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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PS2709
2. Project title: Herbicide resistant broad leaved weeds:research required to address policy needs
3. Contractor organisation(s):

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4. Total Defra project costs (agreed fixed price): £ 466,917
5. Project: start date .... 01 January 2006

   end date .... 31 December 2008

6. It is Defra's intention to publish this form.
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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

This project was carried out in order to increase understanding of the risks to British agricultural and amenity sectors from existing and future cases of herbicide resistant broad leaved weeds. Prior to this project, little was known about the mechanisms of herbicide resistance in these species, and work detailing the prevalence, mechanisms, and cross resistance characteristics of several agronomically important broad leaved species formed a major part of the experimental work. A secondary objective was to raise awareness of the threat posed by herbicide resistant broad leaved weed species in several sectors through various knowledge transfer initiatives. Work was carried out according to a number of agreed objectives and these are presented below, along with a summary of the relevant results:

1. To review the current status of herbicide-resistance in broad-leaved weeds worldwide in relation to their population biology, 'weediness' and response to herbicides, in order to help predict which species are likely to evolve resistance in the UK in the agricultural, horticultural, industrial and amenity sectors.

- A literature review was completed and comprises a wide ranging and detailed assessment of the current and future risks to British agricultural and amenity sectors from broad leaved weed species. The review process also involved collaboration leading to the production of a risk matrix combining biological, herbicide and management factors to determine a numerical "risk value" for the development of herbicide resistance in any particular situation.
- The matrix is particularly suitable for assessing herbicide resistance risks in relation to the registration of herbicides. Edited versions of both the literature review and the risk matrix are currently being prepared with the aim of submission to relevant journals.

2. To determine the resistance characteristics of resistant chickweed (*Stellaria media*) and poppy (*Papaver rhoeas*) populations in order to understand the extent of cross-resistance to herbicides with the same, and different, modes of action and the consequential impact on management strategies.

Several reportedly resistant populations of common chickweed and common poppy were held at Rothamsted prior to the beginning of this project. Many of these were provided by DuPont (UK) Ltd. Testing was performed to assess resistance of these populations to herbicides currently used for control of these weeds.

- Glasshouse dose response and outdoor container experiments revealed that resistance to ALS inhibiting herbicides was common among the five chickweed and thirteen poppy samples held at Rothamsted. No significant resistance was observed to any other class of herbicide for either species.

- Resistance to the sulfonylurea (SU) group ALS inhibitor metsulfuron was present in 11 poppy populations from a total of 13 tested (DK001, DEV001, DEV002, CC001, CC002, CC003, CC004, AMC002, AMC003, CBC001, CAMBS; collected from four counties of England).
- Outdoor container testing revealed that poppy biotypes resistant to metsulfuron were also resistant to tribenuron.
- Resistance to ALS inhibitors was not associated with auxin analogue (MCPA) resistance in any of the British poppy populations tested. This is in marked contrast to the situation in Europe where resistance to both herbicide classes is often found in single populations.
- Good alternative herbicides for SU resistant poppy control include MCPA and ioxynil + bromoxynil mixture. Pendimethalin also provided some control in container experiments.
- Metsulfuron resistance was confirmed in all five chickweed populations tested (CORN, KENT, SCOT, ABER, NI08; collected from five counties of England, Scotland and Northern Ireland).
- One metsulfuron resistant chickweed population (ABER) showed cross resistance to the triazolopyrimidine ALS inhibitor florasulam, whereas the other four populations were fully susceptible.
- No resistance to the alternative non-ALS herbicide fluroxypyr was observed in any ALS inhibitor resistant chickweed populations and this herbicide represents a good alternative for control of resistant populations.
- A single chickweed population (NI08) demonstrated very marginal resistance to the auxin herbicide mecoprop, with several plants surviving application at field rate. No other populations showed any resistance to mecoprop.
- Experiments using sulfonylurea herbicides intended primarily for grass weed control showed that chickweed populations highly resistant to metsulfuron showed only partial resistance to mesosulfuron + iodosulfuron (“Atlantis”) and iodosulfuron (“Hussar”) at field dose rates. Spraying with SU herbicides for grass weed control therefore has the potential to provide some control of certain metsulfuron resistant chickweed populations in field situations.
- The ABER population was not controlled by ALS inhibiting herbicides intended for grass weed control (mesosulfuron + iodosulfuron; iodosulfuron), and also survived application of the broad spectrum imidazolinone herbicide imazapyr (“Arsenal”) up to 750 g a.i. ha^{-1} but showed extensive plant injury and death at higher rates. Imazapyr was the only ALS inhibitor giving reasonable levels of control of the ABER population.
- The demonstrated activity of grass weed ALS herbicides against some metsulfuron resistant chickweed populations also raises regulatory issues about herbicide effects against non-target species and the potential for selection of resistance in those species.

(iii) To determine the biochemical and molecular basis of resistance in broad-leaved weeds in the UK in order to facilitate the development of diagnostic tools for the detection and characterisation of resistance.

Sequencing of two conserved regions of the ALS gene known to be associated with resistance to ALS inhibiting herbicides was performed using leaf material from poppy and chickweed populations showing ALS resistance in glasshouse tests.

- Metsulfuron resistant plants from three poppy populations were sequenced. Resistance to the sulfonylurea herbicides metsulfuron and tribenuron was found to be associated with mutation conferring a predicted Pro-197 amino acid substitution in all three poppy populations.
- Two different predicted substitutions (Pro-197-His and Pro-197-Leu) were observed at the ALS gene 197 position in poppy and all conferred resistance to field rate metsulfuron and tribenuron with no difference observed between plants with different substitutions.
- Resistance to metsulfuron in one chickweed population (SCOT) was associated with ALS mutation conferring a predicted Pro-197-Gln substitution, while cross resistance to florasulam was associated with mutation conferring Trp-574-Leu substitution in a different population (ABER).
- The Trp-574 mutant ABER population showed higher levels of resistance and more extensive cross resistance to different ALS inhibitors than any other resistant population. ABER also showed greater resistance to sulfonylurea herbicides intended for grass weed control (iodosulfuron, mesosulfuron + iodosulfuron) in contrast to the Pro-197 mutant SCOT population which was more susceptible to both at field rates.

(iv) To maintain a “watching brief” on potential new cases of herbicide-resistance and, if appropriate, develop new testing methodologies and procedures so that the extent of any problems can be better quantified.

A watching brief was maintained throughout the project with new cases of resistance being investigated as required.

- A new report of resistance to both ALS herbicides and the auxin herbicide mecoprop in chickweed from Northern Ireland (NI08) was investigated in 2008. In a screening test comparing the NI08 population to the standard resistant populations SCOT and ABER and susceptible standard population UKA, NI08 was the only population with individual plants surviving treatment with mecoprop at field rate, although analysis of variance showed very little difference in percentage fresh weight reduction between populations.
- A sample of mayweed (*Tripleurospermum inodorum* (L.) or *Matricaria inodora*) showing resistance in the field to ALS inhibitors was investigated. A limited number of plants were sprayed with metsulfuron at field rate and these showed substantially reduced control (54 %) compared to a susceptible standard (96 %), confirming resistance.
- The susceptible population was fully controlled while all AMC plants survived. This experiment provides the first evidence of ALS resistant mayweed in the UK.

(v) To undertake technology transfer initiatives to inform suppliers and users of herbicides in the agricultural, horticultural, industrial and amenity sectors of the risks posed by herbicide-resistance and to promote appropriate prevention and management strategies.

Technology transfer was initiated in the following ways:

- A presentation was given at the amenity weed control conference in London on 2 Oct 2008 highlighting the risks presented by herbicide resistance in the amenity sector. The audience consisted largely of amenity sector spray contractors and operatives covering local council, highway and railway vegetation control. A summary of the presentation was reported in the publication “Horticulture Week” on 09 Oct 2008.
- A leaflet was prepared for use on appropriate websites focused on herbicide resistance risks in the UK amenity sector. Content was based on a general introduction to the idea of herbicide resistance along with procedures for good practice for amenity spray operatives.
- An agreement was made to produce a topic sheet in association with HGCA for distribution at Cereals 2009. The topic sheet will provide an update on the current situation regarding ALS resistant chickweed and poppy in the UK and will outline possible alternative herbicides and management strategies to help farmers and agronomists cope with resistant populations.

Implications of the research results

Herbicide resistance in broad leaved weeds does not appear to pose as large a threat in the UK as resistance in grass weeds. This is largely due to resistance being conferred solely by target site mechanisms, with no clear evidence of other mechanisms which commonly occur in grass weeds. Thus alternative (non-ALS) modes of action, such as fluroxypyr on chickweed and ioxynil/bromoxynil on poppy, can provide complete control of resistant populations. However, control of ALS resistant broad-leaved weeds is dependent on the continued availability of effective alternative herbicides. The availability of alternatives to ALS inhibitors is likely to be affected by the current revisions to the EU agrochemical registration directive (91/414). Loss of alternatives is likely to substantially increase the threat posed by ALS resistance, as few effective cultural control options are available. In addition, while ALS resistance has only been confirmed in three weed species in the UK (chickweed, poppy and mayweed) there is no reason why resistance should not evolve in other species. Indeed, increased reliance on a more restricted range of herbicides will increase the risk of resistance in broad leaved weeds, not only in arable crops, but also in horticultural, amenity and industrial weed control situations. The authors recommend that active monitoring is undertaken to detect any new cases of resistance and that the availability of a range of modes of action is maintained to ensure the sustainable control of broad leaved weeds in the UK.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);

- a discussion of the results and their reliability;
- the main implications of the findings;
- possible future work; and
- any action resulting from the research (e.g. IP, Knowledge Transfer).

PS2709: Herbicide resistant broad leaved weeds: research required to address policy needs

Background

Broad leaved weeds represent a particular challenge for the agricultural and amenity sectors in the UK. Their diverse biological characteristics make cultural management practices particularly difficult and herbicides represent the preferred management tool for most broad leaved weed species. Herbicide resistance represents a significant threat to the continued management of broad leaved weeds in agricultural systems, particularly in the amenity sector where fewer alternative herbicide chemistries are available. Common poppy (*Papaver rhoeas*) and common chickweed (*Stellaria media*) are among the most agronomically important broad-leaved weeds in the UK at the present time and resistance to ALS inhibiting herbicides occurs in both species in the UK and in several other countries.

The Pesticides Safety Directorate (PSD) is required to evaluate submissions from companies for registration of herbicides targeting broad leaved weeds. In order to do this the resistance risk and the proposed resistance management strategy relating to any herbicide registration must be evaluated in light of relevant and up to date information and this was the motivation for much of the experimental work carried out on resistant chickweed and poppy. The final resistance risk profile for any new herbicide registration must take into account a number of factors and these must be combined in an appropriate way; a review of resistance in broad leaved weeds and a resistance risk matrix were the outcomes from this part of the project.

Scientific objectives

The overall objective was to carry out research required to address current policy needs for the registration of herbicides targeting broad leaved weed species in the agricultural and amenity sectors. The aim was to produce an effective and easy to use risk matrix for use in the assessment of resistance risk. The specific objectives contributing to this overall goal were as follows:

1. To review the current status of herbicide resistance in broad leaved weeds worldwide in relation to their population biology, ‘weediness’ and response to herbicides, in order to help predict which species are likely to evolve resistance in the UK in the agricultural, horticultural, industrial and amenity sectors.
2. To determine the resistance characteristics of resistant chickweed and poppy populations in order to understand the extent of cross-resistance to herbicides with the same, and different, modes of action and the consequential impact on management strategies.
3. To determine the biochemical and molecular basis of resistance in broad-leaved weeds in the UK in order to facilitate the development of diagnostic tools for the detection and characterisation of resistance.
4. To maintain a “watching brief” on potential new cases of herbicide-resistance and, if appropriate, develop new testing methodologies and procedures so that the extent of any problems can be better quantified.
5. To undertake technology transfer initiatives to inform suppliers and users of herbicides in the agricultural, horticultural, industrial and amenity sectors of the risks posed by herbicide-resistance and to promote appropriate prevention and management strategies.

The report deals with each of these objectives in turn, including the methods used, key results, and conclusions. The final discussion gives an over view of the results in terms of their wider implications and limitations.

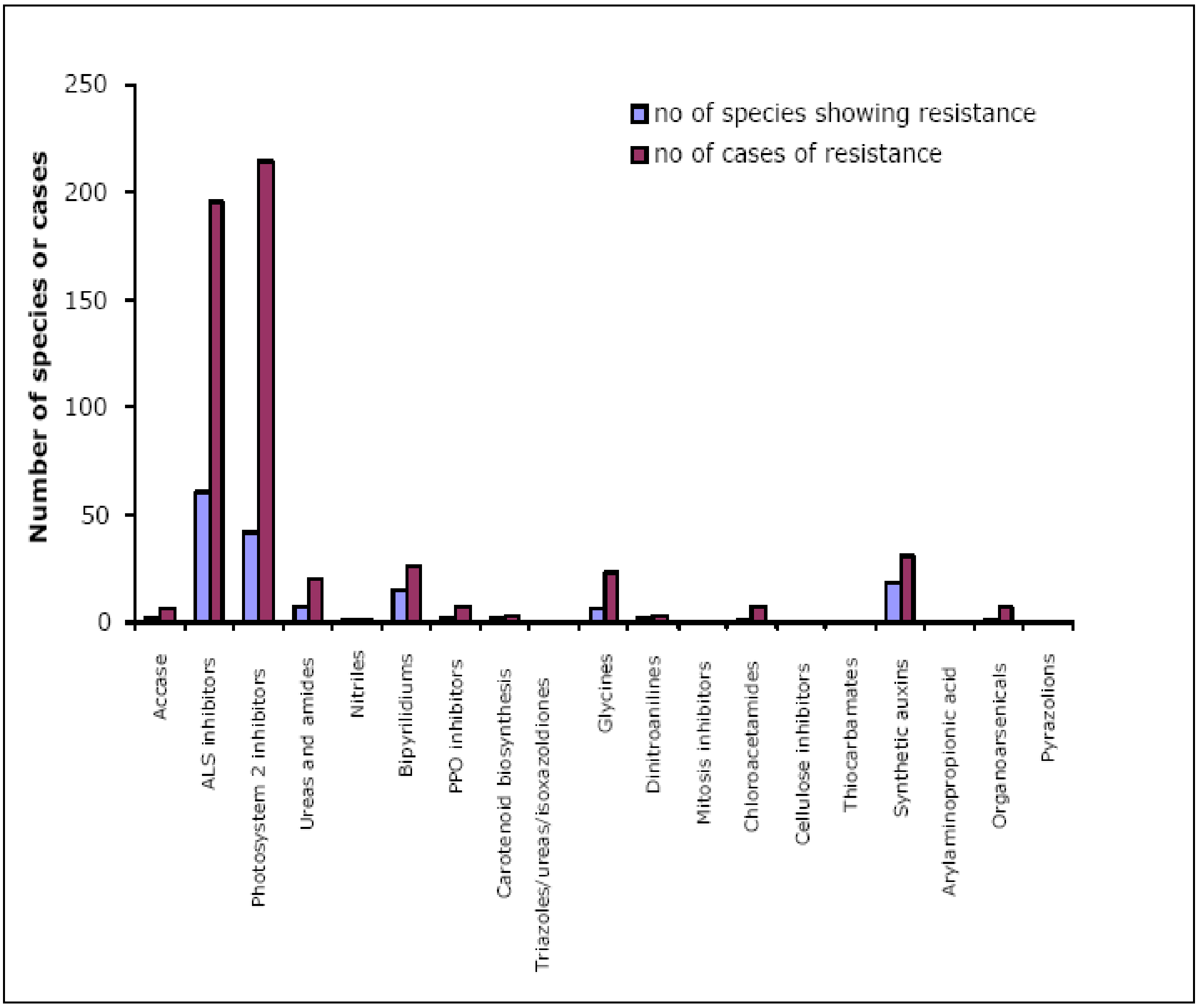
Objective 1. To review the current status of herbicide resistance in broad leaved weeds worldwide in order to help predict which species are likely to evolve resistance in the UK

1.1 Review of broad-leaved weed resistance

A comprehensive review was completed by the end of 2007. The review comprised a history of herbicide resistance in broad leaved weed (BLW) species worldwide, a summary of work done in the UK to date, and an appraisal of resistance risks approached separately from a herbicide standpoint (risks from the chemical) and from the perspective of the particular weed (biological risk factors). The review summarised the current literature on herbicide resistant BLW and used the resulting data to rank the different chemical and biological herbicide resistance risk factors in terms of their estimated contribution to overall risk. An effort was made to achieve objectivity in the ranking process through statistical analysis of the available data in order to show any correlations between number of herbicide resistance cases and specific risk factor. The process did allow some factors to be dismissed as irrelevant for specific consideration in production of the risk matrix.

The BLW resistance review highlighted herbicide chemistry as the single most important factor in terms of resistance risk for broad leaved weed species worldwide and in the UK. Particular chemistry, such as the ALS inhibitor and photosystem II (triazine) group, are disproportionately associated with cases of herbicide resistance in BLW species while alternatives like the synthetic auxins have been in use for over 60 years with relatively few cases overall (Fig. 1). Some herbicide classes were difficult to assess, and the glycines (and in particular glyphosate) defied easy categorisation. This was in part due to the relatively short length of time glyphosate has been available compared to some other herbicide chemistry and the changing picture of global resistance to glyphosate as new cases are discovered. The example of the United States, where glyphosate resistance among *Conyza*, *Ambrosia*, and *Amaranthus* species is increasing rapidly, provided an interesting contrast to the current UK situation. An interesting correlation was observed between the area sown with glyphosate tolerant crops in the USA and the number of glyphosate resistance cases reported in the weed *Conyza canadensis*. With glyphosate tolerant crops unlikely to be introduced on a wide scale into the UK for the foreseeable future, the risk of glyphosate resistance in UK agronomic systems may be lower than in some other countries. Glyphosate is used very widely in the UK amenity sector for total weed control however, and although no cases of resistance have been reported so far, a particular risk was identified as a consequence of increased reliance on this herbicide.

Figure 1. The number of herbicide-resistant broad-leaved weed species and total number of reported cases of resistance for each different herbicide mode of action (Heap 2008)



The biological risk factors affecting herbicide resistance were analysed using data from the International Survey of Herbicide Resistant Weeds database (ISHRW, Heap, 2007), and included 129 broad leaved weeds. The most important risk factors in terms of correlation with the number of cases of reported resistance from the database were judged to be the potential for rapid population increase and the mating system of the weed, with out-crossing species being more likely to show more cases of herbicide resistance. While some of the correlations derived from the analysis were somewhat tenuous due to limited data availability, they did allow several factors such as ploidy and generation time to be ruled out as less important. Overall the analysis of biological factors in the likelihood of resistance development highlighted "previous cases" as the most important factor (Table 1). It makes sense that weed species that have developed resistance to any herbicide class in a particular agronomic or amenity situation present a high resistance risk in similar situations and with similar herbicides. The tendency for certain species to be successful weeds is perhaps best summed up using the term "weediness" rather than by considering every factor independently, and a discussion of this is provided in the review.

Herbicide resistant common chickweed and common poppy are particular problems in UK agriculture and the review provided a summary of research carried out to date on herbicide resistance and general agronomy of these weed species. Poppy is a serious weed in the UK due to its high competitiveness with crop species and its potential to significantly affect yield. Common poppy is an autumn germinating weed which has very high seed persistence and high seed production potential, making it very difficult to deal with using cultural control methods. Consequently herbicides are essential for control of poppy in UK agriculture and any resistance problems are likely to have serious consequences. Herbicide resistance in UK populations of common poppy is currently limited to ALS inhibitor chemistry and was first detected in 2001. At the time of writing the BLW review ALS inhibitor resistant poppy had been detected across at least 7 counties of England, with ALS resistant chickweed being reported in 5 English and 6 Scottish counties. Chickweed is a less competitive weed than poppy, has lower seed production potential, and has less persistent seed, although it is still very prolific. Unlike poppy, chickweed is primarily self pollinating. Chickweed is a very common and diverse weed in British agriculture, adapting itself to a wide variety of different agronomic situations. As with poppy, herbicide resistance in chickweed could constitute a very serious problem in the long term and preventing or slowing the emergence of further cases is a priority.

Table 1. The top broad leaved weed herbicide resistance cases reported worldwide (source: ISHRW, Heap, 2009). Cases refer to individual reports on the ISHRW website. Figures in parentheses show ranking.

			Herbicide type	
Species	Common name	Total cases	C1 (Triazines)	All other herbicides
Chenopodium album	Lambsquarters, Fat Hen	41 (1)	37 (1)	4 (21)
Conyza canadensis	Horseweed	40 (2)	11(7)	31 (1)
Amaranthus retroflexus	Redroot pigweed	36 (3)	25 (2)	13 (5)
Kochia scoparia	Kochia	33 (4)	11 (5)	25 (2)
Amaranthus hybridus	Smooth Pigweed	23 (5)	18 (3)	5 (15)
Amaranthus rudis	Common Waterhemp	22 (6)	8 (8)	17 (3)
Amaranthus palmeri	Palmer Amaranth	17 (7)	4 (11)	13 (5)
Xanthium strumarium	Common Cocklebur	17 (7)	0 (31)	17 (3)
Senecio vulgaris	Common Groundsel	15 (9)	13 (4)	2 (29)
Ambrosia artemisifolia	Common Ragweed	13 (10)	3 (15)	10 (8)
Conyza bonariensis	Hairy fleabane	12 (11)	2 (20)	10 (8)
Solanum nigrum	Black Nightshade	12 (11)	11 (7)	1 (30)
Stellaria media	Common Chickweed	11 (14)	1 (30)	10 (8)
Papaver rhoeas	Corn poppy	5 (20)	0 (47)	5 (15)

The influence of cultivations and cultural control methods was examined in the review as a modifying factor to overall herbicide risk. Cultural control measures such as sowing date, rotations, and cultivations provide a sustainable strategy for mitigating against the development of herbicide resistance but are often highly dependent on particular agronomic systems, weather conditions, and are sometimes ruled out on cost benefit terms. Since cultural control practices vary considerably they do not provide a consistent effect and so are difficult to factor into any risk analysis. It is suggested that cultural recommendations accompanying herbicide registration applications from companies be assessed on a case by case basis in terms of their likely implementation and effects within any particular agronomic system.

1.2 Resistance risk matrix

Building on the analysis and research carried out for the BLW review, a resistance risk report and matrix were produced allowing estimation of resistance risk according to chemical and biological factors previously identified, and of the combined overall risk for any given herbicide and target weed combination. The estimation of total risk involved in the matrix was based on estimations of the relative influence of the different risk factors identified in the review. Compared to the review, the number of risk factors in the matrix was much reduced to include only what were felt to be the most important, with some factors being combined to produce a practical, simple and easy to use system. Estimated risk weightings were tested using real herbicide resistance cases until a working balance was agreed upon with herbicide risk factors given greater weighting overall than weed biology risk factors. An important advantage of the proposed risk matrix is the capacity to give a quantitative judgement incorporating degrees of risk rather than a qualitative "black and white" approach as used in some other risk matrices. We believe this makes it more flexible and user friendly.

As suggested in the review, modifying factors such as cultural control were not included in the formal risk matrix and were instead highlighted in a separate section. Since non-chemical control methods are very situation specific it was felt that including them as part of the matrix would be potentially misleading. A section was included in the risk matrix report explaining how to use modifiers and their relative importance in different situations.

The final output from the risk matrix was a suggested overall risk rating system (Table 2) with four categories ranging from low to very high. Overall the resistance risk matrix is flexible (factors can be removed or modified according to specific situations) and easy to use. Current risk weightings have been tested for a variety of British and European weed and non-weed species and have been found to produce an appropriate prediction of future herbicide resistance development in most cases.

Table 2. Suggested interpretation of combined risk ratings from the risk matrix

			Herbicide risk		
	Score		<15	15-24	>25
		Category	Low	Moderate	High
Weed biology risk	<15	Low	Low	Moderate	Moderate
	15-24	Moderate	Low	Moderate	High
	>25	High	Moderate	High	Very High

Objective 2. To determine the resistance characteristics of resistant chickweed and poppy populations in order to understand the extent of cross-resistance and the consequential impact on management strategies.

2.1 Poppy dose response experiment

A number of resistant poppy populations are held at Rothamsted and we are grateful to DuPont for providing many of these. The dose response experiment concentrated on three populations which showed resistance to ALS inhibiting herbicides in previous pot screening tests: DEV001 from Essex, DEV002 from Sussex, DK001 from Cambridge, and the susceptible standard HERB97. The aim was to quantify the degree of resistance to metsulfuron in the known resistant populations, calculate resistance indices by comparison with the susceptible standard, and investigate any differences between different populations. An additional goal was to investigate the possibility of cross resistance to auxin group herbicides in SU resistant poppy using MCPA. Cross resistance to auxin herbicides is often found in European ALS resistant poppy populations and represents a major threat to the continued control of common poppy in UK cereal crops.

Poppy seeds were sown into trays of potting compost and transplanted into 5 x 5 cm pots. Spraying took place at the 7-12 cm rosette stage using a track sprayer delivering 246 L spray solution ha^{-1} at 210 kPa through a single 'Teejet' TP110015VK flat fan nozzle when 120 plants from each resistant population (8 doses x 15 reps) received metsulfuron ("Ally") at 48, 24, 12, **6**, 3, 1.5, 0.75 and 0.375 g a.i. ha^{-1}, while the same number received MCPA ("MCPA amine 50") at 5000, 2500, **1250**, 625, 312.5, 156.25, 78.125 and 39.0625 g a.i. ha^{-1} (field rates in bold). The susceptible standard population received metsulfuron from 24 to 0.1875 g a.i. ha^{-1}. A total of 35 untreated plants were included per population and pots were randomised after spraying. A plant harvest was completed 24 and 35 days after treatment with metsulfuron and MCPA respectively.

- MLP was used to fit four parameter logistic curves to fresh foliage weight data after treatment allowing calculation of ED_{50} values and resistance indices compared to the susceptible HERB97 population.
- High levels of metsulfuron resistance were observed for all populations compared to the susceptible standard HERB97 (Figure 2). HERB97 ED_{50} was determined to be 2.6 g a.i. ha^{-1} while ED_{50} values for the DEV001, DEV002 and DK001 populations were 177.8, >48 and 36.7 g a.i. ha^{-1} respectively, with calculated resistance ratios of 68, 18 and 14.
- Fitted dose response curves from MCPA treated plants showed little difference between populations based on resistant/susceptible (R/S) ratios. DEV001 showed the highest levels of insensitivity to MCPA which corresponded to an ED_{50} 1.8 times that of the susceptible standard. All populations were well controlled following field rate application of MCPA.

Figure 2. Effect of metsulfuron on poppy fresh weights expressed as a percentage of the mean untreated fresh weight

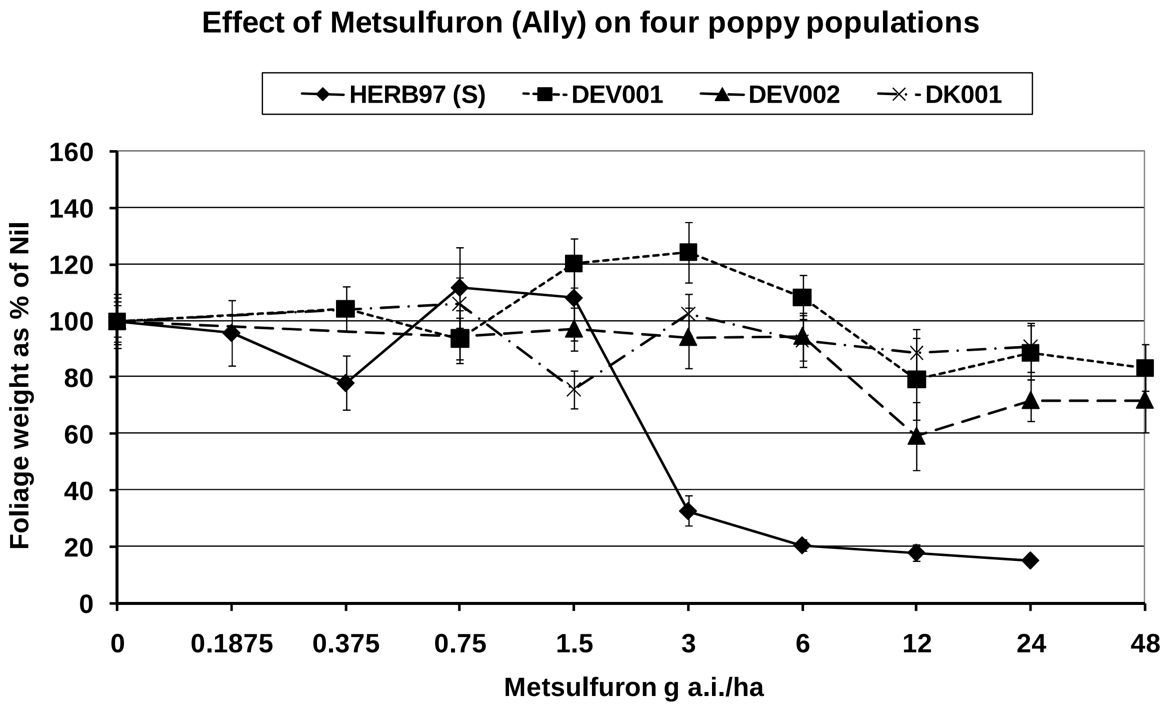
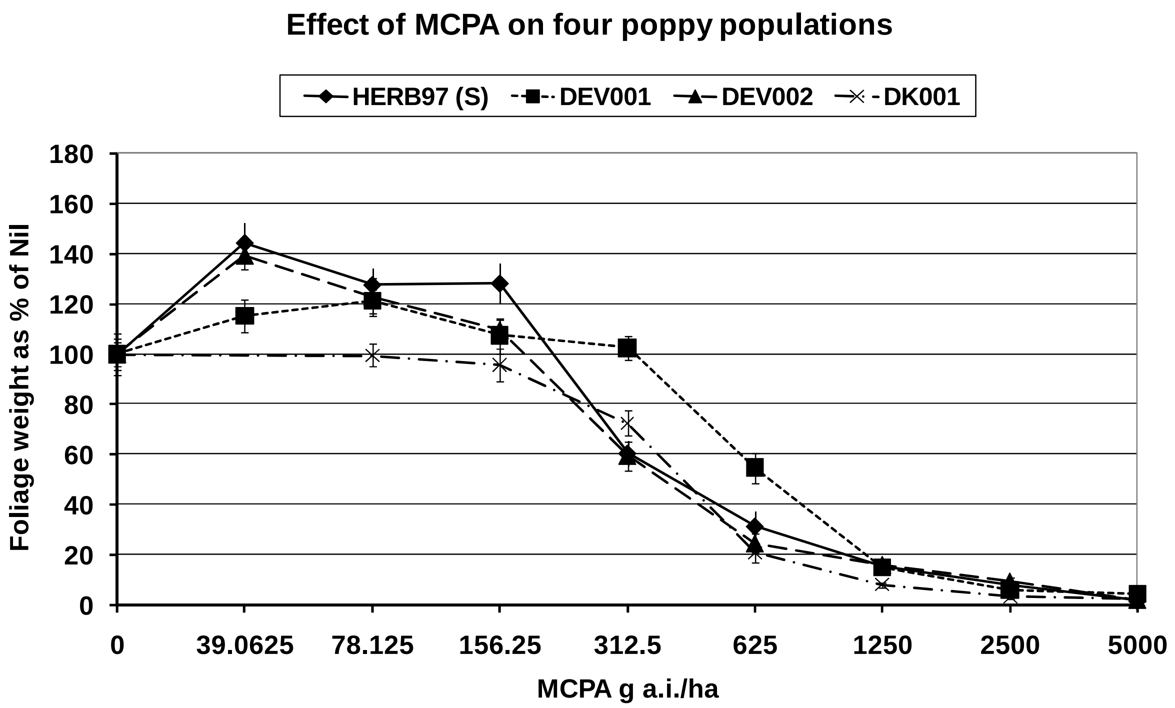


Figure 3. Effect of MCPA on poppy fresh weights expressed as a percentage of the mean untreated fresh weight



2.2 Poppy outdoor container experiment

Poppy dose response work was followed up with a container experiment involving an increased range of herbicides which was aimed at confirming that resistance in the glasshouse transferred to the outdoor conditions and identifying good alternative herbicide options for control of ALS resistant poppy populations. The experiment was set up as a replicate block design with 6 treatments (including untreated), 4 populations, and 3 replicates. Each replicate block contained 24 containers (285mm L x 185mm W x 130mm D) with 12 poppy plants per container in potting compost. Herbicide treatments were applied at the 7-12 cm rosette stage and were as follows: metsulfuron ("Ally") at 6g a.i. ha^{-1}, tribenuron ("Quantum") at 15g a.i. ha^{-1}, MCPA ("MCPA amine 50") at 1250g a.i. ha^{-1}, pendimethalin ("Stomp") at 2000g a.i. ha^{-1} and ioxynil+bromoxynil ("OxytrilCM") at 400 + 400 g a.i. ha^{-1}. The four populations used were the same as in the glasshouse dose response experiment. Containers were randomised by replicate block.

Containers were sprayed on 2 April 2007 using a track sprayer as described in section 2.1. Fresh foliage weights and the number of surviving plants per container were recorded on 2 May 2007. Analysis of variance was conducted using percentage reductions in fresh weights relative to untreated plants.

Table 3. Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants in outdoor containers

Population	Mean % reduction in fresh weight compared to nils				
	Metsulfuron	Tribenuron	MCPA	Pendimethalin	Ioxynil+Bromoxynil
HERB97(S)	92.7	97.1	72.0	64.3	99.4
DEV001	13.0	0.4	46.8	53.0	97.3
DEV002	8.5	20.1	77.3	40.6	99.8
DK001	15.6	8.6	86.2	70.4	99.6
S.E.	6.0				
LSD (5%)	17.1				

- Metsulfuron, tribenuron, and ioxynil + bromoxynil provided high levels of control of the susceptible standard HERB97, with greater than 90% reduction in fresh foliage weight in treated compared to untreated containers (Table 3). MCPA and pendimethalin provided reasonable levels of control with 72% and 64% reduction in fresh weight, respectively.
- The populations DEV001, DEV002 and DK001 which showed high levels of resistance to metsulfuron in glasshouse dose response tests were again highly resistant to both metsulfuron and the alternative sulfonylurea tribenuron. Differences between resistant populations were generally non-significant although a statistically significant difference was observed between DEV001 and DEV002 after treatment with tribenuron. High-level resistance to metsulfuron and tribenuron was present in >90% of plants from all resistant populations.
- DEV001 showed the highest levels of cross resistance to MCPA with 47% reduction in fresh weight compared to untreated plants, supporting the dose response result. Levels of control for other populations following treatment with MCPA were not significantly different.
- DEV001 showed many more survivors than other populations following treatment with MCPA but surviving DEV001 plants were damaged and resistance was not clear cut.
- With the exception of DEV002, no population showed significant differences in response compared to the susceptible standard following treatment with pendimethalin. Levels of control were not greater than 70% for any population, probably due to post-emergent application which was not ideal.
- All populations were well controlled with ioxynil + bromoxynil with >95% reduction in fresh weight compared to untreated containers in all cases and very few or no survivors. Ioxynil + bromoxynil mixture provided the best overall alternative to sulfonylurea herbicides for control of resistant poppy populations with MCPA also providing a viable alternative.
- Results from the outdoor container experiment fully supported those from the glasshouse assay with confirmed resistant populations continuing to show a very high degree of insensitivity to sulfonylureas under outdoor conditions.

2.3 Poppy resistance screening experiment

Following on from the confirmation of sulfonylurea resistant poppy, a larger screening experiment was set up in order to identify the prevalence of resistance to metsulfuron in the ten remaining uncharacterised poppy populations held at Rothamsted, and also to test the larger sample for resistance to MCPA.

The experiment was set up as a randomised block glasshouse trial with 2 herbicide treatments, 12 populations including a sensitive standard and a resistant standard, and 16 replicate pots per treatment for each population. Eight untreated controls were also included per population. Each pot contained a single plant grown from seed in seed trays and transplanted. Herbicide treatments were metsulfuron ("Ally") at 6 g a.i. ha^{-1} and MCPA ("MCPA amine 50") at 1250 g a.i. ha^{-1}. Populations included the susceptible standard HERB97 and the resistant standard DK001 which was tested previously. Other populations were collected from various field sites where control problems were reported. Test population names and locations were as follows: CC001 (Cambs), CC002

(Cambs), CC003 (Cambs), CC004 (Cambs), AMC001 (Yorks), AMC002 (Yorks), AMC003 (Yorks), CBC001 (Cambs), CAMBS (Cambs), and ADAS (Cambs). The experiment was conducted in a glasshouse with supplementary lighting at ambient temperatures. Capillary matting was used to ensure even levels of moisture retention between pots and plants were watered daily.

Spraying was carried out as described in section 2.1 when poppies were at the 7-12 cm rosette stage using a track sprayer as described previously, and pots were harvested 21 days after treatment. A decision was made to harvest metsulfuron pots even though complete control of the susceptible standard had not been achieved because plants were bolting due to very high external temperatures and untreated controls were starting to suffer from nutrient deficiency. Analysis of variance was conducted using percentage reductions in fresh weights relative to untreated plants.

Table 4. Analysis of variance comparing mean percentage reduction in plant weight relative to untreated plants

Population	Mean estimated % reduction in fresh weight compared to untreated controls	
	Metsulfuron	MCPA
HERB97	41.7	92.3
DK001	8.0	87.2
CC001	10.4	90.6
CC002	1.8	77.5
CC003	-13.3	85.0
CC004	5.7	89.7
AMC001	38.9	91.5
AMC002	4.1	63.5
AMC003	-1.6	80.9
CBC001	-27.2	79.9
CAMBS	4.7	85.9
ADAS	25.3	71.7
S.E.	8.7	5.4
LSD (5%)	22.5	14.3

- Metsulfuron at 6g a.i. ha^{-1} did not provide good control of the susceptible standard (41.7 % reduction in fresh weight). This was due to harvesting being carried out too early. Nearly all populations were significantly less well controlled than the susceptible standard after treatment with metsulfuron (Table 4). Only the AMC001 and ADAS populations showed non significant differences in level of control compared to HERB97. All metsulfuron 'resistant' populations with the exception of CC001 showed lower levels of control than the resistant standard DK001. For this reason it is probably safe to assume that they do show some real level of resistance to metsulfuron, despite the inadequate control achieved for the susceptible standard.
- Control of the susceptible standard was achieved with MCPA at 1250g a.i. ha^{-1} (> 90 % reduction compared to untreated controls).
- Three populations (CC002, AMC002 and ADAS) were significantly less well controlled than the susceptible standard after treatment with MCPA. Levels of control with MCPA were comparable to, although slightly higher than, those achieved in previous experiments.
- Overall these results indicate that the majority of the field poppy samples held at Rothamsted are resistant to the ALS inhibiting sulfonylurea herbicide metsulfuron. Most (five) resistant samples came from Cambridgeshire, with Yorkshire contributing three and Essex and Sussex yielding one sample each in total.

2.4 Chickweed dose response experiment

Characterisation of resistant chickweed populations began with a dose response experiment designed to investigate the effects of four herbicides with different modes of action on four different resistant chickweed biotypes from Cornwall (CORN), Kent (KENT), Aberdeenshire (ABER), and Perthshire (SCOT). A susceptible standard population (UKA) was included for comparison. All four of the resistant populations were collected from fields where farmers or consultants reported a failure of control with herbicide. The herbicides selected for dose response characterisation were fluroxypyr ("Starane"), mecoprop-P ("Duplosan"), florasulam ("Boxer"), and metsulfuron-methyl ("Ally"). Herbicides were selected on the basis of being current options for spring control of chickweed in UK cereal crops and to provide a range of different modes of action. Two different ALS inhibitors (the sulfonylurea mesosulfuron-methyl and the triazolopyrimidine florasulam) were included in order to investigate cross resistance to different herbicide groups with this mode of action.

Plants from all populations were grown in the glasshouse to the rosette stage (8-15 cm) before spraying at a range of different doses including the usual field rate (underlined in bold). Treatments were metsulfuron-methyl at 12, **6**, 3, 1.5, 0.75, 0.375 and 0.1875 g a.i. ha^{-1}; florasulam at 10, **5**, 2.5, 1.25, 0.625, 0.3125 and 0.15626 g a.i. ha^{-1}, fluroxypyr at 400, **200**, 100, 50, 25, 12.5 and 6.25 g a.i. ha^{-1}, and mecoprop-P at 2400, **1200**, 600, 300, 150, 75 and 37.5 g a.i. ha^{-1}. A total of 10 reps with a single plant per pot were included at each dose along with a total of 20 untreated controls per population. All plants were sprayed using a track sprayer as described in section 2.1 and were then returned to the glasshouse and randomised in replicate blocks. A plant harvest was carried out 20 days after treatment and fresh foliage weights were recorded. The statistics program MLP was used to fit four parameter logistic curves to fresh weight data allowing calculation of ED_{50} values.

- High levels of resistance to the sulfonylurea herbicide metsulfuron were observed in all resistant biotypes compared to the susceptible standard (Figure 4). The highest dose of twice field rate was not sufficient to cause reduction in fresh foliage weight for all of the resistant biotypes.
- The calculated metsulfuron ED_{50} value for the UKA susceptible standard was 0.25 g a.i. ha^{-1}. No ED_{50} values could be calculated for the four resistant populations, but these were substantially greater than 12 g a.i. ha^{-1}. Consequently RI (resistance index) values for all resistant populations were over 48 compared to the susceptible standard.
- Resistance to the ALS inhibiting herbicide florasulam was observed in the ABER biotype with an ED50 of 163 g a.i. ha^{-1} and an R/S ratio of >1046. ABER was the only biotype demonstrating significant levels of resistance to florasulam. The fact that triazolopyrimidine and sulfonylurea cross resistance is observed in the ABER population but not in any other sulfonylurea resistant population suggests that the ALS mutation conferring resistance in the ABER population is different to that in the other populations.
- No clear evidence of resistance to mecoprop and fluroxypyr was found. R/S ratios were highest for the SCOT biotype (1.3 fold difference after treatment with mecoprop-P, 1.5 fold difference with fluroxypyr) and the ABER biotype (1.4 fold difference with fluroxypyr) respectively.
- The lack of significant levels of resistance to mecoprop-P and fluroxypyr in the ALS inhibitor resistant chickweed biotypes suggests that mutation(s) leading to target site change in the ALS enzyme are the most likely explanation for the observed resistance.

Figure 4. Effect of metsulfuron on chickweed fresh weights expressed as a percentage of the mean untreated fresh weight

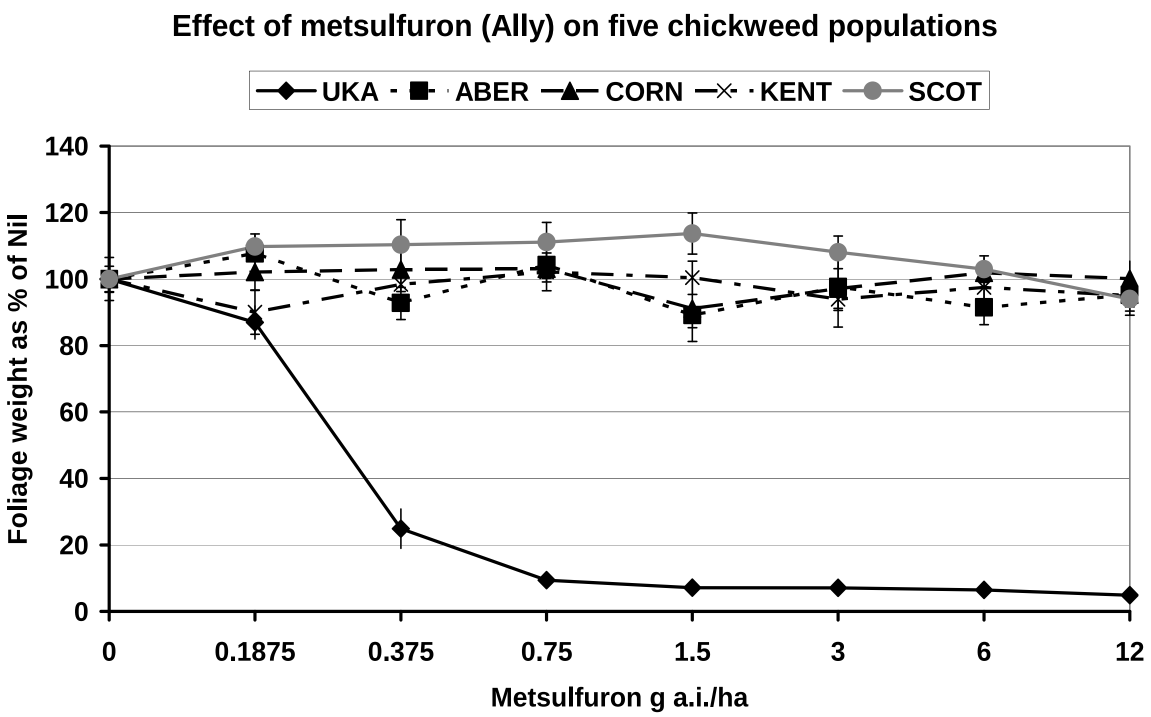
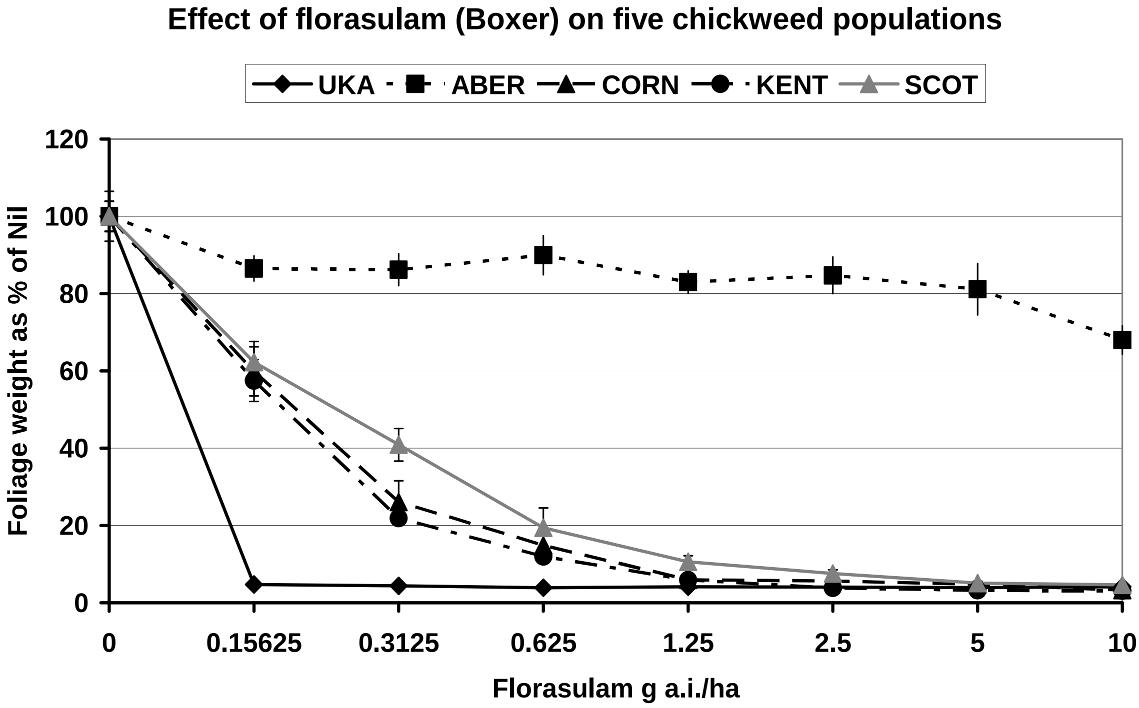


Figure 5. Effect of florasulam on chickweed fresh weights expressed as a percentage of the mean untreated fresh weight



2.5 Chickweed container experiment

The chickweed dose response work was followed up with a container experiment involving the same populations. This experiment was aimed at confirming that resistance in the glasshouse transferred to an outdoor setting and in establishing the reliability of alternative herbicides for control of resistant chickweed populations.

The experiment was arranged on a sand-bed as a replicate block design with 5 treatments (including untreated), 5 populations, and 3 replicates. Twelve chickweed seedlings were planted into each container on 20 Feb 2006 and herbicides (metsulfuron 6 g a.i. ha^{-1}; florasulam 5 g a.i. ha^{-1}; mecoprop-P 1200 g a.i. ha^{-1}; fluroxypyr 200 g a.i. ha^{-1}) were applied at field rate to plants at the 12-20 cm rosette stage using the method described in section 2.1. After spraying all containers were randomised by replicate block.

Plants were harvested 28 days after treatment and foliage weights were subjected to analysis of variance using percentage reductions in fresh weights relative to untreated plants.

Table 5. Analysis of variance comparing mean percentage reduction in plant weight relative to untreated plants

Population	Mean estimated % reduction in fresh weight compared to untreated controls			
	Metsulfuron	Florasulam	Mecoprop-P	Fluroxypyr
UKA	79.8	91.9	88.1	83.5
ABER	-9.9	7.2	83.8	82.6
CORN	-3.5	87.4	89.0	83.2
KENT	14.2	87.3	89.8	88.5
SCOT	19.5	90.4	93.0	89.3
S.E.	5.5	4.3	1.3	0.9
LSD (5%)	17.8	14.1	4.1	3.1

- The susceptible standard UKA population was well controlled by field rate applications of all herbicides. Percentage reduction compared to mean untreated weight was at least 80 % in all cases.
- The ABER, CORN, KENT and SCOT populations were significantly less well controlled after field rate application of metsulfuron than the susceptible standard UKA population (Table 5). Metsulfuron provided

around 80 % reduction in fresh weight for UKA, while the best controlled of the resistant populations was SCOT with 19.5 % reduction in fresh weight. All four resistant chickweed populations were rated RRR (highly resistant) in terms of their response to the sulfonylurea metsulfuron.

- Application of florasulam at 5 g a.i. ha^{-1} provided good control of all populations with the exception of ABER which was significantly less well controlled than the susceptible standard in terms of percentage reduction in fresh weight. ABER was the only population showing cross resistance to both metsulfuron and florasulam.
- Good control of all populations was achieved using field rate applications of mecoprop-P and fluroxypyr. The lack of resistance to these alternative herbicides means that control of ALS resistant chickweed should be sustainable for the foreseeable future.

2.6 Chickweed container experiment with grass-weed herbicides

This experiment was designed in order to examine the resistance profile of confirmed metsulfuron resistant chickweed populations to a variety of grass weed herbicides. The effect of grass weed herbicides on non-target broad leaved weeds in cereal cropping systems is of interest for several reasons including product licensing, selection for resistance in non-target weed species, the possible redundancy of spraying in spring with a specific broad-leaf ALS inhibitor when target weeds have already been controlled, and cross resistance issues (i.e. are metsulfuron and florasulam resistant populations of chickweed also cross resistant to grass weed ALS inhibitors of sulfonylurea chemistry and to other chemical classes; does the resistance spectrum provide any opportunities for control of resistant populations with grass weed herbicides). The non-selective herbicide Imazapyr was included as a representative of imidazolinone chemistry.

The experiment was set up as a replicate block design with 5 treatments (including nils), 3 populations, and 3 replicates. Each replicate block contained 15 containers, with 12 chickweed plants per container. Herbicide treatments (applied as described in section 2.1) were propoxycarbazone-sodium ("Attribut") at 70 g a.i. ha^{-1}, iodosulfuron-methyl ("Hussar") at 9.6 g a.i. ha^{-1}, mesosulfuron-methyl + iodosulfuron-methyl mixture ("Atlantis") at 12 + 2.4 g a.i. ha^{-1} and imazapyr (Arsenal) at 375 g a.i. ha^{-1}. The adjuvants "Biopower" at 0.5 % total volume, and "Comulin oil" at 1 L ha^{-1} were included with the Atlantis and Attribut treatments respectively. Included in the experiment were the confirmed metsulfuron resistant SCOT and ABER chickweed populations, along with the susceptible standard UKA. Containers were randomised by replicate block and the experiment was conducted on a sand bed under protective netting with watering provided daily.

Containers were sprayed on 18 Mar 2008 when plants were at the 12-20 cm rosette stage and all plants were harvested on 8 May 2008. Fresh foliage weights were measured for each container and the number of surviving plants counted. Foliage weight data were subjected to analysis of variance using percentage reductions in fresh weights relative to untreated plants and reduction in plant numbers.

Table 6. Analysis of variance comparing mean percentage reduction in chickweed plant weight relative to untreated plants

	Mean percentage reduction in fresh weight compared to nils (g)			
Population	**Propoxycarbazone**	**Iodosulfuron**	**Meso + iodosulfuron**	**Imazapyr**
UKA	-1.0	99.1	99.2	99.4
SCOT	-2.4	47.2	95.3	99.2
ABER	-0.3	28.2	54.4	95.1
S.E.	4.8			
L.S.D (5%)	14.0			

- Good control of all populations was achieved with imazapyr in terms of fresh weight reduction, while propoxycarbazone offered almost no control (Table 6). Comparing plant numbers showed a significant decrease in control with imazapyr for the ABER population only.
- The grass-weed SU type ALS inhibitors (iodosulfuron and iodosulfuron + mesosulfuron mixture) seem to have greater activity on resistant chickweed than the broad leaf SU herbicide metsulfuron (note that in previous container experiments metsulfuron achieved less than 20% control of both SCOT and ABER populations). The resistant SCOT population was well controlled by the SU mixture mesosulfuron + iodosulfuron, while iodosulfuron alone damaged SCOT plants quite severely but allowed them to grow back. Comparing survivor numbers showed the SCOT population as significantly more resistant to mesosulfuron + iodosulfuron than the susceptible standard UKA, but all surviving SCOT plants were very badly damaged.

- Metsulfuron-resistant ABER plants were not killed by mesosulfuron + iodosulfuron mixture but were badly damaged before growing back (54% fresh weight reduction compared to control). Iodosulfuron alone also failed to control the ABER population but did check growth slightly (28% reduction compared to control).
- The high activity of mesosulfuron + iodosulfuron mixture against resistant chickweed was unexpected and raises issues about activity against non target weeds receiving this herbicide. The mixture proved to be more effective against resistant chickweed than iodosulfuron alone and much more effective than metsulfuron, even against a Pro197 resistant population (SCOT population, refer objective 3). Populations showing Pro197 mutation are usually assumed to be completely resistant to SU chemistry.
- Cross resistance patterns appear to be more complicated than previously thought. It is usually assumed that a mutation conferring resistance to one ALS inhibitor will also provide resistance to other ALS inhibitors of the same chemical class but results show this may not always be the case. Control of confirmed resistant chickweed (and other weed) populations may be possible using mixtures of active ingredients or different chemical classes with the same target site.

2.6 Chickweed dose response experiment with grass-weed herbicides

A second chickweed dose response experiment was carried out in order follow up on the results from the grass-weed herbicide container experiment, focusing more closely on the effect of dose on control of the metsulfuron-resistant populations ABER and SCOT with mesosulfuron + iodosulfuron, imazapyr, and iodosulfuron. A related question of interest was the relative contribution of the different constituents of mesosulfuron + iodosulfuron to metsulfuron-resistant chickweed control.

Populations selected for this experiment were the metsulfuron resistant SCOT population, the metsulfuron and florasulam resistant ABER population, and the susceptible standard UKA. Pre-germinated chickweed seedlings were planted into 5 x 5 cm pots using potting compost. Plants were grown to the 10 – 14 cm rosette stage before spraying at a range of different doses including the usual field rate (underlined in bold). Treatments were mesosulfuron + iodosulfuron mixture (Atlantis) at 0.375 + 0.075, 0.75 + 0.15, 1.5 + 0.3, 3 + 0.6, 6 + 1.2, **12 + 2.4**, 24 + 4.8 and 48 + 9.6 g a.i. ha^{-1}; iodosulfuron (Hussar) at 0.075, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8, **9.6**, 19.2 and 38.4 g a.i. ha^{-1}; and imazapyr (Arsenal) at 5.859, 11.719, 23.438, 46.875, 93.75, 187.5, **375** and 750 g a.i. ha^{-1}. A single field rate (6 g a.i. ha^{-1}) dose of metsulfuron was also included. A total of 10 reps (pots) with one plant per pot were included at each dose for each herbicide and population along with a total of 20 untreated controls. All pots were sprayed using a track sprayer as described in section 2.1 and plants were then returned to the glasshouse and fully randomised in replicate blocks.

A plant harvest was carried out 32 days after treatment and fresh foliage weights were recorded. The statistics program MLP was used to fit four parameter logistic curves to fresh foliage weight data allowing calculation of ED_{50} values and resistance indices compared to the susceptible standard population (see Figures 6 and 7).

Figure 6. Effect of iodosulfuron (Hussar) on chickweed fresh weights expressed as a percentage of the mean untreated fresh weight

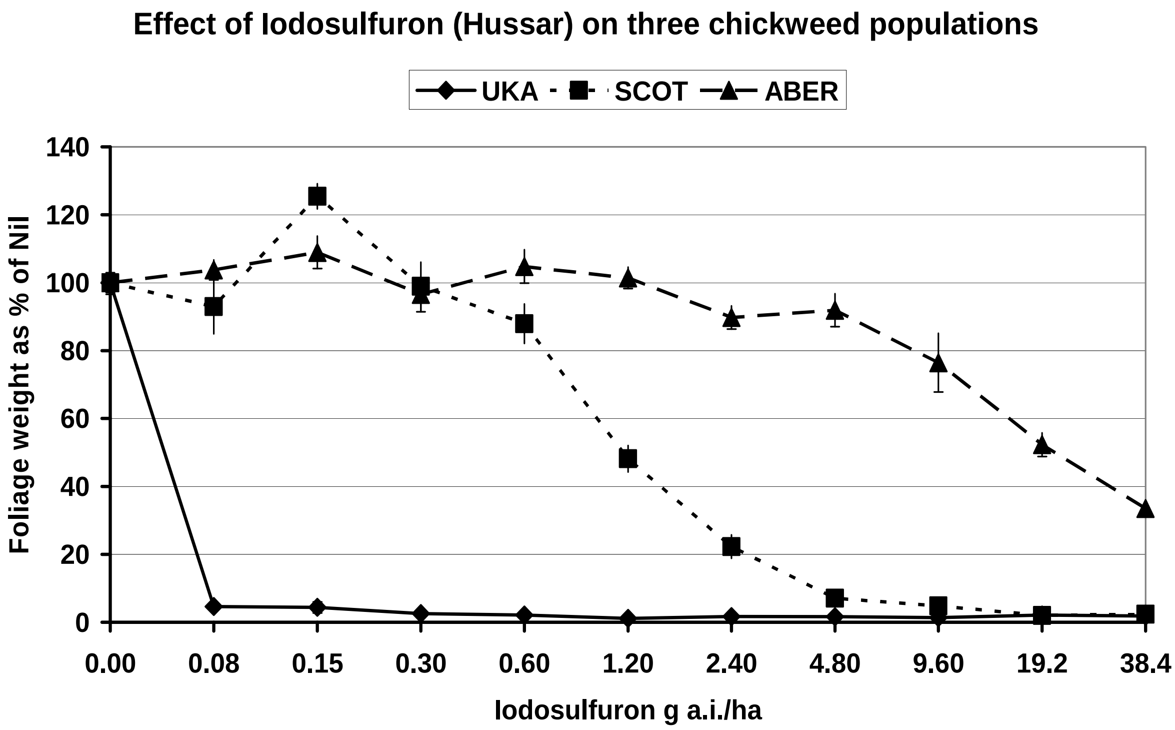
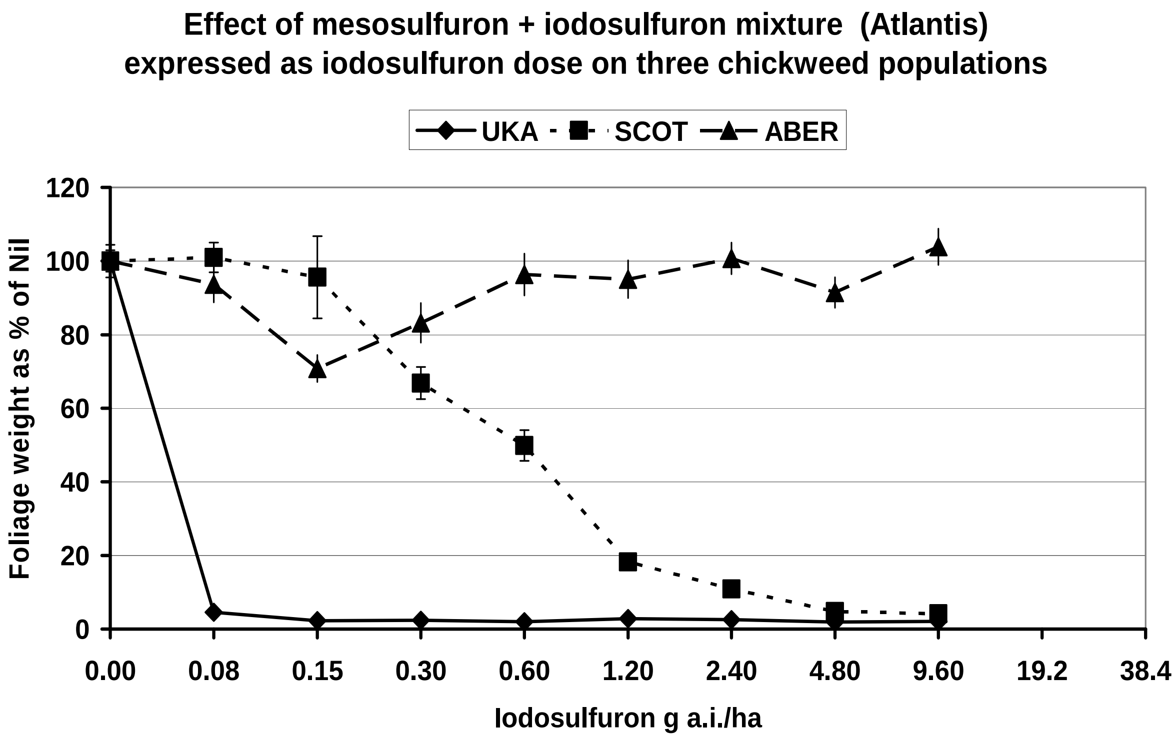


Figure 7. Effect of mesosulfuron + iodosulfuron (Atlantis) on chickweed fresh weights expressed as a percentage of the mean untreated fresh weight



- All herbicide treatments gave good control of the susceptible UKA population at the minimum applied dose; ED_{50} was lower than the minimum dose in all cases.
- Much greater levels of control of the resistant SCOT population were achieved using the grass weed sulfonylurea herbicide mesosulfuron-methyl + iodosulfuron mixture than with metsulfuron. Mesosulfuron + iodosulfuron in particular offered good control of the SCOT population with a calculated ED_{50} value of 2.5 + 0.5 g a.i. ha^{-1}, well below the field rate.

- Control of the ABER population was much poorer with an ED_{50} value above the highest dose of 48 + 9.6 g a.i. ha^{-1}.
- Field rate application of the broad leaf ALS inhibitor metsulfuron (6 g a.i. ha^{-1}) provided >98 % control of the UKA susceptible standard but only 1.9 and 10.4 % control of the SCOT and ABER populations respectively.
- Iodosulfuron alone provided lower rates of control of the metsulfuron resistant SCOT population on an iodosulfuron dose for dose basis with a calculated ED_{50} of 1.2 g a.i. ha^{-1} providing evidence that mesosulfuron provides part of the broad leaf weed activity observed for mesosulfuron + iodosulfuron mixture. Iodosulfuron ED_{50} for the ABER population was calculated as 20.4 g a.i. ha^{-1}.
- The non selective imidazolinone herbicide Arsenal (imazapyr) provided the highest levels of control overall with both UKA and SCOT populations being fully controlled at the lowest dose. ABER was the only population to demonstrate significant resistance to imazapyr with an ED_{50} around 300 g a.i. ha^{-1}. ABER plants at the top two imazapyr doses were severely damaged by the herbicide treatment.

Objective 3. To determine the molecular basis of resistance in broad-leaved weeds in the UK in order to facilitate the development of diagnostic tools for the detection and characterisation of resistance.

3.1 Poppy molecular characterisation

Sequencing of the poppy ALS gene was carried out in order to investigate the mechanism of ALS inhibitor resistance in the three different resistant poppy populations used in the glasshouse and container assays compared to the susceptible standard HERB97. Samples from the DEV001, DEV002 and DK001 populations were taken from plants sprayed with metsulfuron at various doses in the glasshouse dose response experiment described above and stored frozen at -80 °C until extraction and analysis. Samples of around 100 mg each were taken from five individual plants of the following treatment groups: Herb97 metsulfuron 3 g a.i. ha^{-1} (dead plants); DK001 metsulfuron 3 g a.i. ha^{-1} (survivors); DEV001 metsulfuron 6 g a.i. ha^{-1} (survivors); DEV002 metsulfuron 6 g a.i. ha^{-1} (survivors). Three susceptible plants were sampled from the DEV002 population 0.75 and 0.373 g a.i. ha^{-1} metsulfuron treatment groups. No susceptible plants were available from the DEV001 and DK001 populations.

All DNA 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. Primers were designed using the ALS coding sequence for *P. rhoeas* (EMBL accession number AJ577316). Forward and reverse primers were designed to span three of the five conserved domains of the ALS gene in two parts; Domains A and D near the 5' end and Domain B 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 ≥ 20 bp, T_m = 57 - 60°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.

The primer pair L1aR1a (ACCCATTTCCACCACCCACACCACC/ TACTGGACCTGGTCGGCCTGATGTAGC) was used to sequence the region around Doms A and D while the primer pair L3bR3b (GGCGCTATGGGTTTTGGGTTACCTGCTGC/ ACTCGATAAACCAAAAACAAGCCCACACCTTTAGC) was used to sequence the region around Dom B. PCR reactions were carried out using 2x PCR Master Mix (Promega). Reaction mixtures contained primers at 0.8 µM each and ~50 ng of genomic DNA in a total volume of 20 µl. Cycling reactions were performed using a Geneamp PCR System 9700 (Applied Biosystems) with conditions of 94°C for 2 minutes followed by 35 cycles of: 94°C for 30 s, 65°C for 30 s, 72°C for 1 min; with a final extension at 72°C for 10 min. 20µl of each PCR product was mixed with loading buffer and subjected to agarose gel electrophoresis. PCR product bands of the expected size from both the L1aR1a and L3bR3b primer combinations were excised from the gel and extracted using a Qiagen gel extraction kit then direct sequencing of PCR products was performed using a BigDye® Terminator v1.1 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. Analysis of sequence data was performed using Vector NTI Advance 9.0 software (Invitrogen, UK).

- Sequence analysis confirmed that all metsulfuron resistant individuals from the DK001, DEV001 and DEV002 populations showed a single nucleotide polymorphism in the second position of the Pro197 codon of the poppy ALS gene compared to the susceptible standard HERB97 and susceptible individuals of the DEV002 population. All susceptible HERB97 individuals appeared homozygous CCT at position 197 coding for proline.
- Four of five highly resistant individuals from the DK001 population appeared homozygous for a Pro-197-Leu substitution (CTT) while the remaining individual appeared heterozygous. In all individuals single nucleotide polymorphism was associated with high levels of resistance to metsulfuron.

- Three of five resistant individuals from the DEV001 population appeared homozygous Pro-197-Leu while the remaining two appeared heterozygous.
- An alternative target site change was observed in the DEV002 population with all resistant individuals showing Pro-197-His substitution (CAT). Three individuals appeared homozygous while two appeared heterozygous. Three susceptible DEV001 plants appeared homozygous for Pro at position 197 showing segregation of the resistant trait with Pro197 target site change.
- No difference was observed in the Dom B region of resistant plants compared to susceptible individuals. All plants, resistant or susceptible, showed TGG at position 574 coding for tryptophan, indicating no mutation at this position.

According to the International Survey of Herbicide Resistant Weeds (ISHRW) at www.weedscience.org, amino acid substitution at proline 197 of the ALS gene is by far the most common target site substitution conferring ALS resistance in weeds. Both Leucine and Histidine substitutions are common in other weed species and Histidine substitution was reported in resistant common poppy from Italy in 2004. Typically Pro197 mutation confers resistance to sulfonyluea and sometimes tryazolopyrimidine herbicides, but not to imidazolinones. However sulfonylureas are the only ALS inhibitors currently registered for poppy control in the UK and the best course of action available in cases of resistance is to switch to alternative modes of action.

3.2 Chickweed molecular characterisation

A segregation experiment was carried out in order to provide material for later molecular testing using two different resistant common chickweed populations compared to the susceptible standard UKA. Seedlings from the ABER, SCOT and UKA populations were grown to the 10-15cm rosette stage and 48 healthy individuals selected and numbered from each population. 100 mg leaf samples were removed from each plant and all were then sprayed with field rate applications of either florasulam (plants 1-24) or metsulfuron (plants 25-48). All plants were assessed for herbicide injury 3 weeks after spraying using a 1-3 scale. Leaf samples were stored at -80 °C for later DNA extraction and ALS gene sequencing.

DNA extractions and sequencing were carried out as described in section 3.1. Specific *Stellaria* ALS primers were designed from rough sequences obtained using universal primers derived from an alignment of various broad leaved ALS sequences. Universal primers were designed using an alignment of full or partial ALS coding sequences from *Amaranthus tuberculatus* (EMBL accession number EF157819), *Arabidopsis thaliana* (NM_114714), and *Papaver rhoeas* (AJ577316). PCR conditions for the universal primers were 94°C for 1 minute followed by 30 cycles of: 94°C for 30 sec, 55°C for 1 min, 72°C for 1.5 min; with a final extension at 72°C for 10 min, while PCR cycling conditions using *Stellaria* specific primers were 94°C for 1 minute followed by 35 cycles of: 94°C for 40 s, 60°C for 1 min, 72°C for 1 min; with a final extension at 72°C for 5 min. Specific primer sequences were as follows: the primer pair SteF1R1 (TACCCGGGTGGCGCCTCTTTAG/ ATCCCCGTCAATTGAACAAACCTCC) was used to sequence the region around Doms A and D while the primer pair SteF7R6 (GCTGACCTCTAGTGGGCTTG/ GCCTCCCTAAGATCGGACAC) was used to sequence the region around Dom B. Sequencing methodology and analysis were carried out as for the poppy samples.

- This work represents the first full characterisation of ALS resistant chickweed. Several examples are present in the literature with populations in Canada, Ireland, Denmark, and other countries. However the molecular basis of resistance has not been identified in any of these other cases and it is now possible to provide mechanistic explanations for the cross resistance patterns seen in resistant chickweed populations.
- The segregation experiment confirmed results from previous dose response and container experiments. All UKA susceptible plants were completely controlled by field rate application of metsulfuron and florasulam. The SCOT population showed high levels of metsulfuron resistance with 23 of 24 plants surviving undamaged, but no cross resistance to florasulam with all plants completely controlled. The ABER population was highly resistant to both herbicides with all plants surviving undamaged with the exception of two damaged metsulfuron survivors.
- Sequence analysis of confirmed metsulfuron resistant SCOT plants showed Pro-197-Gln amino acid substitution compared to the susceptible UKA (Pro). Analysis of florasulam susceptible SCOT plants also showed a mutation conferring Pro-197-Gln substitution, meaning that Pro197 substitution conferred resistance to the sulfonylurea metsulfuron but no cross resistance to the triazolopyrimidine florasulam in this case. All SCOT plants appeared heterozygous at position 197.
- Plants from the ABER population showed no polymorphism at the Pro197 position (CCG) and were indistinguishable from the susceptible standard UKA at this locus.
- In contrast at position 574 all ABER plants showed TGG/TTG polymorphism conferring Trp-574-Leu amino acid substitution. All plants appeared heterozygous at position 574. ABER plants survived application of both metsulfuron and florasulam and so Trp-574-Leu substitution was associated with cross resistance to ALS inhibitors of both SU and TP chemistry.
- Amino acid substitution at P197 is usually assumed to confer resistance to sulfonylureas (eg metsulfuron), sometimes triazolopyrimidines (eg florasulam), sometimes sulfonylaminocarbonyltriazolinones (eg

propoxycarbazone), and almost never imidazolinones (eg imazapyr); the effect of dose and/or specific active ingredients is rarely taken into account. However by applying the sequencing results to the pot and container experiments with chickweed it can be seen that Pro197 mutation in the SCOT population appears to confer resistance to metsulfuron up to at least 2x field rate (12 g a.i. ha^{-1}), but that resistance is overcome by the grass weed SU herbicides iodosulfuron (Hussar) and mesosulfuron + iodosulfuron mixture (Atlantis) at doses below field rate. This means that P197 substitution does not confer absolute resistance to SU herbicides in this case and that resistance is a function of dose

- Furthermore while W574 ALS mutation is usually assumed to confer high levels of resistance across the spectrum of ALS inhibiting chemistry (see ISHRW website for examples), the case of W574 resistant ABER chickweed shows that dose is again a determining factor, with imazapyr doses above 375 g a.i. ha-1 causing significant reductions in fresh weight compared to untreated controls.
- While metsulfuron and florasulam treatments were not associated with reduction in fresh weight at the highest dose rates tested, the grass weed SU herbicide iodosulfuron at 4x field rate did cause significant foliage weight reduction in ABER plants. Control of a W574 ALS mutant weed population with high dose SU herbicide shows again that resistance is not absolute and that dose and specific active ingredient are the most important factors in determining efficacy.

3.3 Diagnostic tools for the detection and characterisation of resistance in broad leaved weeds

Pot experiments were shown to provide a simple and reliable method of resistance screening for the agronomically important weeds chickweed, common poppy and scentless mayweed. Pot tests can diagnose resistance to any herbicide type and their results are directly applicable to levels of control expected in the field. Seed collection of broad leaved weeds for pot tests can be difficult, and molecular tests on leaf material can therefore be more favourable compared to grass weeds where seed collection is much easier. The advantage of using molecular assays for broad leaved weed species is that resistance is most often due to target site change rather than enhanced metabolism, and molecular assays are particularly suitable for detecting this kind of resistance. Disadvantages of molecular techniques include the time required to develop suitable assays for species where sequence data are not available and their limited applicability allowing diagnosis of resistance to only certain herbicide modes of action. Petri dish tests combine some of the advantages of both pot tests and molecular assays. They are quicker than pot tests and are able to diagnose resistance in time for the next growing season since they are not subject to the same seasonal constrains. Unfortunately broad leaved weeds are less suitable for routine Petri dish assays due to the difficulty in seed collection and small seed size.

- Pot and container experiments provide the most reliable diagnostic test available for initial characterisation of new resistance cases and allow screening of large numbers of plants at relatively low cost.
- Molecular assays based on sequencing of sections of the ALS gene were developed for the detection of resistance to ALS inhibitors in poppy and chickweed. More rapid molecular tests able to process large numbers of samples can be developed easily if required.
- Molecular tests are the most rapid method available for detection of ALS target site resistance and are able to give results for unaffected plants a few days after spraying. However, molecular tests are relatively expensive compared to other methods.
- A protocol was compiled for the collection of chickweed leaf samples from fields where resistance to metsulfuron or florasulam is suspected for later molecular characterisation. The protocol was designed to be used by farmers and consultants and requires that the crop has been sprayed and plants are showing symptoms. Healthy plants are collected and dried in envelopes before being sent for molecular testing and resistance characterisation. The protocol provides a practical method for collection of chickweed samples from farms where resistance is suspected and could provide the basis for a future survey of resistant chickweed.
- Observations on Petri dish assays with resistant chickweed and poppy populations lead us to believe that this method is not favourable for these species compared to grass weed species. Small seed sizes make counting and setting up experiments impractical, and results were often unreliable.

Objective 4. To maintain a “watching brief” on potential new cases of herbicide-resistance and, if appropriate, develop new testing methodologies and procedures so that the extent of any problems can be better quantified.

4.1 Northern Ireland chickweed resistance screening

While ALS inhibitor resistant chickweed is a well established problem in many countries including the UK, resistance to other modes of action is much less common, with single examples of atrazine and mecoprop-P resistant populations being listed on the ISHRW website. In the UK a chickweed population resistant to the synthetic auxin herbicide mecoprop-P was first reported in 1985 from the Bath area. At that time the extent of the problem was unknown and since then other herbicide chemistries have been introduced for the control of chickweed in the UK, including ALS inhibitors, while synthetic auxins have become less important overall. With growing resistance to ALS inhibitors in weed species worldwide and especially in European chickweed populations auxin mimics may become more important again for control of this weed. Establishing the prevalence of auxin resistant populations is therefore increasingly important and contacts with farmers and agronomists were used to gather further information and seed samples of potentially resistant populations where appropriate. Testing of a potential mecoprop-P resistant chickweed population from Northern Ireland (NI08) was carried out under glasshouse conditions in autumn 2008. Seeds were collected from surviving chickweed plants in a barley crop which had been sprayed with both metsulfuron and mecoprop-P in the summer of 2008.

Germination tests were carried out prior to the experiment in order to establish whether any of the original mecoprop-P resistant seed collections from 1985 were still viable for comparison as resistant standards to the recent NI08 sample. Unfortunately the 1985 seed samples failed to germinate and so were not included in the experiment. Instead the NI08 seed sample was tested along with the most resistant population from previous work (ABER) and a susceptible standard population (UKA).

The experiment was set up on 15 Oct 2008 with 6 herbicide treatments (including untreated), 16 reps (pots), and three populations. Herbicide treatments included metsulfuron (“Ally”, 6 g a.i. ha^{-1}), florasulam (“Boxer”, 5 g a.i. ha^{-1}), fluroxypyr (“Starane”, 200 g a.i. ha^{-1}), and mecoprop-P (“Duplosan”, 1200 g a.i. ha^{-1} and 2400 g a.i. ha^{-1}). Pre-germinated seeds were planted into 5 x 5 cm pots using potting compost with a single plant per pot and grown to the 10-15 cm rosette stage before spraying with a track sprayer as described in section 2.1. After spraying all pots were moved to a glasshouse and randomised by treatment. Plants were harvested 27 days after treatment and fresh foliage weights were taken for each pot. Foliage weight data were analysed by analysis of variance using percentage reductions in fresh weights relative to untreated plants.

Table 7. Analysis of variance comparing mean percentage reduction in plant weights relative to untreated plants within herbicide treatments

	Mean percentage reduction in fresh weight compared to nils (g)				
Population	**Metsulfuron**	**Florasulam**	**Fluroxypyr**	**Mecoprop 1x**	**Mecoprop 2x**
UKA	95.2	96.4	97.3	98.3	98.8
NI08	30.7	97.0	96.9	96.7	99.1
ABER	24.1	47.0	97.4	97.9	98.7
S.E.	2.9	2.0	0.3	0.3	
L.S.D (5%)	8.2	5.7	0.8	0.9	

- Metsulfuron offered good control of the susceptible standard population but reductions in fresh weight of NI08 and ABER were both significantly lower. There was no significant difference in the levels of control achieved for the two resistant populations.
- The NI08 population showed no evidence of cross resistance to florasulam and levels of control were not significantly different to those achieved for the susceptible standard population UKA.
- Small though significant differences in control were observed with the mecoprop-P 1x field rate treatment where the NI08 population showed reduced levels of control compared to UKA and ABER, although all populations showed fresh weight reductions of more than 95 % compared to untreated controls. While NI08 plants were badly affected by field rate application of mecoprop-P, they took longer to die and three plants did survive in comparison to the other populations.
- All plants were completely controlled by 2x field rate application of mecoprop-P and by fluroxypyr; there were no survivors from either treatment.
- Overall NI08 showed very marginal levels of reduced control with mecoprop-P when applied at the field rate under glasshouse conditions. It is conceivable that under field conditions this might translate to noticeable levels of surviving plants but it would be unlikely to account for a loss of control or economically damaging levels of chickweed after treatment.

4.2 Mayweed resistance screening

A scentless mayweed (*Tripleurospermum inodorum* (L.) or *Matricaria inodora*) sample (AMC) reportedly showing resistance to the ALS inhibitor metsulfuron was sent to Rothamsted from a farm in Yorkshire for testing. Scentless mayweed is a major weed of UK cereal crops and metsulfuron resistant biotypes could present significant problems for control with few alternative herbicides available. Currently the only report of herbicide resistant mayweed from the UK is from 1975 when low level resistance to MCPA was detected in glasshouse trials.

The experiment was set up on 2 Dec 2008 when 10 Petri dishes of the AMC population with around 80 – 100 seeds per dish were left to pre-germinate in an incubator set to 14 h 17 °C day and 10 h 11 °C night. Each Petri dish contained 3 filter papers and 7 ml of 1 g/L potassium nitrate solution. A susceptible standard population from Herbiseed (HERB) was included for comparison. Seed quality of the AMC population was of a very low level and only 12 seeds germinated overall. These were transplanted into potting compost in 5 x 5 cm pots and transferred to a glasshouse under lights. The HERB population germinated normally and 50 pots were prepared with a single seedling per pot.

Plants were sprayed with 6 g a.i. ha^{-1} metsulfuron (Lorate SX) on 16 Jan 2009 using a track sprayer as described in section 2.1. A total of 4 surviving plants were sprayed from the AMC population, with 4 metsulfuron treated and 4 untreated plants included from the susceptible HERB population. After spraying all plants were returned to the glasshouse. Plants were harvested 28 days after treatment and fresh whole plant weights were taken for each pot after root washing and surviving AMC mayweed plants were transplanted into potting compost to grown on for seed.

- All HERB susceptible standard mayweed plants were completely controlled by field rate application of metsulfuron; there were no survivors.
- The AMC population showed reduced control with metsulfuron when applied at the field rate under glasshouse conditions. Statistical analysis was not performed due to the very low number of treated plants.
- Compared to the untreated HERB control, metsulfuron treated HERB plants showed 96 % mean fresh weight reduction while the AMC population showed only 54 % reduction in fresh weight. Untreated AMC plants were not available for comparison.
- Three out of four AMC metsulfuron survivors showed slight injury after treatment while a single plant was completely undamaged.
- While this experiment did not provide enough data for a statistically significant comparison of the two populations, evidence suggests that the AMC mayweed population is at least partially resistant to the sulfonylurea herbicide metsulfuron. Seed from surviving AMC plants will be collected and tested at a later date to confirm resistance.
- Scentless mayweed is an important weed of cereal crops and ALS inhibiting herbicides are an important part of most mayweed control strategies. This experiment provides the first independent confirmation of ALS resistant mayweed in the UK.

Objective 5. To undertake technology transfer initiatives to inform suppliers and users of herbicides in the agricultural, horticultural, industrial and amenity sectors of the risks posed by herbicide-resistance and to promote appropriate prevention and management strategies.

5.1 Amenity conference

In general, amenity sector vegetation control operatives in the UK are perceived as having less awareness of the threat posed by resistant weeds compared to the agricultural sector where resistance has been recognised as a problem since the 1970s. A presentation to amenity sector weed management personnel and regulators was made at the Amenity Weed Control conference, organised by the Amenity Forum, and held at the Health and Safety Executive offices in Rose Court, London on 2 Oct 2008. The presentation introduced the issue of herbicide resistance and covered the main issues relevant to control of weeds with herbicides in amenity situations. The amenity sector presents unique challenges in that the range of chemicals available for weed control is relatively limited compared to agriculture. Glyphosate is by far the most important chemical for amenity use and the main threat to continued weed control is the development of glyphosate resistance.

The presentation was well received and gave a good opportunity to demonstrate that failure to address the problem of resistance could have potentially serious economic consequences for continued weed control in the amenity sector. Several contacts in rail and highways weed control were made at the conference and these may provide future opportunities for cooperation and investigation of possible herbicide resistant cases. A summary of the presentation was reported in the publication “Horticulture Week” on 09 Oct 2008.

5.2 Amenity sector leaflet

A leaflet was prepared covering the issue of herbicide resistance as it applies to amenity sector weed control in the UK. The leaflet comprises a short introduction on the idea of resistance and then details the specific risk factors and implications of glyphosate resistance for the amenity sector. Checklists detailing best practice in the event of spray failure, what to look for in terms of emerging resistance and practical measures for herbicide resistance mitigation make up the remainder of the leaflet.

The aim is for a PDF version leaflet to be hosted on the Amenity Forum website at http://www.amenityforum.org.uk/.

5.3 HGCA leaflet

An agreement has been reached with the HGCA, the cereals and oilseeds sector of the Agriculture and Horticulture Development Board, to produce a topic sheet detailing recent advances in the understanding of herbicide resistant broad leaved weeds affecting the UK arable sector. The target date for completion of this leaflet is June 2009 and it will initially be available for distribution at the Cereals 2009 event in Cambridgeshire and on the HGCA website.

The HGCA leaflet will provide a concise update on herbicide resistance issues and will concentrate on the recent results explaining the mechanism and cross resistance characteristics of ALS resistance in chickweed and poppy and the implications for management of these weeds in cereal crops. The Cereals event will provide an opportunity to answer questions from farmers and agronomists and to provide advice on alternatives to ALS inhibitors for control of resistant populations.

Overall summary, conclusions and implications of the research

Summary

The confirmation of ALS target site resistant broad leaved weeds in the UK identifies a new threat to crop production in the UK. Resistant poppy in particular may be a problem in cereal crops where fewer alternative herbicides may be available.

- Target site resistance to ALS inhibitors, conferred by either Pro197 of Trp574 mutation, was confirmed for the first time in chickweed. While Pro197 mutation conferred resistance to metsulfuron, cross resistance work showed that florasulam controlled Pro197 but not Trp574 mutant populations. Results also indicated that the grass weed herbicide mesosulfuron + iodosulfuron mixture (“Atlantis”) provided better control of resistant chickweed populations than metsulfuron, even where target site resistance at Pro197 was confirmed, and that iodosulfuron alone (“Hussar”) provided some control.
- No real evidence was found for resistance to alternative broad leaved weed herbicides mecoprop-P and fluroxypyr for control of ALS resistant chickweed and these herbicides provide a good alternative, even though both belong to the synthetic auxin group. A very marginal level of mecoprop-P resistance was observed in a single chickweed population from Northern Ireland and the potential for further development of auxin analogue resistant chickweed certainly exists with previous cases having been identified. The emergence of cross resistance to auxin herbicides in ALS resistant chickweed populations is a major risk factor for future management of this weed in the UK.
- Target site resistance to ALS herbicides was conferred by Pro197 mutation in all of the poppy biotypes tested. Although particular amino acid substitutions varied, there was no observable difference in control with substitution. No evidence of the usually more serious Trp574 ALS mutation was observed in poppy.
- Ioxynil + bromoxynil provided uniformly good post emergence control of all resistant poppy populations and MCPA also provided useful levels of control. The pre-emergence herbicide pendimethalin caused reductions in poppy fresh weights when applied post-emergence and might be expected have greater efficacy when applied at the recommended timing. Pendimethalin provides a very useful pre-emergence option for poppy and widens the options available to growers, taking pressure off herbicides with higher inherent resistance risk.

Implications

Herbicide resistance in broad leaved weeds does not appear to pose as large a threat as that presented by resistance in grass weeds. This is largely due to resistance being conferred by target site mechanisms alone, with no clear evidence of the enhanced metabolism mechanisms commonly occurring in grass weeds. This means that alternative (non-ALS) modes of action, such as fluroxypyr on chickweed and ioxynil/bromoxynil on poppy, can provide complete control of resistant populations. However, control of ALS resistant broad-leaved weeds is dependent on the continued availability of effective alternative herbicides. The availability of alternatives to ALS inhibitors is likely to be affected by the current revisions to the EU agrochemical registration directive (91/414). Loss of alternatives is likely to substantially increase the threat posed by ALS resistance, as few effective cultural control options are available. In addition, while ALS resistance has only been confirmed in three weed species in the UK (chickweed, poppy and mayweed); there is no reason why resistance should not evolve in other species. Indeed, increased reliance on a more restricted range of herbicides will increase the risk of resistance in broad leaved weeds, not only in arable crops, but also in horticultural, amenity and industrial weed control situations. Use of the resistance risk matrix in association with active monitoring is required to detect any new cases of resistance. The continued availability of herbicides with a range of modes of action is also essential. Raising awareness of herbicide resistance should be a high priority so that management strategies can be adopted at an early stage. The presentation made at the Amenity Weed Control Conference, the production of a leaflet targeted at the amenity sector, the production of an HGCA Topic sheet aimed at arable farmers and a planned display at Cereals 2009 should all help raise awareness of the issues.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.