# Session 4D

# Herbicide Mode of Action at the Target Site

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| Session Organiser | Dr P J Ryan |
| Poster Papers | 4A-1 to 4D-7 |

# A PRELIMINARY INVESTIGATION OF HERBICIDE ACTION ON $H^+$-ATPases USING ISOLATED PLASMA MEMBRANE VESICLES.

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## ABSTRACT

Graminicides such as diclofop-methyl are able to act both by inhibiting lipid biosynthesis and also by perturbations at plant cell membranes. Furthermore, auxins such as MCPA and 2,4-D are thought to interact with graminicides at the plant cell membrane. The aim of this investigation has been to use two phase partitioning to prepare a highly purified plasma membrane (PM) from Sugarbeet (*Beta vulgaris* L.) and Blackgrass (*Alopecurus myosuroides* Huds.) and to assay ATPase activities in the presence and absence of these herbicides. Preliminary data suggest a dose-dependent inhibition of the PM $Mg^{2+}$, $K^+$-ATPase by diclofop-methyl in both species.

## INTRODUCTION

A wide diversity of monocotyledon and dicotyledon weeds cause economic loss through a reduction in both yield quantity and quality. A single application of a mixture of herbicides to control a broad spectrum of weeds would therefore be advantageous in reducing the cost of extra labour, time and fuel. However, it is well established that some groups of herbicides are incompatible and cannot be tank mixed. For example, the efficacy of graminicides belonging to the aryloxyphenoxypropionates (e.g. diclofop-methyl, DM) is reduced when they are mixed with broadleaf auxin-type herbicides such as the phenoxyalkanoic acids 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (4-chloro-o-tolyloxyacetic acid) Köcher (1984).

Several theories regarding the mechanism of graminicide antagonism with auxin type herbicides have been proposed including the hypothesis that aryloxyphenoxypropionates are anti-auxins (Shimabukuro *et al.* 1989; Barnwell and Cobb 1994). In addition, graminicides such as DM are able to act both by inhibiting lipid biosynthesis and also by perturbations at plant cell membranes. Shimabukuro and Hoffer (1995) have proposed the collapse of the transmembrane proton gradient by DM which is likely to involve the integral membrane protein $H^+$-ATPase. Such an action by DM will inhibit cell elongation, active membrane transport and cause an increase in cytosolic pH (Shimabukuro *et al.* 1989).

This study has used two phase partitioning to prepare highly purified plasma membrane vesicles (PM) from the dicotyledon sugarbeet (*Beta vulgaris* L.) and the monocotyledon blackgrass (*Alopecurus myosuroides* Huds.). Subsequently, the PM $Mg^{2+}$, $K^+$-ATPase has been assayed in the presence and absence of DM, MCPA and 2,4-D to investigate the effects of auxin-type and aryloxyphenoxypropionate herbicides on PM $H^+$-ATPase activity.

## MATERIALS AND METHODS

### Plant material

Sugarbeet, cv. Celt was sown at a rate of 3 seeds per pot in J. Arthur Bowers potting compost in 100 mm diameter pots. Blackgrass was sown in trays containing the same medium. Plants were raised in glasshouse conditions (20-40°C day, 9-15°C night) at 60-70% relative humidity. A photosynthetic photon flux density (PPFD) of 300-400 $\mu$mol $m^{-2}$ $s^{-1}$ was provided by natural daylight supplemented with high pressure sodium lamps and a 16 h photoperiod maintained throughout the study. The first true pair of sugarbeet leaves and blackgrass plants (1-2 leaf stage, lamina and sheath) were harvested *ca.* 15 d after sowing, frozen in liquid nitrogen and stored at -70°C until required.

### Microsomal membrane preparation

All steps were carried out on ice. Frozen leaves were crushed to a fine powder and first homogenised gently with scissors and second more vigorously with a pestle and mortar in 230 mol $m^{-3}$ sorbitol, 50 mol $m^{-3}$ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP) (pH 8.0), 10 mol $m^{-3}$ KCl, 3 mol $m^{-3}$ ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), 3 mol $m^{-3}$ dithiothreitol (DTT) added immediately prior to extraction. A ratio of 1 g tissue to 10 $cm^3$ homogenisation medium was used. The homogenate was filtered through four layers of muslin and centrifuged at 1,000 g for 5 min and then 10,000 g for 10 min (MSE 21 centrifuge, Beckman Instruments). The resulting supernatant was carefully transfered to Oakridge-type centrifuge tubes and centrifuged at 50,000 g for 45 min (L8-70 Ultracentrifuge, Beckman Instruments). The resulting microsomal membrane pellets were resuspended in phase resuspension medium (350 mol $m^{-3}$ sorbitol, 5 mol $m^{-3}$ potassium phosphate (pH 7.8) and 2 mol $m^{-3}$ DTT).

### Two phase partitioning

Aqueous two-phase partitioning was carried out as previously described (Widell *et al.* 1982). Briefly, a microsomal membrane fraction prepared from 25 g of tissue, suspended in 0.4 $cm^3$ of phase resuspension medium was added to form an 8 g phase system containing 6.2% (w/w) dextran T500, 6.2% (w/w) polyethylene glycol (MW 3,350), 350 mol $m^{-3}$ sorbitol, 5 mol $m^{-3}$ KCl, 5 mol $m^{-3}$ potassium phosphate (pH 7.8) and 1 mol $m^{-3}$ DTT. After mixing by inversion 40 times the phases were separated by centrifugation at 2,500 g for 5 min at 4°C. The procedure was performed for a total of 3 partitions (sugarbeet) and 4 partitions (blackgrass) and the resulting upper (U3, U4) and lower (L1) fractions were diluted approximately 10-fold in ATPase resuspension medium (330 mol $m^{-3}$ sorbitol, 10% (w/w) glycerol, 2 mol $m^{-3}$ HEPES-BTP (pH 8.0) and 2 mol $m^{-3}$ DTT). Following centrifugation at 100,000 g for 45 min and suspension in ATPase resuspension medium, the membranes were frozen in liquid nitrogen and stored at -70°C.

### Enzyme, protein and chlorophyll assays

ATPase activity was measured as the rate of liberation of orthophosphate (Pi) after 1 h incubation at 37°C using the method of Ohnishi *et al.* (1975). The standard reaction medium contained 40 mol $m^{-3}$ 2-amino-2-(hydroxymethyl)-1,3-propanediol(Tris)-2-(N-

morpholino)ethane-sulfonic acid (MES) (pH 6.5), 0.1 mol $m^{-3}$ ammonium molybdate, 0.005% (v/v) Triton X-100, 2 mol $m^{-3}$ $MgSO_4$, 50 mol $m^{-3}$ KCl and 2 mol $m^{-3}$ ATP-BTP (pH 6.5) in a reaction volume of 0.5 $cm^3$. $Mg^{2+}$, $K^+$-ATPase refers to the ATPase activity measured in the presence of magnesium and potassium having subtracted the activity occurring in the absence of these ions. Vanadate sensitive $Mg^{2+}$, $K^+$-ATPase attributable to the PM was measured in the presence of 0.2 mol $m^{-3}$ sodium vanadate. $Mg^{2+}$, $K^+$-ATPase activity attributable to the chloroplast (sodium azide sensitive, 1 mol $m^{-3}$) and tonoplast (potassium nitrate sensitive, 50 mol $m^{-3}$) was measured at pH 8.0. Approximately 5 $\mu$g membrane protein was included in each assay. Assay conditions were optimised with respect to $Mg^{2+}$, $K^+$ and Triton X-100 for each species before herbicide experiments were carried out (data not shown). Protein was determined according to Bradford (1976) using bovine serum albumin (BSA, Sigma Fraction V) as standard. Chlorophyll was measured according to Arnon (1949).

Herbicides

The auxin-type herbicides 2,4-D (99%, British Greyhound, Birkenhead, UK) and MCPA (95%, Aldrich Chemical Co. Ltd. Gillingham, UK) and the aryloxyphenoxypropionate diclofop-methyl (99%, British Greyhound) were dissolved in 50% (v/v) acetone and then diluted with ATPase resuspension medium. Herbicides were added to the standard enzyme reaction medium prior to starting the reaction with 2 mol $m^{-3}$ ATP-BTP (pH 6.5). The final concentration of acetone in the assay medium was 0.5% (v/v) and did not affect $Mg^{2+}$, $K^+$-ATPase activity.

## RESULTS AND DISCUSSION

The aim of this investigation was to use two phase partitioning to purify PM vesicles from sugarbeet and blackgrass in order to assay the PM $H^+$-ATPase in the absence and presence of DM, MCPA and 2,4-D. The methods used follow those published previously for sugarbeet (Bush 1989) except that a second slow centrifugation step was introduced in the extraction procedure. This reduced the amount of chlorophyll and therefore contamination by chloroplasts in the microsomal membrane fraction (data not shown). Riechers *et al* (1994) adapted the same technique to isolate PM from fat-hen (*Chenopodium album* L.). Subsequently, we used the method to isolate PM vesicles from blackgrass.

The purity and quantity of the PM isolated needs to be known before the effect of herbicides on PM $H^+$-ATPase activity can be assessed. The concept of enzyme markers is now well established and it is generally accepted that vanadate, azide and nitrate sensitive $Mg^{2+}$, $K^+$-ATPase activities are good markers for the PM, chloroplast and tonoplast respectively (Hall and Williams 1991). Consequently, the distribution of enzyme markers in the upper phase after phase partitioning was first compared with those in the microsomal membrane fraction (Figure 1). Phase partitioning largely eliminated contamination of the PM by tonoplast fragments and all of the chlorophyll. A 6 fold increase in the vanadate sensitive $Mg^{2+}$, $K^+$-ATPase was found in the upper phase, vanadate sensitivity accounting for 70% of the ATPase activity at pH 6.5 in sugarbeet (Figure 1). In both species, vanadate showed a stronger inhibition of the ATPase activity in the upper phase than in the lower phase (Table 1). Azide and nitrate inhibitions were greater in the lower phase of both sugarbeet and blackgrass (Table 1). The purity of the PM was further studied by measuring pH optimum of the $Mg^{2+}$, $K^+$-

sharp pH profile with an optimum occurring between pH 6 and 6.5 (data not shown). High vanadate sensitivity and a sharp pH optimum around 6.5 are general properties of the PM ATPase (Hall and Williams 1991). This is clearly demonstrated by the characteristics of the ATPase activity associated with the upper phase of both species (Figure 1, Table 2). Any effect of herbicides on $Mg^{2+}$, $K^+$-ATPase in the upper phase will therefore be due to an effect on the PM $H^+$-ATPase.

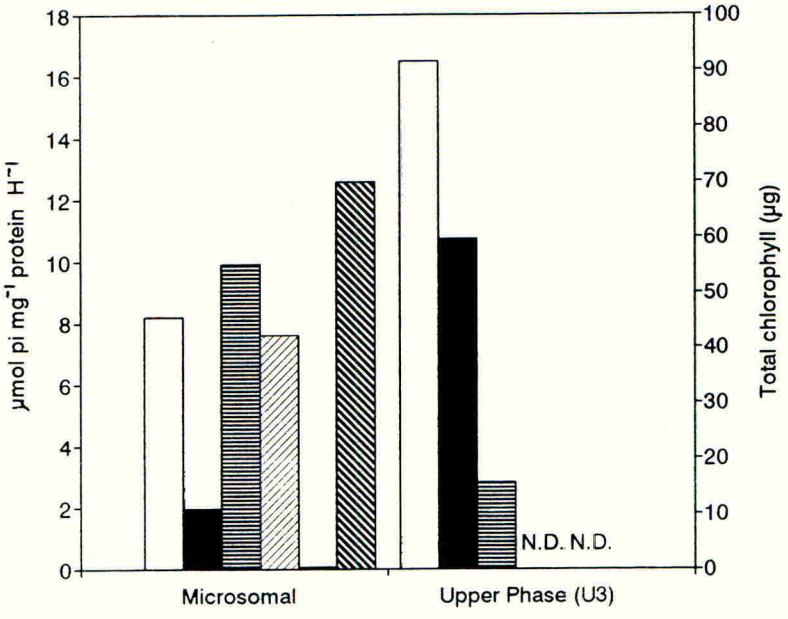


**Figure 1.** Enzyme marker distribution after two phase partitioning of a sugarbeet microsomal membrane fraction. U3 (upper phase after 3 phase partitions). $Mg^{2+}$, $K^+$-ATPase pH 6.5. vanadate sensitive $Mg^{2+}$, $K^+$-ATPase. $Mg^{2+}$, $K^+$-ATPase pH 8.0. azide sensitive $Mg^{2+}$, $K^+$-ATPase. nitrate sensitive $Mg^{2+}$, $K^+$-ATPase. chlorophyll. N.D. Not detectable. Data points are mean specific activities ($\mu$mol $mg^{-1}$ protein $h^{-1}$) derived from 3 separate microsomal/phase experiments. Each enzyme from each experiment was assayed 3 times.

**Table 1.** Effect of inhibitors of $Mg^{2+}$, $K^+$-ATPase activity on upper and lower phases of a sugarbeet and blackgrass microsomal membrane fraction after phase partitioning. Assays were conducted at pH 6.5. Results shown are the means of three enzyme assays conducted on one representative upper and lower phase from both species.

| Species and Reagent | % Inhibition | |
|---|---|---|
| | Upper | Lower |
| **(1) Sugarbeet:** | | |
| Vanadate (0.1 mol $m^{-3}$) | 67 | 29 |
| Azide (1 mol $m^{-3}$) | 0 | 31 |
| Nitrate (50 mol $m^{-3}$) | 5 | 26 |
| **(2) Blackgrass:** | | |
| Vanadate (0.1 mol $m^{-3}$) | 53 | 21 |
| Azide (1 mol $m^{-3}$) | 0 | 22 |
| Nitrate (50 mol $m^{-3}$) | 8 | 38 |

Initial results suggest a dose dependent inhibition of the PM $Mg^{2+}$, $K^+$-ATPase activity by DM between 0.1 - 0.01 mol $m^{-3}$. DM at 0.1 mol $m^{-3}$ inhibited the PM $Mg^{2+}$, $K^+$-ATPase activity by 30% in both sugarbeet and blackgrass (Table 2). These results confirm previous hypothesis that DM may interact with the PM $H^+$-ATPase (Shimabukuro and Hoffer 1995). The nature of this interaction will be studied further in this laboratory. MCPA did not increase PM $Mg^{2+}$, $K^+$-ATPase activity although at 0.01 mol $m^{-3}$ 2,4-D did increase activity by *ca.* 10% in sugarbeet (Table 2). The effect of other graminicides and auxin-type herbicides on the PM $H^+$-ATPase will be examined in the future. In addition, as the tonoplast $H^+$-ATPase becomes enriched in the lower phase (Table 1) this is an opportunity to study herbicide interactions at the vacuolar membrane level.

**Table 2.** Specific activities of $Mg^{2+}$, $K^+$-ATPase ($\mu$mol $mg^{-1}$ protein $h^{-1}$) of the upper phase after two phase partitioning of a sugarbeet and blackgrass microsomal fraction in the presence and absence of diclofop-methyl (DM), MCPA and 2,4-D. Results shown are the means ± s.e. of three enzyme assays conducted on one representative upper phase from both species. Control activities are those measured in the presence of 0.5% (v/v) acetone.

| Species | Control | Herbicide (mol $m^{-3}$) | | | |
|---|---|---|---|---|---|
| | | DM | DM | MCPA | 2,4-D |
| | | 0.1 | 0.01 | 0.01 | 0.01 |
| **Sugarbeet** | 24.13±1.22 | 16.96±2.68 | 19.82±4.5 | 24.89±1.38 | 27.08±1.28 |
| **Blackgrass** | 25.00±0.39 | 17.75±0.72 | 24.39±0.35 | 24.82±0.79 | 26.24±0.37 |

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# CHARACTERIZATION OF SOMACLONES OF SOYABEAN RESISTANT TO IMAZETHAPYR

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## ABSTRACT

The potential for increasing resistance to imazethapyr in soyabean was investigated by an initial *in vivo* screen of 20 selected cultivars at an imazethapyr concentration of $EC_{50}$. Out of this group 5 of the most resistant cultivars were identified. Tissue cultures were initiated from immature embryos of these 5 cultivars, then placed on an organogenesis medium containing imazethapyr ($EC_{90}$) in order to screen *in vitro* for herbicide resistance. The surviving organogenic cultures were transferred to a regeneration medium without herbicide so as to stimulate shoot regeneration. The growth response to imazethapyr of the 2nd generation progeny of somaclones showed that some of the somaclones were 3 times more resistant than their parents under *in vivo* conditions. The resistance was quite stable as it was expressed in the second progeny generation. Comparison between the growth of the parents and somaclones at different stages of the life cycle showed that the parent plants had a consistently higher leaf number than the somaclones. A comparison of leaf chlorophyll showed that four out of five parents had a higher cholorophyll level than the somaclones. However the acetolactate synthase (ALS) activity of the somaclones was still the same as in the parent plants. Increased resistance in the somaclones was not due therefore to alteration in the ALS activity.

## INTRODUCTION

*In vitro* techniques for tissue culture currently play a major role in plant biotechnology. One of the principal applications of this system has been for its use in crop improvement (Duncan and Widholm, 1986) such as herbicide resistance (Chaleff, 1984; Smith and Chaleff, 1990) The potential of these methods is well demonstrated in the recent selection of maize mutants resistant to imidazolinone herbicides with the resistance expressed in regenerated plants and their progeny (Newhouse *et al.*, 1991).

Weeds are controlled by the use of selective herbicides but application rate of the herbicide depends on the level of resistance in the crop (Martin and Staugaard,1985). Soyabean does show resistance to the imidazolinone herbicides but the resistance depends on the particular herbicide-cultivar combination (Wixon and Shaw, 1991). Possible mechanisms of resistance are modification to the site of action, differential

uptake and translocaton or metabolic inactivation of the herbicides (Shaner and Mallipudi, 1991).

The aim of this work was to:
1. Test seed of tissue culture derived regenerants for herbicide resistance.
2. Compare the growth of somaclones and parents.
3. Assess the biochemical basis of any increase in herbicide resistance in somaclones by ALS assay.

## MATERIALS AND METHODS

### *In vivo* screening and *in vitro* selection for herbicide resistance

Five days old seedlings (germinated in vermiculite ) of 20 cultivars of soyabean (Aramir, Ardir, Bonus, Century84, Clark, CN210, Columbus, Cresir, Dakir, Elgin, Elisir, Fiorir, Grangelb, Hadgson, Izmir, Steel, Visir, Wells, Williams, Zane) were screened in 2.5 ppm imazethapyr $EC_{50}$ (Barrentine *et al.,* 1976). After 10 days root length, root and shoot dry weights were measured then the % reduction compared with the control (Wixon and Shaw, 1991). Immature pods from the 5 most resistant cultivars were harvested 3 weeks after flowering and embryos, 4-6mm long, excised and incubated on an organogenesis medium containing imazethapyr at 2ppm as $EC_{90}$ (Barwale *et al.*, 1986). After 6 weeks the surviving cultures were transferred to a regeneration medium without herbicide. Shoots (1cm) were transferred to a rooting medium (Barwale *et al.*, 1986) and the plantlet grown to maturity, allowed to flower, self pollinated and set seed. Since these regenerated plants were small and only produced a limited number of seeds, a proportion of these seed have been germinated and the plants used to increase seed production of the somaclones.

### Morphological and biochemical analyses of somaclones

A proportion of seed from the second generation of each somaclones was sown in the greenhouse to assess the growth response of different somaclones in the absence of herbicide. The branch number, leaf number and the height of plants were recorded before flowering. The chlorophyll content of young leaves from 3 weeks old plants were also measured according to Arnon (1949).

The growth response of the somaclones to imazethapyr was examined *in vivo* to assess for herbicide resistance using the seedling bioassay (Barrentine et al., 1976). Young leaves from selected somaclones and parents were assayed for acetolactate synthase (ALS) activity in the presence of imazethapyr to identify any modification of enzyme activity in the somaclones (Singh *et al.*, 1988).

## RESULTS

Measurement of root length root and shoot dry weight of seedlings of the 20 cultivars grown at $EC_{50}$ for imazethapyr showed that the level of tolerance to imazethapyr of

these cultivars of soyabean varied between 24% and 58%. Tissue cultures of the 5 most resistant cultivars also showed a variation in their capacity to produce viable shoots (6-14%) after screening *in vitro* at an $EC_{90}$ concentration of the imazethapyr (data are not shown).

After the five cultivars were screened at $EC_{90}$ concentration of imazethapyr, the seed progeny of surviving regenerants from this screen were tested as described. A comparison of growth of the five parents and somaclones showed that there was a significant difference between the somaclones and parents in the leaf number except for Ardir. The somaclones appeared to be less vigorous than their parents (fig. 1. a). There was also a significant difference in the level of chlorophyll between the somaclones and parents for Ardir, Izmir and Williams (Fig. 1. b).

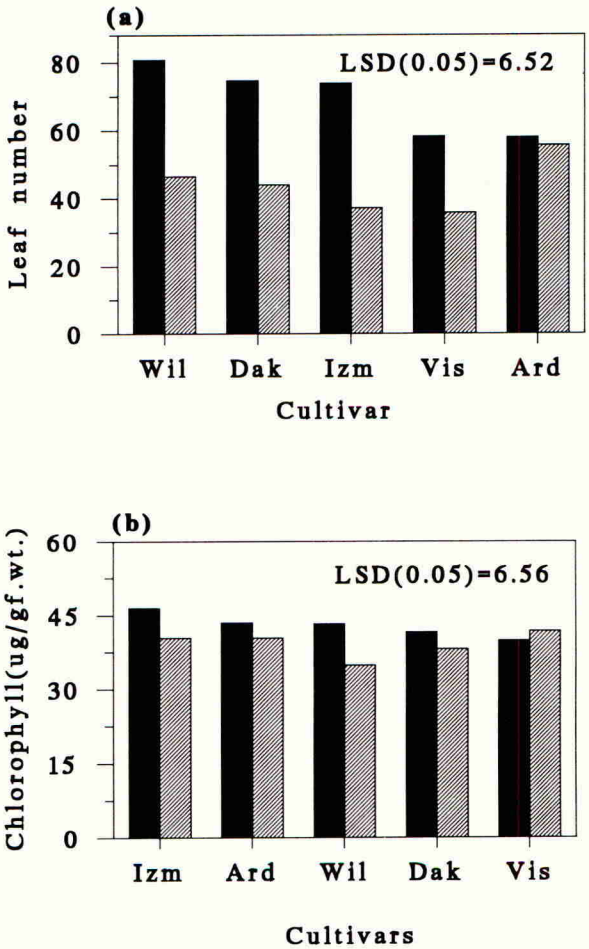


Figure 1. The variation in parent plants (■) and selected somclones (▨) grown in the absence of imazethapyr; (a) leaf number, (b) chlorophyll content.

Seedlings of progeny of the somaclones when tested *in vivo* in the presence of imazethapyr showed a higher level of resistance for each cultivar with a growth reduction of 6-20% (root length) and 17-37% (shoot dry weight) for the somaclones and 14-20% (root length) and 26-34% for the parents (Fig. 2. a and 2. b).

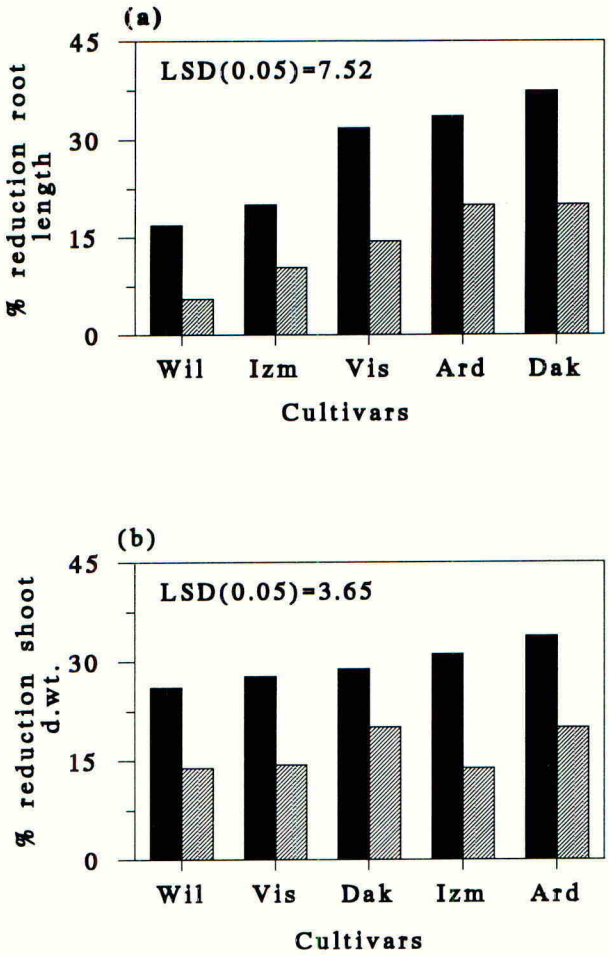


Figure 2. Effect of imazethapyr on growth reduction % as compared with control of parent (■) and selected somaclone (▨) of soyabean seedlings; (a) root length, (b) shoot dry weight.

When the ALS enzyme activity was measured in response to imazethapyr it was found that the ALS activity of the parents was higher than the progeny and showed some

variation as did the progeny (Table 1). The values for $I_{50}$ were also higher in the parent plants (2.91-3.33 uM) than in the progeny (1.28-3.27 uM) suggesting that the ALS enzyme was made more sensitive to the effects of imazethapyr by the tissue culture stage.

Table 1. Effect of imazethapyr on (ALS) activity of parents and somaclones.

| | ALS activity* | | $I_{50}$ (uM imazeth.) | |
|---|---|---|---|---|
| Cultivar | Parent | Somaclone | Parent | Somaclone |
| Williams | 1.04 | 0.88 | 3.24 | 2.87 |
| Izmir | 0.77 | 0.57 | 3.33 | 2.91 |
| Visir | 0.94 | 0.85 | 2.91 | 2.98 |
| Dakir | 0.68 | 0.50 | 3.11 | 3.27 |
| Ardir | 0.77 | 0.77 | 3.15 | 1.28 |

* umol acetoin/mg protein/h.

## DISCUSSION

The variation in resistance to imazethapyr in the parent cultivars of soyabean was surprisingly wide and must obviously be taken in to account when selecting a cultivar and herbicide combination. Such cultivar differences have also been noted in the response of soyabean to other imidazolinone herbicides such as AC263,222 (Wixon and Shaw, 1991).The cultivars most resistant to imazethapyr were also the most resistant *in vitro* which suggested that the tissue cultures reflected the parent characteristics for herbicide response. Although the regenerant plants grew to maturity and flowered, they were smaller than the parents and showed a number of morphological differences, such as reduced number of leaves, flowers and seed production in our experiment. This variation in morphology of somaclones of soyabean was originally noted by Barwale and Widholm (1987). Since a second generation of plants were required to increase the number of seed, the somaclones had been through two seed generation before the progeny was tested. Any herbicide resistance expressed by the somaclones would be genetically stable. The second and third generation of seeds in the progeny test produced smaller seedlings than the parents. The ALS enzyme activity in the somaclones may correlate with general vegetative vigour because it was less active in the herbicide resistant material. The enzyme in the somaclones was also more sensitive than the parent enzyme to imazethapyr. The $I_{50}$ results showed that the *in vitro* selection had not produced ALS enzyme with increased resistance to imazethapyr, which was in contrast to maize somaclones. The

ALS enzyme in maize somaclones was altered by the tissue culture because it showed resistant to the imidazolinone herbicides (Newhouse *et al.*, 1991). Since improvement in the resistance of the somaclones in our experiment was not a result of changes in the ALS enzyme, the increased resistance of somaclones might be due to a modification to either the uptake and translocation of the herbicide or the mechanism of detoxification.

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# THE INFLUENCE OF PHENMEDIPHAM AND OZONE POLLUTION ON CATION LEAKAGE FROM SUGARBEET (*Beta vulgaris* L. cv Saxon).

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## ABSTRACT

Spring-sown crops such as sugarbeet are actively growing at times when ozone episodes are likely to occur. These crops are usually sprayed with post-emergent herbicides. Interactions may occur where photosynthetic inhibitor herbicides are applied shortly after an ozone episode. Laboratory experiments were conducted in which 2-3 leaf sugarbeet plants (*Beta vulgaris* L. cv. Saxon) were exposed to a simulated two day ozone episode (100 nl litre$^{-1}$, 7 h d$^{-1}$). Three days later, the plants were sprayed with field rate phenmedipham (1.14 kg a.i. ha$^{-1}$). Growth analysis indicated an antagonistic interaction. Membrane leakage studies indicated that the response of the leaves treated with ozone and phenmedipham was between that of the herbicide (high efflux) and ozone (low efflux). This effect was not detected until five days after phenmedipham treatment. Determinations of the leachate cation content indicated treatment with phenmedipham increased the leakage of sodium, potassium, magnesium and ammonium from the tissue, whilst ozone had no effect on leakage.

## INTRODUCTION

The major herbicide applied to sugarbeet in the U.K. is phenmedipham which inhibits photosynthesis at PS II in both crops and weeds (Cobb, 1992; Proctor, 1993). Sugarbeet is transiently affected by phenmedipham, seen as a reduction in growth from which the crop recovers within three to four weeks (Cantwell & Norris, 1973). Effects have been shown to be increased when the herbicide was applied during periods of warm and sunny weather as opposed to cool and cloudy conditions (Preston & Biscoe, 1982). In the U.K. in 1995, the majority of sugarbeet was drilled by 8$^{th}$ April (Hollowell, 1995). One month later in early May, at around the time early post-emergence herbicides were being applied, a period of hot weather led to ozone concentrations of up to 120 nl litre$^{-1}$ and the introduction of poor air quality warnings on weather forecasts (Edwards, 1995). A succession of ozone episodes can cause yield reductions in sensitive crops if a critical level of accumulated dose is exceeded during the life cycle of the crop (Fuhrer & Ashmore, 1994).

Pesticides are known to interact with ozone in plants. For example, an antagonistic interaction was observed in *Phaseolus vulgaris* between the fungicide benomyl and ozone resulting in a reduction in ozone injury (Pell, 1976). Herbicides may interact with ozone pollution on some crop plants during or after treatment. The effect of this interaction may be an alteration of the response of the plant to the herbicide (Mersie *et al*, 1989) or the metabolism of the herbicide (Hodgson *et al*, 1974). It is possible that ozone episodes may reduce the safety of the herbicide to the crop resulting in increased crop damage and decreased yields. Alternatively, antagonism

may occur leading to a reduction in ozone and/or herbicide injury. In previous studies, herbicides which inhibit photosystem II have produced antagonistic interactions with ozone, although the interaction observed depended on several factors. For example, tomato plants kept at high light intensities, exposed to 75 nl litre$^{-1}$ ozone for 3 h and treated with metribuzin produced an antagonistic response (Phatak & Proctor, 1976). Similarly, *Zea mays* sown in soil treated with atrazine and exposed to ozone at 300 nl litre$^{-1}$ for a total of 36 h over 3 weeks, showed an antagonistic interaction. However, when exposed to 200 nl litre$^{-1}$ ozone the response was additive (Mersie *et al*, 1990). An antagonistic interaction between phenmedipham and 100 nl litre$^{-1}$ ozone in sugarbeet has been previously described (Sanders *et al*, 1993). The aims of this study were to determine whether there were alterations in membrane leakage and ion content with time in response to exposure to ozone followed by treatment with phenmedipham.

## MATERIALS AND METHODS

Sugarbeet (*Beta vulgaris* cv Saxon, British Sugar, Peterborough, U.K.) was sown in J. Arthur Bowers multipurpose compost on pots of 7 cm diameter and raised in a growth cabinet (Fitotron, Sanyo, 21°C/10°C, 50% relative humidity and 180 µmole m$^{-2}$ s$^{-1}$ photosynthetically active radiation (PAR), 14 h daylength). Prior to any treatments, plants were thinned to two per pot for two pots per treatment, with two replicates of four treatments. Plants were exposed to ozone at the 2-3 leaf stage. Pots were returned to the growth cabinet after treatment.

Plants were exposed to 100 nl litre$^{-1}$ ozone for 7 h d$^{-1}$, for 2 d as described elsewhere (Dixon *et al*, submitted). Three days later, phenmedipham was applied at field rate (1.14 kg a.i. ha$^{-1}$) as Betanal E (AgrEvo Ag UK Ltd), using a laboratory pot sprayer (Mardrive Bioevaluation Unit, Stockport, U.K.; Teejet 80° flat fan nozzle, 240 litre water ha$^{-1}$, 3 bar pressure). Control plants were sprayed with distilled water. Sprayed plants were allowed to dry prior to returning them to the growth cabinet, to prevent cross contamination between the treatments.

### Membrane leakage

Strips (1 cm wide) were cut from the first and second leaves of two plants, avoiding the major veins and the edges of the leaves. These were washed in deionised water for 1 h to remove any debris from the cut surfaces. The water was then decanted off, and the tissue carefully dried and weighed. Twenty mls of deionised water was added to the tissue to give 0.07 - 0.15 g tissue ml$^{-1}$ solution. The flask containing the tissue was placed in an illuminated (180 µmole m$^{-2}$ s$^{-1}$) shaking waterbath at 20°C. Readings were taken 24 h after the final deionised water had been added to the strips. A flow through electrode (glass flow cell, K = 1, Labtech Instruments, Wrexham) was connected to a digital conductivity meter (PTI-18, F.S.A. Laboratory Supplies, Loughborough) calibrated with 2.5 mM potassium chloride. Results were expressed as µSiemens cm$^{-1}$ g$^{-1}$ tissue. One ml samples were taken for later analysis of the cation content of the surrounding solution.

### Ion chromatography

A Dionex DX-100 ion chromatograph with a Dionex autosampler controlled using AI-450 software was used to determine the cation content of the leachate. Samples (0.5 ml) were injected through an Ionpac CG12 guard column (Dionex) onto an Ionpac CS12 ion exchange column (Dionex) using 20 mM methane sulphonic acid as the eluent over a total running time of

10 min with a flow rate of 1.5 ml $min^{-1}$. Cation concentrations were calculated using the standard curves generated by injecting 0, 2, 5, 10, 25 and 50 ppm of each ion onto the column.

Replication and statistics

There were four treatments - charcoal-filtered air (controls), ozone alone, phenmedipham alone and ozone and phenmedipham. Four plants were used for each treatment which was replicated twice within the experiment and experiments were repeated three times. Results are expressed as means ± standard error.

## RESULTS AND DISCUSSION

In this study plants were exposed to ozone for two days, followed three days later by treatment with phenmedipham (Day 0). Growth studies have shown an antagonistic interaction occurs between these two stresses, where plants treated with both ozone and phenmedipham had dry weights which were greater than the expected value when the reductions due to the two treatments were added together (Sanders *et al*, 1993). Conductivity measurements were taken for 6 d after herbicide treatment to show alterations in membrane leakage. Analysis of the leachate was carried out to determine the nature of the ions leaking from the tissue.

Membrane leakage

Plants treated with phenmedipham and ozone followed by phenmedipham showed an increase (277 % and 222 % respectively) in membrane leakage reaching a maximum 2 d after herbicide treatment (Figure 1). Conversely, leakage was unaffected by exposure to ozone.

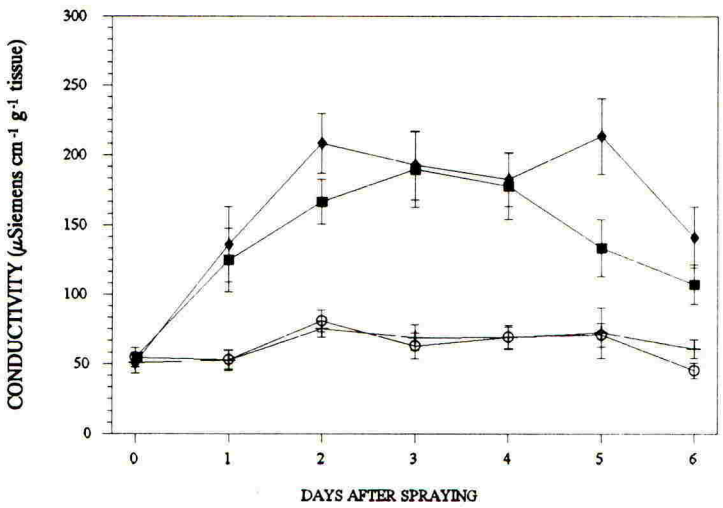


Figure 1. Effects of ozone and/or phenmedipham on the conductivity of bathing medium upon which sugarbeet leaf tissue was floated. Values are means ± standard error. Key control (+), ozone (O), phenmedipham (◆) and ozone and phenmedipham (■).

Plants treated with ozone and phenmedipham showed a trend of slightly lower membrane leakage than those treated with phenmedipham alone. There are few published studies on the effects of either ozone or phenmedipham on the membrane integrity of the plant. Ozone is known to increase the permeability of membranes in susceptible plants, although this has often been measured by $^{86}Rb$ fluxes (Evans & Ting, 1973). The changes observed in leakage are thought to be due to disruptions in the plasma membrane (Heath & Castillo, 1988) and the inhibition of pumps and transporters (Dominy & Heath, 1985). The primary mode of action of phenmedipham is to block photosynthesis, leading to a build up of excitation energy (Cobb, 1992). Transfer of the excitation energy to other molecules such as oxygen leads to the production of active species. These can include singlet oxygen, hydrogen peroxide and superoxide, which result in membrane damage. These effects have been observed for other photosystem II herbicides, such as atrazine and ioxynil (Crowley & Prendeville, 1980).

Ion chromatography

Analysis of cation concentrations in the leachate has given an indication of where the treatments were active within the cell. Ozone alone had no effect on any of the cations measured. Treatment with phenmedipham (alone and with ozone) increased the concentrations of sodium and potassium in the leachate (sodium 630 & 550 % of control respectively, potassium 520 & 530 % of control respectively, Figures 2a & b). Since both of these ions are primarily stored in the vacuole, this implied that the herbicide was having an effect on the tonoplast. Analysis of the nitrate content of the leachate also showed large increases and thus provided further evidence for an effect on the tonoplast (Data not presented).

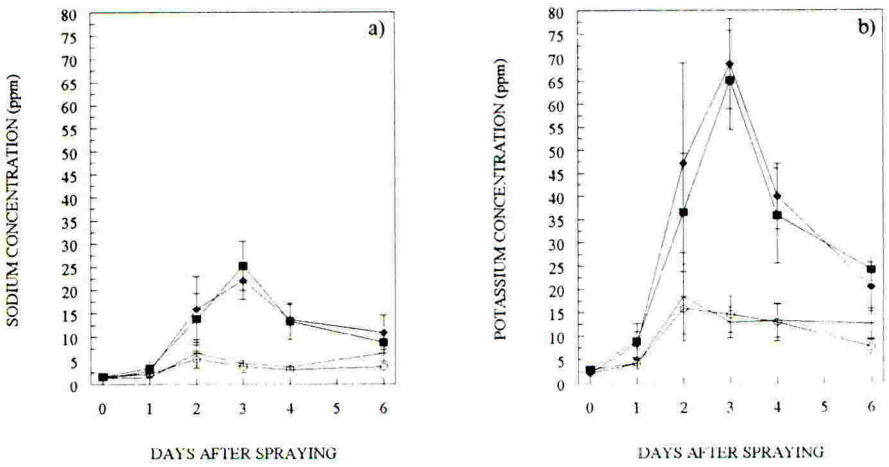


Figure 2. Effects of ozone and/or phenmedipham on the efflux of cations from sugarbeet leaves. a) sodium and b) potassium concentrations in the leachate surrounding sugarbeet leaf tissue. Values are means ± standard errors. Key control (+), ozone (O), phenmedipham (◆) and ozone and phenmedipham (■).

Similar effects were observed in magnesium (525 & 430 % of control respectively) and ammonium (1460 & 1040 % of control respectively) ion concentrations (Figures 3a & b).

Magnesium acts as a metal activator for most enzymes that use ATP or other nucleoside di- or tri-phosphate as a substrate. This cation is found mainly in the chloroplast and would be expected to decrease in concentration in the chloroplast after treatment with phenmedipham, due to the primary effect of the herbicide on this organelle. The observed increase in magnesium concentration into the leachate of phenmedipham treated plants is likely to have represented a loss of integrity of the chloroplast envelope. Ammonium ions are produced in mitochondria during photorespiration by the conversion of two glycine molecules to one serine (Sarojini & Oliver, 1983). Since $NH_4^+$ is toxic, it is incorporated with glutamate by glutamine synthetase almost immediately to produce glutamine (Givan, 1979). The observed increase in $NH_4^+$ concentration following treatment with phenmedipham may thus indicate either a breakdown in mitochondrial membranes or in the detoxification mechanism for this cation. Confirmation of this will be sought through electron microscopy and analysis of glutamine synthetase activity.

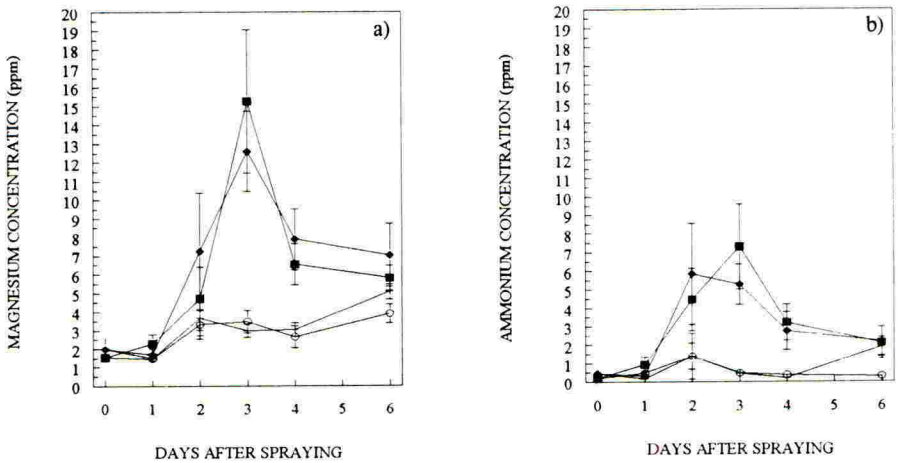


Figure 3. Effects of ozone and/or phenmedipham on a) magnesium and b) ammonium concentrations in the leachate surrounding sugarbeet leaf tissue. Values are means ± standard error. Key control (+), ozone (O), phenmedipham (◆) and ozone and phenmedipham (■).

## CONCLUSION

Ozone had no effect on the leakage of electrolytes from the tissue. The recorded increases in membrane leakage following phenmedipham treatment appears to have resulted from the leakage of ammonium, magnesium sodium and potassium from several organelles within the cell. Treatment with ozone pollution and phenmedipham did not alter the effect of the herbicide on membrane leakage in sugarbeet tissue.

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# CHARACTERISTICS OF ARYLOXYPHENOXYPROPIONATE HERBICIDE INTERACTIONS WITH ACETYL-CoA CARBOXYLASES OF DIFFERENT GRAMINICIDE SENSITIVITIES

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## ABSTRACT

Kinetic analyses of purified acetyl-CoA carboxylase (ACCase) from leaves of maize (two isoforms) or the graminicide-resistant species, *Poa annua* (annual meadow grass), were carried out for inhibition by two aryloxyphenoxypropionate herbicides, quizalofop and fluazifop. Significant differences in cooperativity of herbicide binding were found between sensitive or insensitive ACCases. Like the ACCase activity, carboxylation of propionyl-CoA (PCCase activity) was appreciably inhibited by the aryloxyphenoxypropionates only in the herbicide-sensitive isoforms. Sensitivity to herbicides in the maize isoforms was associated with a high PCCase:ACCase activity ratio.

## INTRODUCTION

Acetyl-coenzyme A carboxylase (ACCase; EC 6.4.1.2.) is a biotinylated enzyme that catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA - the first committed step in lipid biosynthesis (Harwood, 1988). It is the target of two classes of important grass-specific herbicides (graminicides), the aryloxyphenoxypropionates (which includes quizalofop and fluazifop) and the cyclohexanediones (see Harwood, 1991). These graminicides specifically inhibit the multifunctional (*c.*220kDa subunit) form of ACCase present in the chloroplasts of grasses (the *Graminaceae*) but absent in the chloroplasts of dicotyledons (Egli *et al.*, 1993; Alban *et al.*, 1994; Konishi & Sasaki, 1994). Grass crops such as maize (*Zea mays*) are generally susceptible, as are most annual and perennial grass weeds. However, certain grass species, including *Poa annua*, are resistant to examples of these compounds because their ACCases are inherently insensitive (Lichtenthaler *et al.*, 1992; Herbert *et al.*, 1993; Holt *et al.*, 1993).

Multifunctional ACCases with limited graminicide sensitivity exhibit cooperativity in binding aryloxyphenoxypropionates (Dehaye *et al.*, 1994, Evenson *et al.*, 1994). Here, we carried out separate kinetic analyses on two isoforms of ACCase from maize leaves and a *P. annua* ACCase in order to compare their cooperative behaviour in inhibition by quizalofop and fluazifop and their abilities to carboxylate propionyl-CoA.

## MATERIALS AND METHODS

### Plant material and chemical sources

Plant material was as described previously (Herbert *et al.*, 1993). Quizalofop free acid ((RS)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy] propionic acid) was provided by Rhône-Poulenc Agriculture Ltd. Fluazifop free acid ((RS)-2-[4-(5-trifluoromethyl-2-pyridyloxy] propionic acid) was bought from Cluzeau Info. Laboratoire, Ste.-Foy-la Grande, France. Sodium [$^{14}$C]bicarbonate (3.7-37GBq/mmol) was bought from Amersham International, Amersham, UK. Other materials were bought from Sigma, Poole, Dorset, UK, or Pharmacia Biosystems Ltd., Central Milton Keynes, UK.

### Purification of ACCase from maize or *P. annua* leaves

ACCases were purified in a three-step protocol using ammonium sulphate fractionation, Red 120-agarose dye-ligand chromatography and Q-Sepharose anion-exchange chromatography.

### Assay for ACCase or PCCase

Duplicated initial velocities were measured as described previously (Dehaye *et al.*, 1994).

### Preparation of quizalofop or fluazifop solutions

Stock solutions (30mM) were made by dissolving herbicide in acetone and then adding 0.5M Hepes-KOH (pH 8.0) to give an acetone concentration of 9% (v/v). Stock solutions were protected from light, stored at -20°C between experiments and used within 3 weeks. After appropriate dilution in distilled water, solutions were added immediately to assay mixtures. Final acetone concentrations in the assays were always less than 0.3%, a concentration that had no effect on ACCase activity.

### Determination of kinetic parameters

Km values were estimated by fitting data to the Michaelis-Menten equation. Data for inhibition by quizalofop or fluazifop were fitted to two steady-state rate equations (Segel, 1993): The Hill equation,

$$v = \frac{V \cdot K'}{K' + [I]^{n_{app.}}}.$$

where $n_{app.}$, the apparent Hill coefficient, is a measure of the cooperativity between enzyme subunits for the binding of inhibitor, I. A $n_{app.}$ value >1 indicates positive cooperativity; a $n_{app.}$ value <1 indicates negative cooperativity; a $n_{app.}$ value = 1 indicates that there is no cooperativity. K' is an inhibition constant; V is control velocity and v is inhibited velocity.

And an equation for simple hyperbolic inhibition,

$$v = \frac{V}{1 + ([I]/Ki)}.$$

Statistical analysis was performed with SigmaPlot (Jandel Scientific, Corte Madera, California).

## RESULTS

### Purification and characterisation of ACCases from maize or *P. annua* leaves

Two multifunctional isoforms of maize ACCase were separated by anion-exchange chromatography, as observed previously in analogous purifications (Egli *et al.*, 1993; Ashton *et al.*, 1994). A major isoform, ACCase1, represented 80-85% of the total recovered activity and was sensitive to quizalofop or fluazifop (see Table 1). ACCase1 was localized in chloroplast stroma. A minor isoform, ACCase2, was not appreciably sensitive. These results agreed with previous studies (Egli *et al.*, 1993; Ashton *et al.*, 1994). ACCase activity from *P. annua* could not be separated into distinct isoforms. However, purified *P. annua* ACCase contained two biotinylated polypeptides (approx. 220kDa and 210kDa), as did the initial leaf homogenate. Therefore, the *P. annua* ACCase under investigation probably contained two multifunctional ACCase isoforms, by analogy with other grass species (Egli *et al.*, 1993; Konishi & Sasaki, 1994). The different ACCases were estimated by SDS/PAGE and Coomassie blue staining to be 25-40% pure. All were dimers; both maize isoforms were homodimers. The subunit molecular masses and Km values, were in agreement with data for multifunctional ACCases from other plant species (e.g. Egli *et al.*, 1993; Dehaye *et al.*, 1994). No separate PCCase enzyme was detected.

Maize ACCase1 was *c.*2000-fold more sensitive to quizalofop than maize ACCase2 but only *c.*150-fold more sensitive to fluazifop. *P. annua* ACCase was intermediate in sensitivity to quizalofop (see Figure 1). The PCCase:ACCase ratio was lowest in maize ACCase2. Some of the properties of these different ACCases are shown in Table 1.

### Cooperativity of binding of quizalofop or fluazifop to maize or *P. annua* ACCases

Curve-fits for inhibition of the different ACCases by quizalofop are shown in Figure 2. Maize ACCase1, exhibited little cooperativity in binding quizalofop ($n_{app.}$=0.86±0.03) or fluazifop ($n_{app.}$=1.16±0.09). i.e. data fitted only slightly better to the Hill equation than to the equation for simple hyperbolic inhibition. However, the ACCases of reduced sensitivity did exhibit cooperativity: Maize ACCase2 exhibited strong positive cooperativity in binding quizalofop ($n_{app.}$=1.85±0.19) and a lesser positive cooperativity in binding fluazifop ($n_{app.}$=1.59±0.09). *P. annua* ACCase exhibited negative cooperativity in binding quizalofop ($n_{app.}$=0.54±0.02).

## DISCUSSION

The data presented here support previous evidence that insensitivity to graminicide is associated with: (i) cooperativity in graminicide binding and (ii) a lesser ability to carboxylate propionyl-CoA. An explanation is that in sensitive ACCase (e.g. maize ACCase1) both subunits of the ACCase dimer have binding sites that are 'open' to propionyl-CoA and also more accessible to graminicide. In the relatively insensitive ACCases (e.g. maize ACCase2), however, only one site is 'open'. Binding of graminicide to this 'open' site would make the second, 'closed', site more receptive (positive cooperativity) to graminicide. Because isoforms of *P. annua* ACCase were not separated in our purification, the interpretation of the inhibition data and of the apparent negative cooperativity is not as straightforward. However,

Figure 1 Inhibition of maize or *P. annua* acetyl-CoA carboxylases by quizalofop

Duplicate values are shown.

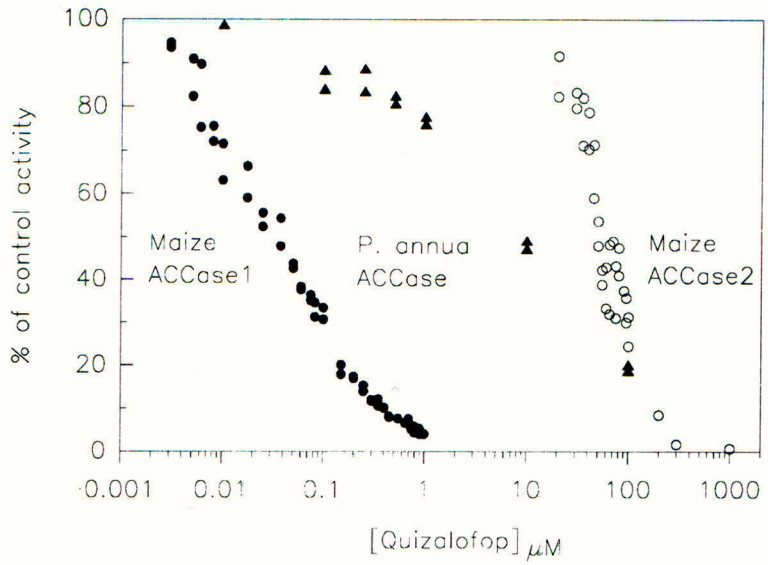


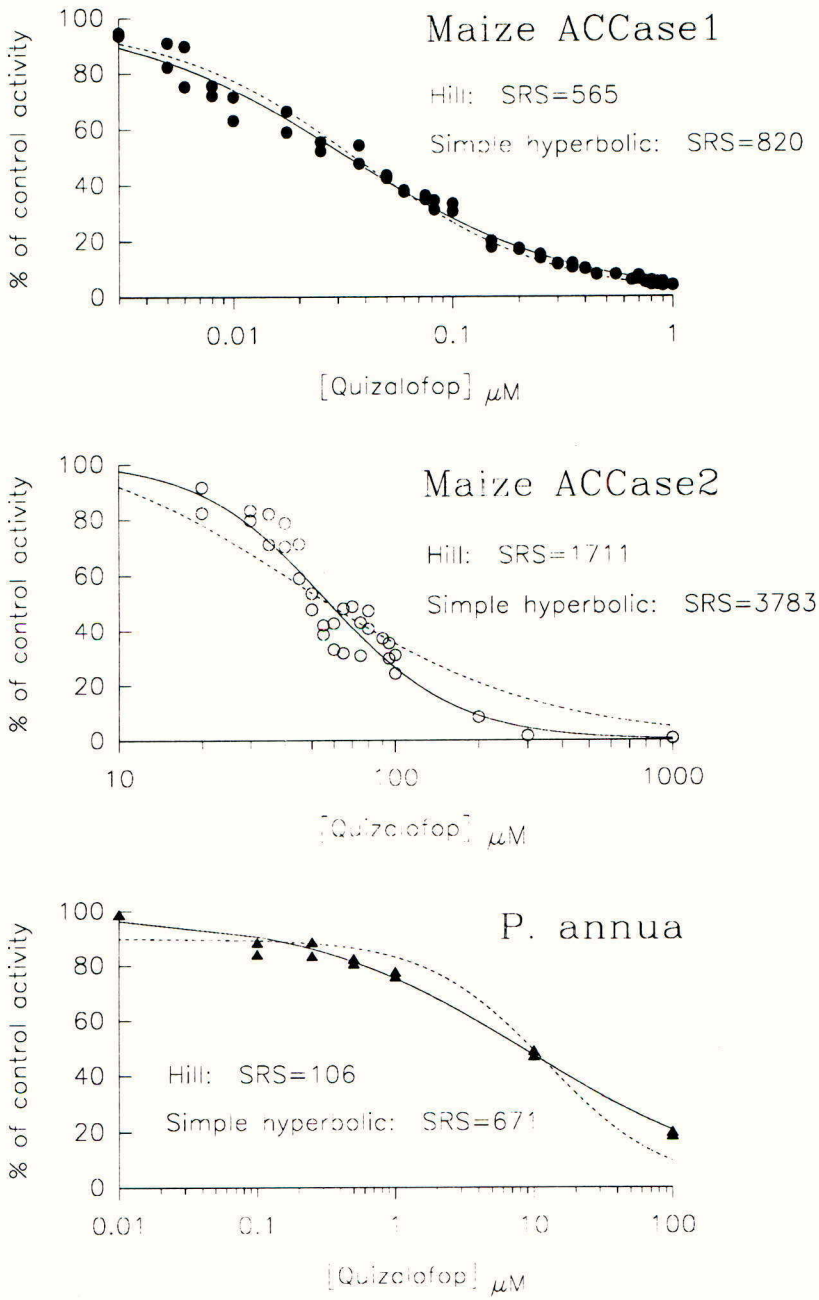

Table 1 Characteristics of purified maize or *P. annua* acetyl-CoA carboxylases

| | Maize ACCase1 | Maize ACCase2 | *P. annua* ACCase |
|---|---|---|---|
| Subunit mol. mass (kDa) | *c.*230 | *c.*220 | *c.*210 & 220 |
| Native structure | dimer | dimer | dimer |
| pH optimum | 8.0 | 7.5 | 8.0 |
| Km AcCoA (μM)* | 122±15 | 113±12 | 66±12 |
| Km ATP (μM)* | 80±9 | 76±8 | 109±29 |
| Km $HCO_3^-$ (μM)* | 1091±242 | 397±60 | 1137±304 |
| $I_{50}$ quizalofop (μM) | 0.03 | 60 | 10 |
| $I_{50}$ fluazifop (μM) | 10 | 1500 | nm |
| PCCase:ACCase | 0.67 | 0.36 | 0.64 |

* ± standard deviation; nm, not measured

Figure 2 Curve fits for cooperative (——) or non-cooperative (-----) binding of quizalofop to maize or *P. annua* acetyl-CoA carboxylases.

SRS = sum of residual squares.



another ACCase preparation that was a mixture of two isoforms, that from a diclofop-resistant biotype of *Lolium multiflorum,* also showed negative cooperativity, in binding the above aryloxyphenoxypropionate ($n_{app.}$ approx. 0.62). Significantly, ACCase from the susceptible biotype showed only weak cooperativity ($n_{app.}$ = 1.2) (Evenson *et al.*, 1994).

The greater difference in cooperative effects between maize isoforms in the binding of quizalofop compared with fluazifop, together with the smaller difference in sensitivity between isoforms toward fluazifop and the greater potency of quizalofop, indicates the highly specific nature of binding of individual graminicides. A full explanation for the different binding characteristics of plant ACCase isoforms must await knowledge of their detailed structure. Such information can then be applied to the rational design of new herbicides.

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# ON THE MODE OF ACTION OF THE NEW, SELECTIVE HERBICIDE QUINMERAC

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## ABSTRACT

Quinmerac (7-chloro-3-methyl-quinoline-8-carboxylic acid) is a new, highly selective herbicide which effectively controls important dicotyledonous weeds, such as cleavers (*Galium aparine*), in sugarbeet, oilseed rape and small grain cereals. Studies using young cleaver plants treated in hydroponics, showed that the mode of action of quinmerac is based on its auxin activity. In the shoot tissue ethylene formation was stimulated through induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis which was accompanied by leaf epinasty and massive accumulations of abscisic acid (ABA). The latter effect resulted in reductions in stomatal aperture, water consumption, $CO_2$ uptake and plant growth. Stem epinasty and local necrosis on the stems appeared to be directly controlled by the auxin activity of quinmerac. In sugarbeet, oilseed rape and wheat corresponding effects were not observed. It is concluded that different tissue/target sensitivity contributes to the mechanism of quinmerac selectivity.

## INTRODUCTION

Substituted quinolinecarboxylic acids, such as quinclorac and quinmerac, represent a new class of highly selective herbicides (Wuerzer *et al.*, 1985, Walter *et al.*, 1994). While quinclorac is used in rice (*Oryza sativa*) particularly to suppress the major grass weed, barnyard grass (*Echinochloa crus-galli*), quinmerac (Fig. 1) effectively controls important dicotyledonous weeds, such as cleavers (*Galium aparine*), in sugarbeet (*Beta vulgaris*), oilseed rape (*Brassica napus*) and wheat (*Triticum aestivum*).

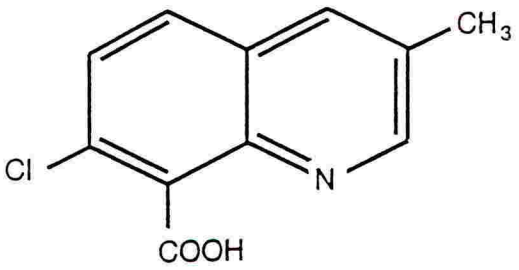


Fig. 1 Structural formula of quinmerac

Characteristic of auxin herbicides, quinmerac induces inhibition of growth in sensitive

plants which is accompanied by epinasty, altered $CO_2$ uptake and water relations, foliar chlorosis and wilting (Berghaus & Retzlaff, 1989). For several decades theories as to how auxinic herbicides induced phytotoxicity remained vague with indistinct conceptions revolving around aberrant nucleic acid biosynthesis and possible effects on plasmalemma ATPases. More recently, attention has focused on a stimulation of ethylene and cyanide biosynthesis (Pallett, 1991, Grossmann & Kwiatkowski, 1995). The latter effect has been suggested to play a key role in the mode of action of quinclorac in barnyard grass (Grossmann & Kwiatkowski, 1995). Therefore, the present study concentrated on possible relations between phenotypical and physiological alterations and changes in the hormonal status of cleaver plants treated with quinmerac.

## MATERIALS AND METHODS

Young plants of cleavers (*Galium aparine*), sugarbeet (*Beta vulgaris* cv. Viktoria), oilseed rape (*Brassica napus* cv. Petranova) and wheat (*Triticum aestivum* cv. Kanzler) were cultivated in 320 ml aerated glass vessels containing 1/2 strength Linsmaier-Skoog medium (3 to 9 plants/vessel, 5 replications) in 16/8h light/dark cycles at 25/22 °C and 75 % relative humidity (according to Grossmann *et al.,* 1994). After 1 d of adaptation, quinmerac was added to the medium in acetone solution (0,1 % final concentration of acetone). Ethylene formation was measured by gas chromatography after transfer of the plants into sealed glass cylinders and withdrawal of gas samples (Grossmann & Kwiatkowski, 1995). For determination of ethylene shortly after quinmerac application, plants were cultivated directly in glass cylinders containing medium and quinmerac. Stomatal behaviour (determined by leaf diffusion resistance), water consumption and growth parameters were also measured. Plant organs from parallel vessels were harvested, immediately frozen and powdered under liquid $N_2$. Determinations of phytohormones were performed by immunoassay after extraction of powdered plant material with 80 % aqueous methanol and HPLC separation, as previously described (Weiler *et al.*, 1986, Grossmann *et al.*, 1994). ACC and malonyl ACC were extracted with 70 % aqueous ethanol and assayed by converting them to ethylene which was quantified by gas chromatography (Lizada & Yang, 1979). For determination of $CO_2$ uptake, plants were cultivated hydroponically in an illuminated glass chamber which received a constant stream of air. The amount of $CO_2$ assimilated per unit time was determined continously from the difference between the $CO_2$ contents of the inflowing and outflowing air streams, as described by Walter *et al. (*1994). Detached shoots and roots of cleaver were incubated in reagent tubes (one explant per tube) with 1 ml 1/2 concentrated Linsmaier-Skoog medium at 25 °C for 24 h with or without light (according to Grossmann & Kwiatkowski, 1995).

## RESULTS AND DISCUSSION

Within 5 h of the hydroponic treatment of cleaver plants at the second whorl stage with 10 µM and 1 µM quinmerac, epinastic symptoms on stems and leaves developed which were followed during the next 20 h by an inhibition of root and particularly shoot growth, and a reduction in water consumption (not shown). After 2 to 4 d the shoots showed red discolorations and necrotic constrictions on the stem accompanied by pro-

gressive chlorosis and wilting. The levels of the direct biosynthetic precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), and ethylene formation began to increase within 3 h (Fig. 2). More slowly, accumulations of abscisic acid (ABA) in root and even more in shoot tissue proceeded (Fig. 2) and reached 70 times the level in untreated plants after 48 h (not shown). This increase in ABA levels was also confirmed by combined gas chromatography-mass spectrometry analysis (W Dreher, BASF, Limburgerhof, Germany; personal communication). The concentrations of gibberellins, cytokinins, and 3-indoleacetic acid (IAA) were only slightly changed (not shown). As a by-product of ethylene synthesis, HCN was increasingly released only after 2 to 3 d of quinmerac application (not shown) which indicated that cyanide was not involved in the initiation of the growth effects. Application of 100 µM IAA also increased ACC and ABA levels in shoot and root tissue of cleaver and induced phenotypic alterations in plants similar to those caused by quinmerac. Likewise, ethephon,

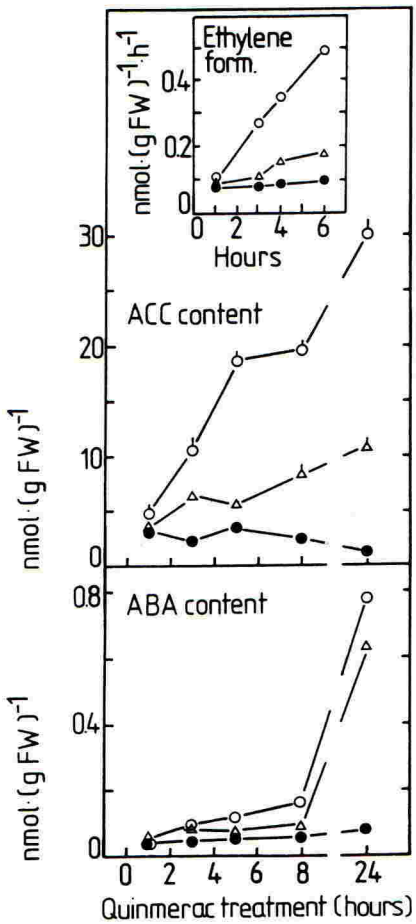


Fig. 2 Time course of the effect of quinmerac on ethylene formation (inset) and the contents of ACC and immunoreactive ABA in shoots of cleaver plants. ●Control, treatment with Δ 1µM, o 10 µM of quinmerac. Vertical bars represent SE of the mean.

which generates ethylene chemically, elicited leaf epinasty and led to an increase in ABA levels and reduction in shoot growth (not shown). Dose-response experiments with quinmerac applied for 24 and 48 h showed that ABA accumulation was in close correlation with reductions in stomatal aperture, water consumption, photosynthetic

$CO_2$ uptake, and shoot and root fresh weight (not shown). Exogenously applied ABA mimicked quinmerac action on these parameters at comparable endogenous ABA concentrations. Later, leaf senescence was promoted, along with red discolorations of stem and leaf areas. Further experiments were carried out to assess whether the effects of quinmerac could be prevented by the ACC synthesis inhibitor PACME, [[(Isopropyliden)-amino]-oxy]-acetic acid-2-(methoxy)-2-oxoethylester (Grossmann *et al.*, 1991) Foliar treatment with 0.1 µM PACME 1 d before root application of 0.03 µM quinmerac decreased herbicide-induced ACC formation, ABA accumulation and reduction of shoot growth (not shown). The effects of quinmerac could partly be restored when plants were additionally sprayed with 100 µM ACC. When isolated shoots and roots of cleaver were treated with quinmerac *in vitro*, only the shoot showed a significant stimulation of ethylene synthesis and ABA levels (not shown).

These results suggest that the biochemical target of quinmerac in cleaver is exclusively localized to the shoot tissue. Here, quinmerac stimulates ACC and ethylene production, possibly by inducing *de novo* synthesis of ACC synthase (Fig. 3). Ethylene triggers leaf epinasty and the accumulation of ABA which is translocated within the

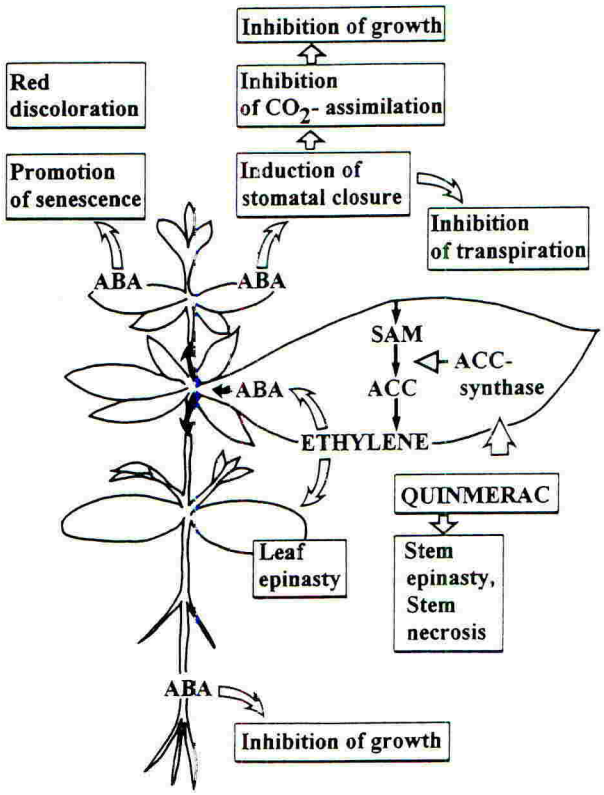


Fig. 3 Proposed model of the mode of action of quinmerac in cleavers. The arrows represent a direct or indirect effect on phytohormone levels and physiological processes. ABA, absicisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, S-adenosylmethionine.

plant. As physiological consequences, ABA accumulation results in reductions in stomatal aperture, transpiration, $CO_2$ assimilation and plant growth, in red discolorations of the shoot tissue and the promotion of senescence. This is the first time that this mechanism has been described for an auxin herbicide. On the other hand, the epinastic stem curvature and necrosis appear to be primarily controlled by the auxin activity of quinmerac, since IAA also induces these symptoms, which are not, however, induced by ethephon, cyanide or ABA.

In sugarbeet, oilseed rape and wheat corresponding effects were not observed (Fig. 4). Thus, the main reason for quinmerac's selectivity lies in the lower sensitivity to the herbicide of the site of action - probably the ACC synthase - in the crop species . In wheat, tolerance to quinmerac is additionally increased by a more rapid metabolism of the compound (Berghaus & Retzlaff, 1989).

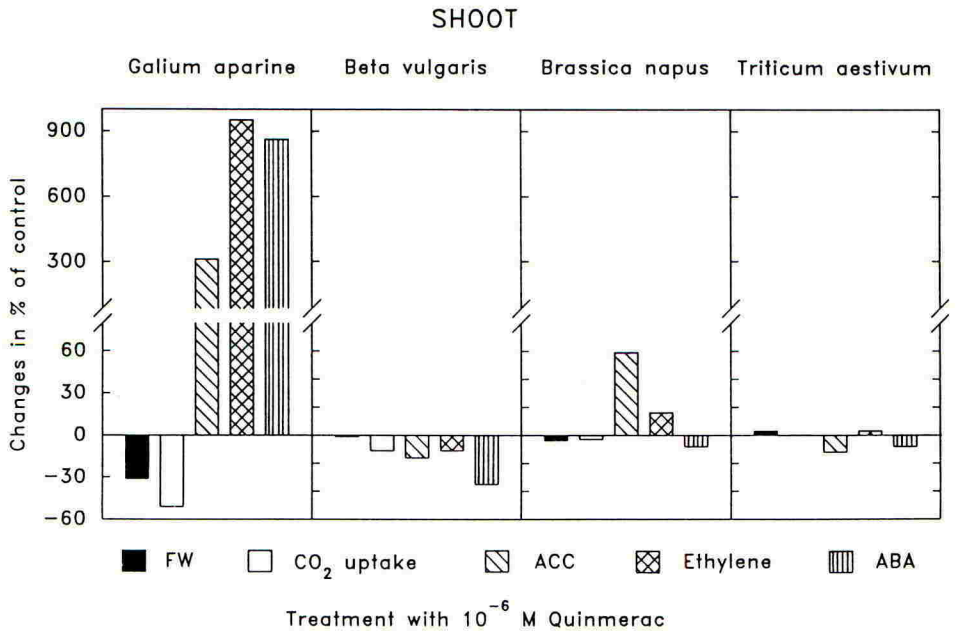


Fig. 4 Influence of quinmerac on fresh weight (FW), $CO_2$ uptake and ethylene formation and levels of ACC and immunoreactive ABA in shoots of cleaver, sugarbeet, oilseed rape and wheat plants after 48 h in hydroponics.

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# NOVEL REDOX MEDIATOR HERBICIDES: HETEROCYCLIC DERIVATIVES OF ISOQUINOLINETRIONES

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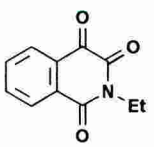
## ABSTRACT

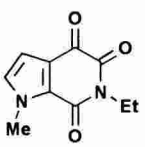
1,3,4(*2H*)-Isoquinolinetrione analogues in which the benzo-ring is replaced with a 5-membered heteroaromatic ring have been prepared and evaluated as herbicides. These compounds have the free-radical properties required to function as efficient redox mediators, as determined by cyclic voltammetry; they are potent stimulators of the light-dependent consumption of oxygen at photosystem I in isolated chloroplasts; and some are fast-acting post-emergence herbicides which produce symptoms of desiccation. The compounds are unstable towards hydrolysis, and this was considered to be the major factor limiting the overall herbicidal effects of some compounds, though other parameters relating to uptake and/or translocation were also found to play a significant role in limiting the full expression of herbicidal activity.

## INTRODUCTION

We recently reported that 1,3,4(*2H*)-isoquinolinetrione derivatives, *e.g.* **1**, constitute a new class of herbicides which act by redox mediation of photosystem I (Mitchell *et al.*, 1995). Our main conclusions from a preliminary structure-activity-property study were that hydrolytic instability is likely to be the key factor which limits the overall expression of herbicidal activity for a series of analogues of **1**, though other factors relating to uptake and/or translocation, particularly high melting point but also high log *P*, play a role in moderating this activity. As part of our continuing search for isoquinolinetrione derivatives with improved herbicidal properties, we were interested in evaluating some analogues in which the aromatic ring is replaced by a 5-membered heteroaromatic ring. Such compounds have received scant attention in the chemical literature; only a handful of substituted tri- and tetra-cyclic isoquinolinetrione analogues have been reported, and simple bicyclic derivatives are unknown. In this paper we report the preparation of the hetero-isoquinolinetrione analogues **2**, **3**, **4** and **5**, and describe their evaluation as redox mediator herbicides.




**1**

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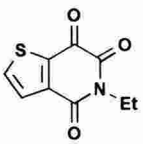


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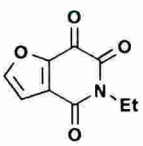
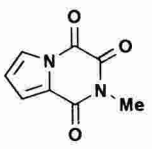


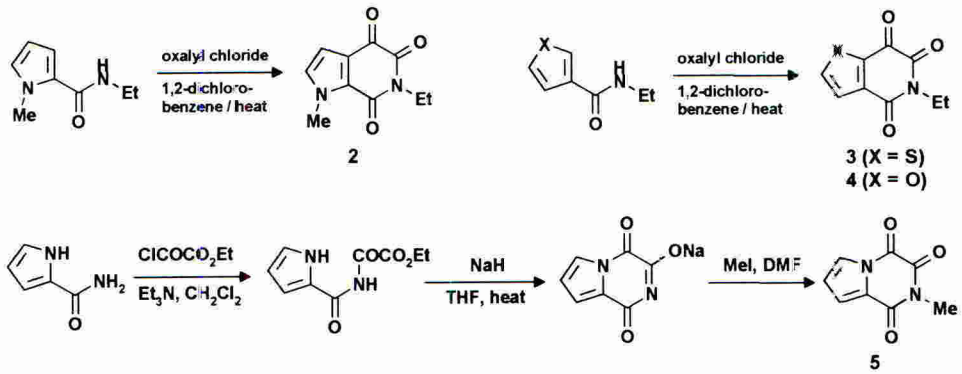

**4**




**5**

## PREPARATION OF HETEROCYCLIC ISOQUINOLINETRIONE ANALOGUES

The methods used for the preparation of the new heterocyclic isoquinolinetrione analogues are summarized in Scheme 1. The pyrrolo-trione **2** was prepared from 1-methylpyrrole-2-(*N*-ethyl)carboxamide and oxalyl chloride (*c.f.* Vekemans and Hoonaert, 1980); analogues **3** and **4** were prepared similarly from thiophene-3-(*N*-ethyl)carboxamide and furan-3-(*N*-ethyl)carboxamide, respectively. Compound **5** was prepared by treatment of pyrrole-2-carboxamide with monoethyl oxalyl chloride, followed by cyclization of the resultant imide with sodium hydride and subsequent methylation using methyl iodide.




Scheme 1. Methods for the preparation of heterocyclic analogues of isoquinolinetriones

## ELECTROCHEMICAL STUDIES AND PHOTOSYSTEM I ACTIVITIES

### Electrochemical Methods

Electrochemical measurements were made by cyclic voltammetry using a HI-TEK DT2101 potentiostat, HI-TEK PPRI waveform generator and Philips PM 8271 XY recorder. A three-electrode cell configuration was used with platinum wire working and secondary electrodes and a saturated calomel reference electrode. Compound (1-2 mg) was added to the cell (4 ml) containing *N,N*-dimethylformamide, with tetrabutylammonium bromide (0.1 M) as the supporting electrolyte, purged with nitrogen at room temperature. Cathodic (*Epc*) and anodic (*Epa*) peak potentials were recorded within the 0 to -2 V range at voltage sweep rates of 0.02, 0.1 and 0.5 V $s^{-1}$, using sodium anthraquinone sulfonate (AQS) as a standard (*c.f.* Mitchell *et al.*, 1995).

### Methods for Determining Stimulation of Oxygen Consumption by Photosystem I

Photosystem I electron transport was recorded as light-dependent oxygen consumption in isolated spinach chloroplasts (Walker, 1980) using an artificial electron donor couple. Measurements were made with a Hansatech oxygen electrode DW-2 at 20 °C under saturating red light from a Schott Cold Light Source (Model KL1500). The reaction mixture (2 ml final volume) contained chloroplasts (20 µg Chl); 1 ml of a double-strength solution of

Resuspension Medium (Walker, 1980); an electron donor couple consisting of 20 µl 100 mM sodium isoascorbate (1 mM) and 5 µl 100 mM *N,N,N',N'*-tetramethylphenylenediamine (0.25 mM); 10 µl 100 mM sodium azide (0.5 mM) and 2 µl 1 mM diuron (1 µM). In setting up the reaction in the electrode cuvette, chloroplasts were added to water (an amount that brought the final volume of the reaction mixture to 2 ml) to burst them osmotically prior to the addition of the other reaction components.

Compounds were initially dissolved in dimethyl sulphoxide to a final stock concentration of 10 mM. Light-dependent oxygen consumption induced by compounds was measured at concentrations between 1 and 20 µM, and the consumption with no compound (Mehler reaction) was subtracted from each value obtained. The rate of oxygen uptake for each concentration was measured over a period of at least 60 seconds, during which time oxygen consumption was linear. The dose responses over a 20 µM range were plotted using a curve-fitting analysis programme to obtain values for 50% stimulation of oxygen consumption ($S_{50}$) and $V_{max}$. In-vitro potencies are expressed as $V_{max}/S_{50}$ ratios (*c.f.* Mitchell *et al.*, 1995).

Results

The electrochemical data and in-vitro potencies for the new heterocyclic isoquinolinetrione analogues are compared to those of the reference compound **1** in Table 1.

TABLE 1. Electrochemical data and in-vitro activities for heterocyclic isoquinolinetriones

| *Compound* | *Epc* (V)[a] | *Epa* (V)[a] | *ipa/ipc* | $V_{max}/S_{50}$ |
|---|---|---|---|---|
| **1** | -0.68 | -0.59 | ~1 | 175 |
| **2** | -0.87 | -0.78 | ~1 | 180 |
| **3** | -0.55 | -0.46 | ~1 | 258 |
| **4** | -0.56 | -0.42 | ~1 | 707 |
| **5** | -0.90 | -0.81 | ~1 | 61 |

[a] vs standard calomel electrode

For each of the heterocyclic compounds **2** - **5**, the cathodic peak potential (*Epc*) of the first reduction step was observed to be independent of both the voltage sweep rate and the potential for switching from cathodic (reduction) to anodic (oxidation) scan. Compounds **2**, **3** and **5** gave anodic peak potentials (*Epa*) 90 mV more positive than their first cathodic peak potentials (*Epc*); for compound **4** the *Epa* was 140 mV more positive than the *Epc*. In each case the anodic to cathodic peak current ratio (*ipa*/*ipc*) was approximately 1. Overall, these electrochemical data indicate that, under the experimental conditions used, the new heterocyclic analogues **2** - **5** generate one-electron reduced species with properties similar to those previously reported for the benzo-compound **1** and the quinone standard AQS (Mitchell *et al.*, 1995; Heinze, 1984), and thus they are likely to have the free-radical properties required to function as efficient redox mediators of photosystem I (Bowyer and Camilleri, 1987).

The in-vitro photosystem I activities confirm that these compounds are indeed efficient redox mediators. Compound **2** is about as active as the parent benzo-compound **1**. Compounds **3** and **4** are more active than compound **1**, with the furano-derivative **4** showing exceptional activity. Compound **5**, however, is less active. Compared to paraquat ($V_{max}/S_{50}$ = 83), all of the compounds show significant stimulation of oxygen consumption by photosystem I.

The new analogues **2** - **5** cover a range of reduction potentials, characterized by *Epc* values in DMF from -0.55 V to -0.90 V. With an *Epc* of -0.87 V, compound **2** is expected to be less readily reduced to its radical-anion than compound **1** (*Epc* -0.68 V). However, the rate of electron-transfer from radical-anion to oxygen could be higher for compounds of lower reduction potential (Wilson *et al*, 1986; Wardman, 1989), and it is therefore likely that in-vitro activity reflects a balance between rate of reduction to the radical-anion and subsequent rate of electron-transfer to yield superoxide. Whilst there is no obvious linear relationship between reduction potential and in-vitro activity, there is a tendency for compounds with the higher (*i.e.* less negative) reduction potentials to be the most potent.

## HERBICIDAL ACTIVITY

### Determination of Herbicidal Potency

The compounds were tested in glasshouse screens for post-emergence herbicidal activity (*c.f.* Mitchell *et al.*, 1995) which contained the following species: *Lycopersicon esculentum*, *Brassica oleracia*, *Chenopodum album*, *Amaranthus retroflexus*, *Avena fatua*, *Alopecurus myosuroides*, *Setaria viridis* and *Echinochloa crus-galli*. The compounds were sprayed at a rate equivalent to 1000 g/ha. Damage to plants was assessed five days after treatment by comparison with untreated plants, on a scale of 0 to 9 where 0 indicates no damage and 9 indicates complete kill.

### Determination of Physicochemical Parameters

Hydrolysis studies were carried out by adding 20 µl of a 1 g litre$^{-1}$ solution of the compound in acetonitrile to a pH 7 buffer solution and measuring compound loss over time by HPLC or spectrophotometric analysis at 25 °C. Half-lives were determined from plots of peak height or absorbance data versus time (*c.f.* Mitchell *et al.*, 1995).

Melting points were measured using a Mettler FP61 apparatus, and are uncorrected.

Octanol-water partition coefficients (log *P*) were measured by an HPLC method using reverse phase columns coated with octanol (*c.f.* Mitchell *et al.*, 1995).

### Results

Compounds **2** - **4** caused rapid desiccation of plants, in a manner similar to that found for the benzo-compound **1** and paraquat (Mitchell *et al*, 1995; Bowyer and Camilleri, 1987); initial symptoms were observed within a few hours of treatment. Compound **5**, however, showed no activity. In contrast to compound **1**, the new compounds **2** - **4** were only significantly active against broad-leaved species, and in order for meaningful comparisons to be drawn

between compounds, herbicidal activity is quoted as the average score against *Lycopersicon esculentum*, *Brassica oleracia*, *Chenopodum album* and *Amaranthus retroflexus*. The reasons for the low grass activity of the new hetero-derivatives are not clear, but may be related to the factors governing activity against broad-leaved species which are discussed below. The broad-leaf herbicidal activities and physicochemical properties of the new heterocyclic isoquinolinetriones **2** - **5** are compared to those of compound **1** in Table 2.

TABLE 2. Broad-leaf herbicidal activities and physicochemical properties of heterocyclic isoquinolinetriones.

| *Compound* | *Herbicidal activity*[a] | *Hydrolysis* $t_{1/2}$ (min) | *m.p.* (°C) | *Log P* |
|---|---|---|---|---|
| **1** | 6.3 | 25 | 95 | 1.1 |
| **2** | 0.3 | 230 | 222 | 0.9 |
| **3** | 7.1 | 18 | 123 | 0.9 |
| **4** | 2.4 | 2 | 226 | 0.4 |
| **5** | 0.0 | 3 | 159 | <1 |

[a] average score against *Lycopersicon esculentum*, *Brassica oleracia*, *Chenopodum album* and *Amaranthus retroflexus* (0 - 9 scale) at 1000 g/ha.

The compounds show widely differing levels of in-vivo herbicidal activity which are not related in an obvious manner to their in-vitro activity. Disappointingly, none shows significantly better activity than the parent benzo-derivative **1**. Nevertheless, some significant observations can be made regarding the extent to which physicochemical properties moderate the overall expression of herbicidal activity within this series of compounds.

The pyrrolo-derivative **2** is the most hydrolytically stable of the new analogues, being about an order of magnitude more stable than compound **1**. Thus, although this compound can by no means be considered to be hydrolytically stable, it is unlikely that hydrolysis is the principal cause of its low herbicidal activity. Compound **2** has a very high melting point (*i.e.* it is highly crystalline) which is likely to adversely affect its uptake and distribution relative to compound **1** following foliar application. This is in keeping with our finding that *N*-alkyl analogues of the benzo-compound **1** with higher melting points all show reduced levels of herbicidal activity relative to compound **1** itself (Mitchell *et al.*, 1995). We believe that this is the major factor limiting the expression of herbicidal activity of compound **2**.

The thiophene **3** is by far the most active of the heterocyclic analogues, showing a level of broad-leaf activity slightly greater than that of the parent benzo-compound **1**. Given that this analogue has physicochemical properties broadly similar to compound **1**, its higher activity, both in-vivo and in-vitro, most probably reflects its more positive reduction potential.

The furano- and pyrrolo-analogues (**4** and **5**, respectively) are each about about an order of magnitude less stable towards hydrolysis than compound **1**. For these compounds it is highly likely that hydrolysis severely limits the full expression of the intrinsic potency in-vivo, being

of greater significance than either melting point (compound **4**) or reduction potential (compound **5**) effects. This is analogous to our finding that derivatives of **1** with hydrolysis half-lives of 2 - 3 minutes or less are virtually inactive as herbicides (Mitchell *et al.*, 1995).

Given the high hydrolytic instability and high melting point of compound **4**, it is perhaps surprising that this compound shows any herbicidal activity at all. This activity is assumed to be due in part to the compounds exceptionally high intrinsic potency (Table 1). However, it also provides an indication that a significant herbicidal effect can be triggered by a redox mediator in a relatively short period of time (perhaps a few minutes?), prior to loss of the xenobiotic, in this case due to rapid hydrolysis.

## CONCLUSIONS

The novel heterocyclic isoquinolinetrione derivatives **2** - **5** have the free-radical properties required to function as redox mediators of photosystem I, and show potent stimulation of oxygen consumption by photosystem I in isolated chloroplasts. However, none of these compounds shows significantly improved herbicidal activity over the parent benzo-compound **1**; indeed, in herbicide tests, only the thieno-derivative **3**, which is the closest of the analogues to compound **1** in terms of its physicochemical properties, showed any significant post-emergence activity at all. As reported previously for a series of analogues of compound **1**, adverse hydrolytic stability and high melting point remain factors which can limit the overall herbicidal activity of isoquinolinetrione derivatives.

## ACKNOWLEDGEMENTS

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# EFFECT OF RIMSULFURON AND ITS MAJOR DEGRADATION PRODUCT ON ALS ACTIVITY AND ON PROTEIN AND STARCH FORMATION IN MAIZE

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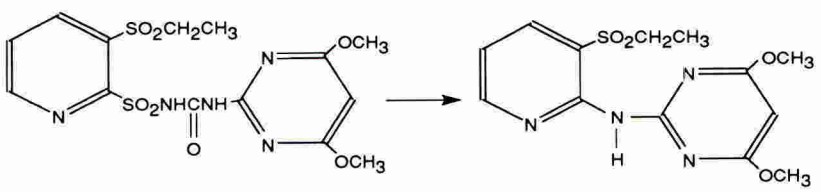
## ABSTRACT

The different toxic actions on maize of rimsulfuron (R) and of its degradation product (M) formed in aqueous and soil environments have been studied. R exerted a greater inhibiting effect on the activity of the target enzyme acetolactate synthase (EC 4.1.3.18; ALS) than M, determining greater indirect effects on protein and starch formation. This difference was confirmed by the effects of R and M on the extractable activity of systems glutamine synthetase (EC 6.3.1.2; GS)-glutamate synthase (EC 1.4.1.14; GOGAT) and phosphoenolpyruvate carboxylase (EC 4.1.1.31; PEP)-ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39; RUBISCO). Therefore, the degradation of R to M could be a factor of maize tolerance to rimsulfuron.

## INTRODUCTION

Rimsulfuron [1-(4,6-dimethoxpyrimidin-2-yl)-3-(ethylsulfonyl)-2-pyridylsulfonyl)urea] (R) is a sulfonylurea herbicide used on maize and potatoes to control a wide variety of grasses and broadleaf weeds. It inhibits ALS, a key enzyme in the biosynthesis of branched-chain amino acids - valine, leucine and isoleucine. R rapidly degrades in soil and aqueous environment, and among its degradation products the major metabolite is [1-(3-ethylsulfonyl)-2-pyridinyl]-4,6-dimethoxy-2-pyrimidineamine (M) (Schneiders *et al.*, 1993). When R-treatment is used, maize tolerance to its major degradation products should also




**R** **M**

be taken into consideration. Therefore a comparative study was carried out on the effects of both R and M on the activity of the target enzyme ALS, and to determine any consequent changes in protein and starch formation. For this purpose ALS activity, protein and starch

contents and the activities of some enzymes involved in $NH_3$ and $CO_2$ assimilation were investigated.

## MATERIALS AND METHODS

Ten-day-old maize seedling (cv Platone), grown in a growth chamber (14-h photo period; 24/16 °C day/night; 75-80% relative humidity; 1/4 strength Hoagland nutritive solution), were treated post emergence. R and M were applied at the usual field dosage (34.8 mmoles $ha^{-1}$) in a spray volume of 500 litre $ha^{-1}$ with a flat fan SS8002E nozzle at 30cm and spray pressure of 275 kPa. Samples of maize shoots and roots were collected every day for five days after treatment.

The ALS activity was assayed according to the procedure by Singh *et al.* (1988).

The protein content was determined according to the procedure by Bradford (1976).

The assays of GS and GOGAT activities were performed according to the procedures described by Lea *et al.* (1990).

The starch was extracted according to Haissig and Dickinson's procedure (1982) and determined following the method used by Cooper and Mc Daniell (1970).

The assays of PEP activity were performed according to the procedure described by Ashton *et al.* (1990).

The assays of RUBISCO activity were performed according to the procedure described by Keys and Parry (1990).

## RESULTS AND DISCUSSION

As sulfonylurea herbicides are known to have ALS as their target site, the primary effect of R and M on the extractable activity of this enzyme in maize seedlings was checked.

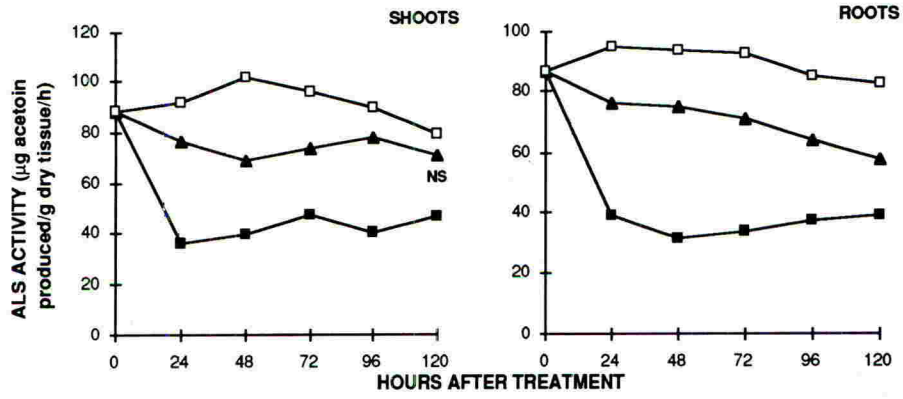



**Figure 1.** Activity of acetolactate synthase in maize seedlings: (□) control; (■) rimsulfuron; (▲) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls ($P<0.05$, on the basis of LSD test).

Figure 1 shows that R-treatment determined an immediate and sharp fall in the enzyme activity compared to the untreated controls. The differences in percent ranged from 61.0 to 41.0 in the shoots, and from 66.6 to 52.6 in the roots. This finding shows that R was able to reduce ALS activity in maize despite the ability of maize to rapidly metabolize the

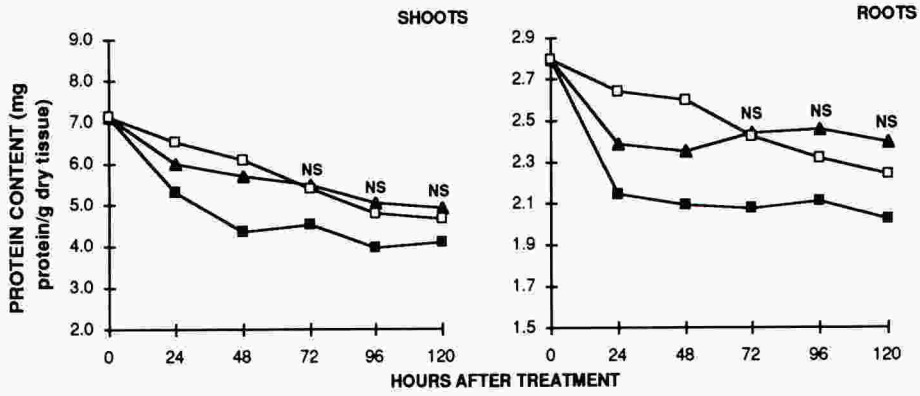



**Figure 2.** Protein content in maize seedlings: ( □ ) control; ( ■ ) rimsulfuron; ( ▲ ) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls ($P<0.05$, on the basis of LSD test).

herbicide (Smith, 1991). A lesser inhibiting effect was determined by the M-treatment, where compared to controls a 32.4 to 11.1% decrease in the shoots and a 30.3 to 19.6% decrease in the roots were observed.

A reduced synthesis of valine, leucine and isoleucine can occur as a result of ALS inhibition resulting in reduced protein formation. Hence, the protein content of the treated and untreated seedlings were compared. The results depicted in Figure 2 show a sharp fall in protein content in the R-treated shoots compared to the untreated controls. Decreases reached a maximum of 28.7% in the shoots and 19.3% in the roots at 48 hours after treatment, after which they decreased to 11.8% in the shoots and 9.6% in the roots. These results show a disturbance in protein formation in the tissues of R-treated seedlings. In order to overcome the lack of valine, leucine and isoleucine an increase in the turn-over rate of pre-existing proteins, rather than a new amino acid synthesis has been hypothesized (Rhodes *et al.*, 1987), which would explain the protein decreases in R-treated samples. A lesser effect was observed in the tissues of M-treated seedlings, where compared to the untreated controls decreases reached a maximum of 6.2% in the shoots and of 9.5% in the roots at 48 hours after treatment, after which no significant differences were recorded.

The extractable activities of GS and GOGAT were also checked as biochemical parameters of nitrogen assimilation. Indeed the GS-GOGAT system provides nitrogen for glutamate which, in turn, acts as a nitrogen source for the synthesis of other amino acids. The results reported in Figure 3 show that R-treatment inhibited the GS activity. Compared to untreated controls, decreases ranged from 24.2 to 11.2% in the shoots and 31.5 to 20.2% in the roots. The inhibition of GS activity in M-treated seedlings was of a lesser extent, varying from 12.0 to 7.2% in the shoots and 21.5 to 8.4% in the roots.

In the R-treated shoots the percent decreases in GOGAT activity (Fig. 4) reached

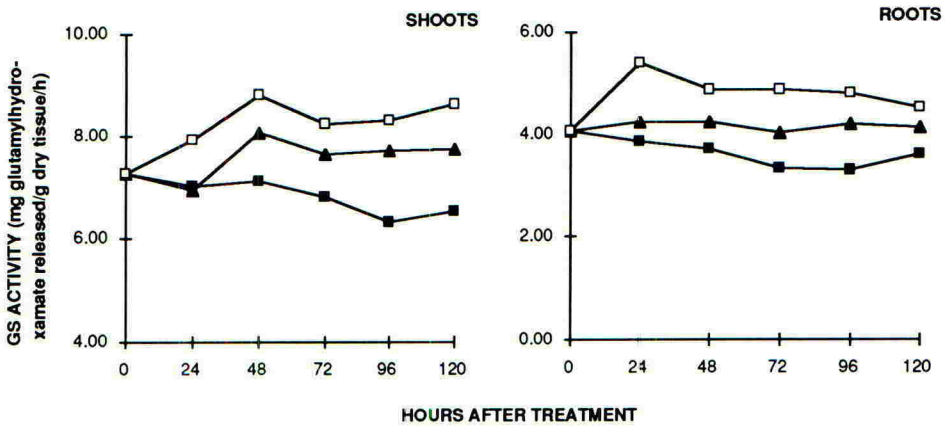



**Figure 3.** Activity of glutamine synthetase in maize seedlings: (□) control; (■) rimsulfuron; (▲) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls (P<0.05, on the basis of LSD test).

values of 42.1 and 39.5 at 24 and 48 hours after treatment respectively, then an average fall of 17.5% was maintained till the end of experimental period. In the R-treated roots, the percent decrease remained virtually constant (18.8) throughout the entire experimental

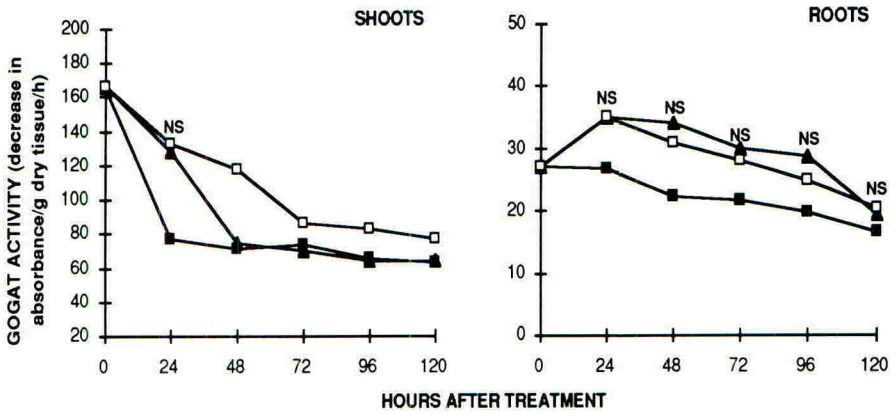



**Figure 4.** Activity of glutamate synthase in maize seedlings: (□) control; (■) rimsulfuron; (▲) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls (P<0.05, on the basis of LSD test).

period. On the other hand, in the M-treated seedlings a significant effect was found only in the shoots, which started at 48 hours with a 36.2% decrease, and an almost constant decrease of 19.6% was subsequently maintained. The behaviour of the GS-GOGAT system showed a reduced ability of the treated seedlings to assimilate nitrogen, which in the M-treated samples was restricted to the shoots.

Among the consequences of ALS inhibition, an increase of neutral sugars and a reduction of photosynthate transport have been observed in response to imazapyr in

maize (Shaner and Reider, 1986). Our data confirm a disturbance of R- and M- treatments on starch formation. R-treatment reduced the starch content to levels ranging from 32.1 to 10.8% in the shoots and to an almost constant level of 27.8% in the roots (Fig. 5). M-treatment resulted in a no significant effects in the shoots during the first 72 hours, after which an average reduction of 10.0% occurred (Fig. 5). In the roots significant decreases ranging from 17.9 to 8.0% were observed between 24 and 72 hours (Fig. 5). According to Devine *et al.* (1990) it might be expected that a blockage of sucrose transport would inhibit $CO_2$ fixation via a feedback mechanism. This hypothesis was supported by the results concerning the extractable activities of PEP and RUBISCO which are key enzymes in the $CO_2$ assimilation in maize shoots. The data obtained indicate varying

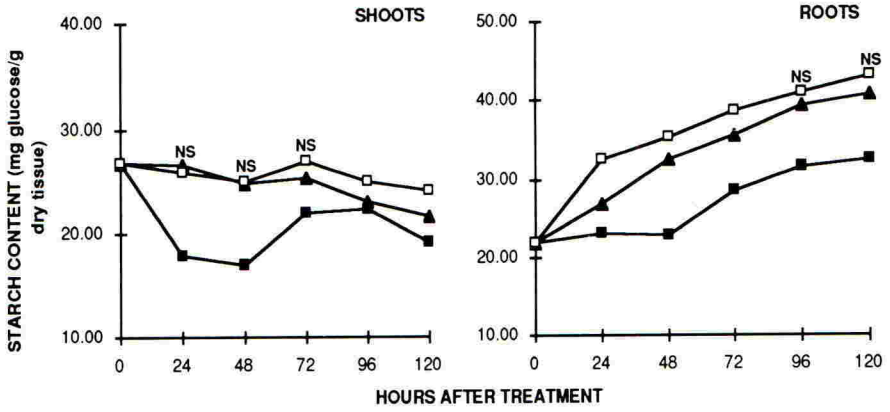



**Figure 5.** Starch content in maize seedlings : ( □ ) control; ( ■ ) rimsulfuron; ( ▲ ) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls (P<0.05, on the basis of LSD test).

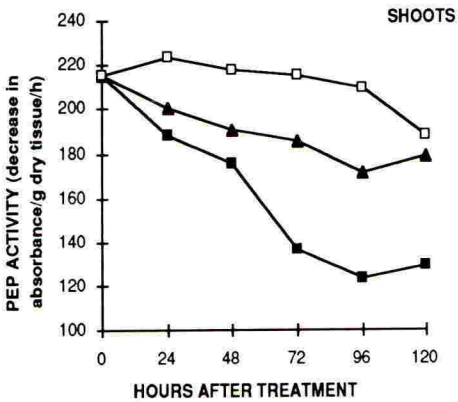



**Figure 6.** Activity of Phosphoenolpyruvate carboxylase in maize seedlings : ( □ ) control; ( ■ ) rimsulfuron; ( ▲ ) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls (P<0.05, on the basis of LSD test).

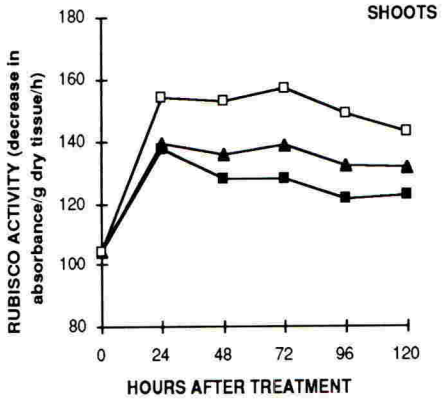



**Figure 7.** Activity of Ribulose 1,5 bisphosphate carboxylase in maize seedlings : ( □ ) control; ( ■ ) rimsulfuron; ( ▲ ) degradation product. Each value is the mean of three determinations. NS, not significantly different from untreated controls (P<0.05, on the basis of LSD test).

degrees of interference from R- and M- treatment. R-treatment resulted in an inhibiting effect of PEP activity ranging from 41.1 to 15.8%, while M-treatment determined an inhibition ranging from 18.4 to 4.8% (Fig. 6). The RUBISCO activity proved to be inhibited by 18.4 to 10.7% after R-treatment, and by 11.8 to 8.2% after M-treatment (Fig. 7).

In conclusion, the results of this research show that M has a less effective action on the target site, ALS, than R, hence lesser indirect effects on protein and starch formation. These findings are further supported by the lesser inhibiting effects of M-treatment on the extractable activities of GS-GOGAT and PEP-RUBISCO systems. Therefore the degradation of R to M in aqueous and soil environments could play a role in maize tolerance to rimsulfuron.

## AKNOWLEDGMENTS

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We would like to thank Prof. Cesare Marucchini for providing the degradation product of rimsulfuron.

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# Evening Discussion Session

# Will Regulatory Pressure Eliminate the Need for New Herbicides?

| | |
|---|---|
| Chairman and Session Organiser | Dr R Briant |
| Paper | ED-1 |

# WILL REGULATORY PRESSURE ELIMINATE THE NEED FOR NEW HERBICIDES?

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ABSTRACT

The new EU policy programme 'Towards Sustainability' and Council Directive 91/414/EEC will set the rules for the future European market for agricultural pesticides. The paper discusses how these rules will interact with those of Sweden as a new Member State in the EU. Sweden has now fulfilled its first round of re-registration and the similarities and differences between that and the new EU programme are discussed.

## WHAT ARE THE REASONS FOR REGULATORY PRESSURE?

Regulatory pressure is generated from actual risks and public opinion, urging politicians and regulators to act. We all know that the history of pesticides contains examples of misjudgements and assurances that pesticides are always safe to use from a health and environmental point of view. When this has proven not to be the whole truth, the credibility of the industry and regulatory agencies has been damaged. This is a history that both industry and the regulatory agencies have to live with. If we do not take away from the market those pesticides that are most harmful to health and the environment, it will not be possible to argue that the benefits from using pesticides override the risks for health and the environment, thereby easing some of the public and political pressure. In other words, the pressure can only be relieved by results.

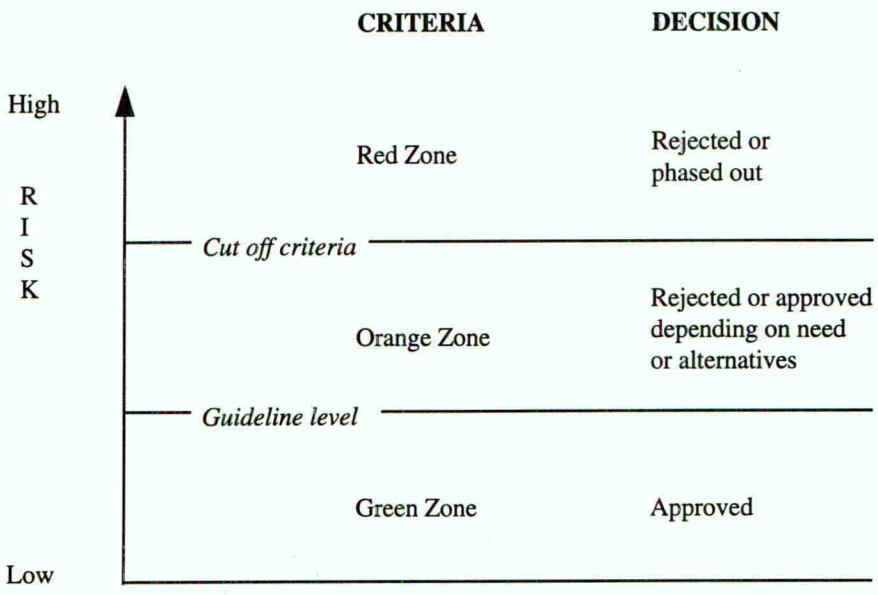
## ARE THERE ANY RISKS TODAY?

Looking at the list of pesticides registered today in one or several of the EU Member States, there are both real risks and potential risks connected with pesticide products. Judging from my experience from the Swedish re-registration programme 1990-1995, at least one third do not have adequate documentation, i.e. carcinogenicity studies and studies on degradability and bioaccumulation (Bernson *et al.*, 1991). The risk of using these substances can therefore not be adequately assessed and compared with more modern pesticides and those with complete dossiers. From a regulatory point of view, such substances must always be regarded as involving potential risks.

The risks can be compared to a traffic stoplight (Figure 1) where pesticides with the highest risks fall into the Red Zone and should be eliminated. Cut off criteria and guideline levels (Figure 1) have been elaborated by Andersson *et al.* (1992) in 'Principles for identifying unacceptable pesticides'. Approximately another 10-15% of EU pesticides approved today would fall into the Red Zone if these were to be defined in the same way as in Sweden, and therefore should be eliminated.

At least in Sweden, a green light means driving with caution. Pesticides which fall into the Green Zone should be used with care by the farmer while regulatory authorities should concentrate their efforts on the Orange and Red Zone pesticides, where there is the greatest potential risk for reduction.

Figure 1: Outline of the principles of the National Chemicals Inspectorate's risk policy for approving pesticides 'A risk stop light'.




In the single case, the principles of substitution are used when significantly less hazardous substitutes are available.

Cut off criteria are today available for the following health effects:
Acute, sub-chronic and chronic toxicity and for carcinogenic and reproductive and teratogenic effects.
Environmental criteria are available for degradation, mobility and bioaccumulation in combination with degradation.

## HOW CAN THE REGULATORY PRESSURE BE MET AND IS THERE AN END TO REGULATORY PRESSURE?

Looking back into the history of pesticide regulations, the documentation requirements have become more and more extensive. Will it ever be possible to fulfil the future need?

Here, I believe that regulatory authorities should use a stepwise approach so that pesticides with less harmful properties, for which the risks can be judged adequately with a standard dossier and where the uncertainty does not have a crucial influence on the decision, can be

handled faster. On the other hand, additional tests may be needed for pesticides with clearly undesirable properties. Such a stepwise system would benefit a manufacturer of a less hazardous pesticide and thus benefit health and the environment by encouraging the development of less hazardous products and by speeding up the regulatory process for such products.

The provision of additional data to today's standard dossier would only marginally improve the decision support and ease the uncertainty and should be left to researchers. Instead, regulators should take decisions based on available data and set predictable rules for authorisation. However, 'new' areas of concern may arise such as immunotoxicology and it could be relevant to ask for data in this field in the future.

## WHY DOES REGULATORY PRESSURE DIFFER BETWEEN COUNTRIES? WILL IT BE HARMONISED IN THE EUROPEAN COMMUNITY BY DIRECTIVE 91/414?

The European Union's policy 'Towards Sustainability':

The policy document to support a stringent environmental policy with respect to agricultural pesticides is already published (Towards Sustainability: A European Community Program of Policy and Action 1992 and a working document by Reus *et al.*, 1994). Some individual EU member countries have ongoing risk reduction programmes for pesticides, i.e. Denmark, the Netherlands and Sweden (see Reference list). The Swedish experience is that it is possible to maintain such a stringent policy towards risks to health and the environment without jeopardising farmers' yield or the possibility for them to earn their living (Emmerman, 1992).

The relevant legal instruments are contained in Council Directive 91/414/EEC and the Uniform Principles Council Directive 94/43/EC, which are the rules according to which the Member States should approve or reject pesticides in the coming re-registration programme and beyond.

In trying to identify any principal differences between the written policy or legal instruments of the Community and the Swedish risk reduction programme from 1986 up to 1995 when we joined the Community, I can only find two major discrepancies. In Sweden we leave the efficacy more to the market to decide on. Of course, we check that the manufacturer presents a product which is effective but we leave the details and instead concentrate on the health and environmental issues.

The second deviation is the use of the substitution principle at the level of authorisation of a product. The concept of substitution means that we do not give approval to a pesticide product for which a less hazardous substitute is available. In this respect, we act as a market force, promoting environmental sustainability and human health. The rule is only used when a substantial risk is at hand and therefore substitution within the Green Zone will usually not be considered.

It is often said that this principle will create great problems for farmers and manufacturers because it is unpredictable and limits the number of products on the market. However, this

is not our experience from using the principle during the Swedish re-registration programme in 1990-1995. During re-registration when we found a product which was in the Red Zone and for which there were presently no alternatives, we announced a phase-out programme. Together with the manufacturer, farmers, and the extension service we tried different strategies which all resulted in new knowledge and usually also applications for a new product. In a way, we created a new market because of the health and environmental demands we raised. However, without the manufacturers who had new products in their pipelines or on other markets ready to apply for them also in Sweden, we would not have been able to fill the gap and they would not have had a market because it was blocked by an old and usually cheap product.

Within the given framework of the Community authorisation system, there is certainly a variability and it is not possible to predict what all the decisions will be after discussions in the Plant Health Committee or whether some decisions will have to be taken up by the Council.

Taking the first stage of the Community re-registration programme, of the list of the first 90 substances (which was later reduced to 87) 40 are approved in products in Sweden today. Twenty-seven have been rejected due to health and environmental problems and the rest have either been withdrawn from the Swedish market by the manufacturer, are unknown or have had applications for approval filed. Therefore, already the outcome of this first stage will be decisive and in 1½ to 2 years time, we will know what that outcome will be.

The substitution principle is not included in Directive 91/414. However, it is included in the Fifth Environmental Programme as a desired policy. In Sweden, the principle should be used at all levels by authorities when approving a pesticide and by users when considering the local conditions. The latter utilisation of the principles will remain and the Swedish Farmers Federation has taken a policy decision to advise their members not to use pesticides which at some time have been rejected in Sweden for health or environmental reasons.

Therefore, even if an active ingredient enters the positive list of approved substances in Directive 91/414/EEC it may not enter the market in Sweden because the use area does not include crops grown in Sweden or it may not have a market in Sweden because of the policy decision of the Farmers Federation.

## CAN PREDICTABLE RULES BE SET FOR THE MANUFACTURERS OF THE NEXT GENERATION OF PESTICIDES?

Another way of putting the same question is the following: Is it possible to have pesticides which are acceptable from a health and environmental point of view?

When developing new pesticides, a number of guiding factors should be taken into consideration:

a) the scientific evidence of the risk a product can pose;
b) an analysis of the uncertainty in the prediction;
c) the level of risk at which one can be certain to build public confidence that the pesticide is safe;

d) the need (benefit) of the product;
e) other means to solve the farming problem.

Science will provide an estimate of the size of the risk according to today's knowledge but it is also important to have the public's view on what is acceptable to society.

What industry and farmers need in order to invest in developing new herbicides, other pesticides and other farming methods is a predictable future. This will most probably occur when the outcome of the first stage of the EU re-registration programme is known. Will the level reached there also be maintained in the future? Probably, but looking back at the figure with the stop light, I would say that if a pesticide has less hazardous properties than those set up by the Swedish guideline levels (limiting the Green Zone), you will be on the safe side even if the substitution principle was to be applied on a broader scale within the Community.

Manufacturers may argue that some of the objectives, i.e. persistence (half-life of the compound) of less than 10 weeks at 20°C counteracts the desired effect from an agricultural point of view which would require a longer period of efficacy in order to suppress the weeds during the major life of a crop. However, this persistence must be achieved by other means than the inherent properties of the active substance, i.e. by the formulation or other forms of slow release.

## IS THERE A NEED FOR NEW HERBICIDES AND HOW CAN THE BENEFITS BE DEFINED?

If herbicides are compared to other classes of pesticides such as fungicides and insecticides, it is the group with the least toxic products from a health and environmental point of view. Fungicides are the most problematic, probably due to the fact that a fungal cell contains much the same constituents as all other cells, including human cells. However, herbicides are of course toxic to other plants and algae and many have an undesirable persistence and mobility. Overall, many of the problems with herbicides are effects concerning the environment.

Table 1 gives a list of herbicides from the first stage of the EU re-registration programme which have been banned/withdrawn or severely restricted in Sweden and where the action was taken due to health or environmental reasons. For those that are severely restricted, lack of alternatives is the main reason for not banning them.

As the final decision to approve a pesticide is based on a risk-benefit analysis, it is not possible to define the benefit once and for all. This means that as less hazardous/risky pesticides are developed, the more use areas can be accepted. On the contrary, if manufacturers do not develop pesticides with less side effects on health and the environment, the use of pesticides will probably be restricted to use areas where professional farming is not possible today without the use of pesticides, i.e. to cure pests.

There is, however, one area for which I am doubtful that it will be possible to develop environmentally safe herbicides and this is herbicides for public paths and gravel paths. The

soil here is made to allow water to pass through and all pesticides applied to these areas will also leach to ground and surface water. I think it is unlikely that a pesticide will be found for this use that would not pollute adjacent water.

Table 1: Herbicides on the first EU list for re-registration, which are banned, withdrawn or severely restricted in Sweden, while approved in one or more of the15 EU Member States (MS).

| Pesticide | Remarks[1] | Approved in X MS |
|---|---|---|
| Amitrole | Banned due to risk of carcinogenic effect on humans according to epidemiological data (1972) | 10 |
| Atrazine | Banned due to its high mobility in soil and potential for contamination of water (1989) | 11 |
| Bromoxynil | Withdrawn. The substance has shown teratogenicity in experimental animals (1994) | 13 |
| 2,4-D | All products containing 2,4-D were withdrawn from the market due to concerns raised by toxicological and epidemiological reports (1991) | 14 |
| DNOC | Banned because of its high acute toxicity (1966) | 7 |
| Loxynil | Severely restricted use. The substance has caused teratogenic effects on experimental animals (1994) | 15 |
| Linuron | Severely restricted use and reassigned to Class 1. The substance is classified as carcinogenic and has high persistence (1994) | 15 |
| Paraquat | Banned because of its high acute toxicity, irreversible effects and imminent risk of accidents (1983) | 10 |

[1]Banned pesticide, a pesticide which has been banned for all use or refused first time use by final regulatory action, for health or environmental reasons. Withdrawn pesticide, a pesticide which has been withdrawn by the industry either from the market or for further consideration in the approval process and where there is clear evidence that such action has been taken for health or environmental reasons.

## CONCLUSION

To my knowledge, Sweden is the only country to have concluded its re-registration of all old pesticides for use in agriculture, horticulture and forestry. During this process, many pesticides have been banned, withdrawn, or severely restricted. It is therefore not a surprise to find that Sweden has banned or severely restricted several of the pesticides on the first re-registration list of pesticides in the EU. It can be foreseen that the European market for herbicides will change in the coming years due to regulatory pressure from the new EU

legislation and policy for registration and re-registration. The market size for herbicides will depend on how successful manufacturers are in developing new herbicides which are less hazardous from a health and environmental point of view. Only those manufacturers which take the increasing public awareness of health and environmental risks as a challenge to develop new, less hazardous pesticides or systems for crop protection will gain. Those that take it as a threat and stick to traditional pesticides will lose.

However, even with acceptable pesticides, there is much for the extension service and farmers to do in using these with care and caution.

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