SESSION 8B

HERBICIDE RESISTANT WEEDS: WHAT'S NEW?

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Papers	8B-1 to 8B-5

International survey of herbicide-resistant weeds: lessons and limitations

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ABSTRACT

The "International Survey of Herbicide-Resistant Weeds" monitors the occurrence of herbicide-resistant weeds throughout the world. Currently there are 222 herbicide resistant weed biotypes (147 unique species) found in 45 countries. Whilst triazine-resistant weeds account for 61 of these cases, ALS inhibitor and ACCase inhibitor-resistant weed species are now of greater economic importance globally. There are 58 ALS inhibitor-resistant weed species found in 14 countries and 19 species of ACCase inhibitor-resistant grasses found in 17 countries. ALS inhibitor-resistant weeds are most problematic in cereal, corn/soybean, and rice production. ACCase inhibitor resistant *Lolium* and *Avena* spp. threaten cereal production in Australia, Canada, Chile, France, Saudi Arabia, South Africa, Spain, the United Kingdom, and the USA. Grasses now comprise 40% of all resistant weed biotypes indicating that this family has the greatest propensity to evolve resistance to herbicides. The incidence of resistance is rapidly increasing in Asia and South America as these regions adopt high input agriculture. Researchers from 60 countries have assisted in completing 583 survey forms to report herbicide-resistant weeds, either via regular mail or over the Internet. The survey is not without its limitations. Estimating the number of resistant sites and the area infested is extremely difficult and is likely to be inaccurate in many cases. Occasionally incorrect identification of species or inappropriate testing procedures has led to retraction of records from the survey database. The survey is brief to encourage participation, and detailed information about the genetics, mechanisms, or even cross-resistances are sought from follow-up questions or the scientific literature and posted along with the survey results at http://www.weedscience.com.

INTRODUCTION

The purpose of the "International Survey of Herbicide-Resistant Weeds" is to monitor the evolution of herbicide-resistant weeds and assess their impact throughout the world. Between 1995 and 1999 survey forms were sent to 560 weed research and extension people throughout the world in 60 countries and 583 forms have been returned (one form for each resistant weed reported), via regular mail or over the Internet. Survey questions are aimed at identifying the species and herbicide(s) involved, when resistance was first identified, how resistance was confirmed, the crop or vegetation management situation involved, the number of sites and area infested, the location of resistant weeds, and the economic impact of resistant weeds.

CURRENT STATUS OF HERBICIDE-RESISTANT WEEDS WORLDWIDE

The 1999 International Survey of Herbicide-Resistant Weeds recorded 222 herbicide-resistant weed biotypes in 45 countries (Table 1). A new resistant biotype refers to the first instance of a weed species evolving resistance to one or more herbicides in a herbicide group. Figure 1 shows the relatively steady climb in the number of new resistance cases (approximately 9 new cases per year) since 1980.

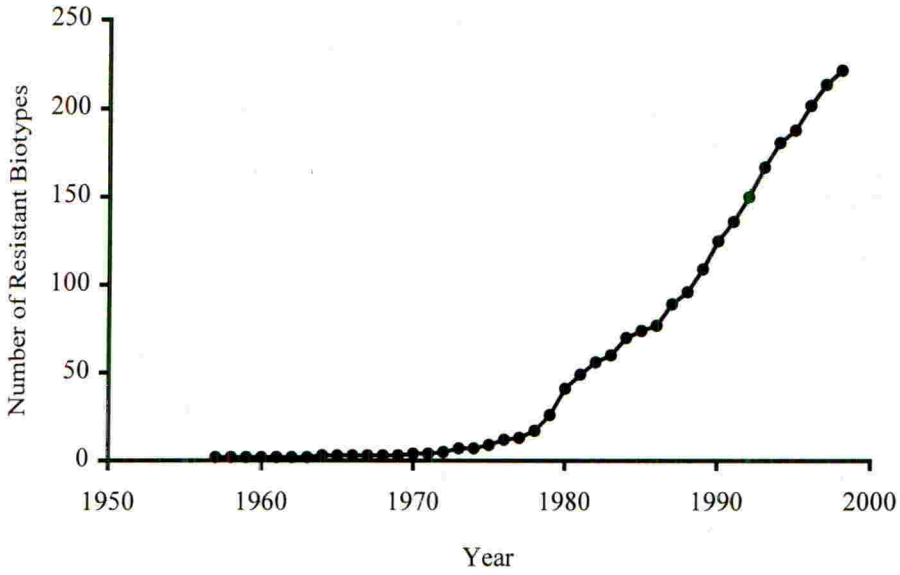

Figure 1. The chronological increase in the number of herbicide-resistant weeds worldwide.

Initially triazine-resistant weeds accounted for much of this nine fold increase in the rate of documentation of herbicide-resistance. In the five-year period between 1978 and 1983 scientists around the world documented 33 new cases of triazine-resistant weeds (Figure 2). More recently ALS inhibitor and ACCase inhibitor resistant weeds accounted for a large portion of the increase in the number of resistant species. In the period between 1988 and 1999 a total of 52 additional species had evolved resistance to ALS inhibitor herbicides. In this same period there were only 14 new triazine resistant species reported (Figure 2).

Several new ALS inhibitor resistant weeds have been reported from Australia including wild radish (*Raphanus raphanistrum*), turnip weed (*Rapistrum rugosum*), African turnip weed (*Sisymbrium thellungii*), and salvation Jane (*Echium plantagineum*). In the USA ALS inhibitor resistance was recently reported in green foxtail (*Setaria viridis*), giant foxtail (*Setaria faberi*), and yellow foxtail (*Setaria lutescens*) from corn/soybean rotations in the mid-west, in mayweed chamomile (*Anthemis cotula*) from wheat production in Idaho, in shattercane (*Sorghum bicolor*) from corn production in Kansas, and in common sunflower (*Helianthus annuus*) from soybean production also in Kansas. Six new cases of ALS inhibitor resistant weeds in Japanese rice production have been identified. Some other notable new cases of resistance are alexandergrass (*Brachiaria plantaginea*) with ACCase inhibitor resistance from Brazil, hood canarygrass (*Phalaris paradoxa*) with ACCase

Table 1. The occurrence of herbicide-resistant weed biotypes to different herbicide groups.

Herbicide Group	WSSA[a] Code	HRAC[b] Code	Example	Resistant Weed Biotypes			Number of[c] Countries
				Dicots	Monocots	Total	
Triazines	5	C1	Atrazine	42	19	**61**	22
ALS inhibitors	2	B	Chlorsulfuron	39	19	**58**	14
Bipyridiliums	22	D	Paraquat	19	7	**26**	12
ACCase inhibitors	1	A	Diclofop-methyl	0	19	**19**	17
Synthetic Auxins	4	O	2,4-D	14	3	**17**	11
Ureas/amides	7	C2	Chlorotoluron	6	11	**17**	18
Dinitroanilines	3	K1	Trifluralin	1	6	**7**	5
Triazoles	11	F3	Amitrole	1	3	**4**	2
Thiocarbamates	8	N	Triallate	0	3	**3**	3
Chloroacetamides	15	K3	Metalochlor	0	3	**3**	3
Glycines	9	G	Glyphosate	0	2	**2**	2
Chloro-Carbonic-acids	26	N	Dalapon	0	1	**1**	1
Organoarsenicals	17	Z	MSMA	1	0	**1**	1
Benzoflurans	16	N	Ethofumesate	0	1	**1**	1
Pyrazoliums	8	Z	Difenzoquat	0	1	**1**	3
Nitriles	6	C3	Bromoxynil	1	0	**1**	1
			Totals	124	98	**222**	

[a]Retzinger & Mallory-Smith, (1997).
[b]Schmidt, (1997).
[c]A country may be counted in more than one herbicide group thus this column adds up to greater than 45.

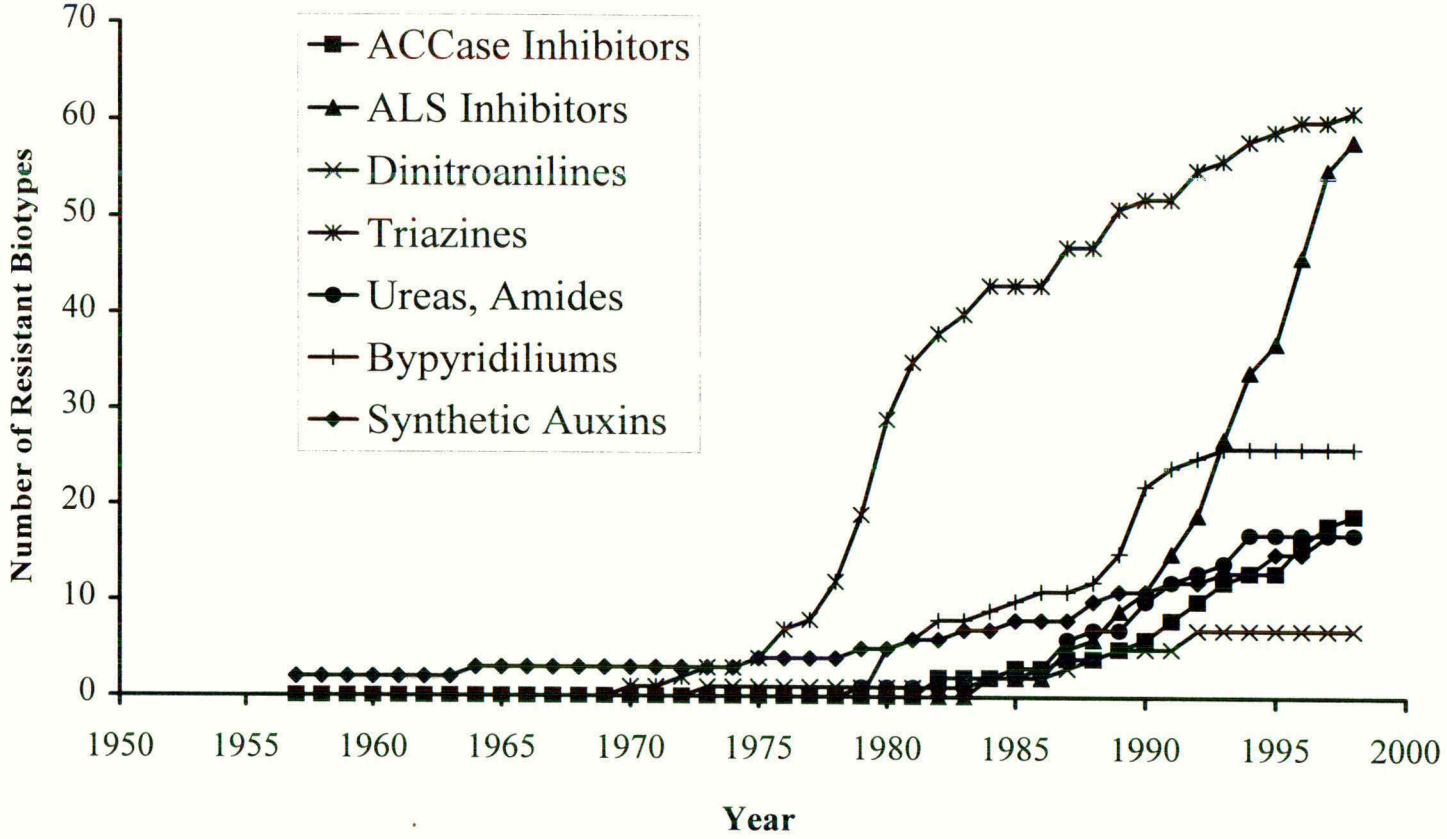

Figure 2. The chronological increase in the number of herbicide-resistant weeds for several herbicide classes.

inhibitor resistance from Mexico, Italian thistle (*Carduus pycnocephalus*) with synthetic auxin resistance from New Zealand, and ALS inhibitor/quniclorac resistance in a population of false cleavers (*Galium spurium*) from Canada.

Populations of multiple-resistance in wild-oats (to fenoxaprop-p-ethyl, imazamethabenz, triallate, and difenzoquat) from Canada forewarn of a serious threat, as there are few remaining herbicides for selective control of these populations. The evolution of glyphosate resistance in rigid ryegrass (*Lolium rigidum*), both in Australia (3 populations) and in the USA (2 populations), as well as glyphosate resistance in goosegrass (*Eleusine indica*) from Malaysia indicate that resistance management strategies will continue to be necessary even after the widespread adoption of glyphosate-resistant crops.

HERBICIDE RESISTANCE ON THE RISE IN DEVELOPING REGIONS

The intensive agriculture practiced by developed countries has, understandably, led these countries to select the greatest number of herbicide-resistant weeds. Figure 3 presents the percentage of new cases of resistance identified from each of seven world regions over 3 decades. In decade 1 (1970-1979) triazine-resistant weeds reported from Europe, North America, and the Middle East (primarily Israel) accounted for most reports of herbicide-resistant weeds (Figure 3). In decade 2 herbicide-resistant weeds began appearing in all other regions, however Europe and North America still produced the greatest number of new resistant biotypes followed by Australia/NewZealand and Asia. It is notable that the number of new resistant weed biotypes declined in North America from decade 1 to decade 2 (due to fewer triazine-resistant species identified) and then increased again in the decade 3 as ALS-inhibitor and ACCase inhibitor resistant weeds proliferated. Europe recorded 47% of the world's resistant weeds in decade 2 and then declined to 10% in decade 3, primarily due to its lower usage of ALS-inhibitor herbicides compared to other regions (Figure 3).

The steady increase in herbicide usage in South & Central America and in Asia has led to a predictable increase in the number of new resistant weed species identified from these regions over the last two decades. Herbicide-resistance, which was once only a problem in the intensive agricultural systems of developed countries, is rapidly becoming a major concern in developing countries.

PROPENSITY OF WEEDS TO EVOLVE RESISTANCE TO HERBICIDES

Some weed species show a propensity to evolve resistance to a wide range of herbicides. Of the 147 weed species that have evolved resistance to one or more herbicide modes of action (MOA), 104 had evolved resistance to only one MOA, 26 species to two MOA, 10 species to three MOA, two species to four MOA, four species to five MOA, and one species (rigid ryegrass) had evolved resistance to eight herbicide modes of action thus giving a total of 222 herbicide-resistant weed biotypes. Rigid ryegrass has evolved resistance to the herbicide modes of action A, B, C1, C2, F3, G, K1 and K3 (letters represent the HRAC herbicide mode of action classification - see Table 1). Other weeds that have evolved resistance to numerous MOA's are wild-oats (*Avena fatua*) to A, B, K3, N, and Z; barnyardgrass (*Echinochloa crus-galli*) to C1, C2, K1, K3, N, and Z; goosegrass to A, B, D, G, and K1; annual bluegrass (*Poa annua*) to C1, C2, D, F3, and N; black-grass (*Alopecurus myosuroides*) to A, B, C2, and K1;

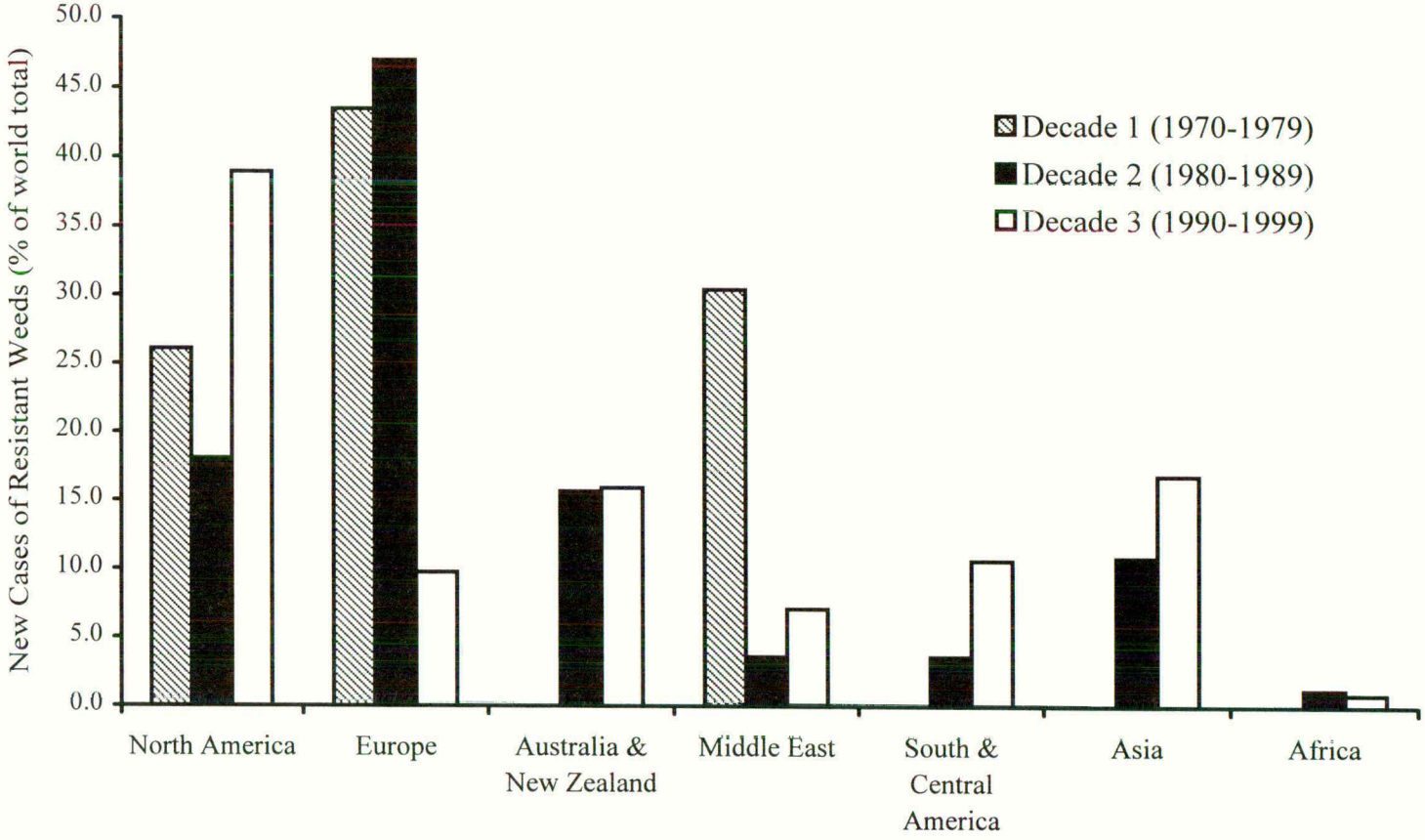

Figure 3. The percentage of new resistance cases identified from each of seven world regions over 3 decades.

and horseweed (*Conyza Canadensis*) to B, C1, C2, and D. With the exception of horseweed these species all belong to the family Poaceae. The 147 resistant weed species belong to 29 weed families, the top 10 are presented in Table 2.

Table 2. The number and percentage of resistant species by family and the percentage of species considered principal weeds by Holm et al. (1991 & 1997) for each of these families.

Family	# Resistant Species	Resistant Species (% of total)	Weed Species (% world's principal weeds[a])
Poaceae	48	33	25
Asteraceae	29	20	16
Amaranthaceae	9	6	3
Brassicaceae	9	6	4
Chenopodiaceae	7	5	2
Polygonaceae	6	4	5
Scrophulariaceae	6	4	1
Alismataceae	3	2	1
Cyperaceae	3	2	5
Solanaceae	3	2	2
19 families pooled	24	16	16
Total	147	100	80[b]

[a] The number of species within a family (as a percentage of total) reported by Holm et al. (1991 & 1997) as being principal weeds of the world.
[b] An additional 20% of the species listed by Holm et al. (1991 & 1997) were in families where no species have evolved herbicide-resistance.

Whilst grasses account for 33% of all resistant species (Table 2) and 40% of all resistant biotypes, they only account for 25% of the world's principal weeds (Holm et al. 1991 & 1997) indicating the high propensity for grass weeds to evolve herbicide-resistance. Other families having a disproportionately high number of herbicide-resistant species (compared to their representation as principal weeds) are Amaranthaceae, Brassicaceae, Chenopodiaceae, and Scrophulariaceae (Table 2).

SURVEY LIMITATIONS AND DISSEMINATION OF RESULTS

The survey does have limitations such as:

1. Resistant species are not always accurately identified. In the 1970's and 80's in the United States smooth pigweed (*Amaranthus hybridus*) was often miss identified/reported as redroot pigweed (*Amaranthus retroflexus*). When in doubt, weed scientists should submit samples to taxonomists for identification.
2. Testing procedures may be flawed. The preferred method of confirming herbicide-resistant weeds is to conduct whole plant dose response experiments on resistant and susceptible biotypes of the same species under greenhouse or growth chamber conditions (Heap, 1994; Moss, 1995). Enzyme based tests or field tests are less desirable but are also accepted provided sufficient care is taken to include susceptible controls. It is left to the researchers to determine if there is sufficient statistical difference between R and S biotypes to warrant calling a population resistant.

3. Many questions require that researchers provide their best estimate based on their own knowledge. Estimating the number of resistant sites and the area infested is extremely difficult. Although this is a limitation of the survey it is better to have an estimate from a well-informed weed scientist than no information at all.
4. Initially an indication of the economic impact was sought from participants in the survey. There are many costs involved with weed control and many avenues to control resistant populations. So few researchers were willing to fill out this portion of the survey that it was dropped from the analysis. The economics of herbicide resistance will probably be best dealt with by targeted economic studies, such as that by Orson & Harris (1997).

The survey is short and primarily serves to identify a new case of resistance. Follow up questions and literature searches are then needed to fill in more detailed information about the evolution, genetics, mechanisms, or management approaches for each resistant biotype.

The dissemination of survey results over the Internet (http://www.weedscience.com) has many benefits over the printed annual report. The information is available to anyone with Internet access. The information is updated on a regular basis and can be searched and sorted to suit the researcher prior to printing. Researchers can first check to determine if a particular resistant weed has been registered for their region, and if not then they can register the new case on-line. Finally the site provides e-mail addresses of the researchers associated with resistance cases to facilitate communication between scientists with similar interests.

ACKNOWLEDGEMENTS

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Modelling strategies to prevent resistance in black-grass (*Alopecurus myosuroides*)

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ABSTRACT

A single dominant mutation conferring resistance to aryloxyphenoxypropionate (AOPP) and cyclohexanedione (CHD) herbicides was incorporated into a quantitative model for the population development of *Alopecurus myosuroides*. The model assumes an initial seedbank of 100 seed/m^2 and that each generation a proportion 10^{-6} of the seedbank mutates to resistance. The model predicts that with annual use of AOPP/CHD herbicides which kill 90% of susceptible but no resistant plants, a threshold of 10 plants/m^2 surviving herbicide ('field resistance') will develop: in 9-10 years if all tillage is by tine cultivation to 10 cm deep; after 28-30 years of continuous ploughing; in 12 years if tine cultivations are interspersed with ploughing once every four years. If AOPP/CHD herbicides are alternated with herbicides with different modes of action, the predicted outcomes depend on the annual kill rate: with 95% kill (of susceptible plants by AOPP/CHDs and all plants by alternative herbicides) and tine cultivation, field resistance develops in 22 years; however with a 90% kill and tine cultivation, field resistance does not develop but there are more than 10 susceptible plants/m^2 surviving herbicide within 10 years. The model predicts that resistance can be delayed indefinitely if three herbicides, each with a different mode of action, are rotated and 95% kill is maintained by each.

INTRODUCTION

Alopecurus myosuroides (black-grass) is one of the commonest grass weeds of winter cereals in England and north-west Europe, and the emergence of herbicide resistance has important consequences for cereal production. AOPP and CHD herbicides act by inhibiting acetyl-coenzyme A carboxylase (ACCase) in lipid synthesis and many populations show complex cross-resistance patterns due to the presence of multiple resistance mechanisms, including enhanced metabolism and target-site resistance (insensitive ACCase) (Cocker *et al.*, 1999). Enhanced metabolism is probably polygenic (G Cavan; unpublished) but target-site resistance to AOPP/CHD herbicides is absolute and appears to be determined by a single genetic locus, with resistance alleles being dominant over a wide range of herbicide dose rates (S R Moss; unpublished). Resistance specific to one mode of action (target-site resistance) should be preventable if herbicides with different modes of action are rotated.

The purpose of this study was to examine the number of years required for single-gene (target-site) resistance to develop under a number of different management regimes, in order to examine the effects of (1) cultivation: mouldboard plough (25 cm deep) *versus* tine cultivation (10 cm deep); (2) herbicide rotation: continuous application of AOPP/CHD herbicides *versus* the rotation of two (or three) different modes of action; (3) herbicide kill:

70% annual kill of susceptible plants *versus* 90% and *versus* 95% kill; and (4) frequency of resistance: mutation rates of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} per generation.

DESCRIPTION OF THE MODEL

The effects of a dominant mutation conferring resistance were incorporated into the *A. myosuroides* lifecycle model of Moss (1990) which was based on data collected from numerous field experiments. The model describes the number of inflorescences produced per m^2, h, to the number of plants per m^2 surviving herbicide treatment, p, by the density-dependent relation:

$$h = 3.88\, p / (1.0 + 0.0018\, p)$$

The model assumes that 55 viable seed are produced on each head and shed onto the soil surface, where 56% of seeds are lost by predation, decay and germination before cultivation. The soil seedbank is divided into two levels: the top 5 cm 'surface seedbank' from which seedlings can emerge and the lower 'deep seedbank' from which they cannot. Seed predation, decay and predation remove 70% of the seedbank annually at both levels. The plough (mouldboard to 25 cm deep) moves 95% of seeds from the surface seedbank to the deep seedbank and 35% from the deep to the surface seedbank. Tine cultivation to 10 cm deep moves 20% of seeds from the surface seedbank to the deep seedbank but does not move any of the deep seedbank upwards. Annually 15% of newly-shed seeds and 30% of seeds that are at least one year old produce seedlings which emerge (from the shallow seedbank); a proportion of susceptible seedlings (set at either 70%, 90% or 95% 'herbicide kill rate') are killed by herbicide before maturing to produce heads. The initial seedbank contains 100 newly-shed seed/m^2, distributed evenly to a depth of 25 cm.

Single-gene resistance was incorporated into the model with a mutation rate of 10^{-6} per generation, conferring total resistance to AOPP/CHD herbicides in both homozygous and heterozygous state but not affecting the kill rate of other herbicide groups. Random, spatially homogeneous pollination was assumed. Infestations of black-grass do not start to impact significantly on cereal yields until they exceed 10 plants/m^2. This level was used as a threshold to define 'field resistance'.

PREDICTIONS OF THE MODEL

(1) Cultivation

Four cultivation regimes were modelled (Figure 1). With annual tine cultivations to 10 cm deep, *A. myosuroides* levels reached 10 plants/m^2 within nine years and only 49% of plants were resistant; whereas with annual ploughing this level was not reached until after 30 years but all plants were then resistant. In a third regime, plough and tine cultivations were used in alternate years, and *A. myosuroides* levels reached 10 plants/m^2 after 20 years with all plants resistant; in a fourth regime the plough was used one year in four with tine cultivation in the three intervening years, and *A. myosuroides* levels reached 10 plants/m^2 after 12 years with 97% of plants resistant.

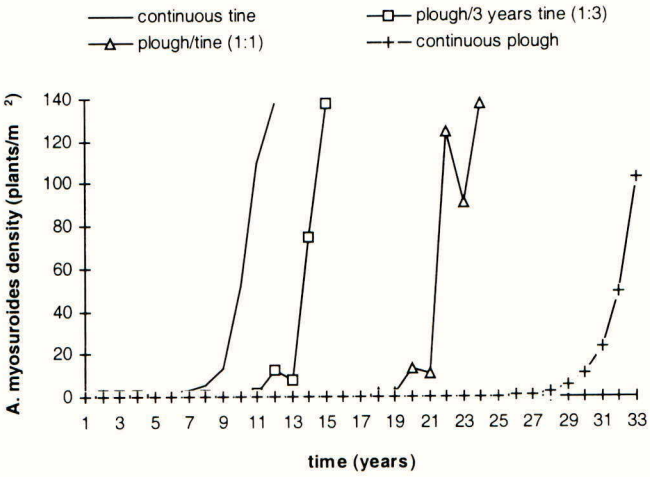

Figure 1. Effect of different cultivation regimes on the build-up of *A. myosuroides*, when AOPP/CHD herbicides are applied each year achieving 90% kill of susceptible plants.

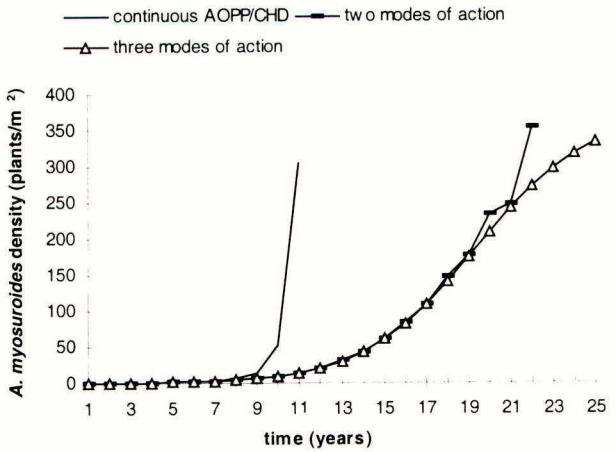

Figure 2. Effect of continuous application of AOPP/CHD herbicides versus rotation of two or three modes of action on the build-up of *A. myosuroides*, with all herbicides achieving 90% kill (with the exception of AOPP/CHD herbicides which do not kill resistant plants).

(2) Herbicide rotation

The application AOPP/CHD herbicides every year ('continuous AOPP/CHD') was compared (Figure 2) with use every second year ('one in two' rotation) or third year ('one in three' rotation). In the 'one in two' rotation, the AOPP/CHD is rotated with a herbicide with a different mode of action. In the 'one in three' rotation, an AOPP/CHD herbicide is applied every third year and non-AOPP/CHD herbicides are used for two years consecutively: if herbicides with same mode of action were used in both of these years then there could be a greater risk of resistance to this alternative mode of action than to the AOPP/CHD herbicides. This risk has not been modelled and so the results for 'one in three' rotation should be regarded as those for the rotation of three different modes of action.

In all regimes (with tine cultivation), *A. myosuroides* levels reached 10 plants/m^2 within 9-10 years. However only in the case of continuous AOPP/CHD use was there a high proportion (49%) of resistant plants in the population. A 'one in two' rotation led to a weed population in which only 0.1% of plants were resistant and 'one in three' rotation did not lead to resistance. These differences became more marked if the regimes were continued until very serious infestations (>300 plants/m^2) developed: with continuous AOPP/CHD use, 300 plants/m^2 was reached in only 11 years (with 95% resistance); with 'one in two' rotation, 300 plants/m^2 was reached in 22 years (with 26% resistance) and with 'one in three' rotation, 300 plants/m^2 was reached in 25 years (with no resistance).

Table 1. Effect of different herbicide kill rates. The model was run over 40 years and terminated when *A. myosuroides*, levels reached 10 plants/m^2. Note: n.a. indicates that kill rates of alternative herbicides are not applicable because AOPP/CHDs were used continuously; AOPP/CHD herbicides gave no control of resistant plants.

Cultivation regime (plough or tine)	Rotation of AOPP/CHD herbicides	% kill by AOPP/CHD herbicides	% kill by alternative herbicides	Time (years) to reach 10 plants/m^2	% of population resistant
plough	continuous	90	n.a.	30	100.0
plough/tine(1:3)	continuous	90	n.a.	12	97.0
tine	continuous	90	n.a.	9	48.7
tine	1 in 2	90	70	5	0.0
tine	1 in 3	90	70	4	0.0
tine	1 in 2	90	90	10	0.1
tine	1 in 3	90	90	10	0.0
plough	continuous	95	n.a.	28	100.0
plough/tine(1:3)	continuous	95	n.a.	12	100.0
tine	continuous	95	n.a.	10	99.7
tine	1 in 2	95	95	22	99.9
tine	1 in 3	95	95	>40	99.5

(3) Herbicide kill rate

The effects of different annual kill rates (70%, 90% and 95%) of both AOPP/CHD and alternative herbicides were modelled in a number of different regimes (Table 1). Regardless of the cultivation regime, continuous application of AOPP/CHDs leads to resistance in similar times regardless of whether herbicide kill rate is 90% each year, or 95%. When herbicides are rotated, 95% annual kill rate delays resistance longer than 90%, but the proportion of resistant plants in the final infestation is much greater.

(4) Frequency of resistance

The times required for field resistance to develop were strikingly insensitive to initial frequencies of resistance. Results were compared for mutation rates set at proportions 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} per generation. Results were also compared for the risks of resistance developing as a result not of mutation but of resistant *A. myosuroides* being present in contaminated wheat seed at rates of 0.01, 0.1, 1 and 10 seed/ha. In both comparisons, a thousand-fold increase in the initial frequencies of resistance caused a less than 50% decrease in the times required to reach 10 plants/m^2 regardless of the management regime.

DISCUSSION

A striking feature of the model is that good weed control is achieved for a number of years but once this is lost the resistant population increases very rapidly (Figures 1 and 2). In practice, populations of *A. myosuroides* are unlikely to be noticed in the field until they approach 10 plants/m^2. Thus resistance may be increasing from a low initial frequency for many years before it is noticed as a field problem ('field resistance'). The model predictions on times required for resistance to develop to a detectable level are in the ranges established for evolution of resistance on real farms, but these data are limited. Heap (1988) studied resistance in *Lolium rigidum*, a species with broadly similar biology to *A. myosuroides*, but included a large number of populations expressing apparently multi-gene as well as single-gene resistance. Bourgeois & Morrison (1997) studied resistant *Avena fatua* whose biology differs from *Alopecurus* (particularly in respect of pollination) but *Avena sterilis* models exist (Gonzalez-Andujar & Fernandez-Quintanilla, 1991; and references therein) into which resistance could be incorporated. Both studies give examples where resistance has evolved in less time than predicted by the model. This is not surprising since resistant populations are over-represented in data from real farms: populations managed the same way but which remain susceptible do not attract attention from the investigator. In addition, measurements of many factors that influence resistance evolution have not been made for individual fields and so the model cannot predict times to resistance on a specific farm.

The value of our modelling is in discriminating between different strategies and so determine which one can best prevent resistance in a majority of real situations: in this respect the model is useful but has two major shortcomings. Firstly, the model is spatially homogeneous and predicts mean population changes. Although *A. myosuroides* is predominantly outcrossing most pollen and seed travel short distances (Paice *et al.*, 1998) and so spatial heterogeneity is likely to reduce the kill rates required for management as predicted by Gonzalez-Andujar & Perry (1995) for the self-pollinating *Avena sterilis*.

Secondly, the model is limited by considering only single-gene, target-site resistance. Although target-site is the most common type of resistance world-wide, strategies that employ reduced kill rates and herbicide rotation to prevent target-site resistance may select for enhanced-metabolism-based resistance. After modelling risks from both types of resistance, Gardner *et al.* (1998) recommend that low kill rates be supplemented with higher levels of control occasionally (e.g. every third year) and a variation on this strategy would be to make use of a containment threshold of 7.5 *A. myosuroides* plants/m^2 (Doyle *et al.*, 1986). Neither strategy works well in our model; our maximum kill rate is 95% and lower rates fail to control *A. myosuroides* adequately even if supplemented occasionally with 95% control. Optimal strategies should be recommended only after the risks of multi-gene resistance are incorporated into the model. Relevant data should be provided from genetic analysis in progress of two *Alopecurus* biotypes expressing multi-gene resistance.

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Resistance to ALS inhibitors in weeds of rice in north-western Italy

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ABSTRACT

In Europe, 10 weed species have developed resistance to acetolactate synthase (ALS) inhibiting herbicides. Currently, the most serious problems with ALS resistance are found in paddy rice. The situation in Italy is presented: two weed species (*Alisma plantago-aquatica* and *Scirpus mucronatus*) in rice, that are among the most sensitive to ALS inhibitors, have developed resistance. The first cases were reported in 1995 and it is now estimated that about 15,000 ha are affected. In greenhouse experiments a total of 53 populations, collected from rice fields where weed control by ALS inhibitors was unsatisfactory, were screened with five ALS inhibitors (four sulfonylureas: azimsulfuron, bensulfuron-methyl, cinosulfuron, ethoxysulfuron; one triazolopyrimidine: metosulam) sprayed at three times the recommended field dose. Only three populations of *A. plantago-aquatica* and six of *S. mucronatus* still appeared to be susceptible to all herbicides. Three populations (one susceptible and two resistant) of each species were then used in two dose-response experiments with two herbicides (bensulfuron-methyl and metosulam) and eight doses ranging from 0 to 64 times the normal field dose. The results indicate that the resistance situation for the two species is similar, with a generalised cross-resistance to all the ALS inhibitors used in rice crops in Italy. The resistance level to the triazolopyrimidine herbicide appears to be lower than that found for the four sulfonylurea herbicides. The available information indicates that an insensitive target site is the resistance mechanism in both species for all the herbicides tested.

INTRODUCTION

Acetolactate synthase (ALS) inhibitor herbicides have been widely used in Europe for nearly as long as in North America, but fewer cases of resistance have been reported where ALS herbicides had been the selecting agent (Kudsk *et al.*, 1995; Heap, 1997; Claude *et al.*, 1998; Chodová & Mikulka, 1999; Heap, 1999). However, several new cases, summarised in Table 1, have recently been reported at workshops of the European Herbicide Resistance Working Group (EHRWG), so taking the number of species involved to 10 (six monocots and four dicots) and indicating that the situation is evolving rapidly. All cases but one (*Kochia scoparia* found along railway lines) come predominantly from monoculture situations (cereals and rice) with intensive use of ALS inhibitors as major or sole mode of action targeting those weed species that consequently evolved resistance.
Although *Stellaria media* was the first species to evolve ALS resistance in Europe, only three populations have been reported so far and the situation seems to have stabilised. This reduced impact is likely to be related with some species' characteristics (e.g. self-pollinating, seed dispersal mechanism).

At present it seems that the largest problem in cereals is related to resistant *Papaver rhoeas* in wheat crops in southern Europe. This was first found in Spain and it is now estimated that about 10% of cereal crops in Spain are infested with this resistant weed (A. Taberner, personal communication). Resistant populations of *P. rhoeas* have recently been reported in two other countries (Greece and Italy) and due to the biology of the species (completely cross-pollinated, protracted germination, production of a large number of persistent seeds), the problem seems to be spreading rapidly.

Resistant *Scirpus maritimus* has recently been found in a few Spanish rice crops. This geophyte species spreads extensively by horizontal creeping rhizomes and stolons with ovoid tubers at the nodes. If this report is confirmed, this would be the first real perennial weed resistant to ALS inhibitors found in rice.

Table 1. Status of resistance to ALS inhibitors in Europe.

Species	Country	Cases	Year	Crop	Mechanism
Alisma plantago-aquatica	Portugal	> 20	1996	rice	target site
	Italy	> 100	1995	rice	target site
	Spain	2	1997	rice	target site
Alopecurus myosuroides	UK	> 100	1984	cereals	metabolic
	France	> 100	1992	cereals	metabolic
	Belgium	> 10	1997	cereals	metabolic
	Germany	> 10	1997	cereals	metabolic
	Netherlands	> 10	1998	cereals	metabolic
	Spain	> 5	1997	cereals	metabolic
Chrysanthemum segetum	Sweden	1	1997	cereals	target site
	Ireland	1	1997	cereals	target site
Cyperus difformis	Spain	2	1997	rice	target site
Kochia scoparia	Czech	2	1998	railway	?
Lolium rigidum	Spain	1	1997	cereals	metabolic
	Greece	1	1998	cereals	metabolic
	Italy(*)	1	1998	cereals	?
Papaver rhoeas	Spain	> 20	1993	cereals	target site
	Italy	2	1998	cereals	?
	Greece	2	1998	cereals	?
Scirpus maritimus	Spain	3	1997	rice	target site
Scirpus mucronatus	Italy	> 100	1995	rice	target site
Stellaria media	Denmark	1	1991	cereals	target site
	Sweden	1	1995	cereals	target site
	Ireland	1	1996	cereals	target site

(*) This population shows intermediate characteristics between *L. rigidum* and *L. multiflorum*.
Most of the unpublished cases are personal communications from EHRWG members (Claude & Cornes, 1999).

There are no reports on grass species in Europe showing ALS target site resistance. However there are some populations of *Alopecurus myosuroides*, *Lolium rigidum* and *Lolium* spp. (having intermediate characteristics between *L. rigidum* and *L. multiflorum*) showing probable metabolic resistance to these herbicides. Most of these latter cases had not been selected by ALS inhibitors.

The worst cases in Europe where ALS inhibitors acted as the selecting agents involve two species, *A. plantago-aquatica* and S. *mucronatus*, infesting rice crops in southern and western European countries (Calha *et al.*, 1996; De Prado *et al.*, 1997; Sattin *et al.*, 1998).

Alisma plantago-aquatica (common waterplantain) belongs to the Alismataceae family and is a wetland rosette-forming species. It is self-compatible and in agricultural environments regeneration occurs mainly by seed; fresh seed exhibit hard-coat dormancy and form a persistent seed bank. The germination is epigeal and initial growth is slow.

Scirpus mucronatus (ricefields bulrush) is a member of the Cyperaceae. In natural environments it is a perennial sedge with short rhizomes, but in paddy ricefields regeneration is mainly by seed. Germination studies report inconsistent results, but fresh seed collected from Italian ricefields show strong physiological dormancy.

Several thousand hectares are infested in Italy, Portugal and Spain, with the worst situation being in Italy where it is now estimated that about 15,000 ha are affected, about 6% of the total area of ricefields in Italy. The most recent information indicates that the area infested by resistant *A. plantago-aquatica* is stabilising, while resistant *S. mucronatus* is still spreading (Sattin *et al.*, 1999).

The aims of this study were: to confirm the presence of resistance to ALS inhibitors in *A. plantago-aquatica* and *S. mucronatus*; to verify the extent of resistance; to check the pattern of cross-resistance to a range of ALS inhibitors and to investigate the degree of resistance with dose-response experiments.

MATERIALS AND METHODS

Seeds of 31 and 22 populations of *A. plantago aquatica* and *S. mucronatus*, respectively, were collected in 1996 and 1997 from rice fields in north-western Italy where weed control by ALS inhibitors was unsatisfactory. Historical records of herbicides use and other agronomic techniques used in the sampled fields were collected from the farmers. Seeds of two susceptible populations of *A. plantago aquatica* were gathered from natural wetland areas near Padova and Novara where they had never been treated with herbicides. The two susceptible populations of *S. mucronatus* were collected from the edges of ricefields in areas (about 250 km away) not yet affected by herbicide resistance. One of rice fields had never been treated with sulfonylureas, while the other had been treated with bensulfuron-methyl or cinosulfuron for the last four years. The population collected from the latter field was also used for the dose-response experiment. The seed samples were cleaned and then stored at ambient temperature.

Screening experiments

Fresh seeds of both species showed strong dormancy, which was removed in different ways for the two species. Seeds of *S. mucronatus* were stratified in Petri-dishes between two layers of wet filter paper for 4-5 weeks in a refrigerator at 4 °C, before being sown in polystyrene trays in the greenhouse. Seeds of *A. plantago-aquatica* were chemically scarified by being dipped in chloroform for 2 min, rinsed and dried using absorbent paper, then immersed in 80% sulphuric acid for 5 min and rinsed well with distilled water. They were then placed in beakers

containing distilled water and left for 5-6 days in a germination cabinet at 12-25 °C night/day with a 12 h photoperiod. When the cotyledon appeared green and well developed, the seedlings were carefully removed and placed in the polystyrene trays in the greenhouse. The trays each contained 40 round cells (55 mm diameter, 64 mm deep). These were filled with a substrate of 60% silty loam soil, 30% sand and 10% peat (by volume). To mimic paddy ricefield conditions the trays were set in 12 cm deep plastic containers and battened down by screwed stainless steel rods to prevent them floating. The water level in the containers was maintained at 1-2 cm below the level of the soil surface until 4-5 days before the herbicide treatment, when it was raised to 1-2 cm above the soil surface. To avoid algae growth 1.5 g of copper sulphate was added to each container (which contained 10-12 litres of water). The experimental layout was a completely randomised design with two replicates of twenty cells apiece (half a tray) for each population. Plants were thinned to 2-3 per cell (40-60 per replicate) providing an average of 100 plants per population for the screening tests.

The populations were screened with ALS inhibitor herbicides used in rice in Italy: four sulfonylureas (azimsulfuron, bensulfuron-methyl, cinosulfuron, ethoxysulfuron) and a triazolopyrimidine (metosulam), each sprayed at three times the recommended field dose (field dose: bensulfuron-methyl 60 g a.i./ha, metosulam 70 g a.i./ha, cinosulfuron 80 g a.i./ha, ethoxysulfuron 60 g a.i./ha, azimsulfuron 20 g a.i./ha). Due to the reduced amount of seeds available, a few populations could not be tested with all herbicides. Only a limited number (five) of selected populations of *S. mucronatus* were screened with azimsulfuron. The herbicide was applied at a water volume rate of 350-450 L ha^{-1} and spray pressure of 150-200 kPa by bicycle sprayer when the plantlets were approx. 30 days old (3-4 leaves for both species). No further water was added to the trays for at least 5 days after the treatment and anyway until the level had returned to the initial level, where it was maintained for the rest of the experiment. The number of surviving plants was recorded 30-35 days after applying the herbicide treatments. Plants that showed no active growth, regardless of colour, were considered to be dead. A total of six screening experiments were conducted.

Most screening tests were done during autumn/winter/spring, so light was supplemented using 400 W metal-halide lamps, which provided a Photosynthetic Photon Flux Density (PPFD) of about 150 $\mu mol/m^2$ s and a 14-hour photoperiod. The temperature varied between 10 and 19 °C and 25 to 35 °C night/day, respectively.

Dose-response experiments

Three previously screened populations of both species: one susceptible (Cervarese and Bonelli for A. *plantago-aquatica* and S. *mucronatus*, respectively), one completely cross-resistant to all sulfonylureas and triazolopyrimidine (Casalino for both species) and one cross-resistant to the sulfonylureas but with a low level of plant survival at three times the field dose of triazolopyrimidine (Quartara and Garbagna), were then tested in two greenhouse dose-response experiments. Two herbicides were used: a sulfonylurea (bensulfuron-methyl) and a triazolopyrimidine (metosulam). The eight doses used were in the range from 0 to twice the field dose for the susceptible populations and from 0 to 64 times for the resistant populations. The number of plants surviving the treatments and shoot fresh weight were recorded 30-32 days after the herbicide treatments.

The experimental layout was a completely randomised design with three replicates for the *S. mucronatus* experiment and four for the *A. plantago-aquatica* experiment, each with 20 plants (half a tray). Identical procedures to those for the screenings were followed, the only difference being that the plants were thinned to one per cell.

The dose-response experiments were conducted during late spring-summer with temperatures varying between 18 and 25 °C and 27 to 37 °C night/day, respectively.
A log-logistic equation was fitted to the data (Seefeldt *et al.*, 1995). The upper and lower asymptotes were forced through the mean of the untreated plants and zero, respectively.

RESULTS AND DISCUSSION

Screening experiments

The screenings showed that only three populations of *A. plantago-aquatica* and six of *S. mucronatus* (without considering the limited number of populations of the latter species treated with azimsulfuron) were still completely susceptible to all five herbicides, showing that the poor control in the field was due to the development of resistance. Very few biotypes of both species proved to be partially resistant (i.e. 21-60% of plant survival - Table 2), so indicating that either the selection imposed by ALS inhibitor herbicides has been acting for several years, or the weed control strategy applied in other situations has successfully prevented resistance development. The number of populations still controlled by ALS inhibitors was slightly higher for S. *mucronatus* (Table 2). Most of the populations showed a high percentage of plants surviving the treatments with bensulfuron, cinosulfuron and ethoxysulfuron at three times the field dose. The opposite was true for metosulam, which at the equivalent dose (3x field rate) controlled almost all the populations of both species. An intermediate response was shown by azimsulfuron, which has been recently introduced onto the market. These results probably reflect what was the selecting agent in the field, most frequently bensulfuron-methyl, with several cases where cinosulfuron appeared to be co-responsible for the selection.

Table 2. Number of populations of *A. plantago-aquatica* and *S. mucronatus* ascribable to three categories based on the percentage of plants surviving herbicide treatment at three times field dose.

Survival (%)	Herbicide				
	bensulfuron methyl	cinosulfuron	ethoxysulfuron	azimsulfuron	metosulam
Alisma plantago-aquatica					
0 - 20	4	4	4	8	28
21 - 60	0	2	0	5	2
61 - 100	26	25	23	13	1
No. of tested populations	30	31	27	26	31
Scirpus mucronatus					
0 - 20	6	7	7	3	19
21 - 60	1	2	2	0	1
61 - 100	15	11	11	2	1
No. of tested populations	22	20	20	5	21

Note. All susceptible standards for both species behaved similarly and proved to be 100% susceptible to all herbicides.

From the screening experiments, it can be concluded that for both species, there is widespread

cross-resistance among all the sulfonylureas that are used in Italian rice crops. There was only one population for each species that showed a complete cross-resistance to all sulfonylureas as well as to the triazolopyrimidine (plant survival was always higher than 92%) and these biotypes came from the same farm (Casalino). The historical records show that these fields have been continuously treated with ALS inhibitors since at least 1990 and for the last four years they received two treatments per year, each with half the recommended sulfonylurea dose. No treatments with metosulam appear from the records, which was introduced on the market about three years ago.

Looking at the geographical distribution of the resistant biotypes, they are spread over almost the entire major Italian rice growing area, which is located between Milan and Turin and covers around 195,000 ha (90% of the national total). The areas where the two species are found only partially overlap: *A. plantago-aquatica* is mainly located in the Novara area and to the N-NE of it, while *S. mucronatus* spreads out to the W-SW, as well as being found in the Novara area. The reasons for this are not yet clear. The distribution appears to be patchy, often only one or a few fields on a farm are affected and these farms are often spaced well apart. It can therefore be inferred that most resistant populations were independently selected. All resistant populations came from fields that had been cropped with paddy rice monoculture for more than a decade and had been repeatedly treated with ALS inhibitors. The field histories suggest that resistance developed after at least three-four years of using an ALS inhibitor, and in some cases even where, as well as ALS inhibitors, other herbicides that partially control these two weeds had been used (e.g. oxadiazon, pretilachlor). Nevertheless, these herbicides had been sprayed at low dose and/or too late to efficiently control germination of *A. plantago-aquatica* and *S. mucronatus*. In fact, where the presence of red rice necessitated stale seed-bed preparation and an early pre-sowing treatment of oxadiazon, in general resistance did not develop.

Dose-response experiments

For both species, the ED_{50} and GR_{50} of the susceptible population were very low and varied between 1/23 to 1/31 and between 1/4 to 1/15 of the normal field dose for *A. plantago-aquatica* and *S. mucronatus*, respectively (Tables 3 and 4).

Table 3. Herbicide dose that causes 50% reduction of the percentage of surviving plants and shoot fresh weight relative to untreated controls (ED_{50} and GR_{50} and relative standard error – S.E.) of ALS-susceptible (S) and –resistant (R) populations of *A. plantago-aquatica*.

Population	PERCENTAGE OF SURVIVING PLANTS					
	Bensulfuron-methyl			Metosulam		
	ED_{50} (g a.i./ha)	S.E.	ED_{50} ratio *R/S*	ED_{50} (g a.i./ha)	S.E.	ED_{50} ratio *R/S*
Cervarese (S)	2.26	0.06		2.22	0.02	
Quartara (R)	> 3840		> 1699	158	8.4	71
Casalino (R)	> 3840		> 1699	4133	11.3	1862
	SHOOT FRESH WEIGHT					
	GR_{50} (g a.i./ha)	S.E.	GR_{50} ratio *R/S*	GR_{50} (g a.i./ha)	S.E.	GR_{50} ratio *R/S*
Cervarese (S)	2.66	0.14		2.43	0.12	
Quartara (R)	> 3840		> 1443	126	6.4	52
Casalino (R)	> 3840		> 1443	4237	382.2	1744

Table 4. Herbicide dose that causes 50% reduction of the percentage of surviving plants and shoot fresh weight relative to untreated controls (ED_{50} and GR_{50} and relative standard error – S.E.) of ALS-susceptible (S) and –resistant (R) populations of *S. micronatus*.

Population	PERCENTAGE OF SURVIVING PLANTS					
	Bensulfuron-methyl			Metosulam		
	ED_{50} (g a.i./ha)	S.E.	ED_{50} ratio *R/S*	ED_{50} (g a.i./ha)	S.E.	ED_{50} ratio *R/S*
Bonelli (S)	16.64	1.66		8.2	1.33	
Garbagna (R)	> 3840		> 231	512	105.5	62
Casalino (R)	> 3840		> 231	> 4480		> 546
	SHOOT FRESH WEIGHT					
	GR_{50} (g a.i./ha)	S.E.	GR_{50} ratio *R/S*	GR_{50} (g a.i./ha)	S.E.	GR_{50} ratio *R/S*
Bonelli (S)	5.9	1.19		4.7	1.31	
Garbagna (R)	> 3840		> 650	355	114.4	76
Casalino (R)	> 3840		> 650	4229	237.7	900

These results confirm that these species are among the most susceptible to ALS inhibitors.
The low value of the standard error of the parameters (ED_{50} and GR_{50}) indicates that the log-logistic equation fitted the data accurately and the range of doses was appropriate.
This was not always true for the resistant populations. Both showed a very high level of resistance with most data points at or near their maximum value, especially to bensulfuron-methyl and it was therefore sometimes impossible to fit the curve. In these cases, in Tables 3 and 4 the ED_{50} and GR_{50} are indicated as higher than the maximum herbicide dose used (64x) in the experiments. The resistance indexes for *A. plantago-aquatica* are generally higher than those calculated for the other species.
The two biotypes from Casalino showed a very high level of resistance to both herbicides, the resistant index being always higher than 1400 and 230 for *A. plantago-aquatica and S. micronatus*, respectively. Although the other two populations, Quartara and Garbagna, proved to be resistant to both herbicides, their resistance level to the triazolopyrimidine was much lower, with the resistance index varying between 52 and 76.
Although the results from the screenings and the dose-response experiments appear to be quite consistent, their analysis suggests that the efficacy of metosulam was lower when the experiments were carried out at high temperatures and, secondly, several populations that appeared to be susceptible to metosulam at 3x, may actually be partially resistant. This suggests that screenings with metosulam should be carried out at a lower dose (1x and/or 2x field rate). However, these results need to be related to the information from the field, which indicates that metosulam does not generally sufficiently control weed populations resistant to sulfonylureas.
The results show that the resistance situation for the two species is quite similar, with a generalised cross-resistance among all the ALS inhibitors used in rice crops in Italy. The resistance level of the triazolopyrimidine appears to be lower than that found for the four sulfonylureas. However, the resistance levels to the various herbicides support the information (J-P Claude, personal communication) that the resistance mechanism involved is a target site.
At the moment most farmers are successfully managing herbicide resistant populations with chemical solutions such as pre-sowing application of oxadiazon and post-emergence

treatments of MCPA (usually mixed with propanil). The real problems are found where farmers have adopted EU regulation 2078, that does not allow the use of MCPA and imposes limits on the number of post-emergence treatments.
Although a simple estimate indicates that the cost of preventing resistance is much lower than that of managing resistance (Orson, 1999), very few farmers have adopted any form of resistance prevention strategy.

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PCR and sequence based strategies for the detection of ACCase inhibitor resistance in grass weeds

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ABSTRACT

The molecular basis of resistance has been established for a number of different herbicides, especially where resistance is due to target site modifications. However, in the case of ACCase-inhibitor resistance the mutations which lead to an insensitive target enzyme are, as yet, uncharacterised. One approach by which such mutations may be identified is the amplification of genomic DNA fragments using the polymerase chain reaction (PCR), and sequencing these fragments in resistant and susceptible lines. The rationale behind this process is described, and the advantages and disadvantage of using molecular biology techniques as an adjunct to traditional diagnostic practices discussed.

INTRODUCTION

Resistance to acetyl coenzyme-A carboxylase inhibiting herbicides, the aryloxyphenoxypropionate ('fops') and cyclohexanediones ('dims') has become an increasing problem in recent years. Almost 20 plant species have evolved resistance in some 18 different countries (Heap, 1997). However, while many resistant populations have been characterised quite extensively, both at the whole plant and the biochemical level, the specific molecular changes leading to resistance have not yet been established for ACCase inhibitors.

In the majority of cases, resistance is attributed to modification in the target site enzyme and is generally found to be conferred by a single dominant, or semi-dominant, nuclear gene (Devine, 1997). Resistance has also been attributed to enhanced metabolism of the herbicides (Hall *et al*, 1997), a mechanism which is likely to be controlled by several interacting genes. However, the molecular basis for this form of resistance will not be explored in this paper.

It can be hypothesised that target site-based resistance is caused by small, heritable changes in the DNA of previously susceptible plants. This subsequently leads to modifications in the three-dimensional structure of the enzyme, thereby altering the response of the plant to selection by a herbicide. The observed patterns of resistance and cross-resistance between

populations vary, suggesting that a number of different mutations have arisen. The aim of this paper is to explain the rationale behind this hypothesis, both by examining the biochemistry of ACCase and the molecular structure of the genes encoding it, and by using examples from other systems where target site resistance has been conferred in this way. We will also evaluate the utility and limitations of molecular approaches for the detection of ACCase inhibitor resistance, using wild-oats (*Avena* spp.) and look at how they may be developed as a diagnostic tool to be used alongside traditional methods.

ACCase - BIOCHEMISTRY AND MOLECULAR BIOLOGY

The biochemical and molecular structure of ACCase, and its interaction with herbicides, give important indications of where the mutations which confer resistance are most likely to have arisen. ACCase catalyses the first committed step of fatty acid biosynthesis, the carboxylation of acetyl CoA to malonyl CoA. The reaction takes place in the stroma of the plastids (Gronwald, 1991). The reaction is catalysed in two steps, each of which occurs within a separate domain on the enzyme. A carboxyl group is first bound to the biotin prosthetic group, which is itself attached to the biotin carboxyl carrier protein. The carboxyl group is then transferred to acetyl CoA, in a reaction catalysed by the carboxyltransferase, resulting in the formation of malonyl CoA.

The carboxyltransferase reaction is the most sensitive to inhibition by the aryloxyphenoxypropionate and cylcohexanedione herbicides. Double inhibition studies indicate that the herbicides are mutually exclusive inhibitors, suggesting that they may share a common binding site, or that their binding sites overlap (Rendina *et al*, 1988; Burton *et al*, 1991). It is thought possible that they may interact with an acetyl CoA binding site (Dehaye *et al*, 1994), or interfere with the release of malonyl CoA (Rendina *et al*, 1990). This suggests an area where mutations may be more likely to influence the response of the plant to selection by a herbicide. However, it is important to emphasise that any change in the amino acid sequence may be sufficient to cause a structural modification in the enzyme which could confer resistance.

Genes encoding ACCase have been cloned in a number of higher plant species, although these have mainly been of the Type II (cytosolic) isoform. However, some genes of the Type I isoform, localised to the plastids and inhibited by "fops" and "dims", have now been cloned. Structurally the genes are broadly similar. All four domains which catalyse the overall reaction are encoded by the same gene, and are arranged in the order biotin carboxylase, biotin carboxyl carrier protein, carboxyltransferase β and carboxyltransferase α (Egli *et al*, 1995). Complete gene sequences for Type II isozymes have been cloned in wheat and alfalfa, and were found to contain 29 and 30 introns respectively. This indicates that the size of the gene is between 10 and 12kb. The cDNAs (the portion of the gene which encodes the protein) for the Type I enzyme encode an additional 100 amino acids at the N terminus of the protein, which appears to be a transit peptide, probably responsible for targeting the enzyme to the plastids.

In the areas encoding the four reaction catalysing domains the genes are highly conserved. This means that there is a high degree of amino acid and nucleotide sequence similarity

between different species. Amino acid identity is ~80% within isoforms and ~60% between the different isoforms.

In hexaploid wheat there are thought to be at least two genes encoding the Type II isoform (Podkowinski *et al*, 1996), but the presence of only 3 cDNAs for the Type I isoform suggests a single copy gene transcribed from all three sets of chromosomes (Gornicki *et al*, 1994). Wild oat is also hexaploid and it is probable that a similar mechanism also operates here.

NATURE OF MUTATIONS

It is most likely that in cases of target site resistance the causal modifications in the enzyme are due to point mutations. These are single nucleotide changes in the gene sequence which result in the substitution of one amino acid for another (Devine, 1997). Given the speed with which resistant populations can arise, it is probable that mutations exist at low frequencies in unselected populations (Devine & Shimabukaro, 1994), clustered in conserved regions of the gene. Inter-population variation in response to herbicides may be a result of several different mutations, each responsible for a characteristic pattern of resistance.

Support for the point mutation hypothesis comes from case of resistance to ALS inhibitors. Mutations have been detected in five highly conserved domains, ranging in size from 4-19 amino acids. Although mutations in only four of these domains are found in the field, in each case a single nucleotide change was sufficient to confer resistance (Boutsalis *et al*, 1999). ALS mutations in previously uncharacterised biotypes have been identified by amplifying these regions by PCR, using gene specific primers and screening sequences for mutations by sequencing the fragments (Boutsalis *et al*, 1999). This should also be possible for ACCase, however this has not been achieved to date, possibly due to the enzymes complex nature.

STRATEGY FOR MUTATION IDENTIFICATION

Regions of the gene which are highly conserved are more likely to be essential for the synthesis of a functional enzyme. Consequently, mutations in these regions are likely to have the greatest phenotypic effect. In addition, the spectrum of use of the herbicides, inhibiting most Type I ACCases, suggests they interact with a conserved region. The Type I ACCase gene has not yet been cloned in a weedy species. Therefore to design oligonucleotide primers for the amplification of relevant gene fragments it is necessary to use the information on conserved regions from other species. By aligning cDNA and genomic DNA sequences from a variety of plant ACCase genes, it is possible to identify regions where sequence conservation is high. Gene specific primers can then be designed based on this consensus sequence, which will not only enable the amplification of the fragment in the template species, but, given the high degree of evolutionary conservation, also in other plants.

Alignment of ACCase Gene Sequences

A number of plant ACCase gene sequences were downloaded in our laboratory from the Genbank Entrez database, including those from maize (Egli *et al*, 1995), wheat (Podkowinski

et al, 1996, Gornicki *et al,* 1994), *Brassica napus* (Schulte *et al,* 1994, 1997) and alfalfa (Shorrosh, 1994). These were aligned using the BLAST Sequence Homology Service of the National Centre for Biotechnology Information.

Design of Primers for Polymerase Chain Reaction (PCR)

Oligonucleotide primers were designed based on the consensus sequence using a specialised computer programme, Oligo5. Although conserved regions of the gene were targeted, sequences with very high degrees of identity between the Type I and Type II isoforms were eliminated in order to prevent the potential amplification of both genes.

Because the ACCase gene is large, and the position of mutation sites can, as yet, only be hypothesised, several primer sets spanning large regions of the gene are necessary. These are then used in PCR reactions to amplify fragments from resistant and susceptible plant genomic DNA extracts (Figure 1). Essentially PCR enables the selective amplification of DNA sequences. When the double stranded DNA is heated, the strands separate. The now denatured DNA is then cooled, so that the single stranded oligonucleotide primers anneal to complementary sequences in the genomic DNA (effectively bridging the region of interest). The primer sequences are then extended by the action of a thermostable DNA polymerase enzyme. This synthesises a copy of the DNA template by sequentially incorporating single nucleotides onto the 3' end of the primer. The cycles of denaturation, primer annealing and extension are repeated 25-25 times. This results in an exponential increase in the number of copies of the desired ACCase fragment.

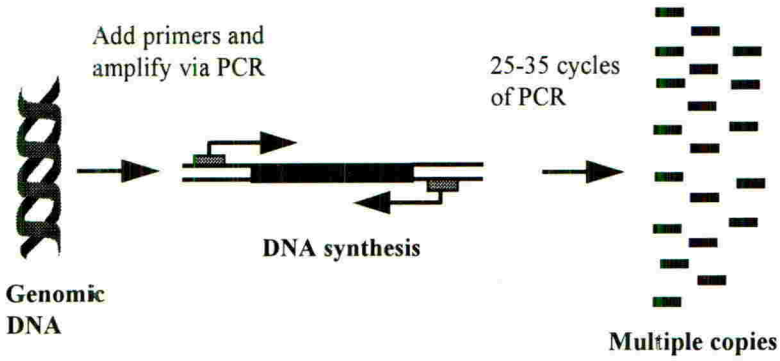

Figure 1: Schematic representation of fragment amplification via the Polymerase Chain Reaction (PCR). Genomic DNA extracted from resistant and susceptible plants. Designed primers are added and the mixture subjected to ~30 cycles of denaturation, annealing and amplification, which produces multiple copies of the fragment spanned by the two primers.

Accordingly, we designed primers to cover a number of regions of the ACCase gene and successfully amplified gene fragments in uncharacterised wild-oats and ryegrass (*Lolium multiflorum*). These fragments span the biotin carboxylase and carboxyltransferase domains,

indicating a conserved sequence primer design approach can be successful for amplifying the ACCase gene in as yet uncharacterised species.

There are however some limitations to this technique. Success is usually dependent upon there being sufficient sequence conservation to make the design of primers possible. This is not the case for the complete ACCase gene, where the region between the biotin carboxyl carrier protein and carboxyltransferase domains is not highly conserved. It is therefore problematic designing primers for this region. Additionally, intron positions have not been fully characterised for the type I genes and as such, may interfere with primer design sequences. Around the biotin carboxyl carrier protein, exons are particularly short, making it difficult to amplify this region successfully.

Sequencing of ACCase Gene Fragments

Assuming a mutation is homozygous, (is present on both alleles of the individual) it should be revealed by sequencing of the PCR fragment from both resistant and susceptible individuals. The success of this approach is dependent on many factors, not the least of which is the species under study. In wild-oats ploidy level may be a considerable hurdle in the detection of mutations by sequencing. As wild-oats are hexaploid ($2n = 6x = 42$), it is likely that only one pair of genes encoding the Type I isoform will contain any single mutation, while the remaining two pairs produce wild-type, herbicide sensitive forms. This means that the mutant fragment will comprise, at most, only one third of the total product of a single PCR reaction. In any subsequent sequencing reaction carried out on the samples, the signal from the altered nucleotide is likely to be masked by that of the two wild types.

Traditional methods for determining herbicide resistance involve glasshouse-based dose-response trials and assaying ACCase activity. This approach is time consuming, costly and has, in the past, been shown to be problematic in certain species (such as wild oats!). Modern molecular methods, such as those outlined here, may represent a viable, alternative, screening strategy. Smaller amounts of sample material are required, the techniques themselves are rapid, which allows more populations to be screened in a shorter time, and with a higher degree of precision. These benefits are, however, based on the need for greater characterisation of the range of mutations which confer resistance upon individuals and the degree to which these vary between populations. When this has been achieved, one of the major potential benefits to be derived from the application of molecular diagnostic techniques, is that it will enable the faster implementation of effective management strategies to control resistant populations. Such methodology will complement the current diagnostic practices by providing the agricultural sector with an informative, precise tool which has no detrimental environmental impact

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Effectiveness of mode of action labelling for resistance management: a survey of Australian farmers

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ABSTRACT

Since 1997, all herbicide labels in Australia have displayed a letter denoting the mode of action (MOA) of the active ingredient. This labelling was part of a strategy to manage herbicide resistant weeds. In 1998 the Herbicide Resistance Action Committee commissioned a survey by the Kondinin Group to determine Australian farmer attitudes and experiences with mode of action lettering on herbicide labels. Based on the results from the survey it appears that there are some benefits in Australia to MOA labeling. Most farmers in Australia are aware of the label and those who have resistance problems are using this designation in planning their weed management programs. However, the survey also showed some of the weaknesses of this system. While the simplicity of the system appeals to farmers, there is confusion in understandings why certain herbicides are grouped together and in interpreting lettering on herbicide mixtures that contain multiple MOAs. This confusion shows that there are is a high potential for misunderstanding the utility of MOA labeling and that MOA labelling alone is not enough. There has to be an effective educational program associated with labelling for this information to be used successfully. In addition, what may work well in Australia may not be as effective in other countries where multiple herbicide mixtures are used.

INTRODUCTION

Herbicide resistance is a worldwide phenomenon with 218 documented cases (Heap, 1999). Selection of herbicide resistant weed populations is often the result of the continuous use of the same herbicide or herbicides with the same mode of action (MOA) (Heap, 1999). Management of herbicide resistance requires an integrated approach utilising various tools to decrease the selection of resistant weeds. One of the key steps in resistance management is to minimise the continuous use of herbicides with the same mode of action through rotations and combinations of products. However, for this technique to be successful the farmer must know which herbicides share the same mode of action. To address this need, the Herbicide Resistance Action Committee (HRAC) developed a classification of herbicides according to their mode of action (Schmidt, 1997). This scientifically based

classification system groups herbicides into various categories designated by different letters. A similar system has been developed by the Weed Science Society of America (Retzinger and Mallory-Smith, 1997) using numbers instead of letters to designate the categories.

Herbicide resistance is particularly widespread in Australia with more than 30% of the cereal fields containing resistant annual ryegrass (*Lolium rigidum*) (Powles, *et al*, 1997). Part of the integrated plan to manage this resistance problem is to encourage farmers to use rotations of herbicide with different modes of action. Labeling herbicide with their mode of action could be part of that programme. In 1997 Australian industry members agreed that all herbicide labels should show the modes of action of the herbicides in order to assist farmers in product selection for their weed management programs (Figures 1 and 2).

It has been recommended that other countries, (e.g. the United States) should also adopt this labelling system (Dyer, 1997). However, there has been some controversy over how effective labelling herbicides by their MOA is in resistance management. The primary concerns are 1) such labelling is too simplistic; 2) MOA labelling does not address metabolism-based resistance; and 3) MOA labelling may not reach the herbicide decision maker. In order to obtain more information on the effectiveness of labelling herbicide by MOA for resistance management, HRAC commissioned a survey by the Kondinin Group in 1998 to determine Australian farmer attitude and experiences with mode of action lettering. In this report, we will present some of the primary results and conclusions from that survey.

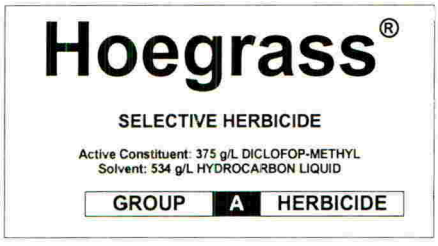

Figure 1. Front panel of the Australian label for diclofop-methyl, displaying an example of Herbicide Mode of Action grouping.

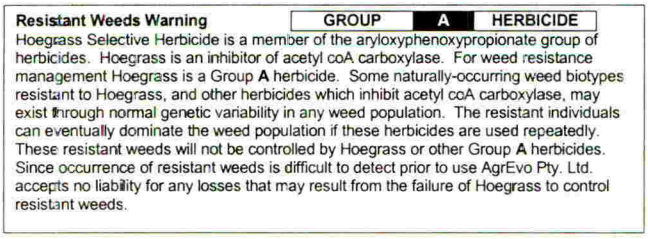

Figure 2. Side panel of the Australian label for diclofop-methyl

MATERIALS AND METHODS

The survey was conducted using a questionnaire of 17 questions that was distributed to 16,000 farmers with the June, 1998 issue of *Farming Ahead* magazine. 1380 responses (8.7%) were received and processed. This survey was followed up with focus groups consisting of farmers and agronomists. The focus groups were run in Wagga Wagga (1), New South Wales (2); Horsham, Victoria (1); Clare, South Australia (1), and Newdegate, Western Australia (1), areas where herbicide resistance was known to be a problem. Selected groups of farmers were invited to attend by local consultants, agronomists and research personnel from the Charles Sturt University. In addition, phone interviews of selected working agronomists were conducted to determine what their opinion was of their clients' attitude to and understanding of the letter code on the herbicide labels. Since this was a relatively informal interview process, no statistical analysis was done on the results and, due to the concentration of the focus groups in areas of known resistance, the attitudes expressed may not be a full representation of the farmers' attitudes in Australia.

RESULTS

Questionnaire Survey Results

Level of resistance and resistance management

The most important weed problems on the farms of the respondents were annual ryegrass (*L. rigidum*) (46%), wild oats (*Avena fatua*) (12%) and wild radish (*Rhaphanus raphanistrum*) (9%). Approximately 26% of the respondents had had their ryegrass population tested for resistance, which corresponds roughly to the report that 30% of the fields in Australia contain herbicide resistant ryegrass (Powles *et al*, 1997).

When asked what farming practices were being used to prevent/reduce resistance rotation of herbicide groups (85%) and crop and variety rotation (85%) were the most cited method followed by chemical pasture topping (77%), improved crop nutrition (75%) and heavy grazing (73%). The least used method was collecting seed at harvest (8%) even though this method has been shown to be a highly effective method for managing resistant ryegrass.

In a question on the factors affecting the development of resistance, farmers responded that the use of low chemical rates, faulty applications, using the wrong mode of action herbicide and poor growing conditions as the most likely causes. The use of high chemical rates was ranked as the least likely factor affecting resistance development. These answers may indicate that farmers were confusing lack of performance with resistance development.

Resistance management and herbicide selection

Forty-nine% of the farmers said they always consider resistance development when buying their herbicides, while 45% sometimes consider this. Only 5% of the respondents never consider resistance. From the standpoint of the effectiveness of MOA labelling, 85% of the

farmers said they were aware of a herbicide's MOA and 85% of the respondents considered MOA an important aspect when making a buying decision. This indicates that herbicide MOA is an important piece of information for many Australian farmers' herbicide buying decisions.

However, when farmers were asked what factors were most important when selecting a herbicide, efficacy was the number one consideration, followed by cost, then mode of action. In terms of who influenced their selection decision, distributor recommendations and other farmer's advice were the most important. On the other hand, 78% of the respondents said that advertising had no influence on their decisions.

Utilisation of MOA label

The majority of the respondents (83%) found the single MOA letter was very or quite easy to understand while only 10% found it confusing. However, when there were multiple MOA letters included (for mixtures of herbicides) 25% of the respondents said they were confused and only 12% found it quite easy to understand, suggesting that the farmers were not sure how to use the information.

One of the purposes for MOA labelling was to make it easier for farmers to record which MOAs they used from year to year. Unfortunately only 39% of the respondents indicated that they always recorded MOA used each year. Fifty-eight % of the farmers never recorded the MOA or only did it sometimes. These responses suggest that there is a need for more education on how MOA labelling fits into a resistance management programme and that knowing the MOA is not only important at the time of purchase but also in the year to year planning process.

When farmers were asked how they'd like to receive additional information on resistance management, they wanted it through field days (69%) labels (59%), chemical distributors (58%) and agriculture department (52%). The least likely source of further information was in training courses (29%), media (33%) and seminars (39%).

Recommendations for improvements of MOA label

There were a number of suggestions for improving the MOA group letter that would assist with the resistance management program. These suggestions included:

- Adding a colour coding with the letter
- Making the letter larger and more defined.
- Including the letter in all advertisements and promotional material
- Increasing education and literature explaining the MOA labelling of herbicides
- Providing a wall chart depicting the herbicide groups and the products in each group
- Including more information on the label by
  - Adding an acronym (e.g. 'fop', 'dim', 'SU' etc) besides the letter
  - Listing the weeds that are most likely to have resistance

- Indicating if a chemical group is in a high risk category
- Including a leaflet summarising the whole code
- Limiting how many times a product can be used consecutively
- Identifying sub-groups (e.g. aryloxyphenoxypropionate vs cyclohexanedione)
- Recommending rotation between groups

Focus Groups and Expert Opinions

The focus groups consisted of farmers, consultants either alone or in mixtures. There were several common themes from these various sessions.

1. Many consultants felt that farmers are aware of the MOA label but there were mixed feelings about how the information was being used. In all cases the leading farmers are aware and using the information.
2. Many of the farmer comments indicate that they don't pay attention to the label until after a problem develops. They then found the labelling very helpful in planning their weed management programme.
3. One common theme was that combining aryloxyphenoxypropionates and cyclohexanediones into the same category (Group A) was not ideal. Although resistance to many of the ACCase inhibitors has occurred, farmers are still able to use clethodim effectively. They felt that this herbicide should not be included with the rest of the ACCase inhibitors or there should be a distinction made between the two classes (e.g. A1 and A2).
4. There was almost universal support for including the MOA letter classification in advertisements and other literature associated with herbicides.
5. Farmers liked having the MOA letter on new products because there is confusion when a new product comes out whether it is actually new or just a new formulation of an existing product.
6. Many farmers depend on their agronomists and consultants to know the MOA of the herbicides they are using and to plan their herbicide programme. To these farmers the MOA label was not perceived as having a lot of value.

In telephone interviews with expert consultants and university personnel, there was almost universal support for the MOA labelling. It was recognised that more education is required to train the farmers to use this information productively. However, these experts saw a lot of benefit for having a simple system for communicating MOA to growers.

CONCLUSION

Based on the results from the survey and focus groups it appears that there is some benefit in having MOA labelling of herbicides in Australia. Most farmers in Australia appear to be aware of the label and it's significance and those who have resistance problems are using it in planning their weed management programs. However, it is also apparent that there is some confusion in how to fully utilise this information. There is a need to educate farmers further in herbicide resistance management and in how to most effectively use MOA labelling.

The results of this survey suggest that the concerns raised by HRAC on the MOA labelling are important. The simplicity of the system does appear to appeal to farmers, but the confusion over interpreting multiple MOAs in mixtures and in understanding why certain herbicides are grouped together shows that there are is a high potential for misunderstanding the utility of MOA labelling. A number of farmers felt that they did not need to know the MOA of a herbicide because they depended on agronomists and consultants to have this information. This indicates that it is important to understand whom the decision-makers are and to be sure that resistance management information is getting to the right audience. Finally, MOA labelling is of little utility for a farmer who had metabolism based resistant annual ryegrass. This aspect was not addressed in this survey, but it is still a weakness of the system.

One suggestion to help understand MOA labelling was to develop a wall chart showing the classification system. HRAC is currently preparing this type of material and will soon be making it available.

It is apparent that MOA labelling alone is not enough. There has to be an effective educational program associated with labelling for this information to be successfully used. This material should extend beyond the label to other educational material. Although there was a call for inclusion of MOA labelling in advertisement literature, the fact that almost 80% of the farmers disregard this material suggests that this is not the ideal way to present this information. Instead there should be more information on the utilisation of MOA information in technical material, research reports and in presentation made by consultants and distributor agronomists, since these are the primary means by which farmers in Australia get their information.

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