SESSION 9B

MODE OF ACTION AND METABOLISM OF HERBICIDES: III

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INDUCTION OF TETRAPYRROLE ACCUMULATION BY DIPHENYLETHER-TYPE HERBICIDES

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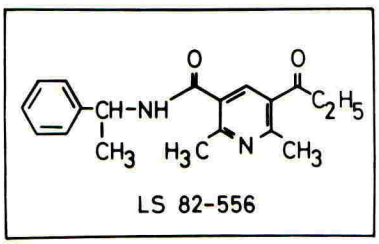
ABSTRACT

The diphenylether herbicide acifluorfen-methyl and the chemically unrelated LS 82-556 (Rhône Poulenc) are toxic to non-chlorophyllous soybean cells. Both herbicides induce the same types of morphological and biochemical symptoms, and their effects are light-dependent. Toxicity of acifluorfen-methyl was observed in soybean cells deprived of carotenoids. Action spectrum showed a peak of activity around 400nm, and notable effects up to 650 nm. Accumulation of tetrapyrroles was observed following a dark treatment with acifluorfen-methyl, and cells could be protected from the herbicidal effect by an inhibitor of tetrapyrrole biosynthesis. Similar conclusions were reached with etiolated cucumber seedlings treated with LS 82-556. These results lead us to propose that the phytotoxicity of diphenylether herbicides is due to their ability to induce abnormal accumulations of tetrapyrroles. These photosensitizing pigments then react with molecular oxygen to produce compounds toxic to living cells.

INTRODUCTION

The diphenylether (DPE) acifluorfen-methyl (AFM) causes bleaching, wilting and dessication of treated plants in a strictly light dependent manner (Orr and Hess 1982). Although belonging to a different chemical family, the pyridine derivative LS 82-556 (Fig. 1) induces exactly the same symptoms (Matringe et al. 1986). Both herbicides are active on green and etiolated seedlings and on non-chlorophyllous cell cultures (Matringe et al. 1986, Matringe and Scalla 1987). In the presence of oxygen, they induce photooxidation of fatty acids, then membrane disruptions and cell death.



LS 82-556

Fig. 1.

In spite of numerous studies, the exact mechanism of the light-activated toxicity is still unclear. Although some experiments support the participation of nitro radicals to the toxic reactions (Draper and Casida 1985), this mechanism is obviously excluded in the case of a chloro-DPE (Orr et al. 1983) and LS 82-556, which nevertheless act in the same way as nitro-DPEs. The nature of the photoreceptor involved is also a matter of controversy. Neither LS 82-556 nor a DPE such as AFM absorb visible light, so participation of cellular photoreceptor(s) has to be postulated. It is commonly accepted that these photoreceptors are carotenoids (Matsunaka 1969). This theory however, does not fit with the sensitivity of treated tissues to red light, i.e. outside the zone of absorption of these pigments (Ensminger and Hess 1985, Matringe et al. 1986).

In order to reexamine the nature of the photoreceptor, we have established the action spectrum for the toxicity of AFM and LS 82-556 on non-chlorophyllous soybean cells and etiolated cucumber seedlings, respectively. As will be shown below, the results of these studies have led us to postulate a participation of tetrapyrrole pigments in the toxicity of DPE-type herbicides.

MATERIALS and METHODS

Cell culture and plant material

Non-chlorophyllous soybean cells were grown in liquid medium as described earlier (Matringe and Scalla 1987). Before experiments, 3-day-old cultures were diluted to 30 mg fresh weight / ml. All chemical treatments were done in the dark during a 14h incubation period. Etiolated cucumber seedlings were grown as described by Matringe et al. (1986).

Norflurazon treatments

Soybean cells were subcultured twice in the presence of 50 µM norflurazon, an inhibitor of carotenoid biosynthesis (Delvin et al. 1976).

Action spectra

Action spectra were obtained using Balzers broad-band interference filters, type Filtraflex-K. The light source was a slide projector delivering a light intensity of 650 $\mu E/m^2/s$ through K 45 to K 70 filters, and 300 $\mu E/m^2/s$ throught the K 40 filter. For white light irradiation, an incandescent lamp (MAZDA PAR, cool beam, 120 W) giving 650 $\mu E/m^2/s$ PAR was used.

Cellular damage

Cellular damage was estimated by three different methods, depending on the plant material. 1: from the amount of ^{86}Rb released by non-chlorophyllous soybean cells into the culture medium, according to Orr and Hess (1982). 2: from the amount of thiobarbituric acid-reacting material (TBARM) in etiolated cucumber hypocot yl, according to Placer et al. (1966). 3: from the amount of electrolytes released from etiolated cucumber hypocothyl sections floating on water (electrolyte leakage was followed using a Hoelzle & Chelius L 17 conductivimeter).

Protective effect of 4,6-dioxoheptanoic acid (DA)

Cell cultures

DA was added twice to cell suspension, at a final concentration of lmM: at the begining of the 14 h dark pretreatment, and one hour before light exposure (650 $\mu E/m^2/s$ PAR). ^{86}Rb efflux was estimated after 8 h in the light.

Etiolated cucumber hypocotyls

They were floated on aqueous solutions of DA (0,5 mM). After 8 h in the dark, they were exposed for 14 h to a light intensity of 400 $\mu E/m^2/s$ PAR. The conductivity of the medium was then determined.

Extraction of protoporphyrin IX

Soybean cells

Extraction was carried out according to Rebeiz et al. (1975). The protoporphyrin content of hexane-washed acetone extracts was determined using a Jobin et Yvon 3D fluorimeter calibrated with a 1 to 8 x 10^{-7} M solution of protoporphyrin IX.

Plant material

Extraction was carried out according to Watson et al. (1960). The protoporphyrin content of 10% HCl extracts was determined by spectrophotometry at 409 nm, with the use of protoporphyrin IX in 10% HCl as a standard.

RESULTS

Effects of AFM and LS 82-556 on the growth of non-chlorophyllous soybean cell cultures.

Treatment by 10 µM AFM in the light resulted in arrested growth and cell death. These effects were not observed when treatment were done in the dark (Fig. 2). LS 82-556 (100µM) induced exactly the same effects (not shown).

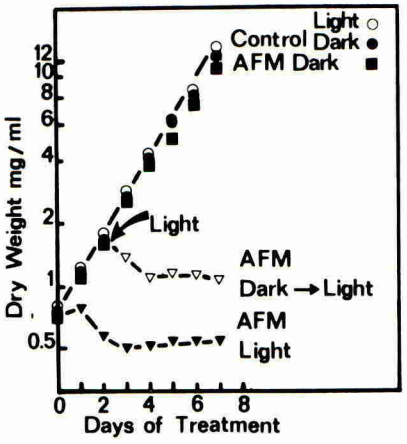


Fig. 2. Response of non-chlorophyllous soybean cells to AFM.

Induction of cellular damage in the absence of carotenoids.

Involvement of carotenoids was examined by monitoring the increase of dry weight / fresh weight ratio in norflurazon-treated cultures, in the presence of AFM or LS 82-556. Although deprived of carotenoids, norflurazon-treated cells remained sensitive to both 10 µM AFM or 100 µM LS 82-556 under white light (Fig. 3).

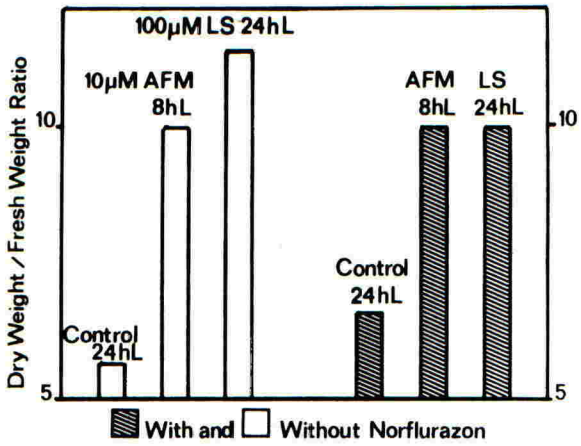


Fig. 3. Effects of LS 82-556 and AFM on soybean cells, either normal or without carotenoids (norflurazon treated). Dry weight/fresh weight ratios were estimated after 8 h (AFM) or 24 h (LS) treatment in the light.

Action spectra of AFM on non-chlorophyllous soybean cells and LS 82-556 on etiolated cucumber hypocotyls.

The action spectrum of AFM on non-chlorophyllous soybean cells was very similar to that of LS 82-556 on etiolated cucumber hypocotyls (Fig. 4). Maximum responses occured in the 350-450 nm region transmitted by a K 40 filter, even though the light intensity delivered by that particular filter was half that of the other ones. Wavelengths between 450 nm and 700 nm also induced toxicity but were less efficient. The light transmitted by the K 70 filter (650-750 nm) did not induce damages, suggesting a drop of activity above 650 nm.

These results indicate that the chromophore(s) implicated in the toxic process strongly absorbs light in the blue region (400 nm) and has secondary zones of absorption at the others wavelengths, ranging from 450 to 650 nm. As the participation of carotenoids was ruled out by the results of Fig. 3, and since soybean cells and etiolated cucumber hypocotyls were devoid of chlorophylls (not shown), a role of these pigments seemed also excluded. Among cellular chromophores, tetrapyrroles have absorption spectra matching the action spectra of AFM and LS 82-556. Therefore, a possible participation of these pigments as photoreceptors was examined.

Protective effect of 4,6-dioxoheptanoic acid (DA)

If tetrapyrroles are the photoreceptors for the light-activated herbicidal toxicity, cells deprived of these chromophores should be tolerant to AFM and LS 82-556. That point was examined using 4,6-dioxoheptanoic acid, which inhibits delta-aminolevulinic acid dehydratase (Meller and Gassman 1981), and consequently stops tetrapyrrole synthesis. As shown in Fig. 5, a 14h pretreatment in the dark with 1 mM DA for non-chlorophyllous soybean cells, or 8 h with 0.5 mM DA for etiolated cucumber hypocotyls, reduced their sensitivity to AFM or LS 82-556, respectively. These results thus

strenghtened the possibility that tetrapyrroles play a role in the toxic process. Tetrapyrrole content of soybean cells treated with AFM and etiolated cucumber seedlings treated with LS 82-556 were thus examined.

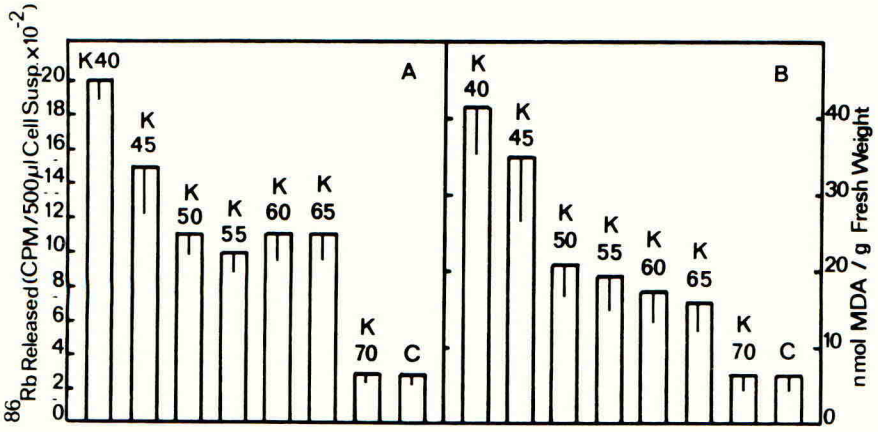


Fig. 4. Action spectra of 10µM AFM on soybean cells (A) and 10µM LS 82-556 on etiolated cucumber hypocotyls (B). Interference filters are designated according to the wavelengths of maximum transmission, e.g. K40 for 400 nm. Half bandwiths are 50 nm. C: control cells (results are the same for all wavelengths). Vertical bars represent standard errors.

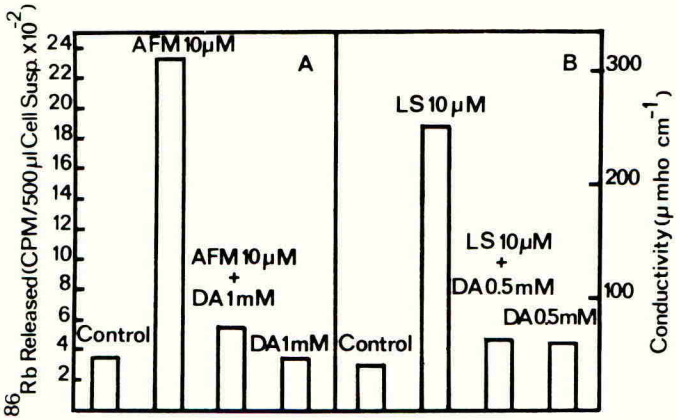


Fig. 5. Effect of 4,6-dioxoheptanoic acid (DA) on the toxicity of AFM on soybean cells (A) and LS 82-556 on etiolated cucumber hypocotyls (B).

Accumulation of tetrapyrroles in AFM-treated cells and LS 82-556-treated cucumber seedlings.

In control soybean cells as well as in etiolated cucumber hypocotyls or cotyledons, the amount of tetrapyrroles was below detection level. By contrast, the extracts of cells or etiolated cucumber seedlings treated with AFM or LS 82-556 in the dark presented fluorescence spectra closely matching the signals of protoporphyrin IX (Fig. 6). Similar accumulation have been found in etiolated cucumber cotyledons (not shown). This accumulation was markedly reduced if AFM treatment was done in presence of DA (Fig. 6).

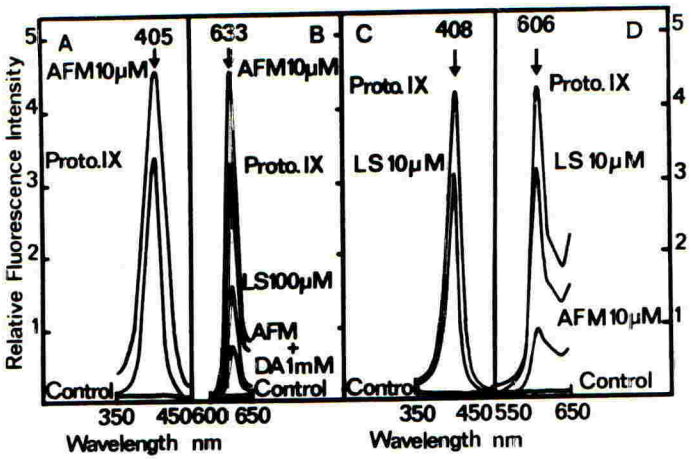


Fig. 6. Fluorescence excitation (A,C) and emission (B,D) spectra of extracts of soybean cells (A,B) and etiolated cucumber hypocotyls (C,D). Spectral differences between the two types of extracts are due to the use of two different extraction solvents (see MATERIALS AND METHODS).

DISCUSSION

Precise identification of the cellular chromophores implicated in the phytotoxicity of DPE could be a clue to the enigmatic mode of action of these herbicides. As mentioned before, the commonly proposed hypothesis is that these chromophores are carotenoids. Since this hypothesis meets some difficulties for chlorophyllous tissues, we have reexamined the question using soybean cell cultures.

AS in green plants, the herbicidal effect of AFM and LS 82-556 on these cells is strictly light-dependent. Our cultures contain carotenoids (0.9 to 1.6 µg / g fresh weight), but are unable to synthesize detectable amounts of chlorophylls, even in the light. For that reason, this material provides a simple and attractive model to reexamine the role of carotenoids. Our results showed that soybean cells deprived of carotenoids remain sensitive to AFM and LS 82-556. Consequently, in non-chlorophyllous cells as well as in green tissues, the role of carotenoid is highly questionable.

Since the action spectrum of DPE in green tissues could be confused by pigments not involved in the toxic process, we have undertaken a reapprai-

sal of this question using our non-chlorophyllous cells. Here again, evidence was not in favour of the participation of carotenoids, but rather of tetrapyrrole-like pigments.

Indeed, spectrofluorometric assays showed that pigment(s) with the fluorescence characteristics of protoporphyrin IX do accumulate in AFM-treated cells. Conversely, cells treated by an inhibitor of tetrapyrrole synthesis were tolerant to AFM.

Several lines of evidence showed that tetrapyrrole accumulation is a characteristic of DPE-type phytotoxicity. First, although AFM and LS 82-556 are chemically unrelated, they exert the same type of phytotoxicity, and similarly induce tetrapyrrole accumulation. Secondly, this last phenomenon is not restricted to cell cultures, but also occurs in plants. Accordingly, these plants are protected if tetrapyrrole synthesis is suppressed, and again the herbicidal action spectrum fits with a participation of tetrapyrroles.

Tetrapyrroles are known as photosensitizers able to generate singlet oxygen in the light (Cox and Whitten 1983). For example, treatment of human erythrocytes by protoporphyrin IX induces photooxidations leading to cell injury (Girotti and Deziel 1983). It has also long been known that in man, metabolic disorders known as porphyrias lead to porphyrin accumulation, and that photosensitivity is one of the main clinical manifestations of these diseases (Schmid et al. 1954). Finally, artificially induced tetrapyrrole accumulation in plants has been shown to result in herbicidal damage, which clearly demonstrate the potential harmful effects of these pigments (Rebeiz et al. 1984).

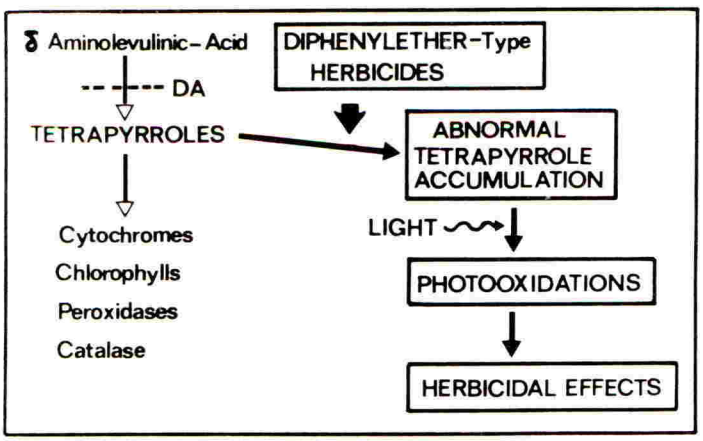


Fig. 7. Postulated mechanism of action of diphenylether-type herbicides.

Our results, together with the photodynamic properties of tetrapyrroles, lead us to propose a new mechanism for the mode of action of DPE-type herbicides (Fig. 7). According to our hypothesis, DPE are not directly involved in any photodynamic reaction. In fact, they would rather induce an accumulation of tetrapyrroles, which would be the actual photosensitizers. The phytotoxic properties of DPE would thus result, not from their elusive photodynamic properties, but from their ability to induce accumulations of these pigments, either in the dark or in the light.

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THE ROLE OF PHOTOSYNTHETIC ELECTRON TRANSPORT IN THE MODE OF ACTION OF NITRODIPHENYL ETHER HERBICIDES

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ABSTRACT

The nitrodiphenyl ether DPEI[1] induces light- and O_2-dependent lipid peroxidation and Chl bleaching in the alga Scenedesmus obliquus. Under conditions of O_2-limitation, inhibition of photosynthetic electron transport by addition of prometryne or diuron, or by mutation, diminishes these effects. Similar symptoms are seen with DPEII, a DPE whose redox properties preclude reduction by Photosystem I. Under conditions of high aeration, diuron does not protect Scenedesmus cells from Chl bleaching induced by DPEI, but does protect against paraquat. These results indicate that the role of photosynthesis in DPE-toxicity in Scenedesmus is not to reduce the herbicide to a radical species which initiates lipid peroxidation but may be to maintain a sufficiently high O_2 concentration. DPEI and DPEII have similar potencies in causing lipid peroxidation in leaves, but while DPEI induces carotenoid bleaching in a non-chlorophyllous "chromoplast" preparation from chrysanthemum petals, DPEII is ineffective, indicating the possibility of multiple bleaching mechanisms in leaves.

INTRODUCTION

Nitrodiphenyl ether herbicides cause a light- and oxygen-dependent membrane lipid peroxidation and pigment bleaching (Orr and Hess, 1982a,b). In the alga Scenedesmus obliquus, the photosynthetic electron transport inhibitor diuron blocks these toxic effects (Kunert and Böger, 1981) and in higher plants partial protection is sometimes observed (Matsunaka, 1969; Orr and Hess, 1982a). These characteristics resemble those of paraquat toxicity, and led Kunert and Boger (1981) to propose that the initial event in nitro-DPE-toxicity is reduction to a radical species. Several lines of evidence argue against this hypothesis. Certain chloro-DPEs which have chemical properties precluding reduction by PSI show similar effects on plants to nitro-DPEs (Ridley, 1983), and plant tissues which are photosynthetically incompetent (non-chlorophyllous soybean cell suspension culture (Matringe and Scalla, 1987); etiolated cucumber seedlings (Duke and Kenyon, 1987); and barley mutants lacking Photosystem I or II (Bowyer et al, 1987)) are highly susceptible to nitro-DPEs. On the other hand oxyfluorfen induces radical formation in a reaction requiring light but inhibited by diuron in isolated spinach thylakoids (Lambert et al, 1984) and the action spectrum of the nitro-DPE aciflurofen-methyl in Chlamydomonas eugametos indicates that light

[1]Abbreviations: DPE, diphenyl ether; DPEI, 5-[2-chloro -4(trifluoromethyl) phenoxy]-2-nitroacetophenone oxime - o - (acetic acid, methyl ester); Chl chlorophyll; PSI, Photosystem I; DPEII, 5-[2-chloro-4-(trifluoromethyl) phenoxy]-methoxyphthalide.

absorbed by chlorophyll can activate the herbicidal effect. A mode of action which does not require photosynthesis, but involves carotenoid in the photo-activation process has been proposed (Orr and Hess, 1982b) on the basis of the absence of nitro-DPE effects on tissue depleted of carotenoid as a result of mutation or treatment with the carotenoid biosynthesis inhibitor nor-flurazon (Matsunaka, 1969). However, reduction of the carotenoid content to undetectable levels by treatment of a soybean cell suspension with nor-flurazon had no effect on the light-dependent toxic action of acifluorfen-methyl (Matringe and Scalla, 1987). It is clear that there are many apparently contradictory results in this field!

In this report we have attempted to clarify some aspects of nitro-DPE action. We show that in Scenedesmus obliquus, the protective effect of diuron does not arise from an inhibition of the photosynthetic reduction of the DPE, but is probably linked to inhibition of photosynthetic oxygen evolution. We also describe a plant system (chrysanthemum petals and "chromoplasts") which is susceptible to a number of nitro-DPEs but which is unaffected by certain DPEs which are potent inducers of light-dependent lipid peroxidation in leaf tissue.

MATERIALS AND METHODS

Algal culture

Scenedesmus obliquus wild type and mutants lacking parts of the photo-synthetic apparatus were obtained from Prof. N.I. Bishop, Oregon State University. Cultures were maintained on an enriched agar medium (modified Kessler's medium (Bishop and Senger, 1971), plus 0.5% w/v glucose and 0.25% w/v yeast extract). Cells in suspension were grown heterotrophically on the same medium at 22°C at a light intensity of 0.27 W/m^2 (100 lux) provided by white fluorescent lights. All cells were grown heterotrophically because the non-photosynthetic mutants are unable to grow autotrophically. Pigment-protein complexes and photosynthetic electron transport chains are produced under these conditions. Cells were grown in 75 ml of medium in 250 ml Erlenmeyer flasks on an orbital shaker rotating at 150 revs/min. They were subcultured every 4 days and each experiment was initiated 4 days after the previous transfer. Cells were harvested by centrifugation and resuspended in fresh sterile growth medium to a concentration of 23 mg wet weight per ml.

Hydrocarbon Formation in Herbicide-Treated Algae

For measurements of ethane formation arising from lipid peroxidation, 5 ml of concentrated cell suspension was placed in a 10 ml Erlenmeyer flask and the required herbicide added. The flask was then sealed with a rubber seal and flushed for 10 min using a hydrocarbon-free synthetic air mixture. The flasks were placed on an orbital shaker rotating at 150 rev/min at 20± 2°C under a light intensity of 330 W/m^2 at the surface of the algal suspension. Illumination was provided by an array of heat filtered 150W floodlights. At intervals 0.5 ml samples of head space were removed from the flasks using a gas tight syringe and analysed for ethane by gas chromatography (Bowyer et al, 1987).

Chlorophyll Bleaching in Herbicide-Treated Algae

5ml samples of algal suspension at 12 mg wet weight/ml in 10 ml conical flasks were set up on an orbital shaker rotating at 200 revs/min at 25°C. The flasks were lightly stoppered with cotton wool, permitting vigorous aeration during the incubation with the herbicide. Illumination for these experiments was provided by an array of 60W bulbs providing an intensity at the surface of the algal suspension of 190 ± 10W/m^2. At intervals, 0.5 ml samples of suspension were removed under sterile conditions, centrifuged,

and pigments were extracted from the algal pellet by heating for 6 min in 96% ethanol at 80°C. The Chl *a* content of the extract was estimated spectrophotometrically (Lichtenthaler and Wellburn (1983)).

Herbicidal effects on Chrysanthemum petals

Horticultural chrysanthemum plants (variety, Bright Golden Princess Ann) in pots were used. For studies on intact petals, detached open blooms with their cut stems in water were coated with a solution of herbicide in 0.01% (v/v) aqueous Triton X-100. Control plants were treated with 0.01% (v/v) Triton X-100. The flowers were exposed to a light intensity of >200 W/m^2 for 51 hours. Damaged was assessed visually. "Chromoplasts" were isolated from mature petals (outer 3 rings of florets) using the method of Falk *et al* (1974) for daffodil chromoplasts. They were visible as yellow bands at the interfaces of the 30%/40% and 15%/30% sucrose layers after centrifugation on discontinous sucrose gradients. The bands were removed, diluted with 5 mM $MgCl_2$, 67 mM phosphate pH7.5 to give a final concentration of 15% sucrose, and pooled. The carotenoid content was determined by centrifuging the chromoplast suspension at 100,000x g for 1 h and resuspending the pellets in ethanol. A white precipitate was removed by centrifugation and an optical absorption spectrum of the ethanol extract taken. An extinction coefficient (E_{1cm} 1%) of 2500 for the predominant peak between 437-440 nm was used to calculate carotenoid content. Comparison of electron micrographs (not shown) of the "chromoplast" preparation and intact petals indicated that the "chromoplast" preparation in fact consisted of large lipid globules probably formed by fusion of the smaller globules within the chromoplasts during homogenisation of the petals. Samples were prepared for electron microscopy as described in Bowyer *et al*, (1987).

General

With the exception of paraquat, herbicide stock solutions were made up in DMSO giving 0.1% v/v DMSO after dilution into the algal suspension. Controls contained 0.1% v/v DMSO. Paraquat was obtained from Sigma Chemical Co. and the other herbicides were kind gifts from agrochemical companies, as listed in the acknowledgements. All results are the means from at least two separate experiments, except where indicated.

RESULTS

The nitro-DPE herbicide DPEI (Bowyer *et al*, 1987) and paraquat caused marked ethane formation resulting from lipid peroxidation in illuminated *Scenedesmus* cells (Fig, 1A, B). DPEI did not induce ethane formation in the absence of illumination (not shown) and the photosynthetic electron transport inhibitors prometryne and diuron both blocked the DPEI and paraquat effects. Neither DPEI nor paraquat induced ethane formation in a mutant which lacked PSI (Fig. 1A). The same result was seen with mutants which lacked either Photosystem II or the cytochrome *b6f* complex (data not presented).

The effects of a novel phthalide diphenyl ether (DPEII) synthesised by the Organic Chemistry Division at Sittingbourne Research Centre are shown in Fig. 2. DPEII induces light-dependent ethane formation and pigment bleaching in leaves of higher plants (P. Camilleri, K. Weaver, J.R. Bowyer and B.J. Hallahan, unpublished observations) and gives a similar primary screen score to DPEI (see Table 1). However, pulse radiolysis studies on DPEII in propan-2-ol/water (1:3 v/v) indicate that it has a one-electron reduction potential of less than -700mV (P. O'Neill, unpublished observa-

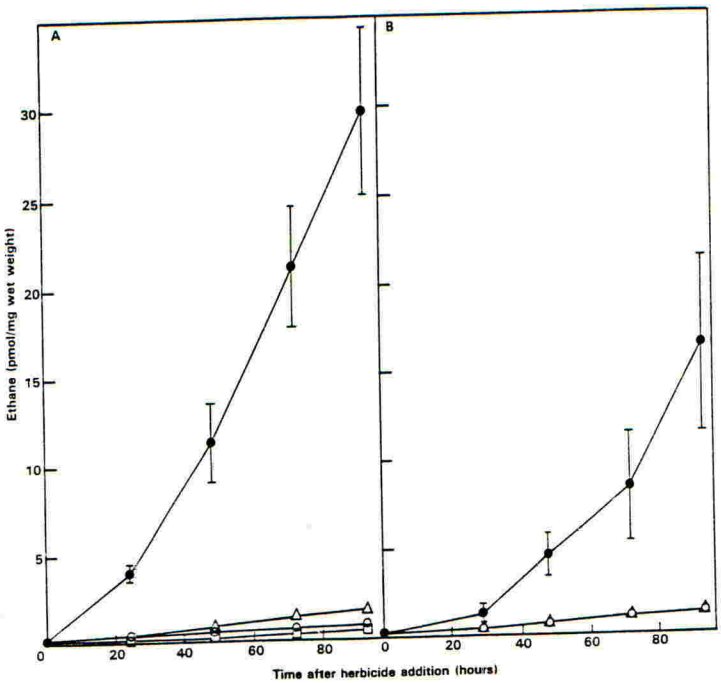


Fig. 1. Effect of inhibition of photosynthetic electron transport on lipid peroxidation induced by herbicides in illuminated Scenedesmus, monitored using ethane formation. A. Effect of 2mM paraquat on wild type (●); effect of 2mM paraquat on mutant lacking PSI (Δ); effect of 2mM paraquat and 10 μM diuron on wild type (O); and control wild type (□). B. Effect of 10 μM DPEI on wild type (●); effect of 10 μM DPEI on mutant lacking PSI (Δ); and superimposed, effect of 10 μM DPEI and 10 μM diuron on wild type (O). The error bars where shown indicate the standard error based on seven measurements.

tions) This property would preclude its reduction by all but the most primary photoreactants of PSI (Hoff, 1982) and reduction by these components can be discounted on kinetic grounds. Fig. 2 shows that DPEII also induced marked ethane formation in Scenedesmus in a light-dependent process, and pre-treatment with diuron provided complete protection. The results in Fig. 2 also indicate that lipid peroxidation induced by both DPEI and DPEII is suppressed when the oxygen concentration is lowered. We have shown that cells suspended in fresh growth medium show a net oxygen evolution rate of around 10μmol O_2/mg Chl/h which increases to 40μmol O_2/mg Chl/h over a period of 2 days. The ethane formation observed in the nitrogen-flushed flasks may therefore be linked to oxygen generated photosynthetically. The results in Fig. 3 show the effects of diuron on chlorophyll bleaching induced by paraquat and DPEI in vigorously aerated cell suspensions. Vigorous aeration was needed to elicit net chlorophyll bleaching by both paraquat and DPEI. Although diuron markedly inhibited the net chlorophyll bleaching induced by paraquat, it did not affect the DPEI-induced bleaching.

Experiments with chrysanthemum petals

In order to further probe the mode of action of nitro-DPEs in a non-chlorophyllous and photosynthetically incompetent plant tissue, we tested the effect of DPEI on chrysanthemum florets. HPLC analysis of extracts of

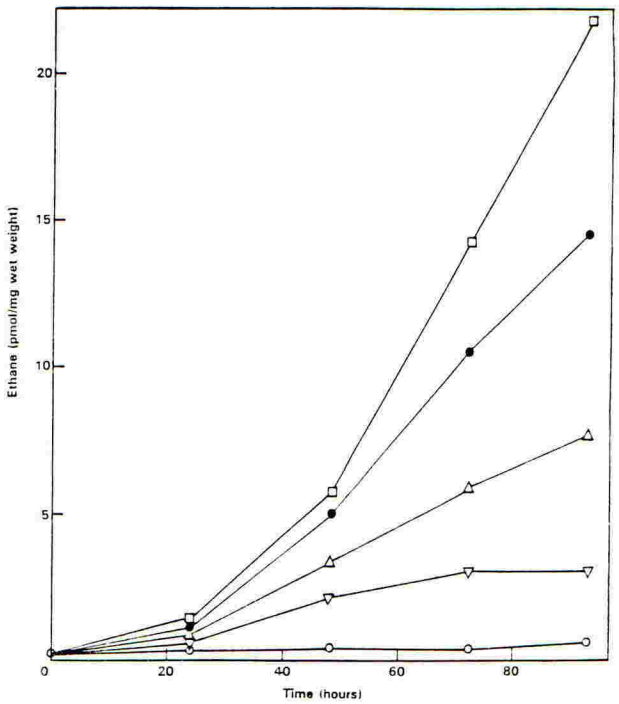


Fig. 2. Effect of O_2 on the ability of DPE's to induce lipid peroxidation monitored by ethane formation in illuminated Scenedesmus cells. At the beginning of the experiment, flasks were flushed with either air or N_2 for 10 minutes. The additions were 10 µM DPEI with air (□); 10 µM DEPII with air (●); 10 µM DPEI with N_2 (▲); 10 µM DEPII with N_2 (▽); 10 µM DPEII with 10 µM diuron and air (○); 10 µM DPEII with air, in darkness, superimposed on (○); and 10 µM diuron with air, superimposed on (○).

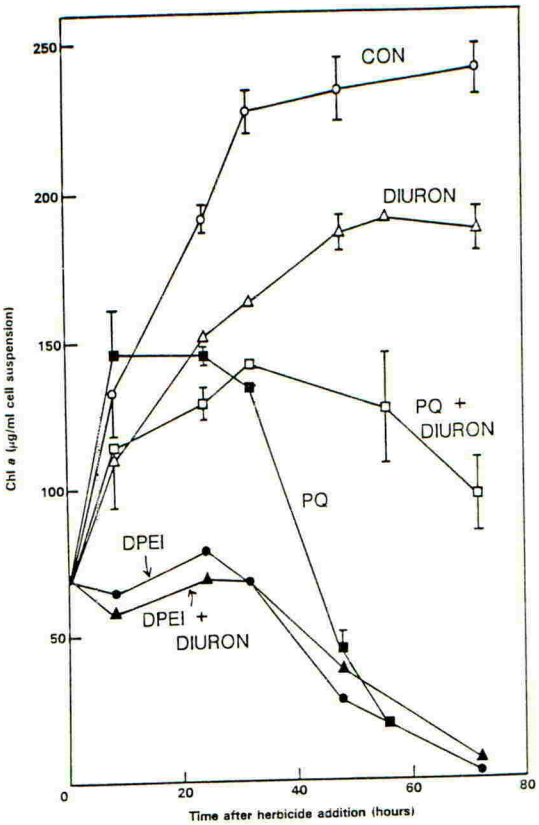
the outer rings of florets confirmed the absence of chlorophylls and revealed a complex mixture of over 40 carotenoid derivatives (not shown). Chrysanthemum flowers treated with DPEI showed necrosis which developed over a period of several days and was strictly light-dependent. Paraquat had no effect on the florets. Electron micrographs of necrotic tissue show that there is extensive membrane disruption which is similar to that seen in DPE-treated leaf tissue (not shown). DPEI also enhanced the light-dependent bleaching of carotenoids in a pigmented fraction ("chromoplasts") isolated from the florets (Table 1). Neither paraquat nor the carotenoid biosynthesis inhibitors norflurazon and diflufenican had any effect on this light-induced bleaching (data not presented). In order to ascertain whether this bleaching and membrane damage were related to the mode of action of nitro-DPEs and related molecules on leaves, we tested a number of different compounds (Table 1). The results show that the structure/activity relationship of the compounds in the "chromoplast" assay was totally different from that on leaves based on a visual assessment of damage in the latter. Of particular interest is that nitrofen was the most potent compound in the "chromoplast" assay, but is a relatively poor nitro-DPE herbicide, whereas the phthalide DPEII which is a very active herbicide with nitro-DPE symptomology, was inactive both in the "chromoplast" assay and also on the intact florets. Both bifenox and nitrofen, although active on the "chromoplast", had no effect on the intact florets (Table 1). α-tocopherol reduced the extent of nitro-DPE induced bleaching in the "chromoplast" suspension, but compounds which would either enhance or reduce the effects of singlet oxygen (deuterium oxide and 1,4-diazabicyclo [2.2.2] octane respectively) or of hydroxyl-radical generating systems (desferrioamine) had no effect (notshown).

DISCUSSION

DPEI and paraquat both induce a light- and O_2-dependent membrane lipid peroxidation in Scenedesmus (cf Kunert and Boger, 1981). This process is

Fig. 3.

Effect of diuron on Chl a bleaching induced by paraquat and DPEI in vigorously aerated illuminated Scenedesmus cells. Effects of 10 μM DPEI (●); 10 μM diuron (▲); 10 μM DPEI with 10 μM diuron (▲); 2mM paraquat (■); 2mM paraquat with 10 μM diuron (□); control (○). The bars where shown indicate the standard error based on at least four measurements.



strongly inhibited when photosynthetic electron transport is blocked either chemically (diuron and prometryne) or by mutation leading to loss of an electron transport component. These effects support the idea that the toxicity of nitro-DPEs may be linked to their reduction by PSI. However, this interpretation can be precluded by the results obtained with DPEII. The redox properties of DPEII prevent its reduction by PSI, but DPEII-induced lipid peroxidation is also inhibited by diuron.

Since lipid peroxidation is suppressed by the removal of O_2, a possible role for photosynthetic electron transport would be to maintain a sufficiently high O_2 content in the medium by water-splitting. Under the conditions of the ethane accumulation measurements, if photosynthetic O_2 evolution is blocked, the respiratory rate of the cells is such that all the oxygen in the flask could be consumed in around 3 hours, and this could therefore be responsible for suppressing lipid peroxidation. In well aerated cultures however, while diuron inhibits the bleaching induced by paraquat, it has no effect on the DPEI-induced bleaching. The inhibitory effect of diuron on the paraquat bleaching can be attributed to an inhibition of the photoreduction of paraquat, an essential step in its toxic action (cf Bowyer et al, 1987). The lack of effect of diuron on DPEI bleaching is consistent with the idea that the role of photosynthetic electron transport is to generate O_2 under conditions when the O_2 concentration may be limiting.

The problem of oxygen supply was not encountered in our own experiments with higher plant tissue (Bowyer et al, 1987) because in the experiments in

TABLE 1

Comparison of the effects of DPE analogues on intact chrysanthemum flowers, and on a "chromoplast" preparation from the chrysanthemum florets, with their herbicide primary screen scores.

Compound	% bleaching induced in chromoplasts[a]	Effect on intact flowers[b]	Primary screen score[c]
Nitrofen	76	-	58
DPEI	43	++	68
Bifenox	30	-	57
DPEII	9	-	70

[a]Bleaching induced by 5 μM compound when added to "chromoplast" suspension at an initial carotenoid concentration of 2.3μg/ml after 18 hours illumination by white light of intensity >200W/m^2. Bleaching is expressed as the carotenoid content as a percentage of that in the control after 18 hours. Typically a 30% bleaching occured in the control during this period.
[b]See Materials and Methods.
[c]Sum of scores on 8 plant species for 1kg/ha foliar spray, max score 72.

which chlorophyll bleaching was monitored in leaf discs, the discs were suspended in water vigorously bubbled with O_2, and in the experiments with barley mutants, the leaves were in contact with the atmosphere during the major part of the herbicide treatment, and only sealed into tubes for 4 hours to measure ethane accumulation. The experimental conditions employed by Kunert and Böger (1981) (autotrophically growing cells bubbled with air) would be less likely to lead to O_2 limitation under conditions of inhibited photosynthesis, but they still observed an inhibition by diuron of oxyfluorfen-induced lipid peroxidation and Chl bleaching. However, the destruction of cytochromes induced by oxyfluorfen was only slightly diminished by diuron, which lead Kunert et al, (1985) to propose O_2-limitation as the cause of the diuron effect. Differences in the degree of protection by diuron would then reflect a balance between the O_2 content of the medium, the toxicity of the DPE, and the O_2 requirement of the particular biochemical effect. Photosynthetic electron transport may play a secondary role in DPE-toxicity by generating lipid radicals, since high concentrations of oxyfluorfen do enhance radical formation in illuminated spinach thlakoids in a diuron-sensitive reaction (Lambert et al, 1984).

The results with the chrysanthemum chromoplasts are of some interest because they suggest that nitro-DPEs can induce light-dependent pigment bleaching by a mechanism not available to other DPEs which are, however, active on leaves. We do not yet know what this mechanism is, but preliminary studies suggest that neither singlet oxygen nor the Fenton reaction are involved. The chemical structures of the non-nitro DPE analogues appear to preclude their conversion by the plant to an active species which would not be generated in the "chromoplasts", The lack of effect of nitrofen and bifenox on intact flowers is presumably due to metabolism or uptake problems.

We do not yet know whether the nitro-DPE bleaching mechanism operating in the "chromoplasts" does occur in leaves, but clearly our results suggest that more than one process may be involved in the bleaching action of DPE herbicides.

ACKNOWLEDGEMENTS

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THE EFFECTS OF ACIFLUORFEN ON MEMBRANE INTEGRITY IN GALIUM APARINE LEAVES AND PROTOPLASTS

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ABSTRACT

Acifluorfen phytotoxicity was investigated in excised leaves and isolated mesophyll protoplasts of Galium aparine. Excised leaf photosynthesis was inhibited by 78% after 15h incubation with 100μM acifluorfen, and chlorophyll breakdown, electrolyte leakage and lipid peroxidation were evident after this time. Furthermore, phytotoxicity was apparent at 8 μmoles/m²/s blue light in the absence of photosynthesis. Protoplast viability and photosynthesis over 2h was sensitive to acifluorfen concentration whilst intactness remained unaffected. However, neither electrolyte leakage nor lipid peroxidation were observed. These results are discussed in relation to the current views on DFE action and favour a primary action that is mediated by blue light and the chloroplast envelope which leads to a disruption of membrane integrity.

INTRODUCTION

Acifluorfen has a mode of action in common with other nitrodiphenyl ethers, chlorodiphenyl ethers (Ensminger and Hess, 1985) and a number of compounds which lack the diphenyl ether (DPE) structure (Matringe et al, 1985; Derrick, 1987). These compounds induce rapid bleaching and necrosis in susceptible plants, probably via a peroxidative destruction of membranes in a light-dependent manner (see Orr and Hess, 1982; Duke and Kenyon, 1987 for review). At first glance this would suggest a parallel between the modes of action of bipyridyls (eg. paraquat) and DPE - type herbicides, in that both participate in electron transfer processes between chloroplast thylakoids and membrane lipids via toxic radical species. Indeed, there is some evidence to suggest that oxyfluorfen may be able to participate in such a system (Gillham et al, 1985). It is unlikely however, that this type of mechanism is generally applicable to DPE - type compounds for a number of reasons. Firstly many DPE's are incapable of being directly reduced (Ensminger and Hess 1985) and some DPE compounds lack a nitro- or other comparable reducable group (Matringe et al 1986; Derrick, 1987) often deemed necessary for such reactions (eg Gillham et al, 1985). Secondly, ultrastructural evidence does not favour a paraquat-type mode of action for DPE's, thylakoid disruption occurring only at a relatively late stage in the

development of DPE - induced toxicity (Kenyon et al, 1985; Derrick et al, 1987), whilst the reverse is true with paraquat (Harvey and Fraser, 1980). Thirdly, photosynthetically incompetent plant tissues eg etiolated tissue, and plants grown in far-red light are susceptible to DPE action (Duke and Kenyon, 1986). Nevertheless, evidence for some participation of photosynthetic electron transport in the action of DPE's has been provided by several research groups (see Duke and Kenyon, 1987) in that electron transport inhibitors suppress DPE activity in a number of plant systems. The mode of action of DPE's therefore remains obscure, but distinct from that of the bipyridyls.

In this laboratory we have studied various aspects of the complex action of DPE - type herbicides in the relevant weed species Galium aparine L. (cleavers), using isolated mesophyll protoplasts and intact leaves. This paper reports the novel use of weed protoplasts to examine the effect of acifluorfen on membrane integrity and discusses the involvement of light quality and lipid peroxidation in the action of this herbicide.

MATERIALS AND METHODS

Plant Material

Galium aparine seedlings were grown in peat-based potting compost at 20-25°C under a 14 h photoperiod of 200-400 μmol/m²/s photosynthetic photon flux density (PPFD) provided by high-pressure sodium lamps. Leaves used for experiments were cut from the second whorl of true leaves when the diameter of this whorl was 45-48mm and that of the third whorl 5-10mm.

Studies on excised leaves

To study the effects of acifluorfen on excised leaves, several processes were monitored over a 30h period at 25°C and 50μmol/m²/s PPFD, including leaf photosynthetic competence, chlorophyll content, electrolyte leakage and malondialdehyde (MDA) release. Leaf photosynthesis was measured polarographically (LD2 oxygen electrode, Hansatech Ltd., Kings Lynn, UK), chlorophyll content by acetone extraction (Arnon, 1949) and electrolyte leakage by increase in bathing medium conductivity (PTI-18 conductivity meter, FSA Ltd). This medium was also assessed for MDA accumulation by colour reaction with thiobarbituric acid (Heath and Packer, 1968).

To examine the effects of light quality, ten leaves were floated abaxial surface down on 100 μM acifluorfen solutions in 5cm Petri dishes in an incubation system maintained at 25°C which only emitted light through a window consisting of a colour light filter. Fluorescent light source/filter combinations used were: (1) for blue light, 'natural white' tubes (GEC) plus Cinemoid no. 19A (Rank Strand) filter giving maximum transmission at 440nm and half bandwidth of 50nm. (2) For red light, 'Deluxe Natural' tubes (Thorn-EMI) plus Cinemoid no. 14 filter transmitting wavelengths greater than 610nm. (3) For green light, 'Cool White' (Thorn-EMI) tubes plus Rosco Supergel no. 90 filter (Rosco, Upper Ground, London, SE1 9PQ, U.K.), giving maximum transmission at 515nm and

half bandwidth of 50nm. Light incident on leaves was at 8 μmol/m²/s PPFD. Physiological damage was detected as a conductivity change in the bathing medium and a reduction in leaf chlorophyll content (determined as above). Four Petri dishes in each of six experiments were assessed per treatment, i.e. 240 leaves for each treatment.

Preparation of protoplasts

The abaxial epidermis was peeled from leaves and floated abaxial surface down on a plasmolysing medium (0.5M mannitol, 0.1% wt/vol polyvinylpyrrolidone, 1mM $CaCl_2$ and 10mM MES-KOH buffer pH 5.5) until sufficient leaf material had been peeled. The plasmolysing medium was then replaced with an enzyme medium containing 1% wt/vol Cellulysin (Calbiochem), 0.05% wt/vol Pectolyase Y-23 (Seishin Pharmaceutical) and 0.25% wt/vol bovine serum albumin, dissolved in plasmolysing medium and the pH readjusted to 5.5 with KOH. Following incubation at 25°C under 50 μmol/m²/s PPFD provided by 'natural white' fluorescent tubes (GEC) for 1.5h, the resulting protoplast suspension was passed through two filters (1mm and 200μm mesh), centrifuged at 100 x g for 2 min and the pellet resuspended in a protoplast storage medium (0.4M mannitol, 1mM $CaCl_2$, 20mM MES-KOH, pH 6.0). This crude protoplast preparation was purified by overlaying the suspension onto a stepped gradient of Percoll (Pharmacia) in storage medium (this consisted of 2ml layers of 35%, 30%, and 25% Percoll) and centrifugation at 150 x g for 10 min. Protoplasts were collected from the 0%/25% interface, diluted four fold with storage medium, centrifuged for 2 min at 100 x g and resuspended in storage medium. The protoplast concentration was adjusted to 5 x 10^5/ml of suspension and stored at 25°C prior to use.

Incubation of Protoplasts with Acifluorfen

Aliquots of protoplast suspension (2ml) were incubated in oxygen electrodes (model DW1, Hansatech Ltd). The stirrer was operated at its slowest speed and a 3mm thick spacer placed between the electrode and stirrer base to reduce the stirrer speed sufficient to avoid excessive protoplast breakage, whilst maintaining a suspension and allowing adequate electrode response to changes in oxygen concentration. Under these conditions, protoplast intactness remained stable for over 3h at 25°C (Derrick, 1987). Acifluorfen (97.5% pure), was added from stock solutions in acetone. Final solvent concentration in the storage medium was 1.25% vol/vol.

Estimation of Protoplast Intactness

Intactness, recorded as protoplast number/ml as a percentage of the original protoplast density, was determined by counting on a haemacytometer grid. Counts were made on two grids per sample for each of six experiments, each experiment being performed on a separate protoplast preparation.

Vital Staining of Protoplasts with Fluorescein Diacetate (FDA).

Intracellular hydrolysis of FDA to fluorescein (which emits a yellow-green fluorescence under u.v. light) was exploited as a measure of protoplast viability since only viable cells hydrolyse FDA (Widholm, 1972). Equal volumes (15μl) of protoplast suspension and freshly made 0.01% wt/vol FDA (A.R. grade Koch-Light Laboratories) in storage medium

containing 1% wt/vol acetone, were mixed on a haemacytometer.

Protoplasts were counted under white light and exactly 2 min later, the same protoplasts counted under u.v. light, scoring yellow-green protoplasts as viable. Four microscope fields of view (each equivalent to 0.628 µl of suspension) were assessed per sample in each of six experiments.

Photosynthetic Competence of Protoplasts

Samples (1.5ml) of protoplast suspension were centrifuged at 100 x g for 2 min, the protoplasts resuspended in 1.5ml of 0.4M mannitol, 5mM $CaCl_2$, 25mM $NaHCO_3$ and 50mM tricine-KOH, pH 7.6 and the suspensions returned to the oxygen electrode well. Following a 3 min dark incubation, the protoplasts were illuminated with 500 µmol/m²/s PPFD and oxygen evolution recorded. The chlorophyll content of suspensions was determined by the method of Arnon (1949), to permit expression of data on a chlorophyll basis.

RESULTS

The effect of 100µM acifluorfen on G. aparine excised leaves over a 30h incubation period is illustrated in Fig. 1, from which a possible sequence of events may be deduced. The first deviation from control values was the steady decline in photosynthetic O_2 evolution, so that by 15h this process was inhibited by 78%. However, chlorophyll breakdown

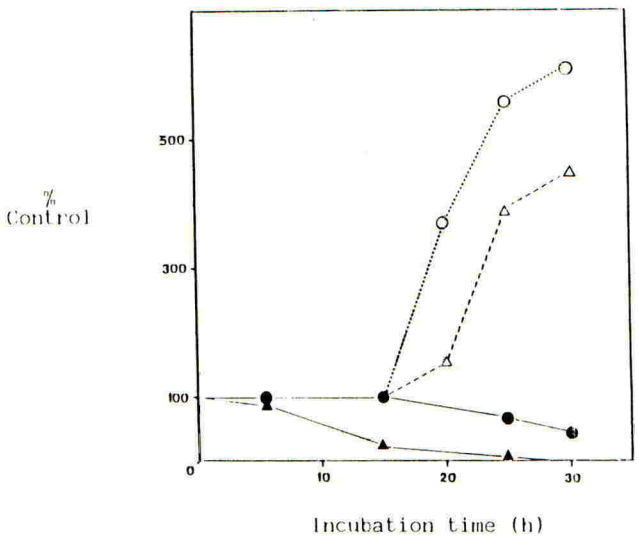

Fig. 1. The effect of 100µM acifluorfen on leaf photosynthesis (▲——▲), chlorophyll content (●——●), electrolyte leakage (O····O) and MDA formation (△- - -△) in excised G. aparine leaves at 25°C and 50 µmoles/m²/s PPFD. Data are expressed as a percentage of control values, which were constant throughout the incubation period i.e. photosynthesis (140 - 150 µmoles O_2 evolved/mg chl/h, determined at 500 µmoles/m²/s, PPFD), chlorophyll (20 - 24 µg/leaf), electrolyte leakage (6µS/cm) and MDA (0.44 nM TBARM after 30h incubation).

only became apparent after this time. Electrolyte leakage markedly increased after 15h, whilst MDA accumulation in the bathing medium followed a similar but lesser pattern. These observations performed with unfiltered white light suggest that photosynthesis in this species is most sensitive to acifluorfen and its inhibition precedes membrane disruption and peroxidation by several hours at 50 μmoles/m^2/s PPFD.

Fig. 2 clearly shows that blue was the most effective light quality in acifluorfen-mediated toxicity after 48h at 8 μmoles/m²/s (PPFD). Photosynthesis was not measurable at such a low flux density and suggests a blue light-sensitive effect that is independent of photosynthesis. These results and others at higher flux densities (Derrick, 1987) are in agreement with those of Ensminger and Hess (1985) who determined an action spectrum for acifluorfen methyl in the green alga Chlamydomonas and found a large peak of activity in the blue region of the spectrum, a minor peak in the red and an inability of green light to generate toxicity.

These observations were further extended by the use of isolated G. aparine mesophyll protoplasts. Fig. 3A illustrates the structural integrity of protoplasts over a 2h incubation period in the presence of 0-750 μM acifluorfen. Intactness was greater than 90% throughout, indicating no significant damage to the plasmalemma in all treatments. However, whilst no acifluorfen - induced lysis was evident, the protoplasts were less able to hydrolyse FDA with increasing acifluorfen dosage, suggesting a decline in metabolic integrity (Fig. 3B). Protoplast photosynthesis was similarly sensitive to inhibition by acifluorfen (Fig. 3C).

Further experiments were also performed incubating protoplasts with acifluorfen to determine lipid peroxidation (by ethane evolution), electrolyte leakage and MDA production. However, none of these products were detected in this experimental system, even after 5h incubation

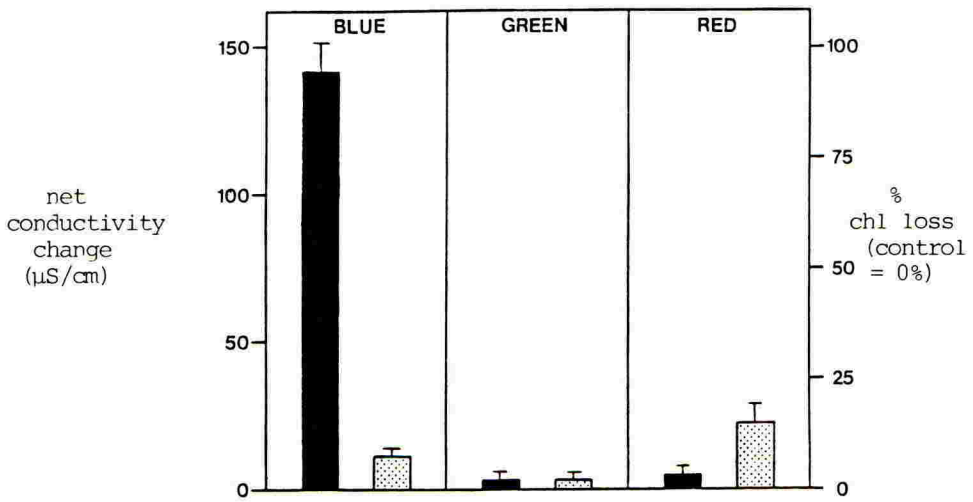

Fig. 2 The leakage of electrolytes (■) and loss of chlorophyll (⊡) from excised G. aparine leaves incubated for 48h with 100 μM acifluorfen under 8 μmoles/m²/s PPFD blue, red and green light. Bars represent S.E.'s.

(Derrick, 1987). Thus, no peroxidative symptoms were detected 3h after a complete loss of photosynthetic activity.

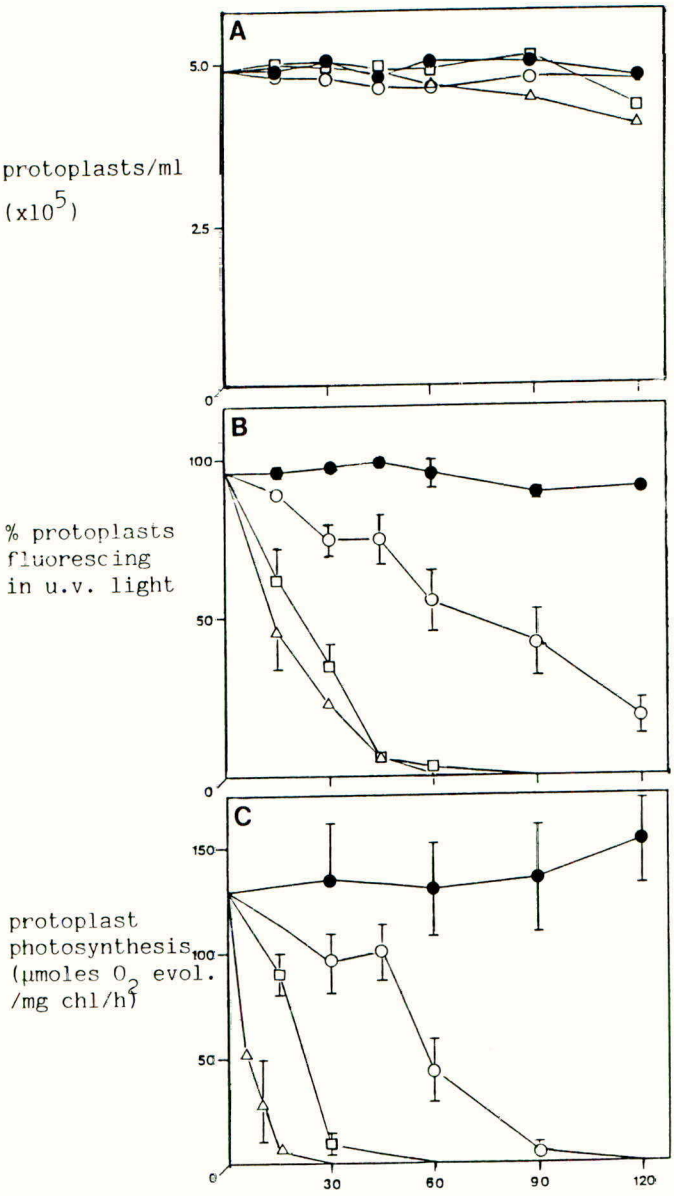

Fig. 3. The effect of 0 (●—●), 375 (○—○), 500 (□—□) and 750 (△—△) μM acifluorfen on protoplast intactness (A), protoplast viability (B) and protoplast photosynthesis (C). Bars represent S.E.'s.

DISCUSSION

Acifluorfen is a post-emergence selective herbicide with a contact action in photosynthetically active tissues of broadleaved weeds, leading to bleaching and necrotic symptoms in these tissues. However, little published information exists on the mode of action of this herbicide in these tissues. This paper reports observations of acifluorfen mode of action in G. aparine leaves and isolated protoplasts and infers a primary effect on membrane integrity prior to lipid peroxidation as shown in Fig. 1. These observations support the findings of an ultrastructural study carried out in this laboratory (Derrick et al, 1988) in which the first observable symptoms of herbicide damage were distorted chloroplast envelopes and later endomembrane disruption. Any such injury to the chloroplast envelope is likely to cause a rapid cessation of photosynthesis as these membranes play a pivotal role in regulating intraplastid homeostasis (see Douce and Joyard, 1979).

Whilst G. aparine mesophyll protoplasts remained intact during acifluorfen incubations over 2h (Fig. 3A), the herbicide clearly had the ability to drastically reduce FDA hydrolysis (Fig. 3B) and photosynthesis (Fig. 3C). As enzymes capable of hydrolysing FDA are presumed present in most cell compartments, such a reduction in FDA hyrolysis suggests a loss of cytoplasmic compartmentation/integrity or a loss of cytoplasmic contents through damaged or a leaky plasmalemma. However, the latter possibility is discounted by the failure to observe electrolyte leakage from protoplasts in the presence of acifluorfen. Furthermore, decreased intactness would have been expected but was not observed.

The finding that acifluorfen induced toxicity is highly sensitive to low flux density blue light (Fig. 2.), indicates the involvement of a chromatophore unrelated to photosynthesis. Indeed, our data strongly suggest that thylakoids are not directly involved in DPE action, but that the initial events occur at the chloroplast envelope. Duke and Kenyon (1987) have recently proposed a model for DPE action in which the herbicide forms a blue-light absorbing photodynamic complex with a 'carotenoprotein' in the chloroplast envelope, which may be able to initiate lipid peroxidation. This study has not detected peroxidative damage in protoplasts and only after a relatively long period after the cessation of photosynthesis in excised leaves. Thus, our data is supportive of the Duke and Kenyon theory but implies a more primary role on membrane integrity rather than peroxidative damage.

ACKNOWLEGEMENTS

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THE MODE OF ACTION OF THE HERBICIDE WL110547

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ABSTRACT

The herbicidal activity of the experimental compound, WL110547, is associated with bleaching of sensitive plants.

More detailed studies of its mode of action were undertaken to establish:

(i) the precise site of action in the chlorophyll or carotenoid biosynthetic pathway;

(ii) any other sites of inhibition which might contribute to the herbicidal effect;

(iii) the reason for variation in activity among closely related analogues.

In vivo and in vitro studies have established that Z-carotene de-saturase and to a lesser extent phytoene desaturase are inhibited, an unusual property among bleaching herbicides.

Linoleic acid desaturase is also inhibited but at relatively high concentrations. This is consequently unlikely to be the more important site for herbicidal activity. Other inhibitors of desaturase enzymes in the carotenoid biosynthetic pathway share this property.

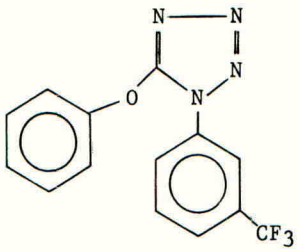
A close analogue of WL110547, WL115531, appears equally active in cell free carotenogenic systems, but is virtually inactive on whole plants. Reasons for this variation have been investigated but a full explanation of the difference has not been obtained.

INTRODUCTION

The experimental herbicide, WL110547 (fig. 1), has shown promise for pre and early post-emergence use in cereal crops. Its herbicidal action is characterised by bleaching of new tissue followed by necrosis. To assist in the optimisation of this compound, we attempted to answer a number of questions:-

a) What type of bleaching is this?
b) What is the precise site of the bleaching action?
c) Are there any additional sites of action?
d) Why are close analogues so much less active?

Fig. 1



WL110547

MATERIALS AND METHODS

Plant material

Barnyard grass seedlings were grown on moist filter paper in covered dishes at 25°C under artificial lights. Capsicum annuum fruits were purchased whilst green and allowed to ripen at 25°C under fluorescent lights (300W/m^2).

Chlorophyll

This was determined from the absorbance of ethanolic extracts at 652nm (Arnon, 1949).

Carotenoids

After saponification of the ethanolic extracts, the carotenoids were extracted into petrol (100-120°C BP) and estimated from their absorbance at 450nm (Tomes, 1963).

Carotenoid Biosynthesis

Incorporation of ^{14}C labelled isopentenyl pyrophosphate into coloured carotenoids was carried out by chromoplasts isolated from ripening fruits of Capsicum annuum (Camara, et al, 1983).

Fatty acid desaturation

Lipids were extracted from plant material (Bligh and Dyer, 1959) and the linoleic and linolenic acid content was estimated by capillary GC.

Catalase and glycolate oxidase

These enzymes were isolated and assayed by the methods of Feierabend and Kemmerich (1983).

Nitrite

This was extracted and assayed by the method of Genichi, et al (1983).

^{14}C labelled compounds

WL110547 (13.6Ci/mole) and WL115531 (16.5Ci/mole) labelled in the phenolic moiety were synthesised at SRC by Dr AN Wright.

TLC analysis

^{14}C labelled compounds extracted from treated plants were separated on silica gel TLC plates developed in chloroform. Radioactive spots were located using a spark chamber, scraped off, and quantified by liquid scintillation counting.

Total ^{14}C content

^{14}C content of treated plant material was estimated by tissue oxidation followed by liquid scintillation counting of the $^{14}CO_2$ evolved.

RESULTS AND DISCUSSION

a) Bleaching of plant tissue may be effected in three ways:-

1) Chlorophyll biosynthesis may be inhibited.

2) Carotenoid biosynthesis may be inhibited, leaving the chlorophyll susceptible to photobleaching.

3) Some form of photoactivation of the herbicide may lead to production of reduced oxygen species or other free radicals which destroy existing pigments.

Treatment of barnyard grass seedlings with WL110547 under low light (.05W/m^2) had little effect on chlorophyll levels but reduced total coloured carotenoids (Table I). Under these conditions, there would be insufficient light to cause photobleaching or photoactivation and so inhibition of carotenoid biosynthesis 2 was indicated.

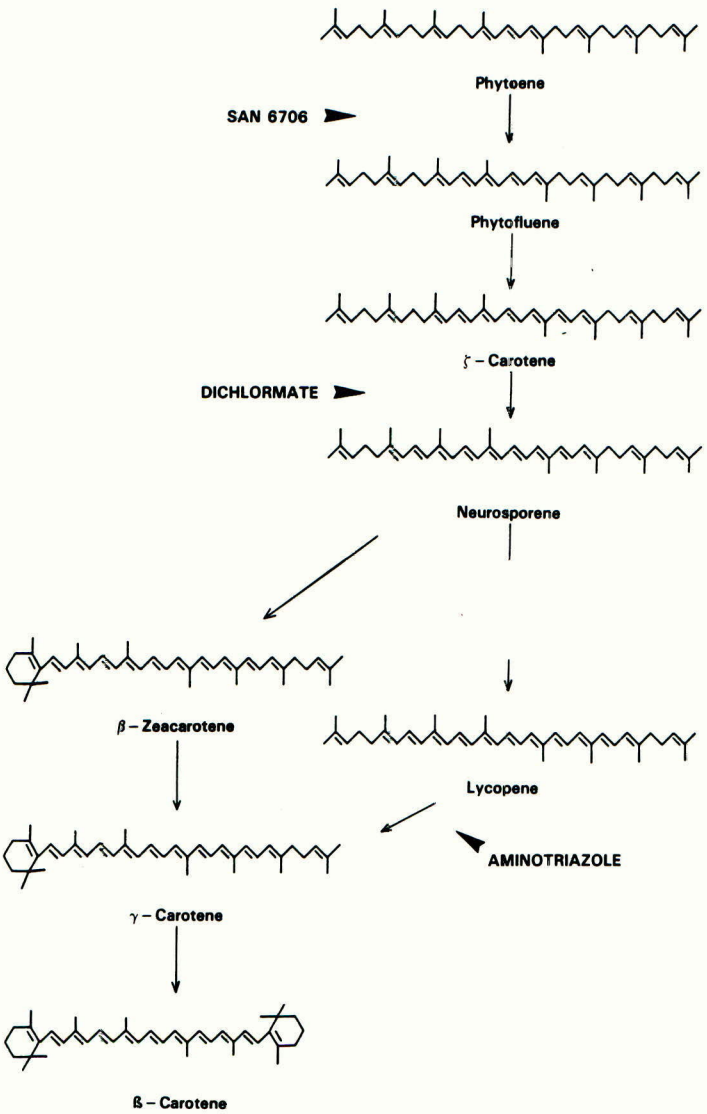
Table I

Effect of WL110547 (10^{-4}M) on pigment content of barnyard grass seedlings after 5 days in low light (0.05W/m^2).

	(average of 2 replicates) chlorophyll (μg/g)	total carotenoids (μg/g)
control	63	11.3
WL110547	69	7.1

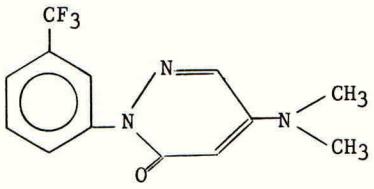
b) The later part of the carotenoid biosynthesis pathway is shown in Fig. 2, together with the sites of action of some well known bleaching herbicides. By far the most common site of action is phytoene desaturase as exemplified by SAN6706 (metflurazon) (Fig. 3).

Fig. 2



Biosynthetic routes for carotenes

Fig. 3



SAN6706

A comparison of the effects of WL110547 and SAN6706 on barnyard grass seedlings showed some differences (Table II). Under low light both compounds caused accumulation of phytoene but WL110547 also caused Z-carotene to accumulate. At higher light intensities, this effect was less clear because the coloured carotenoids were bleached but the loss of chlorophyll by photobleaching was clearly seen.

Table II

Effect of light intensity on pigments in treated barnyard grass seedlings

	% of control values (average of 2 replicates)				
	chlorophyll	phytoene	Z-carotene	β-carotene	lycopene
low (.05W/m^2)					
WL110547.10^{-5}M	97	763	457	38	71
SAN 6705.10^{-5}M	41	1282	12	1	3
moderate (1W/m^2)					
WL110547.10^{-5}M	27	264	135	25	60
SAN6706.10^{-5}M	1	165	5	0	1
high (300W/m^2)					
WL110547.10^{-5}M	29	88	49	23	62
SAN6706.10^{-5}M	1	87	7	0.5	3

SAN6706 is a more active inhibitor of phytoene desaturase and so the absence of Z-carotene accumulation with this compound may have been the result of a total block at the earlier step. At lower SAN6706 and higher WL110547 concentrations, however, the difference was still apparent (Table 3). Thus, WL110547 seems to have a double site of action which is unusual, but not unique.

Table III

Effects of WL110547 and SAN6706 on pigment content of barnyard grass seedlings grown in low light

	% of control values (average of 2 replicates)				
	chlorophyll	phytoene	Z-carotene	β-carotene	lycopene
WL110547					
10^{-5}M	63	230	561	48	82
3 x 10^{-5}M	82	366	918	48	80
SAN6706					
10^{-5}M	67	348	99	39	41
3 x 10^{-6}M	73	447	63	20	14

Using a cell-free carotenogenic system from red peppers, both compounds caused a buildup of phytoene with no evidence of increased Z-carotene. This may be a function of the much shorter incubation time (30 mins. vs. 4-5 days) whereby there was no opportunity for intermediates to leak past the first block.

c) Although WL110547 was some ten fold less active than SAN6706 in inhibiting carotenoid biosynthesis, it was more active herbicidally. The possibility of an additional site of action where it was more active than SAN6706 was therefore explored.

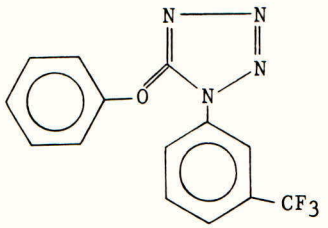
Peroxisomal enzymes such as catalase and glycolate oxidase (Feierabend and Kemmerich, 1983) and also nitrate reductase (Genichi, et al, 1983) are reported to be affected by various bleaching herbicides but WL110547 had no effect on these. Neither did it inhibit photosynthetic electron transport or CO_2 fixation (data not presented).

WL110547 did inhibit fatty acid desaturase activity as exemplified by the conversion of linoleic to linolenic acid (I_{50} = 18uM) but was less active in this system than against carotenoid biosynthesis (I_{50} = 3.5uM).

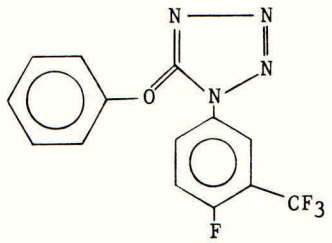
SAN6706 also inhibits fatty acid desaturase (St John, 1976) and is more active (I_{50} = 3μM) than WL110547. Therefore, although this site of action may contribute to the phytotoxic effects of WL110547, it does not explain its greater herbicidal activity than SAN6706. This may be the result of an unknown site of action or superior persistence or distribution in the target plant.

d) A number of close analogues of WL110547 show extremely low phytotoxicity. For example, theoretical calculations show that WL115531 (Fig. 4) is very similar to WL110547 in physical and chemical properties. It is, however, much less active in the herbicide primary screening tests (Table IV). This makes it very difficult to carry out any predictive structure:activity relationship studies. The reason for the low activity of WL115531 was therefore investigated.

Fig. 4



WL110547



WL115531

Table IV

Activity of WL110547 and WL115531 in the herbicide primary screen test at 1kg/ha

(0 = no effect, 9 = total kill)

	maize	rice	barnyard grass	oat	linseed	mustard	sugar-beet	soya
Foliar spray								
WL110547	3	2	7	6	8	8	8	4
WL115531	2	1	2	0	1	2	3	4
Pre-emergence								
WL110547	3	2	9	6	5	7	9	1
WL115531	0	0	0	0	0	0	0	0

Using the cell-free carotenogenic system, WL115531 was shown to be just as active as WL110547. It was argued, therefore, that the difference may reside in:-

a) rates of uptake;
b) rates and pattern of translocation;
c) differential metabolism.

1) Barnyard grass seeds were germinated in the presence of ^{14}C labelled herbicide at 100um in closed vessels in moderate light. Tissue samples were removed at various times up to 6 days and the total ^{14}C content estimated. The seedlings treated with WL110547 were completely bleached whilst those treated with WL115331 were completely green. There was a slightly greater uptake (1.5-2.0 fold) of WL110547 at all times. If the herbicide concentrations were adjusted so that more WL115531 than WL110547 was taken up (Table V), the difference in effect was still apparent.

Table V

Uptake of ^{14}C-labelled WL110547 and WL115531 by barnyard grass seedlings after 6 days in moderate light

	Uptake of ^{14}C (n moles/g. single rep.)	Appearance of seedlings
WL110547		
$5 \times 10^{-5}M$	28.1	very pale green
$10^{-4}M$	42.3	white
WL115531		
$10^{-4}M$	26.2	green
$2 \times 10^{-4}M$	82.7	green

2) Estimates of the distribution of ^{14}C between roots, shoots and seeds of treated barnyard grass seedlings showed little difference between the two compounds (Table VI), although there was again, a slightly greater uptake of WL110547.

Table VI

Distribution of ^{14}C-labelled WL110547 and WL115531 in barnyard grass seedlings after 6 days in moderate light

	% of total ^{14}C taken up (average of 3 reps.)		
	leaf	seed	root
WL110547			
$10^{-4}M$	10	80	10
WL115531			
$10^{-4}M$	12	79	9

3) Extraction and analysis of the ^{14}C labelled components in the treated barnyard grass seedlings showed no detectable breakdown after 3 days. After 6 days, approximately 10% conversion to more polar metabolites was seen, but with little difference between the two compounds (Table VII).

Table VII

Metabolism of ^{14}C-labelled WL110547 and WL115531 in barnyard grass seedlings after 6 days in moderate light

	% of total ^{14}C taken up (single reps.)	
	parent compound	polar metabolites
WL110547		
$5 \times 10^{-4}M$	92	8
$10^{-4}M$	92	8
WL115531		
$10^{-4}M$	89	11
$2 \times 10^{-4}M$	92	8

The reason for the low activity of WL115531 remains elusive unless the carotenogenic system of barnyard grass is very different from that of red peppers.

CONCLUSIONS

Some progress has been made towards understanding the mode of action of WL110547 and this has been helpful to the analogue synthesis programme. Some questions remain unanswered but perhaps it would be naive to hope that all the properties of a herbicide could be explained on the basis of its activity at a single site.

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THE MODE OF ACTION OF DIFLUFENICAN: ITS EVALUATION BY HPLC

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ABSTRACT

The mode of action of diflufenican has been investigated by hplc analysis of pigment extracts of treated plants. The main effect is confirmed as an inhibition of the desaturation reaction of carotenoid biosynthesis, causing the accumulation of phytoene in place of the normal coloured carotenoids. Hydroxy-derivatives of phytoene (and phytofluene) were also present. These may be important in relation to the mechanism of the desaturation reactions. The appearance of β-carotene-5,6-epoxide and a large amount of zeaxanthin suggests that diflufenican also has a minor, photooxidative action. Phytoene was present in all chloroplast pigment-protein complexes, but hydroxyphytoene and zeaxanthin were found only in the free pigment fraction. This information was obtained in only a few days, showing the power of this procedure as a screening method for evaluating the mode of action of potential bleaching herbicides.

INTRODUCTION

Chloroplast carotenoids, especially β-carotene, are essential for protecting plants against the chlorophyll-sensitized photoproduction of singlet oxygen, $^1O_2$, a highly reactive species that can rapidly cause tissue damage (Siefermann-Harms, 1987). Any compound which intereferes with this protective mechanism is therefore potentially useful as a bleaching herbicide. It is already known that many structurally unrelated herbicides cause either white or yellow chlorosis of leaves as a consequence of the total or partial absence of the normal chloroplast pigments, i.e. chlorophylls and carotenoids. These bleaching herbicides can act in two ways, namely by inhibiting the biosynthesis of the carotenoids that are required for photoprotection in the chloroplast pigment-protein complexes (PPC) or by causing the destruction of existing pigments in the PPC following a primary inhibitory action on photosynthetic electron transport.

It is therefore of major importance to have a rapid, reliable and informative screening procedure for elucidating the mode of action of newly synthesized chemicals which cause chlorosis in plants. Much effort is being directed towards the development of model systems, notably algae, plant cell cultures and carotenogenic enzyme preparations from fungi, etc. for use in the evaluation of potential herbicides (Sandmann et al., 1984). We have used an alternative strategy to identify the effects of these compounds directly on the plants. This can be achieved simply by the proper application and interpretation of hplc analysis of chloroplast pigments. This method will immediately distinguish between compounds which act by inhibiting carotenoid biosynthesis and those which interfere with photosynthetic electron transport in a way that leads to destruction of chloroplast pigments. But the method does much more than this. Although we have not yet been able to explore and realize its full potential, it is already clear that this procedure can give information about many aspects of herbicide action, including the site and specificity of inhibition of the biosynthetic pathway, detection of multiple actions, comparison of effects on different plants or of different treatments or doses, specific effects on individual PPC. It also provides a means to study recovery and resistance. A sound first indication of the mechanism by which a new compound acts can be obtained by comparison with other herbicides whose action is well known.

Some of the capabilities of this method are illustrated by the results of a brief evaluation of the effects of diflufenican in comparison with those of other herbicides, e.g. norflurazon, which are believed to have the same primary mechanism of action, i.e. inhibition of the desaturation reactions in carotenoid biosynthesis.

MATERIALS AND METHODS

The plant used was radish (Raphanus sativus L). As a pre-emergence treatment, seeds were soaked overnight in aqueous acetone solutions of diflufenican at the appropriate concentration (O, 1mM, 100µM, 10µM). The seeds were then sown in soil and grown under continuous light (10000 lux light source) for 6 days. Alternatively, for post-emergence studies, seeds were soaked overnight in water and sown in soil and diflufenican was then applied as a suspension in acetone-water (0.5mM) three days after emergence of the radish cotyledons, which were then grown for a further three days in continuous light, as above.

Pigment extraction

Known amounts of leaf material were homogenized in ethanol, the homogenate was filtered through a cotton wool plug and the solvent evaporated under a stream of N_2. The pigment-containing lipid material in the residue was redissolved in diethyl ether, transferred to a clean vial and again evaporated under N_2 ready for hplc analysis.

Hplc analysis

The sample was dissolved in the hplc eluting solvent (acetonitrile-ethyl acetate) and 20µl of this solution injected onto Zorbax-ODS reversed phase hplc column (5µ, 25x0.46 cm). Elution was achieved by a gradient of ethyl acetate in acetonitrile-water (9:1 containing 0.1% triethylamine) as indicated in the legends to the figures. The hplc system used consisted of Kontron pumps and gradient programmer, with a Hewlett-Packard 1040A diode array detector. Chromatograms were monitored simultaneously at 455, 447, 441, 437, 431, 400, 350 and 287nm, and components were estimated quantitatively by integration at each wavelength and by use of established $A^{1\%}_{1\,cm}$ values (Davies, 1976). Compounds were identified by their retention times and absorption spectra. For novel compounds mass spectra and, if possible, ^1H nmr spectra were also determined.

Thylakoid isolation

Thylakoid membranes were isolated as described by Remy et al., (1977), except that Tris-HCl buffer was used throughout the isolation procedure and the isolated thylakoids were resuspended in 0.1M Tris-glycine (pH 9.0).

Separation of pigment-protein complexes

Pigment-protein complexes were separated by our standard polyacrylamide gel electrophoresis procedure. Thylakoid membranes were solubilized for 30 min. at room temperature in 0.1M Tris-glycine (pH 9.2) containing 0.5% (w/v) SDS, to give a final ratio SDS:chlorophyll of 10:1 (w/w). The chlorophyll-carotenoid-protein complexes were then separated by PAGE in 50mM Tris-glycine, pH 9.0, containing 0.06% SDS (w/v) at 3mA/gel for 30 min. Six pigment-protein complexes, plus a free-pigment zone, were separated by the electrophoresis. The pigments were extracted from each individual complex with ethanol and analysed by hplc.

RESULTS AND DISCUSSION

The uptake, transport and metabolism of diflufenican, and its effects on a number of plant species, have been investigated previously (Wightman and Haynes, 1985). The main mode of action has been shown to be on carotenoid biosynthesis. In the present work, the effects of diflufenican on seedlings of radish (Raphanus sativus L) have been investigated. The untreated plants had a normal distribution of coloured carotenoids i.e. β-carotene (approx 25-30% of total carotenoid), lutein (40-45%), violaxanthin and neoxanthin (each approx. 15%), together with chorophylls a and b (chlorophyll a / chlorophyll b approx. 2.0, carotenoid/chlorophyll approx. 0.45). A typical hplc chromatogram of an extract from control (untreated) leaves is illustrated in Fig.1.

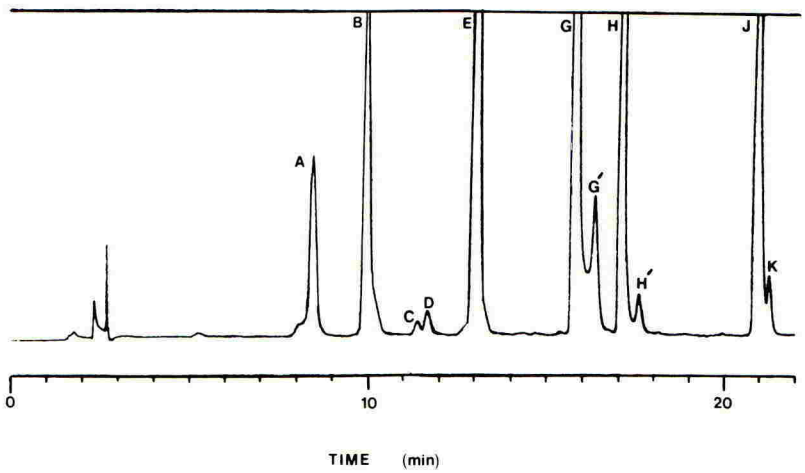

Fig. 1 Reversed-phase hplc chromatogram of an extract from untreated leaves. Monitoring wavelength 445nm. Peak identification: A. Neoxanthin; B. Violaxanthin; C. Lutein-5,6 epoxide; D. Antheraxanthin; E. Lutein; G,G. Chlorophyll b; H,H'. Chlorophyll a; J. β-Carotene; K. cis-β-Carotene.

The effect of diflufenican, applied pre-emergence, was immediately obvious from the reversed-phase hplc chromatograms of the extracts (Fig. 2). The amounts of coloured carotenoids and chlorophylls were greatly reduced, and a substantial amount of the biosynthetic intermediate phytoene was present, thereby confirming that the diflufenican was causing inhibition of carotenoid biosynthesis, in particular blocking the desaturation of phytoene. The chromatographic pattern was easily distinguished from those obtained from plants that had been treated with substances which affect photosynthetic electron transport, such as paraquat and diuron (Fig. 3); in these cases carotenoid levels, especially of β-carotene, were much lower than normal, but no phytoene or other biosynthesis intermediates were present.

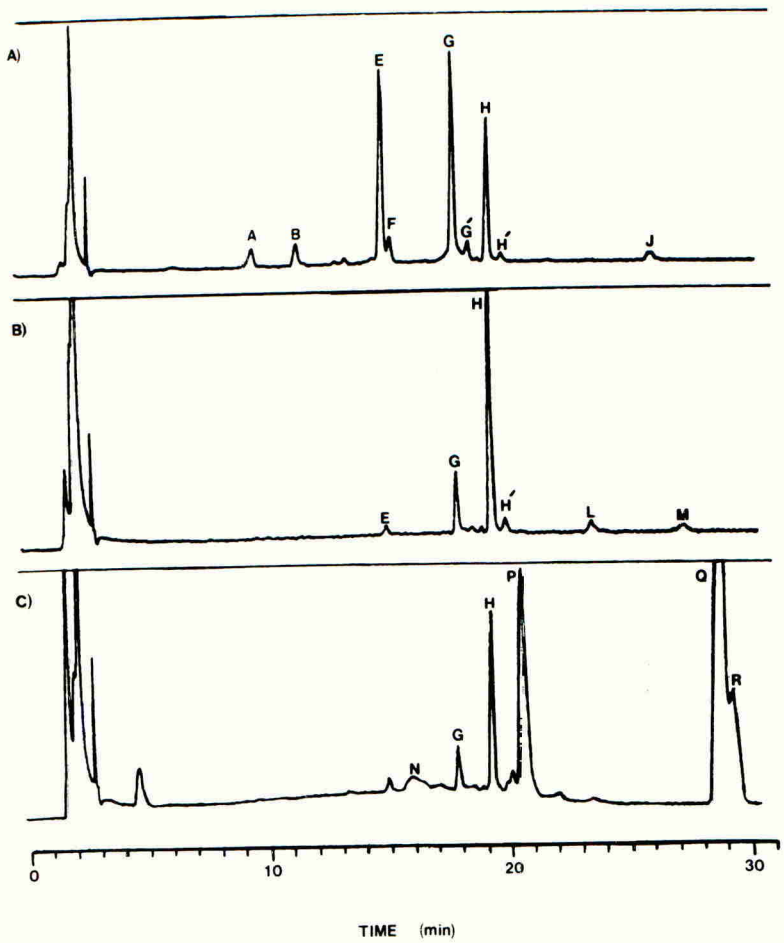

Fig. 2. Reversed-phase hplc chromatogram of diflufenican-treated (pre-emergent) radish. Monitoring at three separate wavelengths a) 445nm, b) 350nm and c) 287nm. Peak identifications: A. Neoxanthin; B. Violaxanthin; C. Lutein; F. Zeaxanthin; G,G'. Chlorophyll b.; H,H'. Chlorophyll a; J. β-Carotene; L. Hydroxy derivative of phytofluene; M. Phytofluene; N. Dihydroxyphytoene; P. Monohydroxyphytoene; Q. 15Z-(cis). Phytoene; R. All-trans phytoene.

The diflufenican-treated plants also contained several other compounds that were not present in the controls, notably ones with absorption spectra identical to those of phytoene and phytofluene. The major one of these compounds has been identified by mass spectrometry and ^{1}H nmr spectroscopy as a hydroxylated derivative of phytoene (probably 12-hydroxyphytoene), and mass spectrometry has also allowed the tentative identification of a dihydroxyphytoene, and a trihydroxy-or dihydroxydidehydro-phytoene, which await full characterization. The phytoene and monohydroxyphytoene were largely the 15Z- (15-cis) isomers. The significance of these hydroxy-phytoenes as possible biosynthetic intermediates remains to be elucidated. It seems likely, however, that they will greatly aid our understanding of the phytoene desaturation reactions.

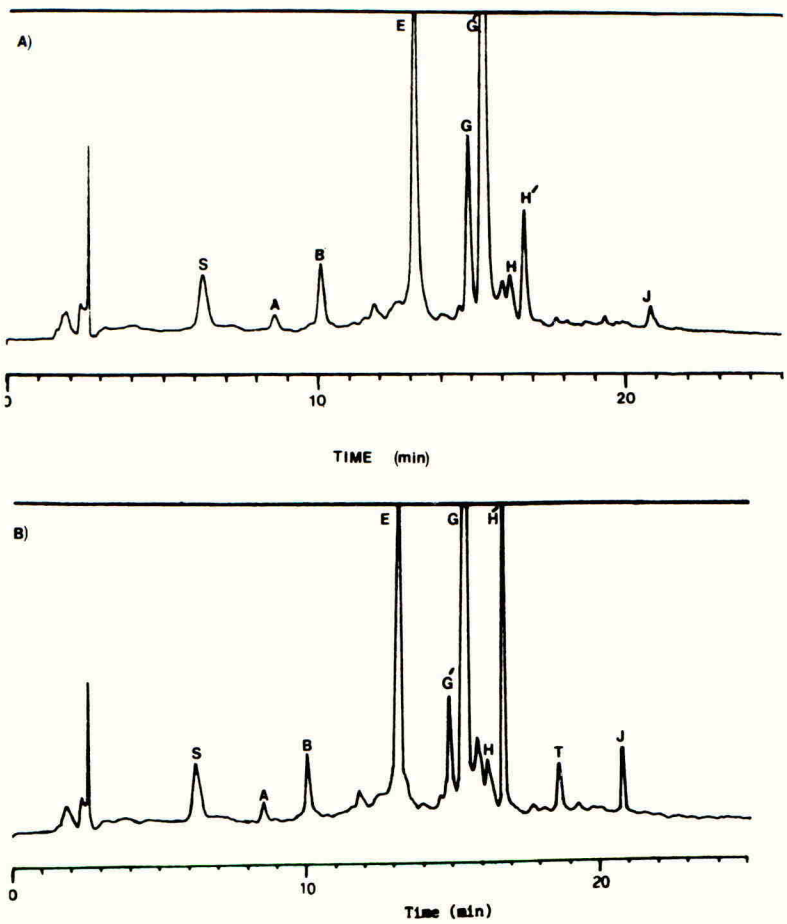

Fig. 3. Reversed-phase hplc chromatogram of barley treated with (a) paraquat and (b) diuron. Monitoring wavelength 445nm. Peak identifications: S. Chlorophyll breakdown product; A. Neoxanthin; B. Violaxanthin; E. Lutein; G,G'. Chlorophyll b; H,H'. Chlorophyll a; T. β-Carotene-5,6-epoxide; J. β-Carotene. Monitoring at 350 nm and 287nm showed that phytoene, phytofluene and their derivatives (compounds L,M,N,P,Q,R) were absent.

Closer examination of the chromatograms showed that, although the pattern of biosynthetic intermediates detected was broadly similar to the pattern obtained for plants that had been treated with other biosynthesis-inhibitor herbicides, e.g. norflurazon, metflurazon, some subtle and possibly diagnostic differences were observed, e.g. in the ratios of cis- to trans-phytoene, phytoene to monohydroxyphytoene or phytoene to phytofluene.

Analysis of plants treated with lower concentrations of diflufenican provided evidence of additional effects on existing pigments. These effects were seen only with diflufenican, not with other carotenogenesis inhibitor herbicides. In

particular, after both pre-emergence and post-emergence treatments and illumination for a few days, small amounts of β-carotene-5,6-epoxide were present (approx. 0.5% of total carotenoid). (Table 1). Our extensive work has shown that this compound is a very sensitive and reliable indicator of the oxidative breakdown of existing β-carotene, the most sensitive carotenoid. Also the 'violaxanthin cycle' (Yamamoto, 1979) appears to be switched on, causing the appearance of abnormally high levels of zeaxanthin and antheraxanthin and a reduction in the violaxanthin content. Both these features are reminiscent of effects of herbicides such as paraquat and diuron which affect photosynthesis and they have not been seen with other inhibitors of carotenogenesis such as norflurazon and metflurazon (data not presented).

TABLE 1.
Comparison of the distribution of carotenoids (expressed as percentage of the total coloured carotenoids) in radish leaves treated, post-em, with diflufenican (DFF) and in untreated leaves (CON).

	β-Car[a]	β-5,6	Lut	Viol	Neo	L-5,6	Anth	Zea	C/X	Non-col	Chla/b
CON	27.8	-[b]	42.5	14.0	12.9	1.9	0.9	-	0.38	-	1.70
DFF	12.4	2.1	51.0	3.2	15.2	1.5	2.5	12.0	0.17	64.0	2.06

[a]Abbreviations: β-Car - β-carotene; β-5,6 - β-carotene-5,6-epoxide; Lut -lutein; Viol - violaxanthin; Neo - neoxanthin; L-5,6 - lutein-5,6-epoxide; Anth - antheraxanthin; Zea - zeaxanthin; C/X - ratio of carotene : total xanthophyll; Non-col - relative amount of colourless carotenoids (phytoene, hydroxyphytoene, etc.) as a percentage of the total; Chl a/b - ratio of chlorophyll a : chlorophyll b.
[b] - indicates below the limit of detection.

The power and scope of this screening procedure become clear when it is realised that the above information about the action of diflufenican was obtained in only a few hours. One analysis is sufficient to give a good indication of the mode of action of any herbicide. This takes only about one hour, including pigment extraction, hplc, and spectral evaluation; several plant species, herbicide concentrations etc. can therefore be examined in a day.

Obviously, for any herbicide a survey of this kind can easily be broadened to investigate, for example, effects on a range of plant species and varieties, plants of different ages and under widely differing growth conditions and also used to look in much more detail at specific features of the inhibition. Thus, over a period of about 5 weeks, much additional information has been obtained about the action of diflufenican. Pre-emergence and post-emergence treatments have been compared and time courses of effects following the application of different herbicide concentrations have been determined. Chloroplasts, thylakoids and individual PPC have been isolated and analysed in an attempt to localize the action (Table 2). The inhibition appears not to be restricted to any particular PPC or carotenoid, although β-carotene was more strongly affected than were the xanthophylls. Appreciable amounts of phytoene were present in all the sub-chloroplast PPC, but hydroxyphytoene was found only in the free pigment fraction. The zeaxanthin that accumulated was also found only in the free pigment. β-Carotene-5,6-epoxide, however, like β-carotene, was localized mainly in the CP1 and CP1a complexes.

TABLE 2.
Percentage distribution of each carotenoid in the chloroplast pigment-protein complexes of untreated radish leaves (CONTROL) and leaves treated, post-em, with diflufenican (DFF).

	CONTROL					DFF			
	CPI/la	LHCPI	CPa/LH2	LHCP3	FP[c]	CPI/la	LHCPI	LHCP3	FP
β-Cara	61.4	8.9	9.3	7.7	12.8	55.9	9.3	12.0	22.8
β-5,6	59.5	6.5	10.2	-	23.8	41.5	12.8	19.0	26.7
Lut	7.5	18.8	5.2	49.1	19.3	12.4	25.7	40.8	21.1
Viol	6.9	7.2	2.1	18.9	64.9	6.6	10.5	26.8	41.4
Neo	3.8	21.6	5.1	62.2	7.3	11.3	30.3	48.6	9.8
L-5,6	11.1	11.4	3.1	25.3	49.1	9.7	13.9	33.9	42.4
Anth	-	-	-	-	-	-	-	41.8	58.2
Zea	-	-	-	-	-	-	-	-	100
Phyt	-	-	-	-	-	18.0	11.2	22.6	45.1
HO-phyt	-	-	-	-	-	-	-	-	100
C/X	2.92	0.17	0.68	0.05	0.16	0.96	0.08	0.06	0.19
Non-col	-	-	-	-	-	19.2	11.2	13.3	35.7
Chla/b	15.08	1.71	3.95	1.46	5.66	5.46	1.57	1.77	3.64

[a]Abbreviations : as in Table l; Phyt - phytoene; HO-phyt - hydroxyphytoene
[b]CPa/LH2 (=CPa/LHCP2) was not detected for the diflufenican - treated plants
[c]FP - Free pigment zone.

CONCLUSIONS

Quite clearly this procedure provides a very powerful means of evaluating bleaching herbicides. In particular it has the potential (i) to distinguish immediately between biosynthesis inhibitors and those which cause reduction in carotenoid levels as a consequence of inhibition of photosynthetic electron transport, (ii) to reveal multiple modes of action and (iii) to detect reliably some features which may be quantitatively minor but which can identify differences or confirm similarities in the action of different compounds which produce the same gross effects. The method is applicable to any potential bleaching herbicide and has the advantages of speed and the amount of information that can be generated, both in terms of breadth and also details of very specific features. It also gives information directly about effects on the plants themselves rather than simply on model systems, and is applicable to any plant species.

When the method has been applied to a wider range of herbicidal compounds, the data base provided will permit deductions to be made about the mechanism of action of any bleaching compound under investigation.

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