanced metabolism standard, along with each of the 11 suspected resistant populations from 2002, was significantly less well controlled than the susceptible standard at 1x field rate. At 2x field rate, all 11 populations with the exception of BoxD showed significantly poorer control than the susceptible standard. The application of 4x field rate flupyrsulfuron reduced the number of populations showing significant resistance to six of the original eleven; these being Berr, Flaw, BoxA, Plum, Sand and BoxB in order of increasing resistance. Pel96 showed significant resistance at both 2x and 4 x field rates. At 4x field rate, all six resistant populations were less well controlled than the Pel96 metabolism standard. Of the populations showing resistance to flupyrsulfuron, BoxA, Flaw, and Sand also showed evidence of some resistance to mesosulfuron+iodosulfuron, along with marginally poorer control with sulfometuron.

3.3.3.3 Mesosulfuron+iodosulfuron

The mesosulfuron+iodosulfuron formulation, gave good control of most populations at field use rate. Populations significantly less well-controlled than the Roth99 susceptible standard included Pel96 at 77%, Flaw at 80% and Sand at 66% reduction in fresh weight relative to untreated plants (see Figure 3.7). The BoxA population also showed evidence, although not statistically significant, of some resistance at 1x field rate ($p \leq 0.05$). In all

populations the resistance was partial, with some individuals less affected than others. All populations were well controlled at 2x field rate.

3.3.3.4 Sulfometuron

More than 98% of all plants treated with sulfometuron showed either severe effects or were dead (see Figure 3.6). No plants showed less than moderate effects. Slightly less than 90% fresh weight reduction relative to untreated plants was observed in Roth99, BoxA, Flaw and Sand populations. The fact that the Rothamsted susceptible standard was among the seed sets showing very slightly less weight reduction compared to untreated plants indicates that the effect observed in other seed sets was probably not linked to any particular resistance mechanism. The metabolism standard population Pel96 was well controlled following application of sulfometuron at 100g a.i. ha^{-1}.

Table 3.7 Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants within herbicide groups at different treatment rates

Seed set	Mean untreated weight (g) per pot	Mean % reduction relative to mean weight of untreated plants					
		Flupyrsulfuron			Mesosulfuron+ Iodosulfuron		Sulfomet-uron
		1x Field	2x Field	4x Field	1x Field	2x Field	100g ha^{-1}
Roth99	7.8	88	90	91	91	93	89
Far95	8.0	78	86	93	96	96	96
Pel96	10.2	27	43	60	77	94	93
BoxA	4.6	-21	26	47	81	90	88
BoxB	8.6	6	42	36	86	93	93
BoxC	9.7	43	55	73	93	94	93
BoxD	8.9	43	64	71	89	94	94
Flaw	8.7	21	59	49	80	90	87
Chan	10.7	21	45	71	90	93	90
Berr	10.5	27	36	51	93	95	93
Plum	9.5	16	33	44	86	94	91
Sand	7.4	-12	21	43	66	92	88
GBB	7.9	6	37	63	89	94	94
GBE	11.6	24	61	79	87	90	92
S.E.±	0.8	10.1			3.8		1.1
LSD,5%	2.2	28.3			10.6		3.1

3.3.3.5 Further analysis

Direct fresh weights were analysed using GenStat in order to check that trends remained constant with different types of analysis. Fresh weights from each herbicide treatment were compared to untreated weights and across treatments within herbicide groups using a two-way ANOVA as before. Unfortunately the residual plots from this analysis were more highly skewed than those from comparison by percentage reduction and the analysis was abandoned. After consultation with a statistician it was decided to attempt a transformation on the percentage reduction data as this was much less skewed than the fresh weight data. The transformations used were $\log_{10}$(100-%reduction+1) and sqrt(100-%reduction). The $\log_{10}$ transformation of original percentage fresh weight reduction data was the more successful of the two and tended to increase the number of seed sets showing significant resistance to each herbicide treatment compared to the Roth susceptible standard. Again Berr, Flaw, BoxA, Plum, Sand and BoxB showed up as the most resistant seed sets along with the metabolism standard population Pel96. Residual plots were less skewed than for untransformed fresh weight data and were comparable to those from percentage reduction analysis.

3.3.4 Outdoor container experiment

3.3.4.1 General observations

Analysis of variance showed significant differences at the population level of analysis and between the 1x and 2x field rate treatments of flupyrsulfuron (see Table 3.8). Roth03, the susceptible standard, was well controlled by all herbicide treatments; no survivors were found in any herbicide treated container. Complete control of Roth03 plants corresponded to fresh weight reduction greater than or equal to 98% relative to untreated plants. Resistance patterns differed between herbicides (see Table 3.8).

Table 3.8 Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants within herbicide groups

Seed set	Mean untreated weight (g)	Mean % reduction relative to mean weight of untreated plants				
		Flupyrsulfuron		Mesosulfuron+iodosulfuron		Sulfometuron
		1x Field	2x Field	1x Field	2x Field	100 g a.i. ha^{-1}
Roth03	100	98	98	98	99	99
Pel96	92	68	50	95	94	88
Berr02	60	24	28	95	98	92
Pel02	82	9	42	59	68.5	67
Pel02SS	83	3	-14	5	10	3
PelRES03	78	2	10	39	49	21
S.E.±	4.8	10.1		5.9		11.7
LSD,5%	14.0	29.7		17.3		37.3

3.3.4.2 Flupyrsulfuron

Flupyrsulfuron at 1x field rate gave complete control of the susceptible standard Roth03 but significantly poorer control of both the Pel96 and Berr02 populations, with Berr02 significantly less well controlled than Pel96. No treatment effects were observed for either population. At 2x field rate flupyrsulfuron gave significantly poorer control of both populations compared to the susceptible standard (see Table 3.8). There was no significant difference between Pel96 and Berr02 at 2x field rate flupyrsulfuron. Flupyrsulfuron did not perform well on the resistant Pel02, Pel02SS and PelRES03 populations, with percentage reduction in fresh weight relative to untreated plants ranging from 2% to 9% at 1x field-rate flupyrsulfuron. Application of 2x field rate flupyrsulfuron showed up some differences between the three resistant populations with significantly poorer control of Pel02SS in relation to the other two populations. PelRES03 was the second most resistant population, whereas Pel02 was not significantly more resistant than Berr02 and Pel96 at this dose of flupyrsulfuron.

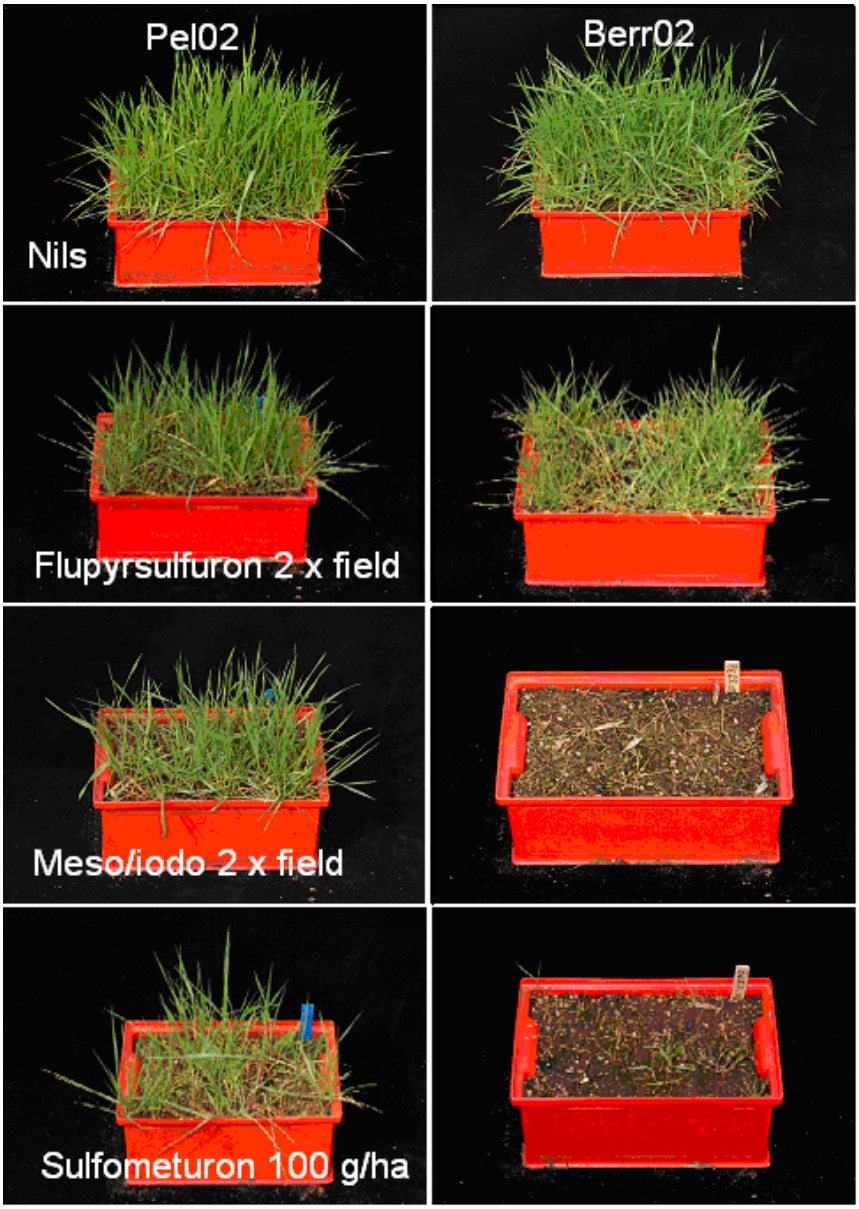

Figure 3.8 Examples of Pel96 and Berr02 containers at evaluation time-point in April 2004. Containers were treated with herbicides in Jan 2004

3.3.4.3 Mesosulfuron+iodosulfuron mixture

Mesosulfuron+iodosulfuron provided much better control in general than flupyrsulfuron. The high metabolism population Pel96 and the flupyrsulfuron-resistant Berr02 did not show any significant resistance to mesosulfuron+iodosulfuron compared to the susceptible standard Roth03 (see Table 3.8 for details). The three more resistant populations segregated in the same order as before with Pel02SS significantly more

resistant than PelRES03, which was in turn significantly more resistant than Pel02. All three populations showed significantly less percentage reduction in fresh weight compared to the susceptible standard Roth03 and the less resistant populations Berr02 and Pel96.

3.3.4.4 Sulfometuron

The sulfometuron based formulation gave very similar results to mesosulfuron+iodosulfuron on all populations (see Table 3.8). Slightly poorer control was achieved on all Peldon derived populations using this herbicide, but the order or resistance was essentially the same. Pel02SS and PelRES03 were the only populations which showed significant resistance to sulfometuron compared to the susceptible standard. It is interesting to note that although good control was achieved in general using mesosulfuron+iodosulfuron and sulfometuron; survivors were found in all populations with the exception of the susceptible standard. The mechanism of resistance in these survivors is an important consideration when deciding on long-term usage strategies with highly selective sulfonylurea herbicides.

3.3.4.5 Additional analyses

Further analyses were conducted in order to verify that the trends detected in population response to herbicides were not artefacts of the analysis method.

Table 3.9 Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants between herbicide groups

Seed set	Mean untreated weight (g)	Mean % reduction relative to mean weight of untreated plants				
		Flupyrsulfuron		Mesosulfuron+iodosulfuron		Sulfometuron
		1x Field	2x Field	1x Field	2x Field	100g a.i. ha^{-1}
Roth03	100	98	98	98	99	99
Pel96	92	68	50	95	94	88
Berr02	60	24	28	95	98	92
Pel02	82	9	42	59	68.5	67
Pel02SS	83	3	-14	5	10	3
PelRES03	78	2	10	39	49	21
S.E.±	4.8	8.9				
LSD,5%	14.0	25.3				

Analysis of variance showed significant effects for treatment, population and population-treatment interaction when all herbicide treatments were analysed together (see Table 3.9). Flupyrsulfuron showed significantly poorer control than mesosulfuron+iodosulfuron and sulfometuron. There was no significant difference in control between mesosulfuron+iodosulfuron and sulfometuron, and no significant overall effect of dose with flupyrsulfuron or mesosulfuron+iodosulfuron. A third ANOVA was conducted using fresh plant weights rather than percentage reduction values. This was done in order to evaluate any potential bias resulting from conversion to percentage values, see Table 3.10 for details. All herbicides were analysed together as treatments using Genstat 7 as before.

Table 3.10 Analysis of variance comparing fresh foliage weights between herbicide groups

Seed set	Mean untreated weight (g)	Mean fresh weight per container (g)				
		Flupyrsulfuron		Mesosulfuron+iodosulfuron		Sulfometuron
		1x Field	2x Field	1x Field	2x Field	100g a.i. ha^{-1}
Roth03	100	1.9	1.7	2.2	1.1	1.3
Pel96	92	30.0	45.9	4.7	5.4	11.4
Berr02	60	45.3	43.0	3.0	1.4	4.6
Pel02	82	75.2	47.8	33.6	28.1	27.4
Pel02SS	83	80.4	94.2	79.1	75.0	80.4
PelRES03	78	77.0	70.1	48.0	40.0	63.9
S.E.±	4.8	7.1				
LSD,5%	14.0	20.1				

The results from fresh weight analysis were similar to the percentage reduction results. All resistance trends were the same with only some minor differences, see Table 3.10 for a summary. For example in the mesosulfuron+iodosulfuron treatments PelRES03 did not show up as significantly more resistant than Pel02. Pel02 also showed significant resistance to sulfometuron compared with the susceptible standard when analysed by fresh weights instead of percentage fresh weight reduction. A final ANOVA was performed using counts of percentage survival as a measure of control; see Table 3.11 for a summary of results. Plants rated 1 or 2 on the injury scale were counted as (undamaged) survivors in this case

Table 3.11 Analysis of variance comparing mean percent survival between herbicide groups

Seed set	Mean untreated plant count	Mean % survival				
		Flupyrsulfuron		Mesosulfuron+iodosulfuron		Sulfometuron
		1x Field	2x Field	1x Field	2x Field	100g a.i. ha^{-1}
Roth03	52	0.0	0.0	0.0	0.0	0.0
Pel96	92	51.8	68.5	1.8	2.2	9.1
Berr02	71	72.8	48.3	1.4	0.0	0.0
Pel02	73	93.6	65.8	35.6	24.7	34.7
Pel02SS	72	104.6	101.8	113.9	104.2	101.8
PelRES03	75	92.5	88.5	52.0	39.1	63.5
S.E.±	3.7	7.7				
LSD,5%	10.8	21.7				

These results give are very similar to those generated by previous fresh-weight analyses. Overall, differences between seed sets are less likely to be significant when analysed in this way, but trends in resistance are the same with only a few minor differences.

3.4 Discussion

This section describes how the results from glasshouse and outside container experiments in 2003/2004 were used to develop a screening methodology using sulfometuron as an indicator of potential ALS target site resistance in *A. myosuroides*. This initial work at the whole plant level also provided important insights into the prevalence and nature of ALS inhibitor resistant black-grass in the UK and provided the basis for later work at the enzyme and molecular levels (refer Chapters 6 and 7 respectively).

3.4.1 Dose response experiment

An unavoidable consequence of using seed samples from the field was that they were mixtures of susceptible and resistant plants. These samples did not yield classic dose response curves and curve fitting was difficult, especially in the case of sulfometuron. In dose response tests with the Peldon seed sets susceptible plants were controlled at very low doses of sulfometuron while resistant plants survived much higher doses meaning it was difficult to fit curves to the data. The choice of MLP as curve fitting software was

helpful because the programme is fairly robust. The problem of poor fits was partly overcome by using mean fresh weights at each dose rate and allowed calculation of an ED50 value of 47.5g a.i. ha^{-1} for the PelRES03 seed set which turned out to be the most highly resistant of those tested. The presence of a consistent proportion of completely undamaged PelRES03 plants, in addition to constant fresh weights with increasing dose, was indicative of a high level resistance mechanism distinct from the metabolic resistance present in the Pel96 seed set. This conclusion was further supported by the flupyrsulfuron dose response curves: ED50 values for flupyrsulfuron were higher for both the Pel96 metabolism standard and the Pel02 field sample than for the PelRES03 seed set. The fact that flupyrsulfuron is subject to resistance by enhanced metabolism in *A. myosuroides* (Moss *et al.*, 2005b) points to a distinct mechanism at work in the PelRES03 plants resistant to sulfometuron.

The ED50 value of around 50g a.i. ha^{-1} for the artificially sulfometuron selected PelRES03 plants was used to set the conservative dose of 100g a.i. ha^{-1} sulfometuron for any further experiments involving screening at the population level for highly resistant plants showing possible ALS target site resistance. It is worth noting that for the high metabolism Pel96 population, only two plants survived at the 50g a.i. ha^{-1} dose with anything less than severe injury or death. At the 100g a.i. ha^{-1} sulfometuron dose only a single Pel96 plant survived and it was highly damaged. It was therefore felt that 100g a.i. ha^{-1} of sulfometuron provided an adequate screening dose to overcome metabolic resistance and select plants with high level resistance mechanisms such as mutation conferring enzyme target site substitution.

The same kind of completely undamaged plants were picked up in the Pel02 field seed set but at a much lower level than in the PelRES03, and not at all at the highest dose rate of 400g a.i. ha^{-1}. The low numbers of plants tested meant that it was impossible to say whether this was a dose effect or just a consequence of high-level resistance being present only at a very low level in the Pel02 seed set. The fact that Pel02 was a field collected set whereas PelRES03 was from crossing of a highly resistant plant followed by selection of progeny with sulfometuron made the presence of plants within the Pel02 population showing the same highly resistant response to sulfometuron more significant. In addition to this Pel02 plants were the most highly resistant of any population to flupyrsulfuron with an ED50 value of around 2400g a.i. ha^{-1} (field rate flupyrsulfuron is 10g a.i. ha^{-1})

and an R/S ratio of 308 when compared to the susceptible standard. These very high levels of flupyrsulfuron resistance, almost twice those of the next closest in PelRES03, indicate that the Pel02 population has a flupyrsulfuron specific resistance mechanism of a different nature to the sulfometuron resistance seen in PelRES03 plants. This is not surprising since *A. myosuroides* in the field at Peldon had been receiving flupyrsulfuron for three years by 2002. The demonstrated ability of sulfometuron to overcome this resistance mechanism, which was most likely due to enhanced metabolism of flupyrsulfuron, makes it an invaluable tool as an indicator of possible target site resistance in *A. myosuroides*.

The results from testing all populations with mesosulfuron+iodosulfuron formulation at 1x and 2x field rates were interesting and showed implications for its future use in the field. High level resistant plants were found to make up a large proportion of both the PelRES03 sulfometuron selected seed set and also the Pel02 field seed set. Even at twice field rate of mesosulfuron+iodosulfuron (800g a.i. ha^{-1}) Pel02 contained 13% resistant plants while PelRES03 contained 50%. These results showed that it was possible for resistance mechanisms to exist in field *A. myosuroides* populations which had not previously been exposed to mesosulfuron+iodosulfuron. Pel96 metabolism standard plants showed no high level resistance to mesosulfuron+iodosulfuron and this further supported the idea of a new high level resistance mechanism in the Pel02 seed set.

3.4.2 Cross resistance characteristics

This experiment was performed in order to provide information on ALS target site cross-resistance between the different groups of ALS inhibiting herbicides using the suspected target site resistant PelRES02 seed set compared to the metabolism standard Pel96 and the Roth susceptible standard. Results showed that both Pel96 and PelRES02 seed sets were equally poorly controlled by all herbicides except sulfometuron. The fact that the PelRES02 plants showed significantly poorer control with sulfometuron than the Pel96 plants supported the dose response work; a proportion of the PelRES02 population displays a high level resistance mechanism distinct from the metabolic resistance present in the Pel96 seed set. Enzyme assays and work at the molecular level were carried out in order to ascertain whether resistance to sulfometuron in the PelRES02 population was due to an insensitive target site or to an enhanced metabolism mechanism. This work is addressed in Chapters 6 and 7 respectively.

Overall these results again support the idea that sulfometuron has potential as an indicator of high level resistance in *A. myosuroides* at the dose of 100g a.i. ha^{-1}. No significant difference was picked up between the response of the Pel96 and PelRES populations to twice field rate applications of flupyrsulfuron, propoxycarbazone, and imazamethabenz, indicating that these herbicides are most likely detoxified by the enhanced metabolism mechanism present in the Pel96 population. Differences between populations might have shown up according to amino acid analysis in leaves or chlorophyll analysis, but these methods were not attempted. Consideration of methods other than fresh foliage weight should be given consideration in future work with ALS inhibitors. Dose response experiments with propoxycarbazone and imazamethabenz were not performed due to time constraints, but might have yielded more information on the cross resistance characteristics of the high level mechanism present in the PelRES seed set. Cross resistance data might have been useful because different ALS target site mutations are often associated with characteristic cross resistance patterns (Heap, 2006; Tranel & Wright, 2002).

3.4.3 Initial screening test with flupyrsulfuron resistant seed samples

Pot screening experiments with a selection of flupyrsulfuron-resistant populations obtained from farmers, consultants and industry representatives, showed that whatever mechanism confers sulfometuron resistance in Peldon *A. myosuroides*, it was not widespread in the UK as of 2002. Flupyrsulfuron resistance was widespread and most likely metabolism based; all statistical data sets were in general agreement regarding resistant populations. Berr, Flaw, BoxA, Plum, Sand and BoxB showed up as the most resistant seed sets along with the metabolism standard population Pel96. Direct analysis of weights was potentially the most useful statistical analysis performed, although a $\log_{10}$ transformation was required before analysis of variance.

Treatment with sulfometuron, a herbicide thought to be less susceptible to metabolism, resulted in near complete control of all eleven flupyrsulfuron resistant populations from 2002. BoxA, Flaw and Sand emerged as the three populations showing greatest levels of resistance to all herbicides, perhaps due to enhanced metabolism. The resistance shown by BoxA and Flaw to the mesosulfuron+iodosulfuron formulation Atlantis WG is interesting since neither of these populations had been exposed to the herbicide before. The Sand population showed greater mesosulfuron+iodosulfuron resistance but had been

sprayed with the herbicide in a previous trial. Overall these results emphasise the prevalence of flupyrsulfuron resistance in British *A. myosuroides* populations and illustrate that the mechanism responsible can be overcome by treatment with sulfometuron or, in some cases, mesosulfuron+iodosulfuron.

3.4.4 Outdoor container experiment

Overall results from the outdoor container experiment support the earlier glasshouse studies in terms of general resistance trends. Of the resistant Peldon seed sets, PelRES was again significantly more resistant in terms of fresh weight comparison than the field-collected Pel02 population and the metabolism standard Pel96 after treatment with sulfometuron. Interestingly a Pel02 sulfometuron selected seed set (Pel02SS) displayed even higher levels of resistance than the PelRES plants. The fact that a heritable resistance mechanism exists in the Pel02 field population and provides very high level resistance to the non-selective sulfonylurea sulfometuron is good evidence for a resistance mechanism distinct from the enhanced metabolism normally present in Peldon seed sets.

Direct comparison of percentage reduction in fresh weight relative to untreated plants showed that glasshouse studies can give slightly misleading results for the magnitude of resistance to sulfonylureas in *A. myosuroides* when compared to outdoor studies. This could be due to reduced levels of cuticular waxes in plants grown under glass compared to those grown outside which would tend to emphasise differences between target site resistant plants and those possessing low-level resistance mechanisms. Particular examples of this difference between glasshouse and outdoor results include the response of the Pel96 metabolism standard population to flupyrsulfuron in outdoor containers; this population was very resistant in the glasshouse but considerably less so in outdoor containers. Similarly Berr02 showed a very similar level of resistance to flupyrsulfuron in glasshouse studies, but proved significantly more resistant than Pel96 outdoors. The more resistant populations show greater consistency in results when glasshouse and outdoor studies are compared indicating that the danger of misinterpretation may be greater with partially resistant populations. One exception to this was the PelRES seed set which showed higher levels of resistance to sulfometuron outdoors than in the glasshouse.

Mesosulfuron+iodosulfuron resistant plants from the Pel02 population were grown on and seeds were collected. These will be tested to investigate whether

mesosulfuron+iodosulfuron produces a similar inherited resistance effect to the one shown in the Pel02SS population. If so then this will have implications for the future use of mesosulfuron+iodosulfuron at Peldon.

3.5 Chapter summary

- Resistance to flupyrsulfuron was widespread in UK *A. myosuroides* populations from the field in 2002.
- Significant resistance to sulfometuron was only present in three seed sets originating from a single farm near Peldon in Essex. Two of these were sulfometuron selected lines (PelRES and Pel02SS) while the third was a field collected sample (Pel02).
- Sulfometuron resistance was characterised by a proportion of very highly resistant plants in each seed set which were largely unaffected by application of high doses of sulfometuron.
- Dose response experiments identified a dose of 100g a.i. ha^{-1} sulfometuron as more than sufficient to overcome metabolism based resistance in the Pel96 population. This was used in later experiments as a screening dose for high level resistance.
- Resistance to the mesosulfuron+iodosulfuron formulation followed the same pattern as sulfometuron resistance with the most resistant seed sets being the PelRES and Pel02SS sulfometuron selected lines and significant resistance also being present in the Pel02 field seed set.
- These results suggest that mesosulfuron+iodosulfuron mixture is a more effective herbicide than flupyrsulfuron for *A. myosuroides* control and that it is able to overcome the common flupyrsulfuron resistance mechanisms found in many UK *A. myosuroides* populations.
- The fact that Pel02 *A. myosuroides* demonstrated a high level resistance mechanism allowing a proportion of plants to survive application of mesosulfuron+iodosulfuron mixture showed that it is possible for resistance mechanisms to develop in *A. myosuroides* populations not previously exposed to this herbicide. This is investigated further in Chapter 5.
- Cross resistance experiments were inconclusive. At the doses used, Pel96 and PelRES were equally resistant to all herbicides with the exception of sulfometuron. It was not possible to say whether resistance in the PelRES plants was due to normal enhanced

metabolism or the apparently separate mechanism conferring sulfometuron resistance.

- To conclude, Peldon black grass from sulfometuron selected Peldon lines, as well as the Pel02 field population, was shown to exhibit a mechanism conferring high level resistance to sulfometuron.

4. Crossing Experiments

4.1 Introduction

Work described in this chapter covers a series of crossing experiments involving progeny from the Pel96 *A. myosuroides* population which showed unusually high levels of resistance to the non-selective sulfonylurea herbicide sulfometuron-methyl (sulfometuron) in a routine screening test performed in the spring of 2002, prior to the start of this project. This preliminary test was aimed at the development of diagnostics for detecting ALS inhibitor resistance in glasshouse pot assays with *A. myosuroides* and compared different growing media (loam and compost). The populations involved included Pel96 (currently used as a high metabolism standard population), Far95 (another metabolism standard population, see Chapter 2 for details), and Roth99 (a susceptible standard). The experiment involved 30 *A. myosuroides* plants per treatment and six ALS inhibiting herbicide treatments overall, as well as untreated controls. Treatments were flupyrsulfuron at 10 and 20g a.i. ha^{-1} (1x and 2x field rate), mesosulfuron+iodosulfuron at 12 + 2.4 and 24 + 4.8g a.i. ha^{-1} (1x and 2x field rate) and sulfometuron at 50 and 100g a.i. ha^{-1}. Of all the plants treated with sulfometuron, only two Pel96 plants survived; one from each sulfometuron treatment group. These two plants were transplanted and grown on but one later succumbed to mildew leaving only a single sulfometuron survivor from the 100g a.i. ha^{-1} treatment group.

Subsequent selfing of the surviving plant followed by further sulfometuron selection led to the establishment of a seed set known as the PelRES high resistance line which contained around 40% of plants with high resistance to sulfometuron. Further crossing experiments were conducted in order to gain insights into the heritability of the sulfometuron resistance trait and to establish additional lines for use in enzyme assays and molecular work and this work is described here. The resistant trait observed in the PelRES line was qualitatively different to the enhanced metabolism based resistance observed in the bulk of the Pel96 population. A large proportion of PelRES survivors showed little or no damage after treatment with 100g a.i. ha^{-1} sulfometuron.

The discovery of this high level resistant trait, picked up in whole plants using sulfometuron at 100g a.i. ha^{-1}, was an important starting point for crossing experiments, enzyme assays and molecular work. Throughout the crossing work sulfometuron was used as an indicator of high level resistance. A dose of 100g a.i. ha^{-1} was used in all screening tests as discussed in Chapter 3 (susceptible populations were controlled at < 10g sulfometuron ha^{-1}).

4.1.1 Genetics of ALS target site resistance

Since the early 1990's when the first cases of ALS target site resistance were discovered in prickly lettuce (*Lactuca serriola*) and kochia (*Kochia scoparia*) (Mallory-Smith *et al.*, 1990a; Primiani *et al.*, 1990), a number of studies have been performed to investigate the inheritance characteristics of resistant ALS alleles in several plant species (Boutsalis *et al.*, 1999; Hart *et al.*, 1993; Mallory-Smith *et al.*, 1990b; Ohmes & Kendig, 1999; Preston & Powles, 2002; Van Eerd *et al.*, 2004; Volenberg & Stoltenberg, 2002). Results show that under herbicide selection, resistant (R) ALS alleles are dominant or semi-dominant over susceptible (S) alleles, with the degree of dominance dependent upon allele and species. This means that resistant alleles are selected in both the homozygous and heterozygous states. ALS target site resistance is determined by a single nuclear gene in most cases and follows normal Mendelian inheritance (Saari *et al.*, 1994).

Several studies have investigated the important question of initial frequency of resistance to ALS inhibiting herbicides, along with mutation rates, in weed and commercial crop species. Mutation frequencies for resistance to sulfonylureas range from to 2.7 x 10^{-8} in *N. tabacum* cell cultures (Harms & DiMaio, 1990) to 0.51 x 10^{-6} in *G. hirsutum* cultures (Rajasekaran *et al.*, 1996). Assuming resistance and susceptibility are determined at a single locus, and that resistance is conferred by a single dominant allele, the expected frequency of resistant plants in populations of out-crossing species like *A. myosuroides* is determined based on Hardy-Weinberg theory (Jasieniuk *et al.*, 1996). Estimations of initial frequency of mutant resistant plants in a weed population are usually based on population genetics theory and are best assumed to be approximate. If resistant mutants are assumed to be almost as fit as susceptible plants then the frequency at equilibrium can be up to two orders of magnitude greater than the mutation frequency (Jasieniuk *et al.*, 1996). This is borne out in field studies with the grass weed *L. rigidum* where initial frequency of individuals resistant to sulfometuron in unselected populations varied

between 2.2 x 10^{-5} and 1.2 x 10^{-4} (Preston & Powles, 2002). Such high initial frequencies in unselected populations suggest that mutations conferring resistance to ALS inhibitors are not associated with a large decrease in fitness compared to susceptible individuals and may provide an explanation for the rapid evolution of target site resistance to ALS inhibiting herbicides since their introduction.

4.1.2 *A. myosuroides* genetics

A. myosuroides is an annual diploid species (2x=2n=14) (Chapman, 1996) and is allogamous, although not completely self incompatible, with the proportion of viable seeds after enforced self fertilisation being variable. The species shows a high level of genetic polymorphism with around 60 per cent of loci being polymorphic (Chauvel & Gasquez, 1994) along with a particularly low level of genetic differentiation between different populations from geographical locations across Europe and the Middle East.

The combination of an out-crossing species with nuclear inheritance of the ALS target site resistance trait has several important implications for the spread of resistance to ALS inhibitors in *A. myosuroides*. Firstly resistance alleles will be transmitted through pollen and ovules, rather than ovules alone as would happen for a cytoplasmically inherited trait and so resistance might be expected to spread more quickly in a population. Secondly, this effect will be enhanced due to the dominance of the ALS resistance trait with heterozygotes able to manifest at least some resistance under herbicide selection.

4.1.3 Scope of crossing work

Crossing experiments using the original sulfometuron resistant PelRES line derived from a single plant were carried out in order to look at segregation patterns in progeny from crosses with susceptible Rothamsted plants after treatment with sulfometuron. Specific aims included determination of the number of genes controlling ALS resistance in *A. myosuroides* and whether resistant alleles were dominant or recessive. Most important was the need to establish whether resistance was a simple monogenic trait giving rise to classical Mendelian ratios in crossings with susceptible plants or whether it was conferred by additive minor genes in a quantitative manner. Inheritance characteristics are important in herbicide resistance studies because certain types of resistance tend to be inherited in different ways. For example ALS target site resistance tends to follow a simple Mendelian pattern as described above whereas enhanced metabolism resistance,

which can confer cross resistance to a wide spectrum of different herbicides, is often inherited polygenically (Mackenzie *et al.*, 1995; Neve & Powles, 2005a).

4.1.4 Seed stocks used in crossing experiments

The following seed sets were used extensively in glasshouse crossing experiments.

4.1.4.1 Roth99 susceptible standard

The Rothamsted susceptible standard was collected at Broadbalk on the Rothamsted Estate as described in Chapter 2.

4.1.4.2 PelRES02 line

Sulfometuron selected line originating from the self pollination of a single resistant plant. The initial work was carried out by Stephen Moss at Rothamsted. Further crossing and screening experiments using PelRES02 plants are discussed later in this chapter.

4.2 Materials and methods

4.2.1 Screening of original PelRES02 progeny with sulfometuron: spring 2003

Following the original self pollination which led to the PelRES02 line, the proportion of viable seeds was low and only a small amount of seed was available. Seeds were sown into Kettering loam in 5cm pots and a total of 35 PelRES02 plants grew to the three leaf stage. These were included in a screening test using sulfometuron at 100g a.i. ha^{-1} along with 35 Roth99 (susceptible standard) plants. Plants were divided into two treatment groups of 15 replicates with five plants left untreated per population. Each group of 15 were then sprayed with either sulfometuron at 100g a.i. ha^{-1} or mesosulfuron+iodosulfuron at 12 + 2.4g a.i. ha^{-1}. Plants were assessed visually for injury after 4 weeks and whole plant weights, including washed roots, were measured for each plant. The aim was to keep the plants for genetic studies, hence the non-destructive method of sampling. This initial phase of the crossing work was carried out by Stephen Moss at Rothamsted in the winter of 2002/2003.

4.2.2 Initial selfing and cross to susceptible: spring/summer 2003

Eight surviving PelRES02 plants with no visible injury from the sulfometuron treatment were each split in half at the pre-heading stage and the two clones re-potted using John Innes potting compost. One clone from each original sulfometuron resistant plant was crossed (R x S) to a Roth susceptible plant in order to produce F_1 progeny while the other clone was isolated and made to self pollinate. The eight crosses were numbered as follows: PelRES26 x Roth5, PelRES27 x Roth74, PelRES28 x Roth71, PelRES30 x Roth72, PelRES31 x Roth79, PelRES32 x Roth2, PelRES35 x Roth1 and PelRES37 x Roth81. Plants for crossing and self-pollination were isolated in separate glasshouse sections and seeds were collected over several weeks after crossing until the end of July 2003. Seed samples from each cross were kept separate on the basis of maternal parent and were later subjected to F_1 segregation screening on this basis. Twelve remaining PelRES02 sulfometuron survivors were poly-crossed to produce a bulk sample of seeds for use in further studies (PelRES03).

4.2.3 F_1 segregation (winter 2003/spring 2004)

Prior to screening of F_1 plants, seeds collected separately from each parent in the R x S crosses were germinated in Petri dishes and planted into 5cm square pots in a glasshouse using pre-wetted Kettering loam. This separation was maintained in order to test for nuclear inheritance of the resistant trait. Two seedlings were initially planted into each pot and these were later thinned to leave one plant per pot. Seeds from the following 2003 crosses were used: PelRES26 x Roth5, PelRES27 x Roth74, PelRES30 x Roth72, PelRES31 x Roth79, PelRES32 x Roth2, and PelRES35 x Roth1. Other crosses were not used because they failed to produce viable seed or came to head at different times. Between 100 and 110 pots were planted for each F_1 seed family; seeds from each parent being treated separately. All seed grew well with the exception of Roth1 and Roth74 where 95 and 20 plants were available for spraying respectively. All plants were sprayed at the three leaf stage with sulfometuron at 100g a.i. ha^{-1} and assessed for injury after four weeks.

4.2.4 Further crossing within injury groups (spring/summer 2004)

Segregation in the F_1 showed a clear separation between live and dead plants but some variation in the range of different phenotypes for survivors. To generate an F_2 generation five plants with injury score 1 or 2 (least affected), and five with injury score 3 or 4

(moderately affected) from each of the following six lines were selected to be grown on and poly-crossed within separate groups. This approach was taken in order to investigate whether the degree of resistance in the F_2 progeny was quantitatively associated with F_1 parental resistance levels. The sulfometuron surviving F_1 plants selected for this cross were those from the following original maternal parents: PelRES26, PelRES31, PelRES32, Roth2, Roth5 and Roth79. Progeny from these particular parents was chosen because all demonstrated an even spread of phenotypes in the F_1 segregation experiment. All plants were re-potted into 24cm pots using John Innes potting compost and grown on in separate glasshouse sections with plants from individual parental groups and injury categories separated in different greenhouse sections to prevent cross pollination (12 separate group crosses in total). Seeds were collected from each group over several weeks, cleaned, and then stored at 30°C to break dormancy as described in Chapter 2.

4.2.5 F2 segregation (winter 2004/2005)

Seeds collected from poly-crossed F_1 survivors from different injury categories were pre-germinated and sown as two plants per pot with a total of 108 pots per seed set. Seedlings were thinned to one per pot then sprayed with sulfometuron at 100g a.i. ha^{-1} at the three leaf stage. Injury assessment was made after four weeks.

4.2.6 Segregation in progeny of selfed resistant plants (spring 2005)

Seeds from self crossed clones of the following resistant plants were germinated in Petri dishes and then planted into Kettering loam: PelRES26, PelRES27, PelRES28, PelRES30, PelRES31, PelRES32, PelRES35 and PelRES37. Resistant plants 28 and 37 were not successfully crossed with susceptible Roth plants but were included to increase the chance of generating homozygous resistant material. The aim was to plant 100 seeds per seed set but germination was very low and this was not possible. After thinning 18, 19, 76, 94, 25, 10, 52 and 68 plants were available from each seed set for spraying respectively. All plants were sprayed with 100g a.i. ha^{-1} sulfometuron at the three leaf stage and then assessed for injury after four weeks.

4.2.7 Overall crossing scheme

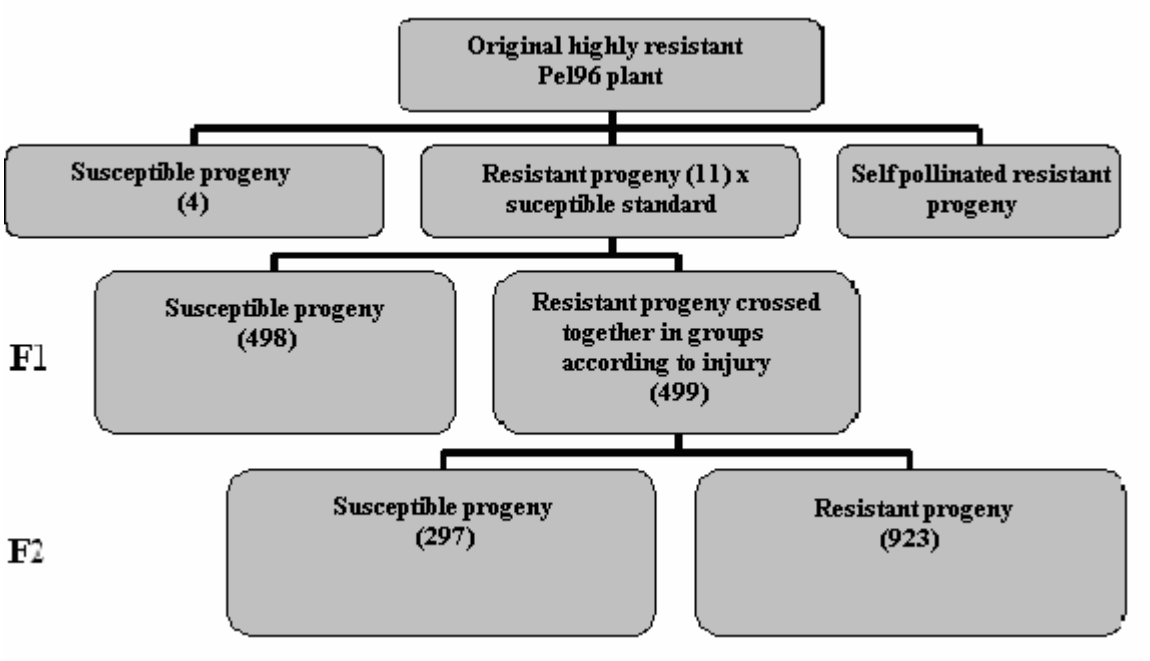

Figure 4.1 Summary of crossing starting with single highly resistant Pel96 plant (total number of plants per category from segregation experiments are in brackets).

4.3 Results

4.3.1 Screening of original PelRES02 progeny with sulfometuron

All Roth99 susceptible standard plants were killed after treatment with sulfometuron or mesosulfuron+iodosulfuron. This was reflected in a greater than 90% reduction in fresh weight for treated Roth99 compared to untreated plants. From a total of fifteen PelRES02 plants treated with sulfometuron, eleven (73%) continued to thrive and were classed as survivors. Injury scores amongst these survivors ranged from 1 to 3 and some were clearly affected by sulfometuron treatment. A similar proportion (67%) survived treatment with mesosulfuron+iodosulfuron. Mean reduction in fresh weight was 18% in sulfometuron survivors and 32% in survivors of mesosulfuron+iodosulfuron.

4.3.2 Initial selfing and cross to susceptible

Not unexpectedly, seeds produced by the self pollinated resistant plants were of a very low quantity and quality. The results with R x S crosses were better. Bulk crosses gave

rise to large samples of seed from PelRES02 plants which survived sulfometuron. These were designated PelRES03 and were used in further whole plant and enzyme experiments (refer Chapters 3 and 6).

4.3.3 F_1 segregation

Each plant was visually rated using a 1-5 injury scale compared to control plants as described in Chapter 2. Results from the initial screening experiment are presented in Table 4.1:

Table 4.1 Numbers of plants falling into each injury category (1-4 resistant; 5 susceptible) from different maternal parents of each cross after screening with 100g a.i. ha^{-1} sulfometuron

Parent Plant (crosses highlighted)	Number of plants per injury category				
	1	2	3	4	5
PelRES 26	10	9	14	13	54
Roth 5	11	10	17	14	48
PelRES 27	15	15	10	4	56
Roth 74	0	0	2	1	17
PelRES 30	27	25	7	2	39
Roth 72	19	12	10	7	52
PelRES 31	16	11	12	14	47
Roth 79	20	12	8	18	42
PelRES 32	18	13	12	7	51
Roth 2	17	15	10	8	51
PelRES 35	3	7	13	18	59
Roth 1	2	7	16	15	55

The plant injury scores were grouped in different ways and subjected to χ^2 analysis. All analyses were conducted including Roth74 and PelRES27 for consistency, but the results were not treated as significant due to very low germination levels in progeny from the Roth74 maternal parent of that cross. A 1:1 segregation of resistant to susceptible plants was tested as the null hypothesis along with 3:1 segregation.

Table 4.2 Results of χ^2 analysis testing 1:1 segregation as the null hypothesis. Hypothesis: 1:1 segregation RS x SS cross 1 d.f. p=0.05. Data from PelRES27 x Roth 74 cross are excluded from total values

Parent Plant (crosses highlighted)	1-4 'alive'	5 'dead'	∑	X^2	Significant Y/N
PelRES 26	46	54	100	0.640	N
Roth 5	52	48	100	0.160	N
PelRES 27	44	56	100	1.440	N
Roth 74	3	17	20	9.800	Y
PelRES 30	61	39	100	4.840	Y
Roth 72	48	52	100	0.160	N
PelRES 31	53	47	100	0.360	N
Roth 79	58	42	100	2.560	N
PelRES 32	50	51	101	0.010	N
Roth 2	50	51	101	0.010	N
PelRES 35	41	59	100	3.240	N
Roth 1	40	55	95	2.368	N
PelRES Total	251	250	501	0.002	N
Roth Total	248	248	496	0.000	N

Excluding the very low germinating Roth74 seed set, there was only one significant deviation from the 1:1 ratio predicted by an RS x SS cross (the PelRES30 offspring). This provides support for the idea that the sulfonylurea resistance observed in the PelRES line is encoded by a single dominant nuclear allele. The total numbers of resistant and susceptible plants summed together illustrate a very convincing 1:1 ratio for both resistant and susceptible parents (251:250 and 248:248 respectively, see Table 4.2). Other segregations, for example classing injury scores 1-2 as 'unaffected' and 3-5 as 'affected', also gave very little significant deviation from the respective null hypotheses but were difficult to explain. Since none of the R x S crosses gave rise to 100% resistant progeny it was concluded that none of the original six PelRES plants were likely to be homozygous for a monogenic dominant or partially dominant trait conferring resistance to ALS inhibitor herbicides.

4.3.4 F_2 segregation

Table 4.3 Numbers of plants falling into each injury category from various different original parents and injury groups after screening with 100g a.i. ha^{-1} sulfometuron

Parent Group (F1 injury category in brackets)	Number of plants per injury category				
	1	**2**	**3**	**4**	**5**
PelRES 26 (1&2)	10	24	33	9	26
PelRES 26 (3&4)	3	26	35	10	28
PelRES 31 (1&2)	24	16	31	8	23
PelRES 31 (3&4)	1	11	21	43	26
PelRES 32 (1&2)	15	39	25	5	18
PelRES 32 (3&4)	1	16	48	10	27
Roth 2 (1&2)	23	27	21	13	18
Roth 2 (3&4)	14	9	31	17	31
Roth 5 (1&2)	14	13	26	22	23
Roth 5 (3&4)	1	25	29	23	24
Roth 79 (1&2)	9	20	21	24	28
Roth 79 (3&4)	13	11	27	26	25

As before the plant injury scores (1-4) were grouped together, this time according to the hypothesis that all parents were heterozygous for a monogenic completely dominant trait conferring resistance to sulfometuron. If this theory was correct, 3:1 segregation of resistant to susceptible plants would be expected. Chi-square analysis was used to test the null hypothesis.

Table 4.4 Results of χ^2 analysis testing 3:1 segregation as the null hypothesis.
Hypothesis: 3:1 segregation RS x RS cross 1 d.f. p=0.05

Parent Plant	1-4 'alive'	5 'dead'	∑	X^2	Significant Y/N
PelRES 26 (1&2)	76	26	102	0.013	N
PelRES 26 (3&4)	74	28	102	0.327	N
PelRES 31 (1&2)	79	23	102	0.327	N
PelRES 31 (3&4)	76	26	102	0.013	N
PelRES 32 (1&2)	84	18	102	2.941	N
PelRES 32 (3&4)	75	27	102	0.118	N
Roth 2 (1&2)	84	18	102	2.941	N
Roth 2 (3&4)	71	31	102	1.582	N
Roth 5 (1&2)	75	23	98	0.122	N
Roth 5 (3&4)	78	24	102	0.118	N
Roth 79 (1&2)	74	28	102	0.327	N
Roth 79 (3&4)	77	25	102	0.013	N
PelRES (1&2) Total	239	67	306	1.573	N
PelRES (3&4) Total	225	81	306	0.353	N
Roth (1&2) Total	233	69	302	0.746	N
Roth (3&4) Total	226	80	306	0.214	N

All chi-square test results in this case showed non-significant values and so this particular set of data provided no evidence contradicting the original hypothesis (see Table 4.4). Ratios were convincing in terms of a 3:1 segregation and the total for all sets summed together was 923 live plants to 297 dead. See Figure 4.2 for an example of a typical tray showing segregation in the F_2 with variation in the response of plants to herbicide. This kind of response was typical and illustrates the difficulty in assigning 'resistant' and 'susceptible' scores to individual plants, particularly in the situation where plants are damaged but not dead.

It was interesting to note that progeny derived from 1 and 2 rated parents contained a greater proportion of 1 and 2 rated survivors than those coming from 3 and 4 rated parents (see Table 4.3). A further chi-square test was performed to examine this effect, see Tables 4.5 and 4.6. Roth and PelRES plants were treated separately.

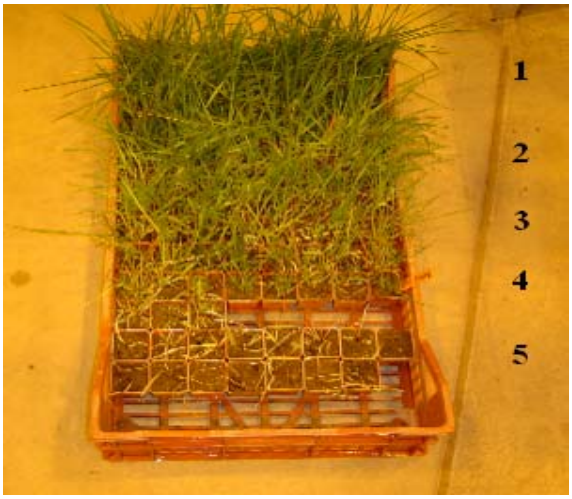

Figure 4.2 A typical F_2 tray showing variation in phenotypic response to sulfometuron with surviving plants falling into injury categories 1-4.

In order to test whether parental injury scores had any effect on the distribution of phenotypes in the surviving progeny, expected values for each injury category were calculated. This was done by assuming no association between injury score in parents and segregation in progeny and calculating expected distribution for each separate row in terms of the row total to be the same proportion as totals for each column compared to the table total. This was assumed to be a valid method based on the good 3:1 ratios demonstrated in the overall analysis (see Table 4.4).

Table 4.5 Results of χ^2 analysis testing no significant difference between expected and observed per category in F_2 plants from original PelRES parents as the null hypothesis, 3 d.f. p=0.05

Group	1	2	3	4	Total
PelRES 1&2 observed (X^2)	49(15.750)	79(1.779)	89(1.010)	22(11.000)	239
PelRES 1&2 Expected	28	68	99	44	
PelRES 3&4 observed (X^2)	5(16.962)	53(1.891)	104(1.064)	63(11.805)	225
PelRES 3&4 expected	26	64	94	41	
Total	54	132	193	85	464

Table 4.6 Results of χ^2 analysis testing no significant difference between expected and observed per category in F_2 plants from original Roth03 parents as the null hypothesis 3 d.f. p=0.05

Group	1	2	3	4	Total
Roth 1&2 observed (X^2)	46(1.684)	60(0.925)	68(1.052)	59(0.254)	233
Roth 1&2 expected	38	53	77	63	
Roth 3&4 observed (X^2)	28(1.778)	45(0.942)	87(1.592)	66(0.258)	226
Roth 3&4 expected	36	52	76	62	
Total	74	105	155	125	459

From the results presented in Tables 4.5 and 4.6 it was possible to reject the null hypothesis (no association between injury score in parents and segregation in progeny) at the 95% level for F_2 plants descended from both PelRES and Roth03 parents. Injury score of original surviving parents had an effect on injury score distribution in the progeny. The effect was less pronounced in plants derived from Roth03 maternal parents where the null hypothesis still held at the 99% level.

4.3.5 Segregation in progeny of selfed resistant plants

Chi-square test results in this case provided no evidence contradicting the hypothesis that segregation for resistance to sulfometuron fitted an expected 3:1 R:S ratio (see Table 4.8). Segregation according to a 1:2:1 distribution was also tested but did not fit the data as well as the 3:1 ratio.

These results were consistent with those from the F_1 segregation and support the hypothesis that resistance to sulfometuron in these particular PelRES lines is conferred by a single dominant nuclear allele. Progeny from the self-pollination of PelRES plants 28 and 37 were not included as part of the chi-square analysis because these were not crossed to susceptible plants. Plant 26 was not included because the number of healthy seedlings following germination was very low.

Table 4.7 Numbers of plants falling into each injury category from different original parents after screening with 100g a.i. ha^{-1} sulfometuron

Parent	Number of plants per injury category				
	1	**2**	**3**	**4**	**5**
PelRES 26	0	2	1	0	7
PelRES 27	0	2	7	1	8
PelRES 30	0	8	6	1	10
PelRES 31	2	11	18	7	14
PelRES 32	1	9	41	10	15
PelRES 35	5	16	49	8	16
PelRES 28*	0	2	14	2	1
PelRES 37*	31	31	6	0	0

*** plants not crossed successfully with susceptibles**

Table 4.8 Results of χ^2 analysis testing 3:1 segregation as the null hypothesis. Hypothesis: 3:1 segregation RS x RS self cross 1 d.f. p=0.05.

Parent Plant	1-4 'alive'	5 'dead'	$\sum$	X^2	Significant Y/N
PelRES 27	10	8	18	3.630	N
PelRES 30	15	10	25	3.000	N
PelRES 31	38	14	52	0.103	N
PelRES 32	61	15	76	1.123	N
PelRES 35	78	16	94	3.191	N
PelRES Total	202	63	265	0.213	N

Unusually high levels of sulfometuron resistance were observed in progeny from the self pollination of original surviving plant PelRES37 (see Figure 4.3). In contrast to other progeny from self pollination of split plants, segregation in the PelRES37 derived seed set was atypical, with all plants surviving sulfometuron treatment at 100g a.i ha^{-1} and very few damaged survivors. Several leaf samples were taken from the PelRES37 progeny and frozen in liquid nitrogen for later sequencing.

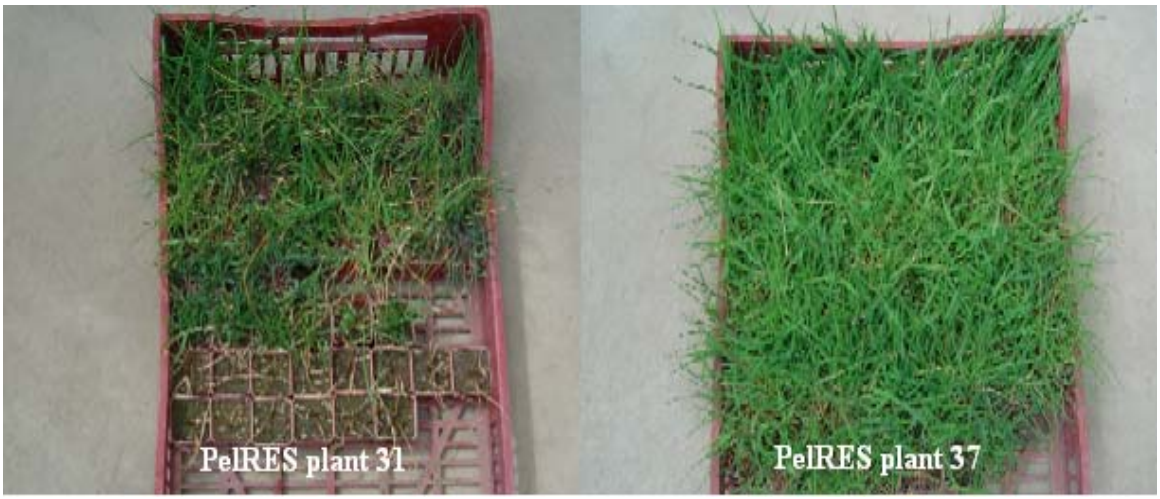

Figure 4.3 Segregation in progeny from self pollination showing high levels of resistance in PelRES37 progeny compared to the more typical PelRES31 progeny

4.4 Discussion

The results support the conclusion that the PelRES line, derived from self pollination of a single Pel96 plant, has a target site mutation or mutations conferring resistance to sulfometuron. The original resistant Pel96 plant was most likely a heterozygote based on the outcrossing nature of *A. myosuroides*, and the very low levels of highly resistant individuals in the Pel96 seed set (around 3-4%). The heritability of the resistant trait was demonstrated through a series of crossing experiments. Chi-square tests were performed using segregation in the F_1 after treatment with sulfometuron to test the null hypothesis that original R x S crosses involved a single dominant allele RS x SS conferring resistance to sulfometuron and leading to a 1:1 R:S distribution. There was only one significant deviation from the 1:1 segregation predicted by an RS x SS cross and this provided support for the idea that the sulfometuron resistance observed in the PelRES line is encoded by a single dominant nuclear allele. Further tests looking at the segregation of resistant and susceptible plants in the progeny from self pollinated resistant parents supported this idea. A single self-pollinated plant, PelRES37, was identified which showed segregation in the progeny suggestive of a parent homozygous for the resistant trait. Samples were taken from this particular plant for molecular analysis (see Chapter 7).

Results from the F_2 segregation experiment showed that the degree of resistance in surviving progeny is seemingly associated with parental resistance levels. Several explanations are available which might explain the observation that variable phenotypic response in survivors has a heritable component. One possibility is that more than a single target site change is present in resistant individuals. Combinations of different mutations have been shown to incrementally increase levels of resistance to ALS inhibitors in tobacco (Creason & Chaleff, 1988). If a number of different point mutations were responsible for resistance in the PelRES line, inheritance would be more difficult to predict and a range of different phenotypes might be expected in progeny of the original resistant individual. Another possibility is that a collection of minor gene effects were responsible for the varying levels of resistance exhibited in survivors in addition to the dominant single gene trait conferring overall resistance. Quantitatively inherited multi-gene traits would explain the association between injury score in parents and progeny in the F_2 selection experiment. Further crossing work would have been useful to explore this idea but unfortunately time constraints ruled this out.

In contrast to earlier work using whole plants (see Chapter 3), it was necessary to class all living plants as a single group in order to arrive at the expected 1:1 and 3:1 ratios in the F_1 and F_2 generations, respectively. In previous whole plant work it was assumed that only those plants falling into categories 1 and 2 on the injury scale demonstrated a unique high level resistance to sulfometuron. The fact that Peldon *A. myosuroides* demonstrates a high level of enhanced metabolism makes this effect difficult to interpret; it is possible that differences in response to sulfometuron are mediated at least partially by metabolic resistance in conjunction with target site change. Dose response experiments demonstrated that sulfometuron at 100g a.i. ha^{-1} was a sufficient discriminatory dose and that increasing the dose did not change phenotypic distribution in the PelRES line.

Australian work using sulfometuron as an ALS inhibitor able to overcome enhanced metabolism in *L. rigidum* used comparatively low doses of the herbicide, often below 20g a.i. ha^{-1} (Preston & Powles, 2002). Use of doses this low in *A. myosuroides* would provide control of completely susceptible plants while tending not to show differences in response of resistant individuals. This would simplify the results from crossing experiments in *A. myosuroides* and make interpretation easier but would have the potential to obscure additional effects like enhanced metabolism or combinations of different mutations with

each conferring different degrees of resistance. There would also be a danger that plants with very high levels of enhanced metabolism might survive lower doses of sulfometuron and give confusing results. In retrospect the 100g a.i. ha^{-1} dose was particularly useful for discrimination of highly resistant plants and also enabled the identification of another possible mechanism conferring variable response in resistant individuals.

Chapter 7 describes molecular work undertaken in order to further investigate the mechanism of resistance in the PelRES and other related populations, particularly the highly resistant field population Pel02. Sampling of leaf material from crosses at each stage would also have been very helpful in gaining a complete picture and would have allowed ALS gene sequencing from each stage of the crossing scheme. Crossing work carried out in conjunction with molecular analysis is a possible direction for future work on resistance to ALS inhibiting herbicides in *A. myosuroides*.

4.5 Chapter summary

- Results from segregation of sulfometuron resistant and susceptible phenotypes in the F_1 suggested that ALS target site resistance in *A. myosuroides* was conferred by a single, dominant nuclear allele.
- Support for this conclusion came from a segregation experiment using progeny of self pollinated clones where a 3:1 ratio was observed.
- Segregation in the F_2 showed an association between parental resistance levels and the degree of resistance in progeny.
- It was concluded that resistance is controlled primarily by a single, dominant gene but additional effects are also present.
- Possible explanations include a contribution from enhanced metabolism resistance or the possibility that more than one resistance mutation was present in the PelRES line.

5. Evolution of Resistance to ALS Inhibiting Herbicides at Peldon

5.1 Introduction

This chapter covers field and glasshouse experiments designed to investigate the evolution of resistance to ALS inhibiting herbicides in *Alopecurus myosuroides* from the Peldon area of Essex in south east England. This was the location where ALS target site resistance was first confirmed in *A. myosuroides* (see Chapters 4 and 7). The Peldon biotype is one of the most difficult to control and has presented unique problems since the early 1980s when resistance to herbicides was first recognised in the field (Moss & Cussans, 1985). Experiments were designed to investigate how resistance to ALS inhibiting herbicides has increased over time at Peldon and results from these were used, in conjunction with herbicide records, to give insights into how resistance might evolve, not only at Peldon, but also in other parts of the country with similar herbicide histories. In addition to this, efforts were made to characterise the spatial distribution of sulfonylurea resistant *A. myosuroides* in the field at Peldon using GPS mapping. This was done in order to gain a better understanding the scale of the problem at the site and provide a resource for future field based studies into herbicide resistance.

5.1.1 Farming in the Peldon area

Peldon, a village near the Essex coast in south eastern England, is surrounded by high quality low-lying arable land which is used mainly for growing winter wheat and oil-seed rape. The soil around Peldon is a heavy clay loam and lends itself especially well to intensive autumn cropping. Many farmers in the Peldon area operate such systems and often sow winter wheat in successive years. This environment is ideal for *A. myosuroides* (Holm *et al.*, 1997) which thrives in autumn sown crops grown on heavy textured soil. Consequently, *A. myosuroides* is a major problem for many farmers in the Peldon area. Because of the minimum tillage farming systems commonly practised around Peldon, there are a limited number of cultural controls available to farmers seeking to control problem populations of *A. myosuroides*. This situation has been exacerbated by the ban on

stubble burning in England since 1992, the trend towards earlier drilling in autumn sown crops, and the move away from crop rotations involving spring sown crops. As a result of these factors, many Peldon farmers rely almost exclusively on herbicides for *A. myosuroides* control and this has led to the development of resistant populations.

5.1.2 History of herbicide resistant *A. myosuroides* at Peldon

The first evidence for a severe herbicide resistant *A. myosuroides* problem in the UK came from a farm in the Peldon area (Moss & Cussans, 1985) where resistance to chlorotoluron was detected in seeds collected from the field in 1984. Marginal resistance was detected prior to this in a sample from Faringdon in Oxfordshire (see Chapter 2 for details). At that time winter cereal monoculture was already common in the area. Herbicides available for *A. myosuroides* control in 1984 included chlorotoluron, isoproturon, pendimethalin and methabenzthiazuron+chlorsulfuron and it was concluded from initial experiments that chlorotoluron resistance at Peldon was a quantitatively inherited multi-gene trait. Since Peldon was the earliest site in the UK with confirmed highly herbicide resistant *A. myosuroides*, much of the later work on resistance was carried out using seed from the Peldon area. Cross resistance to several herbicides with different modes of action was soon confirmed in Peldon *A. myosuroides*; these included phenylureas and triazines (inhibitors of photosynthesis at photosystem II), dinitroanilines (inhibitors of microtubule assembly), aryloxyphenoxypropionates and cyclohexanediones (inhibitors of fatty acid synthesis at the ACCase enzyme), thiocarbamates (non-ACCase inhibitors of fatty acid synthesis) chloroacetamides (inhibitors of cell division) and the ALS inhibitor chlorsulfuron (Kemp *et al.*, 1990; Moss & Cussans, 1987). More recently, resistance to the sulfonylurea herbicide flupyrsulfuron has been observed in Peldon *A. myosuroides* (Moss *et al.*, 2005b).

Research identified two separate enzyme systems implicated mechanistically in herbicide resistant Peldon *A. myosuroides*; cytochrome P450 monooxygenases (Hall *et al.*, 1995; Hyde *et al.*, 1996) and glutathione S-transferases (Cummins *et al.*, 1997; Reade & Cobb, 1999). In these studies the Peldon biotype typically showed elevated P450 and GST activities compared to susceptible standard biotypes, and this was associated with an increased rate of metabolism and detoxification of applied herbicides, see Chapter 1 for further details.

5.1.3 Sulfonylurea resistant *A. myosuroides* at Peldon Hall farm

Peldon Hall farm has for many years been the source of the most resistant *A. myosuroides* samples from the Peldon area. The site was first identified in 1984 and seed samples have been collected from the field annually. Extensive herbicide records also exist for Peldon Hall and this makes *A. myosuroides* from the site an invaluable resource in studies looking at the development of herbicide resistance over time. Presently the farm area is in continuous wheat, with multiple herbicide resistant *A. myosuroides* fairly widespread across many fields. Resistance to the sulfonylurea herbicide flupyrsulfuron first showed up at Peldon in routine screening tests using the Pel96 biotype (collected in the field in 1996), before the resistance mechanism to this herbicide was confirmed as enhanced metabolism (Moss *et al.*, 2005b). The fact that flupyrsulfuron is ineffective against many resistant UK *A. myosuroides* populations (see Chapter 3) meant that detecting resistance at Peldon was not unexpected. However the presence of a high level resistance mechanism allowing a proportion of plants to survive treatment with the non-selective sulfonylurea sulfometuron meant that Peldon was a unique site with regards to sulfonylurea resistance in *A. myosuroides*. Until recently, no efforts had been made to map the population of sulfonylurea resistant *A. myosuroides* at Peldon Hall farm or to evaluate the evolution of resistance in seed samples collected over a period of years. In light of the results from glasshouse studies it was felt that the field site at Peldon merited further investigation, both in terms of the physical distribution of resistant *A. myosuroides* in the field and also regarding the evolution of the high level resistant trait in stored seed samples from different years.

5.1.4 Scope of field work

The aim of the work included in this chapter was to examine how resistance to sulfonylurea herbicides has developed at the Peldon Hall farm site over time and also to investigate the current extent of resistant *A. myosuroides* at the site. In addition, experiments were also conducted on the evolution of sulfonylurea resistance in a susceptible seed set exposed to different sulfonylurea herbicide spray regimes.

- Work began with screening experiments using sulfometuron at 100g a.i. ha^{-1} as an indicator of the high level resistance trait initially picked up in whole plant dose response experiments. This dose of sulfometuron was able to overcome the usual metabolic resistance seen in UK *A. myosuroides* populations and allow identification

of plants with high level resistance mechanisms and possible ALS target site resistance (see Chapter 3).

- Glasshouse screening tests involved seeds collected annually from the same area of one field at Peldon since 1996. The aim was to investigate the rate of build up of high level sulfonylurea resistance between 1996 and 2002. Field work carried out at Peldon focussed on mapping the main patches in the field at Peldon Hall farm using a backpack GPS system and collecting seed for later characterisation in terms of sulfometuron resistance. This was done in order to give some idea of the distribution of high-level resistant *A. myosuroides* and provide possible clues as to the evolution and spread of the trait at the site.
- The final experiment in this section was a field trial over three years investigating the different selection effects in another population of *A. myosuroides* exposed to flupyrsulfuron or mesosulfuron+iodosulfuron. These herbicides were used in order to reflect available sulfonylurea options for UK farmers at the present time. Of particular interest was the type of resistance selected for by each treatment and the time taken for resistance to build up. Both of these questions are of great importance for the continued use of sulfonylurea herbicides in UK agriculture.

5.2 Materials and methods

5.2.1 GPS mapping and resistance screening of Peldon *A. myosuroides* patches

Two mapping trips were made to Peldon; the first was in July 2003 and concentrated on identifying and mapping large patches of *A. myosuroides* in the field at Peldon Hall where annual seed sampling had been done and also the neighbouring Brick House farm. Seeds were collected from each patch and later screened for high level resistance in glasshouse screening tests using sulfometuron at 100g a.i. ha^{-1}. The second mapping trip to Peldon was conducted in July 2004 and focussed on conducting a transect across the field where a large proportion of the seeds collected the previous year displayed possible target site resistance to sulfometuron. This passed through the particular patch where seed has been sampled since 1986.

Figure 5.1 Mapping a field boundary using the Trimble TDC1 backpack GPS

5.2.1.1 July 2003 mapping, sampling and screening

Mapping was performed by walking around the field boundary of Hams field on Peldon Hall farm using a TDC1 backpack GPS system (Trimble, California). Hams field, which covers an area of 800 x 450m approximately, was the area where most of the problem *A. myosuroides* was located and also was the field where seed samples had been collected since 1986. Three large patches of *A. myosuroides* were identified in Hams field (designated Hams A, B and C, Figure 5.2) along with another in the adjacent Twitch field which is not highlighted on the map. An additional two patches were identified with very heavy *A. myosuroides* infestation on the nearby Brick House farm but these were not mapped. Brick House farm patches were designated Ransomes and Church after the fields they were found in. Ransomes field was close to Hams field, and the sampling site was around 200 metres from the edge of that field. The sampling point in Church field was around 1000 metres from both Hams and Ransomes sites.

Mapping was carried out by walking around the Hams field boundary as a reference and

then around patch edges with the backpack GPS. The patch coordinates were related to the field boundary using Pathfinder Office software (Trimble, California). Mean headcounts were performed for each patch using 5 x 0.25m^2 quadrats. While *A. myosuroides* was distributed over the whole field, areas designated as patches typically contained over 70 heads m^{-2}. Density within patches varied but overall were noticeably denser in terms of headcount number than other areas of field. After mapping a seed collection was made from each patch.

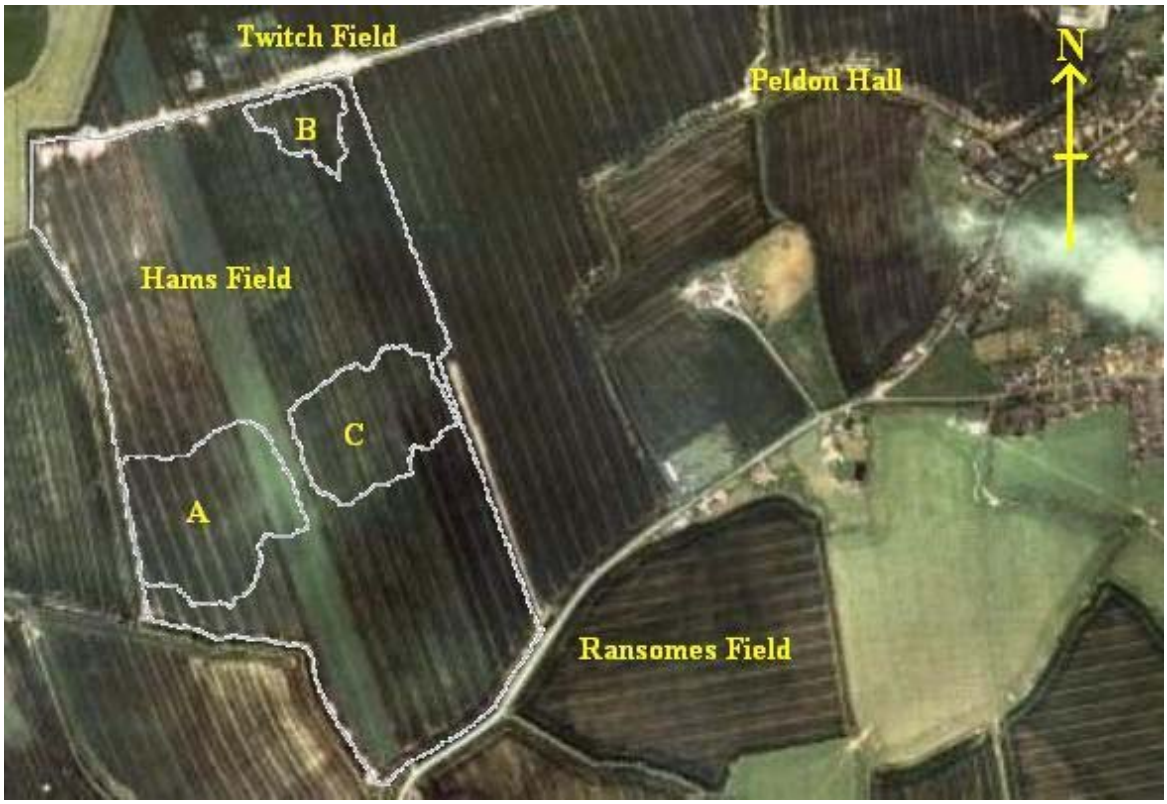

Figure 5.2 Map of *A. myosuroides* patches in Hams field at Peldon, Essex, sampled in 2003. Field boundary and patches A – C are marked in white. Scale 1cm = approx 100m

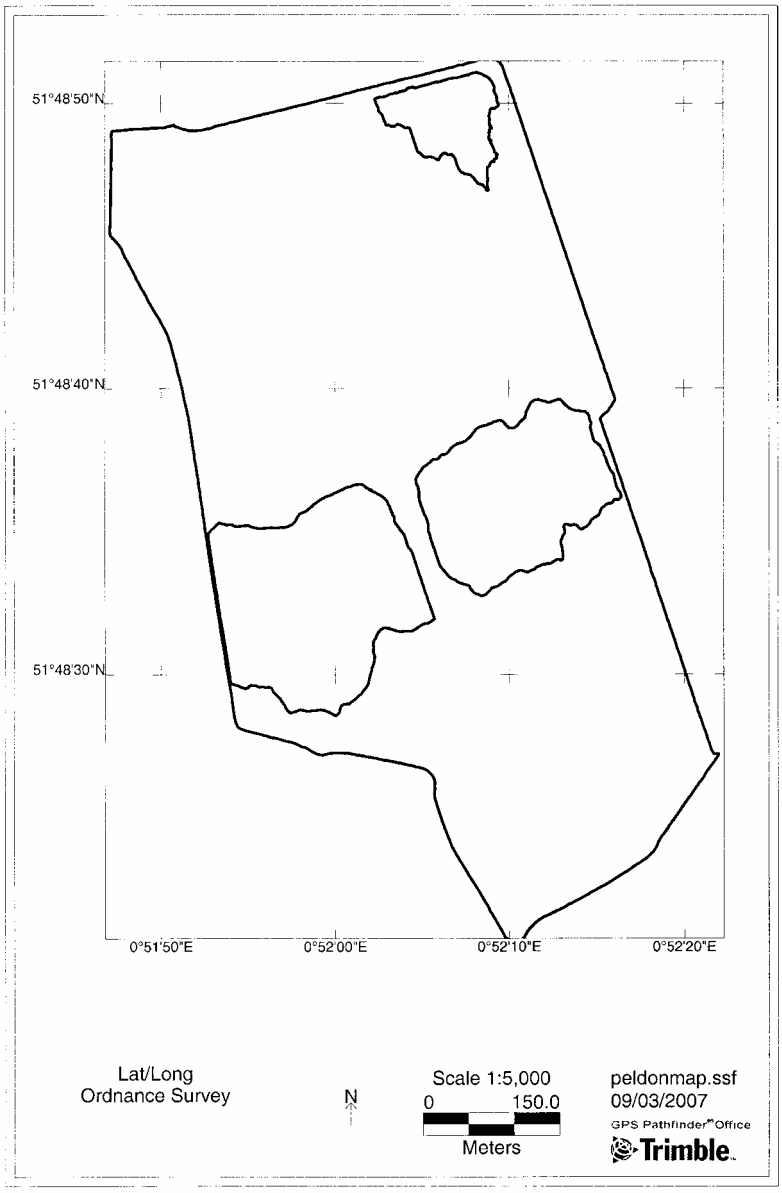

Figure 5.3 Map of *A. myosuroides* patches in Hams field at Peldon with grid references.

Seeds were later dried and cleaned (see Chapter 2), before being subjected to screening tests with sulfometuron at 100g a.i. ha^{-1}. Cleaned seeds were germinated and planted into 20 x 30cm plastic seed trays using Kettering loam with 50-60 plants per tray in four rows. Six trays were prepared per population along with two trays of Roth03 susceptible standard and six trays of Pel02 as a reference standard where the proportion of resistant plant was already known. All plants, which were counted immediately before spraying,

were sprayed with 100g a.i. ha^{-1} sulfometuron at the three leaf stage. The experiment was assessed after five weeks when each plant was individually rated on a 1-3 injury scale which was a simplified version of the 1-5 scale described previously (see Chapter 2). The 1-3 scale consisted of the following categories which are given with the corresponding 1-5 scale values in brackets: 1 = healthy (1-2), 2 = damaged (3-4), 3 = dead (5).

5.2.1.2 July 2004 mapping, sampling and screening

Field work concentrated on more intensive mapping and sampling of *A. myosuroides* from Hams field on Peldon Hall farm. Work from 2003 confirmed that seed from Hams patch C yielded the greatest proportion of plants with high-level resistance to sulfometuron. Seed from the patches A and B also exhibited the trait but as a much smaller proportion of the population. Mapping performed in 2004 built on results from the previous year and the same patch names were used throughout (see Figure 5.4). A transect was taken across the field from the most resistant patch C to intersect with patch A. In total 13 areas were mapped along the transect using the backpack GPS as described in the previous section. An additional two areas were located 60 metres to either side of the transect start point on a perpendicular line. The first seven GPS points along the transect line were spaced 24 metres apart, while the following six were spaced at 48 metres apart. A seed sample was taken from an approximately $4m^2$ area around each GPS point along the transect line and also at the two points out to each side giving a total of 15 seed samples with corresponding mapped points for the Hams field.

Plants grown from seeds were later screened for high-level resistance using sulfometuron at 100g a.i. ha^{-1}. Four trays of 50-60 plants were prepared per sampling point as before, along with four trays of Roth03 susceptible standard. All plants were sprayed with 100g a.i. ha^{-1} sulfometuron at the three leaf stage. A count was performed on the number of plants per tray immediately before spraying and any small or unhealthy looking plants were removed. The experiment was assessed after five weeks using a 1-3 injury scale for each plant as previously described.

5.2.2 Increase in ALS inhibitor resistance at Peldon between 1996 and 2002

The increase in resistance in Hams field at Peldon between 1996 and 2002 was examined by testing seed sampled in each of the seven years for resistance to the non-selective sulfonylurea herbicide sulfometuron and using herbicide application data from those

years to aid in the interpretation of results (see Table 5.4). All seeds were collected from the area defined in 2003 as patch C in Hams field, Peldon Hall farm, Essex. Seeds were germinated and planted into 20 x 30cm plastic seed trays with 50-60 plants per tray using the same methodology as before. Twelve trays were prepared per population along with four trays of the Roth03 susceptible standard. All plants were sprayed with 100g a.i. ha^{-1} sulfometuron at the three-leaf stage. A count was performed on the number of plants per tray immediately before spraying and the experiment was assessed after 28 days when each plant was individually rated on a 1-3 injury scale. In addition to rating each plant, twenty randomly selected plants from each population and injury category were harvested by cutting at the soil surface and weighed.

5.2.3 Selection effects of mesosulfuron+iodosulfuron compared to flupyrsulfuron

A. myosuroides seed was collected from Broadmead field near Woburn, Bedfordshire, in the summer of 1999. Broadmead is a field at Woburn where resistant *A.myosuroides* occurs. Testing was performed at the time and resistance to acetyl-CoA carboxylase inhibitors was identified. In Petri dish resistance screening tests Broadmead seed from 1999 (Broad99) gave following results: fenoxaprop - 53% reduction in growth; pendimethalin - 84% reduction; sethoxydim - 68% reduction. Rothamsted susceptible/Pel96 values for same three herbicides = 98/82%; 99/15%; 100/100%. Broad99 was considered to be a 'typical' resistant population from the UK, showing partial resistance to several different herbicide classes. Prior to sampling, Broadmead had received no applications of grass-weed ALS inhibitors (to the best of our knowledge) and so it was assumed that specific ALS resistance would be unlikely at the site. This experiment was aimed at evaluating any change in the level of resistance in Broadmead seed to the ALS inhibitors flupyrsulfuron 'Lexus' and mesosulfuron+iodosulfuron 'Atlantis' with continued use in the field over two years, and was considered important in light of the recent introduction of mesosulfuron+iodosulfuron and its subsequent wide scale use, replacing flupyrsulfuron as the major sulfonylurea for *A. myosuroides* control in winter wheat. Field scale was used to enable a large number of plants to be exposed to selection and to mimic field conditions in terms of crop and cultivation patterns.

Cleaned Broadmead seeds were hand sown onto two 15 x 15m plots in Highfield on the Rothamsted estate which had been drilled with wheat on 17 Oct 2003. Plots were separated by around 100 m of willow coppice. A weed free discard of 1.5 m wheat was

left around each plot. After sowing with *A. myosuroides* the plots were rolled. The plots were sprayed with flupyrsulfuron at 20g a.i ha^{-1} (Plot1) and mesosulfuron+iodosulfuron at 12 + 2.4g a.i. ha^{-1} (Plot2), representing the maximum allowable amount per year for each herbicide, on 25 Feb 2004. *A. myosuroides* counts were performed on the same day. Further counts were made on 22 April 2004 and 04 June 2004 and seed was collected in mid July. All seed was cleaned and stored at 30°C to break dormancy. Wheat was harvested in September 2004 after which the plots were cultivated shallowly and drilled with wheat as before. This procedure was repeated yearly and is currently in its fourth full cycle. All counts were made using 25 x $0.25m^2$ quadrants with the exception of the first April count which used 25 x $0.1m^2$ quadrants. See Table 5.6 for a full summary of plant count dates and results. Flupyrsulfuron applications were made in the spring and without mixture or sequence partners throughout the selection experiment (contrary to guidelines for best use of this herbicide). The choice of timing was made in order to ensure that the maximum numbers of plants were exposed to herbicide each year. Mixtures were not used because the experiment was focused on the selection effects of single herbicides.

Seed collections from 2004 (year 1) and 2005 (year 2) were tested for resistance to the ALS inhibitors flupyrsulfuron, mesosulfuron+iodosulfuron and sulfometuron alongside the original Broadmead99 seed in glasshouse whole plant experiments. These tests were limited in scale due to restrictions on the amount of seed available from the mesosulfuron+iodosulfuron treated plot where very good *A. myosuroides* control was achieved in all years. From the 2004 collection, 91 plants grown from seed collected from survivors on the mesosulfuron+iodosulfuron plot (Plot2) were screened using sulfometuron at 100g a.i. ha^{-1} as an indicator of high-level resistance mechanisms. Plants were assessed using the normal 1-5 scale after 4 weeks.

The 2005 collection provided more seed and a more extensive screening procedure was performed. Seeds from each plot, the original Broadmead collection, and the susceptible standard Roth03, were pre-germinated and planted into Kettering loam using 5cm pots with one plant per pot before spraying at the three-leaf stage. Treatments were with flupyrsulfuron (1x, 2x, field rate; 10, 20g a.i. ha^{-1}), mesosulfuron+iodosulfuron formulation (0.5x, 1x field rate; 6 + 1.2, 12 + 2.4g a.i. ha^{-1}) or sulfometuron (100g a.i. ha^{-1}). A total of 44 plants were sprayed from each population at each treatment with the exception of Plot2 plants where 44 received sulfometuron, 44 received the higher dose of

mesosulfuron+iodosulfuron and 35 received the lower dose due to low seed numbers. 30 Nils were included from each population. Plants were assessed using the 1-5 injury scale four weeks after spraying and the fresh foliage weight of each plant was determined. Data was analysed using GenStat 7. A general ANOVA was conducted using percentage reduction in fresh weights relative to untreated plants as a measure of control.

5.3 Results

5.3.1 GPS mapping and resistance screening of Peldon *A. myosuroides* patches

Plants grown from the 2003 seed collection, from six different *A. myosuroides* patches on Peldon Hall and the neighbouring Brickhouse farm, were assessed after 34 days allowing percent resistant, intermediate and susceptible plants to be calculated for each replicate and population as described in section 5.2.1.1. Resistant plants corresponded to injury categories 1 and 2 on the 1-5 scale described in Chapter 2, intermediates to categories 3 and 4, and susceptibles to category 5. Refer Table 5.1 for details.

Table 5.1 A summary of percent resistant, intermediate and susceptible plants in plants grown from seed collected from 5 patches of *A. myosuroides* in the Peldon area, 2003

Summary of Percentage Resistance in Peldon *A. myosuroides* Patches, 2003				
Patch	**Number of plants treated with sulfometuron**	**Resistant (1) % of Total (S.E.)**	**Intermediate (2) % of Total (S.E.)**	**Susceptible (3) % of total (S.E.)**
Hams A (Peldon Hall)	313	2.6	1.6	95.8
Hams B (Peldon Hall)	325	4.9	1.5	93.6
Hams C (Peldon Hall)	340	23.5	1.8	74.7
Twitch (Peldon Hall)	322	0	5.9	94.1
Church (Brickhouse)	314	0	5.1	94.9
Ransomes (Brickhouse)	312	0	4.2	95.8
Pel02 (Hams C, Peldon Hall)	343	19.5	1.7	78.8
Roth03 (Susceptible)	123	0	0	100

All Roth03 plants were completely controlled and there were no survivors. The reference standard population Pel02 showed a similar level of resistant individuals compared to previous whole plant work (19.5% vs 25%, see Chapter 3). In comparison, only very low levels of individuals with intermediate resistance were picked up in the other Peldon patches from different fields. Highly resistant seed was found to occur in only one of the sampled fields at Peldon (Hams field). Of the three *A. myosuroides* patches in this field only the Hams C patch showed substantial levels of resistance with 23.5% of individuals surviving sulfometuron at 100g a.i. ha^{-1} without injury. The Hams A and B patches produced seed with 2.6% and 4.9% high-level resistance respectively. Seed from *A. myosuroides* patches on the neighbouring Brickhouse farm displayed no high-level resistance to sulfometuron at all.

Resistance to sulfometuron, as an indicator of potential ALS target site resistance, was confined to Hams field out of those tested in the Peldon area. The GPS map was overlaid onto an aerial photo of Peldon Hall farm (Image copyright at www.getmapping.com) in order to give an idea of the resistant *A. myosuroides* distribution in the field (see Figure 5.2). The aerial photo used in this section was not from the same year as the mapping and was also from a different point in the growing season.

Plants grown from seeds collected in 2004 were assessed 35 days after treatment with 100g a.i. ha^{-1} sulfometuron and scored using a 1-3 scale (Table 5.2). The proportions of resistant, intermediate and susceptible plants from seed samples at each point along the transect were plotted in a graph (Figure 5.4) and the GPS data from the sampling points was overlaid onto the patch map from the pervious year to give a better idea of the distribution of sulfometuron-resistant plants across Hams field (see Figure 5.5).

Table 5.2 A summary of percent resistant, intermediate and susceptible plants in plants grown from seed collected from sampling points (1-13) along a transect in Hams field, Peldon Hall farm, 2004. Points 14 and 15 were approximately 60m to either side from main transect line perpendicular to sampling point 3

Summary of percentage resistance at GPS mapped sampling points, 2004				
Sampling point	**Number of plants treated with sulfometuron**	**Resistant (1) % of Total (s.e.m)**	**Intermediate (2) % of Total (s.e.m)**	**Susceptible (3) % of total (s.e.m)**
1	203	2.9 (0.9)	4.3 (1.9)	92.8 (2.5)
2	211	9.5 (2.1)	11.4 (2.8)	79.1 (4.1)
3	200	9.9 (2.1)	8.4 (1.3)	81.7 (3.4)
4	178	5.4 (1.2)	7.3 (2.1)	87.3 (2.1)
5	200	13.1 (2.0)	11.0 (0.6)	75.8 (2.0)
6	194	23.7 (4.0)	21.1 (1.0)	55.2 (3.7)
7	191	24.6 (4.6)	15.6 (3.7)	59.8 (2.8)
8	187	9.1 (1.7)	10.7 (1.5)	80.2 (1.2)
9	200	2.5 (0.4)	2.5 (0.6)	95.0 (0.6)
10	195	0.9 (0.9)	4.1 (0.8)	95.0 (1.5)
11	179	2.2 (0.8)	5.4 (2.0)	92.4 (1.9)
12	199	4.1 (1.7)	6.5 (1.5)	89.4 (1.1)
13	188	1.1 (0.6)	13.0 (3.8)	85.9 (3.4)
14	176	10.1 (1.1)	11.5 (1.1)	78.4 (0.5)
15	170	5.9 (0.6)	4.1 (1.0)	90.0 (1.5)
Roth03 (susceptible)	194	0	0	100

As before, all Roth03 susceptible plants were completely controlled. Seed samples taken along an east-west transect in Hams field showed that high-level resistance was present across the whole field, although frequency varied considerably, and not restricted to one area. A distinct increase in the proportion of fully resistant seed was observed at sampling points 6 and 7 directly in the middle of Hams patch C (see Figure 5.4) where the proportion was over 20%. A larger proportion of the seed collected from within patch C

was fully resistant overall compared to the other sampling points, reinforcing the results from 2003.

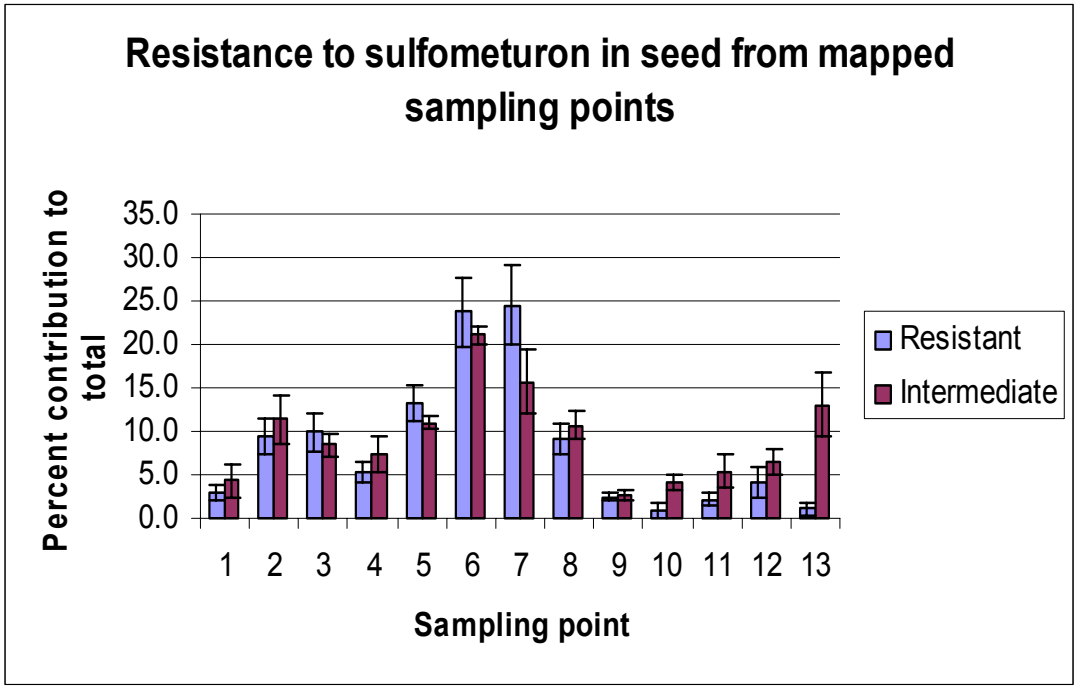

Figure 5.4 Histogram showing distribution of resistant and intermediate plants in seed collected from 13 points along an east to west transect in the Peldon area, summer 2004. Error bars are S.E. of the mean.

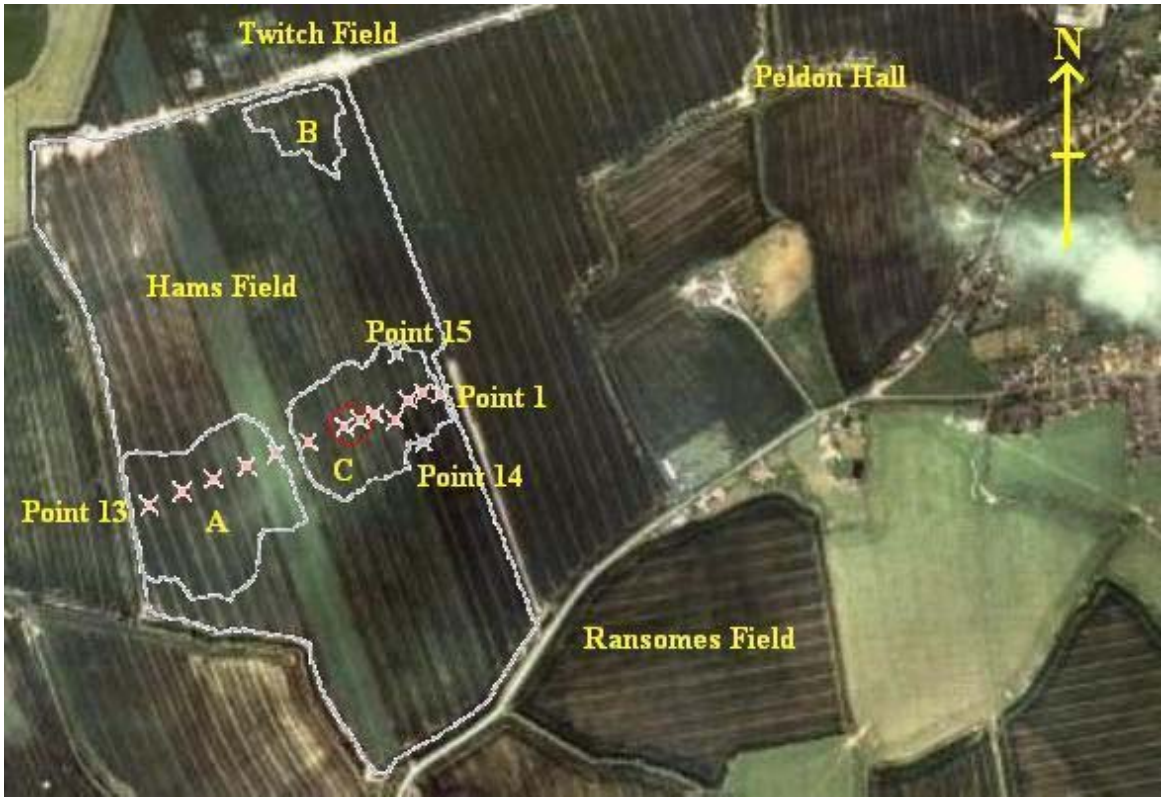

Figure 5.5 Map of sampling points Peldon Hams field at Peldon, Essex, sampled in 2004. Field boundary and patches (A, B and C) sampled in 2003 are shown for reference, sampling points in pink. Points six and seven are highlighted in red. Scale 1cm = approx 100m

5.3.2 Increase in ALS inhibitor resistance at Peldon between 1996 and 2002

All plants were scored using a 1-3 scale prior to harvest allowing percent resistant, intermediate and susceptible plants to be calculated for each replicate and population. Standard errors were calculated for each sampling year and for each level of resistance and the changing levels of resistance over time plotted as a graph. Data was processed to give coefficients of variation for weights of resistant, intermediate and susceptible plants, see Table 5.3.

Table 5.3 A summary of percent resistant, intermediate and susceptible plants in plants grown from seed collected on Hams field patch C, 1996 to 2002

Summary of percentage resistance in Peldon *A. myosuroides*, 1996-2002				
Year	**Total number of plants treated with sulfometuron**	**Resistant % of Total (s.e.)**	**Intermediate % of Total (s.e.)**	**Susceptible % of total (s.e.)**
1996	439	2.1 (0.6)	12.2 (2.6)	85.6 (2.8)
1997	452	0.0	5.6 (1.8)	94.4 (1.8)
1998	397	0.5 (0.4)	7.7 (1.9)	92.0 (2.2)
1999	391	0.0	11.4 (2.8)	88.6 (2.8)
2000	465	3.6 (0.9)	28.3 (4.4)	68.1 (4.4)
2001	407	32.1 (2.5)	13.7 (2.2)	54.2 (2.1)
2002	465	24.9 (1.9)	26.6 (4.6)	48.5 (3.7)
Roth03 (susceptible)	137	0	0	100

All Roth03 susceptible standard plants recorded an injury score of 3 with none surviving sulfometuron at 100g a.i. ha^{-1}. Overall the proportion of plants from Hams field patch C showing high-level resistance was low up until 2000, with the proportion of susceptible plants never falling below 85%. Less than 2% of all plants were highly resistant using seed from any year until 2000. Resistance increased dramatically in the seed from 2000 (see Figures 5.6 and 5.7), with only 68.1% of plants being fully susceptible to 100g a.i. ha^{-1} sulfometuron. Most of the increase was due to intermediate plants however (28.3%), and the proportion of fully resistant plants (3.6%) was not substantially increased compared to previous years. From 2000 onwards, the number of susceptible plants declined steadily, having fallen to 48.5% by 2002. The proportion of highly resistant plants was greater in seeds from 2001 than 2002, but the general trend was a rapid increase in intermediate and highly resistant plants grown from seeds collected since 2000. Variation in weight between plants from different years given the same score on the 1-3 scale showed a reasonable level of consistency in the scoring according to calculated coefficient of variation values (data not shown).

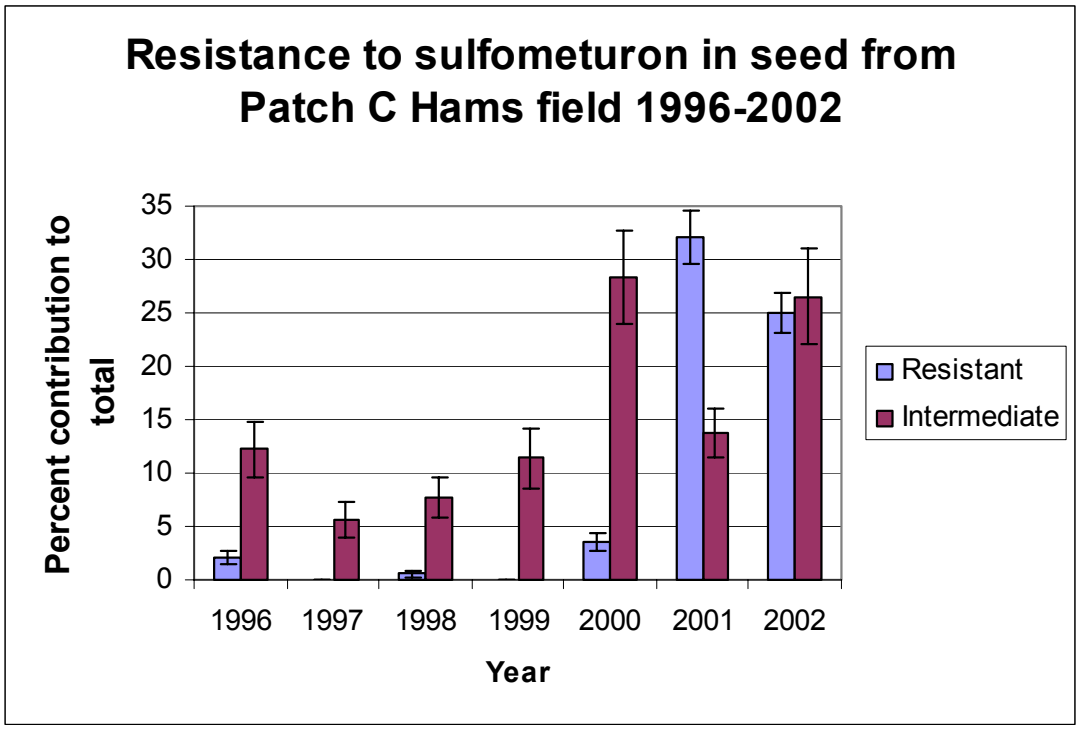

Figure 5.6 Histogram showing the distribution of resistant and intermediate plants in *A. myosuroides* seed collected from Hams field Patch C, Peldon Hall farm, 1996-2002. Error bars are S.E. of the mean.

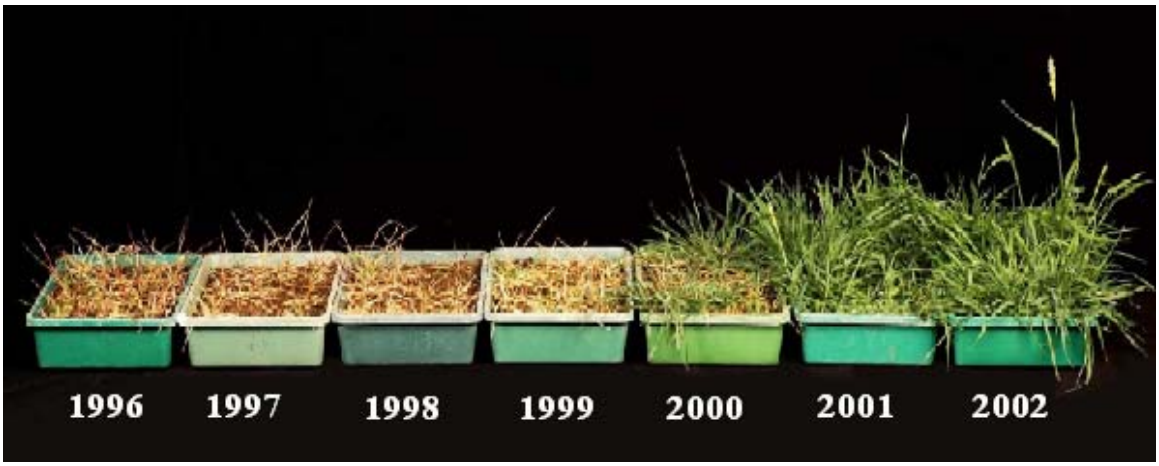

Figure 5.7 Alignment of representative trays from each seed collection year in chronological order, 28 days after treatment with sulfometuron at 100g a.i. ha^{-1} showing differences in the proportions of resistant plants

Herbicide records from Peldon Hall farm Hams field were consulted in order to provide some background for the sudden increase in resistance to sulfometuron in *A. myosuroides* after 2000 and the constant low level of plants showing high-level resistance as far back as 1996. These records showed that flupyrsulfuron was used on the farm for three years from 2000 to 2002. In addition, the sulfonylurea herbicide mixture chlorsulfuron+metsulfuron (Finesse) was used for three years from 1987, and some patch spraying with propoxycarbazone-sodium was carried out, although exact records of the treated areas are not available. It is possible that the earlier use of ALS inhibitors pre-selected for some degree of high-level resistance which remained present in the population or the seedbank. Table 5.4 presents a summary of the various herbicide modes of action applied on Hams field between 1980 and 2002 as a percentage of total herbicide applications made (only herbicides active against *A. myosuroides* are included). This summary highlights the relatively low contribution made to total herbicide application by herbicides in the ALS inhibiting group. Only 8% of all applications were ALS inhibitors and these were limited to chlorsulfuron+metsulfuron and flupyrsulfuron.

Table 5.4 Herbicide applications on Hams field, Peldon, as a percentage of total applied herbicide between 1980 and 2002. Applications of below field rate are treated as a single dose, multiple field rate doses are treated as multiples

Herbicide mode of action	Number of applications 1980-2002	Percentage contribution to total applications 1980-2002
Inhibitors of PSII Eg Chlorotoluron, Isoproturon	31	35
Inhibitors of fatty acid synthesis (non ACCase) Eg Triallate	19	21
Inhibitors of microtubule assembly Eg Trifluralin	14	16
Inhibitors of fatty acid synthesis at ACCase Eg Clodinafop	11	12
PSI electron diversion Eg Paraquat	7	8
Inhibition of amino acid synthesis at ALS E.g. Flupyrsulfuron	7	8

5.3.3 Selection effects of mesosulfuron+iodosulfuron compared to flupyrsulfuron

The first screening experiment using seeds collected in 2004 and screened using sulfometuron at 100g a.i. ha^{-1} yielded no healthy survivors at all. Consequently data was not analysed for this preliminary experiment. In contrast plants grown from 2005 seed provided some interesting differences in response between populations and treatments. Plant weight data were analysed using GenStat 7. A general unbalanced ANOVA test was conducted for all herbicide treatments using percentage reduction in fresh weights relative to untreated plants as a measure of control. The seed collection made in 2005 was from the second year of the experiment when both plots had been subjected to two years of herbicide selection. See Table 5.5 for a summary of results.

Table 5.5 Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants across herbicide treatments for 2005 seeds from flupyrsulfuron (Plot1) and mesosulfuron+iodosulfuron (Plot2) field plots

Seed set	Mean untreated weight per plant (g)	Mean % reduction relative to mean weight of untreated plants				
		Flupyrsulfuron		Mesosulfuron+iodosulfuron		Sulfometuron
		10 g a.i. ha^{-1}	20 g a.i. ha^{-1}	6 + 1.2 g a.i. ha^{-1}	12 + 2.4 g a.i. ha^{-1}	100g a.i. ha^{-1}
Roth	3.76	82.9	87.0	81.8	85.5	85.5
Broad99	3.79	46.1	56.4	75.3	84.8	83.0
Plot1(flup)	3.81	13.2	16.8	54.2	77.9	80.5
Plot2(meso)	3.89	-	-	36.6	66.1	83.2
S.E.±	0.14	3.2				
LSD,5%	0.40	8.4				

General levels of control in this experiment were somewhat lower than usual in terms of fresh foliage weight reduction relative to untreated plants. The experiment was carried out over a period of low temperatures and this may have had some bearing on the reduced levels of control. All susceptible standard plants were eventually killed (rated injury score 5) but grew on for a time after spraying resulting in less reduction in final fresh weight. All weights were analysed relative to the mean weight of untreated plants, and reduced control was consistent across populations. Levels of control for Roth03 susceptible plants corresponded to greater than 80% reduction in fresh foliage weight relative to mean weight for untreated plants in all cases and there were no significant differences between

herbicide treatments. Broad99, the original field population sown onto the plots under herbicide selection, showed significant levels of resistance to flupyrsulfuron at 1 and 2x field rate compared to the susceptible standard but no significant resistance to mesosulfuron+iodosulfuron or sulfometuron (p=0.05). This was expected since flupyrsulfuron has shown poor control of a wide range of *A. myosuroides* populations regardless of herbicide history (see Chapter 3).

Some interesting effects were highlighted following data analysis. Two years of field selection with flupyrsulfuron at 20g a.i. ha^{-1} (Plot1) caused a marked and statistically significant shift in the glasshouse response to the herbicide compared to the original unselected Broad99 seed (13.2% compared to 46.1 % reduction in fresh weight after treatment relative to untreated plants (p=0.05)). Plants from the mesosulfuron+iodosulfuron treated Plot2 were not tested for resistance to flupyrsulfuron due to lack of seed and so it was not possible to compare the difference in selection in this case.

Seed from the flupyrsulfuron treated plot also showed some evidence of selection taking place resulting in a degree of cross resistance to mesosulfuron+iodosulfuron. At the 0.5 x field rate dose, Plot1 plants showed a 54.2% mean reduction in fresh weight relative to untreated plants. This reflected significantly poorer control than was achieved for the original Broad99 population where control was equivalent to 75.3% mean fresh weight reduction (p=0.05). This difference was overcome at 1 x field rate mesosulfuron+iodosulfuron indicating that selection by flupyrsulfuron was most likely based on an enhanced metabolism type mechanism in this case.

Seed collected from Plot2 which was exposed to mesosulfuron+iodosulfuron for two years showed a statistically significant decrease in control at the 0.5 x field rate dose of that herbicide expressed as mean percentage reduction in fresh weight when compared to both the original Broad99 seed and the Plot1 seed (36.6% compared to 75.3% and 54.2% reduction to untreated plants respectively, p=0.05). The level of control was greater at 1 x field rate mesosulfuron+iodosulfuron, with 66.1% reduction in fresh weight of treated Plot2 plants compared to untreated plants. However, even at the 1 x field rate dose, mesosulfuron+iodosulfuron selected Plot2 seed showed significantly poorer levels of control than both Plot1 and Broad99 seed sets.

Sulfometuron at 100g a.i. ha^{-1} provided high levels of control for all seed sets regardless of selection regimes. There was no significant difference between the susceptible Roth seed set and any of the others in terms of fresh weight reduction as percentage of mean control weight with sulfometuron providing greater than 80% reduction in all cases. Sulfometuron was included as an indicator of high level resistance mechanisms such as ALS target site resistance. A count of plant injury scores showed that no plants survived treatment with sulfometuron with anything less than moderate injury (equivalent to a score of 3 on the 1-5 scale). This result indicates a lack of high level resistance mechanisms in all seed sets and means that enhanced metabolism is the most likely explanation for the resistance observed in the Plot1 and Plot2 seed sets after 2 years selection with flupyrsulfuron and mesosulfuron+iodosulfuron respectively.

Continued field counts in both plots performed in spring (pre and post spray) and summer (head count) showed consistently greater levels of control with mesosulfuron+iodosulfuron compared to flupyrsulfuron (see Table 5.6).

Table 5.6 *A. myosuroides* counts in field selection experiment 2004-2006

Date		***A. myosuroides* frequency m^{-2}**		**% reduction compared to pre-spray density**	
		Plot1 Flupyrsulfuron	**Plot2 Mesosulfuron+iodosulfuron**	**Flup**	**Meso +iodo**
Yr1	25 Feb 04	261 (plant count, pre-spray)	321 (plant count, pre-spray)	-	-
	22 Apr 04	15 (plant count, post spray)	0 (plant count, post spray)	94	100
	04 Jun 04	34 (head count)	0 (head count)	-	-
Yr2	17 Mar 05	175 (plant count, pre-spray)	55 (plant count, pre spray)	-	-
	30 May 05	29 (plant count, post spray)	0 (plant count, post spray)	83	100
	23 June 05	324 (head count)	0 (head count)	-	-
Yr3	16 Feb 06	95 (plant count, pre-spray)	5 (plant count, pre-spray)	-	-
	18 May 06	45 (plant count, post spray)	4 (plant count, post spray)	53	20
	20 July 06	372 (head count)	6 (head count)	-	-

It can be seen from the data that although both herbicides have caused much reduced pre-spray plant emergence compared to the first year, mesosulfuron+iodosulfuron has provided consistently higher levels of control than flupyrsulfuron in terms of emerged plants. Also important is the level of control provided in terms of the proportion of emerged plants which survive herbicide application as a measure of the build up of resistance. In 2005 the percent control in pre- vs post-spray counts was 83% with flupyrsulfuron and almost 100% with mesosulfuron+iodosulfuron (some plants were present in the plot overall but were at very low levels and did not show up in counts). In 2006 control was 53% with flupyrsulfuron and 20% (based on a very low population density) with mesosulfuron+iodosulfuron, possibly reflecting an increase in resistance amongst emerging plants. Care is needed with the interpretation of the most recent mesosulfuron+iodosulfuron data since emergence was so low. Although it is too early to draw firm conclusions from field plant counts, it seems that mesosulfuron+iodosulfuron offers greater levels of control overall while, perhaps, selecting more strongly for resistance. The selection experiment is currently continuing into its fourth spray year. It is hoped that future screening experiments and plant count data will provide a better longer term perspective from which conclusions about the different selection effects of flupyrsulfuron and mesosulfuron+iodosulfuron can be drawn.

5.4 Discussion

This section contains a discussion of the results obtained from field sampling at Peldon and the related glasshouse screening experiments. Also included is a discussion of the current results from the continuing field selection experiment on Rothamsted farm where the selection effects of mesosulfuron+iodosulfuron and flupyrsulfuron are compared. An attempt is made to bring these diverse field experiments together and to draw conclusions about the evolution of sulfonylurea resistance in field *A. myosuroides* populations which can be related to the future use of ALS inhibiting herbicides in *A. myosuroides* control.

Mapping and screening experiments performed in the summers of 2003 and 2004 at Peldon Hall farm in Essex provided valuable field scale information about one of the most resistant *A. myosuroides* populations in the UK. Peldon *A. myosuroides* is known to resist a wide range of different herbicides through enhanced metabolism. Applications of the sulfonylurea herbicide flupyrsulfuron were started in 2000 and since that time

resistance to the non-selective herbicide sulfometuron (used here as an indicator of high level resistance mechanisms such as ALS target site resistance) has increased markedly. Mapping revealed several large 'patches' of *A. myosuroides* on Peldon Hall farm but high level resistance to sulfometuron was only detected in a single field (Hams field). Follow-up work including more detailed mapping of sampling points along a transect showed that high level resistance was present at a low level across the whole of Hams field but high proportions of highly resistant seed (>20%) were localised to a fairly small area of the whole field, between sampling points six and seven in Patch C specifically. One possible explanation of these results is that resistance may have arisen in a single area and spread outwards over the whole field. *A. myosuroides* is an out-crossing wind pollinated species and more than 70% of pollen dispersal has been shown to occur within 1m of the pollen donor plant, although distances of up to 60m have been observed (Chauvel & Gasquez, 1994). Seed dispersal is also over a short range for this species (Colbach & Sache, 2001) and these factors together would favour the build up of patches around the location of individuals with mutations conferring an ALS enzyme resistant to sulfonylurea herbicides.

These results have practical implications for the detection and control of ALS target site resistance in *A. myosuroides*. Firstly farmers using ALS inhibitors should be alert for the appearance of any patches where *A. myosuroides* control is noticeably poorer than the control achieved over the whole field. Seeds from such patches should be collected and sent for testing as soon as possible. Secondly, once resistance is confirmed, control of such patches might be more achievable if caught early by spraying off using glyphosate or another non-selective herbicide and monitoring the area for several years, perhaps taking it out of cultivation in the case of a severe infestation.

The evolution of resistance in the area of the highly resistant Patch C was investigated in a time series experiment using seed collected from the patch between the years 1996 and 2002. These collections focussed on the whole area of Patch C and not on the small highly resistant area identified between sampling points 6 and 7 in the mapping experiment. This experiment was aimed at investigation of the adaptive response of the Patch C population over time in response to selection pressures imposed by the introduction of the sulfonylurea herbicide flupyrsulfuron in 2000. The results were very interesting in terms of the speed of the selection process, with selection for high level

resistance mechanisms (as indicated using the non-selective SU sulfometuron) taking place over just 2 years and taking the proportion of highly resistant seed collected from the patch from a low baseline level (0 - 2%) to over 30%. After 2002 the farmer stopped using flupyrsulfuron on Hams field and levels of resistance in seed from surviving plants dropped back slightly as indicated in the mapping experiment. It is thought that the use of the sulfonylurea mixture chlorsulfuron+metsulfuron 'Finesse' for three years from 1987 may have pre-selected for some degree of high-level resistance which remained present in the population and allowed rapid increase in the frequency of ALS alleles conferring resistant phenotypes following the re-introduction of a sulfonylurea herbicide in 2000. Continued monitoring of the proportion of sulfometuron resistant seed from Patch C in Hams field will provide an opportunity to observe the dynamics of the resistant trait in the absence of selection by sulfonylurea herbicides.

One worrying aspect of the development of ALS target site resistance at the Peldon site is that sulfonylurea use was low as a percentage of total herbicide application. Mixtures were used habitually at Peldon Hall for *A. myosuroides* control and sulfonylureas were never relied upon as a single mode of action. This pattern of resistance development needs to be investigated to see if it applies to other sites where sulfonylureas have been used to control *A. myosuroides*. If Peldon is typical then predicting where ALS target site resistance may occur in future will be difficult. Resistant populations may be as likely to emerge in fields with low sulfonylurea application histories as they are in more typical situations where ALS inhibitors have been used with few mixture and sequence partners over several successive years.

Results from the field selection experiment comparing the long term effects of flupyrsulfuron and mesosulfuron+iodosulfuron yielded important information about the nature and speed of development of resistance in a typical field population never exposed to sulfonylureas. Selection for resistance was observed in both 15 x 15m plots after 2 years, with flupyrsulfuron resistance being much more rapidly selected for than resistance to mesosulfuron+iodosulfuron. Seed collected from the flupyrsulfuron treated plot showed selection had taken place resulting in a degree of cross resistance to mesosulfuron+iodosulfuron. Cross resistance was evident at 0.5 x field rate mesosulfuron+iodosulfuron but was overcome at 1 x field rate, indicating that selection by flupyrsulfuron was almost certainly based on an enhanced metabolism type

mechanism, and this was confirmed by the level of control achieved with sulfometuron. Flupyrsulfuron is a less active herbicide than mesosulfuron+iodosulfuron with 15 plants m^{-2} remaining after spraying in the first year of use. Overall control was much greater in the mesosulfuron+iodosulfuron plot meaning that seed return from individuals displaying resistance mechanisms was low, indicated by very low numbers of emerging plants in years 2 and 3 of the trial. Neve & Powles (2005a) showed some evidence that high survival frequencies under recurrent selection with low herbicide doses can allow the accumulation of multiple weak resistance mechanisms, a consequence of selection at many loci of small overall effect (minor alleles). While this work was interesting from a theoretical standpoint, some of the conclusions were fairly speculative, resulting as they did from only a single cycle of selection with diclofop-methyl. More work needs to be done; in particular to understand the genetic basis of this resistance, before solid conclusions can be drawn. The idea of selection for multiple minor resistance alleles under low selection pressure is in contrast to high herbicide doses which tend to allow only those individuals possessing resistance alleles conferring a high level of resistance (major alleles) to survive (Neve & Powles, 2005a, b), and provides an interesting alternative mechanism for resistance development.

These ideas are particularly relevant to the relative evolutionary dynamics of resistance under flupyrsulfuron or mesosulfuron+iodosulfuron. Due to its lower efficacy, flupyrsulfuron might be expected to select for multiple weaker resistance mechanisms which could build up over time in a population, while mesosulfuron+iodosulfuron would be more likely to allow only those individuals possessing major resistance alleles to survive. Consequently it might be expected that resistance to mesosulfuron+iodosulfuron would build up more slowly than flupyrsulfuron resistance due to the low levels of major resistance allele frequencies present in unselected populations (Diggle *et al.*, 2003; Preston & Powles, 2002). Optimisation of control through good timing, mixtures and sequences may help to slow the build-up of enhanced metabolism resistance through the accumulation of multiple weak resistance mechanisms and is a prudent strategy with any ALS inhibiting herbicide. In the case of flupyrsulfuron this can be achieved through the selection of an appropriate mixing partner and careful timing of application. Recent studies at Rothamsted have shown that flupyrsulfuron applied pre-emergence can give better control of *A. myosuroides* populations with enhanced metabolism resistance. It is also worth noting that flupyrsulfuron is almost always used in mixture with other

herbicide modes of action while mesosulfuron+iodosulfuron mixture is more likely to be used with no tank-mix (Bayer, 2005).

No resistance was observed in either seed set to sulfometuron at 100g a.i. ha^{-1} and the results indicated a total lack of ALS target site resistance present in either. This was most likely a consequence of the low numbers of plants under selection on the 15 x 15 m plots used in this experiment. Frequencies of ALS target site resistant mutations have been measured in three different unselected populations of the grass weed *Lolium rigidum* and were estimated at between 2.2×10^{-5} and 1.2×10^{-4} (Preston & Powles, 2002). On a 15 x 15m plot with 321 plants per square metre this would mean the number of individuals expected to have an ALS target site point mutation would be between 1.6 and 8.7 assuming a similar frequency in *A. myosuroides*. The fact that no high level resistance was observed in screening tests means it is likely that the frequency in *A. myosuroides* is towards the lower end of this range or that ALS target site resistant plants have not yet increased to detectable levels.

These results have several consequences for the continued use of sulfonylurea herbicides in the control of *A. myosuroides*. Firstly enhanced metabolism resistance to flupyrsulfuron can develop rapidly in populations which have already been exposed to other herbicide modes of action. Flupyrsulfuron resistance, which is already very common on UK farms, confers a degree of cross-resistance to mesosulfuron+iodosulfuron, most likely through enhanced metabolism mechanisms conferred by a build up of minor alleles, and this may be enough for some individuals in a population to survive and reproduce. Enhanced metabolism resistance to mesosulfuron+iodosulfuron in populations pre-selected with flupyrsulfuron may therefore build up over time. Direct application of mesosulfuron+iodosulfuron to populations previously unselected with sulfonylurea herbicides provides very good control but even here results suggest that metabolism based resistance may build up slowly. The current switch from flupyrsulfuron to mesosulfuron+iodosulfuron is likely to affect the evolution of herbicide resistance by decreasing frequencies of surviving plants and allowing only those individuals with resistance alleles conferring a high level of resistance, or a sufficient build up of minor alleles with additive effect, to survive. In the short term mesosulfuron+iodosulfuron has given very good control of problem *A. myosuroides* populations, but the continued use of this herbicide alone in the spring is likely to strongly select for single gene type resistance

(target site) as well as the build-up of genes with minor additive effects in already flupyrsulfuron resistant field populations due to cross resistance. This effect will be tested in future years by applying mesosulfuron+iodosulfuron to Plot1 and testing seed from survivors with sulfometuron.

The use of herbicide mixtures and sequences along with cultural control methods which reduce the weed population density is the usual advice offered to farmers in terms of reducing the risk of herbicide resistance. However because mesosulfuron+iodosulfuron use is now so widespread in winter wheat production, and since the alternative *A. myosuroides* herbicides are themselves subject to resistance problems, it is probably only a matter of time until resistance builds up to the level previously seen with flupyrsulfuron and effective control is lost in many populations. Whether this will be due to single gene target site resistance or multi gene based enhanced metabolism will probably depend on the level of field population pre-selection by other herbicides on a case by case basis. Monitoring of the changing resistance situation is of crucial importance at the present time in order to predict whether enhanced metabolism or target site resistance will be the major emerging threat to mesosulfuron+iodosulfuron efficacy against *A. myosuroides*.

5.5 Chapter summary

- The situation at Peldon Hall farm in Essex provides one possible model for the evolution of resistance to sulfonylurea herbicides in the weed *A. myosuroides*.
- Distribution of high-level resistant plants across one field was patchy with high proportions of resistant plants concentrated in a small area of a single patch. Lower proportions of resistant plants were picked up across the whole field.
- Farms with similar herbicide records to Peldon Hall are at risk of high level sulfonylurea resistance and would be well advised to minimise the use of the mesosulfuron+iodosulfuron mixture ‘Atlantis’.
- Data from the field selection experiment showed that selection with flupyrsulfuron provided a degree of probable enhanced metabolism cross-resistance to mesosulfuron+iodosulfuron in a population previously not exposed to sulfonylureas.
- Mesosulfuron+iodosulfuron is a more active herbicide than flupyrsulfuron and provides greater levels of control overall. Continued use is likely to strongly select for single gene type resistance as well as the build-up of genes with minor additive effects in

already flupyrsulfuron-resistant field populations.

- The nature of emerging resistance to mesosulfuron+iodosulfuron in field populations of *A. myosuroides* will depend on the previous herbicide history of the populations in question. Widespread resistance can be expected to build up in a short time given current usage practices of spring application and relatively ineffective sequence partners.

6. Enzyme Assays

6.1 Introduction

This chapter covers work done at the enzyme level in order to confirm whether the resistance to sulfometuron and flupyrsulfuron seen at the whole plant level in pot experiments was due to the presence of an enzyme insensitive to inhibition by herbicides. Results from experiments carried out at the whole plant level showed that significant resistance to sulfometuron was only present in three seed sets; PelRES03 which was a sulfometuron selected line originally derived from a single highly resistant plant from the Pel96 population, Pel02 field collected seed, and Pel02SS which was another sulfometuron selected seed set derived from a group crossing of highly sulfometuron resistant Pel02 plants. The enhanced metabolism resistance standard Pel96 contains around 2% highly ALS resistant plants (see Chapter 5).

In whole plant studies sulfometuron at 100g a.i. ha^{-1} was used as an indicator of high level resistance to sulfonylureas. Such resistance was distinct from the more common enhanced metabolism based mechanism assumed to be responsible for the widespread flupyrsulfuron resistance in many UK *A. myosuroides* populations. The resistant PelRES03 and Pel02 seed sets were characterised by a proportion of plants surviving this application with little or no damage in addition to a number of damaged survivors. This is in contrast to susceptible and flupyrsulfuron resistant populations where sulfometuron application resulted in almost total control at much lower doses (<10g a.i. ha^{-1}). Inhibition assays with acetolactate enzyme extracts from susceptible and resistant *A. myosuroides* populations were conducted in order to compare the response of different populations at the enzyme level to flupyrsulfuron and sulfometuron.

6.1.1 Acetolactate Inhibiting Herbicides

Sulfonylureas are part of a group of herbicides known as acetolactate synthase (ALS) inhibitors. Their mode of action was confirmed in 1984 when it was demonstrated that chlorsulfuron acts to inhibit the acetolactate synthase enzyme, preventing susceptible

plants from synthesising the branched chain amino acids leucine, valine and isoleucine (Ray, 1984). It is thought that lack of these essential amino acids is the primary mechanism of plant death, although other explanations including cessation of cell division have been suggested (Schloss, 1989; Shaner & Singh, 1993). Other ALS inhibiting chemistries include the imidazolinone, triazolopyrimidine, pyridinylthiobenzoate and sulfonylaminocarbonyltriazolinone groups.

6.1.2 Resistance to ALS inhibitors

The first examples of target site resistant broadleaved weeds occurred only five years after the introduction of the sulfonylureas (Preston & Mallory-Smith, 2001; Tranel & Wright, 2002) and the number of resistant biotypes has grown consistently since that time. ALS inhibitor resistance is now the most widespread type of resistance in the world with 95 unique resistant weed biotypes (Heap, 2006). Resistance to ALS inhibitors is most often due to target site mutations which prevent herbicide binding at the enzyme target site (Preston & Mallory-Smith, 2001). Target site mutations conferring resistance to ALS inhibitors are usually associated with a single amino acid substitution and often result in enzyme many times less susceptible to inhibition that that from susceptible biotypes. It is well known that different mutations confer different patterns of cross resistance to different ALS inhibiting chemistries (Heap, 2006; Tranel & Wright, 2002).

Typically target site resistance to ALS inhibitors has been identified by a combination of whole plant dose response experiments coupled with inhibition assays where crude acetolactate synthase enzyme is extracted from susceptible and resistant plants and then subjected to a colorimetric end point reaction with different concentrations of ALS inhibiting herbicide as inhibitor (Christopher *et al.*, 1992; Devine *et al.*, 1990; Eberlein *et al.*, 1997; Hall & Devine, 1990; Poston *et al.*, 2002; Saari *et al.*, 1990). This approach has been very successful in pinpointing the mechanism of resistance in many cases, especially in broad-leaved weeds.

In grass weeds there have been fewer confirmed cases of ALS inhibitor resistance due to insensitivity at the enzyme level (Burnet *et al.*, 1994; Novakova *et al.*, 2006; Park & Mallory-Smith, 2004; Saari *et al.*, 1992). A greater number of grass weed species have shown resistance due to enhanced metabolism of herbicide in the target weed when compared to broad-leaved species (Burnet *et al.*, 1994; Carey *et al.*, 1997; Christopher *et*

al., 1991; Christopher *et al.*, 1994; Cotterman & Saari, 1992; Matthews *et al.*, 1990; Menendez *et al.*, 1997; Park *et al.*, 2003; Preston *et al.*, 1996). In some instances both resistance mechanisms have been reported in the same grass weed (Burnet *et al.*, 1994; Christopher *et al.*, 1992). The high frequency of metabolism-based resistance mechanisms in grass weeds compared to broad-leaf weeds complicates the picture in terms of inhibition assays with ALS enzyme as resistance due to enhanced metabolism is not picked up in these assays. This can mean that resistance in populations showing enhanced metabolism as well as ALS target site resistance is underestimated in ALS enzyme assays and shows the importance of always comparing results from work done at the biochemical or molecular level to whole plant data.

6.1.3 Scope of work at the enzyme level

Enzyme assays were performed in order to generate inhibition curves for several standard *A. myosuroides* populations using the herbicides flupyrsulfuron-methyl (flupyrsulfuron) and sulfometuron-methyl (sulfometuron). The populations chosen for characterisation were the susceptible standard population Roth03, the high enhanced metabolism standard Pel96, a field population containing a proportion of plants with a high level resistance mechanism conferring resistance to the non-selective sulfonylurea sulfometuron (Pel02), and a sulfometuron selected line with a higher proportion of sulfometuron resistant plants (PelRES03). Further details of the two standard populations can be found in Chapter 2, (section 2.5), while details of the sulfometuron resistant populations are in Chapter 3 (section 3.1.2). The metabolism standard Pel96 population did not show significant resistance to sulfometuron in whole plant studies at the population level although it did contain a very low proportion of resistant plants (around 2%, see Chapter 5). The inclusion of Pel96 plants was important since this seed set comes from the same area as the Pel02 and PelRES03 resistant populations and is highly resistant to flupyrsulfuron through an enhanced metabolism mechanism (Moss *et al.*, 2005b). It was felt that inclusion of Pel96 in enzyme assays might be used to help separate the enhanced metabolism common in Peldon populations from the additional higher level resistance mechanism seen in the Pel02 and PelRES03 seed sets in whole plant experiments.

6.2 Materials and methods

6.2.1 Plants growth and harvest

The highly resistant Pel02 and PelRES03 populations were assayed alongside the susceptible standard Roth03 and the enhanced metabolism standard Pel96. PelRES03 plants were derived from the seed from a group of original PelRES02 plants which survived 100g a.i. ha^{-1} sulfometuron in 2003 and were poly-crossed (see Chapter 4). Pel02 was included as an example of a field population which contained a proportion of plants showing high levels of resistance to sulfometuron. Cleaned seed samples were germinated in Petri dishes and sown into 20 x 30cm divided trays using potting compost. 40 seedlings were planted per tray with 11 trays prepared per population. The plants were grown at 20°C, 16h day and 15°C, 8h night until the 4 to 6 tiller stage, at which point actively growing healthy green leaf material from the plants was harvested in 50g batches (ca. 40 plants) and extracted as described in section 6.2.2. Extractions were performed as 6 x 50g batches and the extracts were pooled and mixed to ensure consistency before being divided back into 6 separate aliquots. This was done to ensure the largest possible sample of plants per extract and give an accurate representation of each population as a whole.

6.2.2 Crude enzyme extraction method

Crude acetolactate synthase was extracted and assayed from selected *A. myosuroides* populations. Extraction of *A. myosuroides* leaf material was carried out in a cold room at 4°C in order to prevent degradation of enzyme activity. The extractions were performed using a pH 7.5 extraction buffer containing 100mM potassium phosphate, 1mM sodium pyruvate, 0.5mM $MgCl_2$, 0.5mM TPP, 10μM FAD, 1mM DTT, 0.0165% antifoam A, 5% w/v PVPP and 10% glycerol by volume. All chemicals were obtained from Sigma. The extraction buffer was bubbled with nitrogen before use and chilled to just above freezing. 50g of fresh leaf material was harvested and homogenised with cold extraction buffer 4:1 v/w in a chilled Waring blender. The resulting homogenate was filtered through six layers of cheesecloth and centrifuged at 27000g for 20 minutes. Proteins were precipitated from the supernatant using a 50% saturation ammonium sulphate cut and gentle stirring for 30 minutes. The extract was centrifuged again at 27000g, this time for 15 minutes and the supernatant discarded. The pellet was re-suspended in 2.5ml of extraction buffer

containing ammonium sulphate at 55% saturation then frozen in liquid nitrogen and stored at -80°C.

6.2.3 Enzyme assay

The assay method was adapted from Singh *et al.,* (1988), Saari *et al.,* (1990) and unpublished work by Kay Cocker at Rothamsted. All chemicals were supplied by Sigma (Poole, UK), Fisher Scientific (Loughborough, UK) or Promega (Southampton, UK), unless stated otherwise.

6.2.3.1 Assay theory

Acetolactate synthase catalyses the first common step in the synthesis of branched-chain amino acids in plants according to the following two alternative reactions:

$$2\ \text{Pyruvate} \rightarrow \text{Acetolactate} + CO_2 \qquad \text{(a)}$$

$$\text{Pyruvate} + \alpha\text{-Ketobutyrate} \rightarrow \text{Acetohydroxybutyrate} + CO_2 \qquad \text{(b)}$$

Cofactors required for these reactions include a divalent metal ion (eg magnesium), FAD, and thiamine pyrophosphate (TPP). The amount of acetolactate produced in reaction (a) can be measured using an end point spectrophometric assay where the reaction is stopped and acetolactate is decarboxylated to acetoin by heating with acid. Acetoin is then reacted with α-naphthol to give a pink complex which can be detected at 530nm according to the method of Westerfeld (1945).

6.2.3.2 Assay procedure

The pellet from 50g of fresh plant material was thawed in a cold room at 4°C and then centrifuged at 16000g for 15 minutes. The supernatant was discarded and 0.5ml of extraction buffer was added (prepared without DTT, antifoam A and PVPP). The pellet was re-suspended and desalted on a PD-10 column (Amersham Pharmacia Biotech, Sephadex G-25 medium) pre-equilibrated with extraction buffer. The desalted enzyme was assayed in 350µl well microtitre plates with pH 7.0 assay buffer containing 20mM potassium phosphate, 1500mM sodium pyruvate, 50mM $MgCl_2$ and 50µM FAD. 5µl of 3M sulfuric acid was added to the blank wells and the reaction started

by the addition of 40µl of desalted enzyme extract to all wells. After incubation at 37°C for 30 minutes the remaining wells were acidified and the plate heated to 60°C for 15 minutes. 50µl of 0.5% w/v creatine (freshly prepared in water) and 50µl of 5% w/v α-naphthol (freshly prepared in 2.5M sodium hydroxide) were added and the microplate was shaken before heating at 60°C for a final 15 minutes. Finally, the microplate was shaken for 30 seconds and the absorbance determined at 530nm using a Spectramax plate reader. The mean absorbance reading from the blanks was subtracted from each test well reading.

6.2.3.3 Calibration and assay optimisation

Before the assay was performed with ALS inhibiting herbicides, it was necessary to ensure that several parameters were optimised:

- **Calibration curves** were performed according to the method of Westerfeld (Westerfeld, 1945) but adapted to assay volumes as described in section 6.2.3.2. Calibration was important in order to demonstrate a linear relationship between acetoin concentration and absorbance reading over a range of concentrations. Acetoin standard solutions were prepared in assay buffer (refer section 6.2.3.2 for preparation details) as dilutions from a 1mg ml^{-1} stock; concentrations were 20, 40, 60, 80 and 100µg ml^{-1} acetoin. Acetoin was obtained from Acros Organics (Geel, Belgium). Reactions were carried out in 350µl well microtitre plates with six replicate wells at each concentration and blanks consisting of assay buffer alone. The linear nature of the relationship between enzyme concentration and mean absorbance at 530nm was also checked. Desalted enzyme extract from Roth99 plants was diluted using assay buffer without pyruvate to give a range of different enzyme concentrations in 40µl total volume and then assayed as detailed in section 6.2.3.2. Three replicate wells were prepared at each concentration.

- **Apparent Km** is a measure of enzyme affinity for substrate and is often used in ALS inhibition studies to compare binding affinity in resistant vs susceptible enzyme extracts. Estimates of Km can also be used to give some idea of the binding characteristics of inhibitor and enzyme by comparing response to substrate with and without inhibitor. Finally Km curves can be used to estimate suitable substrate

concentrations for inhibition assays. Apparent Km values are derived from fitting the Michaelis-Menten equation to plots of absorbance against pyruvate concentration. The Michaelis-Menten kinetic model is valid only when the concentration of enzyme is much less than the concentration of substrate (i.e., enzyme concentration is the limiting factor) and is summarised according to the equation $y = ax /(b + x)$, where $a = V_{max}$ (maximum reaction veloicity) and b = apparent Km. Prior to commencing inhibition assays with flupyrsulfuron and sulfometuron, it was necessary to determine a suitable substrate concentration to assay the enzyme. This was achieved by assaying enzyme from each population as detailed below with a range of pyruvate concentrations. Each assay was performed in triplicate with pyruvate concentrations of 0, 20, 40, 60, 80, 100, 150, 200, 250 and 300mM and the Michaelis-Menten equation was fitted to the resulting plots of absorbance against pyruvate concentration using Sigmaplot 8.0. Apparent Km values were determined with and without the addition of sulfometuron as inhibitor.

- **Enzyme concentration** in different fractions from the PD-10 desalting column was checked in order to maximise the concentration of ALS enzyme in the desalted extract. 1ml fractions from the Sephadex PD-10 desalting column were assayed without inhibitor according to the method in section 6.2.3.2 and those with the highest ALS activity selected for use in later assays.

- **Acetone** used to prepare herbicide stock solutions was checked as a solvent blank to ensure that it did not interfere with enzyme activity prior to commencement of inhibition assays. A 20µl ml^{-1} stock solution of acetone in assay buffer was prepared and dilutions were carried out using buffer to give a series of acetone standard solutions. These were added to assay buffer and enzyme in 3 replicate wells of a 350µl well microtitre plate to give acetone concentrations in the plate wells identical to those used in assays with herbicide in acetone, then assayed as described in section 6.2.3.2.

6.2.3.4 Inhibition assays with flupyrsulfuron and sulfometuron

Herbicide solutions were prepared as dilutions from an initial stock made up with technical herbicide of greater than 97% purity (supplied by DuPont, UK), rather than commercial formulation. Herbicide stock solution was prepared in acetone and diluted with assay buffer to the required concentration of herbicide. All herbicide solutions were prepared in advance and stored frozen at -20°C until required. 10µl of assay buffer was added to control and blank wells, and 10µl of herbicide spiked assay buffer to the relevant herbicide wells. 5µl of 3M sulfuric acid was added to the blank wells and the reaction started by the addition of 40µl of desalted enzyme extract to all wells as detailed in section 6.2.3.2. After incubation at 37°C for 30 minutes absorbance at 530nm was determined for all wells using a Spectramax plate reader. The mean absorbance reading from the blanks was subtracted from each herbicide well reading.

6.3 Results

6.3.1 Initial calibration curve with acetoin

Mean absorbance values were plotted against acetoin concentration and linear regression performed using Sigmaplot 8.0. All data points were mean values from six separate plate wells containing the same concentration of acetoin. The calibration procedure was performed twice giving two curves with identical gradients. Calibration curves with acetoin standard solutions demonstrated a linear relationship between acetoin concentration and mean absorbance using a Molecular Devices Spectramax 340 microplate reader at 530nm (Figure 6.1).

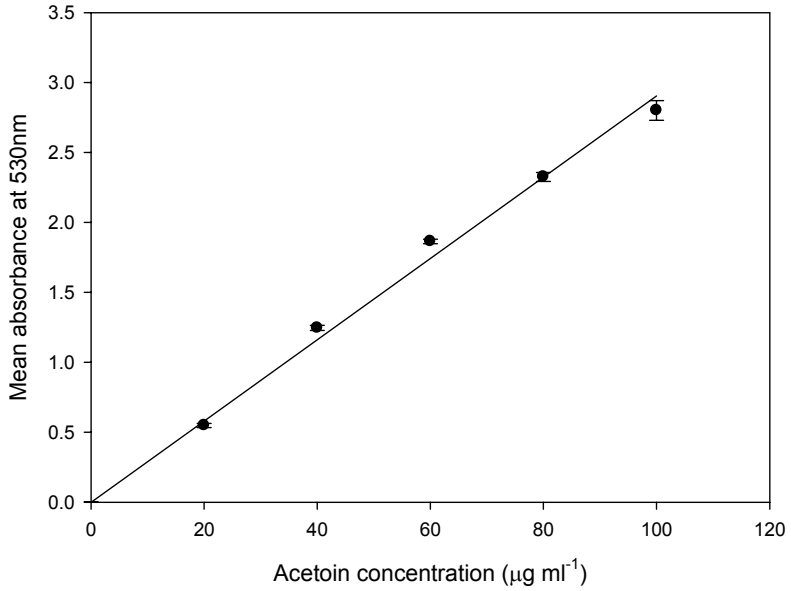

Figure 6.1 A standard curve showing the linear relationship between acetoin concentration and mean absorbance reading from 0 to 100μg ml^{-1} acetoin using a Spectramax 340 microplate reader at 530nm. B = 0.029, Rsqr = 0.989. Error bars are S.E. of the mean.

6.3.2 Determinations of apparent Km

Km (pyruvate) values were determined in standard assay reaction mixtures containing 0 to 300mM pyruvate by fitting the Michaelis-Menten equation using Sigmaplot 8.0. Assays were performed in triplicate and the results treated as replicates at each pyruvate dose. The Km of enzyme extracted from each population (Roth03, Pel96, Pel02 and PelRES03) tended to vary somewhat between populations, with lower Km values in the more resistant populations PelRES03 and Pel02. This is interesting because it suggests that any mutation conferring resistance does not impair pyruvate binding, and may in fact increase enzyme/substrate affinity slightly. It is important to note that this in-vitro observation was not statistically significant, given the error range of these determinations. The results are summarised in table 6.1.

Table 6.1 Apparent Km of ALS extracted from resistant and susceptible populations

Population	Apparent Km pyruvate (mM) ± S.E.
Roth03 (susceptible standard)	43 ± 4
Pel96 (enhanced metabolism standard)	35 ± 4
Pel02 (resistant field population)	32 ± 5
PelRES03 (selected resistant population)	29 ± 6

These results show that crude enzyme extracts from resistant plants do not show impaired pyruvate binding characteristics compared to that from susceptible plants, and so plants expressing resistant forms of the enzyme should not be at a significant disadvantage in terms of this particular measure of enzyme efficiency.

6.3.3 Pyruvate concentration for assay

Km was also determined in assay mixtures containing the inhibitor sulfometuron at a concentration of 0.02μM and enzyme extract from the susceptible standard population. This was done to test sulfometuron binding and so validate that the results were in accordance with what is currently known about the ALS enzyme (McCourt *et al.*, 2006). The results showed a slight, although non-significant, decrease in apparent Km when sulfometuron was added to the assay mixture, indicating that inhibition is not competitive with substrate. Since the errors in the pyruvate saturation graphs were of a similar magnitude to the differences in Km with and without sulfometuron, it was impossible to conclusively demonstrate the type of inhibition. The results obtained with apparent Km values for this experimental system showed that binding was at least not competitive. This information was used to set the pyruvate concentration in the assay to 300mM, ensuring saturation of the ALS enzyme with pyruvate throughout the assay. Refer to Figures 6.2 and 6.3 for pyruvate saturation curves without and with sulfometuron as inhibitor, respectively.

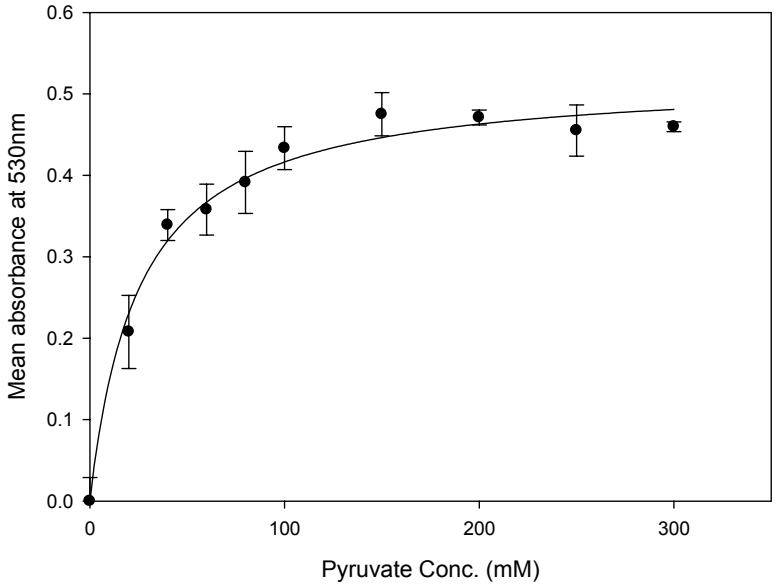

Figure 6.2 Pyruvate saturation curve for Roth99 enzyme extract without inhibitor. Apparent Km pyruvate from fitted Michaelis-Menten curve is 25.2 ± 3.1mM, Rsqr = 0.960. Error bars are S.E. of means.

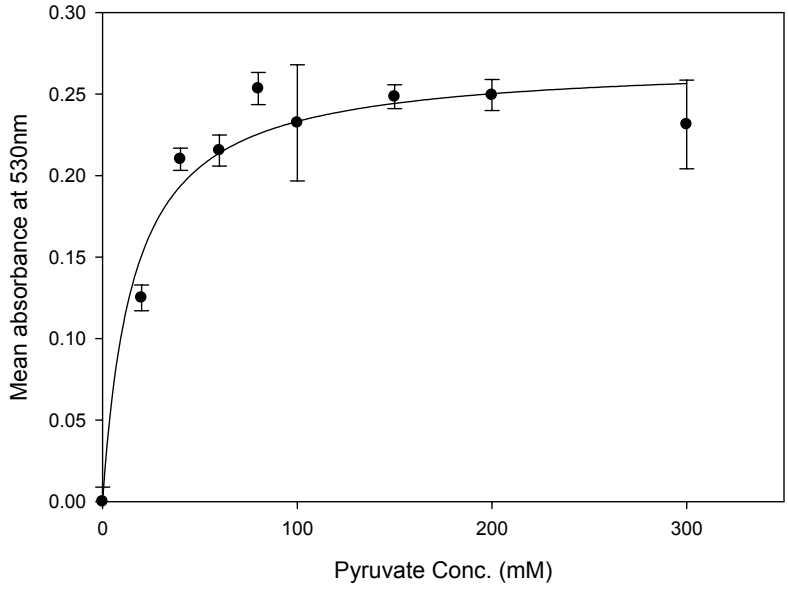

Figure 6.3 Pyruvate saturation curve for Roth99 enzyme extract with 0.02 µM sulfometuron as inhibitor. Apparent Km pyruvate from fitted Michaelis-Menten curve is 20.7 ± 5.8mM, Rsqr = 0.824. Error bars are S.E. of means.

6.3.4 Assay optimisation

Prior to commencement of inhibition assays it was necessary to perform several checks to optimise assay efficiency and ensure that assay results were reliable. These included an analysis of fractions from the PD-10 desalting column to maximise the concentration of ALS enzyme in the desalted extract, a test to ensure that acetone used for herbicide stock solutions did not interfere with enzyme activity, and a check to make sure of a linear relationship between enzyme concentration and mean absorbance at 530nm. The results are presented in Figures 6.4, 6.5 and 6.6, respectively.

- Fractions from the Sephadex PD-10 desalting column were assayed without inhibitor and the results are shown in Figure 6.4. Most of the active ALS enzyme was found to elute from the column in fractions 3 and 4 and so all further assays were conducted using these two fractions combined in order to maximise ALS enzyme concentration in assay mixture.

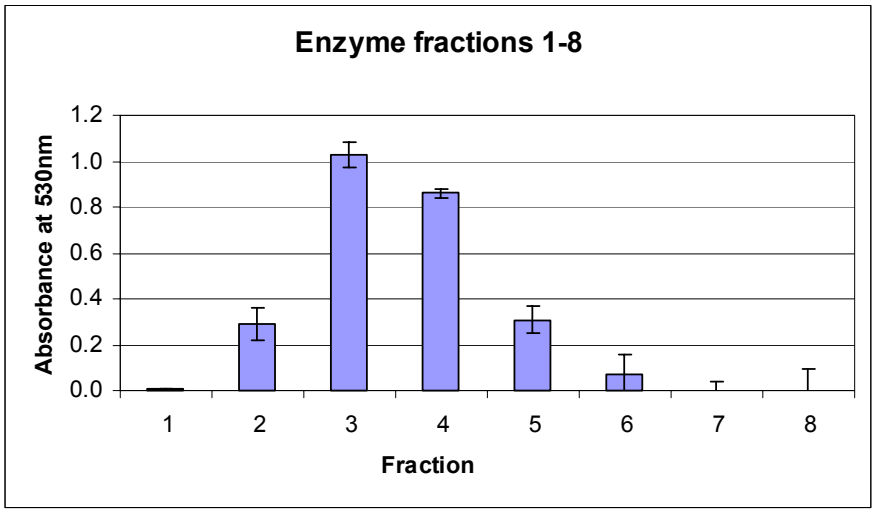

Figure 6.4 ALS assay results for different fractions from Sephadex PD-10 column. Error bars are S.E. of means.

- The possible inhibiting effect of acetone on enzyme conformation was checked and a graph of the results expressed as a percentage of the mean value with no acetone is displayed in Figure 6.5. This demonstrates no inhibition of enzyme due to the presence of acetone at the concentrations required to dissolve herbicide for the assay.

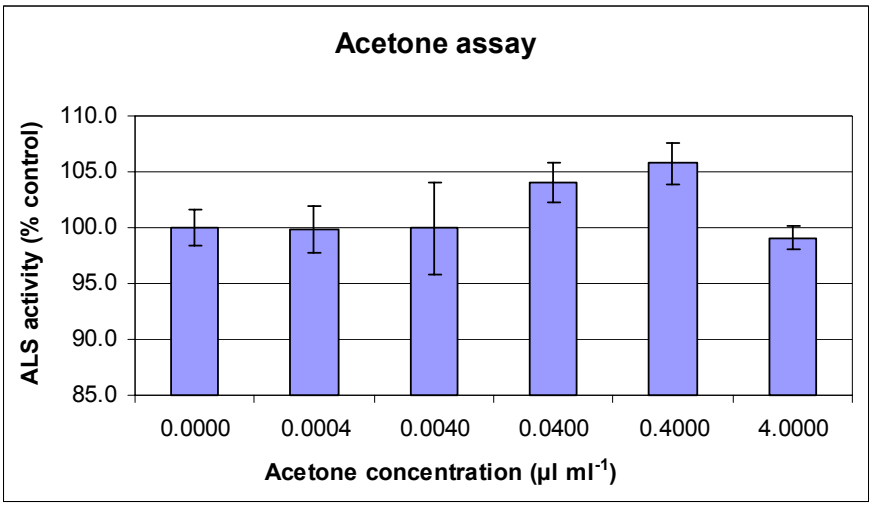

Figure 6.5 ALS activities as percentage of mean control value with various acetone concentrations. Error bars are S.E. of means.

- An assay of enzyme extract from Roth99 plants over a range of different enzyme concentrations was performed as a final check and the results are presented in Figure 6.6. A linear relationship between ALS activity (acetoin production) and enzyme concentration was demonstrated up to the enzyme concentrations used in the assay.

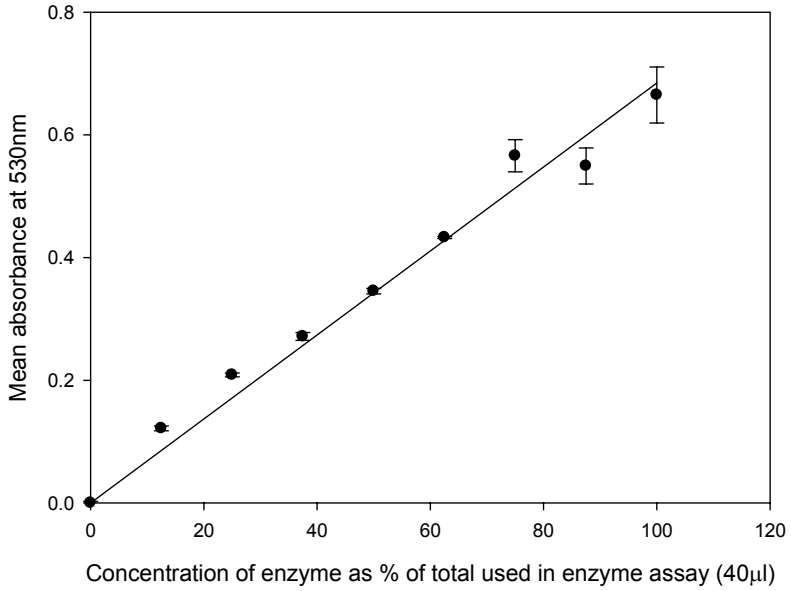

Figure 6.6 Assay with changing enzyme concentration. Error bars are S.E. of means.

6.3.5 Inhibition assay with sulfometuron

All assays were performed with five well replicates according to the method outlined in section 6.2.3.4, and were repeated three times for each population. Analysis was conducted by fitting a four parameter logistic curve to the raw absorbance data expressed as percentage reduction relative to control wells for each plate using MLP v3.09. I_{50} and I_{80} values were determined where appropriate. All curves were fitted with minimum y=0 to allow comparison between populations and R/S ratios were calculated where possible.

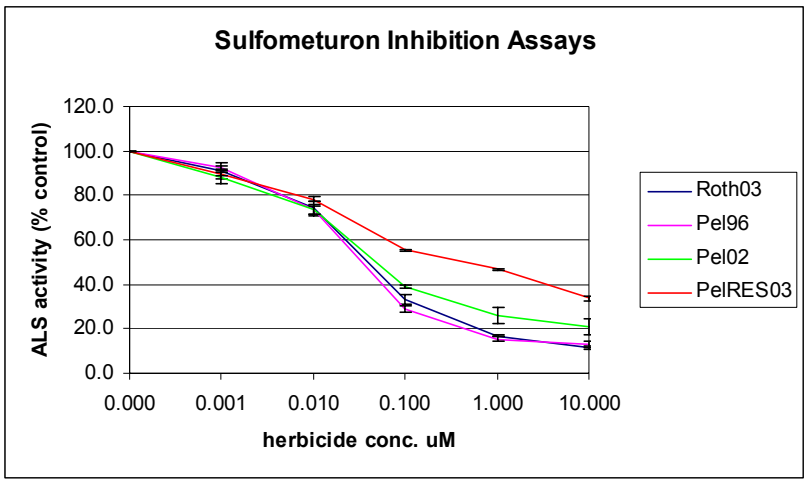

Figure 6.7 Mean ALS activity as percentage control plotted against sulfometuron dose for all populations. Error bars are S.E of means

Enzyme assays confirmed that a form of resistant enzyme is present in the Peldon population. Figure 6.7 clearly shows reduced inhibition by sulfometuron of enzyme extracted from PelRES03 leaf material when compared to that from the susceptible Roth03 plants, notably at higher [herbicide]. ALS enzyme activity in the PelRES03 extract is also less susceptible to inhibition when compared to the Pel96 metabolism standard showing that the additional high level resistance mechanism demonstrated in whole plant experiments using the PelRES03 population may be the result of enzyme insensitivity.

Table 6.2 Summary of sulfometuron I_{50} and I_{80} values calculated using MLP

Inhibitor	Population	$log_{10}I_{50}$ (S.E.)	I_{50} µM	R/S ratio	$log_{10}I_{80}$ (S.E.)	I_{80} µM	R/S ratio
sulfometuron	Roth03	-1.5242 (0.0371)	0.0299	1	-0.1578 (0.0250)	0.6953	1
	Pel96	-1.6419 (0.0359)	0.0228	0.8	-0.2556 (0.0204)	0.5551	0.8
	Pel02	-1.3032 (0.0894)	0.0498	1.7	0.5603 (0.0627)	3.6333	5.2
	PelRES03	-0.3121 (0.0384)	0.4874	16.3	2.0401 (0.0445)	109.6731	157.7

Curve fitting allowed calculation of R/S ratios for the various resistant populations compared to the susceptible standard, Roth03. For I_{50} the ratios were Pel96 (0.8), Pel02 (1.7) and PelRES03 (16.3). For I_{80} the ratios were Pel96 (0.8), Pel02 (5.2) and PelRES03 (157.7). The PelRES03 population was the most resistant to sulfometuron of the three populations tested.

6.3.6 Inhibition assay with flupyrsulfuron

Exactly the same procedure was followed as described in section 6.3.5. All curves were fitted with minimum y=0 and R/S ratios were calculated where possible.

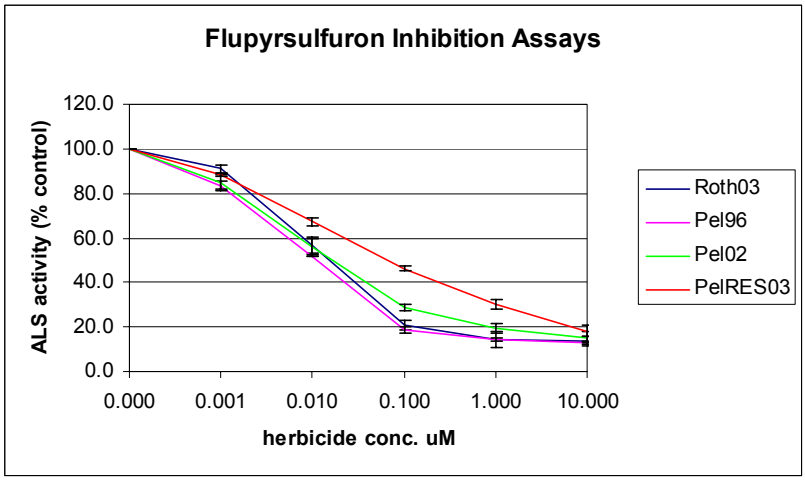

Figure 6.8 Mean ALS activity as percentage control plotted against flupyrsulfuron dose for all populations. Error bars are S.E of means

Table 6.3 Summary of flupyrsulfuron I_{50} and I_{80} values calculated using MLP

Inhibitor	Population	$logI_{50}$ (S.E.)	I_{50} µM	R/S ratio	$logI_{80}$ (S.E.)	I_{80} µM	R/S ratio
flupyrsulfuron	Roth03	-2.0482 (0.0736)	0.0090	1	-0.5209 (0.0504)	0.2569	1
	Pel96	-2.1937 (0.0559)	0.0064	0.7	-0.5800 (0.0356)	0.2630	1
	Pel02	-1.8935 (0.0741)	0.0128	1.5	-0.0475 (0.0435)	0.8964	3.5
	PelRES03	-1.1372 (0.0553)	0.07291	8.3	0.7009 (0.0366)	5.0223	19.6

I_{50} and I_{80} determinations from MLP fitted curves were used to calculate R/S ratios for the various resistant populations compared to the susceptible standard Roth03. For I_{50} the ratios were Pel96 (0.7), Pel02 (1.5) and PelRES03 (8.3). For I_{80} the ratios were Pel96 (1.0), Pel02 (3.5) and PelRES03 (19.6). Again the PelRES03 population was the most resistant to flupyrsulfuron-methyl of the three populations tested.

6.4 Discussion

This section contains a summary of conclusions drawn from the enzyme work carried out using the four standard *A. myosuroides* populations and considers the implications of the results as they relate to previous whole plant work with the same populations. The scope for later work at the molecular level is also discussed in the light of the enzyme assay results.

Initial tests varying the pyruvate concentration showed that the saturation curve for pyruvate is hyperbolic with an apparent Km (pyruvate) of between 29 and 43mM. Enzyme was shown to be approaching saturation above about 200mM pyruvate and this result, along with the demonstration that inhibition is not competitive with substrate, was used to set pyruvate to a concentration of 300mM and ensure that changes in pyruvate concentration did not affect reaction rates during inhibition assays. Since these results were obtained using crude enzyme extract and an end point assay however, when it is known that herbicide inhibition and cofactor activation are time-dependent processes (Duggleby & Pang, 1999; Pang *et al.*, 2002), apparent Km values in particular were

treated with caution. The end point assay has an advantage in sensitivity when compared to the alternative continuous assay (Schloss *et al.*, 1985) and so was used here due to the difficulties encountered in extracting sufficient crude ALS from *A. myosuroides*. ALS is a fairly labile enzyme and occurs at low levels in plant material. A total of 40 plants were required for extraction of enzyme sufficient to perform a single end point assay and it was felt that purification of the quantity required for the continuous assay would have presented too many problems.

Enzyme assays with flupyrsulfuron and sulfometuron demonstrated that resistance to sulfonylurea herbicides occurs at the enzyme level in the laboratory selected PelRES03 *A. myosuroides* population, although without enzyme specific activity data it was not possible to attribute the difference to either enzyme overexpression or altered target site. The PelRES03 line was derived from an original Pel96 plant which showed high level resistance to the non-selective sulfonylurea herbicide sulfometuron; crossing experiments leading to the establishment of this line are discussed in Chapter 4. An R/S ratio of 8.3 was observed between flupyrsulfuron I_{50} values from resistant PelRES03 and susceptible Roth03, with a ratio of 16.3 when PelRES03 was compared to Roth03 I_{50} values in the sulfometuron assay. It is particularly notable that Roth03 and Pel96 show very similar I_{50} values because Pel96 has demonstrated enhanced metabolism of flupyrsulfuron at the whole plant level. This means that any difference between Pel96 and PelRES03 at the enzyme level is most likely due to insensitive ALS and in this respect Pel96 is a better susceptible standard than the unrelated Roth03 population. In addition to this, the PelRES03 line originated from an original selfed Pel96 plant, minimising the potential for genetic variation and strengthening the case for an insensitive ALS enzyme.

R/S ratios for the Pel02 population were smaller with a 1.7 fold increase in I_{50} using sulfometuron and a 1.5 fold increase with flupyrsulfuron compared to the susceptible standard. One possible explanation is that the Pel02 seed set contains a smaller proportion of plants showing the high level resistance mechanism picked up in whole plant screening experiments with sulfometuron at 100g a.i. ha^{-1} than the PelRES03 line (38% of PelRES03 plants show high level resistance to sulfometuron at 100g $a.i.ha^{-1}$ compared to only 13% of Pel02 in whole plant dose-response tests, refer Chapter 3). Thus there is likely to be a substantial dilution effect in Pel02. Dilution with enzyme from susceptible or partially resistant plants could account for the low R/S ratios in Pel02 assays and could

also explain why enzyme from the highly resistant PelRES03 plants showed immediate inhibition by both sulfometuron and flupyrsulfuron. This effect would not be expected if all plants used in the assay were fully resistant. The use of selective germination medium in order to enrich the proportion of resistant plants in the PelRES03 and Pel02 populations prior to extraction for enzyme assay was considered as demonstrated in a previous study (Burnet *et al.*, 1994). However it was decided that the determination of resistant enzyme in lab-selected and natural mixed populations was sufficient, especially when compared to the metabolism standard Pel96 population from the same field at Peldon. When comparison was based on I_{80} values the differences between resistant and susceptible seed sets were more pronounced and I_{80} resistance ratios are perhaps a better method for picking up low levels of resistance in mixed populations.

Differences were observed in R/S ratios for resistant populations depending on whether sulfometuron or flupyrsulfuron was used as the inhibitor. R/S ratios were lower using flupyrsulfuron for all populations tested which is exactly the opposite effect to that observed in experiments with whole plants. This difference between flupyrsulfuron at the enzyme and whole plant levels emphasises the importance of enhanced metabolism effects relating to this particular herbicide and reinforces the decision to use the non-selective sulfometuron, with its ability to overcome most enhanced metabolism resistance, as an indicator of high level resistance due to an altered ALS enzyme target site. Specific activity of enzyme extracts was not determined during the study and this was a weakness when comparing I_{50} values from different populations. In future, enzyme assays determination of specific activity would be a priority in order to rule out differences due to enzyme over expression in particular.

Results from this chapter indicate that resistance in the PelRES03, and to a lesser extent the Pel02 population, is most likely based on an altered enzyme target site; although an alternative possibility is enzyme over expression. The need to use large quantities of leaf material for enzyme assays limited the scope for using better characterised (i.e. confirmed resistant) material. This constraint was a real issue and a major factor in the decision to move forward with molecular techniques requiring much less material. The basis of resistance was investigated in more depth by sequencing regions of the ALS gene from resistant and susceptible individuals and this is described in Chapter 7. The advantage of gene sequencing over ALS enzyme assays was the ability to look at individuals and also

the additional information available in terms of the exact mutation present in resistant individuals making it a more powerful diagnostic tool than enzyme assays. Enzyme assays were however a necessary step and demonstrated the connection between resistance to sulfometuron at the whole plant level and an enzyme less sensitive to inhibition.

6.5 Chapter summary

- Reduced enzyme sensitivity to inhibition by the sulfonylurea herbicides sulfometuron and flupyrsulfuron was demonstrated in the PelRES03 seed set when compared to the susceptible standard population Roth03 and the metabolism standard parent population Pel96 using a crude enzyme extract.
- R/S ratios based on I_{50} comparison of the PelRES03 line and the susceptible standard demonstrated a 16.3 fold decrease in enzyme sensitivity to sulfometuron and an 8.3 fold decrease to flupyrsulfuron.
- R/S ratios for the Pel02 field population (containing around 13% highly resistant plants in whole plant experiments) were 1.7 and 1.5 for sulfometuron and flupyrsulfuron respectively.
- Enzyme assay results confirmed the choice of sulfometuron as an indicator of ALS target site resistance in *A. myosuroides* for whole plant work. Populations showing resistance to sulfometuron in whole plant tests also showed resistance at the enzyme level.
- A lack of specific enzyme activity data made it impossible to rule out enzyme overexpression as a mechanism of resistance in the PelRES03 seed set on enzyme data alone.
- The use of mixed populations as opposed to pre-germinating on a selective medium containing sulfometuron meant that resistant enzyme was diluted in the case of the two resistant populations tested. Due to time constraints it was decided that moving on to molecular work was more important than redesign of the assay protocol.

7. Molecular Studies

7.1 Introduction

This chapter details work done on the molecular basis of resistance to sulfonylurea herbicides in *A. myosuroides* and comprises data from several different experiments. The main goal of the molecular work was to further investigate the differences in enzyme sensitivity previously detected (see Chapter 6) and determine whether a point mutation conferring an enzyme target site change was responsible for the observed differences. Acetolactate synthase (ALS) enzyme target site changes identified to date are restricted to five different point mutations of the ALS gene in the resistant weed species investigated so far (Heap, 2006), and this information provided an obvious starting point for investigation. A secondary goal of this work was to link the use of sulfometuron at 100g a.i. ha^{-1} as an indicator of high level resistance at the whole plant level to mutations conferring enzyme target site changes. Sulfometuron was used extensively in this role because of its supposed lack of vulnerability to enhanced metabolism resistance to ALS inhibitors in grass weeds (Burnet *et al.*, 1994; Christopher *et al.*, 1992; Preston & Powles, 2002). Enzyme work provided support for this idea through the reduced enzyme sensitivity to inhibition observed in the resistant PelRES seed set, but it was felt that confirmation at the molecular level would be more conclusive. Identification of the precise mutation(s) involved in resistance of *A. myosuroides* to sulfonylureas would also be of value in helping predict cross resistance to other ALS inhibitors since different mutations are associated with different cross-resistance profiles (Heap, 2006; Tranel & Wright, 2002).

Populations initially selected for investigation in this section included the PelRES resistant seed set and the Pel02 field population. These populations showed the highest proportion of undamaged plants after treatment with sulfometuron in whole plant experiments (38% and 13%, respectively, see Chapter 3) and also showed resistance in terms of enzyme inhibition with flupyrsulfuron and sulfometuron (see Chapter 6). Primers were developed and used to sequence regions from each of the five conserved domains of

an *A. myosuroides* ALS gene which were then compared using the programme Vector NTI Advance 10 Contig Express (Invitrogen) in order to detect any differences between the sequences of susceptible and resistant individuals, as determined by phenotypic response to sulfometuron at 100g a.i. ha^{-1}.

7.1.1 Acetolactate synthase genes in plants

Acetolactate synthase (ALS) is an enzyme involved in the synthesis of branched chain amino acids in plants, yeast and bacteria. ALS allows the synthesis of acetolactate from two pyruvate molecules which is the first common step in the synthesis of the three essential amino acids valine (Val), leucine (Leu) and isoleucine (Ile). Acetolactate synthase is encoded in the nuclear genome of most plant species although the mature protein is localised in the chloroplasts (Chipman *et al.*, 1998). ALS genes are highly conserved across different plant species and almost all gene sequences from higher plants lack introns, although some differ in length due to non-conserved additions and deletions. The available sequences of ALS genes from monocotyledons range from 1995 bp in *Lolium multiflorum* to 2544bp in *Zea mays* (NCBI, 2006). Only one case of an ALS gene with introns is known to exist at the time of writing, in weeds of the Scrophulariaceae family, genus *Lindernia* ssp. (Uchino & Watanabe, 2002). This highly conserved nature, coupled with the lack of introns even in non-conserved regions of the gene, makes the task of designing primers and sequencing conserved regions easier.

Single copies of plant ALS genes are found in *Arabidopsis thaliana* (L.) Heynh. (Dumas *et al.*, 1993; Mazur *et al.*, 1987), *Beta vulgaris* L. (Wright & Penner, 1998), and *Papaver rhoeas* L. (Scarabel *et al.*, 2004), while multiple copies are found in *Nicotiana tabacum* L. (Keeler *et al.*, 1993), *Brassica napus* L. (Ouellet *et al.*, 1992), *Gossypium hirsutum* L. (Grula *et al.*, 1995), *Helianthus annuus* L. (White *et al.*, 2003) and *Zea mays* L. (Fang *et al.*, 1992). This variation indicates that multiple ALS enzyme forms are not required for general plant growth and development. Possible reasons for multiple ALS copies include the need for variable expression in different plant organs and at different developmental stages, or as a reflection of the evolutionary origins of different plant species (Keeler *et al.*, 1993). Studies with tobacco, an allotetraploid species, showed that two ALS genes were present and both were functionally expressed. Mutations in the conserved domains of both genes were able to confer resistance to ALS inhibitors in mature plants and transformation of sensitive tobacco lines was achievable using cloned resistant gene

segments from either gene (Chaleff & Bascomb, 1987; Lee *et al.*, 1988). *N. tabacum* is thought to have arisen from the hybridisation of two diploid species and DNA blot hybridisation analysis showed that each separate diploid ancestor contributed a single ALS gene to the *N. tabacum* genome (Lee *et al.*, 1988). The pattern of multiple ALS gene copies in allopolyploid species is repeated in *Brassica napus* (Wiersma *et al.*, 1989) and *Zea mays* (Fang *et al.*, 1992), whereas the diploid *Arabidopsis thaliana* and *Papaver rhoeas* contain single copies of the gene (Mazur *et al.*, 1987; Scarabel *et al.*, 2004) suggesting that ALS copy number may largely depend on the level of ploidy.

7.1.2 ALS conserved domains and target site mutation

Acetolactate synthase enzyme has five highly conserved domains (Doms A-E) in higher plants (Boutsalis *et al.*, 1999). The conserved domains according to Boutsalis are as follows: Domain A (AITGQVPRRMIGT), Domain B (QWED), Domain C (VFAYPGGASMEIHQALTRS), Domain D (AFQETP) and Domain E (IPSGG). In each domain substitution at a single amino acid residue (underlined) confers ALS target site resistance. Amino acid changes have been observed at the variable residue in each domain conferring ALS resistance in weed species (Heap, 2006). By far the most common site for amino acid substitution in resistant weed populations is the Proline 197 (Pro_{197}) position in Domain A where every possible amino acid substitution resulting from single nucleotide change has been observed (amino acid numbering based on the *A. thaliana* complete ALS sequence from Genbank).

Cross resistance characteristics of weed species displaying a Pro_{197} amino acid substitution are diverse and predicting patterns of cross resistance based on particular target site changes is difficult. All of those tested displayed resistance to both sulfonylurea and triazolopyrimidine ALS inhibitors, but cross-resistance to the other ALS inhibitor groups (imidazolinones, pyrimidinylthiobenzoates and sulfonylaminocarbonyl-triazolinones) is less easy to predict (Heap, 2006). On the whole, patterns of cross resistance are based more on the particular amino acid substitution at each site rather than the site alone; however there is no single rule which predicts the particular pattern of cross resistance across different species. For example, substitution of a threonine residue at position 197 in *Chrysanthemum coronarium* confers resistance to all classes of ALS inhibitors (Tal & Rubin, 2004) whereas in *Papaver rhoeas* the same target site change results in a plant susceptible to imadizolinone herbicides (Scarabel *et al.*, 2004).

7.1.3 Herbicide binding and resistance

Recent work has provided new information on the structure of *Arabidopsis thaliana* acetolactate synthase bound to the ALS inhibiting herbicides sulfometuron-methyl, metsulfuron-methyl, chlorsulfuron, tribenuron-methyl, chlorimuron-ethyl and imazaquin (McCourt *et al.*, 2006) by x-ray crystallography. Traditionally, most ALS enzyme studies have been conducted using bacterial or yeast ALS due to the relatively low abundance of the enzyme in higher plants. For studies related to resistance and the rational design of herbicides however, plant enzyme is much more relevant. The results from detailed structural work at the enzyme level have backed up many conclusions that were drawn from kinetic studies with yeast enzymes. It is now known that sulfonylurea and imadazolinone herbicides do not function by mimicking the enzyme substrate and so are not competitive inhibitors. Rather they work by blocking access to the enzyme active site and in this way prevent substrate binding.

From a herbicide resistance standpoint, the crystal structure of ALS in complex with its inhibitors allows a greater understanding of how mutations resulting in particular amino acid substitutions can confer resistance to sulfonylureas through particular alterations in enzyme structure. This information can also help to explain why cross resistance to imidazolinones might be more likely with particular substitutions at particular positions based on the structural relationships of amino acid residues and herbicide molecule. At the present time there are only five positions on the ALS gene where single nucleotide substitutions can lead to target site changes where amino acid substitutions have been observed to confer target site resistance in weeds. These are in conserved Domains A (Pro_{197}), B (Trp_{574}), C (Ala_{122}), D (Ala_{205}) and E (Ser_{653}) of the ALS gene as previously discussed (Boutsalis *et al.*, 1999; Heap, 2006). Crystallisation and visualisation of enzyme has shown that ALS is a tetramer made up of four identical subunits and that each subunit is composed of three domains and a C-terminal tail which comes close to the active site. These domains in *A. thaliana* are designated α, β and γ and are composed of amino acid residues 86-280, 281-451 and 463-639, respectively, along with the C-terminal tail consisting of residues 646-668 (see Figure 7.1).

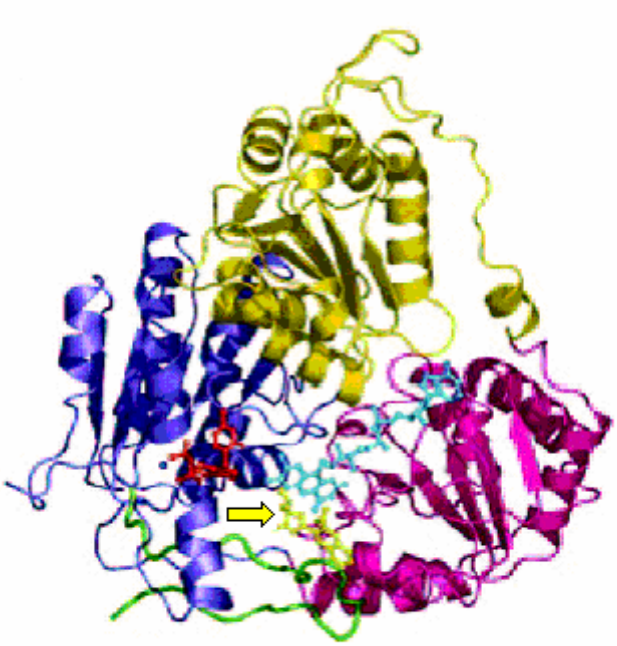

Figure 7.1 A single subunit of *A. thaliana* acetolactate synthase in complex with cofactors TPP (coloured red), FAD (light blue) and the imidazolinone herbicide imazaquin (yellow, arrowed). The individual domains α (residues 86-280), β (residues 281-451) and γ (residues 463-639) are coloured yellow, pink and blue respectively while the C-terminal tail is green. Image taken from "Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase" (McCourt *et al.*, 2006).

Mutations in the five domains of the ALS gene listed above which are associated with herbicide insensitive enzyme translate to amino acid substitutions across all three domains of the ALS protein. Studies in yeast have shown that the sulfonylurea binding site in the substrate access channel is bounded by residues Ala_{122}, Pro_{192} and Ala_{205} from the protein α domain of one subunit along with Trp_{574} from the γ domain of another (Pang *et al.*, 2003). The additional herbicide contact of Ser_{653} from the C-terminal tail is observed in *A. thaliana* when compared to ALS from yeast. Various other herbicide contacts are made but these are not associated with resistance in weed species.

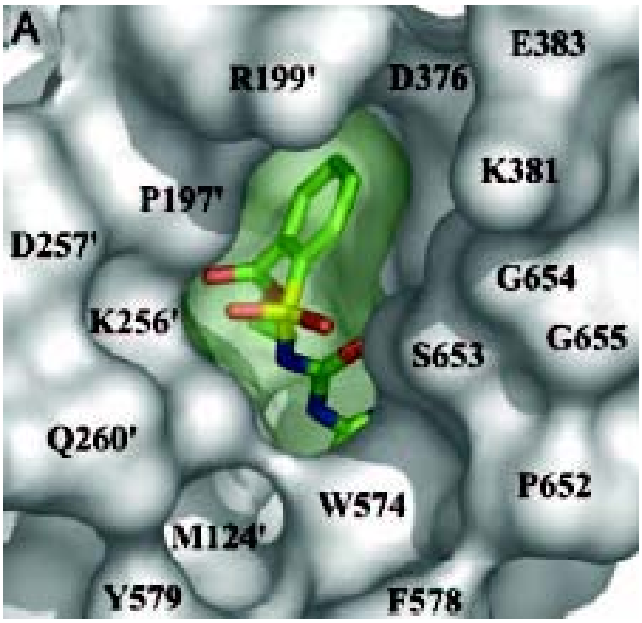

Figure 7.2 Connolly surface with herbicide blocking of the active site channel of *A. thaliana* acetolactate synthase with chlorimuron-ethyl. Image taken from "Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase" (McCourt *et al.*, 2006).

Amino acid substitutions at Pro_{197} are the most common of those associated with resistance due to decreased sensitivity to inhibition. Figure 7.2 shows that this residue is situated at the entrance to the active site channel and that it interacts readily with the aromatic ring of sulfonylurea herbicides. This provides some explanation of the cross resistance patterns seen in Pro_{197} substitutions and shows why almost any substitution at this residue will prevent sulfonylurea access to the channel. Importantly the residue at position 197 only interacts to block imidazolinone molecules when the substituted amino acid is particularly bulky. Another well characterised amino acid substitution takes place at position 574 where tryptophan is substituted for leucine. Tryptophan is a bulky aromatic amino acid and substitution for the smaller non-aromatic leucine results in the loss of several interactions which serve to bind the herbicide in the active channel. New information about structural relationships between herbicide and enzyme is helping to explain why certain amino acid substitutions at the herbicide binding site near the active site of plant ALS confer particular patterns of cross resistance and may in future allow rational design of new inhibitor molecules less prone to the development of resistance.

7.1.4 Scope of work at the molecular level

Primers were designed using alignments of available cereal and grass weed acetolactate synthase gene sequences from Genbank. These were targeted to cover the five conserved domains of the ALS gene where mutations have been shown to confer, in other species, single amino acid substitutions resulting in enzyme less sensitive to inhibition by ALS inhibitors. Direct sequencing was carried out using PCR amplified regions covering conserved Domains A-E. Before sequencing, PCR products were separated on agarose gel and single bands corresponding to the expected size of the amplified DNA fragment were excised and purified. Sequenced fragments were assembled as contigs aligned with the complete ALS gene sequence from *Bromus tectorum* for reference. The populations chosen for molecular work included the PelRES sulfometuron selected line which was later used in crossing experiments, the field population Pel02 which showed 13% highly resistant plants in screening experiments using the non-selective sulfonylurea herbicide sulfometuron methyl at 100g a.i. ha^{-1}, and three field populations in which resistance to the sulfonylurea mixture mesosulfuron+iodosulfuron was identified in screening tests. These last three populations were collected in 2004/2005 near Maidenhead in Berkshire (Maiden05), Little Milton near Thame in Oxfordshire (Thame05), and from a confidential source near the Wiltshire/Berkshire border (Wilts04). All samples came from fields where problems in control using mesosulfuron+iodosulfuron were reported and this was confirmed in glasshouse experiments which are described briefly here. Work on the Pel02 population was the most involved and aimed to link data from whole plant experiments to particular mutations conferring target site resistance in surviving plants. Cloning was carried out using DNA from two highly resistant Pel02 individuals in order to demonstrate the robustness of the procedure and to give further insights as to the nature of ALS in *A. myosuroides*. A 204bp fragment was amplified by a pair of primers covering Domains A and D and then cloned and sequenced. Attempts were also made during the lab based phase of the project to gain additional information by performing a Southern Blot in order to confirm the gene copy number in *A. myosuroides* but unfortunately these were not completed due to time constraints.

7.2 Materials and methods

7.2.1 Plant screening for resistance

7.2.1.1 Pel02

Around 700 Pel02 plants were grown to the 2-3 tiller stage in pots of Kettering loam before being harvested by cutting at the soil surface and flash freezing in liquid nitrogen. Plants were allowed to re-grow until they were about 6cm tall (1.5 weeks) and then sprayed with sulfometuron at 100g a.i. ha^{-1}, and subsequently rated for damage 4 weeks later as described in Chapter 2. The proportion of highly resistant plants rated 1 or 2 on the injury scale (30.9%) was somewhat higher than the usual proportion found in this Peldon population in whole plant experiments (around 13%). This may have been a consequence of the later growth stage and more developed root system in the re-grown plants and was taken into account when interpreting the results. Harvested plants were stored frozen at –80°C in individual plastic bags until required for DNA extraction. Initially the intention was to perform an enzyme assay with leaf material from plants with a target site mutation and compare to an assay of leaf material from those individuals without such a mutation. Unfortunately molecular work took longer than expected and this comparison was not possible. DNA was extracted from selected resistant and susceptible plants as required to allow comparison of ALS gene sequence data.

7.2.1.2 Wilts04

Resistance in the Wilts04 seed set was characterised in routine glasshouse screening experiments. Seeds were pre-germinated and planted into Kettering loam. 70 healthy plants were grown to the 2-3 leaf stage in 5 x 5cm square pots and divided into two groups of 35 plants. A susceptible standard group of Roth03 plants was also included. Wilts04 plants were sprayed with sulfometuron at 100g a.i. ha^{-1} and mesosulfuron+iodosulfuron at 12 + 2.4g a.i. ha^{-1} and assessed for damage using the 1-5 scale described in Chapter 2 after four weeks. The Wilts04 seed set showed a large proportion of plants with high level resistance to mesosulfuron+iodosulfuron (70%) and sulfometuron (14%) in the initial small screening test and a larger test was set up to give material for molecular work. This time 202 plants were grown to the 2-3 tiller stage in Kettering loam then harvested and flash frozen before storage at -80°C. Plants were re-

grown to around 6 cm and sprayed with sulfometuron at 100g a.i. ha^{-1} as described above for the Pel02 population then characterised according to injury score after four weeks. Frozen leaf samples were taken for extraction of DNA from five highly resistant and fully susceptible plants and these were extracted and sequenced according to the methods outlined in sections 7.2.2 to 7.2.8.

7.2.1.3 Maiden05 and Thame05

Pot screening experiments with 25 different populations where resistance to mesosulfuron+iodosulfuron was reported in the field led to the identification of the Maiden05 and Thame05 ALS-resistant populations. These samples were obtained near the end of the project and seed supplies were limited. These constraints led to a simple screening approach for these samples rather than more extensive whole plant work. 150 plants from each population were grown from pre-germinated seed in 9cm diameter pots of Kettering loam with 6 plants per pot. Plants were sprayed at the 2-3 leaf stage with mesosulfuron+iodosulfuron at 6 + 1.2, 12 + 2.4 and 24 + 4.8g a.i. ha^{-1} (0.5 x, 1 x and 2 x field rate), and sulfometuron at 50g a.i. ha^{-1}. There were five replicates of each treatment (plus untreated control) in a randomised block design. Plants were assessed after four weeks as either highly resistant (injury categories 1 and 2) or affected (injury categories 3-5). The Maiden05 and Thame05 populations showed high levels of resistance to all treatments. At 1 x field rate mesosulfuron+iodosulfuron, both had 27 highly resistant survivors out of 30 treated plants across five reps. The proportion of resistance was also high in both Maiden05 and Thame05 populations at 2 x field rate mesosulfuron+iodosulfuron (21/30 and 19/30 resistant survivors respectively), and at 50g a.i. ha^{-1} sulfometuron (27/30 and 29/30 respectively). Fresh leaf samples were taken from five highly resistant plants of each population and these were extracted and sequenced according to the methods outlined in sections 7.2.2 to 7.2.8.

7.2.2 Extraction of total plant genomic DNA for sequence analysis

DNA extractions were carried out using frozen leaf material in most cases although fresh material was used from Thame05 and Maiden05 populations. All extractions from resistant and susceptible plants were performed using the DNEasy plant mini-kit (Qiagen). 100mg of leaf material was ground under liquid nitrogen in a 1.5ml plastic Eppendorf tube and extracted according to the manufacturer's instructions. Extracts were stored at -20°C until required. Extractions were as follows: 14 resistant (rated 1 on the 1-

5 injury scale), 5 intermediate (rated 3) and 14 susceptible (rated 5) Pel02 plants, 5 resistant and 5 susceptible Wilts04 plants rated in category 1 and 5 respectively, 5 resistant Thame05 plants, and 5 resistant Maiden05 plants. In addition to this, 8 leaf samples from the original resistant PelRES plants used in the F_1 cross to susceptibles (see Chapter 4, section 4.2.2) were extracted along with leaf samples from the progeny of the PelRES plant 37 cross (see Chapter 4, section 4.3.5) which showed unusual distribution of phenotypes on treatment with sulfometuron. Extractions were also performed with eight Roth99 susceptible standards as a reference. Following DNA extraction, steps 7.2.3 to 7.2.8 were followed in order with the exception of the cloning step which was only performed using DNA from two highly resistant Pel02 plants.

7.2.3 Primer design and testing

Primers were designed using an alignment of full or partial ALS coding sequences from *H. vulgare* (EMBL accession number AF059600), *L. multiflorum* (EMBL accession number AF310684), ALS-inhibitor resistant and susceptible *O. sativa* (EMBL accession numbers AB049823 and AB049822 respectively) and ALS-inhibitor resistant and susceptible *B. tectorum* (EMBL accession numbers AF487459 and AF488771 respectively). Full ALS genomic sequence from *Z. mays* was also included (EMBL accession number ZMAHAS109) which aligned to show a lack of introns. Forward and reverse primers were designed to span the five conserved domains of the ALS gene in two parts; Domains C, A and D near the 5' end and Domains B and E towards the 3' end. The web based program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to select primers from the consensus sequence with primer length $\geq$ 17 bp, T_m = 59 - 62°C and GC% = 40 – 65%. Suitable primer sequences were subjected to BLAST searching to identify any similar sequences in the Genbank Entrez database and rule out primers not specific to ALS. Finally primers were examined for self complimentarity and the most suitable were ordered from Sigma-Genosys for testing. See table 7.1 for a summary of primers subjected to testing.

Table 7.1 Details of forward and reverse primer sequences derived from an alignment of ALS sequences from various Graminae species

Priming direction	Primer code	Sequence
Forward 5'-3'	F1	CATCACCAACCACCTCTTCC
	F2	TCATCACCAACCACCTCTTC
	F3	TGGTAGCTTCCTCATGAACATT
	F7	TTGGGCAGCACCAGATGT
	F8	CGC(G/C)GACATCCTCGTC
	F9	CAAGGGCGC(G/C)GACATC
	F10	AAGGGCGC(G/C)GACATCCT
Reverse 3'-5'	R1	ATCTGCTG(C/T)TGGATGTCCTT
	R5	CTTCACTC(T/C)TCTTTGTCACACG
	R7	TCCTGCCATCACC(A/T)TCCA
	R8	CTGCCATCACC(T/A)TCCA(T/G)
	R9	TGCCATCACCTTCCATGAT
	R10	(A/G)TCCTGCCATCACC(T/A)TCCA

Testing was carried out using the following primer combinations: F8R1, F9R1 and F10R1 to cover the 5' terminal end of the ALS gene and conserved Domains C, A and D in that order and F7R7, F7R8, F7R9, F7R10, F3R7, F3R8, F3R9 and F3R10 to cover the 3' terminal end of the gene and Domains B and E. Primers were tested by running PCR reactions with different primer combinations and DNA extracts from two Pel02 plants according to the protocol outlined below. PCR products were run on a gel and those producing a single readily identifiable band of the correct size with ethidium bromide staining were cut from the gel and cleaned up (see section 7.2.6). Direct sequencing was performed and those primer combinations producing the cleanest sequences were retained. Very little temperature optimisation was required and the primers selected gave clearly defined bands with little or no evidence of secondary products in the resulting sequences. Primer design and optimisation steps were based on the work of Prado *et al* (Prado *et al.*, 2004). Final primer mixes selected were the F10R1 combination for PCR amplification of Domains C, A and D and the F3R10 primer mix for Domains B and E.

7.2.4 PCR

PCR reactions were carried out using 2x PCR Master Mix (Promega) containing *Taq* DNA polymerase, dNTPs and $MgCl_2$ at optimal concentrations for efficient amplification of DNA templates. All reaction mixtures also contained 4ng μl^{-1} of each primer and ~50 ng of genomic DNA in a total volume of 20µl. Cycling reactions were carried out using a Geneamp PCR System 9700 (Applied Biosystems). Thermocycling conditions were 94°C for 1 minute; followed by 35 cycles of: 94°C for 40 s, 58°C for 1 min, 72°C for 1 min; with a final extension at 72°C for 5 min (Prado *et al.*, 2004). Typically all 20µl of PCR product was mixed with loading buffer and subjected to agarose gel electrophoresis as detailed in section 7.2.5.

Typical PCR reaction mix:

Promega Master Mix	10µl
Primers (100 ng μl^{-1})	0.8µl
DNA	2µl
SDW	to 20µl volume

7.2.5 Agarose gel electrophoresis

DNA fragments were separated according to size using agarose gel electrophoresis. Gels contained between 1 and 1.5% agarose (Invitrogen), 1x TBE buffer and 5µg ml^{-1} ethidium bromide; samples were mixed with an equal volume of gel loading buffer before running through the gel. Gels were run in 1x TBE buffer in a Sub Cell GT DNA Electrophoresis Cell (BioRad, USA) with a BioRad power pack or a GIBCO BRL Horizon 58 Electrophoresis Cell (Life Technologies, USA). Running time was typically between one and two hours at 100 or 120V and DNA was visualised using a 302nm ultraviolet transilluminator (BioRad GelDoc™ 2000 Image Analyser). Images were saved and analysed using Quantity One software (V 4.2.2, BioRad).

1Kb plus DNA ladder (Invitrogen) was used to estimate fragment size where required and was run next to DNA samples on the same gel. DNA quantification was accomplished using known concentrations of uncut lambda DNA (Sigma) and was analysed either visually or using the analysis tools available with Quantity One (V 4.2.2, BioRad). Bands of the expected size from both the F10R1 and F3R10 primer combinations were excised

and gel extracted as described in section 7.2.6.

7.2.6 Gel extraction and purification of PCR products

Following agarose gel electrophoresis, bands were cut from the gel using a razor blade and transferred to pre-weighed 1.5ml Eppendorf tubes. Samples were either stored frozen for several days or extracted immediately. DNA extraction was achieved using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was eluted using 30µl of nuclease free water (NFW), from Promega.

7.2.7 Cloning of PCR products using TOPO® cloning kit

Cloning was carried out with purified F10R1 (Doms C, A and D) PCR reaction products from two highly resistant Pel02 plants determined by screening with sulfometuron at 100g a.i. ha^{-1}. The purified products from these two resistant individuals were inserted into a plasmid vector using a TOPO TA cloning kit for sequencing (Invitrogen) then transformed into One Shot TOP10 chemically competent *E. coli* cells. 50µl and 100µl portions from each transformation were spread onto pre-warmed LB agar plates containing 50µg ml^{-1} ampicillin and incubated overnight at 37°C. Ten colonies were picked from each plate and cultured overnight in LB medium containing 100µg ml^{-1} ampicillin then plasmid DNA was isolated from the best seven colonies from each original plant using a QIAprep Spin Miniprep Kilt (Qiagen) according to manufacturer's instructions. Purified DNA was subjected to PCR using M13 forward and reverse primers as included with the kit and sequencing was performed as detailed in section 7.2.6. All cloning steps were carried out using a TOPO TA Cloning Kit for Sequencing (Invitrogen) and manufacturer's instructions were followed for all steps in the procedure.

7.2.8 DNA Sequencing

Direct sequencing of PCR products was performed using a BigDye® Terminator v1.1 or v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI Prism 3100 Genetic Analyser (Applied Biosystems, USA). All steps were carried according to the manufacturer's instructions:

Typical cycle sequencing reaction mix:

BigDye Terminator RR Mix	2µl
BigDye Terminator 5x Buffer	2µl
Primer (100 ng μl^{-1})	1µl
Template DNA from gel purification step	2µl
SDW	to 10µl volume

Analysis of sequence data was performed using Vector NTI Advance 9.0 software (Invitrogen, UK).

7.3 Results

7.3.1 Pel02 seed set

DNA was extracted and sequenced from 14 highly resistant and 14 susceptible Pel02 plants along with 5 plants which showed intermediate damage four weeks after treatment with sulfometuron at 100g a.i, ha^{-1}. The PCR products obtained using the F10R1 primer set (spanning Domains C, A and D) and the F3R10 set (Domains B and E) are shown in figures 7.3 and 7.4 respectively. In addition PCR products from two highly resistant Pel02 plants were cloned and sequenced using a TOPO TA Cloning Kit (Invitrogen).

Figure 7.3 PCR products from susceptible Pel02 plants with F10R1 primer set, designed to cover Domains C, A and D. Lanes are numbered according to sample. The marked band at around 500bp was cut out of the gel for purification and sequencing. The F10R1 primer combination produced secondary PCR products in some samples of around 300bp and this occurred for both susceptible and resistant individuals.

Figure 7.4 PCR products from susceptible Pel02 plants with F3R10 primer set, designed to cover Domains B and E. Lanes are numbered according to sample. The marked band was cut from the gel, purified and sequenced.

Sequences were obtained from all 14 susceptible and highly resistant Pel02 plants. Domains A-D was sequenced without problems for all plants with the exception of a

single resistant individual, but the Ser653 position in Domain E was only sequenced in a total of 9 susceptible and 4 resistant plants due to its proximity to the R10 primer hybridisation site. Unfortunately sequence homology between different grass family ALS genes breaks down after this point and so time was not spent developing a better primer for this position. Sequence analysis showed a potential protein change in resistant individuals at position Pro_{197}. Susceptible individuals showed a CCC at this position (proline), while resistant individuals showed (A/C)CC, indicating they were heterozygous at the first position.

Several other single nucleotide polymorphisms were detected as heterozygotes but none had the potential for amino acid substitution and none segregated with high level resistance to sulfometuron. The other four common sites for amino acid substitution resulting in target site resistance from Domains C, D, B, and E were also examined in resistant and susceptible individuals. At the Ala_{122} position in Domain C all plants showed a GCC coding for alanine regardless or resistant status. Similarly at the Ala205 in Domain D all plants were either GCC or GC(C/T) with some appearing heterozygous at the last position. In Domain B there were again no mutations segregating for resistance with all plants showing TGG at the Trp_{574} and although only limited sequences were obtained from Domain E, no mutations were found in resistant plants compared to susceptibles and all showed AGC at position Ser_{653}. See diagram 7.5 for a summary of sequences from the conserved domains of resistant and susceptible Pel02 plants with positions of observed nucleotide variation highlighted.

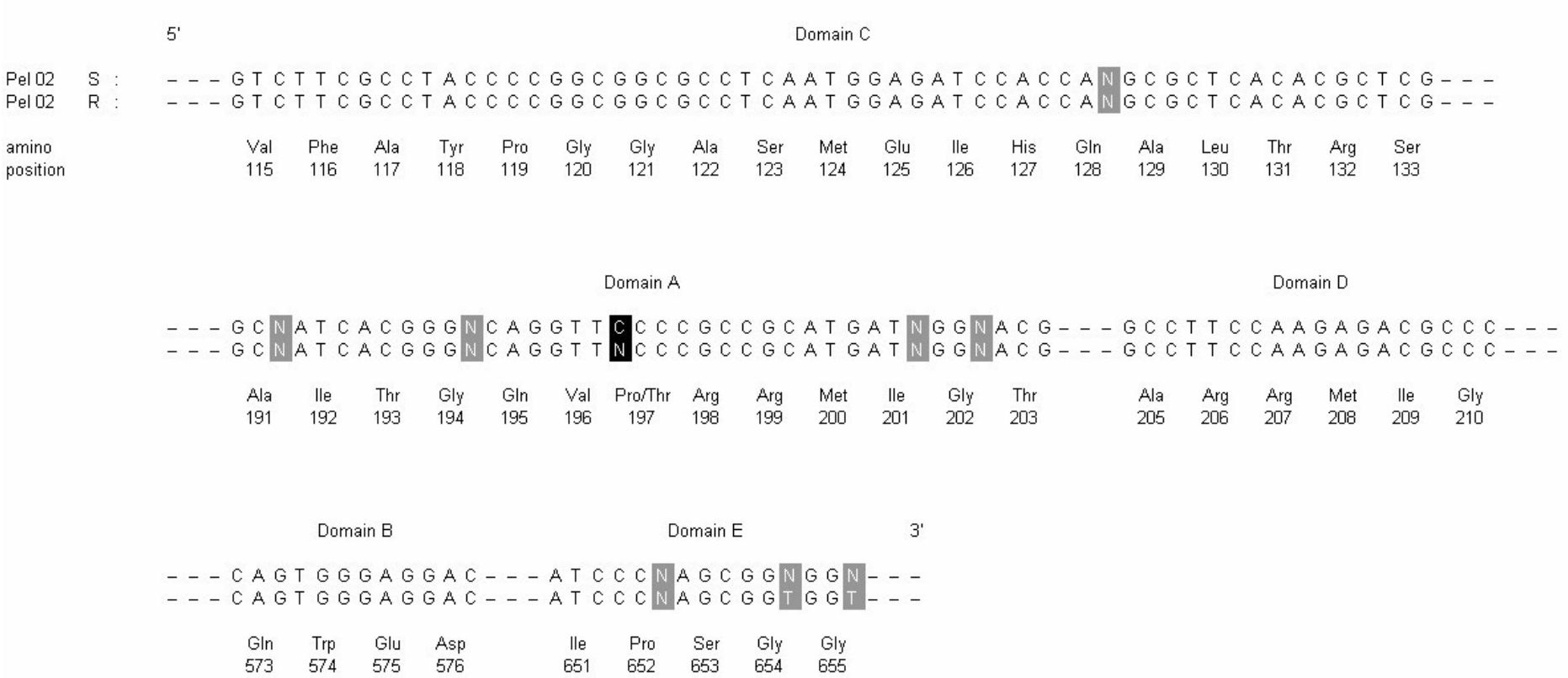

Figure 7.5 Alignment of DNA and amino acid sequences from susceptible and resistant *A. myosuroides* plants, Pel 02 population. Gray boxes show positions where nucleotide variation did not confer potential amino acid substitution and was not segregating with resistance. The black box at position 197 shows the predicted proline to threonine change at this position in resistant plants which appear heterozygous A/C at the first position compared to homozygous C in susceptible plants.

The presence of secondary products after PCR with the F10R1 primer combination was a cause for concern. Several of these bands were cut from the gels and sequenced. Some sequences generated from these bands turned out to be non-specific and were unreadable; however a few produced readable single sequences which after BLAST search were shown to closely match several plant ALS genes. This result raised the question of whether more than a single ALS gene is present in *A. myosuroides*. Cloning into plasmids using the TOPO TA Cloning Kit for Sequencing (Invitrogen) showed that DNA from a single resistant Pel02 plant contained two alleles of the ALS gene in Domain A where the original mutation segregating with resistance was found, see Figure 7.6.

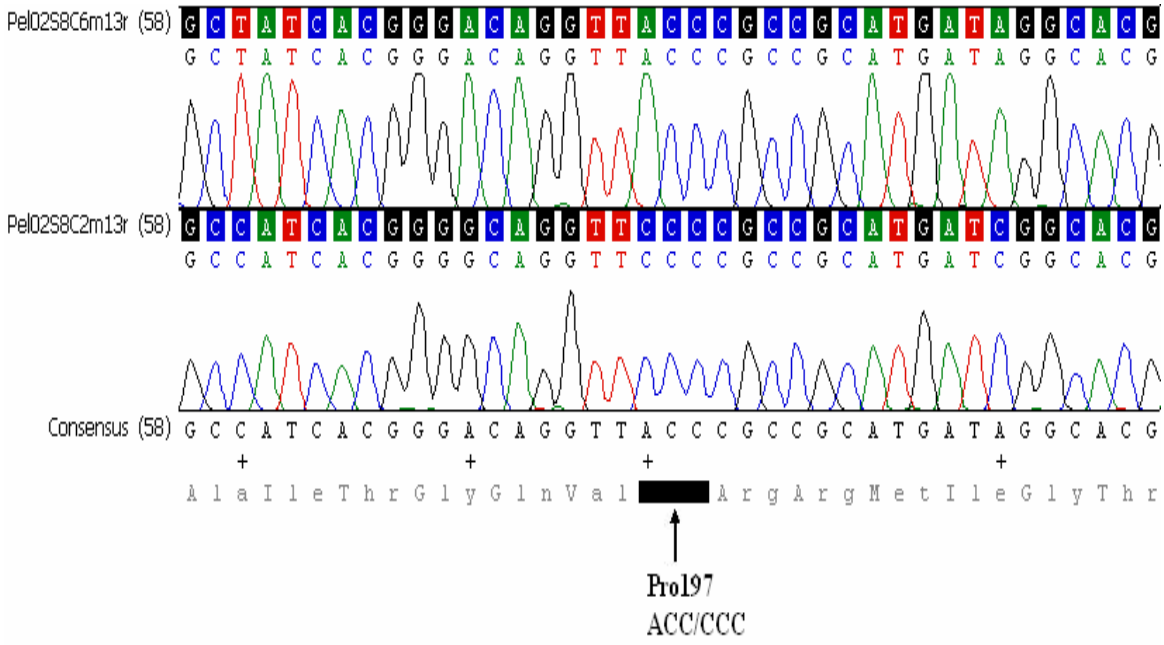

Figure 7.6 Domain A sequence from two plasmid clones from a single Pel02 highly resistant plant showing two different alleles.

Cloning of PCR products from F1R1 primers, spanning only the Domain A and D area, verified that at least two separate ALS alleles were present in a single highly resistant plant. This result could be explained as a diploid heterozygote (2 alleles of the same gene, 2 bases at Pro_{197} SNP position) or as 2 copies of the gene, homozygous at the SNP position. Unfortunately further work to properly characterise the ALS gene(s) in *A. myosuroides* could not be completed due to time constraints and difficulty in extracting

sufficient quantities of high quality DNA from frozen samples.

Direct sequences obtained from the five plants showing intermediate damage (rated 3 on the 1-5 scale, see Chapter 2 for details) four weeks after treatment with sulfometuron at 100g a.i. ha^{-1} showed that none contained the Pro_{197} mutation found in the highly resistant individuals. All of these plants, which survived treatment with fairly extensive damage, had sequences indistinguishable from susceptible plants in all five conserved Domains. None showed possible mutations at any of the five common mutation sites known to confer insensitive enzyme in weed species.

7.3.2 Wilts04, Maiden05 and Thame05 populations

DNA was extracted from 5 resistant and 5 susceptible Wilts04 plants screened according to the same methodology as the Pel02 population. Maiden05 and Thame05 plants were treated differently and were sprayed at the 2-3 leaf stage with DNA being extracted from 5 highly resistant survivors of each population only. This had the disadvantage of not allowing comparison with susceptible material for these populations, but meant that phenotypic classification of highly resistant survivors was probably more accurate since it was done according to the methodology established through dose response testing (see Chapter 3) and not using re-grown plants at a different growth stage. Conserved domains A-E were sequenced for all three populations and in the case of Wilts04 plants, susceptible and resistant sequences were compared.

In the case of Wilts04 resistant and susceptible plants, PCR with F10R1 primers gave the same secondary bands as observed with the Pel02 population but these were only present in a single Thame individual from the Thame05 and Maiden05 populations (see Figure 7.7).

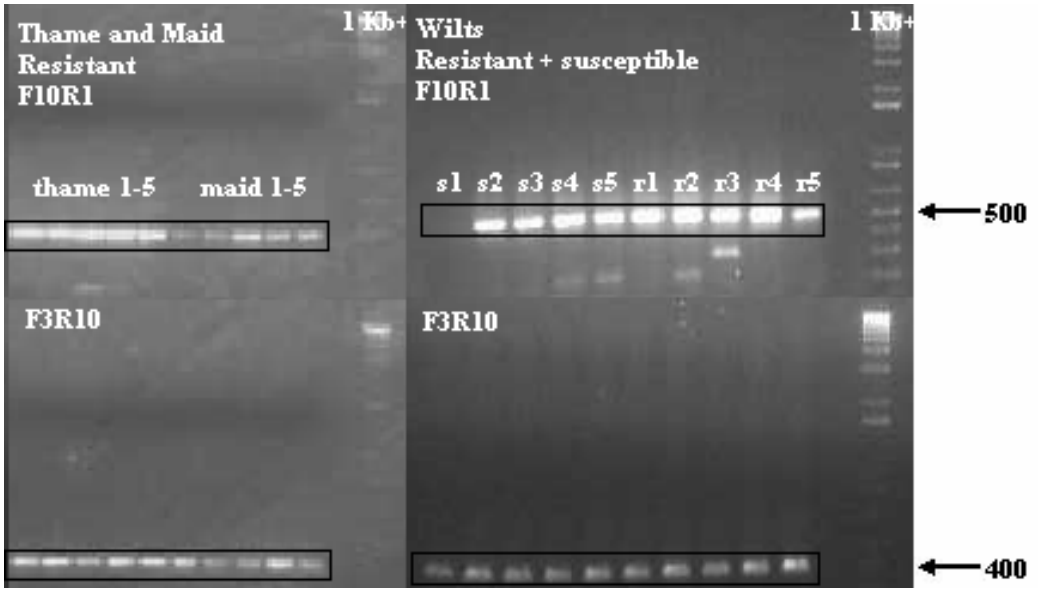

Figure 7.7 PCR products from Wilts04 resistant and susceptible plants, Thame05 and Maiden05 resistant plants with F10R1 and F3R10 primer sets. Marked bands were cut from the gel for extraction and sequencing.

Sequences were obtained from Wilts04 resistant plants across all five conserved Domains A-E. A single susceptible individual (s1) did not amplify using F10R1 primers and it was not possible to obtain sequence covering Domains C, A and D from this plant, even after re-extraction of DNA. Comparison of sequences showed no nucleotide substitutions segregating with resistance and resulting in potential amino acid substitution from any of the five conserved Domains. There was no indication of any difference between susceptible and resistant plants at the Pro_{197} position or the other four positions where mutation has been shown to lead to ALS enzyme insensitivity in wild weed species. All plants showed CCC at position 197 coding for proline.

Sequences from all Thame05 and Maiden05 resistant plants showed the same mutation seen in the Pel02 resistant plants with a predicted amino acid substitution at position Pro_{197}. Resistant individuals showed (A/C)CC, (threonine/proline) at this position, appearing heterozygous. No further nucleotide changes segregating with resistance (compared to Pel02 susceptible sequences) were found in any of the five conserved domains of the ALS gene in either Thame05 or Maiden05 plants. See figure 7.8 for a summary of Domain A sequences from resistant Pel02, Wilts04, Thame05 and Maiden05

plants compared to the susceptible Roth standard.

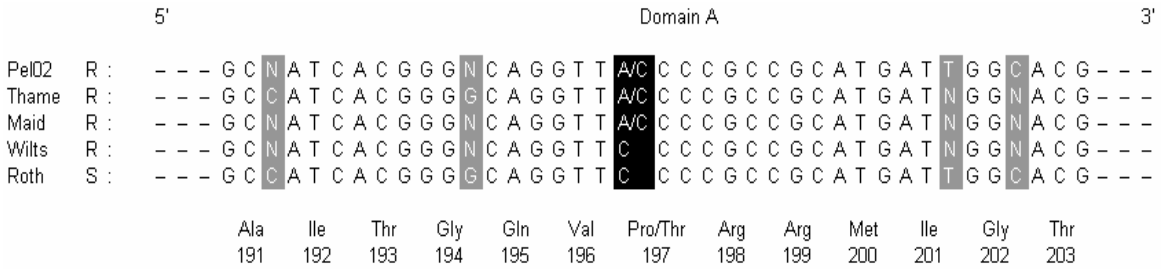

Figure 7.8 Comparison of Domain A sequences from resistant Pel02, Thame05, Maiden05 and Wilts04 populations along with susceptible standard Roth. The black box indicates the single nucleotide change resulting in a predicted proline to threonine substitution at position 197 which was found to segregate with high level resistance. Resistant plants from the Pel02, Maiden05 and Thame05 populations appeared heterozygous A/C at the first position while susceptible plants appeared homozygous C. Wilts04 was the only population for which high level resistance was demonstrated in whole plants tests but not at the molecular level. No nucleotide changes leading to potential target site change were identified in resistant versus susceptible Wilts04 plants.

7.3.3 PelRES seed set and possible homozygous resistant samples from crossing

Eight original PelRES plants from crossing experiments investigating the inheritance of the high level resistance trait were sampled and frozen before extraction of DNA, see Chapter 4 for details. Five progeny from the self pollination of PelRES plant 37 were also sampled and extracted due to the high proportion of highly resistant plants from this seed set in screening tests. Crossing results suggested that plant 37 was possibly homozygous for the resistant allele. PCR was performed using primers F10R1 and F3R10 covering conserved Domains A-E.

Sequences were similar to those from resistant Pel02 plants. Each of the original PelRES plants used for crossing to susceptible standard had a potential target site change at the Pro_{197} position in Domain A, with all showing A/C at the first position and appearing heterozygous. As before it was not possible to say whether the plants were actually heterozygous at a single locus or whether more than a single ALS gene was present.

Results from the PelRES plant 37 progeny were interesting: after self pollination this seed set showed no susceptible plants in a screening test (total of 68 plants) using 100g a.i. ha^{-1} sulfometuron in contrast to other selfed resistant plants which showed an approximate 3:1 ratio of resistant to susceptible individuals in the progeny. Sequence analysis from Domain A showed that PelRES 37 progeny appeared heterozygous at the Pro_{197} position. See Figure 7.9 for an example of sequence from one of these plants. Apart from the potential Pro_{197} change, no other mutations were picked up in any of the crossing samples.

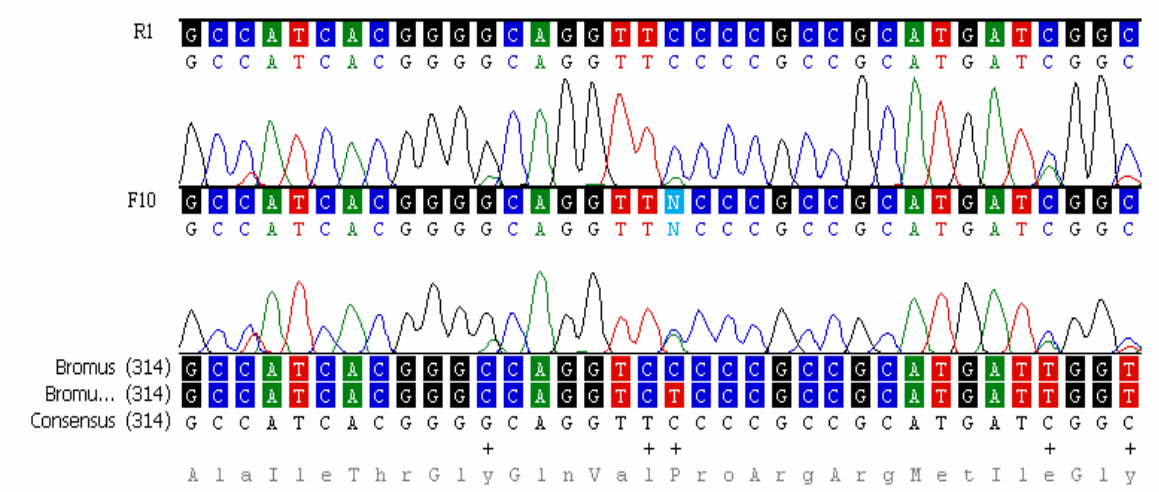

Figure 7.9 Sequence from a plant grown from seed obtained by self pollination of the PelRES 37 highly resistant plant. The chromatogram shows (A/C)CC at the Pro_{197} codon rather than ACC. Taken together with the crossing results, this strengthens the case for two or more copies of the ALS gene being present in *A. myosuroides*. Alignment is made with *B. tectorum* susceptible and resistant ALS sequence for reference.

7.4 Discussion

Sequencing of the conserved Domains A-E of *A. myosuroides* ALS provides the first direct evidence of a point mutation segregating with high-level resistance in Domain A, amounting to the substitution of proline for threonine in resistant individuals. All resistant plants appeared heterozygous (A/C)CC (threonine/proline) at position 197 whereas susceptible plants were homozygous CCC (proline) in all cases. Results from the crossing experiments detailed in Chapter 4, suggesting resistance as a single gene dominant trait,

were very convincing. If this hypothesis is to stand up it would be expected that ALS gene sequences would show some individuals to be homozygous ACC at position 197. Cloning of PCR products into *E. coli* plasmids showed two different alleles at Domain A of the ALS gene. It is not known whether these alleles occupy the same locus (i.e. single ALS gene, heterozygous) or different loci (two ALS genes, homozygous or heterozygous at both loci). The fact that all resistant individuals from all seed sets appear heterozygous at this position, including progeny from the PelRES 37 plant (which appeared homozygous when selfed), suggests that the picture is more complex than previously thought. The possibility that there are two ALS genes in *A. myosuroides* and that in the populations studied here a mutation has occurred in only one of these genes and is sufficient to confer resistance. This would explain all resistant plants appearing heterozygous at position 197 and would be consistent with ALS 1 being ACC homozygous or (A/C)CC heterozygous, and ALS 2 being CCC homozygous in all resistant sequences. Furthermore the idea of two or more ALS genes is compatible with the crossing results since it has been shown in tobacco that target site mutations in a single ALS gene confer full resistance when the other copy is susceptible (Lee *et al.*, 1988).

Another possibility is that the two distinct alleles discovered in duplicate clones are affected by different levels of expression giving rise to different phenotypic responses. This would explain the unusually high proportion of resistant plants in the PelRES 37 progeny but would not account for the complete lack of ACC homozygotes at position 197 in all sequences. The absence of homozygous resistant individuals from screening and crossing experiments suggests that two ALS genes may be present. In future work with these populations Southern blotting is a priority in order to identify the number of ALS genes present in this species and so gain a more complete understanding of the results from direct sequencing and crossing work done to identify mutations in resistant plants.

Segregation of the position 197 mutation with high level resistance was observed without exception in all cases and across three different *A. myosuroides* populations. This result, together with enzyme assays and whole plant screening tests from the PelRES and Pel02 seed sets, shows that a resistant allele of an ALS gene conferring a proline to threonine substitution in the inferred ALS protein is the most likely explanation for the high level

resistance to sulfonylurea herbicides observed in the Peldon, Maidenhead and Thame populations. This is the first evidence of ALS target site resistance in *A. myosuroides* woldwide and only the fourth grass weed where the molecular basis of ALS TSR has been characterised. Other grass weeds where ALS target site resistance has been fully characterised include *Bromus tectorum* (Park & Mallory-Smith, 2004), *Setaria viridus* (Laplante, 2006) and *Lolium rigidum* (Kaundun et al., 2006). Proline to threonine target site changes at amino acid position 197 have been shown to confer resistance to sulfonylurea herbicides in several broad-leaved weed species to date including *Papaver rhoeas, Chrysanthemum coronarium, Raphanus raphanistrum,* and *Kochia scoparia* (Guttieri *et al.*, 1992; Scarabel *et al.*, 2004; Tal & Rubin, 2004; Yu *et al.*, 2003). The proline residue at position 197 of the ALS protein is situated at the entrance to the active site channel and it interacts readily with the aromatic ring of sulfonylurea herbicides in their binding to plant ALS (McCourt *et al.*, 2006). Substitution of proline for threonine at this site creates a difference in amino acid side-chain polarity (and therefore hydrophobicity) and size at that position. The replacement of a proline also removes a constraint on tertiary structure of the protein. Therefore the Pro_{197}Thr substitution has high potential for altering the functional characteristics of the enzyme with regards to the herbicide binding site. These same conditions apply to a large number of other possible substitutions at the 197 site and probably account for the range of different amino acid insertions observed in resistant weed species at Pro_{197}.

Separating the effects of the Pro_{197} mutation from the high level of enhanced metabolism known to occur in the Peldon population was achieved through the use of the non-selective sulfonylurea sulfometuron which has been shown to control metabolism based resistance in whole plant screening tests (see Chapter 3). All of the Peldon plants showing high-level resistance to this herbicide were shown to possess the Pro_{197} mutation. Although no comparison of resistant and susceptible ALS sequences was performed for the Thame05 and Maiden05 populations, the same Pro_{197} mutation was found in all of the highly resistant plants from these seed sets. Comparison of resistant Thame05 and Maiden05 sequences from Domains B-E to susceptible Pel02 sequences showed no detectable nucleotide changes in the resistant plants compared to susceptibles across the other four conserved domains and SNP's giving rise to potential amino acid target site changes were limited to Pro_{197}Thr in all cases.

Sequences from intermediate Pel02 plants scoring 3 on the 1-5 scale after treatment with sulfometuron provided no evidence of any SNP's segregating with intermediate resistance compared to susceptible plants. This result is interesting because it means that results from crossing work are less easy to interpret in terms of a single gene dominant trait (see Chapter 4). The possibility of a completely different additional resistance mechanism such as metabolism cannot therefore be ruled out. On the other hand, the lack of target site mutations in any of the injury score 3 rated plants could be a reflection of the screening methodology used to generate material for molecular work. The use of older plants grown back after harvest of foliage was necessary because the original goal was to perform enzyme assays which require a large amount of leaf material. However the use of larger plants may have allowed susceptible individuals, or those with marginal resistance, to survive treatment with sulfometuron and so skew the injury score scale. This is supported by the high proportion of highly resistant plants in the material grown for DNA extraction (31% compared to the usual 13%). It was felt that this explanation was most likely based on proportions of highly resistant plants and the very convincing ratios obtained in crossing experiment. It is worth mentioning again that Peldon *A. myosuroides* does show a high degree of enhanced metabolism which may have provided some basis for resistance to sulfometuron in older plants without the Pro_{197} mutation. A priority for future work is to sample and extract DNA from a selection of Pel02 plants at the 2-3 leaf stage for spraying and then assess all for whole plant response to sulfometuron at 100g a.i. ha^{-1}.

The use of sulfometuron as a universal indicator of ALS target site resistance in whole plant screening tests did not appear to hold true for the Wilts04 population. A large proportion of Wilts04 plants showed resistance to sulfometuron in whole plant tests but no mutations were found to segregate with high level resistance in any of the five conserved ALS domains. It is possible that such mutations exist and were outside the highly conserved regions of the ALS gene. Such mutations have been found in resistant *Papaver rhoeas* from Spain (Duran-Prado *et al.*, 2004) and it is hoped that further work in future will discover the mechanism of resistance in the Wilts04 resistant population of *A. myosuroides*. The results from the Wilts04 population show that a combination of molecular, enzyme and whole plant work is required for full characterisation of resistance in any weed population. Whole plant assays show resistance regardless of mechanism whereas using a molecular assay alone on Wilts04 plants could have given potentially

misleading results. Sulfometuron whole plant screening tests are obviously still useful in overcoming the type of metabolic resistance regularly found in field populations of *A. myosuroides* and highlighting populations where high-level resistance to sulfonylurea herbicides merits further investigation.

7.5 Chapter summary

- A single point mutation was found in the first position of the Pro_{197} codon of an *A. myosuroides* ALS gene conferring a potential proline to threonine target site change in highly resistant individuals.
- Highly resistant plants were shown to possess two ALS alleles from cloned sequences spanning conserved Domain A.
- Resistant individuals appeared heterozygous at position 197 with an (A/C)CC codon while susceptible plants appeared homozygous CCC at the same position.
- No other mutations segregating with resistance were found across all five conserved domains of the ALS gene.
- Three populations contained resistant individuals possessing the Pro_{197} mutation which conferred potential ALS target site change. These were the Pel02, Thame05 and Maiden05 populations.
- One population (Wilts04) showed high level resistance at the whole plant level but no evidence of differences between ALS sequences from susceptible and resistant plants. The likely cause of resistance in this population is unknown.

8. General Discussion

8.1 General discussion

The aim of this project was to investigate and characterise resistance to acetolactate synthase inhibiting herbicides in biotypes of the grass-weed *Alopecurus myosuroides*, integrating work at the whole plant level with molecular and biochemical characterisation of resistance mechanism. This chapter provides a general review of the results obtained and addresses their implications in the context of the initial project aims and objectives. The consequences for weed management strategies in UK cereal cropping systems are discussed in light of these results, and possible directions for future work are identified.

8.1.1 Sulfometuron as an indicator of ALS target site resistance

Throughout the course of this project, the non-selective sulfonylurea herbicide sulfometuron-methyl (sulfometuron) was evaluated as an indicator of ALS target site resistance in *A. myosuroides* populations showing resistance to flupyrsulfuron-methyl (flupyrsulfuron) and mesosulfuron-methyl plus iodosulfuron-methyl sodium mixture (mesosulfuron+iodosulfuron) in the field. The use of sulfometuron in screening for high level resistance was based on several Australian studies in the grass weed *Lolium rigidum* where resistance to sulfometuron has been associated with ALS enzyme less susceptible to inhibition by sulfonylureas (Gill, 1995). The apparently limited susceptibility of sulfometuron to metabolic breakdown in resistant biotypes of the grass weed *L. rigidum* was demonstrated in an earlier study (Christopher *et al.*, 1992) where the authors highlighted the possible use of sulfometuron as an indicator resistance mechanism in *L. rigidum*. This idea was later confirmed in Petri dish screening methodologies devised by Burnet (1994) and Preston and Powles (2002) which allowed the selection of target site resistant individuals from a mixed population. These studies provided the basis for much of the whole plant work carried out in this project.

8.1.2 Molecular and enzyme summary

Overall the results support the conclusion that sulfometuron can be used as an indicator of probable ALS target site resistance in the grass weed *Alopecurus myosuroides*. The whole plant screening methodology established in Chapter 3 using 100g a.i ha^{-1} of the sulfometuron formulation "Oust" at the three leaf stage in *A. myosuroides* seedlings provided a quick and easy test with the power to discriminate between enhanced metabolism and ALS target site resistance or other high level resistance mechanisms. Crucially the sulfometuron screening test was able to successfully pick out individuals with ALS target site resistance from populations with high base levels of enhanced metabolism. A single point mutation found in the first position of the Pro_{197} codon of an *A. myosuroides* ALS gene, conferring a predicted proline to threonine target site change, was found to be present in all highly sulfometuron resistant Peldon individuals and none of the susceptibles. In addition, enzyme assays demonstrated that crude acetolactate synthase extracted from resistant biotypes was less susceptible to inhibition by the sulfonylurea herbicides sulfometuron and flupyrsulfuron than that from plants not possessing the high-level sulfometuron resistance mechanism in whole plant screening tests. Overall these results show a strong relationship between a single point mutation conferring potential proline to threonine target site change at acetolactate enzyme position 197 and resistance to the non-selective sulfonylurea herbicide sulfometuron. In addition to the Peldon biotype, a further two *A. myosuroides* populations were found to possess the Pro_{197} mutation in highly resistant individuals; the 'Maidenhead' population from Berkshire (Maiden05) and the 'Thame' (Thame05) population from Oxfordshire. No evidence of other mutations conferring potential target site change associated with high level resistance to sulfometuron was found in the other five conserved domains of the ALS gene in any of these populations.

Currently experiments are under way with the aim of establishing a simple Petri dish methodology for quick diagnostic testing of *A. myosuroides* seed samples and initial results are encouraging (Moss 2006, personal communication). The idea of a simple glasshouse or Petri dish screening methodology for ALS target site resistance in *A. myosuroides* follows the precedent set by use of the cyclohexanedione herbicide sethoxydim as an indicator of acetyl-CoA carboxylase target site resistance in *A. myosuroides* and other grass weeds (Moss *et al.*, 2003). It has been observed that sethoxydim is less susceptible to enhanced metabolism type resistance compared to other

ACCase inhibiting herbicides. Molecular work confirms that resistance to sethoxydim appears to segregate with mutations conferring potential target site changes in the ACCase enzyme with few exceptions (Brown *et al.*, 2002; White *et al.*, 2005). Sethoxydim is currently used as part of the Rothamsted rapid resistance test as an indicator of potential target site resistance to cyclohexanedione herbicides. Extending the Rothamsted rapid resistance test to include ALS inhibitors would allow useful diagnostic testing of seed samples from around the country at a time when emerging resistance to ALS inhibitors (particularly mesosulfuron+iuodosulfuron mixture) is a subject of particular importance.

While the sulfometuron screening procedure was useful in picking up individuals with high level resistance mechanisms, there are several issues which remain to be resolved. The first is the presence of damaged survivors following treatment with sulfometuron in some biotypes. These plants were assumed to possess the ALS target site resistance trait based on the segregation ratios from crossing experiments described in Chapter 4, but molecular analysis of five plants showing intermediate resistance failed to show any mutations conferring potential target site change in the five conserved domains of the ALS gene compared to susceptible plants. This inconsistency might be explained by the particular whole plant screening methodology adopted during the molecular part of the project whereby foliage was harvested at the four to six leaf stage and plants then grown back and sprayed with sulfometuron. It is thought that the procedure may have allowed susceptible individuals to survive treatment with the screening dose normally used for 3 leaf stage plants. This explanation is supported by the high proportion of highly resistant plants in the Pel02 material grown for DNA extraction (31% compared to the 13% demonstrated in whole plant assays, see Chapter 3). A priority for future work is to sample and extract DNA from a selection of Pel02 plants at the 2-3 leaf stage for spraying and then assess all for whole plant response to sulfometuron at 100g a.i. ha^{-1} in order to validate this part of the project and better explain the results from crossing experiments.

A second unresolved issue with the use of sulfometuron as an indicator of ALS target site resistance in whole plant assays was the presence of high levels of resistance in the Wilts seed sample. *A. myosuroides* plants from this biotype were highly resistant to sulfometuron at 100g a.i. ha^{-1} but DNA extraction and sequencing showed no significant differences between resistant and susceptible sequence data across the five conserved

domains of the ALS gene. It is possible that mutations exist and were outside the highly conserved regions of the ALS gene which were sequenced in this study, as has been demonstrated in a Spanish biotype of the broadleaved weed *Papaver rhoeas* (Duran-Prado *et al.*, 2004). Further work is required in order to resolve the resistance mechanism in the Wilts population of *A. myosuroides*. These results show that caution is required in the development of quick diagnostic screening tests and that combined molecular, enzyme and whole plant work is necessary for full characterisation of resistance. It is hoped that enzyme assays with Wilts leaf material will provide answers to this particular problem.

At the present time sulfometuron whole plant screening tests have demonstrated their use in highlighting populations where high-level resistance merits further investigation and have so far been successful in highlighting three populations where resistance was due to a mutation conferring potential target site change in resistant individuals. Some further work is required before sulfometuron can be used as a fully reliable indicator of ALS target site resistance in *A. myosuroides*.

8.1.3 Whole plant summary in terms of molecular and enzyme work

Whole plant work focused on selection of the correct sulfometuron dose for use in screening experiments and involved the use of certain well characterised standard populations. The cross resistance characteristics of resistant populations were also addressed along with the frequency of sulfometuron resistance in flupyrsulfuron resistant field biotypes. A sulfometuron dose of 100g a.i. ha^{-1} applied at the three leaf stage was able to effectively discriminate between ALS target site resistance and what was presumed to be resistance due to enhanced metabolism in flupyrsulfuron resistant populations. Overall resistance to flupyrsulfuron was widespread in field populations of *A. myosuroides* while sulfometuron resistance (i.e. probable ALS target site resistance) was very rare. Cross resistance experiments were inconclusive since metabolism standard populations showed high levels of resistance to other ALS herbicide groups and so cross resistance due to the Pro_{197} mutation could not be determined.

Resistance to mesosulfuron+iodosulfuron was strongly associated with sulfometuron resistance. Most of the flupyrsulfuron resistant biotypes tested showed either no resistance or very low level resistance with the exception of the Peldon populations.

Pel96, the enhanced metabolism standard population, showed only low level resistance to mesosulfuron+iodosulfuron indicating that the particular mechanism conferring resistance to flupyrsulfuron was not particularly effective in conferring mesosulfuron+iodosulfuron resistance. These results suggest that mesosulfuron+iodosulfuron mixture is a much more active *A. myosuroides* herbicide than flupyrsulfuron, especially when used against biotypes with some degree of enhanced metabolism resistance.

Further work investigating the inheritance of the high level sulfometuron resistant trait in the PelRES02 sulfometuron selected line of Peldon *A. myosuroides* focused on crossing experiments and segregation of progeny following application of a screening dose of 100g a.i. ha^{-1} sulfometuron. Six sulfometuron resistant plants were split in half with one part being crossed to a susceptible and the other part forced to self pollinate. Results from segregation of sulfometuron resistant and susceptible phenotypes in the F_1 and from self pollinated clones suggested that resistance was conferred by a single, dominant nuclear allele. Segregation in the F_2 showed an association between parental resistance levels and the degree of resistance in progeny and it was concluded that resistance is controlled by a single, dominant gene but that additional effects were also present. Crossing experiments were unable to account for the observed variation in resistant progeny and possible explanations include a contribution from enhanced metabolism resistance (with varying levels present in different plants) or the possibility that more than one resistant allele was present in the PelRES line. Ideally further work on the inheritance of the resistant trait will be carried out to resolve this issue.

Details of the investigation into the evolution of the sulfometuron resistant trait in Peldon *A. myosuroides* are given in Chapter 5. The Peldon biotype presented an ideal opportunity because seed samples exist for every year back to the mid 1980's. In addition to these, detailed herbicide records were provided by the farmer at Peldon (see Chapter 5). Plants grown from seed collected every year from 1996 until 2002 were screened using sulfometuron to determine changing proportions of the high level resistance trait (associated with Pro_{197} to Thr mutation) at Peldon over time. Field experiments were also conducted in order to map the physical distribution of resistance in the field. The results of the time series and spatial screening tests showed that high level resistance was limited to one field on a single farm at Peldon and was unevenly distributed, with higher proportions of resistant plants concentrated in a small area of a single patch and lower proportions of resistant

plants present across the whole field. The speed of selection for the high level resistant trait at Peldon following application of flupyrsulfuron in 2000 suggested the possibility of some pre-selection with sulfonylurea herbicides in the past. The recent introduction of the highly active sulfonylurea herbicide mixture mesosulfuron+iodosulfuron is likely to have consequences for *A. myosuroides* populations like the one at Peldon where herbicide inputs have been heavy and ALS inhibiting herbicides like flupyrsulfuron and chlorsulfuron have been used in the past.

An experiment was set up to investigate selection with flupyrsulfuron compared to mesosulfuron+iodosulfuron on a susceptible *A. myosuroides* biotype over time under field conditions. Results showed that selection with flupyrsulfuron provided a degree of cross-resistance to mesosulfuron+iodosulfuron after only two years of herbicide application. Based on the levels of control achieved with sulfometuron, the cross resistance mechanism was most likely due to enhanced metabolism. Mesosulfuron+iodosulfuron mixture is a more active herbicide than flupyrsulfuron and provides greater levels of overall control. Continued use is likely to strongly select for single gene type resistance as well as the build-up of genes with minor additive effects in already flupyrsulfuron-resistant field populations. The nature of emerging resistance to mesosulfuron+iodosulfuron in field populations of *A. myosuroides* will depend on the previous herbicide history of the populations in question and the management practices adopted by individual farmers. The demonstration of selection for mesosulfuron+iodosulfuron resistant trait(s) after only two years application of flupyrsulfuron is an important finding and suggests that enhanced metabolism might have a significant role to play in emerging resistance. This experiment is currently entering its fourth year and may provide more information on selection and resistance mechanisms in the years to come.

8.2 Summary of important findings

1. In resistant *A. myosuroides* plants a single point mutation was found in the Pro_{197} position of the ALS gene conferring a predicted proline to threonine target site change in the translated enzyme. The Pro_{197} target site change segregated with high level resistance to sulfometuron and was not present in susceptible plants. No other mutations segregating with resistance were found across all five conserved domains of the ALS gene.

2. The same Pro_{197} to threonine mutation was found in highly resistant plants from three separate geographical locations in the UK: Peldon in Essex, Maidenhead in Berkshire and Thame in Oxfordshire. All three biotypes showed varying proportions of plants highly resistant to sulfometuron at the screening dose of 100g a.i. ha^{-1} and the high level resistant trait was associated with Pro_{197} mutation in all cases.
3. These three sulfonylurea resistant *A. myosuroides* biotypes represent the first examples of confirmed ALS target site resistance in a European grass-weed where the molecular basis of the resistance has been characterised, and only the fourth case in grass-weeds worldwide (Kaundun *et al.*, 2006; Laplante, 2006; Park & Mallory-Smith, 2004).
4. Based on crossing experiments the genetic basis of ALS target site resistance in the Peldon biotype seems to be as a single gene dominant trait and this is consistent with results from other species.
5. Work on the evolution of target site resistance at Peldon shows that the trait can build up relatively quickly with continuing flupyrsulfuron treatment, even though enhanced metabolism provides a resistance mechanism which allows plants not possessing ALS target site resistance to survive. Pre-selection with the ALS inhibitor chlorsulfuron is a risk factor in the development of ALS target site resistance. Given the relative efficacy of mesosulfuron+iodosulfuron on enhanced metabolism populations compared to flupyrsulfuron, it is expected that the build up of ALS target site resistance will not be slowed by the widespread adoption of this herbicide.
6. Sulfometuron at 100g a.i. ha^{-1} has potential as an indicator of target site resistance in *A. myosuroides* populations. Sulfometuron has demonstrated the ability to overcome enhanced metabolism type resistance in the Peldon biotype and high level resistance to sulfometuron is associated with a mutation conferring a potential ALS target site change in three out of four resistant biotypes tested.
7. The presence of the Pro_{197} mutation in sulfonylurea resistant biotypes is consistent with data from other weed species and is the most commonly found mutation conferring resistance to sulfonylurea herbicides. Mutations at this position are known to confer high levels of resistance to sulfonylureas but not to imidazolinones in most cases. Use of this group might therefore be expected to provide some level of control on *A. myosuroides* populations where Pro_{197} target site resistance has built up.

8.3 Directions for future work

1. The current frequency of resistance to ALS inhibiting herbicides in the UK is of particular importance, along with knowledge of how this is changing. Continued surveys and monitoring of resistant *A. myosuroides* populations are a priority.
2. The mechanisms involved in emerging resistance to mesosulfuron+iodosulfuron are important in the adoption of effective management strategies. It is important to discover the relative importance of target site resistance and enhanced metabolism.
3. Knowledge of the precise mutation associated with resistance in an ALS gene of *A. myosuroides* might allow the development of high throughput diagnostic techniques for rapid detection of resistance in the field, for example through allele specific PCR methods (Giancola *et al.*, 2006). Another possibility is the development of Petri dish assays for ALS target site resistance which will allow diagnosis of resistance from seed samples sent by farmers before the next cropping year.
4. Continued work is necessary in order to specifically understand the impact and significance of partially sulfometuron-resistant plants and also to determine the resistance mechanism in the Wilts population.
5. Improved prevention and management strategies for resistance to ALS inhibiting herbicides are an important goal for the future. Current work looking at the selection effects of flupyrsulfuron compared to mesosulfuron+iodosulfuron may provide information which will improve current management guidelines.

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Appendix A- Complete list of *A. myosuroides* seed samples

Sample name	Collection year	Details
General reference standards		
Roth99	1999	Susceptible standard. Collected from no herbicides section of the Broadbalk long term experiment on Rothamsted Estate.
Roth03	2003	Susceptible standard. Collected from no herbicides section of the Broadbalk long term experiment on Rothamsted Estate.
Far95	1995	Partial metabolism standard population displaying lower levels of resistance than the Peldon population. Collected from a farm near Faringdon in Oxfordshire.
Peldon field samples		
Pel96	1996	Metabolism standard population showing enhanced metabolism resistance to a wide range of different herbicides including the ALS inhibitor flupyrsulfuron-methyl. Collected from Patch C of Hams field on Peldon Hall farm, Essex. Contains a low proportion of ALS target site resistant individuals (around 2%). A single ALS TSR plant was self pollinated giving rise to the highly resistant PelRES02 seed set.
Pel97, Pel98 and Pel99	1997-1999	Samples collected from Patch C of Hams field on Peldon Hall farm, Essex
Pel00	2000	Sample collected from Patch C of Hams field on Peldon Hall farm, Essex. This was the first year that flupyrsulfuron was used on Hams field. Contains around 4% ALS TSR plants.
Pel01	2001	Sample collected from Patch C of Hams field on Peldon Hall farm, Essex. This was the second year that flupyrsulfuron was used on Hams field. Contains around 32% ALS TSR plants.
Pel02	2002	Sample collected from Patch C of Hams field on Peldon Hall farm, Essex. This was the final year that flupyrsulfuron was used on Hams field. Contains around 25% ALS TSR plants. This seed set was used as a highly resistant field sample in whole plant tests, enzyme assays, and molecular work.

Sample name	Collection year	Details
Highly resistant samples derived from crossing work		
PelRES02	2002	Highly ALS target site resistant seed set collected from the sulfometuron-selected progeny of an original resistant Pel96 plant which survived sulfometuron treatment. See Chapter 4 for further details. Plants grown from this seed set were used in whole plant bioassays, crossing, and molecular work.
PelRES03	2003	Twelve PelRES02 sulfometuron survivors were poly-crossed to produce this seed set containing high levels of ALS TSR. Used in whole plant bioassays and enzyme assays.
Other resistant seed samples- Confirmed ALS target site resistance		
Maiden05	2005	Population containing a high proportion of target site resistant individuals. Collected from a farm near Maidenhead in Berkshire. This seed set was used in molecular work.
Thame05	2005	Population containing a high proportion of target site resistant individuals. Collected from a farm near Thame in Oxfordshire. This seed set was used in molecular work.
Other resistant seed samples- Unknown resistance mechanism		
Wilts04	2004	Population containing a high proportion of very highly sulfometuron and mesosulfuron+iodosulfuron resistant individuals. This seed set was used in molecular work and no target site mutations were found.
Other resistant seed samples- Probable enhanced metabolism. All highly resistant to flupyrsulfuron but susceptible to sulfometuron.		
BoxA	2002	Sample from ADAS Boxworth, Cambridgeshire.
BoxB	2002	Sample from ADAS Boxworth, Cambridgeshire.
BoxC	2002	Sample from ADAS Boxworth, Cambridgeshire.
BoxD	2002	Sample from ADAS Boxworth, Cambridgeshire.
Berr	2002	Sample from a farm near Oundle, Northamptonshire.
Chan	2002	Sample from a farm near Muston, Leicestershire.
Flaw	2002	Sample from a farm near Flawborough, Nottinghamshire.
GBE-01-040	2002	Sample from Dupont (Stevenage, Herts), unspecified test site.
GBB-01-101	2002	Sample from Dupont (Stevenage, Herts), unspecified test site.
Plum	2002	Sample from a farm near Oundle, Northamptonshire.
Sand	2002	Sample from an unspecified test site, mesosulfuron+iodosulfuron trial

Appendix B- List of abbreviations

ACCase	acetyl-CoA carboxylase
Acetoin	3-hydroxy-2-butanone
AHAS	acetohydroxyacid synthase
a.i.	active ingredient
Ala	alanine
ALS	acetolactate synthase
ANOVA	analysis of variance
BBCH	BASF, Bayer, Ciba-Geigy and Hoechst
BCAA	branched-chain amino acid
bp	base pair
dNTP	deoxyribonucleotide triphosphate
Dim	cyclohexanedione
DNA	deoxyribonucleic acid
Dom	domain
DTT	1,4-Dithio-DL-threitol
ED50	effective dose 50
ED80	effective dose 80
F1	first filial generation
F2	second filial generation
FAD	flavin adenine dinucleotide
Flupyrsulfuron	flupyrsulfuron-methyl sodium
Fop	aryloxyphenoxypropionate
GPS	global positioning system
GST	glutathione-*S*-transferase
Ha	hectare
I50	the molar concentration of inhibitor at which enzyme activity is reduced by 50%
IPM	integrated pest management
IWM	integrated weed management

Km	enzyme-substrate affinity
KNO_3^{2-}	potassium nitrate
LSD	least significant difference
M	molar
Mg	magnesium
$MgCl_2$	magnesium chloride
N_2	nitrogen
NFW	nuclease free water
P450	cytochrome P450 mono-oxygenase
PCR	polymerase chain reaction
Pel	Peldon
P	probablility
ppm	parts per million
Pro	proline
PVPP	polyvinylpolypyrrolidone
Roth	Rothamsted
R/S	resistant/susceptible
SD	standard deviation of the mean
SDW	sterile distilled water
SE	standard error
SED	standard error of the difference of the means
SEM	standard error of the mean
Ser	serine
SNP	single nucleotide polymorphism
Sulfometuron	sulfometuron-methyl
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Thr	threonine
Tm	melting temperature
TPP	thiamine pyrophosphate
Tris	2-amino-2-hydroxymethylpropan-1,3-diol
Trp	tryptophan
UK	United Kingdom