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STUDIES ON THE MODE OF ACTION OF TRALKOXYDIM USING CELL SUSPENSION CULTURES OF RICE AND FLAX

by

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SUMMARY

Cell suspension cultures of rice (*Oryza sativa*) and oil-flax (*Linum usitatissimum*) were established and grown in the dark. Sensitivity of these cultures to the cereal selective cyclohexanedione graminicide tralkoxydim (2-[1-(ethoxyimino) propyl]-3 hydroxy-5-mesitylcyclohex-2-enone) was investigated in different stages of the growth cycle by assessing culture fresh weight (FW), dry weight (DW) and settled cell volume (SCV). Both cultures of rice and flax were most tolerant in the early linear phase with an I_{50} of ca. 100 μM, measured by settled cell volume (SCV). In other stages of the growth cycle rice and flax cultures were two to three times more sensitive; the I_{50} was 51 and 72 μM tralkoxydim in the middle and late linear phase for rice and ca. 30 μM in the lag, linear, and stationary phase for flax. Growth of flax was completely inhibited above 25 μM while rice showed growth even at 200 μM tralkoxydim. Individually, the growth parameters of FW and DW were more strongly inhibited compared to SCV in rice while the reverse situation was true for flax cultures with SCV strongly inhibited.

The rice and flax cultures were inoculated with ^{14}C-tralkoxydim, on days 2, 4 and 7 of the growth cycle and incubated for 6, 24 and 72 h periods. Following solvent extraction in hexane/dichloromethane and or adsorption/elution of polar products on C18 columns, extracts of cells, cell debris and media were assayed for ^{14}C-activity and subsequently separated and identified by thin layer chromatography (tlc). The cultures of rice and flax showed a difference in distribution pattern of ^{14}C-activity between three fractions: debris, cells and media. The cell debris of rice and flax contained ca. 10% of the recovered radioactivity. The rice cell and media fractions contained similar amounts of radioactivity (45%) but the flax media fraction contained ca. 75% and the cell fraction ca. 20% of recovered radioactivity. Recovery of radioactivity from the cultures was 50 and 80 % for rice and flax respectively. The difference could have been caused by a reduced number of solvent extraction steps for the flax medium but it is also possible that rice may have been biologically more active in transforming ^{14}C-tralkoxydim. The concentration of radioactivity in the debris fractions decreased with culture age but remained constant in the cell fractions. Radioactivity accumulated in rice but decreased in flax cells with increasing incubation time (6-72 h).

Both rice and flax cell suspension cultures produced at least 8 degradation or transformation products as assessed by tlc, autoradiography and radiochromatogram scanning. The rice and flax cell suspension cultures produced the same metabolites. The limitations of the techniques used did not permit differential rates of metabolism to be distinguished within or between the tested species. Cell and media fractions of both species contained the same metabolites; a strong presence in the cells was often conversely accompanied by a weak presence in the media. The parent tralkoxydim could be transformed abiotically or biotically to yield oxazole and imine derivatives. Biotic transformation also produced hydroxy tralkoxydim and glutaric acid. In addition, cell fractions showed the progressive development of several polar compounds considered to be conjugation products. The differential response of rice and flax to growth inhibition *in vitro* could not readily be explained by the subtle qualitative differences in the transformation products identified nor by quantitative differences in ^{14}C-labelled distribution between cell debris, cell and media extracts.

This *in vitro* study provided a useful technique to investigate the transformation of tralkoxydim and the characterisation of its metabolites. The techniques applied herein could usefully evolve by expanding the species range *in vitro* and the introduction of chemical methods of manipulating metabolism e.g. via cytochrome P_{450} inhibitors and stimulators. Such *in vitro* studies could form valuable adjuncts to the classical *in vivo* studies investigating the mode of action of herbicides.

1 INTRODUCTION

1.1 Plant tissue culture

This is the growth of small pieces of plant tissue, organs or cells (explants) under aseptic conditions in a defined nutrient medium in a controlled environment.

1.1.1 Cell cultures

Cell cultures are morphologically more uniform than whole plants. This makes them useful as model systems for cell biology studies. Most cell cultures consist of de-differentiated cells which contain the same genetical information as whole plants, but do not always exhibit the same characteristics. Cell cultures are either grown on semi-solid medium (callus) or in liquid medium (cell suspension cultures).

Callus, an amorphous mass of de-differentiated cells, is in natural circumstances produced by the plant on wound surfaces or in reaction to stimulators produced by micro organisms (Dodds and Roberts, 1986). *In-vitro* callus can be produced from a wide range of explants including seeds, roots and leaves (Evans *et al.*, 1981). The production of callus may be induced by wounding the explant but often an exogenously supplied growth regulator improves or is necessary for the formation of callus (Allan, 1991). The most commonly used growth regulators are the auxins and cytokinins, the concentrations (usually between 0.1 and 10 mg/l) and mutual ratios of which control the production of callus, shoots or roots from the explant. The auxin: cytokinin ratio regulates cell division, cell elongation and tissue differentiation.

Callus tissues may become heterogenous in cell composition due to re-differentiation. Cells will differentiate when the callus increases in size creating a concentration gradient of nutrients and growth regulators (Allan, 1991). This heterogeneity is seen in established cultures as differences in colour, morphology, structure and growth. Callus is subcultured when it is still healthy, in general every 3-4 weeks.

Cell suspension cultures are usually initiated by transferring callus tissue to a liquid medium, which is then agitated. This medium often has the same composition as that used for callus initiation but does not contain a solidifying agent. Friable callus is a better source for initiation than hard compact callus (Allan, 1991). In time, aggregates will become smaller and fine cell colonies can be separated by sieving (Marshall and Courduries, 1992) or by pipetting (Finch *et al.*, 1991) and thus selectively subcultured. A minimum inoculum density is required to establish a cell suspension culture. A lag phase is avoided if the inoculum is large or if conditioned medium is added (Dixon, 1985). With small but adequate inoculum volumes cell growth will follow a sigmoid curve. Cells have to adapt to the culture media. After this cell numbers will increase logarithmically for a short period, and then linearly until a lack of a certain medium component inhibits growth (or an inhibitor accumulates) and cells enter a stationary phase. If medium is not replaced at this stage the cells will die. However, cultures are usually subcultured during the linear phase (Dodds and Roberts, 1986).

In cell cultures a selection will take place. Cells which adapt fastest to the change in environment and have the shortest doubling time will gradually predominate the whole culture (Allan, 1991). Cell cultures can be genetically unstable due to somaclonal variation. This is caused by culture induced mutations in tissues grown, e.g. chromosomal abnormalities, as well as translocation, deletion and amplification of plant genes, and these mutations may or may not be stable. These changes in the cultures are useful for selection purposes but create problems if a stable culture is demanded.

1.1.2 Methods for growth measurement

In order to compare cell suspension cultures it is important to measure growth frequently. Methods should be quick, cheap, easy to carry out, accurate, comparable between cultures, and growth should be monitored continually without disturbing the culture. Davis *et al.* (1984) compared various growth parameters; settled cell volume (SCV), packed cell volume, absorbance at 525 nm of sonicated aliquots, dry weight and electrical conductivity and pH of the culture medium. They concluded that the most useful methods were the

measuring of the settled cell volume, the conductivity and dry weight (DW). Growth can be measured without removing cells or media (non destructive) or by harvesting a part or the whole culture (destructive). Measuring conductivity and DW are destructive and therefore not useful if growth of growing cultures is to be measured daily. A widely used non-destructive method is measuring the cell volume. This can be done by growing the cultures in special flasks with a graduated side arm. The cultures are shaken and the side arm filled quickly. The cells will settle in this tube and volume can be measured. Gilissen *et al.*, (1983) and Blom *et al.*, (1992) showed that cell volume could be measured using normal flasks. The flasks were placed in a constant angle to let the cells settle. Gilissen measured the 'chord length' and Blom the height of the settled cells. Using known amounts of fluid the chord length or height was measured to create a regression equation with which the length of the chord length or height of the settled cells could be converted into volume. These workers demonstrated a good correlation between SCV and fresh weight (FW) and concluded that FW could simply be estimated by measuring volume.

Cell viability is not really a measurement of growth but more of survival. This is useful if cultures are inhibited by a lack of nutrients or xenobiotics. Membrane intactness can be assessed microscopically (Bolwell, 1985). Membrane integrity can also be measured using staining techniques. Stains can be excluded by living cells e.g. Evans blue, or absorbed, e.g. fluorescein diacetate (Withers, 1985) or tetrazolium chloride (Dixon, 1985).

1.2 Use of tissue culture for herbicide research

Historically, research concerning the fate and behaviour of herbicides in plants has been conducted using whole plants. Cells cultured *in vitro* would be of great use if they showed the same metabolic activity as whole plants. In several reviews and articles the metabolism of herbicides in cell cultures and whole plants was compared (Swisher, 1987, Camper and McDonald, 1989, Owen, 1989, Smeda and Weller, 1991).

When comparing the action of a xenobiotic in whole plants with cell cultures, only two factors are the same; the species and the applied active chemical compound. All other

factors can change and in that way change the response of the cells and effect the efficiency of the herbicide. Sensitivity of cells can change with the cell age and may be different for toxic and non toxic amounts of chemical. Culture media content, pH, nutrients and excreted plant metabolites can increase or decrease the effect of the applied chemical and change the effect in time. Light and temperature can influence cell activity and may also change the form of the chemical. In tissue culture, physiological processes (e.g. absorption, translocation and photosynthesis) can be modified or reduced in importance since there are no intact plant organs.

Most *in vitro* studies are conducted with undifferentiated callus (Davidonis *et al.*, 1978, Lee, 1980) or cell suspension cultures (Irzyk *et al.*, 1990, Sterling and Balke, 1990). Shoot cultures can be used if differentiated tissues are to be studied. In general, tissue cultures show similar phytotoxic symptoms to those shown in whole plants where the herbicides has its mode of action on processes which are not exclusive to differentiated structure, e.g. cell division, protein synthesis, lipid synthesis.

1.2.1 Advantages and disadvantages of cell culture systems

The advantages and disadvantages of cell culture systems are a consequence of the change in environment and tissue. The environment is almost totally controlled; media, light and temperature can be regulated and pathogens excluded. This means that experimental conditions can, theoretically, be precisely repeated anywhere. The use of undifferentiated tissue (callus, cell suspensions) provides the opportunity to examine reactions at the cellular level without plant barriers (cuticular waxes, vascular system). These factors give the advantage of homogeneity of growth, a uniform exposure to the applied chemical, reduced cellular content (Dodds and Roberts, 1986) such as pigments that maybe can complicate analytical measurements, and a more economical use of space, time and chemicals. The changes in environment and tissue also have disadvantages. It is still not possible to grow all species *in vitro*, notably some leguminous and monocotyledonous species. Plant tissue cultures do not necessarily exhibit all the physiological processes which whole plants possess. Absorption, translocation and photosynthesis are often reduced

and external carbon sources required. This means that chemicals which influence these processes *in vivo* are less effective or completely ineffective *in-vitro*.

1.2.2 Absorption of herbicides *in vitro*

Cuticular waxes (Warren, 1991) and vascular systems (Bolwell, 1985) present in whole plants are reduced in meristem cultures and absent in most cell cultures. Cells in suspension are uniformly exposed to herbicides, and barriers to uptake are reduced to simply the cell wall and cell membranes. Differences in the rate of herbicide uptake between plant species *in vivo* and *in vitro* were demonstrated by, Buhler *et al.*, (1985). Haloxyfop uptake by soybean (*Glycine max*) cells in suspension was higher at all harvest times (12, 24 and 72 hours (h)) compared to yellow foxtail (*Setaria glauca*) cell suspension cultures. After 72 h the amount of haloxyfop decreased in soybean cells, but not in the cells of yellow foxtail (Buhler *et al.*, 1985). The uptake of bentazon at 6 h in cell cultures varied both in an inter-specific and intra-specific manner from almost nothing to more than 80% of the applied herbicide (Sterling and Balke, 1989). Alfalfa cells (*Medicago sativa*) absorbed up to 88%, whilst corn (*Zea mays*) 55%, soybean, velvetleaf (*Abutilon theophrasti*), carrot (*Daucus carota*), tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) cells absorbed less than 15% of applied activity. Variation in uptake in species was reported for soybean, between 1.5% and 8%, rice (*Oryza sativa*), between 15% and 50%, and for wheat (*Triticum aestivum* and *T. monococcum*) between 11% and 63%.

Uptake of the herbicide is influenced by the culture media, temperature and light. Absorption can be an active or inactive (diffusion) process, affected by pH and dependent on energy and carriers. Sterling *et al.*, (1990) showed that bentazon movement into velvetleaf cells was via nonfacilitated diffusion but that accumulation was dependent on pH and energy. The non-facilitated diffusion was also shown when media were replaced and bentazon effluxed rapidly.

Cell age can influence absorption. Irzyk *et al.* (1990) showed that 4-day (d)-old cell suspension cultures of Proso millet (*Panicum miliaceum*) absorbed greater amounts of

haloxyfop than 1-day-old cultures. He suggested that this could be a consequence of pH decreasing in the medium of older cultures. Haloxyfop effluxed from 1-d and 4-d-old cells into the media thus reflecting an effect upon membrane integrity.

Absorption of herbicides by the cells is not necessarily linear in time. ^{14}C-haloxyfop was rapidly absorbed in the first hour by the cells of soybean cell suspension cultures compared to yellow foxtail and shattercane (*Sorghum bicolor*) cell suspension cultures, but total uptake did not differ between species after 48 h (Buhler *et al.*, 1985). Tolerant soybean cells absorbed sethoxydim more rapidly than susceptible johnsongrass (*Sorghum halepense*) in suspension cultures. Uptake by soybean cells was at a maximum after 6 h, decreased the next 6 h and was stable from 12-24 h. Uptake by johnsongrass cells increased in time from 6 to 24 h (Swisher and Corbin, 1982).

Uptake of diclofop-methyl by cell suspension cultures of resistant diploid wheat (*Triticum monococcum*) was slower when the concentration of this herbicide was increased to toxic rates (Dusky *et al.*, 1980).

1.2.3 Metabolism

Metabolism is the degradation of the applied herbicide to secondary products. These secondary products can be further detoxified by the formation of conjugates in the cells or the medium where an *in-vitro* system is being studied.

When the same active compound reaches the same biological target site in whole plants and tissue culture, the metabolites produced may be similar. The active compound can be changed *in-vivo* by physical and/or biological systems, e.g. substances on the leaf and in the vascular system. *In-vitro* the media can influence the chemical, forming conjugates or inducing hydrolysis. Abiotic transformation of sethoxydim was reported by Campbell and Penner (1985). Sethoxydim in water or exposed to light for 1 h was transformed to 6 major metabolites. The biological target site for the herbicide may never be reached in whole plants or similarly in a cell culture system. Thus herbicide metabolism in a

meristem culture will show more similarity to that in an intact plant compared to heterotrophic cell cultures grown in the dark. Cultures grown in the dark (no longer autotrophic) will not be influenced by photosynthetic inhibitors, and will express secondary target sites. Non photosynthetic soybean and velvetleaf cell suspension cultures were inhibited by bentazon, which is known to inhibit photosynthesis. This indicates that there was a secondary target site (Sterling and Balke, 1988). Quantitative differences in herbicide uptake and metabolism are difficult to compare between whole plants and cell cultures.

Metabolites produced by whole plants and cell cultures are often the same. Metabolism in whole plants and cell cultures was similar for haloxyfop in soybean and yellow foxtail (Buhler *et al.*, 1985) and for sethoxydim in soybean and johnsongrass (Swisher and Corbin, 1982). Bastide *et al.*, (1990) showed that phenyl-3,4 indole was non-toxic *in-vivo* but toxic *in-vitro* for sycamore (*Acer pseudoplatanus*).

Sterling and Balke (1989) noted a difference in metabolism of bentazon between susceptible and tolerant soybean species in suspension culture. The tolerant species metabolized the bentazon while the susceptible did not. In the same study rice cell suspension cultures metabolized bentazon 25 times faster than the tolerant soybean cells.

Yellow foxtail and soybean cell suspension cultures treated with haloxyfop showed differences in metabolism. The soybean cells contained almost only the added herbicide, haloxyfop-methyl, and significant levels of polar materials. The yellow foxtail cells contained haloxyfop and some haloxyfop-methyl and polar products (Buhler *et al.*, 1985).

The rate of sethoxydim metabolism was greater in tolerant soybean than in susceptible johnsongrass cell suspension cultures (Swisher and Corbin, 1982). Johnsongrass cells contained 4 to 5 times more parent compound than the soybean cells but the soybean cells contained more metabolites. The medium from the johnsongrass cell suspension cultures contained more parent compound than the medium of the soybean cell suspension cultures.

In cell cultures of wild oat (*Avena fatua*) the rate of metabolism of diclofop-methyl was less than in cultivated oat (*Avena sativa*) cell cultures (Dusky *et al.*, 1982). After 2 days the cells of wild oat contained more than 50% of the recovered activity compared to 15% in the oat cells. The distribution of the metabolites was similar in both species.

Dusky *et al.*, (1980) showed that when diclofop-methyl was applied at toxic levels to cell suspension cultures of diploid wheat (*Triticum monococcum*), metabolism and metabolites produced by the cells were similar as in situations where lower non-toxic levels were applied.

1.2.4 Biochemical processes

Cell cultures are extremely useful for investigating the biochemical processes inhibited by xenobiotics in the cells. Examples are the inhibition of acetolactase synthase (ALS) by the sulfonylurea herbicides (Mazur *et al.*, 1987, Schloss *et al.*, 1988), and the inhibition of lipid biosynthesis by the aryloxyphenoxypropionate acids and cyclohexanediones (Secor *et al.*, 1989, Harwood, 1989).

In herbicide mode-of-action studies it is often appropriate to use *in vivo* and *in vitro* methods to identify and characterise the target site. Classical studies have therefore examined the conversion of a xenobiotic into one or more secondary products in control and herbicide treated systems. The use of *in vitro* systems may be especially useful to gain a broad view of the herbicide primary site of action in plant species which are relatively easy to culture, e.g. tobacco, soybean and maize. Herbicides block specific biological processes catalysed by enzymes, causing a deficiency in the subsequent compound or compounds produced in the reaction. To find the influence of a herbicide upon a biochemical reaction a mixture of the substrates is added to the media and the incorporation of each substrate into the final compound(s) is measured. In this way it is possible to determine the nature of the inhibition process. In graminicide mode of action studies, Cho *et al.* (1986) used ^{14}C-labelled sucrose, sodium acetate and L-leucine to investigate the mode of action of haloxyfop in corn and soybean. They placed corn cells

in a growth medium and added radio-labelled substrates and haloxyfop within 1 minute. At harvest, radioactivity in the cells was determined giving an indication how much labelled material was taken up. This was compared with a control without haloxyfop. ^{14}C-acetate incorporation provided a measure of lipid synthesis and ^{14}C-leucine of protein synthesis. The ^{14}C-labelled sucrose was used as a measure of uptake and respiration. Thus selected physiological processes which are key targets for the mode of action of a herbicide may be investigated *in-vitro*.

1.2.5 Tissue culture in future herbicide research

Plant science research continually evolves. This field is so large and comprehensive that the mechanism of many plant processes are still not completely understood in detail. Every step further in the understanding of particular plant processes, e.g. differentiation, cell signalling, cell wall formation, sink-source relations, will provide information about whether herbicides may inhibit or modify plant growth in a quantitative or qualitative manner. A more practical point is that if a herbicide is to be tested *in-vitro* the protocol for culturing a range of species must be available. Further studies are required in several species to attain practical working systems, notably in many monocotyledonous and legume species.

In-vitro techniques, cell and tissue culture, have the potential for screening chemicals with possible herbicidal effects (reviewed by Gressel, 1987), although it will always be necessary to check *in-vivo* as well. Screening protocols should be standardised to make it possible to compare chemicals of various structure. Plant material should cover a large array of plant species, (e.g. monocots, dicots, legumes), and specific plant processes, e.g. autotrophic and heterotrophic cultures, in an environment which minimizes influences upon the chemical before it reaches the cell. Assessments of any effects which the herbicide may produce *in-vitro,* in terms of growth, should be made throughout several intervals of the time course of study.

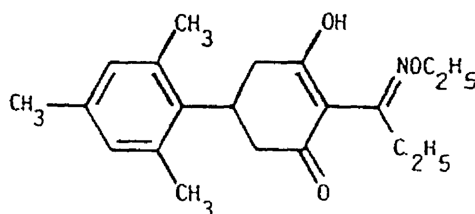
Cell cultures can also be screened with known herbicides to select possible resistant mutants (Marshall, 1991). The *in-vitro* selection of species has produced resistant cell lines, but regeneration to resistant plant varieties, and loss of resistance in whole plants, is often a bottle neck (Smeda and Weller, 1991). Resistant cell lines can be used to investigate the difference in sensitivity between the susceptible and resistant biotype. Gronwald *et al.*, (1989) selected sethoxydim and haloxyfop resistant embryogenic cell cultures of maize. These authors investigated acetyl-CoA carboxylase activity and sensitivity in resistant cell lines.

1.3 Tralkoxydim

Tralkoxydim is a member of the cyclohexanedione family of herbicides, discovered by ICI Australia, code named PP604 and trade named Grasp, Grasp 604 and Splendor (see Warner *et al.*, 1987).

The chemical name is 2-[1-(ethoxyimino) propyl]-3 hydroxy-5-mesitylcyclohex-2-enone. The molecular structure is shown in Fig. 1. The empirical formula is $C_{20}H_{27}NO_3$, with a molecular weight of 329. Tralkoxydim is a white odourless powder with a melting point at 106°C and a vapour pressure of $4*10^{-10}$ kPa at 20°C. Solubility in water at 20°C is 6 mg/l at pH 6.5 and 5 mg/l at pH 5.0. Solubility in other solvents at 24°C include hexane at 18 g/l, toluene at 213 g/l, dichloromethane at > 500 g/l, methanol at 25 g/l, acetone at 8 g/l and in ethylacetate at 110 g/l (Warner *et al.*, 1987).

Figure 1. The molecular structure of tralkoxydim.



1.3.1 Activity and weed spectrum

Tralkoxydim is a post-emergence herbicide selective in wheat and barley for the control of grass weeds. It is very toxic to many grass weeds at doses of 150-350 g ai/ha. Wild oat (*Avena spp*) species are very sensitive but other grass weeds such as ryegrass (*Lolium spp*) species, green foxtail (*Setaria viridis*), canary grass species (*Phalaris spp*), blackgrass (*Alopecurus myosuroides*) and loose silky-bent (*Apera spica-venti*) are well controlled. The herbicide can be applied to cereals from the seedling pre-tillering stage through to stem extension. Tralkoxydim has no significant herbicidal effect on broadleaf weeds or sedges. On non-target species, mammals, birds, fish and insects, the toxicity is very low. The compound hardly irritates, has no chronic, mutagenic or reproductive influences, and rapidly degrades in soil and cereal tissue (Warner *et al.*, 1987).

The recommended rate of use in Australia for the control of wild oat and ryegrass in wheat crops is 100-150 g/ha at the 2-4 leaf stage of the crop. Good control of wild oat was achieved at 75-100 g/ha tralkoxydim, when applied at the 2-leaf stage of the crop, but this treatment did not control large plants satisfactorily. Higher concentrations (150-200 g/ha) were necessary for optimal yield at the early tillering stage. Higher concentrations, > 200 g/ha, or earlier application, < 2-leaf stage, of tralkoxydim in wheat can cause severe crop damage resulting in reduced grain yields (Madin and Martin, 1990). Experiments with tralkoxydim in Canada revealed that green foxtail and wild oat were controlled in wheat and barley using application rates from 150-350 g/ha when applied at the 2-3 leaf stage of the weed. A disadvantage of early application was that late germinating wild oat and green foxtail were not controlled. Wheat and barley were not visibly injured and grain yield not reduced at these rates. The environmental conditions also influenced crop reaction. Tralkoxydim efficacy was reduced under drought stress (Harker and Blackshaw 1991). Both authors emphasize that tralkoxydim activity can vary with weed growth stage and that crop damage, resulting in yield decrease, can occur if tralkoxydim was applied particular early or late in the growth stage of the crop.

1.3.2 Absorption and translocation

The uptake of tralkoxydim is foliar and the herbicide is rapidly transported to all meristematic tissues, where it inhibits growth and probably, as a secondary reaction, induces necrosis. Rainfastness is reached in one hour (Warner *et al.*, 1987). Although no published studies are available on this subject it is understood that the processes are essentially similar to those in other aryloxyphenoxypropionates and cyclohexanediones. Thus like sethoxydim and haloxyfop which were absorbed rapidly via the cuticle and transported in a source-sink direction to accumulate in actively growing areas, apical leaves and root apices of soybean, johnsongrass, yellow foxtail and shattercane (Swisher and Corbin, 1982, Buhler *et al.*, 1985)

1.3.3 Effects on plant growth

Growth in susceptible species is reduced very rapidly. Tralkoxydim moves in the phloem to the growing points and plants cannot form new leaves and existing leaves do not extend. The result is a smaller leaf area and reduced levels of photosynthesis. Fresh weight of wild oat did not increase any more 2 days after foliar application in glasshouse experiments. Necrosis of grasses is reached in 3-4 weeks (Warner *et al.*, 1987). The same symptoms were shown in a histological study of sethoxydim in Johnsongrass. Sethoxydim disorganized apical regions and necrotic cells within the apex and leaf primordia of the shoot. Necrotic zones were also evident at the base of expanding leaves and in root apices 1 day after treatment (Swisher and Corbin, 1982). Wild oat treated with sethoxydim, fluazifop and haloxyfop at the 5-leaf stage reduced stem elongation within 2-5 days and internodes were constricted at the base (Jain and Vanden Born, 1989).

1.3.4 Antagonism

Activity of the aryloxyphenoxypropionate acids and the cyclohexanediones is often reduced when mixed with broadleaf herbicides (McCall, 1988, Bridge *et al.*, 1991, Grichar, 1991),

and tralkoxydim is no exception. Control of wild oats with tralkoxydim was reduced if tank mixed with 2,4 D amine or bentazon. Tralkoxydim activity was normally evident 0.5 hours after bentazon application and 24 hours after 2,4 D amine application (Jensen and Caseley, 1990). Tank mixtures of bromoxynil or thifensulfuron with tralkoxydim reduced tralkoxydim activity against green foxtail and wild oats (Harker and Blackshaw, 1991).

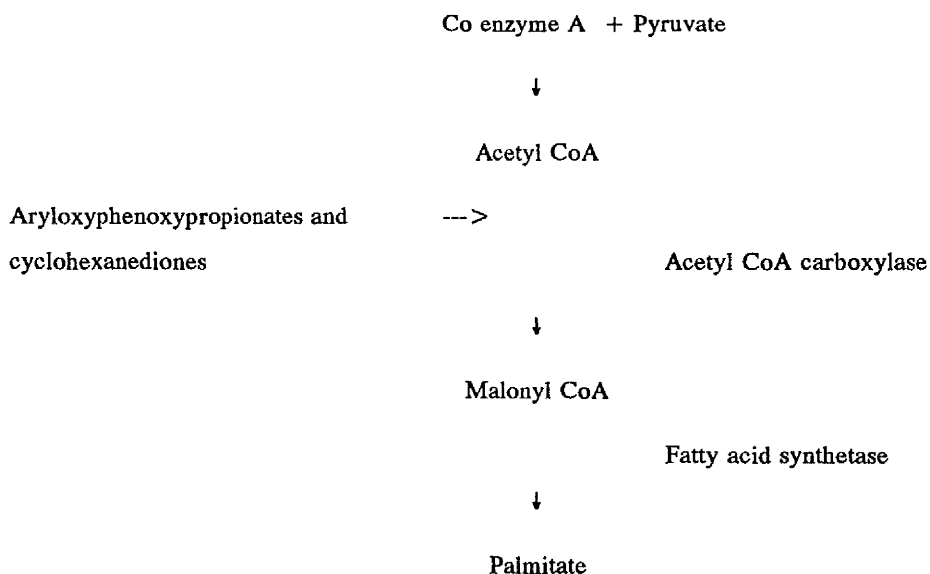
1.3.5 Mode of action

The aryloxyphenoxypropionates and the cyclohexanediones influence the synthesis of fatty acids, (Secor and Csaba, 1988, Rendina *et al.*, 1989, Secor *et al.*, 1989, Gronwald, 1991, Cobb, 1992). More general information about fatty acids in plants is reviewed by Andrews and Ohlrogge (1990), and about the Acetyl-Coenzyme A Carboxylase in particular by Harwood (1989).

In summary, fatty acids are used in plant membranes and cuticular waxes and are synthesized in chloroplast stroma and cytoplasm. Lipids have a three carbon glycerol backbone to which two long chain fatty acids, usually 16 or 18 carbon atoms long, are esterified. The third binding site can be a phosphate or sugar group. The aryloxyphenoxypropionates and cyclohexanediones affect the synthesis of fatty acids. Acetyl Co A is formed by the dehydrogenation of pyruvate; two-carbon acetate units are combined with Co enzyme A (CoA) to form acetyl CoA. Acetyl CoA is carboxylated to malonyl CoA and every malonyl CoA provides two carbon atoms to form palmitate, a 16 C fatty acid. Palmitate has a chain of 16 carbon atoms and can be elongated to stearate (18C) and longer waxes. In Fig. 2 the enzymes involved in fatty acid biosynthesis are shown and the points of aryloxyphenoxypropionates and cyclohexanediones inhibition indicated. The inhibition brought about by the aryloxyphenoxypropionates and cyclohexanediones is in the first step of the process, i.e. the conversion from acetyl CoA to malonyl CoA. Acetyl CoA carboxylase (ACCase) is involved in two steps: the carboxylation of biotin and the transfer of CO_2 from biotin to acetyl CoA. Rendina *et al.* (1989) showed that the second step of the reaction catalysed by acetyl CoA carboxylase, the trans-carboxylase, is inhibited and suggested that the aryloxyphenoxypropionate and

the cyclohexanedione herbicides compete for the same site on the enzyme, and that the inhibition is reversible.

Fig. 2 Simplified schematic of the fatty acid biosynthesis in plants.



While Acetyl CoA carboxylase is universally accepted as the primary site of action for the aryloxyphenoxypropionate and cyclohexanedione herbicides, weeds which have developed resistance to graminicides have not always shown convincing differential enzyme sensitivity. Powles *et al.*, (1990) came to the conclusion that there were no differences in barrier or translocation systems, enzymatic target site, or activity of glutathione S-transferase between susceptible and resistant annual ryegrass (*Lolium rigidum*). Instead herbicide metabolism was considered to be a possible reason for ryegrass herbicide resistance.

1.3.6 Metabolism

Since tralkoxydim is a relatively new compound no literature has yet been published on this topic. However, the metabolism of other aryloxyphenoxypropionate and

cyclohexanedione herbicides in this family may provide a guide to what might be expected. Diclofop-methyl and haloxyfop-methyl were rapidly hydrolysed to diclofop (Shimabukuro *et al.*, 1979) and haloxyfop respectively (Buhler *et al.*, 1985) within the plants. Diclofop was conjugated to an aryl glucoside in tolerant species and to a glucosyl ester by susceptible species.

In addition, studies conducted by ICI Jealott's Hill research station indicate that tralkoxydim was hydroxylated by maize cell suspension. Other possible breakdown products were an imine and an oxazole, further broken down to glutaric acid and sugar conjugates (Dr. S. Hadfield, ICI, Pers. Comm. 1992).

1.4 Aims of the present study

The present study was undertaken to combine investigations in two disciplines (a) plant tissue culture and (b) herbicide mode-of-action studies. Two plant species were chosen; a monocotyledonous plant, rice (*Oryza sativa*), and a dicotyledonous plant, oil flax (*Linum usititassimum*). At the whole plant level rice and flax are considered to be moderately susceptible and resistant respectively to the application of tralkoxydim. Suspension culture systems for rice and flax were used to investigate the effect of various concentrations of tralkoxydim on a range of cell growth parameters: DW, FW and SCV. In addition the effect of incubation period and cell age was determined on the metabolism of ^{14}C-labelled tralkoxydim. The results of this study are intended to contribute towards an understanding of the value of *in vitro* systems in herbicide research.

2 MATERIALS AND METHODS

2.1 Cell suspension cultures

2.1.1 Rice culture

Methods used were essentially those described by Finch *et al.* (1991). Seeds of rice (*Oryza sativa* cv.Taipei 309) were dehusked and surface sterilized (Domestos (20% v/v) for 30 min, 5 washes in sterile distilled water). Seeds were germinated in 9 cm Petri-dishes containing 25 ml autoclaved Linsmaier and Skoog (1965) medium, solidified with 0.4% (w/v) agarose and supplemented with 2.5 mg/l 2.4-D (LS 2.5) and maintained in the dark at 28°C.

Different types of callus were produced on the rice seeds and suspension cultures were initiated after 3-months from soft embryogenic callus. This callus (0.2-0.3 ml) was taken out and inoculated into a 100 ml conical flask containing 20 ml AA2 liquid medium (Abdullah *et al.* 1986) and shaken (120 rpm) in the dark at 28°C. The callus culture was subcultured every 5 days, by replacing 70% of the old medium. After 6 weeks cultures contained many small cell colonies and these were selectively subcultured using a 10 ml plastic pipette (Sterilin). The aliquot taken out contained 1 ml SCV and 6 ml old medium and was subcultured into 21 ml fresh medium, and the culture maintained as before. This subculture procedure was subsequently carried out at weekly intervals.

2.1.2 Flax culture

Methods used were essentially those described by Marshall and Courduries (1992). Seeds of flax (*Linum usitatissimum* L. cv. Norlin) were surface sterilized (Domestos (30% v/v) for 20 min, 5 washes in sterile distilled water) and germinated in 9 cm Petri-dishes containing 25 ml autoclaved tapwater solidified with agar (8% w/v), in the dark at 21°C. Callus was produced from hypocotyl segments (5 days old, 4-8 mm in length) placed

vertically on Murashige and Skoog (1962) MS medium containing 1 mg/l BAP, 0.02 mg/l NAA, 2.5% (w/v) sucrose (MS125), solidified with 0.8% (w/v) agar in 9 cm plastic Petri-dishes and incubated in the growth room at 21°C with a 16 h photoperiod. Callus was subcultured every 4 weeks, maintained on the same medium under the same conditions.

Callus (2 g) cut into small pieces (8-64 mm^3), was inoculated into liquid medium (MS125, without agar), shaken at 21°C in the light and subcultured every week by replacing 75% of the medium. After 10 to 20 weeks, soft white calli were produced from the original hard, compact, green callus. The amount of white callus increased in 6-8 weeks giving rise to finely-divided yellow/white cell colonies. These fine yellow/white colonies were taken out (2 ml SCV in 8 ml media) with a 10 ml plastic pipette (Sterilin) and placed in 21 ml fresh media and subcultured as before. Such cultures could be grown either in the light at 21°C and or in the dark at 28°C but dark grown cultures were used in the present study.

2.1.3 Growth assessments

Culture growth was assessed by measuring SCV, FW, DW and viability.

Estimates of SCV were made by laying the flasks down at a fixed, constant angle for 3 min and measuring the length of the surface line or 'chord length' of the settled cells. This length was converted to volume using a calibration line, constructed using known volumes in identical flasks (Gilissen *et al.* 1983).

Cell fresh weight was determined by harvesting the entire flask by vacuum filtration on Whatman No.1 filter paper. Cells were placed in a pre-weighed tray and reweighed. To determine dry-weight, cells were dried at 60°C to a constant weight (approximately 20-26 h).

Cell viability was determined using the tetrazolium reduction test modified from that used by Dixon (1985). A 5 ml homogenous mixture of cells and medium was taken out of the culture flask, placed in preweighed centrifuge tubes and centrifuged at 1000 rev/min for

5 min. The supernatant was poured off, the pellet and tubes weighed and after adding 4 ml buffered TTC solution (0.6% (w/v) tetrazolium chloride in 0.05 M PO_4 buffer (pH 7.5)) incubated at 28°C in the dark. After 16-20 hours the cells were centrifuged at 2000 rev/min for 5 min and the supernatant removed. The tetrazolium chloride was extracted from the cells with an excess of absolute ethanol (10-15 ml), in a waterbath at 60°C for 15 min. The cells were centrifuged at 5000 rev/min for 1 min and an aliquot of the supernatant measured spectrophotometrically by absorbance at 485 nm. An index of viability was calculated per g FW.

2.1.4 Determination of the growth cycle of rice and flax cell suspension cultures

The growth cycle of the rice and flax cell suspension cultures (subcultured as described above) was determined in order to define the stage in the cell growth curve, (i.e. lag, log, linear or stationary phase) when herbicide was applied. The growth of the cell suspension culture was followed daily by measuring SCV for 7 and 12 consecutive days for rice and flax respectively. To determine FW and DW, three flasks were harvested, daily for rice and on days 0, 2, 5, 7, 9, and 12 for flax. In duplicate experiments only SCV was measured from rice and flax, the flax was harvested on day 9. From experiments 1 and 2 the mean and standard error were calculated for SCV.

2.2 The influence of tralkoxydim on the growth of rice and flax cell suspension cultures

2.2.1 Preparation of tralkoxydim stock solutions

Analytical grade tralkoxydim (99 % pure) provided by ICI was dissolved in absolute methanol and filter sterilized (0.2μm). Different volumes of stock solution were added to the rice and flax cell suspension cultures to give the final treatment concentrations but all treatments and the control received the same volume of methanol.

2.2.2 Assessments

Each treatment contained two or three replicates from which the mean and standard deviation were calculated. Duplicate experiments were averaged and standard error values calculated. Flasks were placed on the shaker in a random design. Calculations were done using a spreadsheet (Supercalc 4); the regression equations were calculated using a statistical program (Minitab).

2.2.3 Treatments of the rice cell suspension cultures

To investigate the sensitivity of rice cell cultures 50, 100, 150, 200 and 250 μM tralkoxydim was inoculated on day 2 of the subculture cycle. SCV was measured daily, and at the end of the experiments, the cells were harvested and FW and DW measured. Growth was compared with a control. The stock solution was prepared by dissolving 2 mg/ml tralkoxydim in absolute methanol.

To investigate the sensitivity of rice cell cultures to tralkoxydim at different stages of the growth cycle, 75, 150, and 225 μM tralkoxydim was added to separate cultures on day 2, 4 and 7 of the subculture cycle. SCV of the cultures was measured daily. On the day of tralkoxydim incubation, 3 representative flasks were harvested and FW and DW determined and 3 days after tralkoxydim incubation all the treated cultures were harvested and fresh and dry weights measured. Growth was compared with a control. All incubations were conducted in the dark to avoid photochemical degradation.

Table 1 Treatments of rice cell suspension cultures with tralkoxydim.

Experiment	Tralkoxydim Range (μM)	Incubation day of growth cycle	Harvest days after incubation
1	50-100-150-200-250	2	12
2	50-100-150-200-250	2	5
3	75-100-225	2,4,7	3
4	75-100-225	2,4,7	3

2.2.4 Treatments of the flax cell suspension cultures

To investigate the sensitivity of flax cell cultures 25, 50, 100, 150 and 225 μM tralkoxydim was inoculated on day 2 of the subculture cycle. SCV was measured daily, and at the end of the experiments the cells were harvested and fresh and dry weights measured. Growth was compared with a control. The stock solution was prepared by dissolving 3 mg/ml tralkoxydim in absolute methanol.

To investigate the sensitivity of flax cell cultures to tralkoxydim treatment at different stages of the growth cycle, 25, 50, 100 and 150 μM tralkoxydim was added to separate cultures on days 3, 6 and 9 of the subculture cycle. SCV of the cultures was measured for 3 consecutive days after tralkoxydim incubation. FW and DW were determined 3 days after tralkoxydim incubation. Growth was compared with a control. All incubations were conducted in the dark to avoid photochemical degradation.

Table 2 Treatments of flax cell suspension cultures with tralkoxydim.

Experiment	Tralkoxydim Range (μM)	Incubation day of growth cycle	Harvest days after incubation
1	50-150-225	2	5
2	25-50-100-150	2	5
3	25-50-100-150	3,6,9	3

2.3 Metabolism of ^{14}C-tralkoxydim

2.3.1 Material

^{14}C-labelled (^{14}C-phenyl) tralkoxydim (1 mg, 1 mBq), was provided by ICI and stored at 0°C prior to and during the use in the project. The radiolabelled tralkoxydim was dissolved in 1 ml ethyl-acetate and purified using a preparative thin layer chromatography (tlc) plate (1 mm silica gel with fluorescent indicator UV^{254}, Camlab) using a solvent

system as follows; ethyl-acetate: acetone: hexane (15:15:10 v/v/v all HPLC-grade). The radioactive sample was spotted completely on to the middle lanes of the preparative tlc plate. The outer lanes were spotted with unlabelled tralkoxydim as standards. Samples were spotted 2 cm from the base of the plate, run to a solvent front of 18 cm, removed from the tank and dried in the dark. The spots of the unlabelled tralkoxydim were visible under UV light (254 nm). The corresponding band with labelled tralkoxydim was scraped off, and the tralkoxydim redissolved in absolute ethyl-acetate. Radioactivity of the stock solution was measured using liquid scintillation spectrometry (paragraph 2.3.4) prior to incubation into the cultures and the required amount of radioactivity added.

2.3.2 Treatments of the rice and flax cell suspension cultures

Radiolabelled tralkoxydim dissolved in absolute ethyl acetate (circa 250.000 dpm) was added to individual flasks containing the flax and rice cell suspension cultures. Cultures were grown as previously described and treated on days 2, 4 and 7 of the growth cycle. Treated cultures were harvested after 6, 24 and 72 hours of incubation. A control, incubated with the same amount ^{14}C-tralkoxydim, was harvested directly (within 5 min), each incubation day, 2, 4 and 7. Controls, incubated with the same amount ^{14}C-tralkoxydim, containing only medium were harvested after 0, 6, 24 and 72 hours on day 2, and extracted in the same way as the treated flasks. Unlabelled tralkoxydim (100 μM), dissolved in absolute methanol (2 mg/ml) was added to the rice cultures only.

2.3.3 Extraction of ^{14}C-metabolites

Cultures were harvested by vacuum filtration on Whatman No.1 filter disks and rinsed with 50 ml distilled water. The cells were processed directly. The filtered media with the rinsing water was stored in the freezer (-15°C). Cells and media extracts were analyzed separately for ^{14}C-labelled materials as described in Figure 3.

Filtered cells were placed in a preweighed bottle, weighed (to calculate dpm per g cell FW) and 30 ml acetonitrile (HPLC-grade) added. Cells were sonicated, using a Decon ultrasonic bath (FS 300b), for 30 minutes (water temperature did not exceed 40°C), and filtered again. The insoluble cell debris was stored in vials in the freezer. The acetonitrile cell extract was evaporated, using a rotary evaporator (Büchi Rotavapor R110) with the waterbath set at < 40°C, and the ^{14}C-labelled materials resuspended by rinsing the round bottom flasks 3 times with 4 ml hexane (HPLC-grade).

The filtered medium was extracted through solvent partitioning, with hexane (HPLC-grade), 4 times 25 ml, and or dichloromethane (DCM) (HPLC-grade), 4 times 25 ml, in a separation funnel. After separation, extracts were filtered through anhydrous sodium sulphate (ca. 10 g) to reduce their water content, evaporated using the rotary evaporator (same conditions as above), and ^{14}C-labelled material resuspended by rinsing the round bottom flask 3 times with 4 ml of the same solvent (hexane or DCM). In addition, Analytichem Bond Elut columns (Varian), bonded phase C18 size, 3cc (supplied by Jones chromatography) were used. These columns contain a chemically modified silica sorbent which can bind a wide range of compounds, from highly non-polar to polar. The columns were activated with 1 volume acetonitrile and rinsed with 1 volume buffer (H-Cl solution pH 1-2). The medium, adjusted to pH 1-2 with H-Cl solution, was passed through the columns (10 ml/min) and the columns rinsed with 1 volume buffer afterwards and dried with higher pressure. The ^{14}C-labelled material was collected by washing the cartridge five times with 1 ml acetonitrile. A summary of the extraction protocols is described in Table 3.

Figure 3 Simplified scheme of the extractions of ^{14}C-metabolites from the cell suspension cultures.

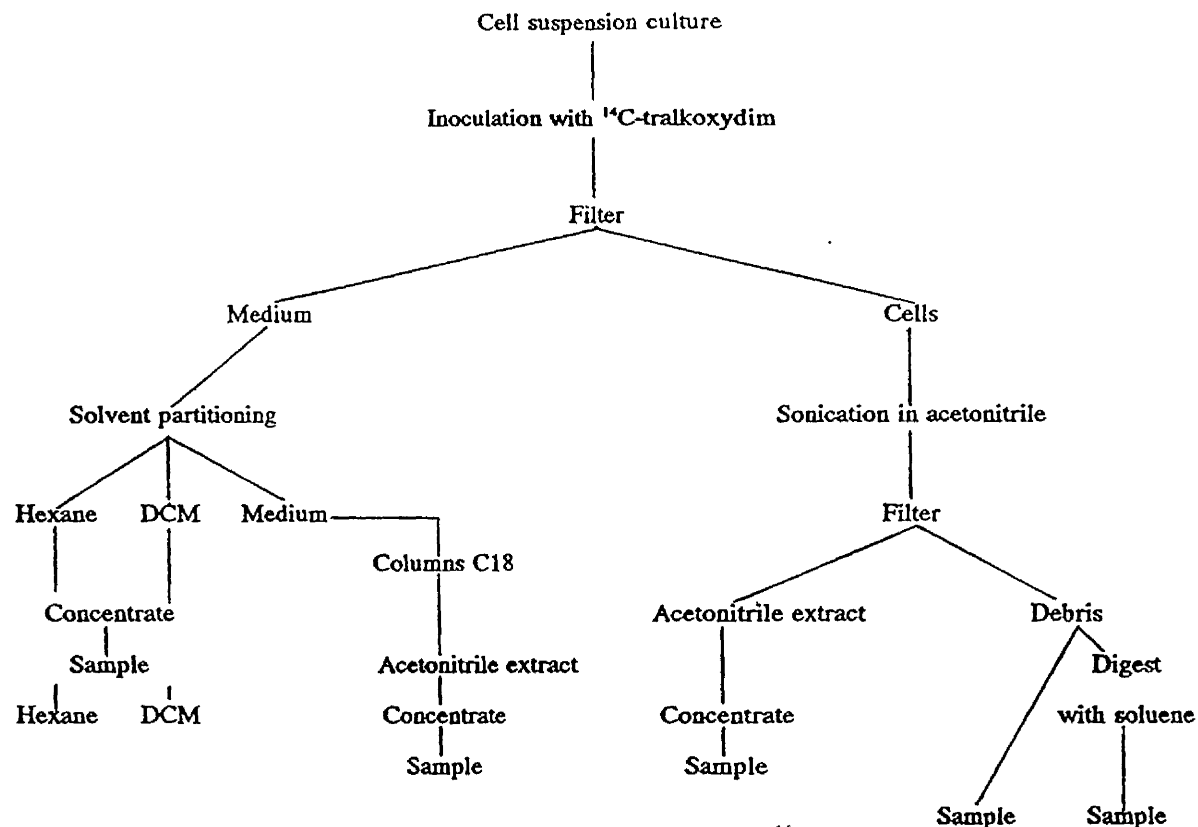


Table 3 Method of extraction of ^{14}C-tralkoxydim and its metabolites from rice and flax media.

Media	Solvent partitioning/extraction		Columns
	Hexane	DCM	Acetonitrile
Rice			
Replicate 1	X		
Replicate 2+3	X		X
Flax			
Replicate 1	X	X	
Replicate 2+3			X

2.3.4 Quantification of ^{14}C-metabolites

Radioactive samples were placed in vials (20 ml econo glass vial Packard), 10 ml of scintillation cocktail (Emulsifier-safe Packard) was added, and the samples were counted using a liquid scintillation analyzer (Packard 1900 TR). Background radioactivity and quenching were measured from 5 vials with the same amount of scintillation cocktail, this value was subtracted from the radioactivity of the samples. Results were produced in disintegration per minute (dpm).

The insoluble flax (1 replicate) and rice cell debris was weighed, placed in scintillant (emulsifier safe) and counted via liquid scintillation spectrometry. In addition, to check the efficiency of ^{14}C-release via direct counting, 2 replicates of cell debris were subjected to 'digestion' followed by scintillation spectrometry. Flax cell debris (replicates 2+3) was digested with soluene 100 (Packard), adding 2 ml of water, and soluene until the cells were bleached (1-2 ml), and put into scintillant (hionic fluor) for counting.

Cell and media extracts were dried under a stream of nitrogen gas and resuspended in a known volume of the same solvent. From these samples subsamples of 20 μl were assessed for radioactivity by placing them in vials, adding 10 ml scintillant (emulsifier safe), followed by scintillation spectrometry.

2.3.5 Analysis of ^{14}C-metabolites

Aliquots of the cell and media extracts were spotted on tlc plates (0.25 mm silica gel with fluorescent indicator UV 254, Camlab). The plates were developed in a dichloromethane (DCM): hexane: acetonitrile: formic acid (60:15:25:3 v/v/v/v, all solvents HPLC-grade) solvent system at 18-22°C in the shade. The samples were spotted 2 cm from the base of the plate and minimal 2 cm from the side. The solvent system was run to 18 cm from the base of the plate and the plates were removed and dried for 3 h in the dark. Each plate contained standard tralkoxydim and several samples, with the same amount of radioactivity spotted per lane (5.000 or 10.000 dpm).

From each treatment 2 or more samples were spotted. From rice and flax all 3 replicates of the cell extracts were spotted. From the rice media extracts of the hexane fraction (all replicates) and in addition the extracts from the columns (replicate 2 and 3) were spotted. From the flax media extracts of the hexane and DCM fraction were spotted separately (replicate 1). Replicate 2 and 3 were only extracted with the columns and these extracts were spotted.

The chromatograms were exposed for 14 days on AGFA X-ray film (OSRAY 3M). The films were developed in AGFA developer (G150, 1:5 v/v), Ilford stop bath (IN-1, 1:40 v/v) and AGFA fixer (G350, 1:4 v/v), for 60, 10 and 60 seconds respectively and finally rinsed in tapwater for several minutes.

Confirmation of the identity of the metabolites was carried out by ICI Jealott's Hill (Dr. S.Hadfield). Metabolites (300 dpm) were spotted and identified by comparison with appropriate chromatographed standards and R_f values of previously determined metabolites. Techniques used were similar as described before with the exception that the solvent was run to 16 cm of the base of the plate. The chromatograms were visualized using the Bio-Imaging Analyzer (BAS 2000, Fuji), and high resolution prints were produced.

3 RESULTS

3.1 The growth of rice cell suspension cultures

The growth of rice cell suspension cultures was an almost linear rise of SCV, FW and DW from the day of subculturing until day 7 (Fig. 4).

SCV inoculum was ca. 0.8 ml. Subsequently, SCV increased with an average of 0.35 ml per day which gave a final volume on day 7 of between 3 and 3.5 ml. The growth of FW, starting with an inoculum of 0.8 g, was most pronounced on the second and third days of the growth cycle with the average increment being 0.15 g per day resulting in a FW of approximately 1.3 g on day 7. DW on the day of subculturing was 0.04 g and accumulated thereafter most strongly during the first 2 days. The average increment was 0.022 g per day resulting in approximately 0.13 g on day 7.

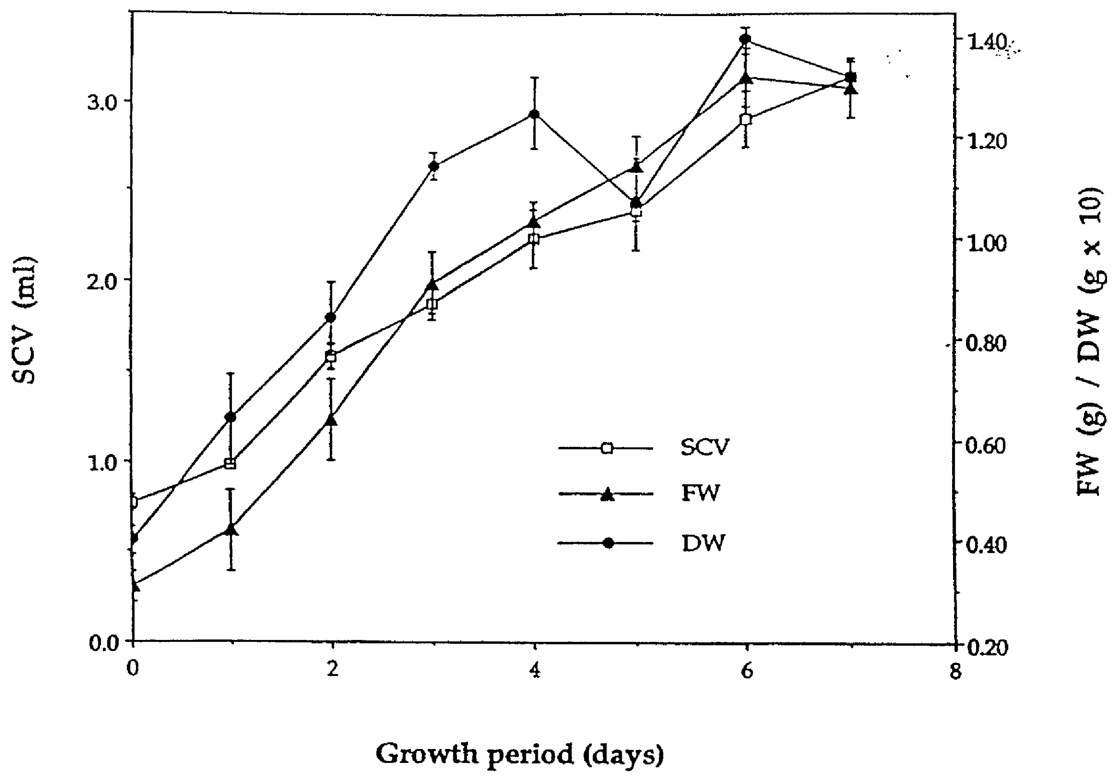
3.2 The growth of flax cell suspension cultures

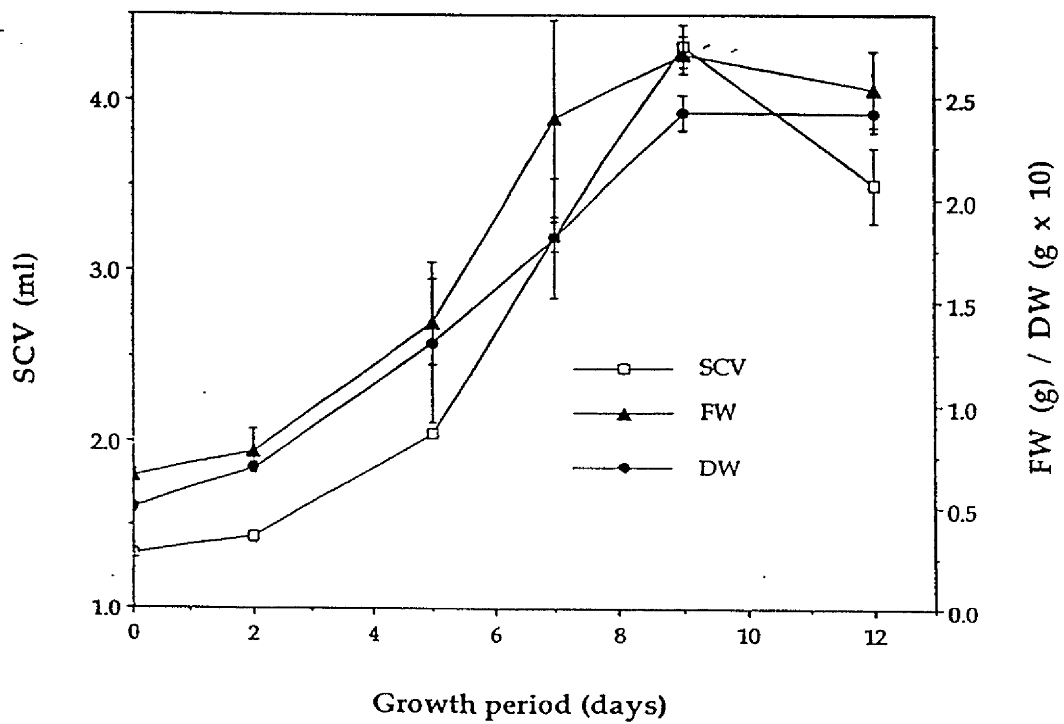
Growth of flax cell suspension cultures, measured by SCV, FW and DW, could be separated into 3 distinct phases; lag (ca. day 0-3) linear (ca. day 4-8) and early stationary (ca. day 9-12) phases (Fig. 5).

SCV declined during the first day of incubation and increased slowly on days 2 and 3. Cells entered the linear phase on day 4, with an average increment of 0.40 ml per day until day 9. Cultures did not increase in volume after day 9 and appeared to become senescent and brown in colour. FW and DW showed a similar growth pattern as SCV, although the different phases were not as strongly defined. Accumulation was greatest from days 2 to 9, with an average increment for FW of 0.3 g and DW of 0.025 g per day.

Figure 4 The growth of rice cell suspension cultures as assessed by fresh weight (FW), dry weight (DW) and settled cell volume (SCV). FW and DW values are the mean of 3 flasks $\pm$ the standard deviation, SCV values are the mean of 2 experiments $\pm$ the standard error.

Figure 5 The growth of flax cell suspension cultures as assessed by fresh weight (FW), dry weight (DW) and settled cell volume (SCV). FW and DW values are the mean of 3 flasks $\pm$ the standard deviation, SCV values are the mean of 2 experiments $\pm$ the standard error.


SCV (ml)
FW (g) / DW (g x 10)
Growth period (days)
SCV
FW
DW
0.0
1.0
2.0
3.0
0.20
0.40
0.60
0.80
1.00
1.20
1.40
0
2
4
6
8


SCV (ml)
FW (g) / DW (g x 10)
Growth period (days)
SCV
FW
DW
1.0
2.0
3.0
4.0
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10
12

3.3 Influence of tralkoxydim on the growth of rice cell suspension cultures

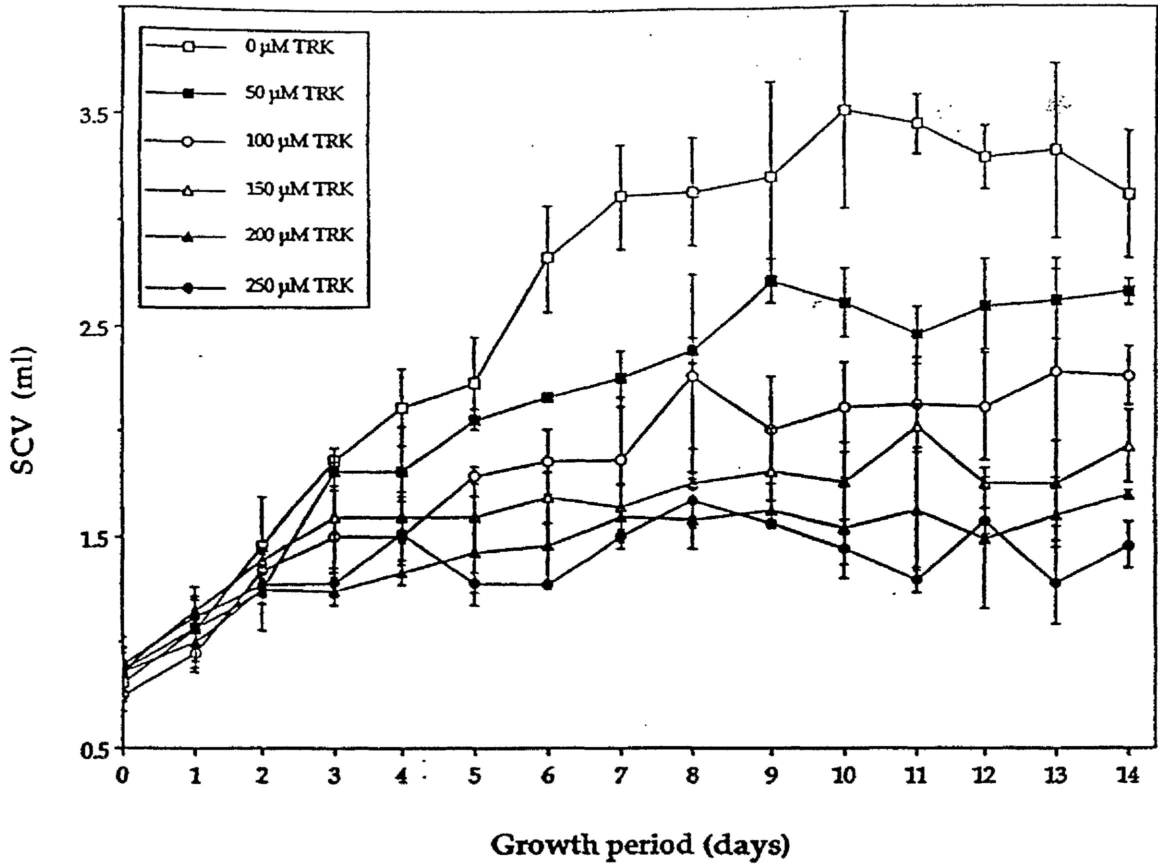
The growth of rice cell suspension cultures treated with tralkoxydim, from the day of incubation to cell harvest, was compared with the untreated control and expressed as the percentage growth inhibition. The inhibition percentages from the cultures treated with a range of tralkoxydim concentrations were used to make a regression equation. With this regression equation the amount of tralkoxydim which reduced growth by 50% (I_{50} as uM tralkoxydim) was calculated. In some experiments a reliable estimate was not obtained. The statistical reliability of the regression equation was a measure of how close the data fitted a straight line relationship.

Tralkoxydim was toxic to rice cultures at rates from 50 - 250 μM. In experiment 1 (Fig. 6), the growth curve of the control was similar to the normal growth curve of rice (Fig. 4). Growth reduction (Table 4) by tralkoxydim was most pronounced from 50-150 μM and higher concentrations did not further reduce the SCV. The I_{50} was calculated at 73 μM tralkoxydim.

In experiment 2, the overall growth (Table 4, exp 2) of the rice cell suspension cultures was less vigourous compared to earlier experiments (Table 4, exp 1). Settled cell volumes on the day of tralkoxydim incubation (day 2), were variable and declined on day 7 for most cultures. To measure the inhibition of growth, SCV was assessed from day 2 through to 6. A control rice cell suspension culture without the methanol addition showed a more vigourous growth than the other control which was incubated with methanol. The percentage inhibition (Table 4) was similar to that recorded in experiment 1 for concentrations of 50 and 100 μM of tralkoxydim but higher inhibition values were recorded for treatments of 150, 200 and 250 μM. Overall, the I_{50} for experiment 2 was higher than that for experiment 1 at 92 μM. FW, DW and viability of the cells in experiment 2 declined when treated with increasing concentrations of tralkoxydim (50 to 250 μM). Lower cell viability was associated with smaller fresh and dry weights (Table 6).

Figure 6 The influence of tralkoxydim on the growth of rice cell suspension cultures as assessed by settled cell volume (SCV) inoculated on day 2. Values are the mean of 3 flasks $\pm$ the standard deviation.

Figure 7 The influence of tralkoxydim on the growth of flax cell suspension cultures as assessed by settled cell volume (SCV) inoculated on day 2. Values are the mean of 3 flasks $\pm$ the standard deviation.


0 µM TRK
50 µM TRK
100 µM TRK
150 µM TRK
200 µM TRK
250 µM TRK
SCV (ml)
Growth period (days)

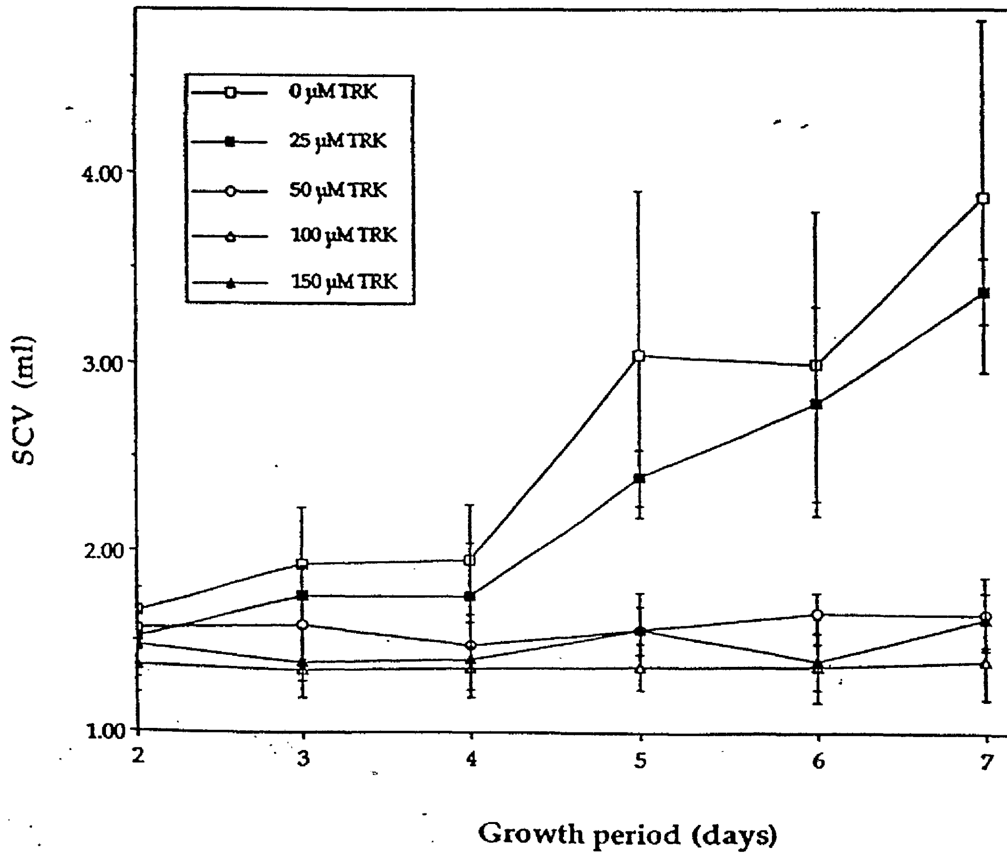

0 µM TRK
25 µM TRK
50 µM TRK
100 µM TRK
150 µM TRK
SCV (ml)
Growth period (days)

Table 4 Effect of various concentrations of tralkoxydim on the growth of 2-day-old rice cell suspension cultures as assessed by settled cell volume (SCV).

Incubation Period (d)	Growth of SCV (ml) / inhibition (%)*						I_{50}
	Tralkoxydim concentration (μM)						
	0 (control)	50	100	150	200	250	
Exp 1							
2-7	1.65 ±0.05 / 0	1.00 ±0.10 / 40	0.52 ±0.29 / 68	0.25 ±0.14 / 85	0.34 ±0.06 / 79	0.23 ±0.04 / 86	73
2-14	1.66 ±0.48 / 0	1.42 ±0.05 / 15	0.92 ±0.15 / 45	0.54 ±0.16 / 68	0.45 ±0.21 / 73	0.19 ±0.05 / 89	129
Exp 2							
2-6	0.59 ±0.03 / 0	0.32 ±0.35 / 46	0.38 ±0.12 / 37	0.03 ±0.11 / 94	-0.08 ±0.09 / 113	-0.05 ±0.09 / 108	92

Table 5 Effect of various concentrations of tralkoxydim on the growth of 2-day-old flax cell suspension cultures as assessed by settled cell volume (SCV).

Incubation period (d)	Growth of SCV (ml) / inhibition (%)*						I_{50}
	Tralkoxydim concentration (μM)						
	0 (control)	25	50	100	150	225	
Exp 1							
2-7	2.11 ±0.82 / 0		0.31 ±0.35 / 85		-0.19 ±0.12 / 104	-0.22 ±0.12 / 111	n/a
Exp 2							
2-7	2.20 ±0.80 / 0	1.86 ±0.13 / 16	0.08 ±0.19 / 96	0.02 ±0.05 / 99	0.14 ±0.07 / 94		36

* means of 3 replicates ± standard deviation of the mean
I_{50} = 50% growth inhibition of the SCV during the incubation period (uM tralkoxydim)

Table 6 Effect of various concentrations of tralkoxydim on the growth of 2-day-old rice cell suspension cultures as assessed by fresh weight (FW), dry weight (DW) and viability.

Tralkoxydim	FW*			DW*			Viability*		
μM	(g)		[%]	(mg)		[%]	%		[%]
0	0.65	±0.06	100	86	±2	100	80	±17	100
50	0.55	±0.08	85	76	±5	89	68	±16	86
100	0.39	±0.02	60	53	±4	62	55	±9	69
150	0.39	±0.03	60	54	±3	63	42	±2	52
200	0.26	±0.02	40	44	±2	51	27	±6	34
250	0.24	±0.04	36	39	±3	46	13	±5	16
Untreated+	1.23	±0	188	113	±5	133	100	±2	126

Table 7 Effect of various concentrations of tralkoxydim on the growth of 2-day-old flax cell suspension cultures as assessed by fresh weight (FW) and dry weight (DW).

Tralkoxydim	FW*				DW*			
μM	(g)		[%]		(mg)		[%]	
Exp 1								
0	2.64	±0.07	100	±27	323	±58	100	±18
50	0.64	±0.31	24	±12	107	±36	33	±11
150	0.42	±0.06	16	±2	81	±6	25	±2
225	0.39	±0.16	15	±6	79	±1	24	±0
Exp 2								
0	2.79	±0.89	100	±32	307	±45	100	±15
25	2.34	±0.34	84	±12	288	±34	94	±11
50	0.89	±0.23	32	±8	145	±30	47	±10
100	0.64	±0.05	23	±2	97	±3	32	±1
150	0.54	±0.08	19	±3	81	±15	26	±5

* mean of 3 replicates, measured on day 7 ± standard deviation of the mean
\+ untreated, cell suspension cultures without methanol
[%] = percentage of the control

Rice cell suspension cultures treated with a range of tralkoxydim concentrations on different days of the growth cycle (2, 4 and 7) were not equally inhibited. Inhibition of cellular growth was measured for SCV, FW and DW, but these parameters were not influenced in the same way by tralkoxydim (Table 8).

SCV was equally inhibited by 75 μM tralkoxydim in 2-d, 4-d and 7-d-old cultures (approximately 50%; Table 8). Inhibition increased in 2-d, 4-d and 7-d-old rice cell suspension cultures when treated with tralkoxydim concentrations increasing from 75 to 150 μM. Inhibition did not increase further when 225 μM tralkoxydim was applied in 2-d and 7-d cultures but increased slightly in 4-d cultures. Growth inhibition of 7-d cultures was significantly greater at rates of 150 and 225 μM tralkoxydim than in 2-d and 4-d cultures. Four day old cultures were slightly less inhibited by 150 μM and slightly more inhibited by 225 μM tralkoxydim than 2-d cultures.

On the day of incubation with tralkoxydim only 3 representative flasks could be determined for fresh and dry weight therefore the value of significance of cell growth assessed by culture FW and DW was limited. Thus the mean values at this starting point could be viewed as a guide only within the cultures. FW growth (Table 8) of 2-d and 4-d cultures treated with 75 μM tralkoxydim was more strongly inhibited than the FW growth of 7-d cultures. When the tralkoxydim concentration was increased from 75 to 150 μM, inhibition percentages increased in all cell suspension cultures. Four-day-old cultures were less inhibited by 150 μM tralkoxydim than the 2-d and 7-d cultures which were equally inhibited. Increase in tralkoxydim concentration from 150 to 225 μM reduced FW further in 7-d cultures, slightly in 4-d cultures but not in 2-d cultures. The 225 μM concentration of tralkoxydim inhibited 2-d and 4-d cultures similarly and 7-d cultures to a greater extent. Cell DW (Table 8) was equally inhibited in the 2-d and 4-d cultures but more strongly in the 7-d cultures. Inhibition increased in all cultures when the concentrations of tralkoxydim increased from 75 to 150 μM. No further inhibition was noted beyond this concentration to 225 μM.

Table 8 Effect of various concentrations of tralkoxydim on the growth of 2, 4 and 7 day-old-rice cell suspension cultures as assessed by settled cell volume (SCV), fresh weight (FW) and dry weight (DW).

Incubation Period (d)	Growth of SCV (ml) / inhibition (%)* — Tralkoxydim concentration (μM)				I_{50}
	0 (control)	75	150	225	
2-5	0.99 ±0.10 / 0	0.55 ±0.06 / 44	0.25 ±0.03 / 75	0.24 ±0.05 / 76	96
4-7	0.86 ±0.02 / 0	0.38 ±0.07 / 56	0.29 ±0.01 / 67	0.12 ±0.00 / 85	52
7-10	0.89 ±0.20 / 0	0.44 ±0.08 / 51	-0.12 ±0.19 / 113	-0.16 ±0.04 / 118	71
		FW (g)*			
2-5	0.30 / 0	0.07 / 77	-0.07 / 124	-0.08 / 126	
4-7	0.58 / 0	0.16 / 72	0.01 / 98	-0.08 / 114	
7-10	0.40 / 0	0.19 / 50	-0.14 / 137	-0.37 / 193	
		DW (mg)*			
2-5	28 / 0	3 / 91	-21 / 176	-18 / 164	
4-7	35 / 0	-3 / 110	-23 / 167	-23 / 167	
7-10	17 / 0	-10 / 160	-33 / 300	-48 / 390	

* mean of 2 experiments ± standard error of the mean

I_{50} = 50% growth inhibition of the SCV during the incubation period (uM tralkoxydim)

3.4 Influence of tralkoxydim on the growth of flax cell suspension cultures

Tralkoxydim was toxic to flax cultures treated on day 2 at rates from 25-225 μM. Growth reduction was most pronounced from 25-50 μM and higher concentrations did not reduce the SCV further (Fig. 7). In experiment 1 the growth reduction was too high to calculate the I_{50}. In experiment 2 only the inhibition values by 25 and 50 μM tralkoxydim were used to make the regression equation and the I_{50} was calculated at 36 μM tralkoxydim (Table 5).

FW and DW growth of the treated cultures was also inhibited (Table 7). Growth from day 2, using FW and DW data measured on day 2 from the normal flax cell suspension cultures (Fig. 5), increased more strongly for the FW compared to the increase in DW. Cultures treated with 150 and 225 μM tralkoxydim reduced both FW and DW. Cultures treated with 50 and 100 μM were reduced slightly in FW and DW. The cultures treated with 25 μM tralkoxydim showed an increase in FW and DW almost equal to the control.

Flax cultures treated with a range of tralkoxydim concentrations on different days during the growth cycle (3, 6 and 9 days) were not equally inhibited (Tables 9 and 10). Inhibition of cellular growth was measured for SCV, FW and DW, by comparison with the control on the day of harvest. The different parameters, SCV, FW and DW, were not influenced in a similar way by tralkoxydim.

Cultures on all incubation days were reduced in SCV with increasing tralkoxydim concentrations, to a small degree in 3-d cultures but to a greater degree in 6 and 9-day-old cultures (Table 9). Cultures treated on day 3 decreased in volume at all the test concentrations of tralkoxydim. Growth inhibition was almost the same in cultures treated with 25, 50 and 100 μM tralkoxydim, and higher in cultures treated with 150 μm tralkoxydim. Growth of cultures treated on day 6 was measured from tralkoxydim incubation to the second day instead of the third day. SCV of the control cultures and the cultures treated with 25 μM tralkoxydim started to decrease after day 3, while the cultures treated with 50-150 μM tralkoxydim showed a recovery in growth from the third day. In these 2 days the control cultures grew the same amount as the control incubated on day

Table 9 Effect of various concentrations of tralkoxydim on the growth of 3, 6 and 9-day-old flax cell suspension cultures as assessed by settled cell volume (SCV).

Incubation Period (d)	Growth of SCV (ml) / inhibition (%)*					I_{50}
	Tralkoxydim concentration (μM)					
	0 (control)	25	50	100	150	
3-6	1.25 ±0.21 / 0	0.89 ±0.10 / 29	0.73 ±0.09 / 42	0.76 ±0.13 / 40	0.46 ±0.12 / 63	104
6-8	1.22 ±0.33 / 0	0.60 ±0.26 / 51	-0.11 ±0.34 / 109	0.18 ±0.38 / 86	-0.22 ±0.31 / 118	29
9-12	-0.8 ±0.20 / 0	-0.5 ±0.08 / 32	-0.66 ±0.10 / 968	-0.25 ±0.07 / 430	-0.51 ±0.21 / 770	

* mean of 3 replicates ± standard deviation of the mean

I_{50} = 50% growth inhibition of the SCV during the incubation period (uM tralkoxydim)

Table 10 Effect of various concentrations of tralkoxydim on 3, 6 and 9-day-old flax cell suspension cultures as assessed by fresh weight (FW) and dry weight (DW).

Incubation day	FW (g) / % from the control*				
	Tralkoxydim concentration (μM)				
	0 (control)	25	50	100	150
3	1.82 ±0.18 / 100	1.66 ±0.21 / 91	1.66 ±0.15 / 91	1.50 ±0.09 / 82	1.24 ±0.30 / 68
6	2.71 ±0.22 / 100	2.40 ±0.25 / 89	1.83 ±0.30 / 68	1.84 ±0.29 / 68	1.37 ±0.19 / 51
9	2.91 ±0.11 / 100	2.90 ±0.09 / 100	3.03 ±0.10 / 104	2.78 ±0.12 / 95	3.07 ±0.20 / 106
	DW (mg) / % from the control*				
3	191 ± 9 / 100	192 ±28 / 101	190 ± 6 / 99	167 ± 6 / 88	139 ±22 / 73
6	323 ±30 / 100	280 ±31 / 87	233 ±32 / 72	238 ±21 / 74	195 ±20 / 60
9	254 ±15 / 100	253 ± 7 / 100	237 ± 7 / 93	251 ±26 / 99	257 ±21 / 101

* mean of 3 replicates 3 days after incubation ± standard deviation of the mean

3. The cultures treated with 25 μM tralkoxydim showed growth (50%) but the cultures treated with higher concentrations (50-150 μM) tralkoxydim were reduced in SCV or grew very little. Cultures treated on day 9 were already turning brown (i.e. senescing) prior to incubation. None of the cultures treated at this stage increased in volume, but concentrations of 50-150 μM tralkoxydim reduced the volume of the cultures while the control and the cultures treated with 25 μM tralkoxydim were stable.

Cell FW was strongly reduced with increasing tralkoxydim concentrations on day 3 and 6 but not on day 9 (Table 10). FW reduction was greater in 6-d than in 3-d cultures. The FW of cultures treated on day 3 was similar when treated with 25 and 50 μM and decreased with 50-150 μM tralkoxydim. FW on day 6 from the cultures treated with 25 μM tralkoxydim was significant higher than FW of the cultures treated with 50 and 100 μM tralkoxydim and these cultures exhibited a greater FW than the cultures treated with 225 μM tralkoxydim. FW of the cultures treated on day 9 did not change with increasing concentrations of tralkoxydim.

Cell DW was not inhibited in the cultures treated on day 9 and in the 3-d cultures at the lower concentrations (25 and 50 μM; Table 10). Inhibition increased in 3-d and 6-d cultures when the concentration of tralkoxydim increased. DW of cultures treated on day 3 was not inhibited by 25 and 50 μM tralkoxydim but was reduced in cultures treated with 100-150 μM tralkoxydim. Tralkoxydim added to 6-d-old cultures reduced DW with all rates increasingly from 25 μM through to 150 μM tralkoxydim. Cell DW of the cultures treated on day 9 was the same as the DW of the control.

3.5 Recovery of radioactivity from rice cell suspension cultures

The percentage of recovered radioactivity (compared to total dose applied) from the rice cell and medium fraction were similar (ca. 50%); the debris fraction contained very small percentages (ca.10%) of radioactivity (Table 11). Overall recovery in the experiment was approximately 50%.

Table 11 Radioactivity in the rice debris, cells and media, following incubation with ^{14}C-tralkoxydim.

Incubation		% of recovered activity*			Total recovery
Day	Period (h)	Debris	Cells	Media	(% from applied)
	0	-	-	100	50.3
	6	-	-	100	45.9
	24	-	-	100	60.1
	72	-	-	100	42.5
2	0	9.6	31.4	59.0	27.1
	6	6.6	31.8	61.6	51.3
	24	8.2	35.5	56.2	47.3
	72	12.6	30.1	57.3	49.2
4	0	6.9	31.9	61.2	46.4
	6	7.7	49.1	43.2	44.4
	24	6.2	47.7	46.1	49.9
	72	6.3	44.5	49.2	41.6
7	0	9.6	69.3	21.1	42.7
	6	6.2	51.0	42.8	51.8
	24	5.9	58.2	35.9	54.1
	72	7.8	48.9	43.3	54.0

* mean of 2 or 3 replicates
- control containing only medium

Radioactivity per g of debris (Table 12a) in the cultures treated with ^{14}C-tralkoxydim on day 2 was twice as high as that recovered on day 4. The debris of the cultures treated on day 7 contained less radioactivity than the debris on day 4. The amount of radioactivity per g of cell debris was not significantly different over the time course (0-72 h) on each day, although there was a slight increase on each day after 24 h.

Accumulation of radioactivity per g of cells (Table 12b) was not significantly different between days 2, 4 and 7 of incubation with ^{14}C-tralkoxydim. Cells directly harvested contained more activity on day 7 than on days 2 and 4. After 6 and 24 h of incubation, cells treated with ^{14}C-tralkoxydim on day 7 contained less radioactivity than the cultures treated on days 2 and 4 but this difference was not likely to be statistically significant. After 72 h the amount of radioactivity was decreased in the cells of all cultures, but this was significant only in cells treated on day 4.

Radioactivity recovered from the medium (Table 12c) was higher in the cell-free medium compared to medium plus cells, and recovery decreased slightly in cultures treated later in the growth cycle.

3.6 Recovery of radioactivity from flax cell suspension cultures

Some 75% of the recovered radioactivity was extracted from the medium fraction, the remainder from the cell and debris fractions (Table 13). Overall recovery declined slightly with an increase in incubation time (0-72 h), from 85 to 60%.

Radioactivity per g flax debris declined in older cultures (Table 14a). In time (0-72 h) radioactive concentration increased to a maximum after 24 h in 2-d and 4-d cultures and after 6 h in 7-d cultures and decreased afterwards. The 'digestion' of the flax debris with soluene revealed that the radioactive content was higher than when debris was not digested, but directly counted. Recoveries in the debris doubled in some cases from 2.5 to 5 % of the recovered activity.

Table 12 Radioactivity in the rice debris/g, cells/g and media/flask, following incubation with ^{14}C-tralkoxydim.

(a) Radioactivity* per g rice debris**

Incubation Period (h)	Day of the growth cycle 2		4		7	
0	64		31		25	
6	78	(8)	28	(2)	17	(2)
24	84	(27)	37	(13)	23	(5)
72	73	(7)	31	(7)	20	(4)

(b) Radioactivity* per g rice cells** (FW)

Incubation Period (h)	Day of the growth cycle 2		4		7	
0	28		33		53	
6	55	(9)	50	(6)	42	(10)
24	50	(7)	50	(7)	44	(11)
72	39	(7)	31	(5)	38	(4)

(c) Radioactivity* in the rice media/flask** (28 ml)

Incubation Period (h)	Control	Day of the growth cycle 2		4		7	
0	125	39		78		24	
6	114	79	(11)	53	(1)	59	(6)
24	149	66	(11)	63	(9)	51	(1)
72	105	70	(11)	56	(0)	62	(7)

* DPM x10^3
** mean of 2 or 3 replicates
values in parentheses = standard deviation of the mean

Table 13 Radioactivity in the flax debris, cells and media following incubation with ^{14}C-tralkoxydim.

Incubation		% of recovered activity*			Total recovery
Day	Period (h)	Debris	Cells	Media	(% from applied)
	0	-	-	100	87.0
	6	-	-	100	79.0
	24	-	-	100	81.5
	72	-	-	100	60.3
2	0	1.3	14.4	84.3	85.0
	6	4.0	9.1	86.9	85.6
	24	8.3	7.7	84.0	79.3
	72	10.4	4.9	84.7	63.3
4	0	2.5	15.7	81.8	60.6
	6	8.1	11.9	80.0	73.9
	24	9.6	6.1	84.3	75.0
	72	6.9	4.5	87.6	61.0
7	0	1.8	23.7	74.5	76.7
	6	6.5	27.4	66.1	86.6
	24	4.3	10.1	85.6	81.1
	72	6.8	5.3	87.9	67.4

* mean of 2 or 3 replicates

\- control containing only medium

Table 14 Radioactivity in the flax debris/g, cells/g and media/flask, following incubation with ^{14}C-tralkoxydim.

(a) Radio activity* per g flax debris**

Period (h)	Incubation Day of the growth cycle 2		4		7	
0	16		12		3	
6	67	(22)	49	(6)	19	(7)
24	157	(15)	76	(14)	8	(1)
72	61	(11)	15	(5)	12	(0)

(b) Radioactivity* per g flax cells** (FW)

Period (h)	Incubation Day of the growth cycle 2		4		7	
0	39		21		15	
6	23	(3)	17	(1)	23	(3)
24	18	(2)	9	(3)	7	(2)
72	5	(1)	3	(0)	3	(0)

(c) Radio activity* in the flax media/flask** (28 ml)

Period (h)	Control	Incubation Day of the growth cycle 2		4		7	
0	214	176		122		139	
6	194	186	(1)	148	(2)	143	(41)
24	199	167	(7)	158	(12)	174	(7)
72	146	134	(11)	133	(12)	148	(6)

* DPM $x10^3$
** mean of 2 or 3 replicates
values in parentheses = standard deviation of the mean

Table 15 Distribution of ^{14}C-label extracted from the rice cell and media fractions; an estimate of the R_f values and of the intensity of the bands.

R_f values+	^{14}C-metabolites in the rice cell fraction and their intensity*												
	Day 2					Day 4				Day 7			
	T#	0	6	24	72	0	6	24	72	0	6	24	72
I 97	S	S	S	S	S	S	S	S	S	S	S	S	S
II 91	T	T				T				T			
III 87	W	T				T				T			
IV 84	W	W				W				T			
V 82			T	T	T		T	T	T		T	T	T
VI 79			S	S	S		S	S	S		S	S	S
VII 76													
VIII 68													
IX 63													
X 58													
XI 9			T	S	S		T	W	S			S	S
XII 5			T	W	S		T	T	W			W	S

R_f values+	^{14}C-metabolites in the rice media fraction and intensity*												
	Day 2					Day 4				Day 7			
	T#	0	6	24	72	0	6	24	72	0	6	24	72
I 97	S	S	S	S	S	S	S	S	S	S	S	S	S
II 91	T	T	T	T	T	T	T	T	T	T	T	T	T
III 87	W	T	T	T	T	T	T	T	T	T	T	T	T
IV 84	S	S	S	S	S	S	S	S	S	S	S	S	S
V 82		T		T		T				T			
VI 79			S	S	W		T						
VII 76							W	W	T		W	W	W
VIII 68		T	W	W	S	T				T		T	
IX 63		T		T	W	T	W	W	S	T	W	S	S
X 58								T	T		T	W	W
XI 9													
XII 5													

* arbitary scale, S = strong, W = weak, T = trace
+ R_f values x 100, mean of two observations
# T = ^{14}C-tralkoxydim standard

Radioactivity per g of cells (Table 14b) was higher in cells treated on day 2 than on day 4 or 7. Radioactivity in the cells declined with increase of incubation time (0-72 h) at all stages of cell treatment. Cells directly harvested contained more radioactivity on day 2 than on days 4 or 7. After 6 h of incubation, cells of cultures treated on day 2 or 7 contained the same amount radioactivity; cells of cultures treated on day 4 contained slightly less. The amount of radioactivity decreased significantly over the 6-24 h incubation period in cells treated on day 4 and 7 but stayed almost constant in cells treated on day 2. The amount of radioactivity decreased significantly in all cultures over the 24-72 h incubation period to less than 2% of applied activity.

Radioactivity recovered from the media (Table 14c) was higher in the cell free medium compared to medium plus cells. Recovery decreased between 24 and 72 h.

3.7 Analysis of ^{14}C-metabolites in the rice cell suspension cultures

The autoradiograms produced from the tlc-plates, spotted with cell and media extracts, revealed ca. 12 different bands, in terms of R_f values. These bands were indicators of qualitative differences in the extracts. Qualitative differences were assumed if such differences were consistent between replicates. In some cases, samples were analyzed by an identical tlc system at ICI, Jealott's Hill Research Station, and metabolite identity confirmed (Figs. 9-12). Since the same amount of radioactivity was spotted on each lane, the intensity of the bands as developed on the autoradiograms was used to give an estimate of quantitative differences between bands. These quantitative differences were described using an arbitrary scale of band intensity: strong (S), weak (W) and trace (T). The R_f values of the metabolites are presented (Table 15) together with an indication of the quantity of the metabolites.

The identity of the bands (I-XII) were described after consultation with Dr. S. Hadfield (ICI, Agrochemicals) taking into account confirmation studies on tlc conducted at Jealott's Hill research station. Bands are believed to represent the following:

Band(s)	: Identity
I-IV	^{14}C-tralkoxydim (in different isomeric forms)
V	the oxazole derivative of tralkoxydim
VI	the imine derivative of tralkoxydim
VII	the hydrolysis product of tralkoxydim
VIII	unknown, probably the same as IX
IX	: glutaric acid
X	unknown, probably the hydrolysed oxazole or imine
XI and XII	unknown, probably conjugation products

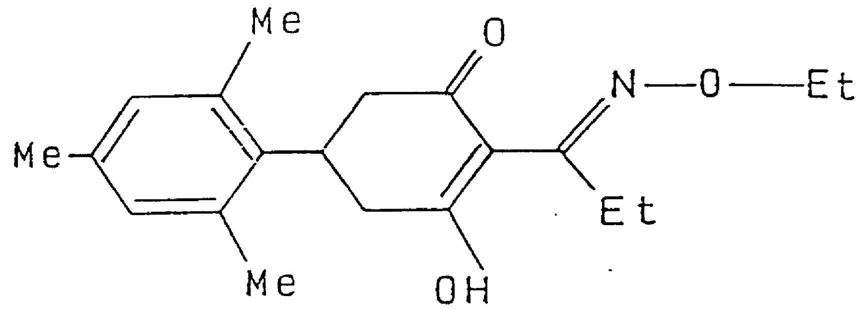
The molecular structure of some of the metabolites is shown in Fig. 8.

The autoradiograms of the rice cell extracts showed 2 major bands (Fig. 9-12, lane 1). The first major band was the parent compound tralkoxydim (I-IV). The second major band contained the oxazole, the imine and the hydrolysed tralkoxydim (V, VI and VII). The conjugates (XI and XII) were also visible. The control cells directly harvested on the day of incubation showed only the parent compound (I-IV). There were no visible changes in the bands or intensity with culture age or in time on the same day in the treated cultures.

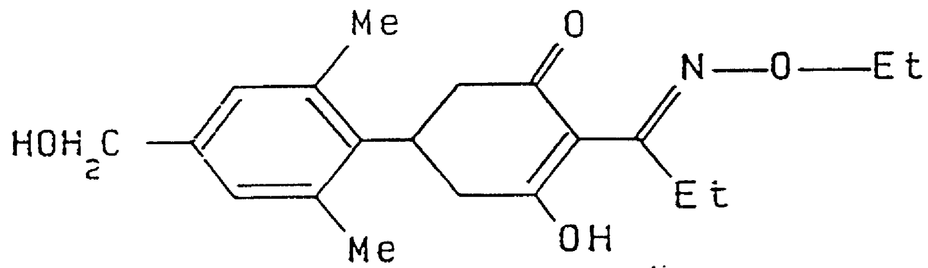
The rice media was extracted first with hexane then eluted or separated on the columns (C18, Varian) and these two extracts were spotted separately. The hexane extract showed the parent compound tralkoxydim (I-IV), the oxazole (V) and the imine (VI) form of tralkoxydim (Fig. 9-12, lane 2). Extracts subject to elution through the columns showed three to five other bands, the hydrolysed tralkoxydim (VII), glutaric acid (IX) and the unknown band X (Fig. 9-12, lane 3). The metabolites extracted with the columns were more polar than those extracted with hexane. Bands I-IV, V and VIII, visible in the treated cultures, were also visible in the controls where media without cells and media from cultures directly harvested were assessed. These bands in the controls were, however, very faint. With culture age there was no change in the quantity and quality of the bands which were visible on the autoradiograms. Band VIII in 2-d cultures was probably the same as

Figure 8 Structure of tralkoxydim and its transformation products, 1 = tralkoxydim, 2 = hydroxylated tralkoxydim, 3 = oxazole, 4 = imine.

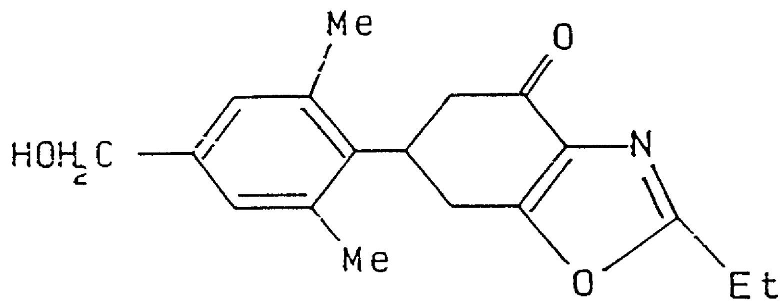
1



2



3



4

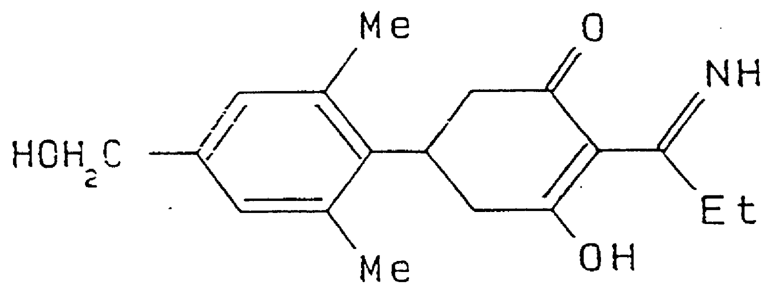


Figure 9 Autoradiogram of a thin-layer chromatogram of radio-labelled materials extracted from rice and flax cell suspension cultures inoculated, on day 2, with ^{14}C-tralkoxydim for 6 h: lane 1 = rice cell extract; lane 2 = rice media hexane extract; lane 3 = rice media column extract; lane 4 = flax cell extract; lane 5 = flax media column extract.

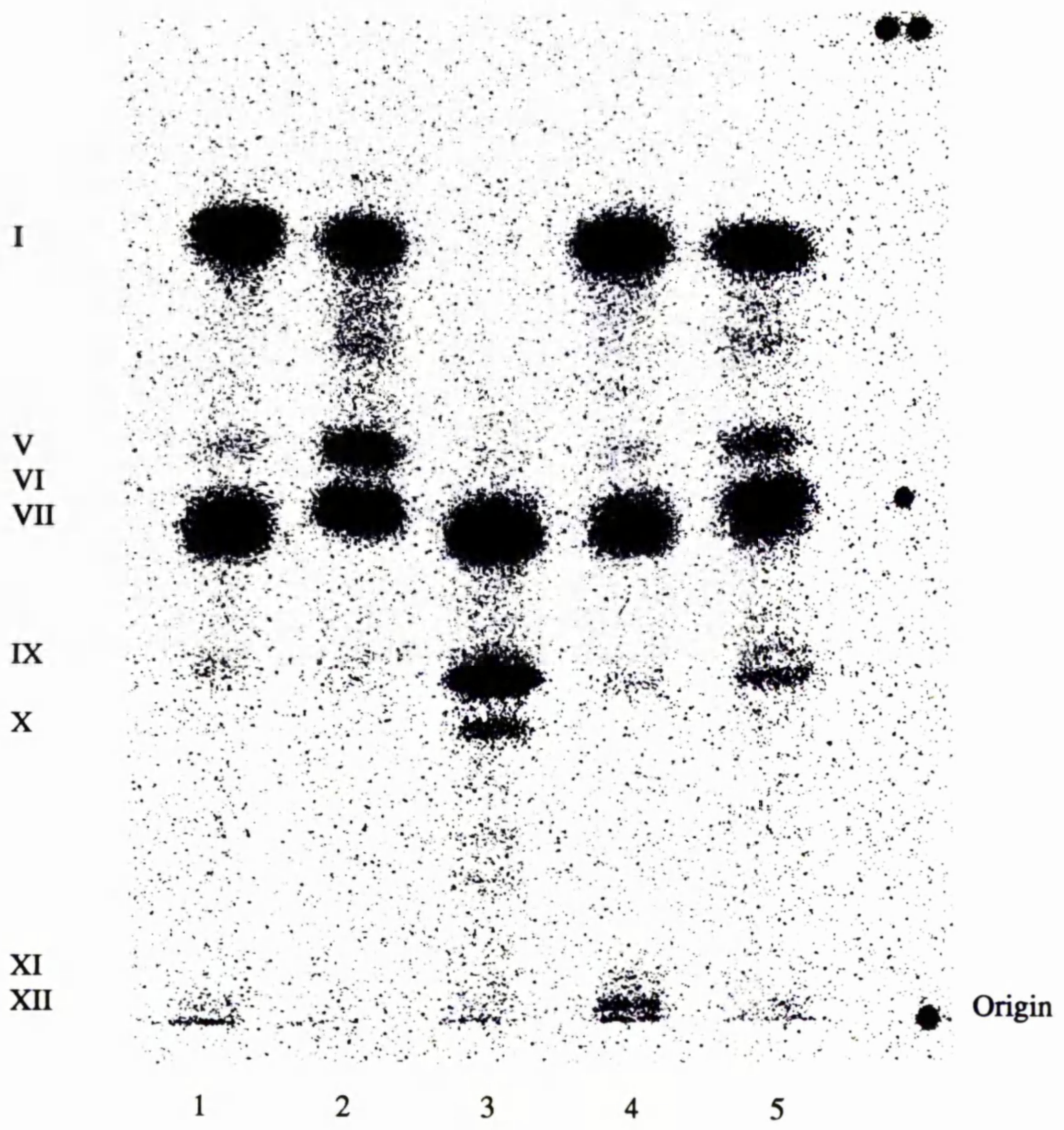
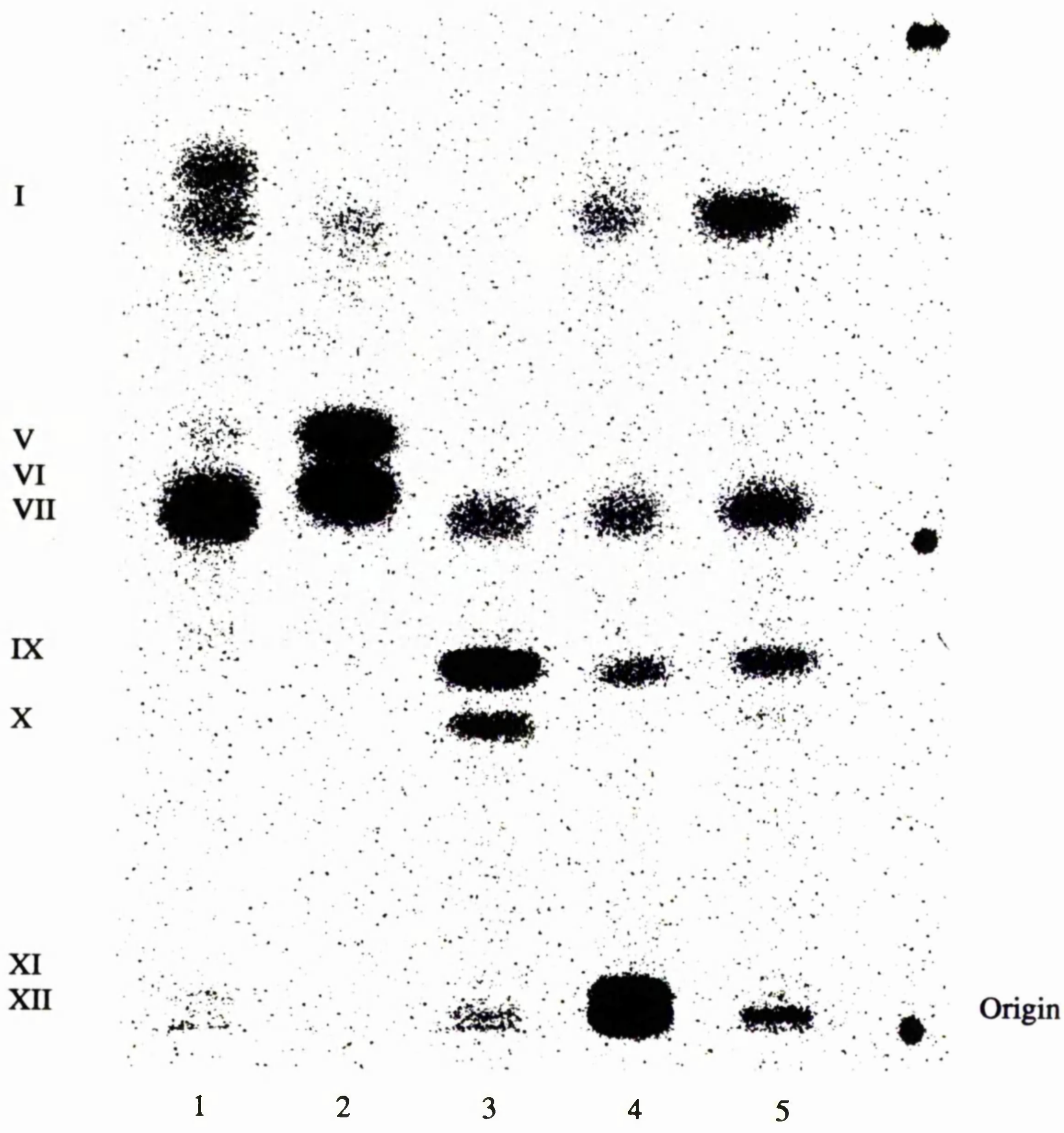

I
V
VI
VII
IX
X
XI
XII
Origin
1
2
3
4
5

Figure 10 Autoradiogram of a thin-layer chromatogram of radio-labelled materials extracted from rice and flax cell suspension cultures inoculated, on day 2, with ^{14}C-tralkoxydim for 72 h: lane 1 = rice cell extract; lane 2 = rice media hexane extract; lane 3 = rice media column extract; lane 4 = flax cell extract; lane 5 = flax media column extract.



I
V
VI
VII
IX
X
XI
XII
Origin
1
2
3
4
5

Figure 11 Autoradiogram of a thin-layer chromatogram of radio-labelled materials extracted from rice and flax cell suspension cultures inoculated, on day 7, with ^{14}C-tralkoxydim for 6 h: lane 1 = rice cell extract; lane 2 = rice media hexane extract; lane 3 = rice media column extract; lane 4 = flax cell extract; lane 5 = flax media column extract.

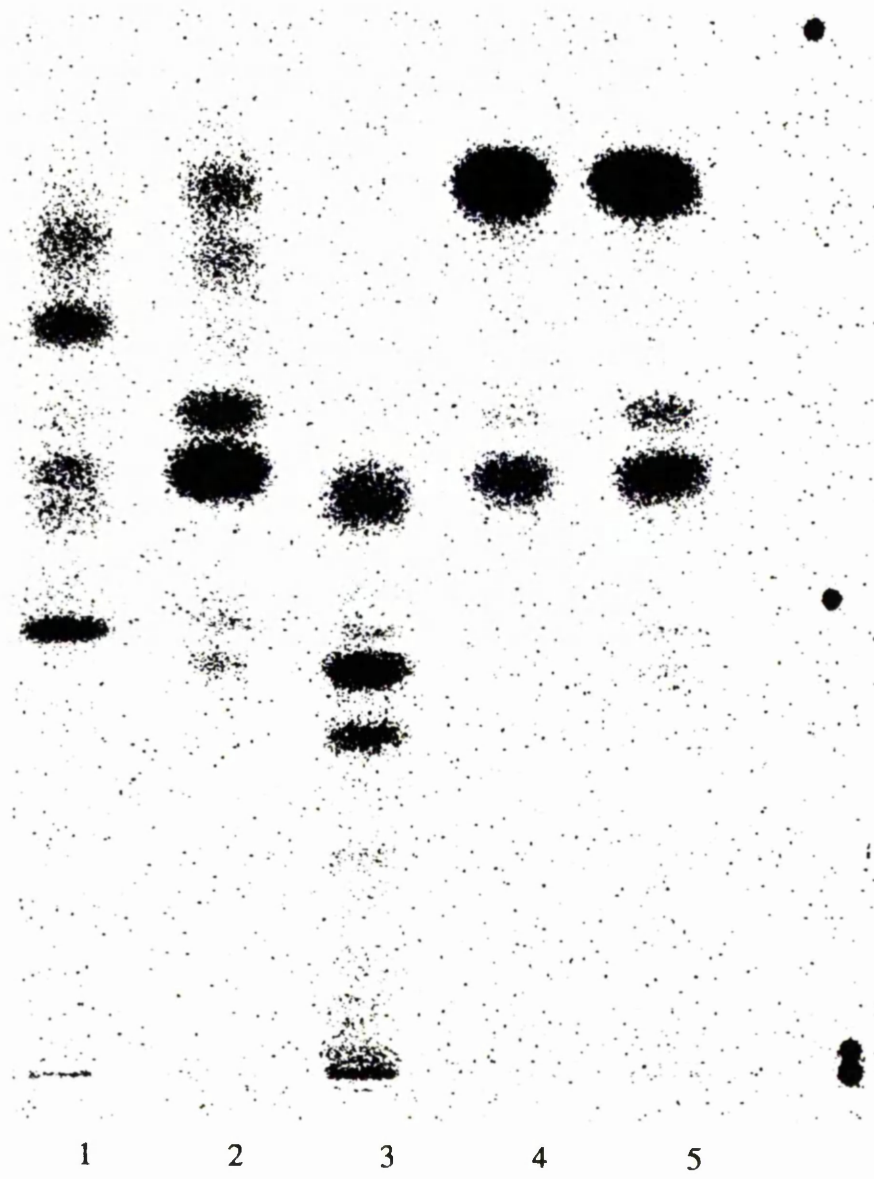
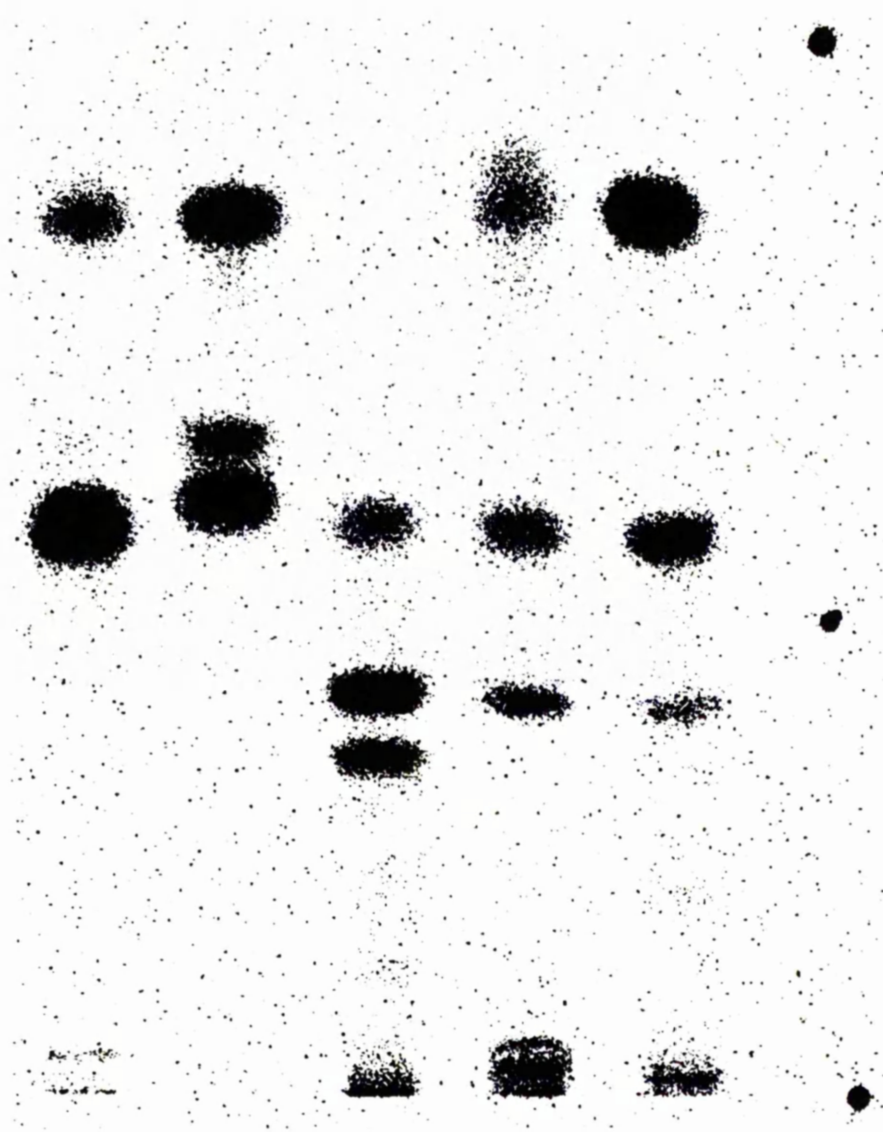

I
V
VI
VII
IX
X
XI
XII
Origin
1
2
3
4
5

Figure 12 Autoradiogram of a thin-layer chromatogram of radio-labelled materials extracted from rice and flax cell suspension cultures inoculated, on day 7, with ^{14}C-tralkoxydim for 72 h: lane 1 = rice cell extract; lane 2 = rice media hexane extract; lane 3 = rice media column extract; lane 4 = flax cell extract; lane 5 = flax media column extract.


I
V
VI
VII
IX
X
XI
XII
Origin
1
2
3
4
5

band IX in 4-d and 7-d cultures. In older cultures there was maybe a small increase in the intensity of all the spots. With incubation time (6-72 h) in the cultures treated on the different days the metabolites extracted became more polar. In all cultures a high amount of tralkoxydim (I) was visible. The bands V, VI and VII were the same at all incubation times. Band IX, glutaric acid, and X became more intense with time.

The cells did not contain the bands VIII, IX and X but contained mainly the bands V, VI and VII, the oxazole, the imine, the hydroxylated tralkoxydim and the conjugates (XI and XII). The bands V, VI and VII were also visible in the media fraction but the media contained also the bands VIII, IX and X, the glutaric acid and probably the hydroxylated form of the oxazole or the imine.

3.8 Analysis of ^{14}C-metabolites in the flax cell suspension cultures

The autoradiograms from the tlc-plates spotted with flax cell and media extracts showed ca. 12 different bands. The R_f values of the metabolites are presented (Table 16) together with an indication of the intensity of the bands, as previously described (3.7).

The autoradiograms of the flax cell extracts showed 3 major bands (Fig. 9-12, lane 4). The first band was the parent compound tralkoxydim (I). The second band contained the oxazole (V), the imine (VI) and the hydroxylated tralkoxydim (VII). The band of glutaric acid (IX) was only faintly visible. The bands of the 2 conjugates, XI and XII, were intense. The distribution of metabolites in the flax cell fraction changed with culture age. The parent compound appeared to be converted faster to more polar products in the 4-d-old cultures. The conjugates, bands XI and XII, were produced in all cultures, but were more abundant in 2-d and 4-d cultures. In time (6-72 h) the parent compound tralkoxydim was less abundant after 24 and 72 h in all cultures. It also appeared that the bands V, VI and VII became less intense in time. In 7-d cultures after 6 h it looked like only the oxazole and or the imine were visible not the hydroxy tralkoxydim. The glutaric acid band (IX) was not visible after 6 h incubation but was visible after 24 and 72 h on all incubation

Table 16 Distribution of ^{14}C-label extracted from the flax cell and media fractions; an estimate of the R_f values and of the intensity of the bands.

R_f values+		^{14}C-metabolites in the flax cell fraction and intensity*												
		Day 2					Day 4				Day 7			
		T′	0	6	24	72	0	6	24	72	0	6	24	72
I	97	S	S	S	S	S	S	S	W	W	S	S	W	W
II	91	T	F											
III	87	W										T		
IV	84	W	W				F				W			
V	82			W	W	T		W	T	T		S	T	
VI	79			S	S	W		S	S	W			S	W
VII	76													
VIII	68													
IX	63				T	T			T	T			T	W
X	58													
XI	9			T	S	S			S	S			T	W
XII	5			T	S	S			W	W			T	T

R_f values+		^{14}C-metabolites in the flax media fraction and intensity*												
		Day 2					Day 4				Day 7			
		T′	0	6	24	72	0	6	24	72	0	6	24	72
I	97	S	S	S	S	S	S	S	S	S	S	S	S	S
II	91	T												
III	87	W		W										
IV	84	W										T		
V	82													
VI	79			W	T			W				S		
VII	76			T	W	W		W	W	W			S	S
VIII	68		T				T				T			
IX	63		T	T	T	W	T	T	T	W	T		W	W
X	58													
XI	9													
XII	5					T			W	W			T	W

* arbitary scale, S = strong, W = weak, T = trace
+ R_f values x 100, mean of two observations
# T = ^{14}C-tralkoxydim standard

days. The conjugates (XI and XII) were characterised by their low R_f values, polar nature and abundance after 24 and 72 h on all incubation days.

The media showed 2 major bands (Fig. 9-12, lane 5), the parent compound tralkoxydim (I) and the band with the oxazole (V), the imine (VI) and the hydroxylated tralkoxydim (VII). Two weak bands were visible, glutaric acid (IX) and the conjugates (XI and XII). From the autoradiograms it was difficult to say if there was a difference with culture age or with incubation time in the mobility or intensity of the bands. It appeared that there was more of the oxazole and or the imine fractions after 6 h in all the cultures, most pronounced on day 7. In time (6-72 h), the amount conjugates also slightly increased in time in all the cultures.

It appeared that the same products were extracted both in the cell and the media fraction. Compounds with a strong presence in the cell fraction were weakly represented in the media fraction.

4 DISCUSSION

4.1 Growth of rice and flax cell suspension cultures

In this study the growth of rice cell suspension cultures appeared to be linear when assessed by measurements of SCV; FW and DW of the cultures increased faster during the first three days of the 7 day growth cycle (Fig. 4). Determination of the growth of flax cell suspension cultures revealed a sigmoid growth curve for SCV, FW and DW (Fig. 5), although the lag phase apparent from FW and DW measurements was not as pronounced as that observed from measurements of SCV. From these data it appeared that cells probably first increased in weight before they increased in volume. The treatment of the cultures with tralkoxydim was determined on days 2, 4 and 7 for rice and on days 3, 6 and 9 for flax. Tralkoxydim was inoculated into rice cultures during growth stages that can only be described as the early linear, the middle linear, and the late linear phases; the difference between the treatments probably being mainly the number of cells present in the cultures. It was possible to inoculate the flax cultures with tralkoxydim during distinct growth phases; the late lag phase (day 2), the log phase (day 3), the linear phase (day 6), and the late linear or early stationary phases (day 9).

Variation in cell-inoculum when subdividing the cell cultures of rice and flax was low. This meant that measuring cell volume in a pipette gave an accurate aliquot and inoculum initially which resulted in a similar growth pattern in all culture flasks. A higher inoculum gave a faster growth especially in the first half of the growth cycle.

4.2 Influence of tralkoxydim on the growth of rice and flax

Rice cell suspension cultures exhibited some sensitivity to tralkoxydim treatments but cells showed growth even at very high concentrations (Table 4). Viability of the cells was still 34% with 200 μM tralkoxydim after 5 days incubation (Table 6). The rice cultures continued growing during 12 days of incubation (Fig. 6). This may have been due to the development of cell resistance or a reduction, with time, in the effective tralkoxydim

concentration. Reduction of tralkoxydim concentration may have been due to herbicide cell wall binding on other cells, the metabolism of tralkoxydim to certain non-toxic metabolites or even the inability of tralkoxydim to reach the ultimate site of the putative target enzyme, the acetyl CoA carboxylase. Rice cultures treated on different days of the growth cycle (2, 4 and 7) showed that 2-d cultures were the most tolerant. The I_{50} values were 96, 52 and 71 uM tralkoxydim on days 2, 4 and 7 respectively (Table 8). Percentage inhibition of the growth by 75 μM tralkoxydim was almost similar on all incubation days as assessed by SCV. This inferred that the smaller number of younger cells were less sensitive or were able to detoxify the tralkoxydim faster than older cultures. It was also possible that regrowth in this stage was so fast that inhibition was not apparent. Inhibition of SCV, FW and DW was not similar in the rice cultures (Table 8). FW growth was inhibited more than SCV. Only cultures treated with the lowest amounts of tralkoxydim (75 μM) showed FW growth. FW could have been decreased by loss of reserve material, starch, to overcome the tralkoxydim stress. DW was reduced in all cultures, indicating a reduction in the cell reserves. The reserves in the cells could have been used for the detoxification of the tralkoxydim.

Flax growth was completely inhibited above 50 μM in 2-d-old cultures (Table 5). From these data it was impossible to say if the inhibition between 25 and 50 μM tralkoxydim was linear or not. Cultures treated with 50-150 μM showed no regrowth during 5 days of incubation (Fig. 7). Treatment of flax cultures on different days in the growth cycle (2, 3, 6 and 9) showed that 3-day cultures were most tolerant as assessed by SCV (Tables 7 and 9). On this day none of the cultures was completely inhibited by 25-150 μM tralkoxydim. In the other stages of the growth cycle growth was completely inhibited above 50 μM tralkoxydim. I_{50} values were 36, 104, 30 and ca. 30 uM tralkoxydim on days 2, 3, 6 and 9 respectively. FW and DW showed a similar inhibition pattern (Table 10). The difference was that SCV growth was inhibited to a greater degree compared to the growth of FW, and FW was more inhibited than the DW growth. It was possible that after the tralkoxydim stress, cultures start to grow in the same way as in a normal growth curve (Fig. 5), firstly showing an increase in DW and FW, and later an increase in SCV.

Rice and flax showed maximum tolerance to tralkoxydim in the early linear phase; the I_{50} was similar in this stage for the two species (Tables 8 and 9). In the other stages rice appeared to be more tolerant than flax although this was difficult to confirm with certainty because rice was not tested in such distinctive growth phases as flax. In future studies it would be desirable to create a sigmoid growth for rice cell suspension cultures to investigate sensitivity in earlier growth stages. This would be done by decreasing the amount of conditioned media, or cells, in the inoculum when subdividing the cultures. To measure inhibition in the stationary phase in rice and flax was difficult. Irzyk *et al.* (1990) showed that tolerance to haloxyfop in proso millet cell suspension cultures was highest in the linear phase (day 7 of the growth cycle). The cultures were twice as sensitive in the early and late linear phases (day 3 and 10 of the growth cycle) and 500 times more sensitive 1 day after subculturing. High sensitivity early in the growth cycle was also reported by Cho. *et al.* (1986) who treated corn and soybean cell suspension cultures with haloxyfop on the day of subculturing. Corn was more sensitive than soybean; FW growth was completely inhibited by 0.2 and 10 μM haloxyfop. Chandrasena (1991) treated maize and soybean cell suspension cultures with tralkoxydim. Both species were very sensitive in the early growth stages and were much more tolerant (> 50 times) in the linear growth phases.

The three previous authors used different methods of measuring growth, after different periods of incubation. Irzyk *et al.* (1990) measured FW of the cultures after 48 h, Chandrasena (1991) measured DW after 96 h and Cho *et al.* (1986) measured FW but calculated the I_{50} using viability data. The present study showed that there can be a large difference between the various growth parameters. Measuring growth after different periods of incubation can change the I_{50} (Table 4) in cultures which show regrowth. Measuring viability was possibly an indication of this regrowth. Cho *et al.* (1986) noted that 10 μM haloxyfop inhibited completely soybean growth but that cell viability was still near 40%. Chandrasena (1991) mentioned that viability was still high even when growth was inhibited completely in maize and soybean. In the present study rice cells were also still viable when treated with concentrations which completely inhibited growth (Table 6). This indicated that some rice cells were not sensitive in certain growth stages and that when cell viability was measured the cells were capable of taking up TTC. This is also

reported for soybean, taking up FDA, (Cho *et al.* 1986) and maize and soybean taking up TTC (Chandrasena, 1991). It would be interesting to know whether these cells would start growing again if they were transferred to fresh herbicide-free medium.

This study has revealed that flax was tolerant of tralkoxydim in a particular stage of growth (early linear) and was completely inhibited in the other stages of growth by much lower concentrations of the herbicide. Rice was also most tolerant in the early linear phase but showed also a high tolerance later in the linear phase. From these data it was impossible to conclude if rice would be more sensitive before the early linear phase. The relative sensitivity of rice and flax *in vitro* did not correspond to expectations based on *in vivo* observations. Chow *et al.* (1983) showed that flax was tolerant of sethoxydim while rice, tolerant of fenoxaprop (Khodayari *et al.*, 1989), was less tolerant of haloxyfop, sethoxydim and clethodim (Carey *et al.*, 1992). Clearly, the *in vivo* and *in vitro* situations are so different physiologically that it is desirable to systematically assess plant species *in vitro* to examine their response to herbicides and xenobiotics where this information is to be used for mode-of-action studies.

4.3 Recovery of radioactivity from the rice and flax cell suspension cultures

The distribution of radioactivity in the rice and flax cell suspension cultures between the three fractions (debris, cells and media) was similar for all treatment days (2, 4 and 7) although the amount recovered in each fraction differed between rice (Table 11) and flax (Table 13). Absolute recovery of radioactivity from rice was 50% and from flax 80%. The difference could have been caused by a reduced number of solvent extraction steps for the flax medium but it is also possible that rice may have been biologically more active in transforming ^{14}C-tralkoxydim. It was possible that radiolabelled compounds may have been absorbed on to the glassware and with every transfer radioactivity was lost. All radioactivity was removed from the media by elution through the C18 columns with the appropriate solvent. It was possible that the columns still contained some of the radioactivity, i.e. that which was not eluted out with the acetonitrile, i.e. bound to the C18 absorbent. The extraction of the flax cell debris by digestion with soluene revealed that

recovery increased in this way by 1-4 % over direct radioassays of debris in scintillant. Digestion of rice debris was not tested. To improve the recovery from cell suspensions in future studies it would be necessary to systematically monitor the loss of radioactivity through every stage of sample preparation and extraction. This could be done by taking subsamples after every extraction step and counting the radioactivity from every fraction.

Radioactivity in the debris fraction of rice (Table 11) and flax (Table 13) was ca. 8%. Similar amounts in the cell insoluble fraction were also reported by other workers. A difference between rice and flax was that rice debris from cultures directly harvested contained similar amounts of radioactivity as debris from cells which were incubated for longer periods (6-72 h) but flax debris contained less radioactivity when directly harvested. Thus ^{14}C-labelled compounds were bound to the flax cell debris with increasing incubation time. Cell suspensions of diploid wheat treated with ^{14}C-diclofop-methyl contained never more than 5% of the total radioactivity recovered in the cell debris after extraction and this was reached 8 h after incubation (Dusky *et al.*, 1980). Cell suspension cultures of oat and wild oat treated with ^{14}C-diclofop contained a maximum of 5 and 12 % of recovered radioactivity respectively in the cell debris and this amount was constant over a 3 week period of incubation (Dusky *et al.* 1982). After extraction of 1-d and 4-d-old proso millet cell suspension cultures treated with ^{14}C-haloxyfop, the debris fraction contained 9 and 3 % respectively of the total amount of herbicide absorbed by the cells. The amount radioactivity increased with incubation time (1-24 h) (Irzyk *et al.*, 1990). Chandrasena (1991) treated maize and soybean cell suspension cultures with tralkoxydim. The cell debris fraction of maize contained more radioactivity than soybean; 3 and 1 % respectively. Radioactivity per g debris from the rice and flax cell suspension cultures decreased with culture age (Table 12a and 14a). The decrease in radioactivity per g debris in rice and flax could be a result of the increase in cell debris weight with time, and thus a dilution of radioactivity. Chandrasena (1991) reported that radioactivity per g debris decreased with culture age in cell suspension cultures of soybean and maize treated with tralkoxydim. In the present study, with incubation time on the same inoculation day, radioactivity per g debris did not decrease in the rice debris but it decreased in the flax debris after 24 h of incubation. In most papers only the absolute amount of recovered radioactivity is shown which leads to the assumption that when radioactivity in the debris

fraction is constant in time, an increase in the culture debris weight will cause a decrease in the radioactivity per g debris. Radioactivity per g often increased until 24 h after incubation and was stable thereafter. This may suggest that binding was complete at this time.

The amount of radioactivity in directly harvested cells was high in both rice (Table 12b) and flax (Table 14b). Rapid uptake was reported by several other workers e.g. 1-day-old proso millet cells accumulated more than 60% of the radiolabelled haloxyfop in the first hour (Irzyk *et al.* 1990). The distribution of the radioactivity between the cell and media fractions was different for rice and flax. In rice, 50% of the total radioactivity recovered was recovered from the cell fraction. In flax, this figure was only 20%. The media showed the opposite picture (Tables 12c and 14c). The absolute amounts in the cell fractions of rice and flax increased with culture age, probably because of the higher numbers of cells, and a decrease in radioactivity in the medium fraction. The rice cultures were inoculated with 100 μM unlabelled tralkoxydim and this might have influenced the distribution of radioactivity. Prelimary work by Chandrasena (with the same rice cultures, Pers. Comm. 1991) showed however that cultures treated with only 5 μM unlabelled tralkoxydim revealed a similar distribution pattern. Thus it can be concluded that tralkoxydim distribution of radioactivity between the cell and media fractions was not influenced by the concentration of tralkoxydim under the range of concentrations listed.

The accumulation of large amounts of radioactivity in the cell fraction of cell suspension cultures has been previously reported in; resistant diploid wheat treated with diclofop (Dusky *et al.*, 1982), proso millet treated with haloxyfop (Irzyk *et al.*, 1990), maize treated with tralkoxydim (Chandrasena, 1991) and soybean treated with haloxyfop (Buhler *et al.*, 1985). Small amounts of radioactivity in the cell fractions are also reported in cell suspension cultures of; soybean treated with tralkoxydim (Chandrasena, 1991), yellow foxtail treated with haloxyfop (Buhler *et al.*, 1985),oat and wild oat treated with diclofop (Dusky *et al.*, 1982) and soybean and johnsongrass treated with sethoxydim (Swisher and Corbin, 1982). It seems that resistant monocotyledonous species can accumulate graminicides more rapidly than some susceptible species and that uptake decreases with time in resistant species but continues in susceptible species (Dusky *et al.*, 1982; Swisher

and Corbin, 1982). The dicotyledonous species absorbed very little herbicide with the exception of soybean (Buhler *et al.* 1985).

Recovered radioactivity per g rice cells was almost the same on the three inoculation days (Table 12b), and decreased slightly in time (6-72 h). Flax showed a different accumulation pattern (Table 14b). Similar amounts were recovered on the three inoculation days but with time radioactivity decreased significantly, notably in the 4-d and 7-d-old cultures. It was possible that the amount of radioactivity in the cell fraction of rice did not decrease as rapidly as in the flax because of the higher concentration of unlabelled tralkoxydim (100 μM). Chandrasena (1991) noted that radioactivity in the cell fraction of maize cell suspension cultures treated in the linear phase with tralkoxydim decreased in time, most pronounced from 48 to 72 h. Radioactivity in the cells of proso millet cell suspension cultures decreased in 1-d and 4-d-old cultures from 1-24 h (Irzyk *et al.*, 1990). Absorbtion of sethoxydim by soybean cells in suspension reached a maximum after 6 h of incubation, the amount radioactivity decreased the following 6 h and was stable from 12-24 h. In the same study, johnsongrass cells accumulated more radioactivity in time during 6-24 h incubation (Swisher and Corbin, 1982). Soybean and yellow foxtail cell suspension cultures treated with haloxyfop contained more radioactivity per g cells than yellow foxtail. The amount of radioactivity in the cell fraction was constant in the yellow foxtail cells and in the soybean cells from 12-24 h but decreased from 12-72 h (Buhler *et al.*, 1985).

Thus rice and flax cultures rapidly absorbed ^{14}C-tralkoxydim. In flax the balance of radioactivity was progressively transferred from cells to the medium but in rice, radioactivity per g cells remained constant. In both species radioactivity in the cell debris decreased with time.

4.4 Metabolism

The metabolites produced appeared to be the same in rice and flax cell suspension cultures (Table 15 and 16, Figs. 9-12). The metabolites extracted from the cell suspension cultures were not visible or less abundant in the controls indicating a minimal transformation effect

of tralkoxydim in the media. The parent tralkoxydim was detected in different isometric forms (bands I-IV) and was converted to a hydroxy derivative (VII). This conversion was probably the first step in the metabolism. Hydroxylation has also been reported for other graminicides e.g. diclofop (Dusky *et al.*, 1982) and haloxyfop (Buhler *et al.*, 1985). The oxazole (V) and imine (VI) derivatives of tralkoxydim can be formed abiotically through photolysis or spontaneous breakdown (Dr. S. Hadfield, ICI, Pers. Comm. 1992). The formation of glutaric acid (IX), due to the cleavage of the ring structure of tralkoxydim, was considered to represent a biological transformation. The identity of band VIII was unknown and was perhaps the same as band IX because it only appeared on one tlc plate and was not found by workers at ICI. Only rice media contained band X, probably the hydroxy form of the oxazole or imine derivative of tralkoxydim. The route of formation is unknown. The bands XI and XII are probably conjugates of some of the metabolites with sugars or amino acids produced by the cells. The formation of conjugates in graminicide metabolism *in vitro* has been noted previously (Dusky *et al.*, 1980, Dusky *et al.*, 1982). Generally, conjugation is considered to be a principal mechanism of herbicide detoxification. In *in vivo* studies with wheat, conducted by ICI, the oxazole, imine and hydroxy derivatives of tralkoxydim were also found (Dr. S. Hadfield, ICI, Pers. Comm. 1992).

There were some small qualitative differences between the metabolites found in the cell and media fractions. There were only traces of the oxazole and imine form of tralkoxydim in the cell fraction compared to an abundant amount in the media fraction. This suggested that these two metabolites were not taken up by the cells or that they are rapidly converted to another metabolite in the cells. Because these products are also formed by photolysis it would be interesting to know if these substances are also phytotoxic. Hydroxy-tralkoxydim (VII) was found in the cell and the media fraction, but appeared to be more abundant in the cell fraction. It is possible that the hydroxy-tralkoxydim was released by the cells into the media perhaps from lysed cells or was converted by enzymes in the media. Such a transformation could be confirmed by incubating tralkoxydim with a cell-free extract from the cell suspension cultures (as previously attempted by Dusky *et al.*, 1980). Glutaric acid mainly appeared in the media. The glutaric acid was either produced in the media by external enzymes (cell-secreted) or the cells released glutaric acid into the

media without storing it in the same form in the cells. The conjugates XI and XII were only produced in cell extracts. This was perhaps not surprising since physiologically active cells are undoubtedly a rich source of substrate for subsequent conjugation activities. This result was in accordance with the work of Dusky *et al.* (1982) where large amounts of diclofop conjugates accumulated in the cells of oat and wild oat grown in suspension.

It was difficult to say if there was a change in the rate of metabolism with culture age, defined as loss of ^{14}C-labelled material in the bands I-IV, the parent tralkoxydim, and an increase in the intensity of the bands V-XII (the metabolites). From the autoradiograms of rice it was not possible to see a difference in the intensity of the bands with culture age. It was possible that younger cells metabolized the tralkoxydim faster but that older cultures compensated for a reduced metabolizing-potential per cell simply by virtue of their greater cell number and biomass. In flax the rate of metabolism seemed most rapid in 4-d-old cultures. In 7-d-old cultures it seemed that after 6 h almost no conversion had occurred but after 72 h it seemed that the bands were the same as on other days. It is possible that enzymatically-induced metabolism of the tralkoxydim was much less active initially in the 7-d-old cultures but required a lag phase to be stimulated before tralkoxydim was converted. In future studies similar amounts of cells (SCV) should be treated and tested for quantitative differences. The incubation period should not be too long because the cells can start to adapt and perhaps induce their detoxifying systems in some way. Thus in time tralkoxydim was converted to more polar products, in both the rice and the flax cell suspension cultures. The C18 columns proved to be a simple, practical and effective method for the extraction of the more polar conjugation products from the media. In the future the speed of the transformation reactions, the determination of the end products and their localisation (in cells or in media) could be achieved in studies using low concentrations of tralkoxydim and an excess of cells grown in suspension culture.

4.5 General

The differences in sensitivity between rice and flax cell suspension cultures did not appear to be related to the metabolites produced. There was a difference in the amount of ^{14}C-

labelled material which accumulated in the cells but this did not affect the insensitivity as assessed by growth. The rate of metabolism may have been different in rice and flax but it was difficult to conclude this from the bands on the autoradiograms since these were only a guide to quantitative aspects of metabolism. Clearly, further detail could be added to this type of study by developing an efficient system to elute and assess the radioactivity located in individual chromatogram bands. The principal factor which influenced the sensitivity of cells to tralkoxydim treatment was their stage of growth. Typically, young, actively dividing and developing cells (early linear phase) were least sensitive, perhaps a consequence of their ability to detoxify tralkoxydim. In all cases the conversion of tralkoxydim was accomplished by a series of abiotic and biotic transformations. The oxazole and imine derivatives could be formed by abiotic and biotic means while the hydroxylation of tralkoxydim, the ring cleavage of tralkoxydim to glutaric acid and the formation of conjugates were considered to be biotic mechanisms (Fig. 8). Thus both species provided a rich source of tralkoxydim derivatives *in vitro* which would benefit greatly from further chemical and biological characterisation.

In-vitro herbicide treatment of rice and flax revealed that this method was extremely useful for the analysis of metabolites produced by the cells. An advantage of this technique over classical *in vivo* studies was that only small amounts of the particular herbicide were required, the incubation period was short and the extraction was relatively simple. Rice and flax were selected as examples of a monocot and a dicot. In this study rice was tolerant to tralkoxydim and therefore did not represent a susceptible monocot as previously expected. By comparison, flax was much less tolerant *in vitro* than expected. Therefore future *in vitro* studies should involve a wide range of monocot and dicot species since it was evident that metabolism studies would benefit from a diverse range of responses (resistance vs susceptible). With rice and flax, subtle differences were apparent eg. the extent to which each species accumulated tralkoxydim in the cell extract. Such a difference in herbicide/metabolite localisation could be important with respect to mode of action.

In addition to all the advantages and limitations of an *in vitro* approach to herbicide mode-of-action studies, previously outlined in chapter 1, this work using rice and flax could be extended to include chemical means of influencing herbicide metabolism. For example in

graminicides it may be appropriate to consider the use of cytochrome P_{450} inhibitors and stimulators (Jones, 1991). This approach could be valuable because cytochrome P_{450} is known to be involved in the hydroxylation, dealklylation and oxidation of herbicides.

This study with rice and flax has demonstrated many of the practical merits and limitations of an *in vitro* system. The ability to control culture age/development, environment, herbicide treatment, extract protocols and the efficient use of time, space and material provided a good reason to develop an *in vitro* approach. It is wise however to link such *in vitro* studies to an *in vivo* approach in order that the value of both systems to investigate herbicide mode-of-action can be maximised.

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