THE BCPC CONFERENCE

Weeds 2001
Volume 2

SESSION 7A

INTEGRATED CROP AND WEED MANAGEMENT IN GRAIN CROPS

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Papers: 7A-1 to 7A-4

Propoxycarbazone-sodium (BAY MKH 6561) – A key tool in integrated *Bromus* management in Germany

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ABSTRACT

Bromus species are serious arable weeds in Germany, becoming more prevalent in winter cereals. Not commercially controlled by standard selective cereal herbicides, farmers commonly used metribuzin (Sencor). This usage however, has been forbidden since July 1st, 2001.

Propoxycarbazone-sodium (Attribut) can be applied post-emergence in spring to winter wheat, rye and triticale at rates of 28 to 70 g a.i./ha against grasses such as *Bromus* species. Single applications of propoxycarbazone-sodium showed moderate to good control. By adding adjuvants, or applying propoxycarbazone-sodium in a split application or sequences following an autumn standard treatment, good to very good control could be achieved. No single measure or herbicide can solve the *Bromus* problem on its own. Propoxycarbazone-sodium however offers a highly effective treatment as an important part of an integrated *Bromus* management strategy.

INTRODUCTION

Bromus sterilis and other *Bromus* species are serious arable weeds in Germany. Based on a survey carried out by Bayer Vital GmbH in 1999, more than 100,000 ha of the winter cereal acreage were found to be heavily infested. *Bromus* is mainly a problem in winter wheat and winter barley (75 % and 15 % respectively of the total infested area). The situation may even become worse as current trends in cereal production such as minimum tillage, early drilling and narrow crop rotations favour *Bromus* occurrence.

Whereas *Bromus* can be controlled in sugar beet and oil seed rape by applying graminicides based on aryloxyphenoxy-propionate or cyclohexanediones, there is no standard selective herbicide providing commercial control in cereals. Farmers commonly used metribuzin in combination with other herbicides like flufenacet & diflufenican (Herold), providing temporary suppression of *Bromus*, in order to manage *Bromus* species in winter barley and winter wheat (Balgheim & Kirchner, 1998). Although highly effective (Table 1), the usage of metribuzin was restricted due to the risk of crop damage under certain climatic and soil conditions. However, its off-label usage was forbidden by law as of July 1st, 2001.

In the absence of any herbicidal solution for autumn post-emergence application the control has to rely on spring applications. Herbicides which can be used as early as possible and in many cereal crops as possible are requested.

Table 1. Herbicidal efficacy of metribuzin and flufenacet & diflufenican on *B. sterilis* in winter cereals at different timings and different rates (Germany 1993-1999)

Treatment	Rate (g a.i./ha)	Timing	Efficacy (%)	n
Metribuzin	280	post-em. autumn	87	6
Metribuzin	350	post-em. spring	77	20
Flufenacet & diflufenican	240 & 120	post-em. autumn	61	10
Flufenacet & diflufenican	240 & 120	post-em. autumn		
+ metribuzin	140		81	4
fb metribuzin	140	post-em. spring		

(& = ready mix; + = tank mix; fb = followed by; n = number of field trials)

METHODS AND MATERIALS

Propoxycarbazone-sodium (BAY MKH 6561) is a sulfonylaminocarbonyl-triazolinone herbicide discovered and developed by Bayer AG (Feucht *et al.*, 1999). Its mode of action is inhibition of the enzyme ALS. Uptake by plants is via leaves and, in the absence of adjuvants, predominantly via roots. The compound provides some residual activity, so weeds emerging during the few weeks after application are also controlled. Propoxycarbazone-sodium will be registered in Germany for applications post-emergence in winter wheat, rye and triticale at rates of 28 to 70 g a.i./ha. The application window lasts from growth stage 13 to 29 (BBCH) of the crop. Target weeds are *Alopecurus myosuroides* Huds., *Apera spica-venti* (L.) P.B., *Bromus sterilis* L. and *Elymus repens* (L.) Gould. It has been tested against *B. sterilis* in several series of trials. All trials were conducted according to EPPO-guidelines using a WG formulation (700 g/kg propoxycarbazone-sodium). Only the final efficacy assessments after heading of the weeds are presented.

RESULTS

Propoxycarbazone-sodium has been tested in winter cereals in Germany since 1993. It was highly selective in winter wheat, rye and triticale, but must not be applied in winter barley due to a strong risk of crop damage.

Rates against *Bromus* species vary from 42 to 70 g a.i./ha, depending on species, growth stage, application timing, soil type and soil conditions. The core rate of propoxycarbazone-sodium for *Bromus* control was 42 g a.i./ha, resulting in good control of all important *Bromus* species. In greenhouse experiments conducted by Bayer AG and Augustin (2000) as well as under field conditions the least susceptible *Bromus* species was found to be *B. sterilis*

(Table 2) representing 80 to 90 % of *Bromus* infestations in Germany. The rate was established for the control of *B. sterilis* as all the other species will also be controlled.

Table 2. Herbicidal efficacy of propoxycarbazone-sodium on different *Bromus* species (42 g a.i./ha, applied at different weed growth stages (BBCH), greenhouse experiment, Bayer AG 2000)

Species	Herbicidal efficacy (%) Weed growth stage (BBCH)			
	00	11	13	21
B. arvensis	100	95	90	90
B. commutatus	98	95	90	80
B. japonicus	98	98	98	95
B. mollis	98	98	95	90
B. secalinus	95	95	90	80
B. sterilis	90	80	70	70
B. tectorum	95	90	70	70

In field trials, single applications in spring achieved on average 76 % and 81 % control respectively at rates of 42 and 70 g a.i./ha propoxycarbazone-sodium (Figure 1). Splitting of the maximum rate in early and late treatment was more effective in controlling *B. sterilis* than single sprays, achieving 89 % control on average. In more than 90 % of all trials an efficacy higher than 73 % was observed. In the modified box and whisker plot the box shows

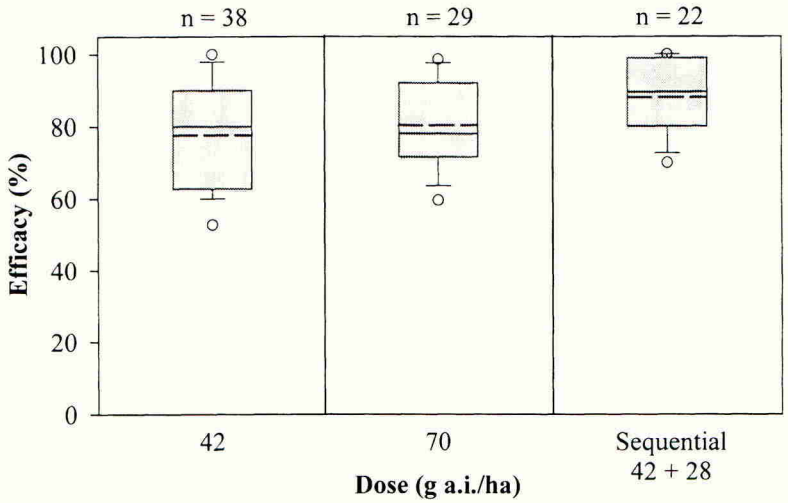

Figure 1. Herbicidal efficacy of propoxycarbazone-sodium on *B. sterilis* in winter wheat (applied in post-emergence in spring, Germany 1994-2001, n = number of trials)

the interquartile range, the horizontal bar the median and the medium dash the arithmetic mean. The whisker extends from the ends of the box to the 10 % and 90 % quantile excluding the 5 % and 95 % quantile, which are shown as detached points form the whisker.

Analysing the observed variability by clustering the results to the growth stage of the weed and plotting them against weed control it can be seen that final control depends on the growth stage at application (Figure 2): The more advanced the growth stage, the less susceptible the weed resulting in a lower final control. To maximise herbicidal efficacy it is crucial to apply at early growth stages. Application should be made before mid-tillering.

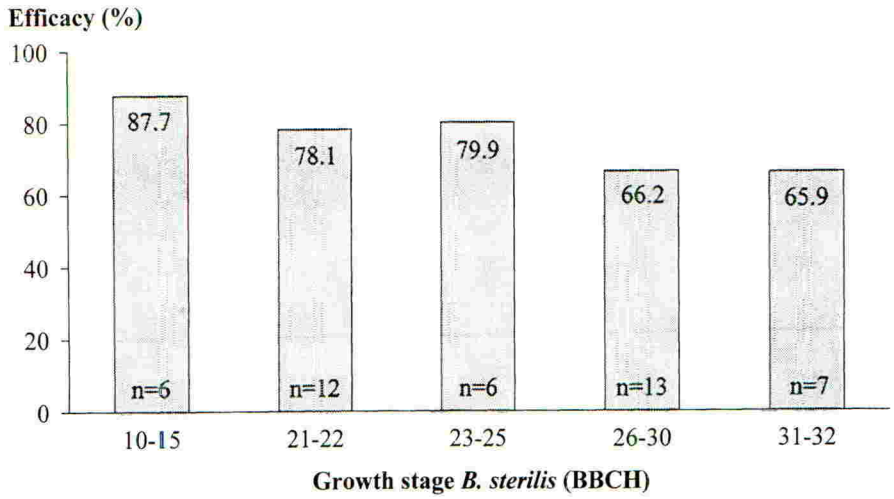

Figure 2. Herbicidal efficacy of propoxycarbazone-sodium on *B. sterilis* at different growth stages (42 g a.i./ha, applied post-emergence in spring, Northern Europe 1995-2000, n= number of trials)

As *Bromus* species can emerge in flushes, different growth stages being more or less susceptible might be found side by side in one field. Sequences of sprays increase the chance to hit the weeds at their most susceptible stage and to avoid the regrowth of advanced plants which are not fully controlled by an single treatment. It is recommended to apply the first spray as early as possible in spring and to apply the second when the regrowth starts, i.e. two to three weeks after the first one.

Propoxycarbazone-sodium predominately acts via the roots. Therefore, it is more effective in controlling weeds when soil is moist. Under dry conditions it recommended to increase leaf absorption by adding appropriate adjuvants or applying in fluid urea ammonium nitrate fertiliser (UAN), which is quite common in Germany. Adding an adjuvant is of advantage as well, if high crop or weed densities prevent spray solution reaching the ground.

In field trials the efficacy of propoxycarbazone-sodium on *B. sterilis* was increased about 5 %, if applied in UAN (Figure 3). Comparable results were achieved by the addition of the

rape seed oil Rako-Binol. By far the strongest effect was observed, when the tallow amine ethoxylate Frigate was used. At a rate of 42 g a.i./ha an average increase in *B. sterilis* control of approximately 13 % was achieved.

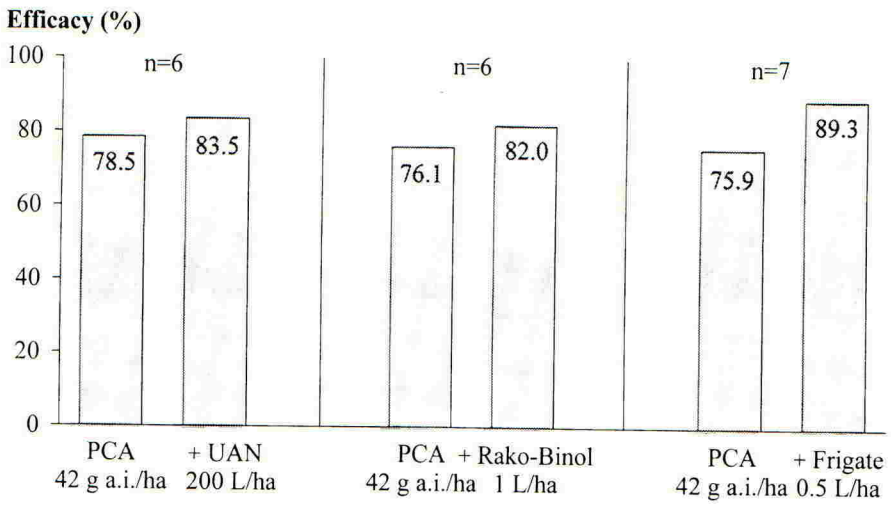

Figure 3. Herbicidal efficacy of propoxycarbazone-sodium (PCA) alone on *B. sterilis* and in combination with UAN or different adjuvants (applied post-emergence in spring, Germany 1999-2001, n = number of trials)

DISCUSSION

Propoxycarbazone-sodium, applied at the recommended rate, timing and in combination with an adjuvant has proven to be a highly effective tool for *B. sterilis* control in winter wheat, rye and triticale, provided the weed is not too far developed in spring. In early drilled winter cereals however, *B. sterilis* growth stage in spring might be beyond the most susceptible stage, causing less consistent control. Therefore, a two-pronged approach using cultural and chemical methods is still the best way to keep *B. sterilis* at manageable levels.

In addition to crop rotation ploughing deeper than 15 cm is one of the best measure to control *Bromus* species. Where ploughing is not practical, drilling of subsequent crops should be delayed in order to gain more time for stubble cultivation. *B. sterilis* populations generally have low dormancy (Peters *et al.*, 1993), thus the majority of seed will germinate more or less immediately after harvest if sufficient moisture is available. Cultivating as soon as possible after harvest in order to cover *B. sterilis* seeds prevents the onset of light enforced or induced dormancy and encourage germination. The first flush of seedlings then can be destroyed by further cultivations or using a non-selective herbicide based on glyphosate.

Subsequently, in later drilled cereals less *Bromus* seedlings emerge expressing less competition to the crop as temperature requirements for germination favour the cereal crop in later

autumn. In addition a later seeding date of the crop will result in less advanced *B. sterilis* plants in spring, which are more susceptible to propoxycarbazone-sodium.

For in crop control of *Bromus* species in pre- or post-emergence in autumn no herbicide is registered in Germany. If an early drilling is preferred due to economical reasons, farmers have to rely on flufenacet & diflufenican providing some suppression on *B. sterilis* (Table 1). By controlling parts of the *B. sterilis* infestation and reducing at least the vitality of the other, this treatment supports spring applications in early drilled winter cereals as the *B. sterilis* development gets delayed.
For winter barley however, there is no chemical solution available, neither in the fall nor in the spring. *Bromus* control totally relies on cultural methods.

CONCLUSION

Ten years experience of *Bromus* management in Germany have shown, that no single measure in cereals can solve the *Bromus* problem. An integrated approach has to be taken adjusting all available tools like crop rotation, drilling date and different herbicides in different crops. As metribuzin is forbidden in winter cereals since July 2001, there is in short term no herbicide available for *Bromus* control in autumn and especially in winter barley. The use of propoxycarbazone-sodium however will provide new opportunities for *Bromus* control in winter wheat, rye and triticale and will allow a further increase of minimum soil tillage systems.

ACKNOWLEDGEMENTS

The authors would like to take the opportunity to express their gratitude to their Bayer colleagues throughout the world and acknowledge their efforts in developing propoxycarbazone-sodium.

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The effect of climatic factors on the activity of isoproturon and clodinafop-propargyl on *Alopecurus myosuroides* (black-grass) in winter wheat

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ABSTRACT

Field trials were set up at ADAS Boxworth on a clay soil, for two seasons, 1997-1998 and 1998-1999 to assess the activity of the herbicides isoproturon and clodinafop-propargyl on *Alopecurus myosuroides* in winter wheat. The herbicides were sprayed at 4 different rates, on 8 and 10 occasions between 29 October 1997 to 25 February 1998 and 23 November 1998 to 14 April 1999 respectively. There was a considerable variation in the efficacy of the herbicides on the different dates and between seasons, with the ED_{50} as a fraction of the recommended dose varying between 0.28 and 1.17 for isoproturon and 0.22 and 1.51 for clodinafop-propargyl. The effects of the weather factors were investigated using multiple regression techniques for the period of 1 to 14 days before and after spraying. Significant ($p<0.001$) regressions were produced for each herbicide in each season, however the climatic factors identified in each season were not the same.

INTRODUCTION

The unnecessary application of pesticides has become an increasingly important issue from both an economic and environmental view point. Rapidly growing weed plants are generally expected to absorb herbicides more efficiently and translocate them more easily (Legg, 1983), hence lower doses can be more active. The objective of this study was to optimize herbicide dose and to understand the optimum climatic conditions for herbicide activity (Kudsk & Kristensen, 1992; Collings *et al*, 1999).

Two herbicides were chosen for this study. Isoproturon (IPU) is largely active through uptake from the soil, and hence enters the plant through the roots. In contrast, clodinafop-propargyl (CP) enters the plant solely through the foliage.

The results of field trials on winter wheat during two seasons (1997-98 and 1998-99), in which a range of herbicide doses were sprayed on a number of different occasions throughout each season are reported, and the relationship with climatic factors are discussed in this paper.

MATERIALS AND METHODS

Layout and drilling

Each season the field trials were marked out on a heavy clay soil of Hanslope series, in a different field at ADAS Boxworth, known to be relatively free of natural populations of grass-weeds. Plots measured 6 x 3 m. In 1997/1998 there were three blocks in a fully randomised design. In 1998/99 there were four blocks in a randomised block split plot, with herbicide timings as the main plots.

A. myosuroides seed (of know non-resistant source) was broadcast by hand at a rate of 400 seeds m^{-2} prior to drilling winter wheat (cv. Equinox) on the same day, on 2 October 1997 and 19 October 1998 respectively.

Treatments

The herbicide clodinafop-propargyl was applied at a rate of 15, 30, 60 (full rate) g a.i.ha^{-1}, and isoproturon at 625, 1250, 2500 (full rate) g a.i.ha^{-1}, with an untreated control for each date. Sprays were applied on eight occasions between 29 October 1997 to 25 February 1998 and on ten occasions between 23 November to 14 April 1999 respectively (Figure 1).

A. myosuroides growth stage at treatment ranged between 1-2 leaves at the first timing up to 2-3 tillers at the last timing in each season.

Herbicides were applied using a knapsack sprayer and 3m boom, operating at a pressure of 2.0 bars delivering 225 litres ha^{-1} through 02 F 110 nozzles set at a height of 35cm above the target leaf.

No other grass-weed herbicides were applied to the trials, but normal farm practice occurred for all other inputs, in both years.

Assessments

Effectiveness of the herbicides was assessed by counting panicles of *A. myosuroides* on 19 June 1998 and 22 June 1999 respectively, using 5 x 0.1m^{-2} quadrats per plot.

Analysis of data

Estimation of ED_{50}

The mean percentage reduction in *A. myosuroides* panicle numbers from the untreated control plots were calculated for each herbicide at all doses and timings. Log fractions of label dose to response curves were fitted to these data using combined controls and the Whadley's problem variant of probit analysis (Ross, 1987). From the fitted curves the log dose and its 95% confidence limits required to kill half the *A. myosuroides* plants (ED_{50}) was calculated and the values back transformed to fractional dose. In practice much higher levels of kill are

required but these higher levels are near the asymptotes, and thus can only be very poorly estimated and are unsuitable for further analysis.

Meteorological data

Data collected were daily totals of rainfall (R, mm), solar radiation (I, MJ m^{-2}) windrun (W, km) and daily maximum (T_X, °C) and minimum (T_N, °C) temperatures and 10 (T_U, °C) and 20cm (T_L, °C) deep average daily soil temperatures. Dry Bulb (T_D, °C) and wet bulb (T_W, °C) temperatures were recorded at 0900 daily and vapour pressure (V, mbar) and relative humidity (H, %) calculated. Potential evaporation (P, mm d^{-1}), was also calculated using standard methods (Thompson *et al.*,1981). The data were collected from a meteorological station within 1 km except T_D and T_W which were collected at Cambridge Botanic Gardens 10 km from the site. All the above factors, summed into periods of up to 14 days before and after spraying were then included in a stepwise multiple linear regression analysis. The factors explaining most of the variation in ED_{50} for each of the two herbicides were identified.

RESULTS

The mean number of *A. myosuroides* panicles in the untreated control plots for the two seasons was 244 and 260m^{-2} respectively.

The percentage reduction in *A. myosuroides* panicles, compared to the untreated controls by the full dose of IPU and CP (Figure 1), illustrated a wide variation in the level of control with both herbicides in both seasons.

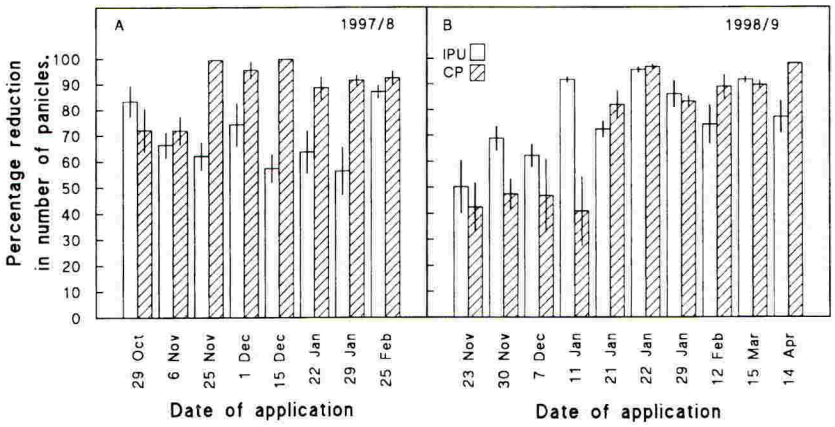

Figure 1. The % reduction from the untreated controls of *A. myosuroides,* sprayed with isoproturon (IPU) and clodinafop- propargyl (CP), at range of spray timings in 1997/98 and 1998/99. Standard errors are shown as a vertical line where greater than the thickness of the edge of the histogram bar.

In 1997/98; IPU, at the field rate, gave between 56-87% control, whereas CP gave between 71-99% control. The most effective spray timings for IPU were 29 October 1997 (date 1) at 83% and 25 February 1998 (date 8) at 87%. For CP, also at the field rate, the most effective spray timings were 25 November 1997 (date 3), 1 December 1997 (date 4) and 15 December 1997 (date 5), giving 99.4%, 95.4% and 99.8% respectively.

In 1998/99 IPU was slightly more effective giving between 52-95% control, but CP varied greatly between 27-98% control. The three most effective spray timings for IPU were 15 December 1998 (date 4) at 92%, 22 January 1999 (date 6) at 95.8% and 15 March (date 9) at 90.8%. Between the 23 November 1998 to 15 December 1998 (dates 1,2,3,4) CP gave less than 50% control. Between 29 January 1999 to 15 March 1999 (dates 7,8,9) control from CP ranged between 80-89%. The most effective spray timings for CP in 1998/99 were 22 January 1999 (date 6) at 96.9% and 14 April 1999 (date10) at 98.0%.

A multiple linear regression analysis was done on the ED_{50} values, for a period of 14 days before and after each application of herbicide for both seasons. The range of efficacy of the two herbicides, in 1997/98 was reflected in ED_{50} values varying between 0.42 and 0.94 for IPU and 0.22 and 0.67 for CP (Figure 2).

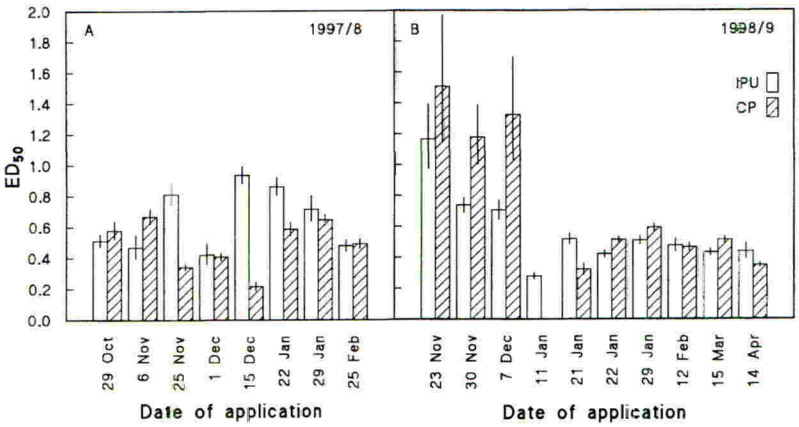

Figure 2. The ED_{50} values for clodinafop-propargyl (CP) and isoproturon (IPU) applied to *A. myosuroides*, at a range of spray timings in 1997/98 and 1998/99.

For IPU in 1997/98 the procedure resulted in a highly significant ($p<0.001$) regression which accounted for 96.8% of the total variation in ED_{50}, with the following relationship:

$$ED_{50} = 1.126 - 0.0780\ P_{A9} - 0.0357\ T_{U\,B2} + 0.00474\ T_{N\,A8}$$

For CP in 1997/98 the procedure also identified a significant ($p<0.001$) regression with only one driving variable which accounted for 86.3 % of total variation:

$$ED_{50} = 0.645 - 0.0257\ R_{A5}$$

In 1998/99 there was again a range in ED_{50} between timings from 0.28 and 1.17 for IPU and 0.32 and 1.51 for CP (Figure 2). There was a pattern of lower activity from both herbicides encountered early in the season compared with later in the season.

For IPU the regression was again highly significant ($p<0.001$), accounting for 95.1% of the total variation in ED_{50}, with the following relationship:

$$ED_{50} = 0.280 - 0.0759\ R_{B8} + 0.00239\ W_{B1} + 0.0359\ R_{B12} - 0.0234\ T_{N\,B1}$$

For CP a significant ($p<0.001$) regression accounting for 96.4 % of the variation in ED_{50} was found, with the following relationship:

$$ED_{50} = - 0.563 + 0.153\ R_{A2} - 0.0190\ R_{A7} - 0.000208\ W_{B14} + 0.00512\ H_{B5}$$

DISCUSSION

This series of experiments demonstrated that the performance of CP and IPU varied both between and within seasons, even at full field rate, which would have been chosen to even out variation and be consistent.

As these experiments were done at the same site, the main differences between timings would be climatic factors and weed growth stage. The climatic factors were over-riding, since better control occurred on early and late application timings, when plants were either small or large. In 1998/99 plant counts in fixed quadrats established that there was no significant emergence after the first spray date and hence poor control cannot be attributed to late emerging plants. This was perhaps not surprising, as the *A. myosuroides* used in these trials was a planted population, resulting from similar aged seed, sown at a similar depth.

The analysis of ED_{50} values was done to identify key variables and good fits were achieved for each herbicide in each season. These factors were not the same in both seasons. Soil temperature was identified as a positive factor for IPU activity and not for CP, which is perhaps not surprising considering the mode of action of IPU. Other factors that were identified as having positive effects for IPU activity were potential evaporation and air temperatures. These factors would all promote the uptake of IPU, as these are some of the conditions favourable for active plant growth. At this stage it was difficult to see any pattern of weather factors associated with CP activity. These data are being investigated in more detail and in conjunction with results from a third season of field experiments. The regression across all three seasons will be compared.

Climatic variables pre-spray can be taken into account at the time of spraying; for example, IPU works better when the soil is moist (Blair *et al*, 1994). Post-spraying climatic variables are more difficult to immediately incorporate into recommendations, although weather forecasting is improving all the time. IPU which is predominately soil acting, will require soil moisture and an actively growing plant to optimise herbicide uptake through the plant roots. In contrast, CP which enters through the foliage, could potentially be washed off the plant, if rainfall occurred shortly after spraying.

A better understanding of the principles should enable us to identify sets of conditions for optimum herbicide application dose and timing. If we know the herbicide will work well, there is a potential to reduce the full rate of herbicide, or equally importantly, if conditions are unfavourable more suitable alternatives could be used. It may be that this will need to take the form of a risk analysis, looking at particular weather patterns.

ACKNOWLEDGEMENTS

We would like to thank the Ministry of Agriculture, Fisheries and Food for funding this project. We thank our colleagues in ADAS for their help in the field. We are very grateful to Cambridge Botanic Gardens for providing us with additional meteorological data.

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Long-term trials with reduced herbicide doses

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ABSTRACT

Two series of experiments on herbicide application strategies, each with five field trials, were initiated in 1987 and 1988 in the south and centre of Sweden. The trials comprised herbicide application at a full dose and at reduced doses every year, alternation between full and reduced doses during different years and the use of guidelines for choice of dose. In 1998, i.e. one year after the last herbicide application, and in comparison with an untreated control, the weed density was reduced at all 10 sites by 47%-80% when a full herbicide dose was used each year. Compared with a full dose, a 75%-dose decreased the weed density at one site, while the density was increased at another. A significant weed density increase as a response to a dose reduction from 75% to 50% was found only at one sites. Changing every second year between a full and a zero herbicide dose caused higher weed plant density than most other treatments. Where guidelines were used, the mean amount of herbicides applied as a percentage of a full dose varied at different sites between 20% and 70%, with 50% as an average over years and sites. The development of the weed flora was more influenced by different herbicide application strategies than were the grain yields. The use of guidelines proved to be a fruitful way of adapting the dose reduction to the specific conditions at different sites, thereby avoiding a stereotypical application strategy.

INTRODUCTION

In Sweden, the debate concerning the use of herbicides and pesticides within agriculture started at the end of the 1960s and gradually led to a programme for reducing the risks connected to the use of pesticides, starting in 1986 (Bellinder et al. 1994) and initiated by the Swedish government. Thereafter, another two programmes on reduced herbicide risks started in 1990 and 1997, respectively. The total reduction in use of these chemicals (a. i.) so far has been 2900 tons, from the average use 1981-1985 until 1998, which corresponds to a reduction of about 65%. The desired reduction goal is 75% of the average used between 1981-85.

Besides reduced herbicide use, all the programmes comprise the following measures: change over to pesticides with fewer health and environmental risks, regulation of training and information on safer handling of pesticides and control of pesticide residues in food and water. A large part, 75%, of the pesticide reduction obtained was due to reduction in herbicide use. Around 70-80% of the reduction in herbicide use in cereals can be explained by an increased use of sulfonylureas, a decrease in arable area and a changeover to organic farming. The remaining 20-30% of the reduction in herbicide use can therefore be attributed to better dose adaptation through increased research development, voluntary tests of sprayers, integrated weed

management and improved advisory services.

Essential constituents in dose adaptation research and development have been long-term trials with different dose strategies combined with non-chemical methods and in different crop rotation systems. Here, we will present results on the development of the weed flora and on grain yields in continuous cereal cropping as a response to the long-term use of herbicides at reduced doses and using guidelines for choice of herbicide dose, which were developed for field use.

MATERIALS AND METHODS

Treatments

Two series of experiments, each with five field trials, were initiated in 1987 and 1988 in the south and centre of Sweden. The experiments comprised different herbicide application strategies as follows: (1) herbicides applied at a full dose (the dose recommended by the manufacturer) each year (100%), (2) alternation between a full dose and no herbicide application every second year, respectively ($100\%_{1/2}$), (3) half of the full recommended dose every year (50%), (4) a quarter of a full dose each year (25%), (4) half the full dose during two out of three years and a full dose the third year ($50\%_{2/3}$), (5) a quarter of the full dose during two out of three years and a full dose the third year ($25\%_{2/3}$), (6) dose application according to guidelines and (7) an untreated control. The treatments were applied to the same plots between the years 1987 and 1997 in Series I and between 1988 and 1997 in Series II. Herbicide application was carried out post-em. in spring.

Between the years 1987 and 1993, a commercial mixture of bromoxynil+dichlorprop-P+ioxynil+MCPA (165; 120; 54; 36 g a.i. l^{-1}) was used at 2.8 l ha^{-1} as a full dose. Since Swedish authorities forbade this mixture in 1993/1994, the herbicides were changed in 1993 and thereafter to a commercial mixture of dichlorprop-P+MCPA (285; 265 g a.i. l^{-1}) in Series I. In Series II, a herbicide rotation was initiated in 1993 and alternated every second year between dichlorprop-P+MCPA (285; 265 g a.i. l^{-1}) and tribenuron+a wetting agent (750 g a.i. kg^{-1}; 0.1%). A full dose of dichlorprop-P plus MCPA was 684 + 636 g a.i. ha^{-1} and a full dose of tribenuron was 6 g a.i. ha^{-1}.

The crop rotation included only spring-sown cereals: oats, wheat and barley. In the two treatments $25\%_{2/3}$ and $50\%_{2/3}$, a full dose was applied to wheat, i.e. the least competitive crop species, while reduced herbicide doses were used in the more competitive barley and oats. The experimental design was a completely randomised block with four replicates. The plot-size was 6 m ×15 m. At all 10 experimental sites, herbicides had been regularly used during the last 20 or more years before the commencement of the study.

Guidelines

During the first 3 (Series II) or 4 (Series I) years, the herbicide dose in the guideline treatment was chosen according to weed plant density: a full dose was applied at a weed plant density > 250m^{-2}, 75%, 50% and 25% of a full dose at densities 150-249 m^{-2}, 100-149 m^{-2} and 50-99 m^{-2}, respectively, while no herbicides was used at plant densities < 50 m^{-2}.

In 1991 and thereafter, printed guidelines for use of herbicides in spring-sown cereals directly in the field was adopted. The guidelines were developed at the Swedish University of Agricultural Sciences, based on results from a very high number of Swedish field trials on herbicide efficacy performed during the past 10 years. Based on: crop performance at the time of herbicide application; weed plant density; density of weed species which are difficult to control; competitive ability of species present; and actual weather conditions, the guidelines recommended herbicide application in the dose-steps 0, 13%, 25%, 50%, 75% or 100% of a full dose.

Assessments

In 1998, i.e. one year after the last herbicide application, weed counts were made in three quadrants of 0.08-0.25 m^{-2} each. The size of the quadrant was adjusted according to weed density. All weeds were separated into species. Crop grain yields were estimated in 1996 and 1997 in areas varying between 21 and 44 m^{-2} at different sites and years.

Weed species were classified as either easy (90% efficacy) or difficult (<90% efficacy) to control by the commercial mixture of bromoxynil+dichlorprop-P+ioxynil +MCPA. This classification was based on data from the 'Official Programme for Herbicide Approval' in Sweden.

RESULTS AND DISCUSSION

The herbicides used did not have any effect on the grass weed *Elymus repens*, hence this species was excluded from the data. Since site×treatment interactions were found ($P<0.01$) for weed plant density and percentage of difficult-to-control weeds in 1998 and for barley grain yield in 1996, results are reported separately for each site.

Experimental series

One year after the last herbicide application, treatments influenced the total number of emerging weeds significantly ($P < 0.05$) at all sites. However, no indication was found of any response to the herbicide rotation initiated in 1993 in Series II. It is possible that any differences between the two series in treatment response was concealed by long-lived seeds in the seedbank and this indicates that the experiment should have been continued for a few more years.

The untreated control vs. a full dose

Frequent precipitation and favourable temperatures during the spring of 1998 favoured seed germination at the 10 experimental sites and probably resulted in weed densities that did not diverge from normal.

In plots where herbicides had not been used during the previous 10-11 years, the weed density was 135-250 plants m^{-2} at three sites, 335-380 plants m^{-2} at two sites and 525-715 plants m^{-2} at five sites (Table 1). In Sweden, the weed density at sites treated with herbicides since the 1960s usually varies between 200 and 300 annual weeds m^{-2}, i.e. close to the five sites in this study with the lowest weed densities. This shows that in competitive crops with rapid canopy

Table 1. Impact of herbicide application strategy on total number of weeds (log+1) m^{-2} and on proportion of difficult-to-control species (%) in the spring of 1998 and grain yields (log+1) kg ha^{-1} in 1996. Herbicides were used at a full (100%) or at reduced rates. An untreated control was included.

Site and Series[a]	Control	100%$_{1/2}$	25%	25%$_{2/3}$	50%	50%$_{2/3}$	75%	100%	Guide-lines	LSD	Signi-ficance
Weed density											
Bjällösa I	22.82	21.91	18.41	14.79	14.75	12.14	12.22	11.94	20.05	3.46	***
Brunnby I	19.36	17.38	15.02	16.54	13.99	13.66	14.18	11.71	13.78	2.70	***
Lanna I	15.74	10.91	12.81	11.69	9.72	10.56	8.32	7.24	12.45	1.64	***
Lönnstorp I	26.67	18.90	19.04	17.91	15.41	15.86	13.25	13.05	16.22	2.63	***
Stenstugu I	24.44	19.66	17.68	14.77	17.13	12.96	12.69	16.04	15.35	3.08	***
Bränneberg II	11.51	9.41	7.43	8.13	7.76	7.76	8.41	6.32	7.77	1.49	***
Endre II	24.17	23.31	17.40	18.70	14.48	14.97	14.03	15.23	18.01	4.53	***
Kloster II	26.50	23.58	20.26	19.66	16.02	14.61	14.55	14.02	19.07	4.01	***
Lysekil II	18.23	15.53	16.37	15.14	13.75	14.79	13.51	13.19	13.63	2.07	***
Saleby II	15.66	9.80	9.77	9.11	8.20	7.65	7.26	6.94	8.52	1.36	***
Proportion of difficult-to-control species											
Bjällösa I	38.6	42.2	57.4	41.7	63.5	68.6	62.6	58.2	46.6	17.4	**
Brunnby I	50.4	47.5	45.9	41.5	43.2	42.4	34.6	41.6	51.2	9.3	*
Lanna I	48.2	50.1	67.4	51.7	46.8	44.8	53.0	49.9	50.2		n.s.
Lönnstorp I	20.1	16.4	22.8	26.3	25.6	29.2	29.4	27.1	23.3		n.s.
Stenstugu I	6.4	12.6	19.9	22.5	25.6	25.3	23.6	26.4	21.7	12.5	*
Bränneberg II	18.1	12.7	25.0	11.1	14.1	14.9	13.5	21.3	15.2		n.s.
Endre II	20.3	25.0	27.6	46.4	47.3	37.6	45.8	44.1	27.2	15.6	*
Kloster II	60.1	70.2	65.0	67.9	48.5	76.4	63.8	61.8	59.8		n.s.
Lysekil II	69.4	71.8	77.9	86.7	77.0	76.7	81.4	87.1	75.8		n.s.
Saleby II	45.6	45.5	60.6	63.7	72.2	74.1	83.6	71.4	63.3	14.6	***
Grain yield											
Bjällösa I	8.41	8.43	8.52	8.53	8.51	8.51	8.52	8.49	8.50	0.10	***
Brunnby I	8.67	8.68	8.67	8.69	8.67	8.68	8.69	8.70	8.69		n.s.
Lanna I	8.60	8.64	8.68	8.68	8.65	8.68	8.64	8.67	8.67	0.04	***
Lönnstorp I	8.52	8.50	8.46	8.47	8.46	8.44	8.47	8.47	8.41		n.s.
Stenstugu I	8.54	8.52	8.59	8.56	8.48	8.57	8.57	8.49	8.60		n.s.
Bränneberg II	8.69	8.70	8.69	8.71	8.71	8.70	8.71	8.69	8.71		n.s.
Endre II	8.53	8.50	8.54	8.48	8.52	8.48	8.52	8.45	8.53		n.s.
Kloster II	8.52	8.50	8.55	8.50	8.57	8.57	8.52	8.50	8.48		n.s.
Lysekil II	8.50	8.53	8.56	8.62	8.55	8.59	8.58	8.49	8.56		n.s.
Saleby II	8.69	8.72	8.73	8.74	8.73	8.72	8.73	8.70	8.74	0.04	**

[a]The trials had been treated with herbicides during 1987-1997 in Series I and during 1988-1997 in Series II. Index 2/3 denotes that the reduced rate was used during two out of three years while a full dose was used the third year. Index 1/2 denotes that the application rate alternated every second year between a full dose and a zero dose. LSD = least significant difference, n.s. = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The species *Elymus repens* is excluded from all data.

closure, like in spring-sown cereals, it will take some time before an extreme increase of the weed population can be observed.

A full dose resulted in weed densities varying between 50 and 270 plants m^{-2}. Compared with the untreated control, a full dose each year reduced weed density significantly ($P<0.05$) at all 10 sites, by 47%-80%.

Alternation between a full dose and zero dose every second year

In the one treatment where it was changed every second year between a full and zero herbicide dose the weed density compared with the control was significantly reduced ($P<0.05$) at six sites. At these sites the reduction compared with the control varied between 28% and 61%.

Taken as an average over two years, the same amount of herbicides is used in this treatment as when a 50%-dose is used every year. Despite this, alternation between a full dose and a zero dose every second year generated higher weed densities than the 50%-dose at six sites ($P<0.05$).

Reduced dose every year

Compared with a full dose, a 75%-dose influenced weed density significantly only at two sites. At one of these site, Stenstugu, the full dose contained more weeds than the 75%-dose. A significant weed density increase as a response to a dose reduction from 75% to 50% was found only at one sites($P<0.05$). That herbicides may be used now and then at reduced doses without accumulation of weed problems has been pointed out by others (Salonen 1992, Richards et al. 1997). At seven sites, a dose-reduction from 75% to 25% of a full dose caused the weed density to increase by 48-137% ($P <0.05$).

Reduced dose during two out of three years

Only at two sites were statistically significant differences obtained in weed response between the treatments $25\%_{2/3}$ and 25% or $50\%_{2/3}$ and 50%. At both these sites a reduced dose during two out of three years and a full dose the third year caused the weed density to increase in comparison with a reduced dose every year.

Guidelines

The dose recommended by the guidelines differed not only between sites but also between years within the same site. The mean amount of herbicides applied as a percentage of a full dose varied at different sites between 20% and 70%, with 50% as an average over years and sites. Only at two sites were significant differences obtained between the guideline treatment and 50% dose each year. At nine sites, the use of guidelines reduced the weed density by 38-71% when compared with the untreated control.

Weed species which are difficult to control

Herbicide application influenced the proportion of difficult-to-control weeds significantly at five sites ($P<0.05$) (Table 1). At four of these sites, the proportion increased where herbicides were applied each year at doses 100%, 75% or 50% compared with the control, the increase varying

between 20 and 31 percentage units. Where guidelines were used, the proportion increased compared with the control at two sites, the increase being 15-18 percentage units. In contrast, at Brunnby, the proportion was higher in both the control and in the guideline treatment than in 75%-dose. At three sites, a 50%-dose generated a higher proportion of difficult-to-control weeds than when alternations were made every year between a full and a zero dose ($P<0.05$), although the same amount of herbicides are used when summarized over two years.

Crop yields

In 1996, the barley yields in the untreated control varied between 3700 and 6700 kg ha^{-1} at different sites, while in 1997 the yields of oat varied between 4500 and 5500 kg ha^{-1}. These yield levels are well in line with yields from herbicide-treated crops in the same area (Anonymous 2000). Treatments did not significantly influence the grain yield of oats in 1997. In 1996, herbicide application increased the barley yields at three sites ($P<0.05$) (Table 1). At two of these sites, the yields were lower in the control than in all other treatments while yields at the third site were lower both in the control and in $100\%_{1/2}$ than in all other treatments.

CONCLUSIONS

The development of the weed flora was more influenced by different herbicide application strategies than were the grain yields. There was a rather close connection between the sum of the amount of herbicides applied during the experimental time and the density of weeds one year after the last application. A herbicide rotation initiated in 1994 did not significantly influence the weed flora when compared to treatments where the same herbicides had been used since 1987. Changing every second year between a full and a zero herbicide dose caused higher weed plant density than most other treatments. The proportion of difficult-to-control weed species also increased when herbicides were used every year when the dose reduced to 50% or 75% of a full dose. However, compared with the control, the total weed density was reduced, and the density was reduced more at high than at low doses. The use of guidelines proved to be a fruitful way of adapting the dose reduction to the specific conditions at different sites, thereby avoiding a stereotypical application strategy.

ACKNOWLEDGEMENTS

The study was financed by the Swedish Board of Agriculture.

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The effect of propoxycarbazone-sodium on jointed goatgrass (*Aegilops cylindrica* Host)

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ABSTRACT

Propoxycarbazone-sodium will be registered in the United States for the selective control of annual grasses including *Bromus tectorum*, *B. secalinus*, *B. rigidus*, *B. japonicus*, *Avena fatua*, *Phalaris minor*, *P. paradoxa* in wheat. The product also can be used to selectively suppress *Aegilops cylindrica*. Best efficacy on *A. cylindrica* was observed after two sequential treatments of 30 g a.i./ha + non-ionic surfactant in autumn and spring. Single applications of 45 g a.i./ha made in autumn or spring provided less consistent suppression than sequential applications. Yields responded favorably to *A. cylindrica* suppression and increases varied from 5 to 71 % of the untreated control depending on environmental conditions and efficacy reached.

INTRODUCTION

In the USA *A. cylindrica* infests 2 million hectares of winter wheat and 1 million hectares of fallow land and is increasing by 20 000 hectares or more every year. The infestation level varies with environmental conditions and is promoted by warm and dry conditions. Currently no herbicide for the selective control of *A. cylindica* is registered in the US and growers depend on the use of various cultural practices to limit the impact of the weeds on the crop. Most emerging chemical control technologies are based on the use of glyphosate or imazamox on herbicide resistant crops. Propoxycarbazone-sodium has demonstrated the potential to selectively suppress *A. cylindica* in wheat. The product is the active ingredient of the herbicides Olympus and Attribut, which was submitted for registration for wheat in the US in 1999. The use pattern will include a single application of up to 45 g a.i./ha, or two sequential treatments of up to 30 g a.i./ha in both autumn and spring. Mixture with a minimum of 0.25 % (v/v) of a non-ionic surfactant will be required.

METHODS AND MATERIALS

Field experiments were conducted 1993 – 2000 throughout the winter wheat producing areas of the western half on the United States. Trials were conducted in

growers' fields using natural infestations of the target weed. Replicated small plot (25 m^2) trials were used for rate and timing studies. To determine suitability under commercial conditions, and to account for inherent variations in soil type and weed pressure, large plot strip trials (200 m^2, 2 replications) were conducted as well.

Equipment used for small replicated trials consisted of knapsack sprayers, while strip trials were applied with tractor or all terrain vehicle mounted sprayers. In both cases spray volume reflected commercial practices common in the US and ranged from 110 to 220 litres/ha.
Spray solutions were prepared using a 70 % WG formulation of propoxycarbazone-sodium and contained 0.25 % (v/v) non-ionic surfactant and were applied post-emergence. Efficacy in field tests was rated visually as % biomass reduction. Only the final ratings at heading of the weed are represented.
Yields were measured after the center portions of the strip plots were harvested using a small plot combine. The relative difference in yield of the treatments was expressed as percent of the untreated control.

RESULTS AND DISCUSSION

Different levels of sensitivity of *Triticum aestivum*, *Bromus secalinus*, *Bromus tectorum*, *Bromus japonicus* and *A. cylindrica* to propoxycarbazone-sodium were demonstrated in a greenhouse study and could be confirmed under field conditions. All three *Bromus* species were highly susceptible to the herbicide. *T.* aestivum was not affected by the product confirming excellent crop tolerance. *A. cylindrica* exhibited intermediate susceptibility.

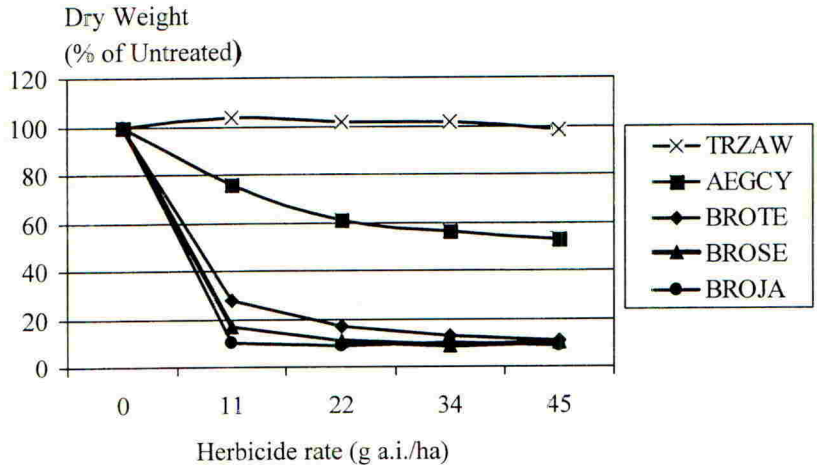

Figure 1. Differential plant response after post-emergence application of propoxycarbazone-sodium in the greenhouse

Dose response studies showed inconsistent efficacy against *A. cylindrica* with herbicide doses ranging from 30 to 90 g a.i./ha applied as a single treatment in autumn or spring. The arithmetic mean values of control ratings taken after autumn application showed an almost flat dose-response curve. Individual ratings ranged from 0 to 73 % control with yield increases of up to 14 %. The efficacy of spring treatments increased slightly. Individual ratings ranged from 0 to 85 % control and yield increases of up to 71 % were observed. Sequential treatments starting with an autumn application, which then was followed by a second application in spring provided better and more consistent performance than single applications. Within the limits of the label, which will permit the application of a total dose of 60 g a.i./ha per growing season in two sequential treatments, *A. cylindica* control never was lower than 25 %. The average control rated over 13 trials was 60 % and a maximum level of 94 % control was reached.

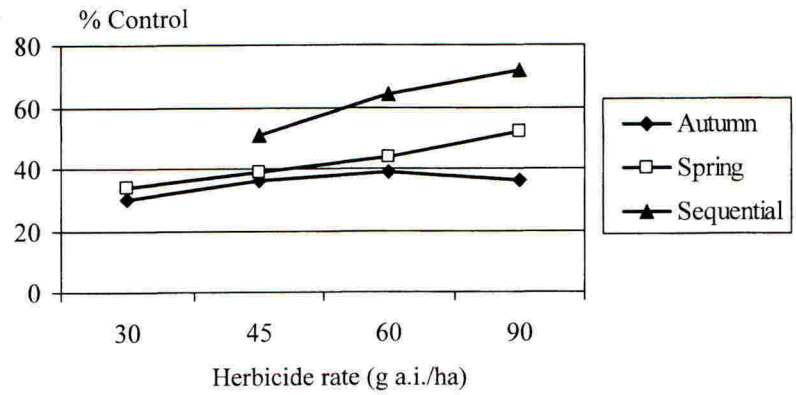

Figure 2. Response of *A. cylindica* to different propoxycabazone–sodium rates and timings of application

Average efficacy against *A. cylindica* based on 91 ratings from 31 different experiments in which propoxycarbazone-sodium was applied at proposed label rates as a single application in autumn, spring or as sequential treatments, was 51 % with a standard deviation of 20 %. Single applications in autumn or spring were rated at 32 and 36 % suppression respectively. Sequential applications provided 65 % suppression.

Quantitative research on the impact of propoxycarbazone-sodium on *A. cylindrica* propagation is still under evaluation. Visual assessment of the development of plants, which showed suppression, but survived the herbicide treatment indicates several effects of the herbicide. Elongation growth and numbers of tillers per plant are reduced and the biomass production is decreased. Seed heads are smaller and frequently show malformations (kink). Number of seed heads per plant and number of seeds per seed head appear to be reduced. Seeds harvested in the field from suppressed plants were viable and germinated when tested in the greenhouse.

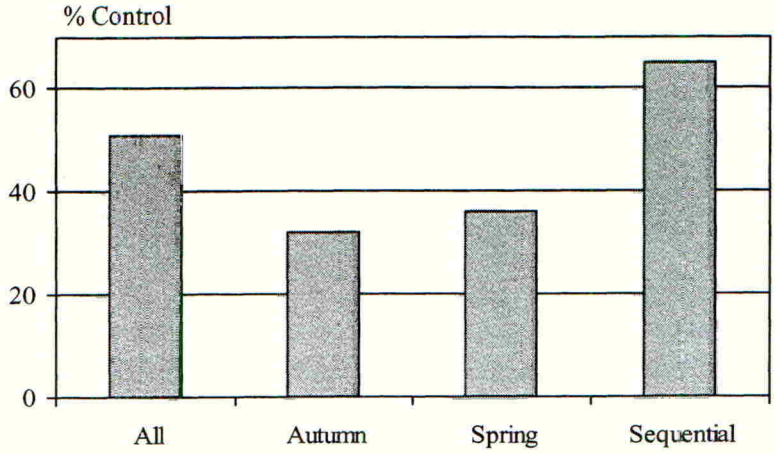

Figure 3. Average efficacy against *A. cylindrica* based on 91 observations in 31 trials

To analyze consistency of performance, efficacy ratings were grouped into five categories: Low (0 – 30 %), Visible (31 – 50 %), Suppression (51 – 70 %), Acceptable (71 – 85 %), and Good (> 85 %). More than two thirds of all ratings taken after single applications fell into the "low" or "visible" efficacy categories. After sequential applications over 70 % of all treatments provided suppression, acceptable, or even good control of *A. cylindrica*.

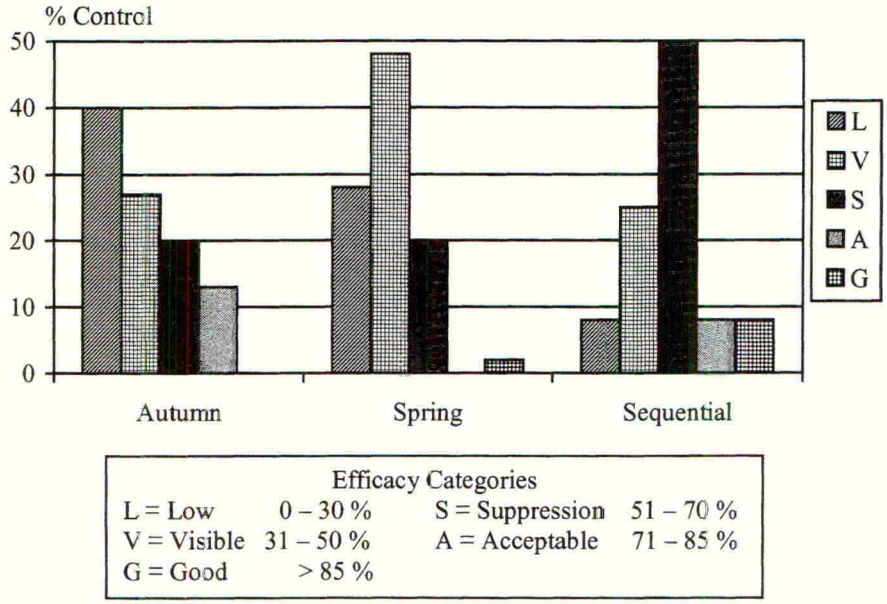

Figure 4. Distribution of efficacy ratings over efficacy categories

Risk of treatment failure was analyzed using a box and whisker plot. Sequential treatments provided best efficacy. Only 10 % of all treatments resulted in less than 40 % suppression. Spring treatments were superior to autumn applications. For all treatments "Mean" and "Median" values of were almost identical indicating a nearly normal distribution of the results. The width of the box containing 50 percent of all results indicated almost identical consistency of performance (on different absolute levels) of spring and sequential treatments. The results of autumn treatments were clearly more variable.

Yields taken in test plots depended on density of weed infestation, timing of application, and environmental conditions all influencing the level of efficacy achieved. Yield increases varied from 5 to 71 % compared to untreated control plots. Yield increases were minimal when efficacy was low and reached the maximum when 85 % control of a dense *A. cylindrica* infestation was achieved.

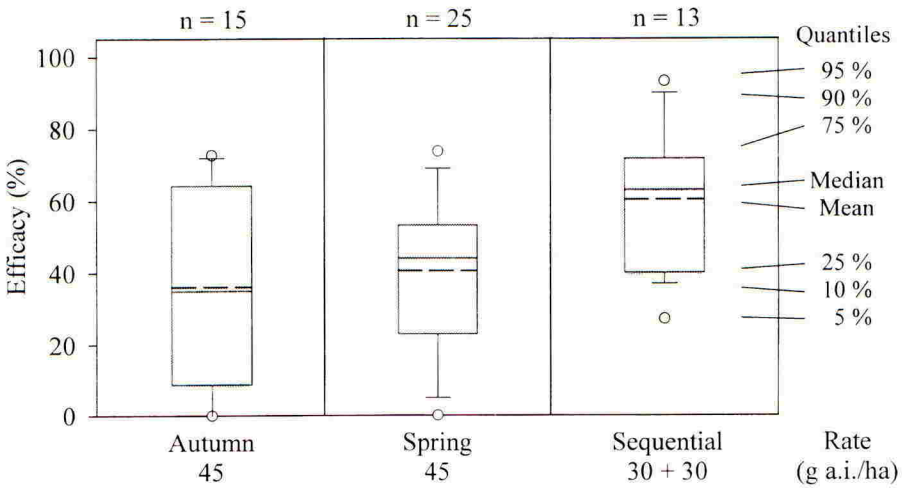

Figure 5. Analysis of consistency of performance of propoxycarbazone-sodium against *Aegilops cylindrica* at different application timings.
n = number of observations

CONCLUSIONS

Propoxycarbazone-sodium is active against *A. cylindrica.* Consistency of efficacy is not sufficient to label the herbicide for control, but supports labeling for suppression. The product is seen a one additional tool to selectively manage *A. cylindrica* in the wheat crop and it does not depend on the production of a herbicide resistant crop. The herbicide offers the potential to reduce crop competition and increase yields. It can reduce the propagation of the weed and the expansion of the infested area. It will

assist wheat producers in dealing with the *A. cylindrica* problem until improved technologies are commercially available and accepted by consumers.

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SESSION 7B

INFLUENCE OF WEATHER ON HERBICIDE PERFORMANCE

Chairman & Session Organiser: Dr J C Caseley
IACR-Long Ashton, Bristol, UK

Papers: 7B-1 to 7B-4

How to investigate the influence of environmental factors on herbicide performance

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ABSTRACT

It is widely recognised that climatic conditions before, at and after herbicide application can affect the efficacy of herbicides and can be the cause of much variation in herbicide performance. Most studies on climatic conditions have been done in controlled environment chambers under controlled or semi-controlled conditions allowing the manipulation of one climatic parameter while the others have been kept constant. Such studies provide a better understanding of the plant-herbicide interactions and allow for a ranking of the climatic parameters but their relevance to the more complex field situation where climate parameters fluctuate and interact are questionable. As a matter of fact despite the abundance of information on the role of climatic parameters on herbicide performance the information has not been widely used in dose recommendations. A significant improvement is to replace the traditional controlled environment chambers, where climatic parameters can only be examined at fixed levels, with climate simulators where the natural diurnal fluctuations and interactions can be simulated. In common with field experiments one compares climate scenarios rather than studying the impact of individual climatic parameters, but in contrast to field experiments, climatic conditions will be the only parameter varying. Another approach is to conduct field experiments under contrasting climatic conditions recording all relevant climatic parameters and then subsequently establish correlations between herbicide activity and climatic conditions before, during and after spraying. This approach is costly and rarely produces conclusive data partly because many other factors besides the climatic conditions inevitably will vary and this tends to confound any correlation. A recently published approach is to use a so-called spline method to analyse data from field experiments where climatic conditions at and around the time of application have been recorded. This method holds promise for improving the interpretation of field data and to develop recommendations on basis of a set of field data.

INTRODUCTION

Herbicide activity is influenced by many complex interactions involving weed flora, growth stage of weeds, environmental conditions and competitive ability of the crop. Under favourable conditions satisfactory weed control can be obtained with doses several times lower than the recommended dose while under unfavourable conditions not even the highest dose recommended on the label may provide satisfactory weed control.

Herbicide labels often contain information on what is considered optimum conditions for application and what is considered unfavourable conditions where the herbicide should not be applied. The labels, however, rarely contain information advising reduced doses under optimum conditions.

Generally, the performance of foliage-applied herbicides is influenced more by environment than is the performance of soil-applied herbicides; the effects of the latter being primarily affected by soil moisture (Kudsk & Kristensen, 1992) and consequently most studies on environmental effects on herbicide performance have been on foliar-applied herbicides. The pre-spraying climatic conditions primarily affect the development and physiological status of the plants, e.g. the cuticular characteristics of the shoot and root development which may subsequently affect the response of weeds and crops to the herbicide. The climatic conditions during and immediately after spraying are very important to the effect of foliage-applied herbicides as temperature, humidity and rain may have a significantly influence on herbicide uptake. Climatic conditions in the long-term post-spraying period will determine the growth of weeds and crops which may affect, e.g. herbicide translocation, the ability of weeds to recover from application of non-lethal herbicide doses and crop competitiveness.

Studying the influence of environment on herbicide performance has been the subject of numerous studies and different approaches have been applied. Broadly classified four different experimental approaches have been applied *viz.* controlled environment chambers, climate simulators, modified conditions in field experiments and field experiments repeated in time and space. The purpose of this paper is to give an overview of the methods that have been used and to discuss the pros and cons of these approaches. Previous reviews on experimental approaches to study the influence of environmental conditions on herbicide performance have been written by Caseley (1979) and Devine (1988) and the present paper is an update on these reviews describing new approaches and including recent data.

CONTROLLED ENVIRONMENT CHAMBERS

The majority of the studies have been conducted in controlled environment chambers or growth chambers. In these experiments pot-grown plants, either raised in the glasshouse or in the controlled environment chamber, are subjected to typically two or three levels of one climate parameter while the other climate parameters are kept constant. The parameters most frequently studied are temperature, humidity and light intensity.

Controlled environment chambers are very suitable to study the influence of individual climate parameters and effects of pre- and post-spraying environment can easily be separated but there are also a number of methodical problems associated with the use of controlled environment chambers.

Although the advances in lighting technology have made it possible to avoid some the growth anomalies observed in earlier days on plants grown in controlled environment chambers the quality of artificial light is different from natural light. Furthermore, in most studies light intensity is considerably lower than that experienced in the field during the spraying season. The main reasons for this is that increasing light intensity is expensive and will produce excessive heat which is costly to remove from the chambers and would make temperature control more difficult. Improvements in reflector technology have made it

possible to increase light intensity without increasing costs. Low light intensity and differences in light quality can have a pronounced effect on the morphological and physiological appearance of the test plants, e.g. leaf size, leaf angle and stem length and may also affect the cuticular characteristics and the number and size of stomata (Davies and Blackman, 1989). An unsuitable spectral quality may also lead to radiation stress resulting in increased leaf temperatures (Omrod & Krizek, 1977).

Often a 16h photoperiod is used and although different levels of temperature and humidity are applied during day and night time the climate regimes are very different from the fluctuating temperature and humidity in the field. The lack of natural fluctuations in temperature and humidity will also contribute to that plants are morphologically and physiologically different from plants in the field (Devine, 1988).

The problems associated with unnatural light, temperature and humidity conditions in controlled environment chambers can be partly overcome by raising pot-grown plants outside and only keep them in the controlled environment chambers during the period when they are exposed to different climatic conditions. Besides outdoor-grown plants will be exposed to wind and rain damaging and eroding of the epicuticular wax which can promote herbicide retention and uptake (Caseley, 1989). This approach has rarely been used probably because such experiments are not reproducible.

Another limitation to controlled environment chambers is the restricted root volume. Plants are watered frequently as it is normally the intention to avoid soil moisture stress. In contrast, in the field plants will typically experience periods with at least a mild moisture stress. Mild moisture stress will affect leaf and cuticular development as well as the growth of primary roots (Davies & Blackman, 1989). The limitations of a restricted root volume can be assumed to be most prevalent in experiments using plants at later growth stages. The differences in root volume may also have implications if the purpose of an experiment is to examine the effect of soil moisture stress. Pot-grown plants have been found to be more susceptible to soil moisture stress than field grown plants, e.g. both leaf expansion and photosynthesis decline at a much lower plant water potential in pot experiments than in field experiments (Begg, 1978). Similarly, much lower plant water potential was required to cease leaf expansion and photosynthesis of plants in the field than in pots.

In controlled environment chambers it is necessary to maintain a relatively high constant air flow otherwise temperature and humidity cannot be controlled adequately. The air flow can be either across the plants or from the bottom of the chambers. The air flow is generally not sufficient to cause major movements of the plants and mimic wind abrasions as it is experiences in the field but due to the lack of a crop canopy the air flow may affect the climate immediately around the leaf surface. Under field conditions it is expected that humidity is close to 100% RH on the leaf surface due to transpiration but with a constant air flow around the plants this may not be the case in a controlled environment chamber. An indication that this can alter herbicide performance is the findings of Savory & Hibbitt (1972) that the activity of ioxynil and bromoxynil was considerably lower in controlled environment chambers than in the glasshouse and that a similar difference in effect could be produced in the glasshouse by using a fan to blow air over the plants. Uptake of the water-soluble salt formulation of bromoxynil has been shown to be strongly influenced by humidity in contrast to the lipophilic ester formulation (Savory *et al.*, 1975) and accordingly the effect of wind was more pronounced with salt than with the ester formulations of ioxynil

and bromoxynil. The constant air flow will also have the effect that despite a humidity close to 100% r.h. dew will never form on the plants. Plants in the field will often be wet due to either dew or fog and re-wetting of leaves within the first days after herbicide application has been shown to promote the activity of many foliage-applied herbicides (Behrens, 1977). The lack of dew may also diminish the herbicidal effect.

Controlled environment chamber studies can provide a better understanding of the principles of plant-herbicide interactions and allow for a ranking of the climatic parameters but their relevance to the more complex field situation where climate parameters fluctuate and interact are questionable. As a matter of fact despite the abundance of information in the literature on the role of individual climatic parameters on herbicide performance the information has not been widely incorporated in dose recommendations.

CLIMATE SIMULATORS

In this context climate simulators are defined as advanced controlled environment chambers where natural climates can be simulated. The only example in the literature of the use of climate simulators in herbicide research is at our department. In the climate simulators we can change climate parameters every fifth minute and consequently natural diurnal fluctuations in temperature, humidity and light intensity can be accurately simulated (Kristensen, 1992). Rather than studying the influence of individual climatic parameters, as done in controlled environment chambers, climate simulators are developed to study the performance of herbicides under various climate scenarios.

In principle any set of climatic data can be used to run the climate simulators but in the majority of the experiments a set of standard climate scenarios have been used. The standard climate scenarios were developed using a simple model assuming that water vapour pressure is constant throughout the day, i.e. that the daily water vapour pressure is determined as the saturated water vapour pressure at the lowest temperature. This assumption is normally valid in a period without rain but in case of rain the water vapour pressure will increase. Assuming constant water vapour pressure means that the fluctuations in relative humidity are determined by the difference between daily maximum and minimum temperature. Another assumption made developing the standard climate scenarios was that temperature fluctuations can be described using sinus curves. The model was verified by comparing observed and calculated values for temperature, humidity, water vapour pressure and water vapour deficit (Mathiassen *et al.*, 1994).

The standard climate scenarios were selected by plotting daily temperature differences versus daily mean temperature collected at two locations over several years during the peak seasons for herbicide application (Figure 1). Neglecting any observation with temperatures below zero nine climate scenarios were selected covering the whole spectrum of environmental conditions that farmers may experience during the time of the year when herbicides are applied in Denmark.

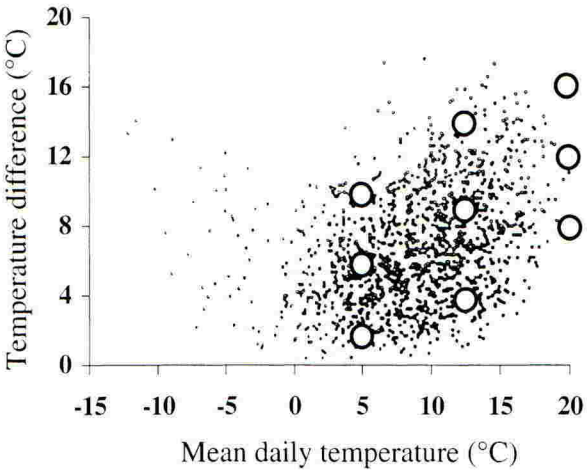

Figure 1. Daily temperature difference plotted against mean daily temperature at two locations in Denmark from 1 April-31 May and 1 August-15 November in 1987 to 1989. The nine selected climate scenarios are marked as circles (from Mathiassen *et al.*, 1994).

The climate simulators have been used to generate information on the impact of variations in climatic conditions on the performance of numerous foliage-applied herbicides. Plants are raised outside, moved to the climate simulators one day prior to application and moved outside again 5 days after application. In some experiments all nine climate scenarios were studied while in others only three of the nine climates were included. The purpose of the experiments has been to examine if weather conditions within the period covered by the weather forecasts will affect herbicide performance and the information generated has been incorporated in the Danish decision support system on crop protection providing adjustment of herbicide doses according to environmental conditions around the time of application (Kudsk, 1999).

The effect on herbicide performance of natural diurnal fluctuations in temperature and humidity compared to fixed day and night levels was examined by Mathiassen & Kudsk (1996). Three herbicides were applied at six climate scenarios. Three of the climates had fixed day and night levels of temperature and humidity while the other three climates were characterised by having the same average temperature and humidity as the other three climates but temperature and humidity was following a diurnal fluctuation. It was concluded that the responses to temperature were less pronounced with diurnal temperature fluctuation whereas the response to humidity tended to be more significant. Bethlenfalvay & Norris (1977) studying desmedipham injury on sugar beet found no phytotoxicity when temperature, humidity and light intensity were held constant irrespectively of the time of day of application while morning applications injured sugar beet more than late afternoon applications when temperatures were cycled.

In a kind of validation study the climate simulators were running the same climate scenario as outside and herbicide performance in the climate simulators was compared to the

corresponding effect on pot-grown plants placed outside and field-grown plants (Mathiassen *et al.*, 2000). Only minor differences were observed between the activity on pot-grown plants in the climate simulators and outdoor indicating that outdoor conditions can accurately be mimicked. In contrast the effect on field-grown plants of the same species was significantly lower. Soil moisture stress in the field around the time of application was suggested as a likely cause of this difference highlighting the limitations imposed by growing plants in pots with limited root volume and frequent watering.

Due to the lack of a crop canopy and the constant air flow the microclimate around the plants will be almost identical to the macroclimate registered above the plants. In the field the micro- and macroclimate can differ significantly (Legg, 1989) and from an extrapolation point of view it would be more correct to simulate the microclimatic conditions. However very little is known about the relation between macroclimate above the crop and the microclimatic conditions in the crop canopy. Anyhow many herbicides are applied at the early crop growth stages when differences between macro- and microclimate can be expected to be minor.

In principle the experimental approach adopted using climate simulators is very similar to doing field experiments repeated in time or space. The approach has some of the same limitations as mentioned for controlled environment chambers, e.g. the restricted root volume but in contrast to field experiments climate will be the only parameter varying between experiments. Climate simulators also allow study of the influence of time of day of application which may also be an important parameter to consider in studies on the influence of environmental factors.

FIELD EXPERIMENTS

Two different approaches have been used when studying the influence of environmental factors in field experiments. Either the growing conditions are modified or field experiments are repeated in time or space collecting relevant climatic information and subsequently it is determined which environmental factors contribute to the observed variation in herbicide performance.

Modifying field conditions

To a certain extent it is possible to grow plants in the field and modify the environmental conditions the plants are growing under. Shading can be used to study the effects of light intensity and rain before and after herbicide application. Minkey & Moore (1996) used a rain shelter catching 50 % of the rain to established different soil moisture levels. The rain caught by the shelters was used to irrigate another plot, i.e. three levels of soil moisture was created. The relative water contents of wheat leaves at the time of application varied from 85 to 65%. resulting in ED_{90} doses of glyphosate varying from 113.8 to 1292.4 g a.e./ha. Permin (1988) used a shelter put up in the evening before application to study the influence of dew on the performance of the growth regulator ethephon but found no effect of dew.

It is only possible to modify a limited number of climatic parameters including light intensity, rain and soil moisture (shading or irrigation). Although done under field conditions caution is required when interpreting the results because a shelter may also influence other

climatic parameters such as temperature and humidity. It is therefore imperative to monitor other relevant climatic parameters in such studies.

It should be mentioned that modifying growing conditions is also possible using outdoor-grown pot plants but this approach will have some of the limitations mentioned for experiments in controlled environment cabinets and climate simulators.

Field experiments repeated in time or space

The influence of environmental conditions have also be studied in field experiments by replicating the same experiment in time and space. If all relevant information concerning environment, plant factors (e.g. weed flora and growth stage) and soil factors is collected then, in theory, it should be possible to determine the influence of environmental factors on herbicide performance by statistical analyses. If the main objective of a study is to assess the influence of environmental factors all other factors should be kept as constant as possible but this is rarely possible. Conducting this type of study is costly but has become easier in recent years with the development with portable weather stations which can automatically record data on, for example, an hourly basis. A similar approach can be used with pot-grown plants as exemplified by the study of Savory *et al.* (1975). They conducted continuous outdoor pot experiments over a 4-year period examining the influence of climatic factors on the efficacy of ioxynil and bromoxynil and found that solar radiation and humidity affected the activity of the salt formulations whereas the corresponding ester formulations were less affected by climatic conditions.

Devine and Vanden Born (1998) studied the performance of a number of foliage- and soil-applied herbicides in spring-sown crops at five locations in Alberta in Canada over a 4-year period. They recorded a number of environmental parameters using data loggers. They found good weed control in all experiments and were consequently not able to find any correlations between herbicide performance and environmental conditions. Only the recommended dose was included in the experiments and the lack of any correlations can most likely be attributed to the fact that the efficacy of the applied dose was so high that not even adverse climatic conditions resulted in any significant reduction in performance.

Lundkvist (1997a) conducted field experiments with two cereal broadleaf herbicides at six sites in Southern Sweden over a 4-year period. In contrast to Devine and Vanden Born (1988) the herbicides were applied at reduced rates (1/8, 1/4, 1/2 and 3/4 of the recommended dose). The herbicides were applied at three occasions in each experiment *viz.* the cotyledon stage, 7 days following the first application and 7 days following the second application. The timing of the second and third application was flexible to accommodate as contrasting conditions as possible. A number of climatic parameters were recorded at an hourly basis. General linear models were used to explain the influence of individual climatic parameters on herbicide efficacy. The effect of environment was analysed for seven different periods (7, 2 and 1 day(s) before and after application and the day of application), as suggested by Caseley & Coupland (1985). Although the reduced doses resulted in less than full effect and ED_{80} doses were estimated only few significant interactions were found. The most pronounced effects of environment were found on the day of application, suggesting a strong influence of environment on herbicide uptake, and the day before application. The mixture of the two phenoxy alkanoic herbicides dichlorprop and MCPA generally responded more to environment then the sulfonylurea herbicide tribenuron-methyl.

In an attempt further to explain the observed differences in herbicide performance Lundkvist (1997b) correlated herbicide performance to growth rate of the weeds at the time of application. Growth rates calculated for 17 of the experiments were classified into four classes. The statistical analyses revealed that the two herbicides generally performed better when applied to weeds having an increasing growth rate and it was suggested that on-line calculations of growth rate could provide a means for determining the optimum time of application.

Minkey & Moore (1998) conducted a number of dose response experiments with glyphosate, paraquat + diquat and diclofop-methyl over a 3-year period in Western Australia targeting a limited number of weed species. For each experiment the ED_{90} dose was estimated using non-linear regression. Very pronounced differences were found in the ED_{90} doses which most likely reflects that the conditions in western Australia in comparison to Northern Europe can be very variable and sometimes harsh. The ED_{90} doses varied by a factor of ca. 30, 20 and 8 for glyphosate, paraquat + diquat and diclofop-methyl, respectively and highly significant correlations were found between herbicide performance and, e.g. mean degree-days and days since receival of 5 mm rain. A prototype decision support model was develop on basis of linear regression models. Based on input of environmental and plant factors the decision support system will provide farmers with a herbicide dose that is expected to produce satisfactory weed control.

The above-mentioned studies were based on experimental series specifically designed to produce the requested information. These experiments are costly and, with the exception of the study by Minkey and Moore (1998), have so far not produced conclusive results which could improve label recommendations.

When a new herbicide is introduced numerous experiments are undertaken by the companies and governmental institutes to test the herbicide under the conditions it is intended for. These experiments will typically run for several years and in contrast to the experimental series discussed above the experimental lay-out in terms of formulations, doses etc. may change during the years. Increasingly such experiments are conducted according to standard guidelines as this is a required for registration in many countries. Another approach to study the influence of environmental conditions on herbicide performance could therefore be to explore such sets of data. The only example of this hitherto is the study by Medd *et al.* (2001) who collated information from 59 Australian experiments conducted by the producer of clodinafop-propargyl on *Avena* spp. in wheat. A spline method was used to analyse a set of observed and interpolated covariates and they found significant correlations between clodinafop-propargyl performance and environmental parameters as well as application factors supporting and complementing the label recommendations. Using a spline method with the logarithm of dose a natural smoothing spline bears much similarity to fitting logistic dose response curves; thus information concerning adjustment of doses according to environmental conditions around the time of application can be generated. So far the study by Medd *et al.* (2001) is the only publication using the spline method but considering the numerous experiments conducted with new herbicides the approach holds promise for improving the interpretation of data from field experiments where climatic conditions before, during and after application are recorded.

CONCLUSION

Controlled environment chambers will probably continue to be a popular tool for studying the influence of environmental conditions on herbicide activity because such experiments are easy to conduct and relatively inexpensive. Due to the limitations of controlled environment chambers only very simple label recommendations can be generated but they can be used to confirm or disprove the importance of specific climatic parameters. Climate simulators provide a significant improvement in simulating natural climate and some of the complex interactions occurring in the field but still have some of the limitation of controlled environment chambers mainly because pot-grown plants are used. Climate simulators are costly and expensive to operate and most likely very few institutes will establish such a research facility. Field experiments specifically designed to study the influence of environmental factors on herbicide performance are expensive and have rarely produced conclusive data and are not a viable approach. Specific experiments studying the influence of individual environmental parameters could however produce valuable information. In contrast, analysing existing data using a spline method or similar statistical procedures holds promise for extracting additional information out of field experiments conducted for other purposes, but to be successful it requires a certain standardisation regarding collection of relevant climatic data. If this approach was combined with experiments in controlled environment chambers or even better in climate simulators, using the field data to validate findings from controlled conditions or *vice versa*, it would provide a very strong experimental set-up for developing more precise label recommendations on herbicide doses under contrasting climatic conditions.

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Influence of weather on the performance of acetolactate synthase inhibiting herbicides

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ABSTRACT

Weather can strongly influence acetolactate synthase (ALS) inhibiting herbicides when applied to the soil or plant foliage. The weather factors influencing herbicidal activity are rainfall, humidity, temperature, light, soil moisture, and wind. Weather conditions can influence ALS-inhibiting herbicide performance before, during, and after application. Generally, weather conditions that favour weed growth will favour herbicide uptake, translocation, and overall activity. Stressful weather conditions often inhibit ALS-inhibiting herbicide weed control and may increase crop injury. Cold and dry conditions also can reduce degradation in the soil and influence rotational crop safety. Much work is done to understand these conditions and define recommendations on herbicide labels that ensure consistent and satisfactory performance. Wind, rainfall, moisture, and temperature conditions are often specified on ALS-inhibiting herbicide labels. Although these factors can strongly influence ALS-inhibiting herbicide performance, this class of herbicides is not particularly susceptible to weather conditions compared to other herbicide classes. Official label restrictions on their use are not strict. This paper reviews our current understanding of the influence of weather conditions on ALS-inhibiting herbicide performance.

INTRODUCTION

Scientists working at DuPont and American Cyanamid independently discovered acetolactate synthase (ALS) inhibiting herbicides in the 1970s. ALS-inhibiting herbicides rapidly became important tools worldwide for weed control because of their versatility, ability to control many difficult-to-manage weeds, high safety to crops, low toxicity, and recognized safety to the environment. Currently there are approximately 56 different ALS-inhibitor herbicides in five chemical classes: sulfonylureas (35), imidazolinones (8), pyrimidinylthiobenzoates (5), sulfonylaminocarbonyltriazolinone (2), and triazlopyrimidines (6). Despite concerns over market saturation, loss of patent protection, and weed resistance, companies are still developing new ALS-inhibiting herbicides.

ALS-inhibiting herbicides are generally weak acids with intermediate water solubility. Making these herbicides salts or formulating at neutral or high pH increases their water solubility. Their formulations range can be dry or liquid and range from very concentrated to very dilute. ALS-inhibiting herbicides enter plants through the roots and shoots and move in both the xylem (apoplast) and phloem (symplast). Some are formulated with activator adjuvants to enhance foliar activity while others must be tank-mixed with adjuvants to ensure activity.

When companies guarantee weed control with ALS-inhibiting herbicides, they are essentially guaranteeing the weather. Such guarantees discourage growers from paying close attention to label recommendations that specifically define weather conditions to ensure weed control and crop safety. Weather conditions before, during, and after application are important. Weather conditions that favour weed growth usually favour herbicide uptake, translocation, and activity. Stressful weather conditions often reduce weed control and increase crop injury. ALS-inhibiting herbicides are generally most effective when humidity is moderate to high, temperature is moderate, and diurnal temperature fluctuations are modest.

RAINFALL AND HUMIDITY

Rainfall and irrigation frequency and amount influence ALS-inhibiting herbicide activity by changing soil moisture, humidity, dew, and rainfastness.

Soil Moisture

ALS-inhibiting herbicides require significant rainfall to activate and must be in soil solution for roots to absorb. When applied to the foliage, soil moisture helps ensure that weeds will be growing and susceptible to these herbicides. Adequate soil moisture ensures ALS-inhibiting herbicide translocation throughout the weed and subsequent control (Olson *et al.*, 1999), metabolic inactivation and crop safety (Olson *et al.*, 2000). Soil moisture also determines uptake of organophosphate insecticides that are commonly used in-furrow in maize and thus affects their interaction with ALS-inhibiting herbicides that often results in crop injury (Bailey & Kapusta 1994). Overabundant soil moisture reduces activity by promoting leaching out of the zone of greatest effectiveness (near the soil surface) or enhancing degradation. Effects of rainfall on leaching are well understood. Degradation in the soil generally increases as soil moisture increases, reducing the herbicide available for weed control (Dinelli *et al.*, 1998). Dry soil has the reverse effects. Weeds stop growing and are more difficult to control and less water is available to solubilize the herbicide for root absorption. Dry soil slows chemical and microbial breakdown processes and may increase crop rotation intervals.

Rainfastness

Rainfall soon after application reduces spray residues on leaves and herbicidal activity. The most important characteristics of rainfall are amount, intensity, drop size, and time interval after application. The general rule is that the more hydrophilic the herbicide, the longer the required rainfree time interval. The rainfree time interval after application is usually the most important factor (Kudsk *et al.*, 1990, Malefyt & Quakenbush 1991), but specific studies have shown gradually reduced activity as rain increased up to 4 mm (Nalejawa *et al.*, 1991). Although many ALS-inhibiting herbicide are dry particulate formulations and thus potentially more vulnerable to wash-off, their uptake is fairly rapid and labels require only a few rainfree hours for maximum activity. Rainfastness is not considered a serious limitation.

High Humidity

Some ALS-inhibiting herbicide labels address temperature and humidity with the following recommendation: "When making applications under hot and dry conditions, set up equipment

to produce larger droplets to reduce effects of evaporation." Relative humidity describes the "drying power" of air and is a function of actual water vapour and temperature. Herbicides are generally more active under high humidity because spray droplets dry more slowly and the cuticle is more hydrated aiding penetration. Kudsk and Streibig (1993) showed much higher herbicidal activity at high relative humidity with and without surfactant (Table 1). Cereal ALS-inhibiting herbicides often successfully control young dicotyledonous weeds under cool and humid conditions without surfactant. Surfactant is always necessary when weeds are large and conditions are dry and sunny.

Table 1. Effect of relative humidity on the relative potency of chlorsulfuron on white mustard (*Sinapis alba*) (adapted from Kudsk & Streibig 1993).

Relative Humidity	Relative Potency	
	Without Surfactant	With 0.1% v/v Surfactant
35%	1.0	4.9
85%	5.4	8.0

Dry Conditions

Under dry conditions weed problems do not dry up, but become progressively harder to control. Weeds grow slowly and create barriers to conserve moisture that also serve as barriers to herbicide uptake. Dry conditions slow weed growth and thus reduce the visually apparent effect of ALS-inhibiting herbicides. The dry conditions that make weeds more difficult to control also minimize risk of crop injury. The highest recommended herbicide rates and the most potent adjuvant systems can then be used safely.

Plants grown under low humidity and dry soil conditions tend to have smaller leaves, thicker cuticles with more trichomes, and a more compact leaf structure (Caseley 1989). These attributes reduce herbicide interception, retention and penetration. Changing the humidity before application can change the amount of wax and alter its morphology and chemical composition; during application, humidity affects droplet formation and size; and after application, humidity affects the physical form and drying time of spray deposits.

Dry conditions change the adjuvant requirement needed for optimum activity. For example, Hull *et al.* (1975) found lipophilic surfactant performed better during dry growth conditions while hydrophilic surfactants were better during the humid season. The hypothesis is that ALS-inhibiting herbicides can penetrate better through hydrophilic pathways with hydrophilic adjuvants under humid conditions when cuticles are thin, but would need lipophilic adjuvants to penetrate thick, waxy cuticles. Small weeds are much easier for ALS-inhibiting herbicides to control and a practical way to improve efficacy under dry conditions.

Dew

Many ALS-inhibiting herbicides are formulated as dry particles and often their spray deposits dry to particles on the leaf surface. Dew, aided by the adjuvants in the spray deposit, can solubilize these particles into small concentrated droplets. The more concentrated these droplets, the greater the absorption and herbicidal activity (Al-Khatib *et al.*, 1994). Dew may

also reduce herbicide activity by increasing spray runoff when high spray volumes are used. Most companies only recommend application only to dry surfaces.

TEMPERATURE

The effect of temperature can be dramatic when ALS-inhibiting herbicides do not control weeds under cold temperatures or injure crops under hot temperatures. Plant systems must be functioning normally to achieve expected weed control and crop tolerance. Varying the temperature from 1° to 20° C increased the potency of flumetsulam nearly 100-fold (Madafiglio *et al.*, 2000). The effect of temperature was greatest at the lowest and highest temperatures. Planting crops adapted to local climate fluctuations and using safeners minimizes crop injury due to temperature conditions (Berzsenyi *et al.*, 1997). Temperature also influences chemical and microbial degradation rates in the soil and can change rotational crop intervals.

Cold

Extended cold weather alone can cause symptoms that may be attributed to ALS-inhibiting herbicides by slowing growth and causing leaves to yellow. Frost immediately before or after application stops normal metabolic inactivation and translocation processes. Frost dramatically increases crop injury for ALS-inhibiting herbicides that depend on metabolic inactivation. Practise dictates that application should not be made when temperatures can decrease below freezing. If the plants are already damaged, applications should be delayed until better weather returns and the plants have recovered. Cold weather slows crop recovery, but can also reduce efficacy by slowing translocation to meristems and delaying the expression of injury.

The herbicidal activity of acid imidazolinones is generally greater at cooler temperatures because acid imidazolinones are more rapidly deactivated via metabolism at warmer temperature (Malefyt & Quakenbush 1991). Herbicide safeners can help overcome ALS-inhibiting herbicide injury in monocotyledonous crops by stimulating metabolic deactivation, but safeners would not help when extreme temperatures have stopped metabolism.

Heat

Similar to cold temperatures, hot temperatures can slow or stop metabolic inactivation and other plant processes. The temperature of the leaf tissue is most important because leaves are the primary organs that deactivate ALS-inhibiting herbicides. In practice, effects of sunlight, humidity, and temperature are difficult to separate. Bright sunny conditions can raise leaf temperatures significantly higher than ambient air temperature. Transpiration can cool leaf temperatures below ambient air temperature when soil moisture is plentiful and humidity is low. However, leaves usually have higher temperatures. Leaf temperatures with a canopy exposed to sunlight are often 2° C hotter than ambient leaf temperatures and can be over 10° C hotter (Sharkey 1996). Leaf temperature shifts can have a dramatic effect on crop physiology and herbicide tolerance. For example, maize does not metabolically inactivate rimsulfuron at temperatures greater that 35° C (data not given) and whole plant studies showed it is six times more sensitive at 35° C compared to 20° C (Figure 1).

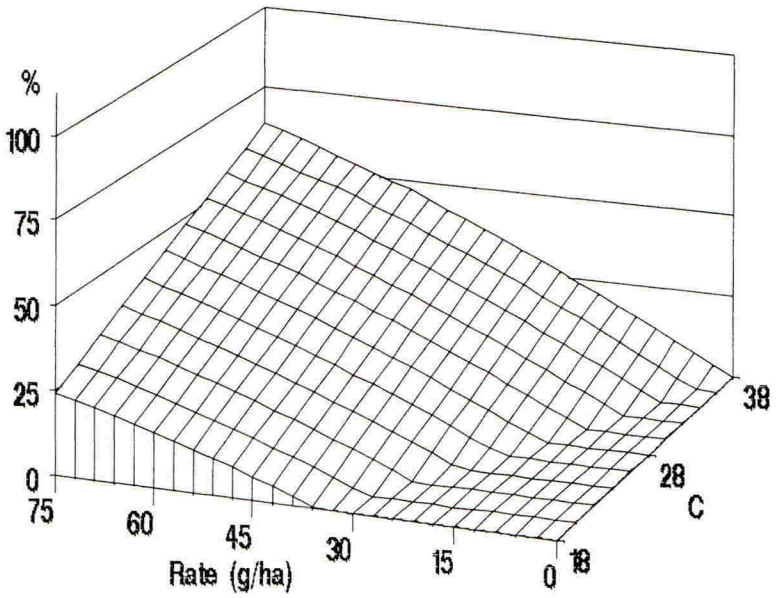

Figure 1. Effect of greenhouse temperature immediately following application on rimsulfuron inhibition of maize (*Zea mays*).

ALS-inhibiting herbicides have low vapour pressures, among the lowest ever reported for agricultural chemicals (Shaner & O'Connor 1991; Schmuckler *et al.*, 2000), and are not generally subject to drift or loss through vapourization, even at high temperatures.

LIGHT

Sunlight influences herbicide activity in the plant and on the soil by changing degradation rates, plant growth habit, cuticle thickness, and translocation. Light is essential for photosynthesis to create the energy and chemicals needed to drive translocation, metabolism, and growth and development processes in a healthy crop.

Cuticle

When light conditions are low, cuticles are thin and easy for ALS-inhibiting herbicides to penetrate. When relative humidity is high, an activator adjuvant may not even be needed (Kudsk *et al.*, 1990). In contrast, when light intensity is high and humidity is low, the cuticles become thick and the weeds become very difficult to control. Growth chamber experiments that varied light and humidity clearly showed that adjuvants enhanced activity under all situations (Table 2). High light and low humidity made the weed more difficult to control and together these factors created a much more difficult situation than either situation alone. Under the most difficult conditions for control, high light and low humidity, nonionic surfactant (NIS) was not effective with the 35 g/ha nicosulfuron and 1% v/v modified

vegetable oil concentrate (MVO) was required. These results support a common recommendation in the U.S. to use MVO with ALS-inhibiting herbicide when conditions are sunny and dry. Oil concentrates generally help when spray deposits dry fast and the cuticles are thick.

Table 2. Interaction of light and humidity on giant foxtail (*Setaria faberi*) with nicosulfuron under different adjuvant conditions (adapted from Green & Casini 1998).

Environmental Conditions		Adjuvant Conditions		
Light Intensity ($\mu E/m^2/s$)	Relative Humidity (%)	No Adjuvant	0.25% v/v NIS	1% v/v MVO
		———	50% Control Rate (g/ha)	———
125	85	16	0.9	0.7
	25	55	2.4	0.6
500	85	56	6.6	2.6
	25	533	37	5.6

Uv degradation

Some ALS-inhibiting herbicides, particularly imidazolinones, can degrade rapidly when exposed to *uv* light in aqueous solution (Mallipudi *et al.*, 1991), but photodecomposition has not been a serious use limitation. Photodegradation in soil is much slower than on the surface of leaves or dry soil. Degradation rates are slow enough to allow incorporation into the plant or soil and special protective measures have not been needed.

Leaf Angle

Diurnal leaf movement is a significant factor for the control of some weeds with some herbicides (Anderson & Koukkari 1978). The vertical orientation of many monocotyledonous weeds usually makes them more difficult targets for herbicide sprays than the broader and more horizontal leaves of most dicotyledonous weeds. However, during the night or under heavy cloud cover or shade many dicotyledonous leaves also reorient their leaves. The solution to this problem is to encourage mid-day spraying when wind velocity is often highest. Fortunately, the weeds most likely to show this behaviour are usually very susceptible to ALS-herbicides and this issue has not been a major problem.

WIND

You cannot control wind velocity, direction, and atmospheric stability. In windy conditions, proportionally less herbicide is deposited on the targeted weeds and more moves off-target. Cold and humid conditions allow wind to carry fine spray droplets significantly farther as there is minimal spray droplet evaporation. The high potency of ALS-inhibiting herbicides under field conditions, particularly the sulfonylurea herbicides, has created the perception that this class is particularly susceptible to spray drift. However, when non-target plant sensitivity is considered as a proportion of the rate applied and other exposure and environmental fate

factors, sulfonylurea herbicides are not generally more hazardous than other herbicides (Obrigawitch *et al.*, 1998).

To combat spray drift, labels restrict spraying herbicides under windy conditions. Such restrictions are easy to make but difficult to follow when conditions are constantly windy. To reduce spray drift, labels usually recommend: spray slowly with ground equipment when wind speed is 5 to 16 km/h; use high spray volumes (> 150 L/ha) and low pressures (< 200 kPa); replace worn nozzles; and use spray drift control adjuvants. The spray drift control adjuvants increase spray droplet size and do not reduce activity.

Drift potential also increases when wind speeds are low (less than 5 k/h) because wind direction varies and there is an increased potential for temperature inversions. Inversions often begin when the sun sets and continue into the morning if the air is very stable and there is no mixing between air layers. The lower warmer air can rise and act as a blanket over cooler air trapped underneath. Particles and droplets suspended in the cooler air can form a concentrated cloud and move laterally over a long distance. Eventually, the suspended cloud will encounter a downdraft and return to the surface. The best way to avoid drift associated with temperature inversions is to monitor conditions closely and spray when there is some air movement.

Some ALS-inhibitor herbicide labels warn against application on dry, powdery soils where wind can erode the soil surface and carry soil particles to off target areas. Unwanted injury to susceptible crops may result when contaminated soil particles are moved by the wind. Wind also can abrade leaf surfaces by causing leaves to rub against each other and by blowing soil particles against leaf surfaces. Wind abraded leaf surfaces in sugar beets are known to be more sensitive to herbicides.

SUMMARY

Weather conditions such as rainfall, humidity, temperature, light, rainfall, soil moisture, and wind influence ALS-inhibiting herbicide performance. Generally, conditions that favour plant growth favour weed control while conditions that stress plants reduce control and increase crop injury. ALS-inhibiting herbicides are usually most effective when humidity is moderate to high, temperatures moderate, and diurnal temperature fluctuations modest.

Our understanding of weather effects is still growing and we cannot yet make precise adjustments in rates and other application parameters based on weather conditions. Adjuvants can help weatherproof herbicides by reducing drift, making applications rainfast, adding humectancy, and increasing penetration through thick cuticles under dry conditions. Although weather can strongly influence ALS-inhibiting herbicides, label recommendations are specifically defined to ensure performance and are not strict compared to other herbicides.

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The effect of environmental factors on the activity of glufosinate

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ABSTRACT

Research work dealing with the effect of environmental factors on the activity of the foliar-acting herbicide glufosinate is reviewed. High light intensities at and after glufosinate application tend to increase herbicidal efficacy. Darkness immediately after herbicide application delays symptom development, but final herbicidal action under normal day/night cycling is not dependent on the time of day at which the herbicide is applied. Glufosinate is a water soluble compound and the post-application period required to obtain full rainfastness of this herbicide is dependent on the weed species and the intensity of rainfall. High relative humidity increases foliar uptake and herbicidal efficacy. Low temperature after glufosinate application delays the appearance of visible damage symptoms. The temperature factor appears to be less crucial for the efficacy of glufosinate than the role of relative humidity.

INTRODUCTION

The compound DL-homoalanin-4-yl(methyl)phosphinic acid (glufosinate), in the form of the monoammonium salt, was initially developed as a non-selective post-emergence herbicide. Non-selective uses include directed spray to weeds between the rows in crops such as fruit trees, plantation crops and vines and end of season application to crops such as potato and oilseeds for desiccation purposes. More recently, glufosinate-tolerant transgenic crops have been developed, e.g. rapeseed and maize, which permits selective weed control by spraying the herbicide "over the top" of the tolerant crop.

Glufosinate acts by inhibition of the enzyme glutamine synthetase (GS). This enzyme catalyzes the synthesis of glutamine from glutamate and ammonia and plays a central role in plant nitrogen metabolism, particularly in the process of ammonia assimilation into organic N. Inhibition of GS by glufosinate results in a rapid build-up of high ammonia levels and a concomitant depletion of glutamine and other amino acids. These effects are accompanied by a rapid decline of photosynthetic CO_2-fixation, and are followed by chlorosis and desiccation of the shoot tissue.

Since 1981, when glufosinate was introduced as a new herbicide, the investigation of the influence of environmental factors, mainly light, relative humidity, rain and temperature, on the activity of this herbicide have found continued interest. The aim of this paper is to evaluate the information which has accumulated in this field to date.

LIGHT

Early mode of action studies revealed, that plants kept in permanent darkness immediately after application of glufosinate showed a retarded development of visible damage symptoms and much less accumulation of ammonia in the leaf tissue than plants kept under a normal day/night cycle. For example, visible damage on soybean plants kept after treatment for 48 hours under a day/night cycle (light intensity 40 W/m^2) was 35%, and the ammonia level in the leaf tissue was 1060 µg NH_4-N/g fwt. After the same period under continuous darkness, however, no visible damage was observed and the ammonia level was only 144 µg NH_4-N/g fwt. Damage ratings and ammonia values at reduced light intensities of 8.5 and 2.5 W/m^2 were significantly lower than at 40 W/m^2 (Köcher, 1983). The observed influence of light on glufosinate effects can be explained by the fact that the main ammonia-generating process in green plants is the photorespiratory conversion of glycine to serine, hence a process being dependent on light. While the released ammonia is normally reassimilated by GS, the inhibition of this enzyme by glufosinate results in a rapid build-up of high ammonia concentrations in the plant tissue and a depletion of organic N compounds, leading finally to a general disruption of cellular functions. In a recent study with *Galium aparine* and *Brassica rapa* (Petersen & Hurle, 2001) plants were placed, beginning 5 days prior to glufosinate treatment, in a growth chamber under two different light regimes (300 and 130 $\mu Em^{-2}s^{-1}$, day/night rhythm of 12 h). Similar to the findings mentioned above, the low light intensity resulted for both species 10 days after herbicide application in roughly a doubling of the ED_{50}, hence in a lowered susceptibility to glufosinate. In agreement with these findings field observations point to a higher activity of glufosinate at high than at low light intensities.

When plants (e.g. *Sorghum halepense*) were kept after spraying with glufosinate for one day under continuous darkness and then exposed to a day/night cycle, ammonia levels and damage symptoms, being retarded in the dark period, increased rapidly after the beginning of the day phase and soon reached the same level as in plants kept under a day/night cycle immediately after treatment (Köcher, 1983). This shows that darkness does not prevent or reduce the activity of glufosinate, but just causes a transitory delay. Similar trends were seen for barley in a growth chamber trial where final injury ratings were about the same when plants were treated with glufosinate at either the beginning or end of a 16 h-photoperiod. Visual injury in *Setaria viridis* was even slightly higher when glufosinate was applied at the end of the photoperiod (Anderson *et al.*, 1993a).

A different situation arises, when weeds grow up from emergence under shady conditions, e.g. in matured plantations. Trials in rubber plantations in Malaysia revealed that under conditions of strong and uniform shade *Paspalum conjugatum* was controlled more effectively by glufosinate than under higher light conditions (Purusotman *et al.*, 1985). These data and further observations of better glufosinate performance within matured plantations suggest that a lowered efficacy of glufosinate by low light conditions may be compensated or even overcome under the permanently shady plantation situation. This is probably a result of increased weed susceptibility, owing to a shade type plant morphology and/or higher relative humidity in the plantation compared to the open field.

RELATIVE HUMIDITY

Data on the role of relative humidity (r.h.) for herbicidal efficacy of glufosinate were first published by Langelüddeke *et al.* (1988). Barley plants were raised outside and placed in two growth chambers maintained at 95% and 40% r.h., respectively, for two days immediately following glufosinate treatment. Thereafter, both groups were placed together into a greenhouse until the end of the experiment. The plants subjected for 2 days to continuous 95% r.h. were significantly more damaged by glufosinate 17 days after application (ED_{80} = 180 g/ha) than the plants kept for 2 days continuously at 40% r.h.(ED_{80} = 550 g/ha). It was interesting that addition of ammonium sulfate or of the wetting agent sodium C_{12}/C_{14}-fatty alcohol-diglycolether sulfate (FAEO-sulfate) to the application solution effectively increased the efficacy of glufosinate under the low humidity regime. The observed decrease of glufosinate efficacy at low humidity can be attributed to a lower rate of foliar penetration, while penetration is stimulated by ammonium sulfate or the wetting agent (Köcher, 1989).

Anderson *et al.* (1993) studied in barley and *Setaria viridis* the influence of relative humidity on glufosinate efficacy. The plants were precultivated at 52% r.h. and 22/17°C (day/night) and, starting 3 days prior to herbicide application, subjected in growth chambers to different humidity regimes (95% vs. 40% r.h., continuously during day and night). A dose of 800 g/ha was lethal to barley at 95% and sublethal at 40% r.h. The same dose was lethal to *Setaria viridis*, regardless of the humidity regime, due to the high glufosinate susceptibility of this species. At the low dosage of 100 g/ha *Setaria viridis* survived at 40% r.h., but was killed at 95% r.h. In the same paper these authors also reported on the effect of temperature (see below) and came to the conclusion that the humidity factor had a much higher impact on glufosinate efficacy than temperature. Petersen & Hurle (2001) tested the impact of different humidity regimes, starting 5 days prior to glufosinate application, on control of *Galium aparine* and *Brassica rapa*. When 80% r.h. was compared with 55% (both continuously during day and night), the ED_{50} for *Galium aparine* was 142 g/ha at the high, and 398 g/ha at the low humidity regime. For *Brassica rapa* it was 30 g/ha at the high, and 72 g/ha at the low humidity. When the plants were exposed to a regime of 55/80% r.h. (day/night), an ED_{50} of 290 g/ha was obtained for *Galium aparine* and 44 g/ha for *Brassica rapa*, hence this regime, which is closer to natural conditions than continuously low or high humidity, resulted in intermediate efficacy.

Recently Ramsey & Hall (2001) demonstrated that high relative humidity (95% vs 40%) increased the effectiveness of glufosinate on *Avena fatua*. In subsequent experiments these authors looked at the reaction of *Avena fatua*, when the continuous 40% r.h. regime was interrupted by a short period of high humidity beginning prior to and after spraying. Even when the plants were exposed to only very short periods (40 min before and after spraying) of >95% r.h., the efficacy of glufosinate was higher than at continuous 40% r.h. Efficacy was increased to the same extent, when the plants were exposed to 40 min of high humidity only after spraying, while the same period of high humidity given only prior to spraying did not increase herbicidal effectiveness above the level obtained at continuous 40% r.h. These results indicate the importance of the post-treatment period for herbicidal effectiveness. Furthermore, Ramsey & Hall (2001) showed that there was greater foliar uptake of [^{14}C]glufosinate in plants grown at 40% r.h. with an interruption at >95% r.h. for 30 min before and after [^{14}C]glufosinate application than in plants grown continuously at 40% r.h. Hence, humidity conditions resulting in improved herbicide efficacy were correlated to foliar uptake of this herbicide.

Mathiassen & Kudsk (1993) simulated in growth chambers natural climates by diurnal cycling programs for humidity and temperature. Barley plants, cultivated outdoors, were transferred to the growth chambers 2 days before spraying and kept under 6 different climate types. All high humidity climates resulted in higher efficacy of glufosinate than the low humidity climates. The relative potency of the herbicide under high humidity climates increased with increasing temperature (5, 11 and 17°C on average). Under low humidity regimes the relative potency of the herbicide increased when the average temperature was raised from 5 to 11°C, but decreased again at an average temperature of 17°C. The authors concluded that the limitation of herbicidal activity by the high vapour pressure deficit at 17°C overcame the promotion of herbicidal activity by increasing temperature. These authors found, similar to Langelüddeke *et al.* (1988), that the lowered herbicidal effectiveness under low humidity regimes could partially be overcome by addition of ammonium sulfate (2%) or of the wetting agent FAEO-sulfate (0.5%) to the spray solution.

Hence all research data gathered so far points to an increase of glufosinate effectiveness with an increase of relative humidity. The positive effect of high humidity on foliar uptake of this herbicide may be explained by an increased rate and prolonged period of herbicide diffusion from the spray deposit at the leaf surface into the cuticle. This happens possibly in conjunction with increased hydration of the cuticle, which would be expected to improve the cuticular permeation of the highly hydrophilic glufosinate molecule.

RAIN

The question regarding the influence of rain on the herbicidal effectiveness of glufosinate is of particular interest, considering the high water solubility of this herbicide. Published data and in-house experience on the rainfastness of glufosinate resulted in the use recommendation that glufosinate should not be applied if there is a risk of rain in a period less than 6 hours after spraying the weeds. This recommendation will usually cover the situations occurring in agricultural practice. In rain simulation experiments the period required to obtain rainfastness can differ widely, depending on herbicide dosage, susceptibility of plant species and rain regime. This can be illustrated by a study with barley and *Setaria viridis* by Anderson *et al.* (1993a), who simulated rain of 4, 9 and 22 mm between 10 min and 12 hours after glufosinate application at dose rates of 200, 800 and 1200 g/ha. Generally the minimum rain-free period after spraying, required to prevent loss of efficacy, increased as the intensity of rainfall increased, and decreased with increasing dose rate. At a dose of 800 g/ha barley required a rain-free period of 1-8 hours, depending on rain volume, while *Setaria viridis*, a species much more susceptible to glufosinate, required less than 20 min of rain-free period at the same dose rate. Several research programs paid attention to the question whether the rainfastness could be improved with the use of spray additives. Langelüddeke *et al.* (1988) reported that to some extent FAEO-sulfate wetting agent (0.2%) and more effectively ammonium sulfate (10 kg/ha) could compensate the reduced efficacy of glufosinate caused by 10 mm of artificial rain in barley, spring rape, *Ottochloa nodosa* and *Paspalum conjugatum*. Similar results were obtained with these additives by Mathiassen & Kudsk (1993) in rainfastness trials with barley, *Sinapis alba* and *Veronica persica*. The improved rainfastness is attributed to the already mentioned ability of these additives to increase the rate of foliar penetration of glufosinate.

TEMPERATURE

Temperature can modify herbicidal activity in plants via an influence on herbicide uptake, translocation and degradation as well as on plant metabolism in general. In growth chamber studies a significant influence of the temperature regime on glufosinate performance was observed in dicotyledonous weed species (*Chenopodium album, Galium aparine*), when exposed in the post-application period to different temperature regimes. High temperatures (26/18°C, day/night) resulted in optimum weed control, whereas at low temperatures (10/2°C, day/night) the herbicidal effectiveness was markedly reduced, resulting in ED_{80} values 3-5 fold higher than at 28/18°C, 3 weeks after treatment. When the grass weeds *Cynodon dactylon* and *Sorghum halepense* were kept under differential temperature regimes (32/14°C vs. 18/10°C, day/night) after glufosinate application, the ED_{80} values were not significantly influenced by temperature. However, when these weed species were kept under the same regimes in the pre-application as well as in the post-application phase, they needed about double the herbicide dosage at 18/10°C compared to 32/24°C, to obtain an equal herbicidal effect. Similar tests were carried out with *Agropyron repens*, but even the lowest temperature regime (10/2°C) did not significantly reduce the efficacy on this species. A general observation made in these tests was that damage symptoms appeared after only 1-2 days under a 18/10°C regime, but took about 4-7 days to appear at a regime of 10/2°C (Donn, 1982).

When barley and *Setaria viridis* were kept at constant relative humidity (60%) and were subjected to temperature regimes of 8/5°C, 15/10°C and 22/17°C (day/night), beginning 1 day prior to glufosinate treatment, the visual injury to both species was significantly delayed as temperature decreased. After 12 days, however, there was little difference in the level of injury among these temperature regimes (Anderson *et al.*, 1993). Studies with *Galium aparine* and *Brassica rapa* (Petersen and Hurle, 2000) gave a contrasting finding. Plants which were kept continuously under 80% r.h. and subjected to differential temperature regimes (24/16°C vs. 16/12°C, day/night), beginning 5 days prior to glufosinate application, were controlled with about doubled efficay at the high compared to the low temperature regime.

As already pointed out, Mathiassen & Kudsk (1993) simulated natural climates in the growth chamber by diurnal cycling of humidity and temperature. At high humidity regimes the activity of glufosinate on barley increased with temperature (5°C < 11°C < 17°C on average), while at low humidity regimes the potency of glufosinate was higher at 11°C than at 5°C or 17°C on average. Though there was an effect of temperature, the authors derived from the results of their climate simulation trials, that the temperature factor was less crucial for the efficacy of glufosinate than the relative humidity.

CONCLUSIONS

With the exception of rain simulation trials growth chamber techniques were used to study the influence of environmental factors on glufosinate activity. There is no doubt that the complex interaction of environmental factors in nature cannot be satisfactorily mimicked by growth chamber techniques, which usually compare different levels of one or two factors while leaving the others constant. But despite of such limitations, growth chamber experiments can provide us with information which of the environmental factors play a significant role for the performance of a herbicide.

In the case of glufosinate such studies led to the conclusion that high humidity and high light intensity increase the activity of this herbicide. These findings are supported by the evaluation of glufosinate performance in field trials and their correlation with weather recordings at the time of herbicide treatment (Bickers, 2001). As to the temperature factor, obviously increasing temperatures speed up the appearance of visible glufosinate symptoms in dicotyledonous and grass weeds. Furthermore, it can be concluded from the available growth chamber data that in dicotyledonous species also the final herbicidal efficacy increases with temperature. Why such a temperature dependence was less apparent in grass species, remains an open question.

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Drought-induced tolerance to aryloxyphenoxypropionate herbicides in blackgrass (*Alopecurus myosuroides*) and wild oats (*Avena fatua*)

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ABSTRACT

The herbicides, fenoxaprop-P-ethyl and clodinafop-propargyl are used to control grass weeds in cereals at rates of 55-68 g a.i./ha. Reductions in their activity have been reported under drought conditions. Studies were undertaken to reproduce this phenomenon and quantify the level and duration of drought likely to reduce activity against blackgrass (*Alopecurus myosuroides*) and wild oats (*Avena fatua*). Shoot fresh weight data showed that for both species, plants exposed to drought conditions were significantly less susceptible to foliar applications of fenoxaprop-P-ethyl and clodinafop-propargyl than plants grown under normal soil moisture conditions. In some cases, the dose needed to cause 50% inhibition was up to 200% greater for drought-stressed plants. Results also showed that the level of drought-induced tolerance was greatest at doses below recommended application rates and was typically dependent on the duration of drought prior to spraying rather than after spraying. Furthermore, plants subject to drought periods that were alleviated prior to herbicide treatment, or imposed immediately after treatment, did not show increased tolerance to these herbicides. Therefore, the occurrence of drought-induced tolerance in the field may be minimised by ensuring the use of maximum recommended rates, spraying at the onset of a drought period or delaying applications until drought has been alleviated.

INTRODUCTION

The aryloxyphenoxypropionoate herbicides, fenoxaprop-P-ethyl and clodinafop-propargyl, are widely used to control grass weeds in cereals. These herbicides are foliar-acting, fatty acid biosynthesis inhibitors, intended for use at rates of 55-68 g a.i./ha. However, their activity against several weed species has been reported to be reduced under drought stress conditions (Rossi *et al.*, 1994; Lemerle & Verbeek 1995). This paper reports the results of studies designed to quantify the level and duration of drought likely to reduce activity of these herbicides against blackgrass (*Alopecurus myosuroides*) and wild oats (*Avena fatua*).

MATERIALS AND METHODS

Blackgrass and wild oat seeds were germinated for 7-10 days and transplanted into sealed pots containing a weighed quantity of sandy loam soil mixed with grit and slow-release fertilizer. Soil was maintained at 75% field capacity (FC) by daily gravimetric addition of water *via* a perforated tube in the centre of each pot. Plants were maintained in controlled environment

rooms with a 14-hour daylength and day-night temperature and relative humidity of 10-16°C and 75-85%, respectively. Drought was imposed by with-holding water until the soil moisture content dropped to the specified level. During this period, air circulation was increased and relative humidity dropped to 65-75% to accelerate moisture loss. Once attained, the moisture level was maintained by daily watering. After the required stress period, soil moisture content was returned to 75% FC.

The herbicides, fenoxaprop-P-ethyl (Cheetah Super) and clodinafop-propargyl (Topik) were applied at 20, 40, 60, 80 and 100% of recommended field rates to plants between GS12 and GS31. Stock solutions of the formulated products were prepared in deionized water and applied as foliar sprays, using a track sprayer calibrated to deliver 200 L/ha at 200 kPa. After 3 weeks, plants were harvested for measurement of shoot fresh weights. Material was then dried at 60°C for at least 48 hours before measurement of dry weights.

Experimental design and statistical analysis

The effects of moisture stress on herbicide dose responses were investigated in randomised factorial experiments incorporating 5 or 6 herbicide doses, including control, applied at several levels of drought stress. Drought treatments were defined according the duration and timing of the drought period relative to herbicide treatment. All experiments included a control herbicide dose response in which plants were maintained continuously at 75% FC. Experiments incorporated 3 or 4 replicate pots containing 2 plants. Analysis of variance (ANOVA) followed by Student's Protected t-test, were used to detect significant effects of herbicide treatments and soil moisture stress, on fresh and dry weight data. Significant dose responses were described by a logistic curve to enable estimation of EC_{50} (concentration required to cause 50% effect) parameters for each drought treatment.

RESULTS

Fenoxaprop-p-ethyl *x* blackgrass

In experiments investigating the effects of drought on fenoxaprop activity in blackgrass, drought was initiated 8 days before spraying such that soil moisture content dropped to 30% FC 4 days before herbicide application. Drought was alleviated 5 days after spraying.

This drought treatment did not significantly reduce the fresh or dry weight of untreated plants but did increase the tolerance of blackgrass to fenoxaprop (Table 1). Comparison of mean fresh weights showed that fenoxaprop activity at doses up to 41.3 g a.i./ha was not significantly altered by drought treatment. However, unstressed plants treated with the recommended dose of 55 g a.i./ha, suffered 81% reduction in fresh weight while those exposed to drought suffered significantly smaller reductions of only 56%. Dose response models fitted to fresh weight data, confirmed that the EC_{50} was significantly increased from 33.2 g a.i./ha in unstressed plants, to 51.7 g a.i./ha in drought- stressed plants.

Fenoxaprop-p-ethyl *x* wild oats

In experiments investigating the effects of drought on fenoxaprop activity in wild oats, drought periods were initiated 8 or 4 days before spraying such that soil moisture content dropped to 20% FC 3 and 0 days before herbicide application, respectively. Drought was then alleviated 2 or 6 days after treatment.

While drought periods of 8+2 and 4+2 days did not cause significant reductions in fresh or dry weight of untreated plants, drought periods of 8+6 and 4+6 days significantly reduced fresh weight by 25 and 21%, respectively. All drought treatments reduced fenoxaprop activity against wild oats. The magnitude of the reduction was correlated with the duration of drought such that longer periods caused greater reductions in activity (Table 2).

Drought periods of 4+2 and 4+6 days significantly increased the EC_{50} for fenoxaprop from 32.3 g a.i./ha in unstressed plants to 45.6 and 51.3 g a.i./ha, respectively. Fresh weight data confirmed that unstressed plants treated with doses of 41.3 g a.i./ha, suffered a 68% reduction in fresh weight while those exposed to drought beginning 4 days before spraying, suffered significantly smaller reductions of 37-45%. However, neither drought treatment significantly reduced the activity of fenoxaprop at the recommended doses of 55 and 68.8 g a.i./ha.

Drought periods of 8+2 days and 8+6 days further increased the EC_{50} for fenoxaprop to 55.4 and >68.8 g a.i./ha, respectively. Comparison of mean fresh weight data showed that unstressed plants treated with doses of 41.3 g a.i./ha, suffered a 68% reduction in fresh weight while those exposed to drought periods beginning 8 days before spraying, suffered significantly smaller reductions of 20-22%. Furthermore, while the fresh weight of unstressed plants was reduced by 83% following treatment with 55 g a.i./ha, the fresh weight of plants subject to these drought periods was reduced by 37-46%. The duration of the stress period after spraying did not significantly affect this response. However, at the recommended dose of 68.8 g a.i./ha, only those plants subject to a drought period of 8+6 days were less susceptible to inhibition by fenoxaprop. In this case, unstressed plants suffered 87% reductions in fresh weight while those exposed to a drought period of 8+6 days suffered 55% reductions in fresh weight.

Table 1. Effect of drought stress on fenoxaprop-P-ethyl activity against blackgrass

Dose (g a.i./ha)	Fresh weight (% reduction from untreated control)	
	Drought stress duration (days before + after spraying)	
	0	8+5
0	0	15
13.8	4	20
27.5	38	34
41.3	64	54
55.0	81	56*
Estimated EC_{50}	33.2	51.7*
SE	3.6	10.3

* indicates significant difference from unstressed means at same herbicide dose
df=40; SED= 12.3%; LSD (p=0.05)= 24.8%

Table 2. Effect of drought stress on fenoxaprop-P-ethyl activity against wild oats

Dose (g a.i./ha)	Fresh weight (% reduction from untreated control) Drought stress duration (days before + after spraying)				
	0	8+2	8+6	4+2	4+6
0	0	8	25*	3	21*
13.8	4	11	23	13	15
27.5	16	-1	20	15	15
41.3	68	22*	20*	45*	37*
55.0	83	46*	37*	76	70
68.8	87	85	55*	80	77
Estimated EC_{50}	32.3	55.4*	>68.8*	45.6*	51.3*
SE	2.0	2.7	n.d.	2.4	2.9

* indicates significant difference from unstressed mean, treated with same herbicide dose
df=90; SED= 10.3%; LSD (p=0.05)= 20.4%; n.d. not determined

Clodinafop-propargyl *x* blackgrass

In experiments investigating the effects of drought on clodinafop activity in blackgrass, drought periods were initiated 9,7 or 5 days before spraying such that soil moisture content dropped to 20% FC 3, 2, and 1 days before herbicide application, respectively. Drought was then alleviated either immediately or 3 days after treatment.

Drought stress treatments did not significantly affect the fresh or dry weights of untreated plants but did significantly reduce clodinafop activity against blackgrass (Table 3). EC_{50} parameters were significantly increased from 29.8 g a.i./ha in unstressed plants to 42.3-52.3 g a.i./ha in stressed plants. The magnitude of reduction in activity was not correlated with the duration or timing of the drought treatment and comparison of dose response models, fitted to fresh weight data, showed that there were no significant differences between the level of tolerance induced by each treatment. Fresh weight data confirmed that, with the exception of plants deprived of water for 7+3 days, drought stressed plants were significantly less susceptible to clodinafop applied at 36 and 48 g a.i./ha. In particular, unstressed plants, treated with 36 g a.i./ha clodinafop, suffered a 81% reduction in fresh weight while those exposed to drought for any length of time, suffered significantly smaller reductions of 20-42%. Furthermore, with the exception of plants deprived of water for 7+3 days, treatment with 48 g a.i./ha clodinafop caused a 91% reduction in the fresh weight of unstressed plants but only a 37-53% reduction in drought stressed plants. Plants subject to drought periods of 7+3 days did not show increased tolerance to 48 g a.i./ha clodinafop and, for all drought treatments, stressed plants were not significantly less susceptible to clodinafop doses of 60 g a.i./ha. In a separate experiment, a 7-day drought period, beginning immediately after spraying, did not significantly affect the response of blackgrass to clodinafop applied at doses between 12 and 60 g a.i./ha (data not shown).

Clodinafop-propargyl *x* wild oats

In experiments investigating the effects of drought on clodinafop activity in wild oats, drought periods were initiated 9, 7 and 4 days before spraying such that soil moisture contents dropped to 20% FC 3 days before spraying, 20% FC 2 days before spraying and 30% FC on the day of application, respectively. Drought was then alleviated either immediately, 1 or 3 days after

treatment. In the case of the drought period beginning 9 days before spraying, moisture content was returned to 75% FC, 1 day before spraying.

Drought treatments did not significantly affect the fresh or dry weights of untreated plants but several treatments did reduce clodinafop activity against wild oats (Table 4). The magnitude of reduction was correlated with the duration or timing of the drought treatment such that those treatments initiated 7 days before herbicide application caused greater reductions in activity. In particular, drought periods of 7+0, 7+1 and 7+3 days significantly increased the EC_{50} for clodinafop from 38.5 g a.i./ha in unstressed plants to >60, 55.4 and 50.1 g a.i./ha, respectively. Fresh weight data confirmed that unstressed plants treated with doses of 36 g a.i./ha, suffered a 53% reduction in fresh weight while those exposed to drought beginning 7 days before spraying, suffered significantly smaller reductions of 10-24%. Furthermore, while the fresh weight of unstressed plants was reduced by 72% following treatment with 48 g a.i./ha, the fresh weight of plants subject to drought stress for 7 days before spraying was reduced by up to 47%. Significantly greater reductions in activity were seen when drought was alleviated immediately after spraying. This trend was also seen at the recommended dose of 60 g a.i./ha, where only those plants subject to a drought for 7+0 days were less susceptible to inhibition by clodinafop. In this case, unstressed plants suffered 81% reductions in fresh weight while those exposed to a drought period of 7+0 days suffered 53% reduction in fresh weight. Continuation of the drought period for 3 days after herbicide application did not significantly increase the tolerance of wild oats to 60 g a.i./ha clodinafop.

In contrast, fresh weight data showed that clodinafop activity was not affected by a 8-day drought period that was alleviated 1 day before spraying. However, plants subject to drought periods of 4+1 and 4+3 days were significantly less susceptible to clodinafop, but only when treated with 36 g a.i./ha. For example, unstressed plants suffered a 53% reduction in fresh weight while those exposed to drought beginning 4 days before spraying, suffered significantly smaller reductions of 22-26%. Neither treatment significantly affected clodinafop activity at doses of 48 and 60 g a.i./ha. Comparison of dose response models showed that EC_{50} parameters were not significantly increased by drought treatments of (9-2)+0, 4+1 or 4+3 days.

CONCLUSIONS

Shoot fresh data showed that, for both species, plants exposed to drought conditions were significantly less susceptible to foliar applications of fenoxaprop-P-ethyl and clodinafop-propargyl, than plants grown under normal soil moisture conditions. In some cases, the EC_{50} was up to 200% greater for drought-stressed plants. Results also showed that the level of drought-induced tolerance was greatest at doses below recommended application rates and was typically dependent on the duration of drought before, rather than after, spraying. Furthermore, plants subject to drought that was alleviated prior to herbicide treatment, or imposed immediately after treatment, did not show increased tolerance to these herbicides. Therefore, the occurrence of drought-induced tolerance to these herbicides in the field may be minimised by ensuring the use of maximum recommended rates, spraying at the onset of a drought period or delaying applications until drought has been alleviated.

Table 3. Effect of drought stress on clodinafop-propargyl activity against blackgrass

Dose (g a.i./ha)	Fresh weight (% reduction from untreated control)						
	Drought stress duration (days before + after spraying)						
	0	9+0	9+3	7+0	7+3	5+0	5+3
0	0	-2	19	-3	-2	-6	31
12	7	17	21	22	7	16	2
24	21	15	8	21	5	32	-13
36	81	23*	27*	42*	20*	28*	35*
48	91	48*	49*	42*	82	37*	53*
60	93	73	72	90	75	91	92
Estimated EC_{50}	29.8	50.9*	52.3*	48.7*	42.3*	50.4*	45.7*
SE	3.0	4.2	4.6	4.2	3.3	4.3	3.7

* indicates significant difference from unstressed means at same herbicide dose
df=144; SED= 18.5%; LSD (p=0.05)= 36.6%

Table 4. Effect of drought stress on clodinafop-propargyl activity against wild oats

Dose (g a.i./ha)	Fresh weight (% reduction from untreated control)						
	Drought stress duration (days before + after spraying)						
	0	(9-1)+0	7+0	7+1	7+3	4+1	4+3
0	0	3	7	-11	8	-11	20
12	11	-5	12	8	6	14	-2
24	5	2	14	20	4	-12	5
36	53	43	24*	17*	10*	22*	26*
48	72	53	11*	26*	47*	64	73
60	81	88	53*	69	77	79	75
Estimated EC_{50}	38.5	42.3	>60*	55.4*	50.1*	44.2	43.9
SE	2.1	2.2	n.d.	3.0	2.6	2.2	2.4

* indicates significant difference from unstressed means at same herbicide dose
df=126; SED= 10.4%; LSD (p=0.05)= 20.6%; n.d. not determined

ACKNOWLEDGEMENTS

IACR receives grant-aided support from the Biotechnology and Biological Research Council of the UK. This work was funded by DEFRA, UK.

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POSTER SESSION 8A
MODE OF ACTION AND METABOLISM

Session Organiser: Dr T R Hawkes
Syngenta, Bracknell, UK

Poster Papers: 8A-1 to 8A-8

The herbicide safener MG-191 enhances the expression of specific glutathione *S*-transferases in maize

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ABSTRACT

The herbicide safener MG-191 (2-dichloromethyl-2-methyl-1,3-dioxolane) and its structural analogue dichloromethyl dioxolanone (NO-17; 2-dichloromethyl-2,5-dimethyl-1,3-dioxolane-4-one) were tested for their ability to differentially enhance the expression of members of the glutathione *S*-transferase (GST) superfamily in maize. Maize seedlings were treated *via* root application with the safeners at a final concentration of 10 μM. The GSTs in root and shoot tissue were then resolved by gel electrophoresis and detected by Western blotting, using antisera raised to specific GST isoenzymes. MG-191 and to a lesser extent NO-17 selectively enhanced the expression of tau class *Zm*GSTU1 in both root and shoot tissues after one day of treatment. Addition of cycloheximide to the treatment solutions suppressed the enhancement of expression of *Zm*GSTU1 in the roots. It was concluded that MG-191 is a more specific inducer of maize GSTs than other compounds commonly used to safen thiocarbamate or chloroacetanilide herbicides in maize.

INTRODUCTION

Herbicide safeners protect crop plants from herbicides without altering their toxicity to weeds. A strong correlation between the ability of a safener to increase GST activity and its efficacy in protecting maize from herbicide injury has been demonstrated (Davies & Caseley, 1999).

MG-191 is a highly active, non-dichloroacetamide safener used in safening maize against thiocarbamate and to a lesser extent chloroacetanilide herbicides (Dutka *et al.*, 1987a). The MG-191 induced elevation of glutathione synthesis and glutathione-related enzyme activities such as cytosolic and microsomal GSTs as well as glutathione reductase in protected plants have been shown as major factors in its protective mechanism of action (Dutka & Komives, 1987b).

GSTs are multifunctional enzymes, each composed of two subunits which catalyse conjugation of broad range of electrophilic substrates with glutathione (Marrs 1996). Analysis of the isoenzyme profile of maize GSTs revealed that phi(F) class of GSTs predominate, with *Zm*GSTF1 as the major subunit which is present constitutively and shows high specificity to 1-chloro-2,4-dinitrobenzene (CDNB) substrate (Dixon *et al.*, 1997). A second phi type GST termed *Zm*GSTF2 accumulates following treatments with herbicide safeners. These subunits can

dimerise together to form *Zm*GSTF1-1 and *Zm*GSTF2-2 homodimers as well as *Zm*GSTF1-2 heterodimer. In addition to these three phi GST isoenzymes a phi type GST *Zm*GSTF3 and three tau (U) class GSTs *Zm*GSTU1, *Zm*GSTU2 and *Zm*GSTU3 are present in lower amounts (Dixon *et al.*,1998 and Dixon *et al.*, 1999).

The objectives of our study were to test MG-191 and its less active structural analogue (NO-17) for their ability to differentially enhance the expression of members of the glutathione *S*-transferase (GST) superfamily in maize.

METHODS AND MATERIALS

Chemicals

MG-191 and NO-17 were prepared in our laboratory from 1,1-dichloroacetone and purified by distillation (Dutka 1991). Cycloheximide (CH) and all other chemicals were purchased from Sigma.

Plant material and enzyme isolation

Seeds of maize (Pioneer 3394) were planted in trays of sterile vermiculite and maintained at 25 °C under 16-h photoperiod at a light intensity of 510 µmol m^{-2} s^{-1}. Plants were watered as required. Ten uniformly sized 5-day-old green maize seedlings were selected and transferred to plastic rectangular jars (80 x 80 x 110 mm) containing the solution (40 ml) of MG-191 (10 µM), NO-17 (10 µM), and MG-191 or NO-17 (10 µM) plus cycloheximide (1 µM). The treated plants were grown in light and harvested 1 and 2 days after treatment.

After weighing, the harvested plants were cut into shoot and root tissues. Shoot and root tissues were frozen with liquid nitrogen and homogenized with 1 g of sand in a mortar and pestle then extracted with 5 volumes of Tris-HCl buffer (100 mM, pH 7.5) containing 2 mM EDTA, 1 mM dithiothreitol, and 5 % (w/v) PVPP. The homogenates were filtered through two layers of Miracloth and the filtrates were centrifuged at 10,000 x *g* for 10 min. The supernatants were collected and the protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to 80% saturation. After centrifugation at 10,000 x *g* for 30 min aliquots of the protein precipitates resuspended in 20 mM of potassium phosphate buffer (pH 6.5) were desalted by gel filtration (PD-10, Pharmacia).

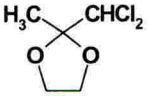
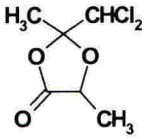
Analysis of GSTs

Glutathione *S*-transferase activities of desalted enzymes were determined photometrically using CDNB substrate and expressed as nmol product formed per second (nkat) per mg protein (Dixon *et al.*, 1998). Protein contents were measured by a commercial Bio-Rad dye-binding assay with (γ-globulin as reference protein as recommended by manufacturer. The polypeptide composition of the GST preparations were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was carried out using antisera raised to *Zm*GSTF1-2 and *Zm*GSTU1-2 (Dixon *et al.*, 1998).

RESULTS

In safening efficacy experiments the more lipophilic MG-191 was more effective to EPTC and acetochlor than its structural analogue NO-17 at a 1:10 molar ratio to the herbicide (Table 1). The effect of chemical treatments on root and shoot GST enzymes 1 and 2 DAT was analyzed using the non-herbicide CDNB substrate. The crude freshly desalted enzyme preparations of both untreated and safener-treated roots contained higher GST activities than did the respective

Table 1. Safening effectiveness of dioxolane derivatives in maize to EPTC and acetochlor

Safener	logP	Protection[a] (%) in maize against herbicides	
		EPTC[b]	Acetochlor[c]
MG-191 	1.24	104 ± 7	64 ± 6
NO-17 	0.65	62 ± 5	23 ± 5

[a] Based on shoot length; protection (%) = 100 x [(herbicide + safener)] – herbicide]/[control – herbicide];

[b] at 100 μM concentration of EPTC and 10 μM concentration of the safener;

[c] at 50 μM concentration of acetochlor and 5 μM concentration of the safener

shoot extracts (Table 2). Both safeners elevated GST activities by 50% only in shoot tissues one day after treatment at 10 micromolar concentrations of these chemicals. A day later this transient elevation in activity had faded and, in the case of NO-17, had declined to a level which was no longer detectable. Co-addition of a low concentration (1 μM) of the protein biosynthesis inhibitor cycloheximide resulted in a decrease in the shoot and an increase in the root GST activities. Previously, Miller *et al.* (1994) showed that much higher concentrations (10 and 100 μM) of cycloheximide caused significant reductions in both basal and benoxacor-induced levels of GST(metolachlor) activities as well as inhibit the incorporation of [^{3}H]leucine into total soluble protein.

Previously it was shown that *Zm*GSTF1-2 made a minor contribution to the total level of GST in untreated shoot extracts. However, in untreated roots approximately 40% of the total GST(CDNB) activity was associated with *Zm*GSTF1-2. In addition, treatment with the safener dichlormid resulted in an increase in *Zm*GSTF1-1 as well as increasing *Zm*GSTF1-2 in both root and shoots (Dixon *et al.*, 1997).

Table 2. Effect of chemical treatments on GST(CDNB) activities (mean ± SE, n=3) in crude extracts of roots and shoots of maize

Time	Treatment	Enzyme activity (nkat mg^{-1} protein)			
		shoot	treated/control	root	treated/control
1 DAT	control	1.96 ± 0.16	-	4.60 ± 0.34	-
	MG-191	2.98 ± 0.19	1.52	4.59 ± 0.32	1.00
	NO-17	2.94 ± 0.24	1.50	3.59 ± 0.30	0.78
2 DAT	control	2.23 ± 0.20	-	4.37 ± 0.28	-
	MG-191	2.82 ± 0.18	1.30	5.62 ± 0.36	1.29
	NO-17	2.11 ± 0.15	0.95	4.28 ± 0.21	0.98
	control + CH	1.97 ± 0.19	-	4.47 ± 0.18	-
	MG-191 + CH	2.05 ± 0.17	1.04	6.81 ± 0.29	1.52
	NO-17 + CH	1.64 ± 0.17	0.83	5.14 ± 0.31	1.15

In order to further clarify which maize GSTs were induced by the dioxolane derivatives the polypeptide compositions of the GSTs in, treated and untreated, root and shoot tissues were resolved by SDS-PAGE and Western blotting experiments. The resulting blots were probed using antisera raised to *Zm*GSTF1-2 and *Zm*GSTU1-2 (Dixon *et al.*, 1998). When an antiserum raised to the heterodimer *Zm*GSTF1-2 was used, it selectively recognized 29 kDa polypeptide expressed in both untreated and safener-treated shoot (Figure 1a) and root (Figure 1b) tissues. Cycloheximide treatment did not influence expression of this isoenzyme at 2 DAT. While the expression of *Zm*GSTF2 was enhanced by auxins, herbicides, the herbicide safener dichlormid and glutathione, the *Zm*GSTU1 subunit was induced more selectively, only accumulating significantly in response to dichlormid treatment (Dixon *et al.*, 1998). Although *Zm*GSTF2 has been considered more active in detoxifying metolachlor and alachlor than *Zm*GSTF1 it is far less abundant (Rossini *et al.*, 1996).

Blots using anti-*Zm*GSTU1-2 serum indicated that MG-191 and to a lesser extent NO-17 selectively enhanced the expression of tau class *Zm*GSTU1 in both shoot (Figure 1c) and root (Figure 1d) tissues after 1 DAT. Addition of cycloheximide to the treatment solutions suppressed the enhancement of expression of *Zm*GSTU1 only in the roots. *Zm*GSTU1 has previously been shown to play a key role in metabolism of nitrodiphenyl ether type herbicides (Cole *et al.*, 1997). However, since both dioxolane derivatives safen maize to only chloroacetanilides and thiocarbamates, the importance of *de novo* synthesis of the isoenzyme *Zm*GSTU1 in their safening action is difficult to explain. The complex mixture of GST isoenzymes present makes it difficult to know whether a combination or a single form of GST is predominantly responsible for herbicide detoxification.

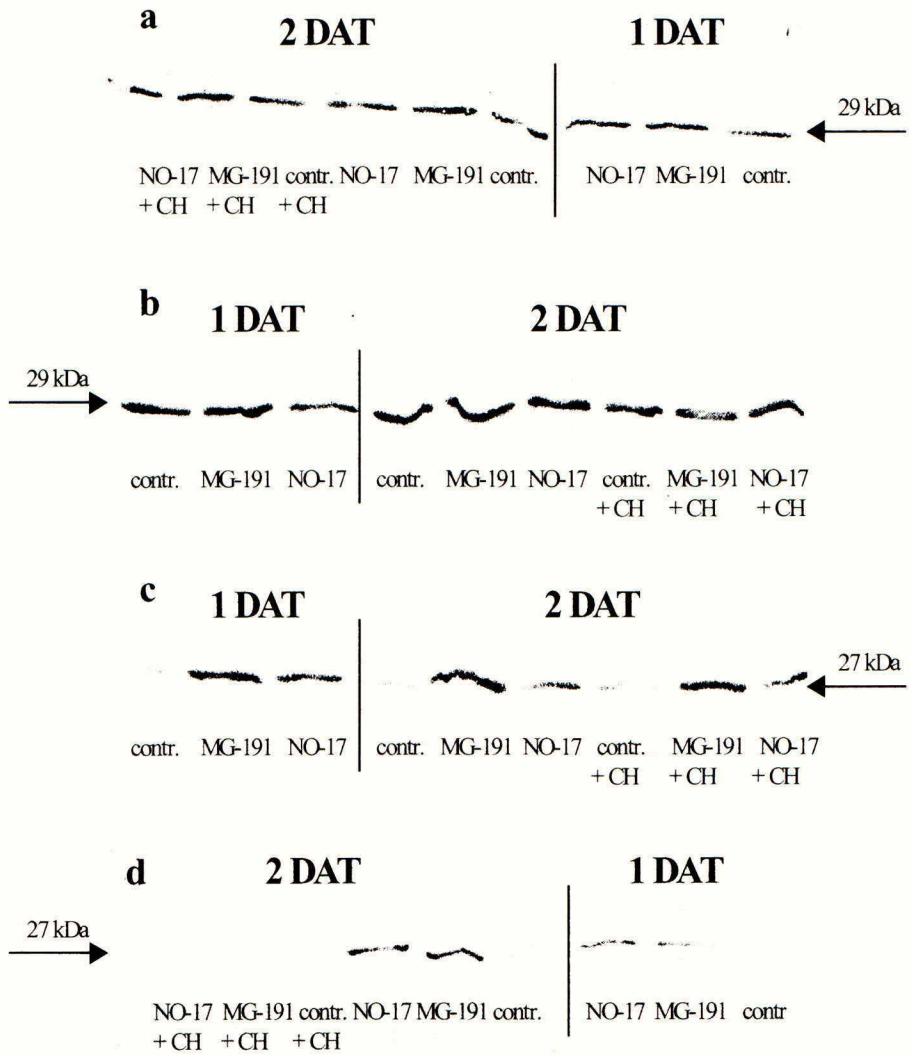


Figure 1. Western blots of crude GST extracts from maize roots and shoots
(a) Analysis of GSTs from maize shoots using the anti-*Zm*GSTF1-2 serum.
(b) Analysis of GSTs from maize roots using the anti-*Zm*GSTF1-2 serum.
(c) Analysis of GSTs from maize shoots using the anti-*Zm*GSTU1-2 serum.
(d) Analysis of GSTs from maize roots using the anti-*Zm*GSTU1-2 serum.

Nevertheless these results indicate that MG-191 is a more specific inducer of maize GSTs than other compounds commonly used to safen thiocarbamate or chloroacetanilide herbicides in maize.

ACKNOWLEDGEMENTS

This work was partially supported by the Hungarian-British Bilateral Intergovernmental Science and Technology Cooperation (GB-30/98).

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Glucosyltransferases active in pesticide metabolism in soybean, maize and *Arabidopsis* ?

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ABSTRACT

Enzymes with *N*-Glucosyltransferase (NGT) activity toward 3,4-dichloroaniline and *O*-glucosyltransferase (OGT) activity toward 2,4,5-trichlorophenol and other phenols have been extracted and assayed from seedlings and cell cultures, of soybean, maize and *Arabidopsis thaliana* Both NGT and OGT activities were optimized with respect to assay conditions and found to be stimulated by the inclusion of thiol reducing reagents and inhibited following exposure to salt and high temperatures. The formation of conjugated products was found to be strictly dependent on protein content and incubation time. By comparing NGT and OGT activities in different organs and cultures of the three species it was concluded that, although the majority of studies to date have concentrated on the use of soybean as a model plant source for conjugating activities involved in pesticide metabolism, *Arabidopsis* root and *Arabidopsis* – derived suspension cultures may be preferable sources of these activities to use in future.

INTRODUCTION

Glucosyltransferases (GTs) conjugate xenobiotics with glucose and are key enzymes of phase II pesticide metabolism. Although relatively little is known about the GTs in crops involved in pesticide detoxification, a number of GT activities directed toward the hydroxyl groups of phenols (*O*-glucosyltransferases, OGTs) and the amino groups of anilines (*N*-glucosyltransferases; NGTs) have been described and partially purified from soybean (reviewed by Cole and Edwards 2000). Soybean remains the best characterized source of pesticide metabolizing GTs with enzymes with conjugating activity toward 2,2-bis-(4-chlorophenyl)-acetic acid (Wetzel and Sandermann, 1994) and pentachlorophenol and 3,4-dichloroaniline (Sandermann *et al.*, 1991) being partially purified and characterized. OGTs active in the metabolism of the herbicide bentazone have also been described in soybean cell cultures (Leah *et al.*, 1992). Interestingly, these and other studies suggest that the OGTs and NGTs responsible for metabolising phenolics and anilines respectively are distinct enzymes (Sanderman *et al.*, 1991; Gallandt and Balke, 1995). Using soybean as a reference species, we are now interested in extending these observations, further characterizing the OGT and NGT activities in soybean plants and cell cultures and comparing these enzymes with those seen in the model dicotyledenous plant *Arabidopsis thaliana* and the monocotyledonous crop maize. Having identified optimal sources of the OGT and NGT activities of interest it is then intended to purify and characterize the respective enzymes and relate their roles in pesticide metabolism to their functions in natural product metabolism in different plant species.

METHODS AND MATERIALS

Seeds of soybean (Glycine max L.) cv. Chapman and maize (Zea mays L.) were obtained from Aventis crop science and imbibed in water for 1 h prior to sowing on vermiculite. Seedlings were then either grown in the light or in darkness as described previously (Dixon *et al.*, 1998). Soybean suspension cultures were maintained as described by Lao *et al.*, (2001). Root cultures of *Arabidopsis thaliana* Columbia were initiated from sterile seeds as described by Reiter *et al.* (1992) with cell suspension cultures of Arabidopsis (Landsberg erecta) maintained on MS medium (Murashige and Skoog, 1962). For induction studies, 5 mM stock solutions of formononetin and 3,4-dichloroaniline were prepared in methanol and added to the culture medium at a 1: 100 dilution for 24 h. Prior to assay, whole plants were weighed and frozen in liquid nitrogen prior to storage at –80°C. Cultured plant material was collected by filtration, rinsed in distilled water prior to freezing.

Tissue was homogenised with a pestle and mortar and extracted with 3 v/w ice cold 0.2 M Tris-HCl, pH 8.0 containing 2 mM dithiothreitol (DTT) and 5% w/v polyvinylpolypyrrolidone. After centrifugation (8,500 g, 15 min), protein in the supernatant was precipitated by adding ammonium sulphate to 70% saturation. Prior to assay, protein was suspended in 0.2 M Tris-HCl pH 8.0 containing 2mM DTT and desalted on a Sephadex G-25 column (PD-10, Pharmacia). The protein content was then determined using a Coomassie dye binding reagent (BioRad) using gamma-globulin as the reference protein. The desalted protein preparations (40-100 µg protein) were incubated in a total volume of 75 µl with 66 µM phenolic substrate and 50000 dpm UDP-[^{14}C-glucose] (specific activity 205 mCi mmol^{-1}) as described by Parry and Edwards (1994). Reactions were incubated at 30°C for 20 min. When assaying OGT activity toward phenolic substrates, the reaction was stopped with 125 µl of 0.3 M HCl prior to partitioning against 200 µl of water saturated ethyl acetate. When assaying with aniline substrates, after the 20 min incubation, 125 µl of buffer was added and the reaction contents immediately partitioned against 200 µl of water saturated ethyl acetate. In both cases the radioactive conjugates in the organic phase were quantified by liquid scintillation counting (Parry & Edwards 1994). Enzyme activities were expressed as pmol product formed min^{-1} mg^{-1} protein after correction for the radioactivity present in the organic phase in the absence of added substrate which resulted from the conjugation of contaminating endogenous metabolites present in the crude protein preparation.

RESULTS AND DISCUSSION

As a prelude to comparing OGT and NGT activities in the different plant species, glucosylating activities toward a range of xenobiotics and natural products were characterized and optimized using soybean cell suspension cultures as a source of enzyme activity. The enzyme assays were based on quantifying the amount of [^{14}C-glucose] incorporated into the conjugated reaction product after resolving the radioactive conjugates from the UDP-[^{14}C-glucose] at the completion of the incubation by partitioning with ethyl acetate and radioassaying the organic phase by liquid scintillation counting (Parry and Edwards 1994). An initial screen for conjugating activity revealed that of the xenobiotics, 2,4,5-trichlophenol and 3,4-dichloroaniline were good substrates for OGT and NGT assays respectively and these were then used to optimize incubation conditions. With respect to optimal pH, both activities were unaffected by varying the pH between pH 7 and pH 8.5. Finally pH 8.0 was used in OGT assays and pH 7.5 in NGT assays. Both activities were increased by reducing agents, and 2 mM dithiothreitol was included in all buffers used to extract and assay the GTs. The formation of radioactive products was found to

be strictly dependent on protein content in the range 20-100 μg protein per assay and in this range product formation was directly proportional to incubation time for up to 60 min. For comparative assays protein contents were standardized and maintained within the range 40 - 100 μg protein per assay and incubation time limited to 20 min. The enzymes were also found to be sensitive to temperature and to the presence of salt. Using ammonium sulphate precipitated protein preparations from soybean cell cultures, under standard assay conditions NGT activity toward 3,4-dichloroaniline was determined to be 0.13 pmol product formed min^{-1} mg^{-1} protein and OGT activity toward 2,4,5-trichlorophenol 1.87 pmol min^{-1} mg^{-1} . After a 30 min exposure to 40°C, NGT and OGT activities were reduced by 30% and 20% respectively, with both activities being abolished by a 30 min incubation at 50°C. The two activities were also sensitive to the presence of salt, with 0.5 M NaCl reducing NGT activity by 75 % and totally inhibiting OGT activity. Following these studies, careful attention was paid in the preparation and assay of the GTs with respect to incubation temperatures and thorough desalting of all preparations prior to assay.

After optimizing the enzyme assays, OGT and NGT activities were compared in soybean seedlings and cell cultures (Table 1). NGT activities were determined with 3,4-dichloroaniline (3,4-DCA) and OGT activities with the xenobiotics 4-nitrophenol and 2,4,5-trichlorophenol (2,4,5-TCP) and the natural products quercetin and coumestrol. When the foliage of light-grown soybean plants was assayed, no OGT or NGT activities could be assayed in extracts (data not shown). As it is well documented that light grown soybean seedlings actively *N*- and *O*-glycosylate a range of xenobiotics *in planta* (Schmidt *et al.*, 1995), this result suggests that the respective NGTs and OGTs were inactivated in the course of their extraction from green tissue. This conclusion was partly reinforced by the observation that dark grown soybean seedlings contained extractable OGT activity but not NGT activity (Table 1). NGT and OGT activities could be readily determined in the roots and suspension cultured cells of soybean.

In order to compare these activities with those determined in a monocot crop, light grown and etiolated maize seedlings were also assayed for OGT and NGT activities (Table 1). Light grown maize shoots apparently contained a limited range of OGT activities only. In contrast, etiolated maize shoots contained high OGT activities toward 4-nitrophenol, 2,4,5-trichlorophenol and 3,4-dichloroaniline. As compared with dark-grown soybean seedlings, etiolated maize contained higher activity toward 4-nitrophenol and 3,4-dichloroaniline and lower activity toward 3,4,5-trichlorophenol.

Table 1 : OGT and NGT activities in seedlings and cultures of soybean, maize and *Arabidopsis*. Results refer to the mean ± variation in replicates of duplicates extractions. ND= None detected, NT=Not tested.

	GT enzyme activity pmol product $min^{-1}mg^{-1}$protein				
Tissues sources	p-nitrophenol	2,4,5-TCP	Coumestrol	Quercetin	3,4-DCA
Soybean cell culture	ND	1.87±0.87	ND	1.08±0.66	0.13±0.07
Soybean root	0.15±0.08	0.26±0.01	0.04	1.18±0.09	0.32
Soybean etiolated	0.08±0.01	5.51±0.11	3.28±0.91	0.50±0.15	ND
Maize shoot	ND	0.07±0.02	ND	0.11±0.01	ND
Maize etiolated root	0.30±0.15	0.37±0.04	0.43±0.07	ND	0.20±0.09
Maize etiolated shoot	1.58±0.01	2.34±0.47	0.42±0.07	ND	3.08±0.05
Arabidospis suspension culture	NT	8.09±1.43	NT	3.79±0.23	11.30±1.60
Arabidopsis root culture	NT	3.71±1.39	NT	0.69±0.36	6.71±0.11

As a major objective of the project was to characterize NGTs active in 3,4-dichloroaniline conjugation, it was of interest to screen other plant sources for this enzyme activity. As *Arabidopsis thaliana* is known to contain a diverse range of GT-like sequences in its genome (Li *et al.*, 2001), root cultures and suspension cultures of Arabidopsis were screened for NGT activity. The root cultures contained 6.2 pmol product formed min^{-1} mg^{-1} protein, while the suspension cultures contained 10.9 pmol min^{-1} mg^{-1}, making Arabidopsis cultures the optimal source of NGT activity of all the sources tested. Subsequent analysis showed that the Arabidopsis cultures also had high OGT activities toward 2,4,5-trichlorophenol and quercetin (Table 2). It was therefore opportune to further study these GT activities in cultures of *Arabidopsis* rather than in soyabean.

There are a number of reports of OGT activities toward natural products being enhanced following exposure of plants to their natural substrates (reviewed by Vogt and Jones 2000). It was therefore of interest to use the Arabidopsis culture system to address the question of the enhancement of OGT and NGT activities following exposure to a natural product, the isoflavone formononetin and the xenobiotic 3,4-dichloroaniline. Root cultures and suspension cultured cells were treated for 24 h in duplicate with either 50 µM formononetin, 50 µM 3.4-dichloroaniline or a 1% v/v solution of methanol and then extracted an analysed for OGT activity towards quercetin and 2,4,5-trichlorophenol and NGT activity toward 3,4-dichloroaniline (Table

2). In the root cultures, relative to the untreated controls, none of the treatments altered OGT activity toward quercetin. Activity toward 2,4,5-trichlorophenol were increased in the root cultures by all three treatments, with formononetin giving a significantly greater response than 3,4-dichloroaniline or methanol alone. Formononetin treatment also resulted in a modest increase in activity toward 3,4-dichloroaniline, with the other treatments being ineffective. In the suspension cultures treatment with either formononetin or 3,4-dichloroaniline resulted in no effect on enzyme activity toward quercetin and a decrease in activity toward 3,4-dichloroaniline and 2,4,5-trichlorophenol (Table 2).

Table 2 : Enhancement of NGT and OGT activities towards quercetin, 2,4,5-trichlorophenol (2,4,5-TCP) and 3,4-dichloroaniline (3,4-DCA) in *Arabidopsis* root cultures and suspension cultures following treatment with 50μM formononetin, 50μM 3,4-DCA, or 1% v/v methanol. Results refer to mean of duplicate treatments ± variation in the replicates.

		Enzyme activity pmol product $min^{-1}mg^{-1}$protein		
		Quercetin	2,4,5-TCP	3,4-DCA
Root culture	Control	0.7±0.4	3.7±1.4	6.7±0.1
	Methanol	0.9±0.1	5.3±0.3	7.3±0.5
	Formononetin	1.0±0.2	7.1±0.4	8.1±0.3
	3,4-DCA	1.0±0.4	5.2±0.8	7.5±0.4
Suspension culture	Control	3.8±0.2	8.1±1.4	11.3±1.6
	Methanol	3.9±0.2	9.1±1.7	11.3±1.5
	Formononetin	3.3±0.1	4.0±0.2	4.2±0.4
	3,4-DCA	3.7±0.2	4.4±0.3	4.3±0.5

Our results demonstrate that while previous studies on GTs involved in xenobiotic detoxification have largely concentrated on soybean, notably on soybean suspension cultures, that root cultures and suspension cultures of Arabidopsis contain significantly higher GT activities than either maize or soybean. Although under-utilized to date, *Arabidopsis sp.* may well prove to be a useful biochemical model model organism for studying xenobiotic detoxification.

ACKNOWLEDGMENTS

Caroline Loutre is supported by a studentship funded by Aventis Crop Science UK Ltd.

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Herbicide safeners induce glucosyltransferase activity in wheat

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ABSTRACT

O-Glucosylation, catalysed by *O*-glucosyltransferases (OGTs), is a major route of phase II metabolism of pesticides in wheat, but little is known about the enzymes involved or of the factors which regulate their activities. OGT activities toward flavonoids and xenobiotics were identified in crude extracts from wheat shoots. When wheat shoots were incubated with UDP-[^{14}C-glucose], together with either quercetin, 4-nitrophenol or 2,4,5,-trichlorophenol, formation of single major radioactive glucose conjugates was determined in each case. Product formation was found to be strictly dependent on protein content and incubation time. To determine whether or not OGT activities in wheat were enhanced by chemical treatments known to induce other herbicide detoxifying enzymes, seedlings were treated with the wheat herbicide safener cloquintocet mexyl and the maize safener dichlormid. Both safeners enhanced OGT activities toward a range of xenobiotics and flavonoids, with cloquintocet mexyl being the most effective inducing treatment. As determined by HPLC, cloquintocet mexyl also gave rise to subtle modifications in the content of glycosylated UV absorbing natural products in wheat shoots as compared with untreated controls. Our results demonstrate that herbicide safeners perturb the conjugation of both xenobiotics and natural products in wheat.

INTRODUCTION

Plants are able to metabolise a broad range of xenobiotics such as herbicides by a combination of phase I reactions (oxidations, hydrolyses) followed by phase II conjugation reactions (glutathionylation, glycosylation). Significantly, the more rapid detoxification of herbicides in crops as compared with competing weeds is a primary determinant of selectivity (Owen 2000). In wheat, a major mechanism for the detoxification of herbicides involves oxidation, mediated by cytochrome P450 mixed function oxidases (CYPs), followed by glucosylation of the oxidised herbicide by *O*-glucosyltransferases (OGTs) (Cole & Edwards 2000). A good example is seen in the metabolism of the sulphonyl urea herbicide metasulfuron-methyl, which is first hydroxylated and then glucosylated, the latter step rendering the herbicide inactive (Anderson & Swain 1992). Despite their obvious importance in pesticide metabolism, relatively little is known about the OGTs which conjugate xenobiotics in wheat (reviewed by Cole & Edwards 2000). With an interest in extending our understanding of the OGTs in wheat involved in herbicide metabolism, we have identified OGT activities toward phenolic compounds of both natural and synthetic origin in wheat

seedlings. We have also been interested in how these OGT activities are affected by herbicide safeners, compounds which enhance herbicide tolerance in cereals largely due to their ability to enhance the expression of enzymes involved in herbicide detoxification (Davies & Caseley 1999). Although safeners are well known to induce glutathione transferases (GSTs) and CYPs in wheat, the effect of safeners on OGT activities has not previously been reported.

MATERIAL AND METHODS

Plant studies

Wheat seedlings (cv. Hunter) were grown for 10 days under a regime of constant treatment with either dichlormid or cloquintocet mexyl, both at a concentration of 10mg litre^{-1} or with 0.1% v/v aqueous acetone. Growth conditions and harvesting was as described previously (Cummins *et al.*, 1997).

Analysis of OGT activities

Tissue was homogenised with a pestle and mortar and extracted with 0.2 M Tris-HCl, pH 8.0 containing 2 mM dithiothreitol (DTT) and 5% w/v polyvinylpolypyrrolidone. After centrifugation (8500 *g*, 15 min), protein in the supernatant was precipitated by adding ammonium sulphate to 70% saturation. Prior to assay, protein was suspended in with 0.2 M Tris-HCl pH 8.0 containing 2mM DTT and desalted on a Sephadex G-25 column (PD-10, Pharmacia). The protein content was then determined using a Coomassie dye binding reagent (BioRad) using gamma-globulin as the reference protein. The desalted protein preparations (40-100 µg protein) were incubated in a total volume of 75 µl with 66 µM phenolic substrate and 50000 dpm UDP-[^{14}C-glucose] (specific activity 205 mCi mmol^{-1}) as described by Parry & Edwards (1994). Reactions were incubated at 30^{0}C for 20 min, then stopped with 125 µl of 0.3 M HCl prior to partitioning against 200 µl water saturated ethyl acetate. The radioactive conjugates in the organic phase were quantified by liquid scintillation counting as well as being analysed by radio-HPLC (Parry & Edwards 1994).

Flavonoid analysis

Wheat shoots were sequentially extracted with ice-cold acetone (10 v/w) followed by 10 v/w acetone:methanol (1:1) using a pestle and mortar with acid-washed sand as an abrasive. After filtration the combined extract was concentrated to near dryness under reduced pressure. Samples were then resuspended in 0.15 M citrate phosphate pH 5.0, in the presence or absence of 1mg ml^{-1} cellulase. After incubation at 30^{0}C for one hour, samples were partitioned with ethyl acetate, dried down and resuspended in methanol. Natural product profiles were determined by reversed phase HPLC as described previously (Parry & Edwards 1994), with the eluant monitored for UV-absorbing metabolites at 287 nm.

RESULTS AND DISCUSSION

OGT activities in wheat

Preliminary experiments indicated that there was significant OGT activity in wheat shoots extracts towards, 4-nitrophenol and the flavonoid quercetin. These two substrates were used to optimise OGT assays with respect to incubation conditions. Enzyme activity was found to be markedly enhanced in the presence of thiol reducing agents, so 2 mM DTT was included in all assay and extraction buffers. Optimal pH conditions were found in the range pH 7.5 to pH 9, with pH 8.0 used in all standard assays. The formation of radioactive conjugates was found to be strictly dependent on protein content in the range from 40μg to 200μg of protein per assay and, at 30^{0}C, on incubation time up to 30 minutes. In all subsequent assays with wheat shoot preparations, reactions contained 40-100μg of protein and were incubated at 30^{0}C for 20 minutes.

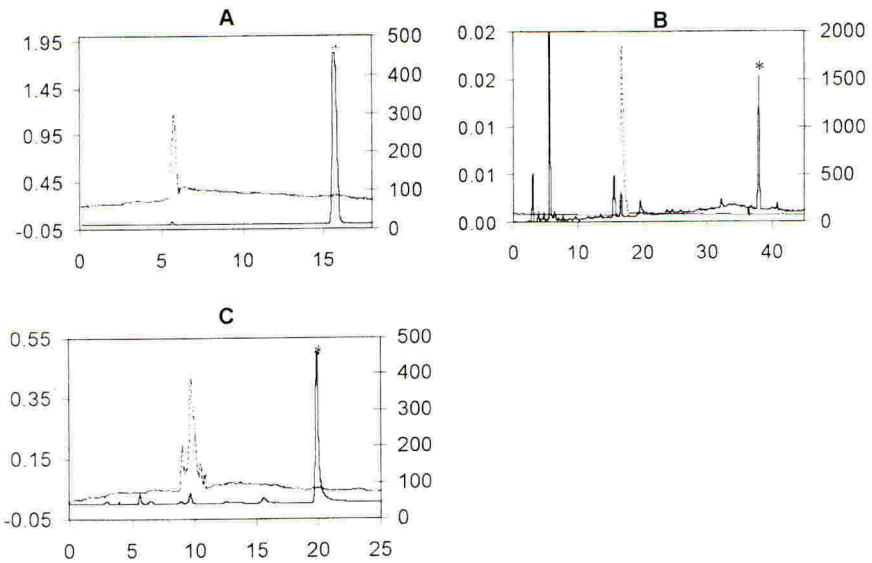

Figure 1. Analysis by HPLC of reaction products of 4-nitrophenol (A), 2,4,5-trichlorophenol (B) and quercetin (C) (aglycones labelled *), in crude wheat shoot extract. The left hand y-axis refers to UV absorbance at 287nm ( ) and the right hand y-axis corresponds to radioactivity (dpm). The x-axis refers to retention time (Rt, min)

A range of xenobiotic phenols and flavonoids were then assayed as potential substrates of wheat OGTs. These included the pesticides chloramben, picloram, 2,4-dichlorophenoxyacetic acid and pentachlorophenol as well as the pesticide-related metabolites 4-nitrophenol, 2,4,5-trichlorophenol, and 4-hydroxyphenylpyruvic acid. Amongst these, significant OGT activity was found towards 4-nitrophenol (1.37 pmol min^{-1} mg^{-1} protein) and 2,4,5-trichlorophenol (2.55 pmol min^{-1} mg^{-1}) only. Natural product substrates tested included benzoic acids, coumarins, phenylpropanoids and flavonoids. Amongst these, OGT activity appeared to be limited to the flavonoids quercetin, and luteolin, and the isoflavonoids genistein

and coumesterol. To confirm the nature of the reaction products formed, the [^{14}C-glucosyl]-conjugates formed from the aglycones 4-nitrophenol, 2,4,5-trichlorophenol and quercetin were analysed by radio-HPLC (Figure 1). A single radioactive peak was identified for both 4-nitrophenol and 2,4,5-trichlorophenol corresponding to UV-absorbing peaks eluting at 5.5 min and 16.7 min respectively. With quercetin one major radioactive peak was also observed at 9.5 min, though, with this substrate, additional minor radiolabelled products were also observed. These studies validated the use of the OGT assays for subsequent studies examining the regulation of these activities by herbicide safeners.

OGT activity in safener treated wheat shoots and roots

Wheat seedlings were treated with either cloquintocet mexyl, a wheat safener, used to enhance tolerance to clodinafop propargyl (Kreuz *et al.*, 1991) or the maize safener dichlormid which enhances crop tolerance to chloroacetanilide herbicides (Dixon *et al.*, 1998). The effect of safener application on OGT activity in wheat was measured using the six model substrates (Table 1). In shoots, treatment with cloquintocet mexyl increased OGT activity toward all substrates, notably toward luteolin and comesterol. Treatment with dichlormid gave only a minor enhancement in OGT activity in shoots and a reduction in activity towards quercetin (Table 1). In roots, a modest increase in OGT activity towards 4-nitrophenol and 2,4,5-trichlorophenol was seen with both safener treatments, with dichlormid slightly less effective than cloquintocet mexyl (data not shown).

Table 1. OGT activities in 10 day wheat shoots following treatment with the safeners dichlormid and cloquintocet mexyl.

	OGT activity pmol product min^{-1} mg^{-1} protein		
Substrate	Control mean ± SE	Dichlormid mean ± SE	Cloquintocet mexyl mean ± SE
4-Nitrophenol	0.67 ± 0.00	0.93 ± 0.13	1.65 ± 0.03
2.4.5-Trichlorophenol	1.65 ± 0.01	2.77 ± 0.16	3.46 ± 0.13
Quercetin	3.79 ± 0.17	2.14 ± 0.14	7.11 ± 0.42
Luteolin	0.37 ± 0.02	0.41 ± 0.01	1.32 ± 0.08
Genstein	0.54 ± 0.01	0.69 ± 0.00	1.38 ± 0.07
Coumesterol	1.01 ± 0.06	1.64 ± 0.19	3.74 ± 0.31

The results of these assays clearly demonstrate that OGT activities in wheat can be enhanced following treatment with safeners. The safeners appear to have differing affects with cloquintocet mexyl, the wheat safener, producing increases in activity in both shoots and roots whereas dichlormid had a more limited effect, which was restricted to the roots. OGT activity towards the flavonoid compounds luteolin, genistein and coumesterol was enhanced to a greater extent than with the other substrates, suggesting that, as is the case with with GSTs (Cummins *et al.*, 1997), safeners are selective with respect to the sub-set of OGT isoenzymes that they induce.

Profile of glycosylated natural products in safener treated wheat shoots

The concentrations of glycosylated flavonoids and other UV-absorbing natural products in wheat are known to change in response to subtle variations in their environment (Estiarte *et al.*, 1999). It was therefore of interest to determine if the content of glycosylated natural products in wheat was affected by treatment with the herbicide safeners which enhanced OGT activities toward natural products. HPLC analysis of the natural product profile of shoots and roots in untreated and cloquintocet mexyl treated wheat was performed. To simplify the analyses, the plant extracts were treated with cellulase to release the aglycones. The UV absorbing peaks which appeared following hydrolysis are indicated (Figure 2). Root extracts revealed very little variation in control and safener treated plants profiles. In shoots, after digestion with cellulase, three new peaks appeared (peaks 1, 2 and 3). While peak 3 was present at similar levels in untreated and treated shoots, peaks 1 and 2 were significantly increased in the extracts from cloquintocet mexyl treated plants. Compound 4 was unaffected by cellulase digestion suggesting it was not glycosylated. Interestingly, cloquintocet mexyl treatment depressed the levels of this compound at least ten-fold. These results demonstrate that cloquintocet mexyl perturbs the metabolism of glycosylated and other endogenous natural products in wheat.

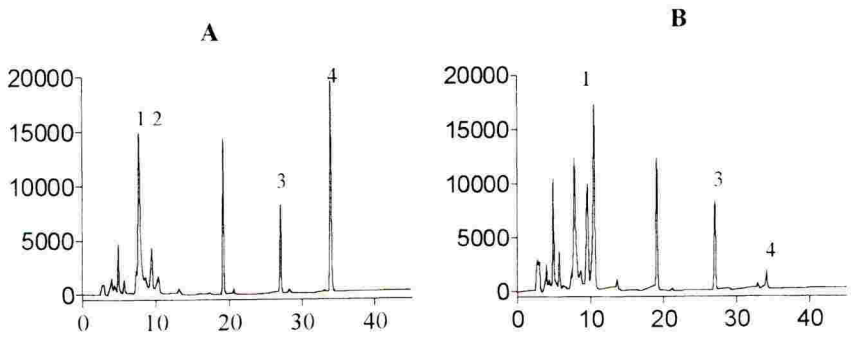

Figure 2. HPLC profile of cellulase treated extracts from A. untreated and B. cloquintocet mexyl treated wheat shoots. The absorbance (y-axis) was measured at 287nm and the x-axis refers to retention time (min).

ACKNOWLEDGEMENTS

M Brazier is supported by a CASE studentship jointly sponsored by the Biotechnology and Biological Sciences Research Council and Aventis Crop Science UK Ltd.

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Is diclofop-methyl resistance in *Lolium rigidum* associated with a lack of penetration?

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ABSTRACT

Diclofop-methyl, an aryloxyphenoxypropionate herbicide, has been used for many years both alone and in mixture with the substituted ureas, chlorotoluron or isoproturon, for the control of many types of grass weeds in the north-east and south-east of Spain. Since the early 1990s, the control of some *Lolium rigidum* populations has become increasingly difficult in winter wheat. Growth chamber experiments indicated the existence of a range of different biotypes exhibiting different levels of tolerance to diclofop-methyl. The most highly resistant, biotype R3, contained an altered isoform of ACCase. In biotype R2 which exhibited a medium level of resistance there was an increased rate of oxidation of the aryl ring of diclofop, a reaction most likely catalysed by a cytochrome P450 enzyme. Lastly, a new biotype with a moderate level of resistance (R1) to diclofop-methyl was found. This biotype did not present any differences in its diclofop-metabolism or any mutation at the target site. However [^{14}C]-diclofop-methyl penetration into R1 was significantly less than observed in the resistant (R2 and R3) and susceptible (S) biotypes. Analysis of the leaf cuticle surface by scanning electron microscopy showed a greater wax density in the leaf cuticles of biotype R1 than in the other biotypes. Thus, it is suggested that biotype R1 is tolerant to diclofop methyl because the increased wax content of its cuticle permits less penetration of herbicide into the plant.

INTRODUCTION

Diclofop-methyl (DM), an aryloxyphenoxypropionate herbicide, has been used for many years both alone and in mixture with the substituted ureas, chlorotoluron or isoproturon, for the control of many types of grass weeds in the north-east and south-east of Spain (De Prado *et al.*, 1997). Since the early 1990s, the control of some *Lolium rigidum* populations has become increasingly difficult in winter wheat. Growth chamber experiments indicated the existence of a range of different biotypes exhibiting different levels of tolerance to to diclofop-methyl (De Prado & Menendez, 1996). Three mechanisms of resistance to DM need to be considered in these populations. The most highly resistant of these biotypes, R3, contained an altered isoform of ACCase having an altered affinity for herbicide while the

medium level of resistance appeared to correspond to an enhanced rate of herbicide metabolism and/or to an enhanced rate of recovery of membrane potential following herbicide treatment (De Prado *et al.*, 1999). The latter mechanism is sometimes found by itself or sometimes alongside one or both of the other two mechanisms. The objectives of thie current study were: (a) to quantify the level of sensitivity of the diclofop-resistant and -susceptible *L. rigidum* biotypes to DM; (b) to evaluate the role of differential penetration into leaves as a determinant of resistance to diclofop; and (c) to compare the waxy cells of R and S biotypes.

MATERIALS AND METHODS

Chemicals

[^{14}C] Diclofop-methyl (specific activity 95.5 kBq μmol^{-1}) was provided by Dr. H. Köcher (Hoechst AG, Germany). Commercial formulation (Iloxan; 36% w/v EC) of this herbicide used for growth assays was supplied by Aventis Ibérica S.A.

Plant material

L. rigidum seeds were collected from wheat fields where recommended rates of diclofop-methyl, either alone or in combination with chlortoluron or isoproturon, had failed to control weeds. Seeds were collected between 1995 and 2000 from three winter wheat fields in Spain. *L. rigidum* resistant biotype (R2) was collected from a winter wheat field which had been treated annually with a mixture of diclofop-methyl plus chlortoluron or diclofop-methyl plus isoproturon for at least 10 years. The other resistant biotypes (R1 and R3) were collected from fields treated annually with diclofop-methyl alone also for at least 10 years. Control seeds of a susceptible biotype were collected from nearby olive tree groves which had never been treated with herbicides (Table 1).

Table 1. Characteristics of *L.rigidum* biotypes.

Biotype	Crop	Herbicide	Mechanism of resistance	Reference
S	Olive trees	Non treated		
R1	Wheat	Diclofop-methyl	?	-
R2	Wheat	Diclofop-methyl + isoproturon or chlortoluron	Enhanced diclofop metabolism and recovery of membrane potential	Menendez *et al.*, 1996 De Prado *et al.*, 1999
R3	Wheat	Diclofop-methyl	Diclofop insensitive ACCase and enhanced recovery of membrane potential	De Prado *et al.*, 1997 De Prado *et al.*, 1999

R and S biotype seeds were germinated in petri dishes with a blotter disk moistened with distilled water. The petri dish cover was sealed with parafilm and seeds were germinated in a growth chamber at 23/18 °C (day/night) in a 16-hr photoperiod at 80% relative humidity. Four or three pre-germinated seeds (for growth and absorption/translocation assays, respectively) were planted per pot (7 cm diameter, 7 cm high plastic pots) in a peat/soil mixture (1/2, v/v). Plants were grown in a growth chamber under the same conditions as for germination.

Comparative herbicide tolerance assays

At the two- to three- leaf stage, the R and S biotypes of *L. rigidum* were sprayed with a commercial formulation of diclofop-methyl at several concentrations (S: 0.1, 0.2, 0.4 and 0.8 kg a.i./ha and R1, R2 and R3: 1, 2, 4, 6 and 12 kg a.i./ha) using a laboratory track sprayer (Tee-Jet 8001 flat-fan nozzle) delivering 200 litres/ha at 200 kPa. Treatments were replicated three times and shoot fresh weight was evaluated after 21 days for each treatment. The concentration of herbicide that caused a 50% decrease in growth with respect to the control (ED_{50}) was determined for each biotype.

[^{14}C] Diclofop-methyl penetration

[^{14}C] Diclofop-methyl was mixed with commercially-formulated diclofop-methyl to prepare emulsions with a specific activity of 25000 dpm/µl and a diclofop-methyl concentration of 6.6 g/litre. This formulation of labelled herbicide was applied to the adaxial of the second leaf of each plant in four 0.5-µl droplets using a microapplicator (Hamilton PB-600). A total of 50000 dpm were applied to each plant.

Plants were harvested in batches of three at several time intervals after herbicide application (0, 3, 6, 12, 24 y 48 h) and separated into treated leaves and the remainder of the shoots. Roots were discarded, as diclofop-methyl herbicide translocation from leaves to roots was reported as being undetectable in wheat (Brezeanu *et al.*, 1976). Unabsorbed [^{14}C]-diclofop-methyl was removed from the leaf surface by washing the treated area with 1.5 ml of acetone. Washes from each batch were pooled and analysed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA). Plant tissue was dried at 40 °C for 72 h and combusted in a sample oxidizer (Packard 307). The $^{14}CO_2$ evolved was trapped and counted in 10 ml of Carbosob/Permafluor E$^+$ (3/7 V/V) (Packard Instruments Co.). The radioactivity was quantified by LSS and expressed as a percentage of the recovered radioactivity, according to the following formula:

% absorption=[^{14}C in combusted tissue/(^{14}C in combusted tissue + ^{14}C in leaf washes)]x100

The experiment was repeated three times.

Scanning electron microscopy (SEM)

Small pieces of fresh *L. rigidum* leaves were cut off with a sharp razor blade and fixed in glutaraldehyde 2% (v/v) in phosphate buffer 0.2 M, pH 7, overnight at 4°C. As described by Casado & Heredia (2001), the samples were thoroughly rinsed in fresh phosphate buffer and dehydrated through an ethanol solution series: 20, 40, 60, 80 and 100% (v/v) and

increasing times, from 15 minutes to 1h 30 min. The pieces were placed on a metallic holder using a double-faced adhesive and coated with a 0.05 µm thin film of gold. A JEOL JSM-840 scanning electron microscope operated at 10-20 kV was used for the examination of the samples.

RESULTS AND DISCUSSION

Comparative herbicide tolerance assays

The growth responses of *L. rigidum* biotypes to DM treatment were markedly different. Whereas the S biotype was killed 15 days after treatment at a field rate of DM (Iloxan 2.5 litre/ha) the R1, R2 and R3 biotypes were 7.20, 13.00 and 36.60, respectively, less sensitive to DM than the S biotype control (Table 2). These different levels of tolerance appear to reflect different underlying mechanisms of resistance. The resistance of the R2 biotype to diclofop-induced catabolic breakdown has previously been shown to be related to a combination of its decreased sensitivity to membrane depolarization (De Prado *et al.*, 1999), and its enhanced ability to form a nonphytotoxic polar metabolite of diclofop via aryl hydroxylation (Menendez *et al.*, 1996). The R3 biotype contains a diclofop-tolerant form of ACCase and, at the same time, exhibits an ability to rapidly recover the electrogenic membrane potential following herbicide-induced depolarisation (De Prado *et al.*, 1997 and 1999). As shown herein, the R1 biotype represents an entirely novel uptake-related mechanism of resistance. The relative herbicide tolerances of the different biotypes have not previously been evaluated. On the basis of this study it would appear that, in general terms, 'target site' alterations in ACCase confer the highest level of resistance to diclofop, enhanced oxidative metabolism the next and uptake, apparently, the least.

Table 2. Effect of DM on growth of different *L. rigidum* biotypes.

Biotype	ED_{50} (kg a.i./ha)	$ED_{50}R/ ED_{50}S$
S	0.25 ± 0.02	-
R1	1.80 ± 0.06	7.20
R2	3.25 ± 0.3	13.00
R3	9.15 ± 0.7	36.60

Note. Data are means of three experiments ± SE

[^{14}C] Diclofop-methyl penetration

There were no significant differences in the penetration of [^{14}C]diclofop-methyl into the R2, R3 and S *L. rigidum* biotypes; however, the R1 biotype showed a lower penetration rate than the other biotypes (Figure 2). After 24 h of application, about 75% of the recovered radioactivity had penetrated into the leaf tissue of the R2, R3 and S *L. rigidum* biotypes, while only 50 % had penetrated into the R1 biotype. Further studies (data not shown) indicated that the R1 biotype was no different from the susceptible in terms of the rate of metabolism of [^{14}C]diclofop-methyl or diclofop tolerance of the ACCase activity. These results are consistent with a decreased rate of herbicide penetration being the major determinant of resistance in biotype R1.

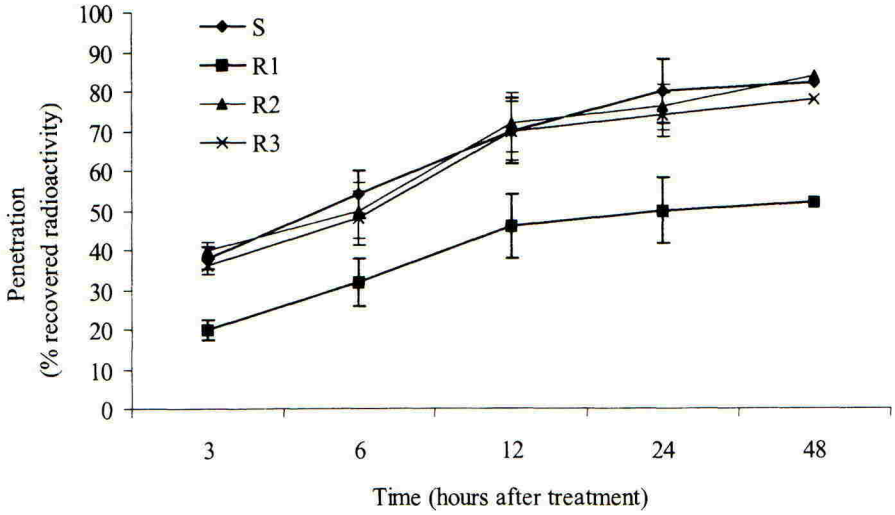

Figure 2. Penetration of DM into different biotypes of *L. rigidum*.

Morphology of leaf cuticle surface

The outer surface of the cuticle of leaves of the different biotypes of *L. rigidum* were studied by SEM. Significant morphological differences were found in the adaxial side of the leaves. Figure 3 shows scanning electron micrographs of the outer surface of the S and R1 biotypes. The cuticle appears as a thin, continuous and smooth layer with a noticeable amount of wax in the form of isolated platelets thickly distributed over the outer surface of the cuticle. This epicuticular wax morphology and ultrastructure has been observed in a wide variety of plant species (Barthlott *et al.*, 1998). Figure 3 also shows a significant difference between the two biotypes: the amount of wax platelets per unit of cuticle area is significantly higher being perhaps 2-4 fold denser in the case of the R1 biotype. Potentially, this would provide the cuticle of the R1 biotype with a more effective hydrophobic molecular barrier to chemical diffusion. Comparative thin layer chromatography of the isolated cuticular waxes of the two biotypes indicated no major compositional differences and that wax esters were the main components of the wax of both biotypes (data not shown). Plant wax esters, together with wax alkanes, constitutethe most hydrophobic components of plant waxes (Barthlott *et al.*, 1998). These results permit the hypothesis that this waxy barrier was responsible for the marked herbicide tolerance and reduced herbicide penetration exhibited by biotype R1 relative to the susceptible wild-type of *L. rigidum*.

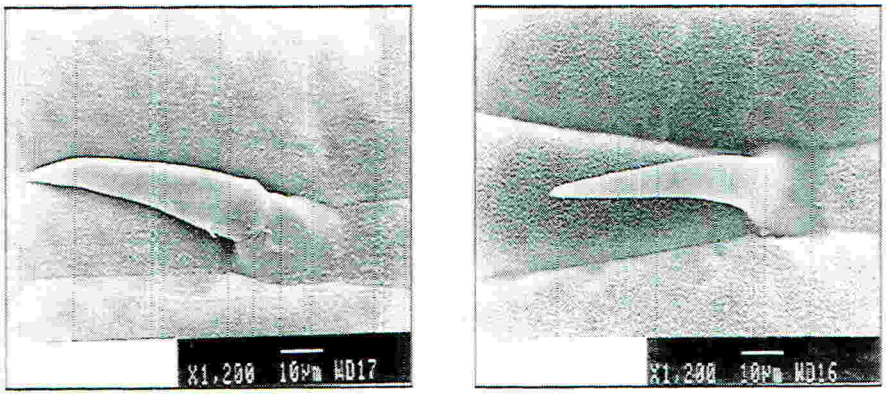

Figure 3. Scanning electron micrographs of the outer surface of the S (left) and R1 (right) biotypes of *L. rigidum*.

ACKNOWLEDGEMENTS

Financial support for this research was provided by CICYT (Project IFD 97-1675).

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Metabolite profiling by NMR for high-throughput mode of action identification of screen hits

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ABSTRACT

A key challenge in modern agrochemical discovery is characterising the large number of hits identified using high throughput screening (HTS) methods. Early identification of the modes of action (MOA) of screen hits can highlight issues which may cause a lead to fail during development. Traditional methods of MOA determination rely upon multiple separate enzyme assays, making it too costly to determine the MOA of a large number of leads. This paper describes an alternative approach, which is to use ^{1}H NMR to monitor changes in concentration of a wide range of endogenous plant cell metabolites. Pattern recognition methods are then used to interpret the large number of changes in the metabolites that are observed following herbicide treatment. Different types of ALS inhibitor induce similar metabolite changes to one another but which are different from changes caused by other herbicide MOA. This demonstrates how metabolite profiling can identify the MOA of screen leads as the same as, or different from, known standards. Multivariate regression analysis of ^{1}H NMR data also allows the identification of endogenous metabolites which accumulate during herbicide treatment and from this, novel MOA can be identified. This approach is successful for ALS and EPSPS inhibitors.

INTRODUCTION

Whole-plant screening has historically been the most successful method of herbicide discovery; almost all of the main commercially successful herbicides were discovered in whole-plant screens (Harrison 1999). This success has prompted the development of whole-plant HTS methods which can screen hundreds or thousands of compounds per day. This approach has greatly increased the rate of discovery of compounds with herbicidal activity. Unfortunately, whole-plant screening gives little information about the MOA of herbicidal compounds. MOA has implications for toxicity, spectrum and market size so it is crucial information for the decision on whether or not to develop a herbicidal lead.

The rate of herbicide discovery by HTS poses a serious challenge for MOA identification. The traditional methods of MOA identification are specialised enzyme assays which are costly, not always suitable for high-throughput and can give ambiguous results. An alternative approach is to examine the changes in endogenous metabolites within a plant when it is treated with a herbicide. Many herbicides inhibit or disrupt a specific aspect of metabolism so different classes of herbicide treatment cause different changes in metabolite levels (Sauter *et al.*, 1991). These changes occur in a wide range of different metabolites due to both the direct toxic effect of the herbicide, and plants' attempts to counter the toxic effect. Because of this combination, identifying changes in individual metabolites which are specific to individual MOA would be time-consuming and would discard useful information about

plant responses to herbicides. Instead, pattern recognition methods can be used to compare complete sets of metabolite profiles with limited user intervention. This approach has been successfully applied in mammalian toxicology (Anthony *et al.*, 1994).

MATERIALS AND METHODS

Plant cultivation and treatment

Maize plants (*Zea mays* var. Samsara and Pioneer 3394) were cultivated individually in potting mix and treated with herbicides at the 1.25 to 1.5 true leafs growth stage. Application rates were chosen to give approximately 80% kill within two weeks of treatment, except for 2,4-D which was applied at 125 g/ha. Herbicides were formulated in 50% acetone, 1% Tween-20, 49% water. Plants were harvested 2-4 days after treatment.

Extraction of metabolites and NMR spectroscopy

Plants were freeze-dried and homogenized in ethyl acetate (10ml) on ice. The extract was centrifuged and the pellet was resuspended in water (10ml). Particulate matter was removed by centrifugation and filtration through centrifuge filters. Potassium hydroxide was used to adjust the pH to 7 and samples were freeze-dried. After freeze-drying, the samples were resuspended in water (1ml, 10% D_2O, 0.001% d_4-trimethylsilylpropionic acid, TSP) and centrifuged and 650µl was transferred to a 5mm NMR tube.

NMR spectra were recorded at 23°C on a Varian INOVA 500MHz NMR spectrometer. For 1D proton experiments, 256 transients were acquired giving a total duration of 25 minutes per sample. Spectra were baseline-corrected and divided into 256 equal regions. The regions were integrated and those containing the solvent and reference compound (TSP) were discarded. This reduced the size of the NMR spectrum from 32768 to approximately 200 data-points per spectrum.

Metabolites were identified using standard 1D and 2D ^1H and ^1H,^{13}C NMR experiments together with published assignments (Fan, 1996).

Multivariate data analysis

The data-reduced spectra were analysed using Pirouette (Infometrix, Woodville, WA. USA) and SIMCA-P (Umetrics AB, Umeå, Sweden). The following statistical methods were used: Principal Components Analysis (PCA), Soft Independent Modelling of Cluster Analogy (SIMCA) and Partial Least Squares - Discriminant Analysis (PLS-DA) (Manly, 1996; Eriksson *et al.* 1999).

DISCUSSION

Figure 1 shows a typical 1D proton NMR spectrum of a maize plant extract. Even though it has only been acquired for a relatively short time, hundreds of peaks can be observed, so the spectrum contains information on the concentration of tens or hundreds of different metabolites present within the plant.

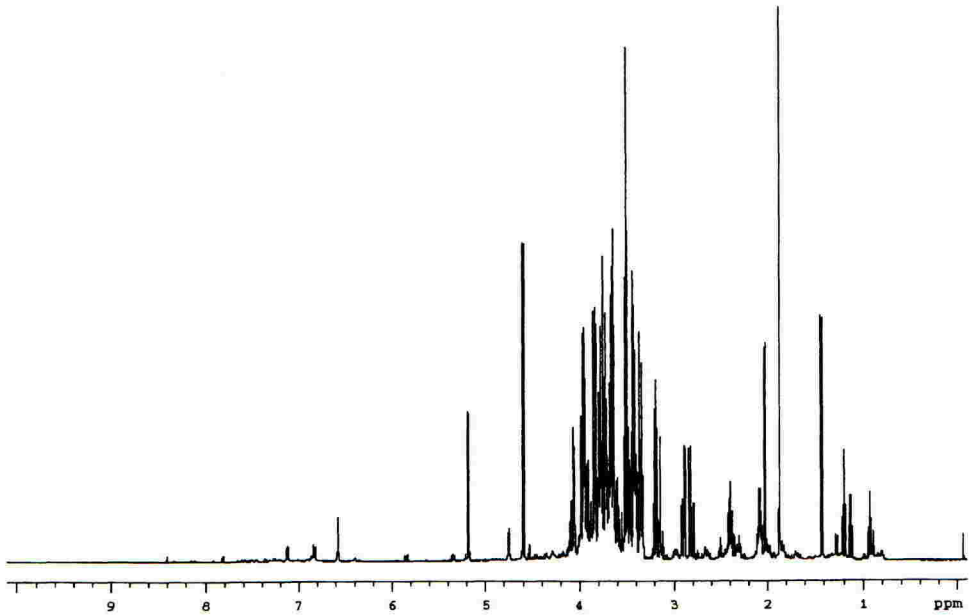

Figure 1 Proton NMR spectrum of the water extract of a maize plant

This wealth of information suggests that NMR can give useful information regarding the metabolic state of the plant, but also poses a problem. For MOA profiling it will be necessary to compare tens of MOA for hundreds of screen hits. Comparing such a large number of NMR spectra would be impractical. An alternative approach capable of doing this is data-reduction followed by multivariate data analysis. In the data-reduction step, the number of variables needed to represent the spectrum is reduced from several thousand to approximately 200 by reducing the spectral resolution. Multivariate data analysis methods can then be used to compare and contrast large numbers of the data-reduced spectra.

To demonstrate this approach, herbicides with 5 different MOA were used to treat 24 different plants. The metabolites were extracted and NMR spectra were acquired as described above. The data-reduced spectra were then analysed using PCA. PCA extracts information from a data-set by identifying variables which are correlated. In this way, it is possible to replace many different correlated variables with a much smaller number of variables referred to as principal components (PCs). With the data from these 24 plants, very little information is lost by using PCA because about 83% of the original variation in the data-set is captured in 3 principal components.

Figure 2 is a graph of the first 3 principal components from the set of 24 plants; each point represents the data reduced NMR spectrum of one plant. Untreated plants cluster at the top right of the graph. The treated plants cluster separately from the control plants, but are also separated according to their MOA. This shows that herbicide treatment causes changes in metabolites compared with the control plants, and that different herbicide MOA cause different changes. One of the MOA (2,4D) clusters close to the controls. This is expected because 2,4D is a herbicide for broad-leaved weed control which does not control maize at the application rate used.

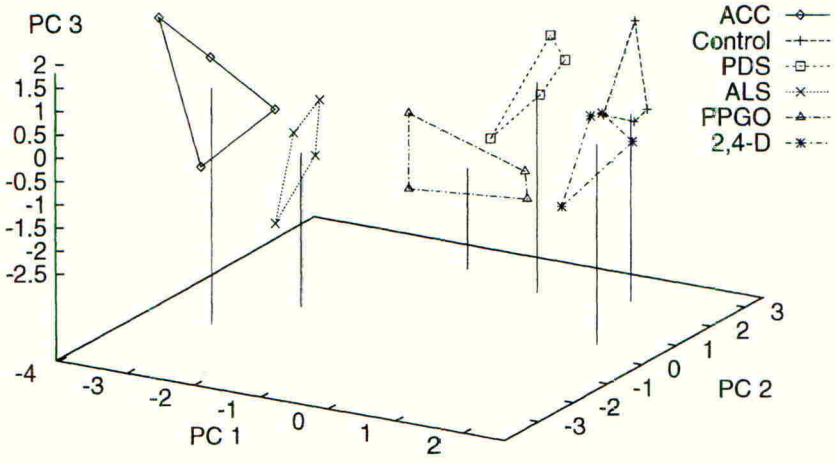

Figure 2 Plot of the first 3 PCs of metabolite profiles of herbicide-treated plants. To help visualisation, plants treated with the same herbicide are linked and there is a line from the centre of each treatment to the baseplane.
CON: control plants. 2,4-D: 2,4-D-2-ethylhexyl ester. PDS: Norflurazon, PPGO: Oxyfluorfen. ALS: Sulfometuron-methyl. ACC: Fluazifop-P-butyl.

Classification of known mode of actions

A second experiment was run to show that metabolite profiling can automatically classify MOA. 24 plants were treated with glyphosate, with formulation blank or with one of a range of ALS herbicides. Metabolite profiles of the plants treated with sulfometuron-methyl were used to generate a SIMCA model (SIMCA is an extension of PCA used for classification). Metabolite profiles of plants treated with other herbicides were then tested against this model to calculate the probability they were different from the sulfumeturon profile (see Table 1).

Table 1. SIMCA classification of metabolite profiles from treated plants. The SIMCA model contained 1 component and probability was calculated using the supplemented sample residual, as implemented in Pirouette

Treatment (n=4)	*p*	*Total (p < 0.8)*	*MOA*
Control	0.93,0.91,0.91,0.91	0	–
Glyphosate	0.90,0.90,0.91,0.95	0	EPSPS
Chlorsulfuron	0.61,0.55,0.65,0.63	4	ALS
Metsulfuron methyl	0.77,0.44,0.74,0.83	3	ALS
Imazaquin	0.46,0.81,0.49,0.78	3	ALS

SIMCA successfully classifies the metabolite profiles of other ALS inhibitors as being similar to that of sulfometuron-methyl and classifies those of glyphosate and no treatment as being different. This example only uses two MOA but the method can be scaled up to include other types of MOA. Further advantages of metabolite profiling are that it is relatively unaffected by dose, and is reproducible over time (Hole *et al.*, 2000). These features make metabolite profiling a practical approach for screening large number of HTS leads.

Identification of unknown modes of action

The method described in the previous section will identify herbicides that are similar to known MOA and so, by elimination, will highlight MOA which may be novel. Metabolite profiling has the potential to identify novel MOA as well. The observation that different herbicides have different metabolite profiles suggests that metabolite changes are directly related to the process which is affected by the herbicide. If this is the case, then from the metabolite changes it will be possible to identify the process which the herbicide is inhibiting – in other words, to identify the MOA.

This approach was tested using two herbicides with known MOA, sulfometuron-methyl and glyphosate. Data-reduced spectra from plants treated with these herbicides and control plants were compared using PLS-DA. This identifies changes which are correlated with one another but which differ between the treatments and control. The results of PLS-DA are regression vectors which contain a loading for every variable in the data-reduced spectra; high loadings suggest variables which change upon treatment. Both sulfometuron-methyl and glyphosate treatments were included in the same PLS-DA calculation so that general changes caused by plant death could be distinguished from specific changes caused by individual herbicides. Because the original NMR spectra were data-reduced, the PLS-DA loadings identify regions of the 1D NMR spectra which are affected by treatment rather than specific metabolites. However, the NMR signals which are causing these changes can be identified by comparing the parts of the 1D spectra which have high PLS-DA loadings and the NMR signals can then be assigned to specific metabolites using information from other NMR spectra. Previously known metabolites are usually identified relatively quickly from published assignments, but NMR also has the potential to identify metabolites which have not been previously described. This is an important advantage of NMR over mass spectroscopy for metabolite profiling.

Figure 3 shows parts of 1D spectra of extracts from a control and a sulfometuron-methyl treated maize plant, together with the PLS-DA loadings for the same region. A high positive loading is seen for a number of variables. Comparison with the NMR spectra shows that most of these are from bulk metabolites, but the NMR signal at 0.98ppm is only observed in sulfometuron treated plants. Further work identified this peak as the methyl signal of 2-aminobutyrate. This is the transamination product of 2-oxo-butyrate, a substrate of acetolactate synthase, so the accumulation can be directly related to sulfumeturon inhibiting acetolactate synthase. Therefore, it is possible to identify the MOA of sulfometuron-methyl by metabolite profiling.

Metabolite profiling of glyphosate treated plants highlighted an accumulation of shikimate,which can also be directly linked to the known MOA. These two successes suggest that in some cases it will be possible to identify novel MOA using metabolite profiling. However, in both of these test cases other herbicide specific changes were observed which could not be related to the known MOA (for example, in fumaric acid, alanine and glucose). These unrelated changes complicate the use of metabolite profiling for MOA identification.

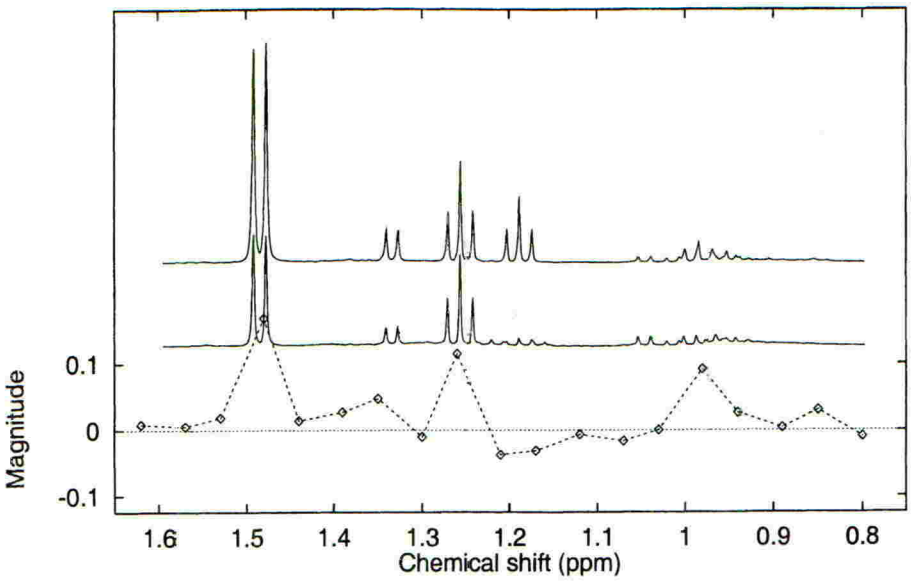

Figure 3: Parts of proton NMR spectra of sulfometuron-methyl treated (top) and control (middle) plants, together with the PLS-DA loadings for data-reduced spectra of sulfometuron treated plants (bottom). The triplet at 0.98ppm is only observed in sulfometuron treated plants.

This work shows that NMR is an effective method for metabolite profiling, and that metabolite profiling has the potential to identify the MOA of herbicides whether the MOA is known or new. We are now investigating other methods of quantifying metabolites to see whether they can further improve the throughput of the metabolite profiling technique.

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Mechanism of action of sulcotrione in mature plant tissues

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ABSTRACT

Sulcotrione acts by inhibiting 4-hydroxyphenyl pyruvate dioxygenase (HPPD) an early step in the biosynthesis of plastoquinone (PQ). Kim *et al.* (1999) proposed that, especially in developed tissues, the ultimate herbicidal effect of sulcotrione might result more from indirect inhibition of photosynthetic electron transport than of carotenoid biosynthesis. Here it is shown that treatment of mature cucumber cotyledons with, alternatively, diuron, fluridone, or sulcotrione, resulted in distinct perturbations in the relative levels of chlorophyll and of total carotenoids. Four days after herbicide treatment, the residual chlorophyll/carotenoid ratios were 5.24, 7.02 and 5.88, respectively. Sulcotrione caused a much more rapid decrease in the *in planta* quantum yield of photosystem II (PS II) as monitored by chlorophyll fluorescence than did fluridone. Furthermore, measurements of 2,6-Dichlorophenolindophenol reduction in extracted thylakoids indicated that sulcotrione was a more effective inhibitor of the Hill reaction in cucumber, a herbicide sensitive species than in corn, a herbicide-insensitive species. Overall, these results are consistent with the notion that the major herbicidal effect of sulcotrione in mature green tissue is to indirectly inhibit electron transport *via* a reduction in PQ levels.

INTRODUCTION

Sulcotrione is one representative of a new class of herbicides which act by inhibition of HPPD (Secor 1994). This causes significant reductions in the PQ and tocopherol contents of treated plants, which further leads to decreases in phytoene desaturase activity and in the carotenoid content of chloroplast (Prisbylla *et al.*, 1993). Loss of carotenoid leads to bleaching and plant death.

However the pattern of plant bleaching associated with inhibition of HPPD is distinctive and is not identical to that observed with herbicides such as norflurazon and fluridone which inhibit phytoene desaturase directly (Mayonado *et al.*, 1989; Sandman *et al.*, 1990).

This difference may be significant and indicative of some further underlying difference in mode of action. Kim *et al.* (1999) proposed that the reduction in PQ levels caused by sulcotrione treatment should lead to a decrease not only in phytoene desaturase activity but also to a decrease in photosynthetic electron transport and especially so in mature green tissues. Here, this suggestion is further investigated.

MATERIALS AND METHODS

Measurements of the *in planta* content of photosynthetic pigments and of the quantum yield of photosynthetic electron transport

Cucumber seeds were planted in a pot and grown in a greenhouse for 8 d. Cotyledons at 80% of full growth were treated with three different herbicides at rates calculated to give approximately equivalent levels of herbicidal effect e.g. 30 g a.i./ha of diuron, 100 g a.i./ha of fluridone, and 500 g a.i./ha of sulcotrione. The treated plants were grown for 4 d in a controlled growth chamber at 25°C with a photoperiod of 14 h and light intensity of 55 µmol $m^{-2}s^{-1}$. Photosystem II (PS II) quantum yield was measured at 24 h intervals, using a pulse amplitude modulation fluorimeter (PAM-2000, Walz, Effeltrich, Germany). Chlorophyll and carotenoids were extracted 4 d after herbicide treatment with methanol and quantitated by the method of Lichtenthaler (1987).

Herbicidal activity after foliar treatment

350 cm^2-area pots were filled with sterilized, sandy loam soil (pH 6.0) including 1.0% organic matter and appropriate amount of fertilizers. Cucumber (*Cucumis sativa* L.) and corn (*Zea mays* L.) were sown and grown in a greenhouse at 30/20 °C (day/night) using a 14 h-photoperiod. Herbicides were dissolved in acetone and diluted with water including a nonionic surfactant (Tween 20) and sprayed, 10 d after inoculation, at 4,000 liter/ha. Final concentrations of acetone and of Tween-20 in the solutions were 50% and 0.1%, respectively. The biological activity of herbicides was rated visually 6 d after treatment using a scale between 0 and 100 where '0' represented no herbicidal effect and '100' represented complete death.

Inhibition of the Hill reaction

2,6-Dichlorophenolindophenol reduction assays were conducted according to the method of Anderson *et al.* (1994) at a final volume of 10 ml.

RESULTS AND DISCUSSION

Herbicide-induced changes in the contents of photosynthetic pigments and in the quantum yield of photosynthetic electron transport

In order to better understand the mode of action of sulcotrione its effects on the pigment content and photosynthetic quantum yield of mature cucumber cotyledon tissue were compared with those of other herbicides yielding visually similar symptoms such as fluridone, a known inhibitor of phytoene desaturase and diuron, a known inhibitor of electron transport in PS II. Experiments were carried out under low light conditions in order to better clarify the causes of bleaching. After 4 days all herbicide treatments resulted in decreases in the contents of both chlorophyll and carotenoids but to differing and distinct extents (Table 1). Sulcotrione treatment resulted in a chlorophyll/ carotenoid ratio of 5.88, diuron treatment resulted in a ratio of 5.24 (the same as in untreated controls) whilst fluridone resulted in a relatively greater reduction in carotenoids and yielded a final ratio of 7.02 Qualitatively, by this measure, sulcotrione is more 'diuron-like' than 'fluridone-like'. Furthermore, analysis of

herbicide-induced changes in chlorophyll fluorescence indicated that sulcotrione exerted a much more rapid inhibitory effect on the quantum yield of PSII than did fluridone (Figure 1). The rate of inhibition of quantum yield by the three herbicides paralleled the extent to which they reduced the chlorophyll content *relative* to carotenoids (Table 1). Thus, although carotenoid contents were less diminished four days after treatment with sulcotrione than with fluridone, PS II quantum yield was, nevertheless, inhibited more rapidly following treatment with sulcotrione.

It has been reported that indirect inhibition of PS II by fluridone can be related to an effect of the carotenoid deficiency which follows inhibition of phytoene desaturase (Sandman *et al.*, 1996; Trebst & Depka, 1997). If sulcotrione-mediated inhibition of PS II were mediated solely by a such a mechanism then it would be expected that sulcotrione should inhibit PS II less than fluridone. However, as indicated in Figure 1, the converse was observed.

Table 1. Changes in photosynthetic pigment contents of cucumber cotyledons 4 d after treatment of with diuron, fluridone and sulcotrione.

Herbicides	Pigment contents (μg/ml)		
	Chlorophylls (A)	Carotenoids (B)	A/B
Control	115.8±5.9	19.7±1.1	5.88
Diuron	81.7±1.6	15.6±0.4	5.24
Fluridone	89.9±2.2	12.8±0.2	7.02
Sulcotrione	96.8±4.5	16.5±0.8	5.88

Data represent the mean±SE from three replicates.

Relation between herbicidal activity *in vivo* and inhibition of the Hill reaction

The herbicidal activity of sulcotrione observed in the greenhouse was compared with the extent of inhibition of the Hill reaction. Consistent with the relative tolerance of corn to herbicide treatment. Following treatment with sulcotrione the Hill reaction was much less inhibited in thylakoids extracted from corn than in cucumber thylakoids (Figure 2). These results are consistent with the major effect of sulcotrione in mature tissue being related to inhibition of electron-transport in PSII. although other explanations are also possible.

Considering that PQ is a mediator of photosynthetic electron transport as well as an essential cofactor for phytoene desaturase (Norris *et al.*, 1995), it is to be expected that sulcotrione should, indirectly, affect both processes. *A priori* it would seem most likely that sulcotrione should exert a relatively greater effect on photosynthetic electron transport in mature green tissues in which rates of *de novo* carotenoid biosynthesis are relatively low but rates of photosynthesis remain relatively high. This notion is consistent with the observation that the typical bleaching (whitening) symptoms induced by sulcotrione which correspond to carotenoid deficiency-induced degradation of chlorophyll (Sandmann *et al.*, 1990; Böger, 1996) are observed in the developing rather than mature tissues of treated plants.

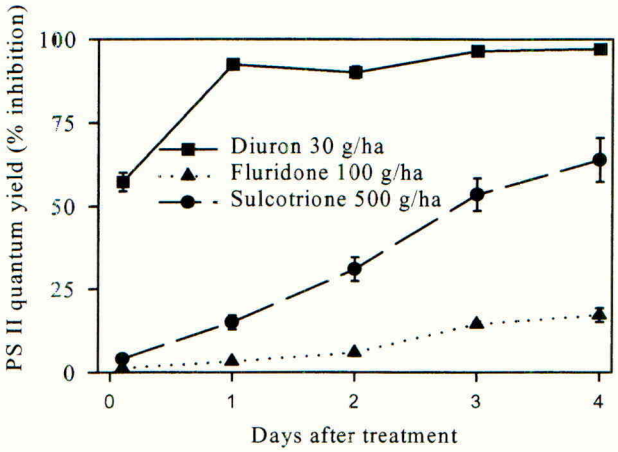

Figure 1. Inhibition of PS II quantum yield determined by chlorophyll fluorescence in cucumber cotyledons treated with diuron, fluridone and sulcotrione.

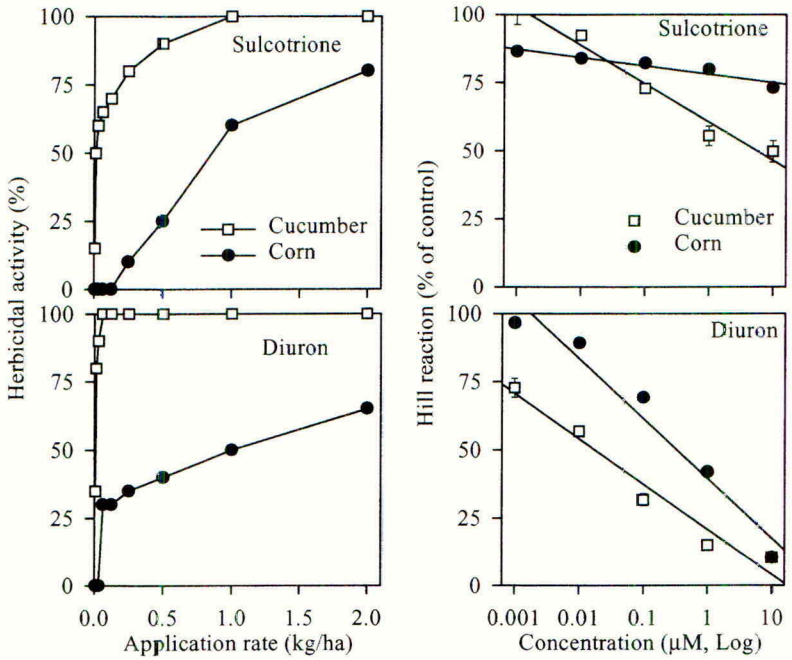

Figure 2. Effects of sulcotrione and diuron on herbicidal activity and on the Hill reaction in thylakoid membrane extracted from cucumbe or corn. Herbicidal activity was determined at 6 d after application.

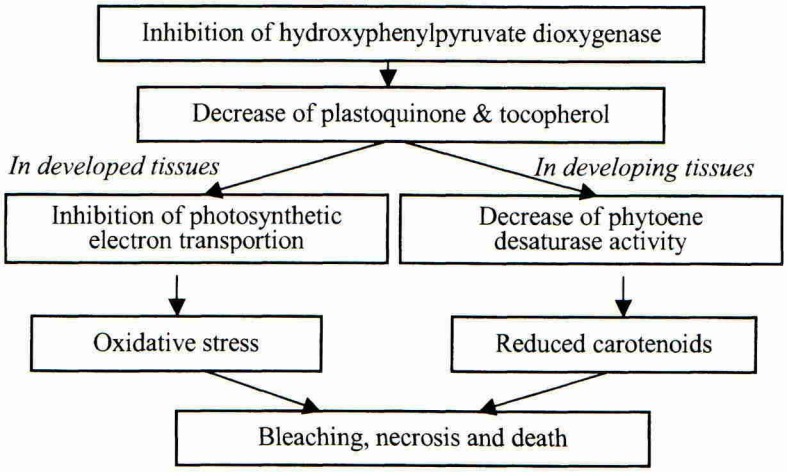

Figure 3. Possible action mechanism of sulcotrione herbicide in different developmental stages of plants

In conclusion, we propose that the major herbicidal consequences of sulcotrione inhibition of HPPD differ according to the developmental stages of plant tissues. In developing tissues such as new leaves the major effect is on inhibition of carotenoid biosynthesis whereas in older leaves and other mature tissues the major effect is on inhibition of photosynthetic electron transport (Figure 3).

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Mesotrione: Mechanism of herbicidal activity and selectivity in corn

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ABSTRACT

Mesotrione, a herbicide that acts by inhibition of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) step in plastoquinone biosynthesis, is highly effective for broad-leaved weed control in maize. The basis of its efficacy at low use rates is shown here to lie in its exquisite potency (e.g. K_d 15 pM) and slow rate of dissociation (t ½ c. 2d) from its HPPD target site in dicotyledonous weeds. Safety to corn is shown to derive not only from a relatively low rate of uptake but also from a relatively fast rate of cytochrome P450 catalysed 4-hydroxylation. The significance of this to the prevention of corn injury is underscored by the observation that sweetcorn lines, which are impaired in their ability to perform this hydroxylation, either by mutational variation or by addition of malathion, a cytochrome P450 inhibitor, exhibit significantly increased susceptibility to mesotrione damage. Mesotrione is also a much less potent inhibitor of the HPPD in monocotyledonous plants than of that in dicotyledonous plants. It is, for example, several hundred fold weaker an inhibitor of HPPD from wheat (K_d 7 nM, t ½ for dissociation of the enzyme/ inhibitor complex c. 10 min) than of HPPD from *Arabidopsis thaliana*. Tobacco, a species normally damaged by less than 2 g/ ha of mesotrione, is rendered entirely insensitive to the application of several hundred g/ ha after it is transformed to express the HPPD gene from wheat. This illustrates that inherent resistance at the level of the monocot HPPD target enzyme has an important role in determining the robust safety of mesotrione to corn.

INTRODUCTION

Mesotrione (Figure 1) is the active ingredient of Callisto, a herbicide recently developed by Syngenta for the selective pre- and post-emergence control of mainly broad-leaved weeds in maize (Wichert *et al.*, 1999). It is a member of the benzoylcyclohexane-1,3-dione family of herbicides, which are chemically derived from a natural phytotoxin obtained from the Californian bottlebrush plant, *Callistemon citrinus*. Mesotrione acts by competitive inhibition of the enzyme HPPD, a component of the biochemical pathway that converts tyrosine to plastoquinone and α-tocopherol (Prysbilla *et al.*, 1993).

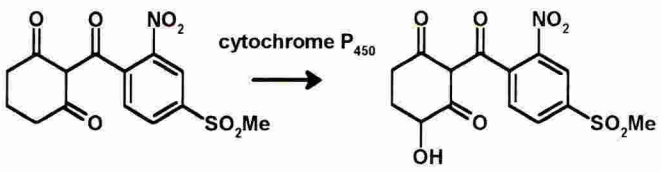

Figure 1. Structure of mesotrione and of its 4-OH metabolite

The observed crop selectivity of mesotrione may be due, at least in part, to foliar uptake being slower into maize than into weed species (Mitchell *et al.*, 2001). Mesotrione is a weak acid, with a dissociation constant (pK_a) of 3.12 at 20°C resulting in a pH dependency for water solubility ideal for uptake into, and translocation within plants. Comparative data regarding the extent of uptake of ^{14}C mesotrione, into the foliage of treated leaves of a range of weed species indicated relatively rapid uptake (c. 75-90% after 6 hours) relative to maize (c. 50%). Here we explore further aspects of the interaction of mesotrione with HPPD and the mechanisms that underpin its high degree of safety for use in corn.

MATERIALS AND METHODS

Mesotrione was prepared > 95% pure and uniformly ^{14}C-labelled to a specific activity of 1.12 GBq/ mmol (Wichert *et al.*, 1999). *Arabidopsis thaliana* and wheat HPPD genes were obtained *via* 'One-step' RT-PCR (Qiagen) of mRNA purified using the Oligotex method (Qiagen) from total RNA prepared by Tri-Zol extraction (Life Technologies) of 5d old seedlings. Primers to obtain full length coding sequences were designed based upon the known DNA sequences (Norris *et al.*, 1998; Derwent Geneseqn accession AAA29169) and included 5' *Nde*1 and 3' *Bam* H1 restriction enzyme sites. The authentic products obtained were cloned into pCR2.1TOPO (Invitrogen), excised using *Nde* 1 and *Bam* H1 (where the *Nde*1 site corresponds to the first codon), ligated into similarly restricted pET-24a (Novagen) and transformed into *E.coli* BL21(DE3). Cells were grown in a fermenter in L-broth containing 100 µg/ ml kanamycin and induced for expression for 2h before harvest, washing, resuspension in ice-cold 0.1M Hepes/ KOH buffer at pH 7.5 and disruption in a Constant Systems Basic Z cell disrupter. The resultant extracts were clarified by centrifugation for 2h at 40000 *g* and the supernatant exchanged down Sephadex G25 into the same buffer. For tobacco transformation, the *Nde*1-*Bam*H1 wheat HPPD fragment was first cloned into vector pMCJA, identical to pMJB1 (Thompson *et al.*, 1998) but with the *Nco*1 site at the translation initiation codon replaced by *Nde*1. The thus created expression cassette, comprising a double enhanced 35S cauliflower mosaic virus promoter/ tobacco mosaic virus translational enhancer/ wheat HPPD coding sequence/ nopaline synthase 3' terminator region was excised as a *Hind*III/ *Eco*R1 partial digest, cloned into pBIN19 (EMBL accession PPU09365), transformed *via Agrobacterium tumefaciens* LBA4404 into plant tissue of tobacco variety Samsun, selected and regenerated into plants as described by Thompson *et al* (1998). PCR positive primary transformants , single-copy segregants from later generations and control plants were grown in small pots in the glasshouse and, at the 5-7 leaf stage, were sprayed with 300 g/ ha mesotrione formulated in 0.5% Turbocharge ™ surfactant/ water at 200 litre/ ha (Figure 3). The extent of damage visible as bleaching of meristems, bleaching of leaves, necrosis and stunting of growth was assessed visually at 18 DAT by reference to unsprayed controls.

Wheat HPPD and Arabidopsis HPPD extracts exhibited K_m values of 10.1 µM and c. 3.5 µM hydroxyphenylpyruvate and, based on measured titres of active site ^{14}C inhibitor-binding, k_{cat}/K_m values of 1.1 x 10^6 and 1.35 x 10^6/ s/ M, respectively. Inhibitor binding experiments were all carried at 25°C in Bis-Tris propane buffer at pH 7.0 containing 25 mM sodium ascorbate and 2 µg/ ml of highly pure catalase (Sigma C3155). HPPD assays were carried out in 50 mM Bis-Tris propane buffer at pH 7.0 as described by Viviani *et al.* (1998) with initial rates obtained by curve fitting to time points taken at 10s intervals up to 70s. HPPD active-site titres in extracts were calculated from the ratio of bound inhibitor/ amount of extract as derived

from titration (between 0 and c. 0.9 μM) versus a fixed concentration of ^{14}C-inhibitor (c. 0.3 μM). Bound and free inhibitor were rapidly separated by gel filtration and quantitated by scintillation counting. Calculated titres accounted for the fact that inhibitors bind rapidly to half the available HPPD sites (Garcia *et al.*, 2000) and then slowly with the remainder. Values of HPPD/ inhibitor dissociation rates, k_{off}, were obtained from the rate of the exchange reaction observed (Figure 2) after addition of 50 μM of unlabelled inhibitor (I) to c. 0.35 μM HPPD/ ^{14}C inhibitor complex (EI*) preformed after reaction for > 3 h at 25°C between HPPD (E) and a slight excess of ^{14}C inhibitor (I*). Aliquots of bound and free ^{14}C label were removed at different times, separated by gel filtration and counted. The data obtained were fitted numerically to the scheme EI* + I ⇆ EI + I* to obtain best fit values to the rate constant, k_{off}, governing the dissociation EI* → E + I* (Schloss 1989). Values of HPPD/ inhibitor association rates, k_{on}, were a) similarly obtained but using unlabelled excess mesotrione to rapidly quench the binding reaction between HPPD and ^{14}C mesotrione or b) calculated from the rate of decline in enzyme activity at 25°C of HPPD reacted for different times with different concentrations of mesotrione and then simultaneously trapped and assayed by rapid quenching *via* 10 fold dilution into 150 μM HPP.

For metabolism studies, c. 1 μg of ^{14}C mesotrione was spotted as microdroplets (20 x 0.2 μl) to the middle of fully expanded leaves of 18 d old soybean (*Glycine max.*) and sweetcorn plants (*Zea mays* cv. 'Landmark' or 'Merit', as indicated). At 6 and 24 h time points, the treated area was excised and compound on the leaf surface removed into 2 ml of acetone. The leaf segment was powdered in liquid nitrogen, extracted into 10 ml of acetone, lyophilized, resuspended in acetonitrile and applied to a 2D Sorbsil C-30 tlc plate run with (15:15:5:1) chloroform/ ethyl acetate/ methanol/ formate in the first dimension and (20:8:1) chloroform/ methanol/ ammonium hydroxide in the second dimension. Radioactive spots were identified by reference to the R_f values of authentic mesotrione and 4-OH mesotrione in the first dimension of 0.25 and 0.50 and, in the second, of 0.56 and 0.9, respectively.

RESULTS AND DISCUSSION

Following foliar uptake, ^{14}C-mesotrione is translocated both acropetally and basipetally with some metabolism occurring in all plant species but particularly rapidly in maize (Mitchell *et al.*, 2001). Here, the possibility that metabolic inactivation of mesotrione is a major determinant of safety in corn was further pursued through comparative study of the early metabolism of mesotrione in "Landmark" corn and soybean, a highly sensitive species. A single leaf of each species was treated with radiolabelled mesotrione at a rate equivalent to 72 g ha^{-1}, and the extract of the treated leaf was analysed by tlc at 6 and 24h. After 24 h, less than 40% of the parent mesotrione was found in corn whereas > 85% remained in treated soyabean (Table 1). Mesotrione was not only taken up less rapidly into corn but was also metabolised much faster than in soybean. The major early metabolite formed was 4-hydroxy-mesotrione (Figure 1). Overall, the combined effect of these differences in uptake and rate of metabolism between the two species would be to significantly reduce the relative amount of mesotrione in the sensitive tissues of corn plants.

'Merit' is a line of sweetcorn, which is peculiarly susceptible to mesotrione injury. It is about 6-7 fold more susceptible than typical corn lines, exhibiting an equivalent level of transient injury at c. 70 g/ ha as, for example, 'Landmark' at 500 g/ ha of mesotrione. Comparative studies of uptake and metabolism, similar to table 1, indicated that, whereas there was no

significant difference in uptake, Merit was comparatively deficient in its ability to form the 4-OH derivative of mesotrione with c. 60% survival of parent and only c. 5 % conversion to 4-OH after 24h as compared to c. 13 and c. 40%, respectively in Landmark corn.

Table 1. Comparative uptake and metabolism of mesotrione in soybean and corn.

Sample/ Leaf Content	After 6h	After 24 h
Soya/ total(pmol)	283	256
Soya/ % parent	78	66.5
Soya/ % 4-OH	0	2
Corn/ total(pmol)	80	125
Corn/ % parent	55	13.5
Corn/ % 4-OH	13	40

In a further experiment to investigate the importance of mesotrione hydroxylation to crop safety, ^{14}C mesotrione was applied in droplet form, at a rate equivalent to 70g/ ha, to leaves of Landmark and Merit corn lines after first pre-spraying with 400 or 800 g/ ha of malathion, a known inhibitor of cytochrome P450 hydroxylase enzymes. Analysis by tlc confirmed that malathion did indeed inhibit the formation of the 4-OH metabolite of mesotrione in Landmark corn. Individually, neither malathion nor mesotrione caused injury to Landmark corn; however the combination caused Landmark to suffer mesotrione injury to a similar extent as the Merit susceptible line (c.10-15 % damage at 14 DAT with obvious partial leaf bleaching). Thus oxidative conversion of mesotrione to 4-OH mesotrione is probably cytochrome P450 hydroxylase catalysed and is an important determinant of mesotrione safety to corn.

Mesotrione is generally more active against broad leaved than against grass species. Further experiments were carried out to understand whether some difference in inherent susceptibility at the level of the HPPD target enzyme might contribute to the relative safety of mesotrione in corn. Figure 2 depicts data from one experiment to measure the dissociation rates of the complexes formed between mesotrione and HPPD from wheat, a representative monocotyledonous plant, and between mesotrione and HPPD from *Arabidopsis thaliana*. The experiment shows that mesotrione forms a much less stable complex with HPPD from wheat (t ½ c. 10 min, k_{off} c. 1 x 10^{-3}/ s) than it does with HPPD from *Arabidopsis thaliana* (t ½ c. 2 d, k_{off}, 3.3 x 10^{-6}/s). Dissociation rate is an important component of herbicidal activity since it determines how long the inhibitor can 'stick' to its target and maintain an effective block after spraying. It may be noted that, in Figure 2, c. 25% of the mesotrione initially bound to *Arabidopsis thaliana* HPPD exchanged relatively rapidly. In separate experiments we showed this fraction to represent inactive enzyme and it is disregarded in the determination of k_{off} that is calculated from the major slow phase. In this context it should be noted that, since the wheat enzyme was highly active and, moreover, since HPPD inhibitors with high levels of graminicidal activity similar to 2-[2-chloro-4-methanesulphonylbenzoyl]-4,4,6,6-

tetramethylcyclohexane-1,3,5-trione (Mitchell *et al.*, 2001) exchanged only slowly (<1.5 x 10^{-5}/s) from wheat HPPD, there was no question of the fast exchange of mesotrione from wheat HPPD being an artefact of enzyme inactivation. Mesotrione bound about equally rapidly to *Arabidopsis thaliana* and wheat HPPD with k_{on} values of 2.5 x10^5/ s/ M and 1.8x10^5/ s/ M, respectively. Overall K_d values were thus, c. 15 and 7000 pM, representing a > 400 fold difference in inherent susceptibility to inhibition by mesotrione. Even allowing for the c. 3 fold difference in Km value such a large difference in susceptibility to inhibition could readily explain why mesotrione is predominantly a broad leaved weed herbicide.

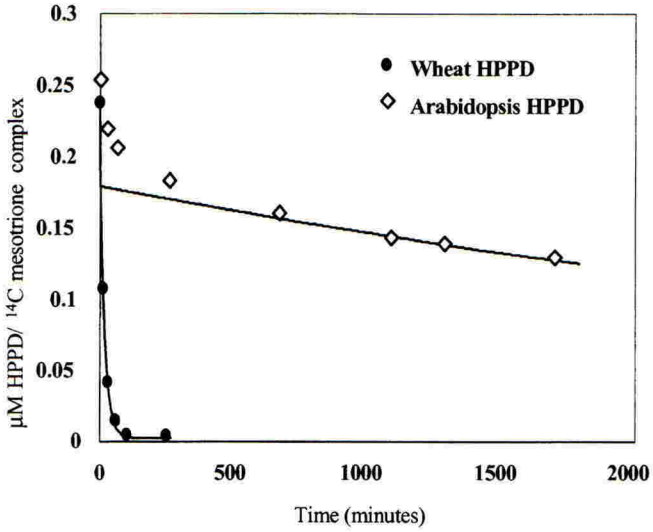

Figure 2. Exchange of ^{14}C mesotrione from wheat and from *Arabidopsis* HPPD

A further experiment (Figure 3) dramatically illustrated the contribution of intrinsic activity to crop safety. Tobacco plants were transformed to express wheat HPPD to c. 0.1% of the total tobacco leaf protein (estimated by Western analysis). At spray rates up to 300 g/ha as many as a third of primary transformants showed no visible signs of damage in contrast to untransformed tobacco which showed significant damage at less than 2 g/ ha. Resistance was even higher in homozygous plants of later generations. In comparable experiments wherein the *Arabidopis thaliana* HPPD gene (Norris *et al.*, 1998) was similarly expressed, but in *Arabidopsis*, we also observed an enhancement in resistance to mesotrione but relatively modest and < c. 8 fold in even the best individual events.

In summary, mesotrione is an effective herbicide because of its very high affinity for its HPPD target site. It is exceptionally safe to corn not only because 1) it is taken up more slowly into corn than into the majority of target weeds but also because 2) at least judged from wheat, the HPPD target enzyme of monocotyledonous plants is several hundred fold inherently less sensitive to mesotrione than that in broad leaved species and 3) corn has the ability to rapidly metabolise mesotrione to inactive derivatives and, in particular, *via* P450 catalysed 4-hydroxylation at the 4-position of the cyclohexanedione unit.

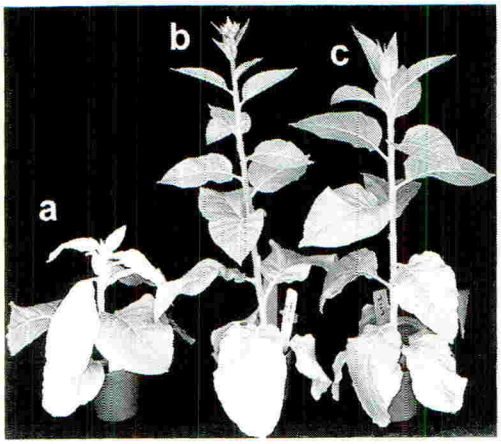

Figure 3. Control (a) and transgenic (c) tobacco expressing wheat HPPD 18 DAT after treatment with 300 g/ ha mesotrione as compared with unsprayed control tobacco (b).

ACKNOWLEDGEMENTS

The authors especially thank Peter Sutton and Matt Cordingley in weed biology and the radiochemistry teams at both Jealott's Hill and the Western Research Center, CA, USA

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Histological investigations into the mode of action of the novel grass herbicide oxaziclomefone

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ABSTRACT

Oxaziclomefone is a new grass herbicide offering excellent control of *Echinochloa crus-galli* and other grass weeds in rice. Preliminary investigations into its mode of action have shown it to have no inhibitory effects on established herbicide target sites and therefore a novel mode of action is implicated. Histological analysis shows that the mode of action of oxaziclomefone can be distinguished from those of dichlobenil and oryzalin, but there are similarities to cinmethylin. However, initial studies have shown oxaziclomefone not to share the biochemical site of action of cinmethylin.

INTRODUCTION

Oxaziclomefone (MY-100; 3-[1-(3,5-dichlorophenyl)-1- methylethyl]-2,3 dihydro-6-methyl-5-phenyl-4*H*-1,3-oxazin-4-one) (Figure 1) is a novel herbicide developed by Aventis CropScience for the control of *Echinochloa* spp. in paddy rice and transplanted rice (Jikihara *et al.*, 1997). In combination with a number of sulfonylurea herbicide partners, oxaziclomefone has been launched this year in Japan in the one-shot application market for weed control in rice, this sector accounting for 70% of the weed control market for rice in Japan. Oxaziclomefone offers growers season-long residuality, a wide window of application, low application rates and excellent selectivity for the control of *Echinochloa* spp. plus sedges and certain broad-leaved weeds, when applied pre- to early post-emergence.

Of special interest is the fact that oxaziclomefone brings a new type of mode of action to this market, complementary to that of sulfonylureas. Oxaziclomefone causes stunting and chlorosis of weeds in the greenhouse but clearly possesses a mode of action new and distinct from that of established herbicide classes. We have undertaken a histological and microscopic characterisation of the action of this herbicide and here present the results in comparison to certain growth-inhibiting reference herbicides *viz.* cinmethylin, dichlobenil and oryzalin. These reference herbicides are reportedly a pro-inhibitor of asparagine synthetase (Romagni *et al.*, 2000), and inhibitors of cellulose biosynthesis and mitosis respectively.

MATERIALS AND METHODS

Treatment of wheat tissues with herbicide

Wheat seeds were germinated in the dark until the root system was 20 - 30 mm long. Seedlings were then grown hydroponically in Long Ashton mineral nutrient solution

under standard glasshouse conditions, keeping the solution continually aerated and maintaining the roots in the dark and the shoots in the light. When plants reached the two-leaf stage they were treated with the herbicide under investigation by adding it to the nutrient solution. The concentration of herbicides used were such that the wheat plants showed symptoms three to four days after treatment yet remained alive for at least eleven days after treatment.

Light microscopic analysis of tissues

Sections of root tip were fixed in 2 % glutaraldehyde for two hours at room temperature. The sections were then infiltrated with methacrylate and embedded in resin. Longitudinal sections 4 µm thick were cut and stained with either Toluidene Blue, which stains cellulose cell walls, cytoplasm and nuclei blue, or Ruthenium Red which stains pectin red.

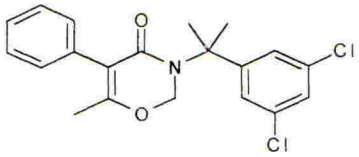

Figure 1. Structure of oxaziclomefone.

RESULTS

When wheat plants at the two-leaf stage were treated with 24 nM oxaziclomefone root and shoot growth ceased although the plants remained alive (Figure 2b). Protuberances resembling developing lateral roots appeared in the region 0 – 5 cm back from the root tip three days after treatment. As treatment continued these became more obvious although ceased to enlarge further after eleven days following treatment (Figure 2d). No discoloration of these 'lateral-root-like' protuberances was observed. Untreated control plants showed continued root and shoot growth and no root abnormalities developed (Figure 2a and 2c).

Microscopic analysis of sections from control roots showed no cellular abnormalities. The cells at the root tip were dense with the degree of vacuolation increasing as the cells aged (Figure 3a). Nuclei and nuceoli were present and all stages of mitosis were clearly visible in the zone of cell division (Figure 3b and 3d). No stages of mitosis were found in comparable regions of the root when plants were treated with 24 nM oxaziclomefone for 24 h although nuclei and nucleoli were present (Figure 4b). There were no differences in the shape of the roots of oxaziclomefone-treated plants (Figure 4a) compared with the control plants (Figure 3a).

When stained with Toluidene Blue and Ruthenium Red, the cell wall of control plants stained as a solid boundary (Figure 3b and 3c). In root sections from oxaziclomefone-treated plants, there was little or no staining of the wall with Toluidene Blue (Figure 4b). Staining of the cell walls of oxaziclomefone-treated plants by Ruthenium Red gave thin

lines of stain around the perimeter of the cells, the remainder of the cell wall showing no staining (Figure 4c).

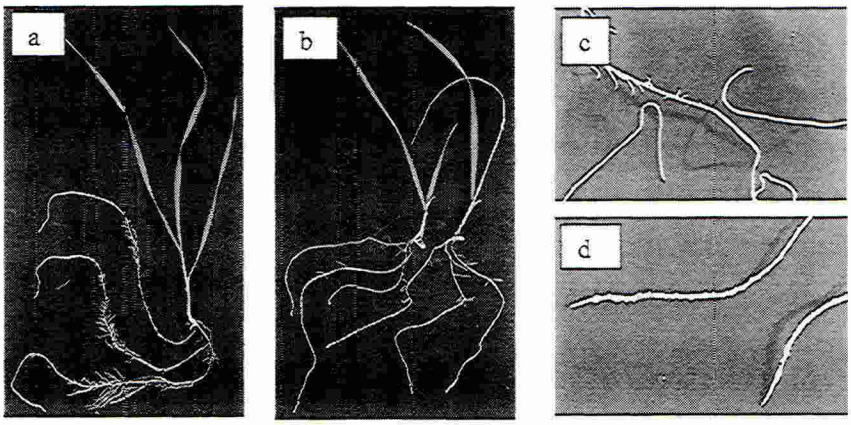

Figure 2. Cessation of root and shoot growth and root abnormalities caused by 24 nM oxaziclomefone. Wheat plants were treated via the nutrient solution at the two-leaf stage and grown in herbicide-amended media for eleven days. (a) And (c) are control plants; (b) and (d) were treated with oxaziclomefone.

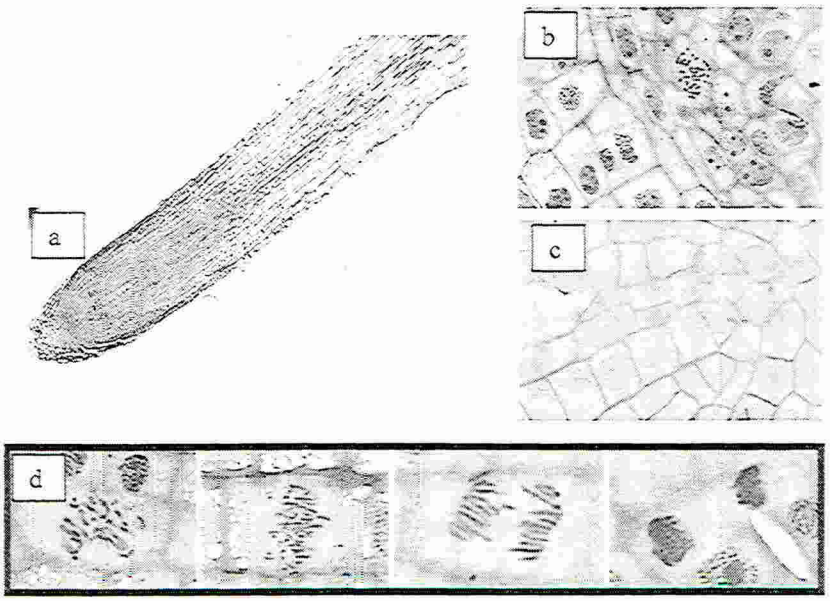

Figure 3. Longitudinal sections of the roots of untreated control plants stained with Toluidene Blue (a), (b) and (d), and Ruthenium Red (c). Stages of mitosis are clearly identifiable in the cells of control roots. Magnification (a) x10; (b), (c) and (d) x100. Encircled region in (a) indicates area examined under higher magnification in (b) and (c).

This unusual staining pattern with Toluidene Blue and Ruthenium Red was also observed when plants were treated with 18 nM cinmethylin (Figure 5c and 5d) and 100 µM dichlobenil (Figure 6c and 6d).

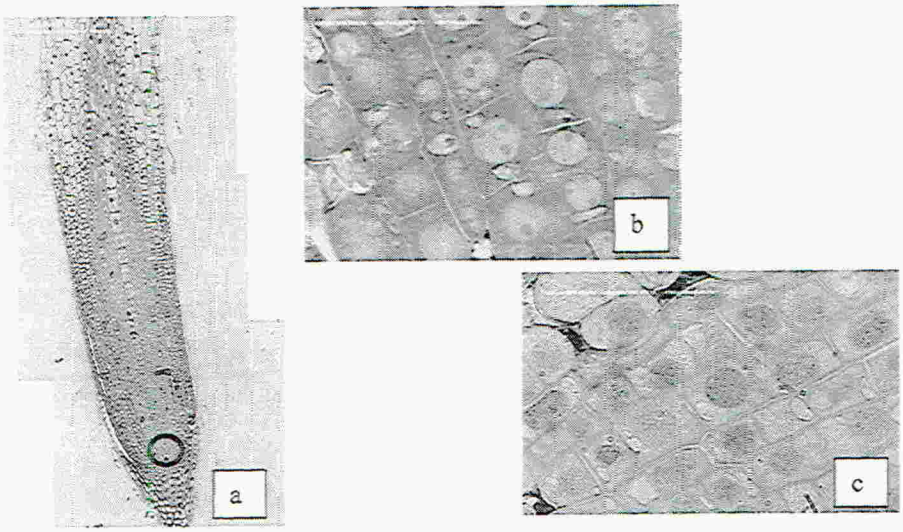

Figure 4. Longitudinal sections of the roots of wheat plants treated, via the nutrient solution, at the two-leaf stage with 24 nM oxaziclomefone for 24 h. Sections stained with Toluidene Blue (a) and (b), and Ruthenium Red (c). Encircled region in (a) indicates area examined under higher magnification in (b) and (c). Magnification (a) x10; (b) and (c) x100.

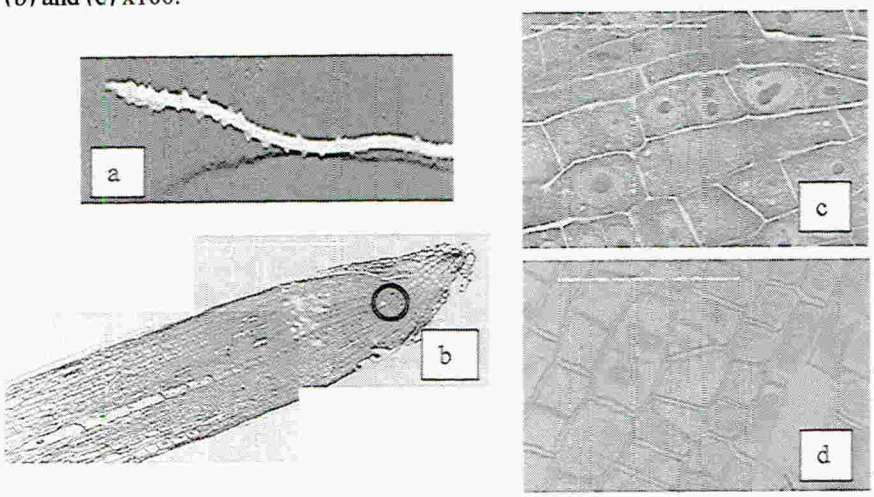

Figure 5. Effect of 18 nM cinmethylin on the roots of wheat plants treated via the nutrient solution at the two-leaf stage and grown in herbicide-amended media for either eleven days (a) or 24 h (b), (c) and (d). Longitudinal sections of the roots were stained with Toluidene Blue (b) and (c), and Ruthenium Red (d). Encircled region in (b) indicates area examined under higher magnification in (c) and (d). Magnification (b) x10; (c) and (d) x100.

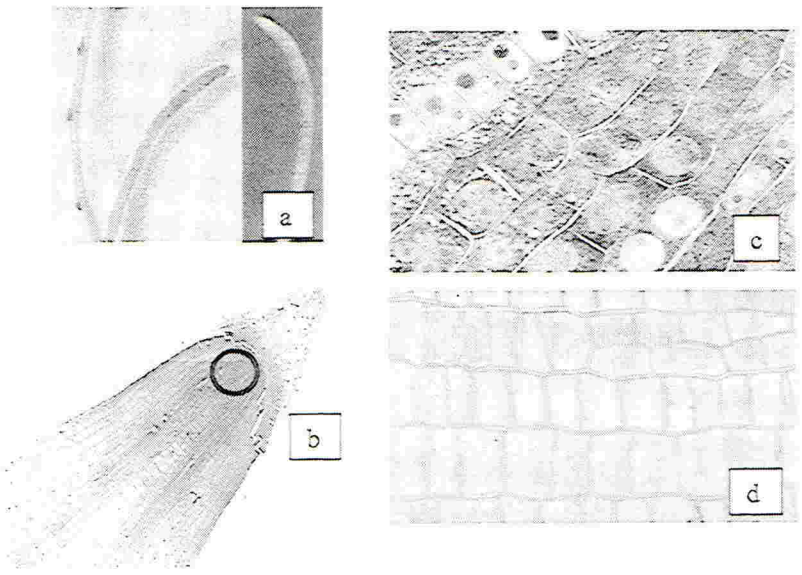

Figure 6. Effect of 100 µM dichlobenil on the roots of wheat plants treated via the nutrient solution at the two-leaf stage and grown in herbicide-amended media for either eleven days (a) or 24 h (b), (c) and (d). Longitudinal sections of the roots were stained with Toluidene Blue (b) and (c), and Ruthenium Red (d). Encircled region in (b) indicates area examined under higher magnification in (c) and (d). Magnification (b) x10; (c) and (d) x100.

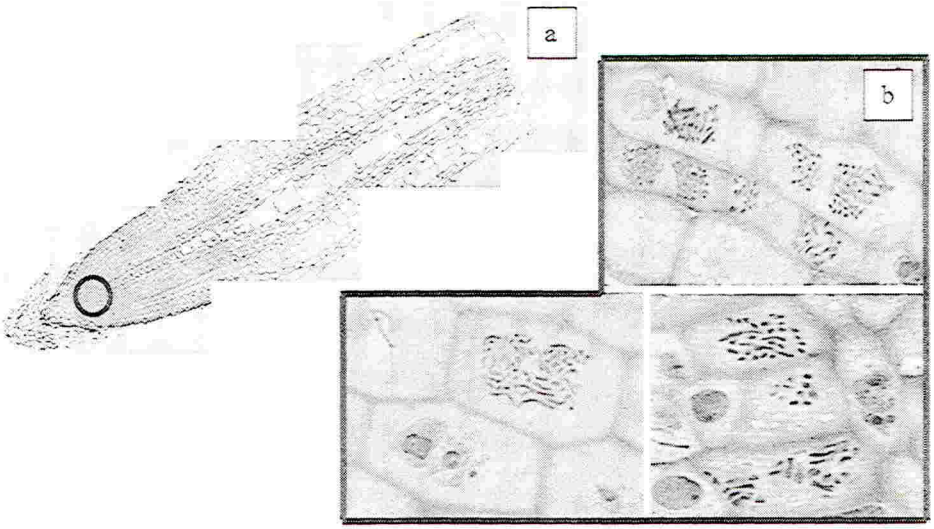

Figure 7. Effect of 10 µM oryzalin on the roots of wheat plants treated via the nutrient solution at the two-leaf stage and grown in herbicide-amended media for 24 h. Longitudinal sections of the roots were stained with Toluidene Blue. Aberrant stages of mitosis are clearly visible (b). Encircled region in (a) indicates area examined under higher magnification in (b). Magnification (a) x10; (b) x100.

As with oxaziclomefone, treatment of wheat plants with 18 nM cinmethylin via the nutrient solution caused the cessation of root and shoot growth although the plant remained alive. The protuberances that developed on the roots of cinmethylin-treated plants (Figure 5a) were similar in appearance and position to those caused by oxaziclomefone (Figure 2d). In contrast to treatment with oxaziclomefone, later stages of mitosis were occasionally seen in roots of cinmethylin-treated plants (Figure 5c and 5d).

When wheat plants were treated with 100 µM dichlobenil for eleven days, the first leaves showed signs of wilting, developing lateral roots were discoloured and the root tips were swollen (Figure 6a). In contrast to the roots of oxaziclomefone and cinmethylin-treated plants, there were no 'lateral-root-like' protuberances occurring near the root tip. Wheat plants treated with 100 µM dichlobenil (Figure 6b) and 10 µM oryzalin (Figure 7a) for 24 h showed obvious swelling of the cortical cells. Many cells in those roots treated with oryzalin showed aberrant mitotic figures as expected (Figure 7b).

DISCUSSION

These results demonstrate that some herbicide modes of action can be easily identified using histological studies. Although the swelling of root tissue caused by oryzalin and dichlobenil may appear similar, the two compounds are clearly distinguishable by the presence, as with oryzalin, or absence, as with dichlobenil, of aberrant mitotic figures. The lack of enlargement of cortical cells and aberrant mitotic figures in the roots of oxaziclomefone-treated roots further supports the biochemical studies suggesting that neither cellulose biosynthesis nor mitosis are directly affected by oxaziclomefone.

The similarities observed between the roots of oxaziclomefone and cinmethylin-treated plants did initially suggest that they may have the same mode of action. However, initial results have shown that oxaziclomefone does not inhibit asparagine synthetase as has been reported for cinmethylin (Romagni *et al.*, 2000).

An unexpected result seen in these studies is the disruption to the cell wall that has occurred in roots of those plants treated with oxaziclomefone, cinmethylin and dichlobenil. From the staining of the cell wall it appears that both the cellulose and pectin components of the cell wall are either not formed as in the control plants or that they undergo some chemical change that renders them insensitive to the stain. Further studies would be needed to determine what the cause of this is and whether is it characteristic of herbicide treatment of plants.

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