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Use of plant cell cultures to study graminicide effects on lipid metabolism

Lindsey J. Price[a], Derek Herbert[a], David J. Cole[b], John L. Harwood[a,*]

[a]School of Biosciences, Cardiff University, PO Box 911, Cardiff CF10 3US, UK
[b]Aventis Crop Science UK Ltd. (formerly Rhone-Poulenc Agriculture Ltd.), Ongar, Essex CM5 0HW, UK

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Abstract

Graminicides belonging to the cyclohexanedione and aryloxyphenoxypropionate classes are well established to act by disrupting acyl lipid biosynthesis via specific inhibition of acetyl-CoA carboxylase. Species of grass inherently resistant to such herbicides, or biotypes of grassy weed species which display acquired resistance to recommended rates of graminicide application, are known to possess an altered plastidic multifunctional acetyl-CoA carboxylase showing reduced sensitivity to these herbicides in vitro. Studies reported here demonstrate that cell suspension cultures of maize, a graminicide-sensitive species and *Poa annua*, a graminicide-insensitive species, display a similar differential sensitivity of acyl lipid biosynthesis as tissue from corresponding intact plants. Acyl lipid biosynthesis in *P. annua* can be inhibited if sufficiently high concentrations of graminicide are used. The major plastidic form and the minor cytosolic forms of acetyl-CoA carboxylase were successfully purified from maize cell suspensions, were compared to those from leaf tissue and were shown to be differentially inhibited by graminicides in a similar manner to their counterparts from leaf tissue. These studies demonstrate that cell suspensions are useful for studying the mode of action of graminicides, especially in view of the limited amount of material obtainable from many grassy species which are very fine-growing.

Keywords: Graminicides; Acetyl-CoA carboxylase; Tissue cultures; Lipid synthesis; Test system for herbicides

1. Introduction

Graminicides are herbicides which selectively control grass (graminaceous) species and, hence, are used extensively in broad-leaved crops (Harwood, 1999). Two chemically-distinct groups of graminicides are the aryloxyphenoxypropionic acids (AOPPs) and the cyclohexanediones (CHDs). Together they account for about 10% of the total world herbicide market (Harwood, 1999). The AOPP chemistry was first developed in the mid 1970s for potential use in the pharmaceutical industry but it was discovered later that some of the compounds were highly herbicidally-selective against grasses and herbicides based on such chemistry were then marketed in the mid 1980s (Rendina et al., 1989). The CHDs were developed later but found to produce similar herbicidal symptoms in grasses, while being tolerated in dicotyledons and other monocotyledons (Harwood, 1991).

Herbicide selectivity in the field often relies on differential uptake, translocation or metabolism of the active compound in the plant. However, measurement of these parameters in AOPP or CHD-resistant or sensitive species failed to reveal consistant differences and it became clear that the basis of selectivity depended generally on variations in the target site protein. Further work revealed that the latter was acetyl-CoA carboxylase (Rendina et al., 1989; Secor et al., 1989; Walker et al., 1989).

Acetyl-CoA carboxylase exists as a multifunctional protein in some organisms but a multiprotein complex in others. In dicotyledons, with the exception of members of the family Geraniaceae (Christopher and Holtum, 2000), plastids contain a multiprotein complex which is used for de novo synthesis of fatty acids. Dicotyledons also contain a high molecular mass (200–240 KDa) multifunctional protein which is outside the plastids and believed to be cytosolic (Harwood, 1996; Konishi et al., 1996). By contrast, grasses contain two different multifunctional protein forms of acetyl-CoA carboxylase. Whilst it is easy to understand why

* Corresponding author. Tel.: +44-1222-874108; fax: +44-1222-874116.
E-mail address: harwood@cardiff.ac.uk (J.L. Harwood).

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there might be a difference in graminicide sensitivities between markedly different enzyme structures, the basis for sensitivity in the different multifunctional forms is probably due to various point mutations (Devine, 1997). A particular change that has been identified recently is a substitution of isoleucine with leucine in the carboxyltransferase domain (Brown et al. 2002; Zagnitko et al. 2001). On the other hand, detailed enzyme characterisation with acetyl-CoA carboxylase preparations from resistant and sensitive biotypes of *Lolium multiflorum* (Everson et al., 1994) or with plastid and non-plastid isoforms from maize (Herbert et al., 1996) failed to reveal any significant differences in the nature of the enzymatic reaction.

Regular and repeated use of AOPP or CHD herbicides in agriculture has given rise to increasing acquired resistance problems in some important grassy weed species which fail to be controlled at recommended use rates. Almost without exception, these resistant biotypes contain an acetyl-CoA carboxylase insensitive to these herbicides (Devine, 1997). There are also a few grassy weeds (e.g. *Poa annua*) that have inherent resistance (Herbert et al., 1995). In order to study such resistant species in greater detail then it is, of course, necessary to have a suitable test system. Unfortunately, many of the grasses of interest have fine leaves and show variable germination and growth. On the other hand, acetyl-CoA carboxylase has been extracted successfully from a number of tissue cultures in the past (Egin-Buhler and Ebel, 1983; Egli et al., 1993). Therefore, we have explored the possibility of using tissue cultures of grasses for further biochemical studies and results from such experiments are reported here.

2. Results and discussion

2.1. Studies with maize cultures

In order to compare the behaviour of suspension cultures towards graminicides with whole plants, we carried out experiments with representatives of each of the graminicide classes. The free acid derivatives of quizalofop and, sometimes fluazifop, were used as representatives of the AOPPs and sethoxydim for the CHDs. The data for inhibition of cell mass were fitted to an equation for simple hyperbolic inhibition and yielded I_{50} values for quizalofop and sethoxydim of 0.014 and 6.97 μM respectively (Fig. 1). These are comparable to in vivo values for maize which are about 0.02 μM for quizalofop and 4 μM for sethoxydim (data not shown). Furthermore, they also compare to I_{50} values for the sensitive plastid isoform of acetyl-CoA carboxylase which were 0.04 and 10 μM, respectively, for these two herbicides (Herbert et al., 1995). Therefore, it is likely that maize cultures contain a pattern of acetyl-CoA carboxylase isoforms which closely resemble those of leaves and also have similar sensitivity to the purified leaf enzymes (Herbert et al., 1996).

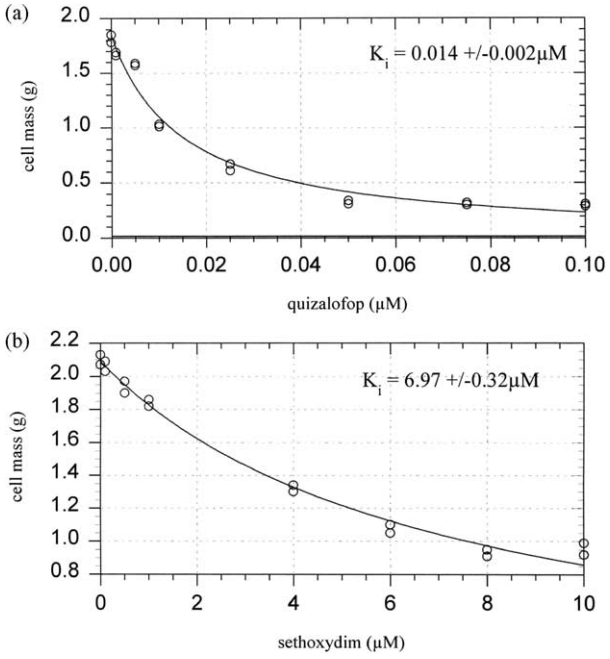

Fig. 1. Inhibition of maize cell suspension growth with different graminicides. The estimated I_{50} values for quizalofop (a) and sethoxydim (b) are shown after fitting data to an equation describing simple hyperbolic inhibition using MicroMath Scientist. Results show duplicate values of cell mass for each treatment.

In order to validate further the use of tissue cultures, we carried out radiolabelling experiments. [1-^{14}C] Acetate was used as a precursor for lipid synthesis since this radiolabelled compound has been shown to be a suitable and effective precursor of plant lipids (Roughan and Slack, 1982). It has an advantage in that the radiolabel is almost exclusively located in the acyl chains of membrane acyl lipids, thus simplifying interpretation of data. Table 1 shows that three different graminicides each effectively reduced total lipid labelling in young maize leaves. This result is, of course, consistent with the general action of graminicides on lipid labelling in sensitive grasses (Hoppe, 1985; Focke and Lichtenthaler, 1987) and with the pathological symptoms of treatment where necrosis of the growing meristematic tissue is prominent (Walker et al., 1989). As expected, increasing concentrations of both sethoxydim (a CHD) and two different AOPPs, quizalofop and fluazifop, gave better inhibition. Quizalofop was more effective than fluazifop or sethoxydim at inhibiting lipid labelling, in keeping with previous data on the relative efficacy of these compounds (e.g. Herbert et al., 1996).

It will be clear from the data shown in Table 1 that the action of the herbicides on lipid labelling is not secondary to a reduction in precursor uptake. There were no statistically significant effects on the labelling of the aqueous fraction. Moreover, the inhibition of lipid

Table 1
Graminicides inhibit lipid labelling from [1-^{14}C]acetate in leaves from maize (*Zea mays*)

Treatment	Total uptake (d.p.m.× 10^{-5}. g^{-1})	Aqueous fraction (d.p.m.× 10^{-5} g^{-1})	Lipid labelling (d.p.m.× 10^{-5} g^{-1})
None (control)	16.4±0.6	6.4±0.6	10.0±0.4
0.05 μM sethoxydim	17.8±1.4	6.3±1.0 (98)	9.5±1.6 (95)
0.5 μM sethoxydim	11.0±3.2	5.8±2.1 (91)	5.2±1.2* (55)
0.05 μM quizalofop	8.9±0.9*	5.2±0.7 (81)	3.7±0.5* (37)
0.5 μM quizalofop	7.4±0.8*	5.7±0.6 (89)	1.7±0.3* (17)
0.05 μM fluazifop	15.6±1.4	6.3±1.3 (98)	9.3±1.9 (93)
0.5 μM fluazifop	11.7±2.8	5.5±1.4 (86)	6.2±0.6* (62)

Three-day-old post-emergence maize leaves were used. Means±S.D. shown ($n=3$). Figures in parentheses show % of control values.
* $P<0.05$, significant difference from the control.

Table 2
Uptake of [1-^{14}C]acetate and incorporation of radiolabel into total lipids of 3-day-old maize cell cultures

Treatment	Total uptake (d.p.m.× 10^{-5} g^{-1})	Aqueous fraction (d.p.m.× 10^{-5} g^{-1})	Lipid labelling (d.p.m.× 10^{-5} g^{-1})
None (control)	20.1±4.4	3.3±0.8 (100)	16.8±4.3 (100)
10 μM sethoxydim	7.8±0.5*	3.2±0.5 (97)	4.6±0.4 (27)*
20 μM sethoxydim	7.4±1.4*	2.9±0.8 (87)	4.5±1.6 (26)*
0.01 μM quizalofop	16.3±0.9	3.8±0.9 (115)	12.5±0.7 (74)
0.02 μM quizalofop	9.0±0.9*	2.6±0.6 (79)	6.4±0.3 (38)*

Results as means±S.D. ($n=3$ independent experiments). Figures in parentheses show % control.
* $P<0.05$, significant difference from control.

labelling was always greater than the reduction in total uptake. This is seen clearly for the most effective graminicide, quizalofop (Table 1). It should also be borne in mind, when considering total lipid labelling, that some of the radioactive lipids produced are not acyl compounds. In particular, biosynthesis of pigments and, less important quantitatively, other terpenoids or sterols and their derivatives is not affected by graminicides because acetyl-CoA carboxylase is not involved (e.g. Hoppe, 1985). Thus, the maximum inhibition of total lipid labelling that can be achieved from [1-^{14}C]acetate is limited to about 80%, depending on the tissue (Hoppe, 1985; Walker et al., 1989).

We compared the effect of graminicides on lipid labelling in maize leaves with that in tissue cultures. In order to obtain a more obvious effect of sethoxydim we used higher concentrations than for leaf tissues. The data in Table 2, show that both sethoxydim and quizalofop cause significant reductions in total lipid labelling. It is also clear that, as with leaf tissues, the inhibition of lipid labelling is not due merely to a reduction in tissue uptake of [1-^{14}C]acetate. Maximal inhibition of lipid labelling (by sethoxydim) appeared to be around 75% which was of the same order as discussed above and reflects the labelling of non-acyl lipids which is largely unaffected by graminicides. It is of note that the labelling rates per gram fresh weight of tissue in both leaves and cell cultures were comparable and that the majority of the label taken up in both controls was incorporated into lipids. Furthermore, the relative sensitivity of lipid labelling to inhibition by quizalofop was similar in the two tissue types. These results show that maize tissue cultures behave similarly to leaf tissue with regard to graminicide effects on both growth and lipid synthesis.

2.2. Alterations in lipid class labelling

When considering the inhibition of lipid labelling by graminicides (Tables 1 and 2), we discussed above the fact that, while such herbicides would be expected to effectively block labelling of acyl lipids (through their effect on acetyl-CoA carboxylase), they would not affect other biosynthetic pathways if their action was specific. Accordingly, we separated total radiolabelled lipids from maize cultures by TLC. A polar lipid solvent system was used (see Experimental) so that the total pigments and neutral lipids were unresolved and migrated as two large bands (Fig. 2). This allowed us to check the various membrane acyl lipids to evaluate whether the graminicides inhibited generally the labelling of such compounds. From Fig. 2 it will be clear that the effect of 5 μM sethoxydim was to reduce acyl lipid labelling without altering that of pigments (which were the main non-acyl lipids labelled). Higher concentrations of sethoxydim reduced acyl lipid labelling further (data not shown) and total reduction of labelling by 10 μM sethoxydim was nearly 70% which was comparable to the data shown in Table 2. Furthermore, in the extracts from control tissue (Fig. 2A) the labelling of pigments represented about 25% of the total radioactive lipids, thus accounting for the maximal inhibition of lipid labelling that could be achieved with graminicides (see Section 2.1 and Table 2).

2.3. Experiments with a resistant grass, P. annua

We succeeded in establishing cell suspension cultures from *P. annua*, which is a common horticultural weed in temperate regions (Tottman et al., 1982). Early studies showed that this grass exhibited resistance to the AOPPs (Walker et al., 1989) and, indeed, graminicide binding studies revealed that acetyl-CoA carboxylase

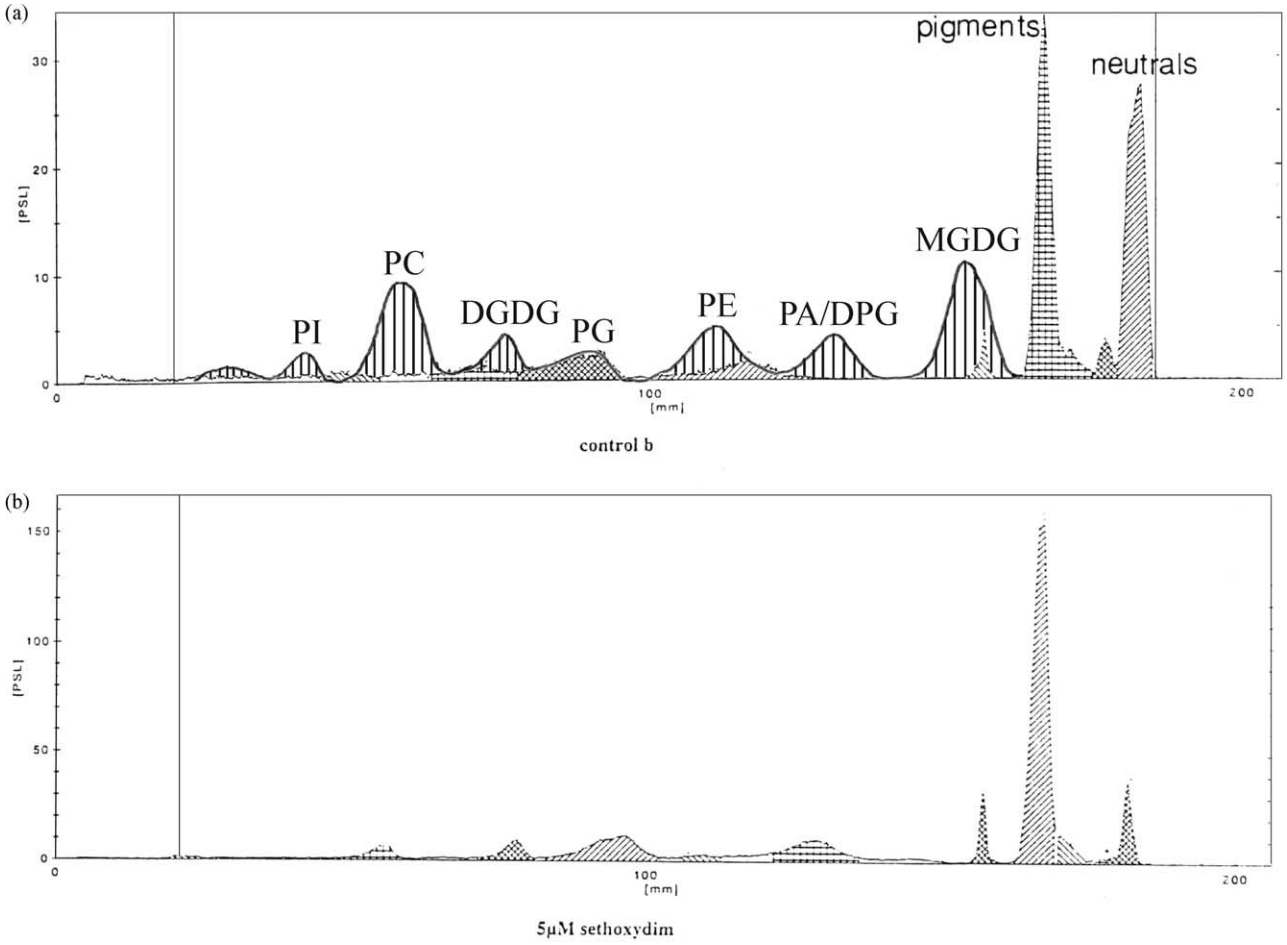

Fig. 2. Distribution of radioactivity between lipid classes after treatment of maize cells with 5 μM sethoxydim. Separation of the lipids, labelled for [1-^{14}C]acetate, was made by TLC and radioactivity measured by radioluminology. Major peaks are labelled. PC, phosphatidylcholine; DGDG, digalactosyldiacylglycerol; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA/DPG, phosphatidate/diphosphatidyl glycerol. PSL stands for photostimulable luminescence which is directly proportional to total radiation energy. (a) = control, (b) = treated with 5 μM sethoxydim. For herbicide treatment and lipid labelling see Section 3.3.

preparations from *P. annua* leaves were 300-fold less sensitive to quizalofop than the susceptible maize enzyme (Herbert et al., 1995, 1996). Thus, *P. annua* represents an example of (inherent) target-based resistance as discussed by Devine (1997).

Because of the inherent insensitivity of *P. annua* acetyl-CoA carboxylase we had to use high concentrations of graminicides in order to produce any effects. I_{50} values for *P. annua* growth were about 1 and 25 μM for quizalofop and sethoxydim respectively (data not shown) which compared with in vitro values of 0.2 and 40 μM for acetyl-CoA carboxylase preparations (Herbert, 1995). Thus, *P. annua* was about 100 times less sensitive than maize seedlings to inhibition by quizalofop (Section 2.1), in keeping with its known resistance to AOPPs (Walker et al., 1989).

Accordingly, we used high relative concentrations of graminicides, especially quizalofop, in order to test for activity against lipid labelling (Table 3). While lipid labelling was reduced by both herbicides in a concentration-dependent manner, total uptake of [1-^{14}C]acetate into the cells was unaffected. Thus, as with maize tissues, any effect of the herbicides on lipid labelling

Table 3
Graminicides reduce lipid labelling from [1-^{14}C]acetate in *Poa annua* cell cultures

Treatment	Total uptake (d.p.m.× 10^{-5} g^{-1})	Aqueous fraction (d.p.m.× 10^{-5} g^{-1})	Lipid labelling (d.p.m.× 10^{-5} g^{-1})
None (control)	14.2±0.8	11.3±0.7 (100)	2.9±0.3 (100)
20 μM sethoxydim	13.8±0.2	11.8±0.1 (104)	2.0±0.1 (69)
40 μM sethoxydim	15.2±0.4	13.5±0.4 (120)	1.7±0.2 (59)
5 μM quizalofop	13.8±0.5	11.7±0.4 (103)	2.1±0.1 (72)
10 μM quizalofop	13.2±0.5	11.5±tr. (102)	1.7±0.5 (59)

Three-day-old cells were used. Results as means±S.D. (n=2 independent experiments). Figures in parentheses show % control. tr., trace (<0.05).

was not merely secondary to a reduction in the available radiolabel. One difference that could be seen, however, was that although the total uptake of acetate into *P. annua* cells (Table 3) was similar to maize cells (Table 2), less labelling of lipids on a fresh weight basis was seen.

As discussed above, the inhibition of lipid labelling by graminicides in sensitive grasses, such as maize, is confined to the acyl lipids. Therefore, it was of interest to see what the labelling patterns were in *P. annua* when high concentrations of graminicides had to be used. In Fig. 3 the polar lipid classes, as separated by TLC, are shown. It will be clear that sethoxydim reduced the labelling of all the membrane acyl lipids while that of the pigment band was unaffected. Quizalofop produced a similar result (data not shown). Thus, in the inherently-resistant grass, graminicides (if provided at sufficient concentrations) are able to inhibit acyl lipid biosynthesis while leaving other lipids unaffected.

2.4. Acetyl-CoA carboxylase activity in the cultures

Since acetyl-CoA carboxylase is the target for graminicides (Rendina et al., 1989; Walker et al., 1989), we wished to check the properties of the enzyme from tissue cultures to make sure that the latter could be used to study herbicide effects on acetyl-CoA carboxylase in more detail. Cells of both maize and *P. annua* were grown in suspension culture and total acetyl-CoA carboxylase activity was measured at different times during the growth cycle, which was 7 days (see Experimental). Fig. 4 shows that, in both cases, good acetyl-CoA carboxylase activity (on a protein basis) was found at the beginning of the exponential phase of cell growth. Based on these data we used 3-day-old cells for acetyl-CoA carboxylase studies, as we had for the lipid labelling (above).

We used a protocol for the purification of acetyl-CoA carboxylase which was based on the method that we had developed for maize leaves (Herbert et al., 1996). A comparison of purification from the two tissues is shown in Table 4. We found that the acetyl-CoA carboxylases from suspension cells showed very similar properties to those from leaves and recovery from the various steps was similar. For the cell cultures we were able to dispense with the Blue Sepharose step. In both cases, two peaks of activity were clearly resolved on the Q-Sepharose column. The first, minor peak, representing 4–12% of the total recovered activity, eluted at about 210 mM KCl while the major fraction eluted at about 250

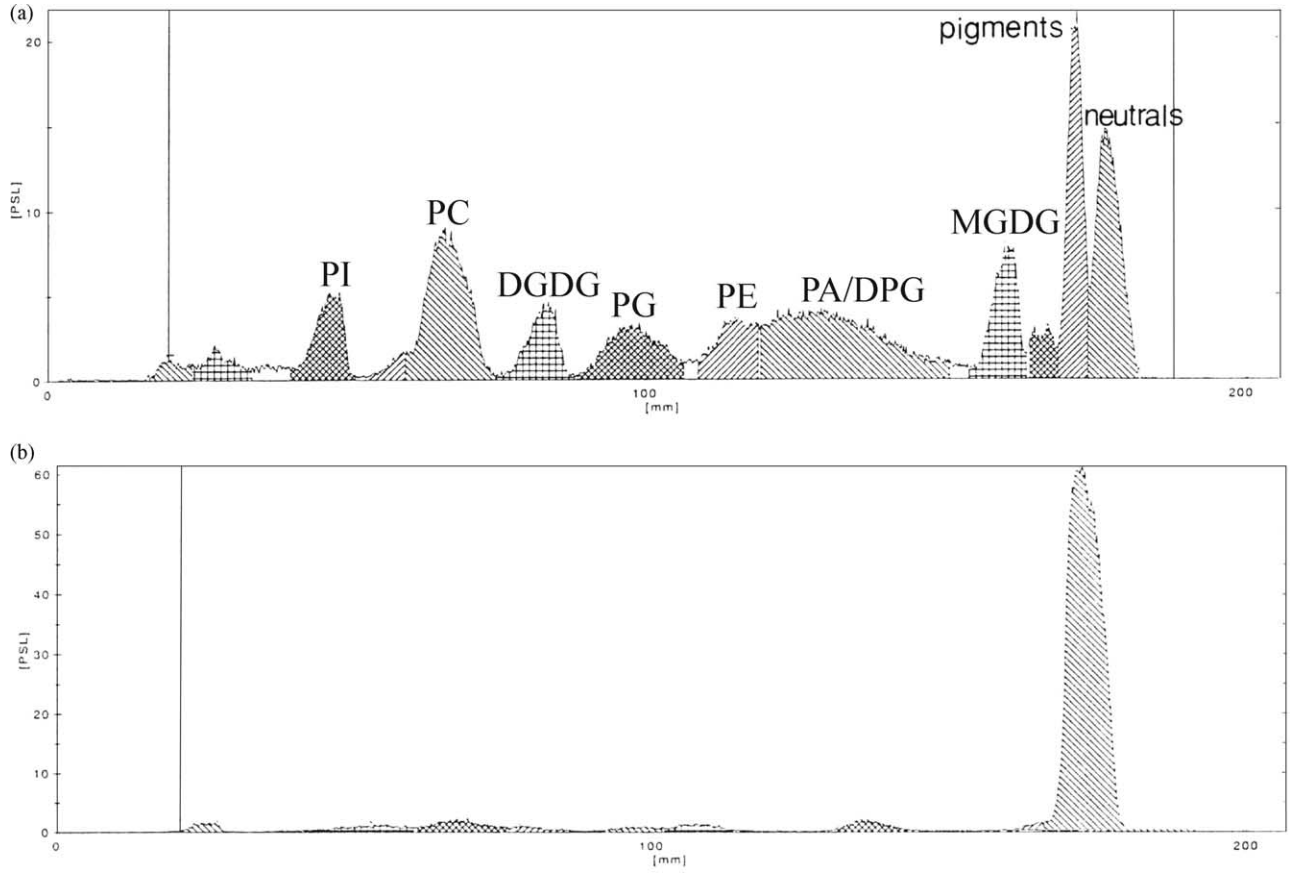

Fig. 3. Changes in the relative labelling of lipid classes after treatment of *Poa annua* cells with 40 μM sethoxydim. For other details see Fig. 2. (a) = control, (b) = treated with 40 μM sethoxydim.

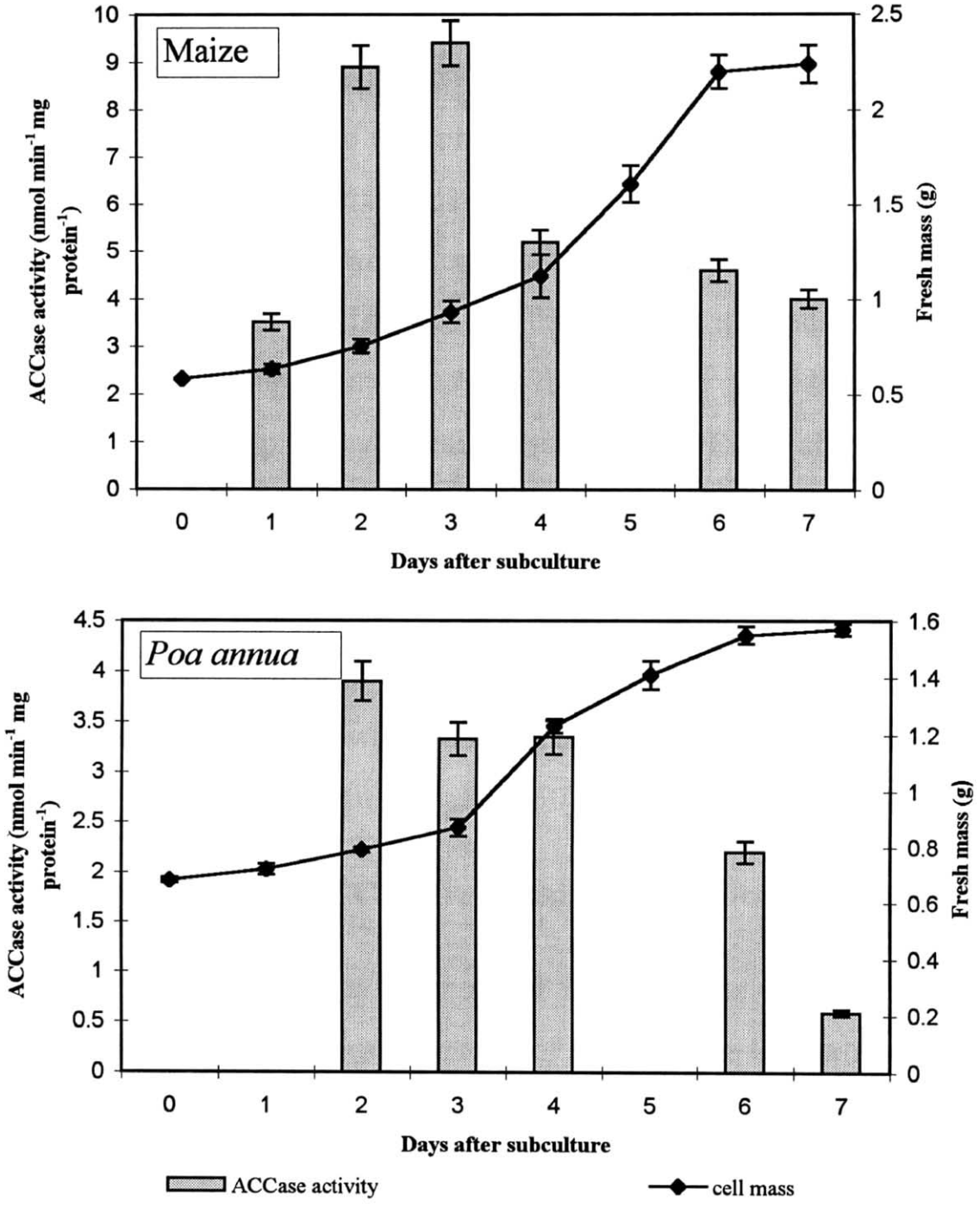

Fig. 4. Acetyl-CoA carboxylase activity and cell mass during the growth cycle of *Zea mays* or *Poa annua* cell suspensions. Results show means±S.D. for triplicate samples.

mM KCl in the salt gradient. The major isoform, for both tissue sources, had the highest specific radioactivity and, assuming that the major non-plastid isoform represented about 20% of the original total tissue activity (Harwood, 1996; Herbert et al., 1996), had a better recovery. The only noticeable difference was that the specific activity of acetyl-CoA carboxylase from suspension cells was about 20 times that of leaves on a protein basis. Although the maize cultures were photosynthetic, they were only pale green. Thus, it is likely that proteins which are normally abundant in leaves, such as Rubisco, were present in much lower amounts in the suspension cells. This may have contributed to the difference in specific activity.

We examined the separated isoforms from the two maize sources in order to compare their enzymatic

Table 4
Comparison of the purification of acetyl-CoA carboxylase isoforms from maize leaves or maize cell suspensions

Step	Total protein (mg)	Yield (% recovered activity)	Specific activity (μmol min^{-1} mg $protein^{-1}$)	Purification
Leaf tissue				
Homogenate	306	100	0.02	1.0
$(NH_4)_2SO_4$ cut	173	50.9	0.02	1.0
Sephacryl	82	12.9	0.02	1.6
Red agarose	1.42	6.4	0.33	16.5
Blue agarose	0.11	3.8	2.54	127.0
Q-sepharose	0.04	0.3	0.39	97.5[a]
Peak 1				
Peak 2	0.03	2.3	4.84	302.5[a]
Suspension cells				
Homogenate	517.0	100	1.2	1.0
$(NH_4)_2SO_4$ cut	129.5	28.3	1.3	1.1
Sephacryl	60.2	22.0	2.2	1.9
Red agarose	3.2	17.4	34.1	28.2
Q-sepharose	0.2	0.68	34.9	144.2[a]
Peak 1				
Peak 2	0.4	14.8	217.0	224.2[a]

[a] Calculated from the original homogenate assuming that the minor isoform accounted for 20% of the total activity.

properties. In both cases, their estimated molecular masses by SDS-PAGE were 219 kDa for the minor isoform and 227 kDa for the major isoform; the data for the leaf isoform agreeing with results from Herbert et al. (1996). In terms of substrate K_m values the acetyl-CoA isoforms from the two sources were also indistinguishable, being about 100 μM for ATP and acetyl-CoA and 1 mM for bicarbonate (plastid isoform) and 0.4 mM for the minor isoform (data not shown; Herbert et al., 1996). In maize leaves, as discussed in Section 2.1, the sensitivity of whole plants towards graminicides agreed well with the I_{50} for the plastid acetyl-CoA carboxylase isoform. Furthermore, as discussed, the extra-plastid minor isoform is rather insensitive to graminicides in general, despite also being a multifunctional protein. A critical test of the validity of using cell cultures to study herbicide activity was, therefore, whether the two acetyl-CoA carboxylase isoforms, which had been separated, showed distinct differences in their sensitivity. In Fig. 5, inhibition curves for acetyl-CoA carboxylases purified from cell suspensions are shown. Clearly, the minor isoform was very much (>100 times) less sensitive than the major (plastid) isoform. The I_{50} values for the two isoforms from cell suspensions were 0.04 and 48.2 μM compared with 0.03 and 60 μM for the equivalent leaf isoforms, respectively (Herbert et al., 1996). Thus, there was excellent agreement in the properties of acetyl-CoA carboxylases purified from maize cell suspensions with those from leaves.

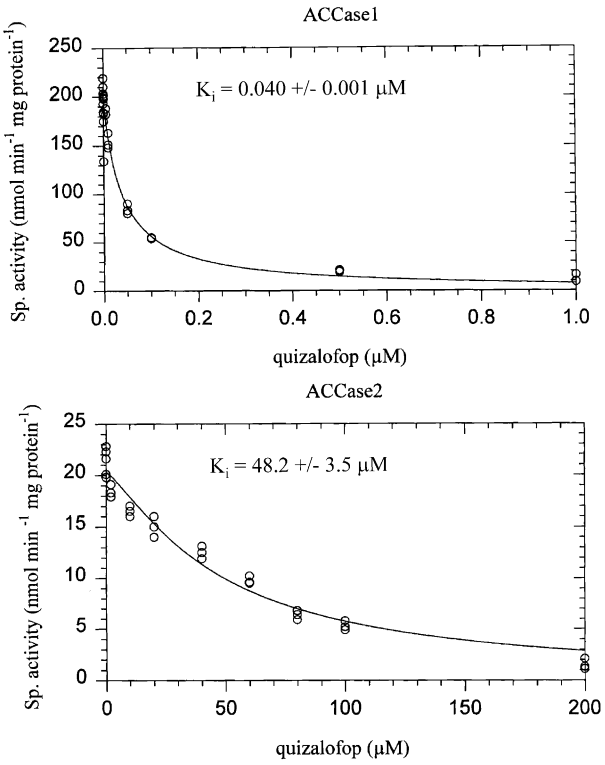

Fig. 5. Inhibition of purified acetyl-CoA isoforms by quizalofop. Purified isoforms from maize suspension cultures were incubated with quizalofop in the assay medium and the inhibition data fitted to a simple hyperbolic equation using MicroMath Scientist. K_i values were estimated from the equation using data from triplicate assays.

2.5. Conclusions

In this paper, we have shown that it is possible to use cell suspension cultures to study the effects of graminicides, as well as to use them as sources from which to purify acetyl-CoA carboxylases. The reproducible nature of cell cultures, as well as the possibility of generating large amounts of tissue and, therefore, protein means that they would be particularly useful for studies on morphologically fine grasses that often represent problem weeds with inherent or acquired graminicide resistance.

3. Experimental

3.1. Plant materials and growth

Maize plants were grown using maize (*Zea mays* cv. Celebration) seeds from Nickerson Seeds Ltd., Lincoln, UK. They were grown in soil-less compost at 20 °C with a 14-h light period of 650 μE m^{-2} s^{-1} and watered daily.

Maize cell cultures (cv. Black Mexican Sweetcorn) were from the laboratories of Aventis Crop Science

(UK) Ltd. *Poa annua* cultures were from the same source and were of root origin. Cultures were maintained on Murashige and Skoog basal salt medium (Sigma, Poole, Dorset) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 20 g/l sucrose and 8 g/l agar. Cultures were maintained by sub-culturing into fresh medium every 3 weeks. For suspension cells, a 15% inoculum was placed in 50-ml of the above medium without agar, in 100-ml flasks, agitated at 200 r.p.m. on an orbital shaker at 27 °C with illumination. Cells were sub-cultured every 7 days. For graminicide I_{50} value determinations, 50-ml flasks containing 25 ml medium were used. For enzyme extractions, 500 ml flasks with 250 ml medium were used.

3.2. Graminicide treatment of cell cultures

Quizalofop free acid [RS 2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid] was obtained from Aventis Crop Science (UK) Ltd. Fluazifop free acid (RS)-2-[4-(5-trifluoromethyl-2-pyridyloxy)[propionic acid] was bought from Cluzeay Info. Laboratoire, Ste-Foy-La Grande, France and sethoxydim 2-(1-ethoxyiminobutyl)-5-[2-(ethylthio)propyl]-3-hydroxycyclohex-2-enone from Greyhound Chem. Service, Birkenhead, Merseyside.

Growth curves were established by using triplicate flasks each time and filtering the cells under vacuum through a Whatman No. 1 filter. When dry weights were required, cells were placed in a pre-weighed container in an oven at 60 °C for 48 h or until a constant mass was observed.

Inhibition of cell growth by graminicides was measured in terms of a reduction in cell fresh or dry weight. Herbicides were made up in a stock solution using acetone and were added to the cell medium at a final acetone concentration of 0.1% (v/v). This concentration was found not to be inhibitory but 0.1% (v/v) acetone was added to controls in any case. Cells were incubated for 1 week under normal growth conditions before harvesting. The results were fitted to a simple hyperbolic inhibition curve by MicroMath Scientist and I_{50} values estimated.

3.3. Lipid labelling and analysis

148KBq [1-^{14}C]acetate (1.88–2.29 MBq/mmol; Amersham-Nycomed, Amersham, Bucks, UK) were added to each flask containing 3-day-old cells. Incubations were usually for 4 h under the normal growth conditions. At the end of the incubation, the cells were washed thoroughly and then ground in a pestle and mortar in hot *iso*-propanol. Extraction used the method of Garbus et al. (1963) as modified by Smith et al. (1982). Samples of the aqueous and lipid phases were taken for counting using Optifluor Scintillant (Packard) and a Beckman 1209 Rackbeta (Beckman Instruments Inc., Fullerton, CA, USA) liquid scintillation counter. Quench corrections was made automatically by the external standard channel ratios method.

For herbicide treatments, leaf blades were pre-incubated overnight by standing in the appropriate graminicide solution with a good draught (5 m s^{-1}) to ensure satisfactory uptake. 148 KBq of [1-^{14}C]acetate was then added and incubation continued for 4 h. The leaves were rinsed thoroughly and lipid extraction carried out as above. Cell suspensions were also pre-incubated overnight with the appropriate herbicide concentration before incubating for 4 h with radiolabelled substrate. In all cases, the graminicide were added in acetone to a final concentration of 0.1% (v/v) and control incubations had acetone alone added.

Total lipid extracts were separated on silica gel G plates (Merck, Darmstadt, Germany) by TLC using chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Lipid bands were revealed by spraying with 0.02% (w/v) methanolic 8-anilinonaphthalene-4-sulphonic acid (ANSA) and exposing to UV light. The identity of various bands was routinely made by reference to standards (Sigma, Poole, Dorset, UK) but they were more fully identified using differential staining (Kates, 1986). Radiolabelled bands were revealed by autoradiography in a spark chamber (Birchover Instruments, Hitchin, Herts, UK) or by imaging using a Fajix Bas 1000 photoimager (Fuji) and analysed with TINA software.

3.4. Acetyl-CoA carboxylase assay

This enzyme was analysed using the method of Burton et al. (1987) by measuring the incorporation of radioactivity from $NaH^{14}CO_3^-$ into malonyl-CoA. The standard assay conditions, which were found to be optimal, were 100 mM HEPES–KOH (pH 8.0) buffer, 5 mM $MgCl_2$, 2 mM ATP, 20 mM $NaH^{14}CO_3$ (37 MBq/mmol; Amersham-Nycomed) and 1 mM acetyl-CoA. The enzyme preparation was pre-incubated with the assay solution, minus acetyl-CoA, for 10 min at 30 °C before addition of the latter and incubation for 20 min. Reactions were stopped with 12 M HCl and acid-stable radioactivity measured, after drying in Optifluor scintillation fluid. The identity of the malonyl-CoA product was checked by TLC (Baldet et al., 1993).

3.5. Purification of acetyl-CoA carboxylase

For maize leaf isoforms the method of Herbert et al. (1996) was used. For maize cell suspension cultures, cells were grown in 500 ml conical flasks and harvested at day 3 of their growth cycle, when acetyl-CoA carboxylase levels were high. Cells were harvested by vacuum through Whatman No. 1 paper and frozen rapidly in liquid nitrogen. They were ground with a

small pestle and mortar until a fine powder was obtained. This was then mixed with extraction buffer (Herbert et al., 1996). Purification then proceeded as for maize leaves except that the Blue Sepharose step was omitted. Thus, the de-salted Red Agarose fraction was loaded directly on the Q-Sepharose column to yield two peaks of activity.

Protein in the fractions was estimated by the Coomassie blue dye-binding method (Bradford, 1976) using Bio-Rad protein assay reagent (Bio-Rad Labs, Munich, Germany) and bovine serum albumin as standard. Proteins were separated by SDS-PAGE using 5% stacking gels and 5 or 7.5% running gels. Proteins were revealed with Coomassie blue. Western blotting used up to 24 h to transfer proteins onto nitrocellulose membrane (Hybond-C-Super; Amersham-Nycomed). Acetyl-CoA carboxylase was revealed by immunodetection using antibodies raised to the multifunctional isoform from pea and alkaline phosphatase conjugated second antibodies. The latter were revealed as pink bands using nitro blue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate developer.

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