NOTTINGHAM POLYTECHNIC
CLIFTON CAMPUS
CLIFTON LANE
NOTTINGHAM NG11 8NS

NOTTINGHAM POLYTECHNIC
CLIFTON CAMPUS
CLIFTON LANE
NOTTINGHAM NG11 8NS

AN INVESTIGATION INTO THE MODE OF ACTION OF THE HERBICIDE M&B 39279.

By

PETER MICHAEL DERRICK

Trent Polytechnic

in collaboration with Rhone - Poulenc Agriculture

A Thesis submitted to the Council for National Academic Awards in partial fulfilment of the requirements of the degree of Doctor of Philosophy. Submitted July 1989.

DECLARATIONS

The author has not been a registered candidate nor an enrolled student for another award of the CNAA or other academic or professional institution during the research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the author's individual contribution (with the exception of data depicted by Figures 60 and 61). The author has attended appropriate lectures, seminars, conferences and visits to other laboratories in partial fulfilment of the requirements of the degree.

Signed P.M. Dent.

(Candidate)

Signed J.A.M. COBB

(Director of Studies)

A C K N O W L E D G M E N T S

I wish very much to thank Prof Andy Cobb and Dr Ken Pallett for obtaining funding for this project, for their supervision during the course of the work and their advice during preparation of this thesis. I would also like to use this opportunity to acknowledge the excellent quality of their teaching during my undergraduate days at Trent Polytechnic which provided me with a sound basis for research in plant sciences.

I would also like to thank the research staff at Trent for their friendship while sharing the laboratories (and coffee room !), especially Lorna, Paul, Pete, Penny, Gerry, Gina, Jane, Richard, Mike, Chop, Linda and Debbie.

I am also indebted to the Scottish Crop Research Institute, Dundee, for the use of computing and printing facilities during preparation of this thesis and to Jackie for help with typing.

I would lastly very much like to thank Anne for being very patient with me during writing of this thesis - I can come out to play now.

This thesis is based on work made possible by a grant from Rhone - Poulenc Agriculture (formerly May and Baker Agrochemicals), Ongar, Essex, U.K.

Publications arising from this study

P.M.DERRICK, A.H.COBB and K.E.PALLETT (1987) The effects of acifluorfen on membrane integrity in *Galium aparine* leaves and protoplasts. Proceedings, British Crop Protection Conference-Weeds (1987) pp 997-1004

P.M.DERRICK, A.H.COBB and K.E.PALLETT (1988) Ultrastructural effects of the diphenyl ether herbicide acifluorfen and the experimental herbicide M&B 39279. Pesticide Biochemistry and Physiology 32 153-163

Copies of these papers are held in the pocket at the back of this thesis.

C O N T E N T S

L I S T O F I L L U S T R A T I O N S

Figures

Tables

AN INVESTIGATION INTO THE MODE OF ACTION OF THE HERBICIDE M&B 39279

By Peter Michael DERRICK

ABSTRACT

This thesis describes an investigation into the mode of action of the novel herbicide M&B 39279 in two target weed species.

The herbicide acifluorfen was investigated alongside M&B 39279 in experiments to enable comparison of the activity of M&B 39279 with acifluorfen, a nitrodiphenylether herbicide which generates similar toxic symptoms in plants to those induced by M&B 39279. These two herbicides behaved indistinguishably throughout the study. It was proposed that these compounds share a common mode of action.

Both herbicides were shown to be light dependent, contact herbicides which caused necrosis of plant leaves. These toxicity symptoms were not accompanied by any changes in primary metabolism beyond those expected of moribund plants.

Blue and red light were shown to be the most active regions of the visible spectrum with these herbicides and low light intensities were sufficient to mediate their toxicity.

Diuron suppressed activity of M&B 39279 and acifluorfen, although these compounds had no significant direct effect on thylakoid electron transfer and photophosphorylation.

The sequence of effects of M&B 39279 and acifluorfen on subcellular morphology was established by electron microscopy. Vesiculisation of chloroplast envelopes was the most rapid symptom of damage on an ultrastructural level in Galium aparine. Ultrastructural changes were accompanied by a decline in photosynthetic capability, loss of chlorophyll, leakage of electrolytes and malondialdehyde in leaflets maintained under identical conditions implying the occurrence of a peroxidative destruction of membrane lipids.

A decline in photosynthesis was the most sensitive response of excised G.aparine leaves and protoplasts to M&B 39279 and acifluorfen. Although photosynthesis declined after a short time lag, it preceeded evolution of short chain hydrocarbons by at least four hours in protoplasts.

Evidence is presented to support recent proposals suggesting that acifluorfen and M&B 39279 act via inhibition of protoporphyrinogen oxidase leading to an accumulation of the photosensitiser protoporphyrin IX, and thereby initiating lethal lipid peroxidation.

C H A P T E R I

GENERAL INTRODUCTION

1.1 THE RELEVANCE OF HERBICIDE MODE OF ACTION STUDIES IN AGRICULTURE AND THE PLANT SCIENCES

This thesis records the findings of an investigation into the mode of action of a novel potential agricultural herbicide. The compound in question, M&B 39279, is a representative of many thousands of such compounds generated annually by chemical companies. New, potentially herbicidal compounds are screened for activity on a wide range of crop and weed species. These glasshouse - based trials indicate (a) any herbicidal potential and (b) any selective properties of new compounds. This process is used to screen tens of thousands of new compounds annually by agrochemical companies in attempt to discover new herbicides. Following preliminary selection of potentially useful compounds, laboratory, glasshouse and field trials are conducted to evaluate efficacy, selectivity, toxicity and environmental fate. Studies into the mode of action of novel herbicides generally take low priority and until recently, no novel herbicides have been developed following a study of potential herbicidal targets. Rebeiz et al (1984, 1987, 1988) however, described and successfully developed a herbicidal system utilising a natural plant constituent (5-aminolaevulinic acid) in conjuction with synthetic stimulators of porphyrin biosynthesis (metal chelators). This combination resulted in the generation of photosensitising chlorophyll precursors in treated plants. By altering the formulation of this herbicide, different selective properties were obtained. Although 'designed' herbicides such as this have not been previously derived, there is potential in following a study of herbicide mode of action to design further and more effective compounds which may act on the same biochemical target. Studies of structure - activity relationships

amongst chemical analogues of novel herbicides are, however, cheaper and more effective than a study of the mode of action. Mode of action studies can, however, provide information which is useful to agrochemical / plant breeding / plant physiological institutions. For example, the discovery of enzyme targets for certain herbicides, herbicide detoxification systems and the genetics involved in these systems has yielded a variety of marker genes which have been introduced into and used as a means to select plant genetic transformants eg. the 5-enol-pyruvyl- shikimyl-3- phosphate synthase, acetolactate synthase, bromoxynil nitrilase and 2,4-D monoxygenase genes (see Ratner, 1989). Herbicide tolerant plants can also be produced by such means. Furthermore, studies into the mode of action of herbicides can provide impetus for research into poorly understood areas of plant metabolism.

Herbicides currently in use have a wide variety of modes of action, a detailed description of which is beyond the scope of this thesis. A comprehensive review has been published by Fedtke (1983). Considerable advances have been made in several areas of herbicide mode of action research since publication of Fedtke's book and more recent, smaller reviews and papers should be consulted. Kishore and Shah (1988) for example, described recent advances in research into the modes of action of herbicidal inhibitors of amino acid biosynthesis ie. glyphosate, the sulphonylureas and phosphinothricin, a broad group of compounds which have recently become widely used in U.K. agriculture. The most intensively studied mode of herbicidal action to date however, has been the inhibition of photosynthetic electron transfer by a large group of compounds including the substituted ureas, triazines, uracils and hydroxybenzonitriles. These herbicides bind to a site on a 32kd polypeptide constituent of

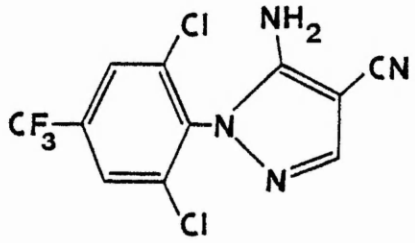
photosystem II (the so - called D1 protein), to which also binds bicarbonate and plastoquinone. The result of herbicide binding to this protein is a disruption of thylakoid electron transfer and subsequent oxidative damage. Also dependent on chloroplast electron transfer for activity are the bipyridyl herbicides which accept electrons from photosystem II and initiate lipid peroxidation following re - oxidation of the reduced herbicide. A very large group of herbicides including 2,4-D, mecoprop and fluroxypyr appear to mimic natural auxin plant growth regulators (2,4-D is widely used as a synthetic auxin in in vitro plant tissue culture). How these compounds act herbicidally however, is not well understood, research being limited by uncertainty regarding the mode of action of natural auxins. A further, widely used group of herbicides typified by norflurazon, difunon and diflufenican, act by inhibiting isoprenoid desaturation, thereby disrupting carotenoid biosynthesis. Other targets for various herbicides include fatty acid synthesis (eg. diclofop and fluazifop) and microtubule synthesis and organisation (eg. trifluralin).

1.2 RATIONALE AND OBJECTIVES OF THIS STUDY

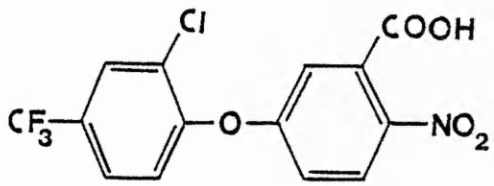
This study was initiated to investigate the biological activity in plants of 5-amino-4-cyano-(2,6-dichloro-4-trifluoromethylphenyl) pyrazole (Fig. 1), a compound developed by May and Baker Agrochemicals (now Rhone - Poulenc Agriculture) as a potential new selective herbicide and designated M&B 39279.

Preliminary studies into the nature of the herbicidal properties of M&B 39279 were carried out by May and Baker Agrochemicals and by Dr K.E. Pallett at Trent Polytechnic (unpublished) prior to commencing this study. This work revealed a number of similarities between the activity of M&B 39279 and properties typical of nitro - diphenylether (DPE) herbicides. It was proposed from this that M&B 39279 and diphenylether herbicides may share a common mode of action in plants and consequently, the DPE herbicide acifluorfen (Fig. 1) was incorporated into all investigations herein, allowing comparisons to be made.

In summary, the objectives of this study were the following: (1) To gain insight into the mode of action of M&B 39279. (2) To compare the activity of M&B 39279 with that of the DPE compound acifluorfen. (3) To investigate some herbicidal properties of DPE herbicides in general.

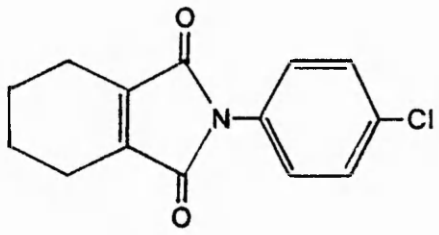


M&B 39 279

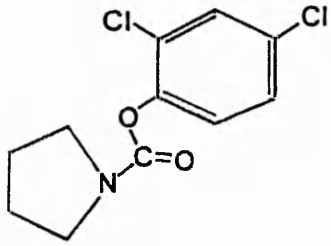


ACIFLUORFEN

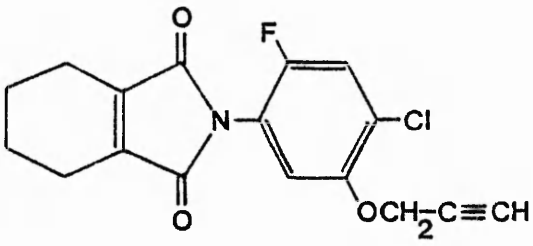
Figure 1. Structures of herbicides investigated in this study.



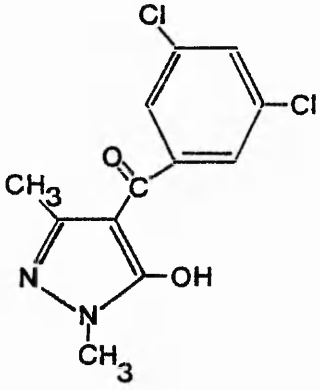
Chloropthalim (MK 616)
(Wakabayashi et al, 1978)



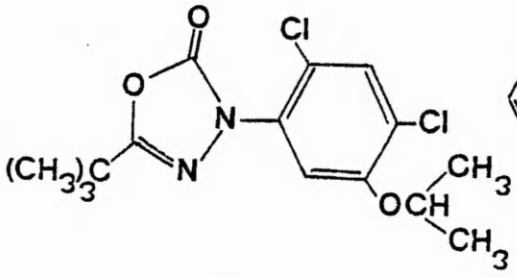
Phenopylate
(Wakabayashi et al, 1986)



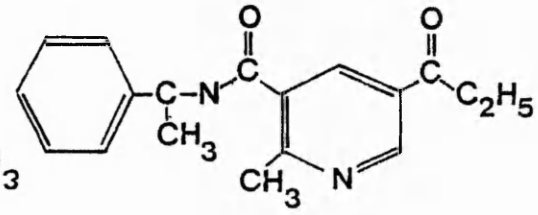
S-23142
(Sato et al, 1987)



Pyrazolate (DTP)
(Wakabayashi et al, 1986)



Oxadiazon



LS 82-556
(Matringe et al, 1986)

Figure 2. Structures of non - phenoxybenzyl DPE - Type compounds.

1.3 SUMMARY OF PUBLISHED LITERATURE ON THE MODE OF ACTION OF DIPHENYLETHER HERBICIDES.

1.3.1 FOREWORD

The ensuing overview is a summary of published literature surrounding the mode of action of diphenylether (DPE) type herbicides, ie. the research relevant to the mode of action of acifluorfen and that thought likely to be relevant to the mode of action of M&B 39279. Before considering this literature summary as a part of this thesis, however, several points are relevant to the reader. Firstly, although the original research described herein was carried out between September 1984 and September 1987, the literature summary is based on a comprehensive and to the best of my knowledge, complete survey of relevant material published before June 1989. It should be borne in mind, therefore, that a substantial proportion of the following literature citations are of papers which were not available during design, planning and execution of experiments described in Chapters 2 to 5. The following literature summary should therefore, be regarded in the context of this thesis being both a contribution to the literature in this field, an illustration of the development of the literature and clearly, more than the basis for experimental design. Secondly, the purpose of the following literature summary is descriptive rather than interpretative (although a degree of interpretation is inevitable during collation of published work). The literature will be discussed alongside original data in the final chapter of this thesis. Thirdly, it is shown both by evidence presented in this thesis and literature published elsewhere, that the mode of action of DPE's appears to be shared by numerous compounds

which are structurally distinct from DPEs as depicted in table 1 (see section 1.3.4 and Fig. 2). The term "DPE-type" is consequently used throughout this thesis to refer collectively to nitro-DPE's and non-phenoxybenzyl compounds having an apparently similar mode of action. This approach and terminology will be seen to be justified upon further reading of this thesis.

1.3.2 USES OF HERBICIDAL DIPHENYLETHERS (DPE's)

Nitrofen, introduced around 1964 (Fryer and Makepeace, 1977), was the first commercially available herbicidal DPE and its introduction was followed by the development of further similar compounds, notably chlornitrofen, bifenox, oxyfluorfen and acifluorfen (Table 1). Nitrofen and chlornitrofen were introduced as broad spectrum herbicides for the control of dicotyledonous and graminaceous weeds, particularly barnyardgrass (Echinochloa crus-galli) in rice fields. The more recently developed DPE's however, are used in a wide variety of crop situations such as cereals, certain vegetables, cotton, ornamentals and large seeded legumes (Richardson, 1984) as listed in Table 2. The DPE's are very effective in controlling many weeds which are important in the soybean and cotton growing regions of the United States, such as morningglory (Ipomoea spp), redroot pigweed (Amaranthus retroflexus), velvetleaf (Abutilon theophrasti), common cockleburr (Xanthium strumarium) and jimsonweed (Datura stramonium) (Mangeot et al, 1977 ; Lee and Oliver, 1982 ; Godley and Kitchen, 1986 ; Worthing, 1987 and Higgins et al, 1988). As many of the crops in which DPE's are used include several major world crops (soybeans, maize, rice, groundnuts) and crops of high value (eg. cotton), the DPE's have become a commercially important group of compounds.

SUBSTITUENT				HERBICIDE NAME
R1	R2	R3	R4	
Cl	Cl	H	H	Nitrofen
Cl	Cl	H	Cl	Chlornitrofen (CNP)
CF_3	Cl	H	H	Nitrofluorfen
CF_3	Cl	COOH	H	Acifluorfen
CF_3	Cl	COONa	H	Acifluorfen-sodium
CF_3	Cl	$COOCH_3$	H	Acifluorfen-methyl
Cl	Cl	$COOCH_3$	H	Bifenox
Cl	Cl	OCH_3	H	Chlomethoxynil
CF_3	Cl	OC_2H_5	H	Oxyfluorfen
CF_3	NO_2	H	H	Fluorodifen
CF_3	Cl	$CONHSO_2CH_3$	H	Fomesafen

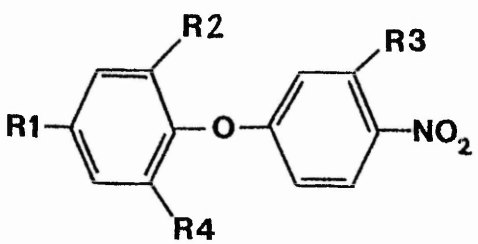


TABLE 1. DIPHENYLETHER STRUCTURES

HERBICIDE	CROPS	REFERENCES
nitrofen	brassicas rice	Hawton and Stobbe, 1971; Matsunaka, 1969; Fryer and Makepeace, 1977; Hyakutake and Ishizuka, 1975
chlornitrofen	rice	(as for nitrofen)
fluorodifen	rice soybeans	Fryer and Makepeace, 1977 Pollak and Crabtree, 1976
oxyfluorfen	soybeans	Oakes et al, 1981 Worthing, 1987
	Groundnuts cotton sugarcane onions	Worthing, 1987
bifenox	soybeans rice maize	Leather and Foy, 1978
	sorghum	Worthing, 1987
acifluorfen	soybeans	Mangeot et al, 1977; Yih et al, 1978; Oakes et al, 1981; Ritter and Coble, 1981; Lee and Oliver, 1982; Worthing, 1987
	rice groundnuts wheat	Lee and Oliver, 1982
Fomesafen	soybeans	Higgins et al, 1988

TABLE 2. USES OF DIPHENYLETHERS

1.3.3 SYMPTOMS OF DIPHENYLETHER TOXICITY

DPE's are contact herbicides and their application to susceptible plants rapidly induces the appearance of pitted water - soaked patches on the foliage (Gorske and Hopen, 1983; Komives and Casida, 1983). Such leaves wilt, become desiccated, chlorotic / bleached and necrotic (Bowyer et al, 1987b; Duke and Kenyon, 1987; Halling and Peters, 1987).

Physiologically, Orr and Hess (1981a and b, 1982a) described the initial response to DPE's as a general increase in membrane permeability on the basis of leakage of $^{86}Rb^{+}$, $^{36}Cl^{-}$, $^{45}Ca^{2+}$, 3-O-methyl - [^{14}C]-glucose and ^{14}C-methylamine from pre-loaded cucumber cotyledons treated with acifluorfen - methyl. Other symptoms elicited by DPE's include stimulation of ethylene synthesis (Bugg et al, 1980), promotion of phenylalanine ammonia lyase activity (Komives and Casida, 1983) and an increase in chlorophyll precursor content (Matringe and Scalla, 1987a, 1988b; Witkowski and Halling, 1988). Boger, (1984) listed effects of DPE's on treated plants as inhibition of photosynthetic electron transfer, carotenogenesis, ATP synthase, and respiratory electron transport, a stress response and lipid peroxidation. The inhibition of carotenogenesis however, is characteristic of m-phenoxybenzamide DPE's, the herbicidal properties of which differ greatly from those of p-nitro DPE's with which this study is concerned and so are not discussed herein.

Although a number of reports have been published on ultrastructural changes induced by DPE's (Orr and Hess, 1982a; Kenyon et al, 1985; Duke and Kenyon, 1986, 1987; Matringe et al, 1986; Bowyer

et al, 1987b; Kenyon et al, 1988), only that of Kenyon et al (1985) describes a detailed chronological sequence of changes. These workers noted that structural abnormalities of chloroplast envelopes, chloroplast swelling and disruption of the plasmalemma occurred rapidly following acifluorfen treatment of cucumber cotyledons. Shortly afterwards, intra - plastid vesicles were observed near to the chloroplast envelope and a loss of cytoplasmic integrity was observed, (ie. tonoplast disruption and cytoplasmic vesiculation). Chloroplast thylakoid disruption followed chloroplast envelope lysis. Earlier work of Orr and Hess (1982a) had reported initial acifluorfen - induced injury to cucumber cotyledons in the chloroplast / etioplast envelope. These workers also noted the persistance of thylakoid membranes. Thus, although the symptoms induced by DPE - type herbicides are macroscopically similar to those induced by the bipyridyl herbicides, the sequence of their effects on an ultrastructural level differ considerably. Although both classes of herbicide induce rapid chloroplast swelling and tonoplast / plasmalemma disruption, bipyridyls usually also induce rapid disruption of thylakoid membranes (Harris and Dodge, 1972; Dodge and Lawes, 1974; Harvey and Fraser, 1980), this not being true for DPE - type compounds. Furthermore, following a comparative study of the bipyridinium paraquat, acifluorfen and the "DPE - like" LS 82-556 (Fig. 2) on thylakoid activities in intact chloroplasts, Tissut et al (1987) concluded that the similar behaviour of LS 82-556 and acifluorfen was quite different from that of paraquat, supporting the ultrastructural observations described above.

1.3.4 DIPHENYLETHER STRUCTURE - ACTIVITY RELATIONSHIPS

Attempts to correlate DPE structure and activity have been made

investigating herbicide activity in photosynthetic electron transfer inhibition, lipid peroxidation and photosynthetic phosphorylation (Van den Berg and Tipker, 1982; Lambert et al, 1983a and b). Lambert et al (1983a) showed a necessity for the presence of a 4'-nitro, -nitroso or hydroxylamino group for activity in photosynthetic electron transfer inhibition and Van den Berg and Tipker (1982) showed that lipophilicity is an important factor in determining photosynthetic electron transfer inhibiting capability of DPEs. No relationship correllating gross herbicidal activity with inhibition of photosynthetic electron transfer has, however, been established and so this aspect of DPE activity appears unlikely to be of primary importance.

Lipid peroxidation is elicited by p-nitro, nitroso and hydroxylamino DPE's, their relative activities being similar (Lambert et al, 1983a and b). Ortho- and meso- nitro DPE's showed little or no ability to induce lipid peroxidation. The most potent inducers of lipid peroxidation were 2-chloro,-4-trifluoromethyl-4'-nitro DPE's with substituents adjacent to the 4'-nitro group (eg. carboxyl in acifluorfen, ethoxy in oxyfluorfen). Again, lipophilicity was found to be of great importance in determining activity (acifluorfen - methyl was far more active than acifluorfen - sodium in inducing ethane evolution in Scenedesmus). There also appeared to be a good correlation between herbicidal activity and the ability to induce lipid peroxidation. This suggested that lipid peroxidation was likely to be a crucial part of the mode of action of DPE's.

Lambert et al. (1983a and b) showed that in isolated thylakoids, photophosphorylation was inhibited by DPE's carrying a substituted nitro group on one ring and chloro substituents on the other phenyl ring, the 2 or 4 - chloro and 2,4 - dichloro - DPE's

being most effective. This was not however, deemed to be a very important mechanism in the mode of action of DPE's.

Since the introduction of most of the DPE herbicides listed in Table 1, a number of compounds have been developed which although structurally distinct, induce herbicidal symptoms characteristic of DPE's (Wakabayashi et al, 1978; Matringe et al, 1986). Clearly, the phenoxybenzene skeleton of DPE's appears to be unnecessary for this mode of herbicidal action.

1.3.5 THE INVOLVEMENT OF LIGHT IN DPE - TYPE ACTION

It is widely accepted that light is essential to the activity of herbicidal DPE's (e.g. Orr and Hess, 1982a; Fedtke, 1983; Duke and Kenyon, 1987 and publications cited by these authors). Of the few papers which reveal any herbicidal activity in darkness, Pereira et al, (1971) reported injury to nitrofen treated cabbage plants maintained in darkness and Matsunaka (1969a and b) reported some dark activity of certain (albeit commercially unimportant) DPE's. In all other reports, phytotoxic symptoms did not develop if treated plants were maintained in darkness and symptom development was arrested if treated, illuminated plants were transferred to the dark (Orr and Hess, 1981a and b; Gillham and Dodge, 1987a; Matringe and Scalla, 1987b). This light requirement has led to the term "photoactivation" in the literature as a somewhat ambiguous description of the stages of the DPE mechanism of action (eg. Matsunaka, 1969b; Orr and Hess, 1982a). Photoactivation has been used to describe both a theoretical light - driven modification of the DPE molecule, generating a toxic DPE derivative and to describe a putative light dependant system with which DPE's interact to initiate a lethal chain of events. Because of the confusing nature of this term, "photoactivation" will not be used

in this thesis.

Since light appeared to be essential to DPE action, identification of the regions of the light spectrum active in permitting DPE toxicity have been sought in a number of investigations, in order to implicate certain constitutive photoreceptors in the toxic process. Attempts at constructing action spectra for DPE activity have revealed peaks of activity across the visible spectrum (Matsunaka, 1969b; Ensminger and Hess, 1985a; Matringe and Scalla, 1987a; Sato et al, 1987b and Gaba et al, 1988). Most of these workers found that the most effective wavelengths for mediating DPE -type herbicide injury were in the blue regions of the spectrum. Sato et al (1987b) also found peaks of activity in the blue regions of the spectrum (for acifluorfen - ethyl and S-23142 [see fig.2]) but the major peak of activity was at 550nm. Ensminger and Hess (1985a), Matringe and Scalla (1987a) and Gaba et al (1988) also found activity in yellow/green wavelengths but these were much smaller than blue peaks. Ensminger and Hess (1985a) and Sato et al (1987) additionally found large peaks of activity in the red part of the spectrum.

The publication of action spectra and other related data has prompted many researchers to speculate as to the identity of a number of blue-light absorbing photoreceptors which may be involved in DPE action. Carotenoids for example, have been proposed, since many reports show that chemicaly or genetically modified plants unable to synthesise chlorophylls and plants grown under conditions preventing chlorophyll synthesis are susceptible or hypersensitive to DPE's, whereas white (carotenoid - free) plants are resistant (Matsunaka, 1969a; Fadayomi and Warren, 1976; Orr and Hess, 1982a; Devlin et al., 1983; Duke and Kenyon, 1986; Halling and Peters, 1987). Flavins have also been proposed as possible mediators of DPE toxicity as they

absorb blue light, possess characters favourable for generation of singlet oxygen and catalyse many intermolecular oxidations as part of normal metabolism (Orr and Hess, 1982a; Galland and Senger, 1988). Furthermore, several DPE's have been implicated in interacting with the putative flavin - cytochrome ("cryptochrome") system involved in the blue light - dependant phototropism of Avena coleoptiles (Leong and Briggs, 1982; Briggs and Iino, 1983) and blue light - induced sporulation of Trichoderma harzianum. The Trichoderma system lacks both carotenoids and chlorophylls (Gaba and Gressel, 1987). Most recently, a number of publications suggesting a role for chlorophyll precursors (i.e. tetrapyrroles) have emerged. Kawakubo et al (1979) showed DTP (a hydroxypyrazole with DPE - type activity, see Fig. 2) to stimulate synthesis of protoporphyrin, uroporphyrin and coproporphyrin which were proposed as being photosensitising pigments of de-etiolating radish cotyledons. Duke and Kenyon (1987) hypothesised that chlorophyllide / protochlorophyllide contained within chloroplast envelopes and thylakoids may act as photoreceptive initiators of DPE toxicity. Halling and Peters (1987), presented evidence with acifluorfen - methyl action on developing cucumber cotyledons which may be explained by DPE interruption of normal chlorophyll - protein complex formation or the accumulation of photodynamic chlorophyll precursors. Matringe and Scalla (1987a, 1988) showed an accumulation of a pigment tenatively identified as protoporphyrin IX in acifluorfen - methyl and LS 82-556 treated cucumber seedlings, which the authors suggested, could initiate photodynamic damage. These findings were substantiated by the similar data of Witkowski and Halling (1988) who found acifluorfen - methyl induced increases in protoporphyrin IX, concomitant decreases in protochlorophyllide and that acifluorfen - methyl induced membrane

damage could be arrested with the 5-amino-laevulinic acid synthesis inhibitor gabaculine. Lydon and Duke (1988) found similar results with acifluorfen, oxyfluorfen and nitrofen and inhibited their activity in cucumber cotyledons, Amaranthus retroflexus, and Abutilon theophrasti with gabaculine and 4,6-dioxoheptanoic acid . Activity of these herbicides was stimulated by 5-aminolaevulinic acid and 2,2'-dipyridyl, known stimulators of porphyrin biosynthesis. Kouji et al (1988) furthermore showed that a tobacco cell homogenate prepared from cells pre-treated with acifluorfen - methyl, oxyfluorfen, nitrofluorfen, chloropthalim (MK 616), S-32142 or oxadiazon posessed a light - dependent oxygen consuming capability which correlated with the appearance of lipid hydroperoxides. This capability was heat stable and tentatively identified as being due to the presence of large amounts of protoporphyrin IX.

1.3.6 LIPID PEROXIDATION AS A MECHANISM OF ACTION FOR DPE - TYPE HERBICIDES

As indicated in the previous section on DPE structure - activity relationships, it is widely believed that by whatever means, these herbicides exert their effect on plant tissues via initiating a light - dependant free radical reaction involving membrane lipids (see Orr and Hess, 1982a; Fedtke, 1983; Duke and Kenyon, 1987). Considerable evidence has been presented suggesting the occurrence of a photo - oxidative process during DPE -type herbicide induced toxicity. The products of oxdative damage have been detected by measuring the evolution of short chain hydrocarbons (Lambert and Boger, 1981; Lambert et al, 1983a and b; Kunert, 1984; Kunert and Boger, 1984; Finckh and Kunert, 1985; Matringe et al, 1986 Bowyer, et al ,1987a, b and c;Lambert et al, 1987; Gillham and Dodge, 1987a;

Matringe and Scalla, 1987a; Sato et al, 1987a; Wakabayashi et al, 1988) and malondialdehyde (Orr and Hess, 1982b; Ensminger and Hess, 1985b; Matringe et al, 1986; Gillham and Dodge, 1987b). The generation of malondialdehyde and short chain alkanes have long been regarded as good indicators of lipid peroxidation in biological systems (see Pryor, 1978 and Slater, 1984). Measurements indicating the effects of lipid peroxidation on membrane permeability have also been made (Vanstone and Stobbe, 1979; Orr and Hess, 1982a; Duke and Kenyon, 1986; Halling and Peters, 1987).

The implications of detecting lipid peroxidation products in DPE - treated plants have been further substantiated by discovering that herbicides effect a rapid depletion of glutathione reductase, dehydroascorbate reductase, superoxide dismutase, ascorbate oxidase, ascorbate free - radical reductase, peroxidase and catalase (Kenyon and Duke, 1985) in treated plants. These enzymes are known to be involved in protection from photooxidative damage (eg. Elstner, 1987; Salin, 1987). Depletion of the endogenous antioxidants ascorbate, glutathione and α-tocopherol has been similarly observed (Kunert, 1984; Kenyon and Duke, 1985). Furthermore, correlations between leaf content of ascorbate, α-tocopherol, and plant susceptibility to DPE phytotoxicity, have been shown in a range of higher plant species (Boger, 1984; Finckh and Kunert, 1985). Finckh and Kunert (1985) additionally demonstrated that the ratio of ascorbate to α-tocopherol present in the leaf was critical to the degree of a plant's resistance to DPE's. Additionally, exogenously applied antioxidants have been shown to lessen or retard the effects of DPE's. The natural, lipophilic free - radical scavenger α-tocopherol and the synthetic antioxidants ethoxyquin, N,N'-diphenyl-1,4-phenylene diamine and 2,6-di-tert-butyl-p-cresol have given protection against DPE toxicity

in a number of experiments (Boger, 1984; Duke et al, 1984; Kunert and Boger, 1984) further suggesting the likelihood of the involvement of lipid peroxidation in the action of DPE's. The most direct evidence for lipid peroxidation being central to the mode of action of DPE - type herbicides has been provided by Kouji et al (1988). These workers showed a close correlation between activity of a range of DPE - type compounds and the ability to induce an oxygen consuming capability in cultured tobacco cells (see section 1.3.5). This light dependant oxygen consuming capability was closely correlated to the generation of lipid hydroperoxides.

As described above, there appears little doubt that DPE - type herbicide mediated toxicity proceeds via a photo - dependant peroxidative destruction of membrane lipds leading to cellular decompartmentalisation and ultimately death. The mechanism of this photo-oxidation is not, however known. In searching for a mechanism, a number of groups have failed to detect superoxide or its dismutation product hydrogen peroxide in chloroplast and thylakoid preparations (Lambert et al, 1984; Wettlauffer et al, 1985; Gillham and Dodge 1987b), the excessive production of which would be lethal (Pryor, 1978; Elstner, 1982, 1987). Ensminger and Hess, (1985b) also failed to reduce toxicity of DPE's towards cucumber cotyledons using the synthetic dismutating catalyst, copper penicillamine. Ridley (1983) however, showed generation of superoxide by illuminated thylakoids in the presence of fomesafen. Upham and Hatzios (1987), attempted to demonstrate the generation of alkoxyl and hydroxyl radicals in DPE - treated Pisum thylakoids, by measuring methional oxidation to ethylene. Of the DPE's tested, only oxyfluorfen stimulted methional oxidation. Acifluorfen, acifluorfen - methyl, nitrofen and nitrofluorofen did not show this effect, suggesting that this process

in conjunction with thylakoids was not directly related to the general mode of action of DPE herbicides. Nevertheless, pathologic lipid peroxidation usually requires the presence of radicals or other energetically excited species and further attempts at identifying the putative radical initiators of lipid peroxidation reactions have been made with electron paramagnetic resonance measurements. Draper and Casida, (1983) demonstrated using "spin trapping" techniques that nitroso derivatives of nitrofen and CNP can form stable adducts with tetramethylethylene (an unsaturated hydrocarbon used as an analogy to unsaturated lipid molecules) which auto-oxidise to form stable free radicals. Similar results were found reacting nitroso - DPE's with a range of natural lipids. Extracts of excised sugar beet leaves treated with 10 - 100mM nitrofen in very bright light also revealed nitroxide radical electron paramagnetic resonance signals similar to those generated *in vitro* using nitroso - DPE's. Similarly, Takahashi and Mason (1987) showed that nitrofen and chlomethoxynil dissolved in 90% methanol and irradiated with a xenon arc lamp, can form anion radicals. These data imply that such a mechanism could be involved initiating DPE - mediated peroxidation. Lambert *et al* (1984) also sought to detect paramagnetic species generated by DPE treatment of chloroplasts using spin - trapping techniques. Although radical species were evident in acifluorfen - methyl and oxyfluorfen treated spinach chloroplasts, it was suggested that these were alkyl radicals. No transient radicals of DPE molecules similar to those reported by Draper and Casida (1983) were detected in this study. Furthermore, although the mechanism of Draper and Casida (1983) provides a plausible mechanism for peroxidation initiation, it does not explain the activity of herbicidal chloro-DPE's (Ensminger *et al*, 1985b) which have physicochemical properties incompatible with radical

formation in biological systems, nor does it explain the activity of non - nitro "DPE-like" compounds such as LS 82-556 (Matringe et al, 1986). Infact, in spite of the numerous propositions suggesting that DPE induced peroxidation is initiated via a herbicide anion / radical (eg. Kunert and Boger, 1981; Draper and Casida, 1983; Lambert et al, 1983a and b, 1987; Gillham and Dodge, 1987a and b), the data of Draper and Casida (1983) and Takahashi and Mason (1987) form the only direct evidence to support such an idea.

The most recent attempt to identify the toxic species which mediates DPE - induced photo - oxidation (Haworth and Hess, 1988) demonstrated that sub-micromolar concentrations of oxyfluorfen are capable of generating singlet oxygen, a very reactive oxygen species which can initiate lipid peroxidations (Pryor, 1978; Elstner, 1982, 1987; Konings, 1984; Knox and Dodge, 1985a; Salin, 1987) in Pisum thylakoid preparations. Although the generation of singlet oxygen by oxyfluorfen was dependant on the presence of intact thylakoids, photosynthetic electron transfer through photosystem I was not directly linked to its production.

1.3.7 THE INVOLVEMENT OF PLASTIDS IN THE DIPHENYLETHER - TYPE MODE OF ACTION

As DPE's are light dependant herbicides, plastids have naturally been targets for investigating the mode of action of these compounds. In considering plastid functions, much effort has been devoted to studying the inhibitory effects of DPE's on photosynthetic electron transfer even though symptoms induced by DPE's differ markedly from those resulting from photosynthetic electron transfer - inhibitor herbicide (eg. diuron) treatment. The pursuit of this line of inquiry infact probably reflects the availability of established

techniques rather than a likelihood that such inhibition is important in DPE action.

DPE herbicides generally inhibit photosynthetic electron transfer with 50% inhibitory values (I_{50}) of approximately or greater than 10^{-5}M (Moreland et al, 1970; Lambert et al 1979; Bugg et al 1980; Pritchard et al 1980; Huchzermeyer 1982; Van den Berg and Tipker 1982; Lambert et al 1983a; Alscher and Strick 1984; Bowyer et al 1987b; Moreland and Novitzky 1987; Tissut et al 1987) and the site of interaction with the chain of electron carriers has been moderately well studied. Bugg et al (1980) showed nitrofluorofen inhibition of methyl viologen and ferricyanide - mediated Hill reactions but via only photosystem I or photosystem II. Furthermore, the rate of dark reduction of cytochrome-f was slowed by this herbicide. Other similar reports (Boger, 1984; Van den Berg and Tipker, 1982; Draber et al, 1981; Moreland et al, 1970; Ridley, 1983) contributed to a general agreement that DPE's can inhibit photosynthetic electron transfer in the plastoquinone - cytochrome b_6/f complex region of the chain. Inhibition of photosynthetic electron transfer nevertheless appears to play only a small, if any, part in the DPE type mode of action, some very potent DPE's such as acifluorfen - sodium being very poor photosynthetic electron transfer inhibitors (Pritchard et al, 1980; Van den Berg and Tipker, 1982; Lambert et al, 1983; Tissut et al, 1987).

Although it is generally agreed that DPE - type inhibition of photosynthetic electron transfer is insignificant in their mode of action, an interaction with or necessity for photosynthetic electron transfer has been proposed by several research groups. These workers showed that inhibitors such as diuron and atrazine suppress DPE - type

effects on plant cells (Kunert and Boger, 1981; Lambert et al, 1983a and b, 1984; Ridley, 1983; Gillham et al, 1985, 1987; Matringe et al, 1986; Tissut et al, 1987; Haworth and Hess, 1988; Nurit et al, 1988; Matringe et al, 1988a). Contradicting these findings however, Matsunaka (1969a), Duke et al (1984), Ensminger and Hess (1985b) and Duke and Kenyon (1986) showed such inhibitors to be ineffective in reducing DPE activity. The activity of DPE's in chlorophyll free and photosynthetically incapable plants (Matsunaka, 1969a; Fadayomi and Warren, 1976; Duke et al, 1984; Duke and Kenyon, 1986; Halling and Peters, 1987; Matringe and Scalla, 1987b) also sugested that photosynthetic electron transfer does not play a direct role in DPE type activity. The reason for the apparent disagreement of experimental evidence on the question of photosynthetic electron transfer involvement is unclear. Kunert et al (1985) however, suggested that diuron suppression of oxyfluorfen activity in Scenedesmus acutus L may be explained in terms of a reduction in photosynthetic electron transfer - coupled oxygen evolution, diuron therefore, indirectly retarding lipid peroxy radical formation through lowering oxygen availability. Bowyer et al (1987c, 1989) presented experimental evidence supporting this idea.

A number of reports have been published showing DPE's acting as "energy transfer" or photophosphorylation inhibitors (Moreland et al, 1970; Lambert et al, 1979; Pritchard et al, 1980; Ridley, 1983; Boger, 1984). Additionally, Huchzermeyer (1982), Lambert et al (1979) and Ridley (1983) demonstrated competitive inhibition of chloroplastic ATP synthase between DPE's and ADP, though the relevance of this DPE property is not clear. Ridley (1983) however, postulated that binding of DPE's to thylakoid ATP synthase, which is

closely associated with photosystem I may disturb the flavin moiety of ferredoxin - NADP and / or ferredoxin - thioredoxin reductase in such a way as to enable interaction of the flavin with oxygen, generating activated species.

In comparison with chloroplastic electron transport, very little work has been performed to examine DPE effects on carbon fixation and assimilation, although DPE's rapidly decrease carbon fixation when applied to susceptible plants. Matsunaka (1969a) found that carbon - dioxide dependant oxygen evolution ceased when half of the photosynthetic electron transfer capacity remained. Performing work on intact chloroplasts and leaf discs, Wettlauffer et al (1985) found that acifluorfen is inhibitory to those photosynthetic functions which require a functional chloroplast envelope. Previous work by this group (Alscher and Strick, 1984) led them to suggest that an envelope situated photoreceptor may be involved in DPE activity. Their studies on fructose - 1,6 - bisphosphate and glyceraldehyde - 3 - phosphate dehydrogenase (NADP) additionally implied that acifluorfen toxicity leads to inactivation of these light activated enzymes, via a pathway which uses oxygen as a terminal oxidant and involves thioredoxin and ferredoxin - thioredoxin reductase.

Finally, the recent publication of evidence suggesting that DPE - type compounds interfere with porphyrin biosynthesis leading to an accumulation of protoporphyrin IX (see section 1.3.5), clearly implicates the involvement of plastids in the DPE - type mode of action. It remains to be shown however whether plastids are the sole source of accumulated protoporphyrin IX in vivo.

1.3.8 INVOLVEMENT OF ORGANELLES OTHER THAN PLASTIDS

The only organelle which has been studied with respect to DPE action apart from plastids has been the mitochondrion. Moreland et al (1970) demonstrated nitrofen to be effective as an inhibitor of mitochondrial electron transport with I_{50} concentrations between 2.7 and 7.4 x 10^{-5}M depending on the substrate used. Similarly, Hoagland et al (1986) showed 50% inhibition of succinate - dependant respiration in isolated soybean mitochondria by $1x10^{-5}$M acifluorfen - sodium. Duke et al (1984) using ultrastructural cytochemical methods, showed some appearance of superoxide and hydrogen peroxide within mitochondria (but not plastids). The respiratoy electron transport inhibitor antimycin A has furthermore been shown to decrease DPE - induced toxicity (Duke et al, 1984; Matringe and Scalla, 1987b). However, the significance of these results is not at all clear. As however, respiratory inhibition by acifluorfen was as effective in darkness as in light (Hoagland et al 1986), it is unlikely that this property of DPE's is of direct importance in their mode of action.

No reports have been made proving that porphyrin biosynthesis inhibition in mitochondria is of importance to the DPE - type mode of action although Matringe et al, (1989a and b) showed inhibition of both plastidic and mitochondrial protoporphyrinogen oxidase, which these authors proposed as the primary target of DPE - type herbicides.

1.3.9 METABOLISM OF DIPHENYLETHERS IN PLANTS

Few detailed studies of the metabolism of DPE herbicides have been performed, although differences in rates of metabolism have been suggested to partially account for differences in selectivity of these compounds. Eastin (1972) and Ritter and Coble (1981) showed that susceptibility to DPE's was inversely related to the ability of several species to metabolise these compounds.

The most detailed studies of DPE metabolism were published by Eastin (1972); Frear and Swanson (1973); Shimabukuro *et al* (1973); Shimotori and Kuwatsuka (1978) and Frear *et al* (1983). These workers showed that the major means of DPE metabolism in plants are: (1) cleavage of the diphenyl ether bond; (2)reduction of the nitro moiety to form amino - DPE's (it should be noted however, that amino - DPE's are herbicidally active (Lambert *et al* 1983a and b); conjugation of metabolites to sugars and peptides. Frear *et al* (1983) proposed the metabolic pathway depicted in Fig. 3 for metabolic detoxification of acifluorfen in soybeans.

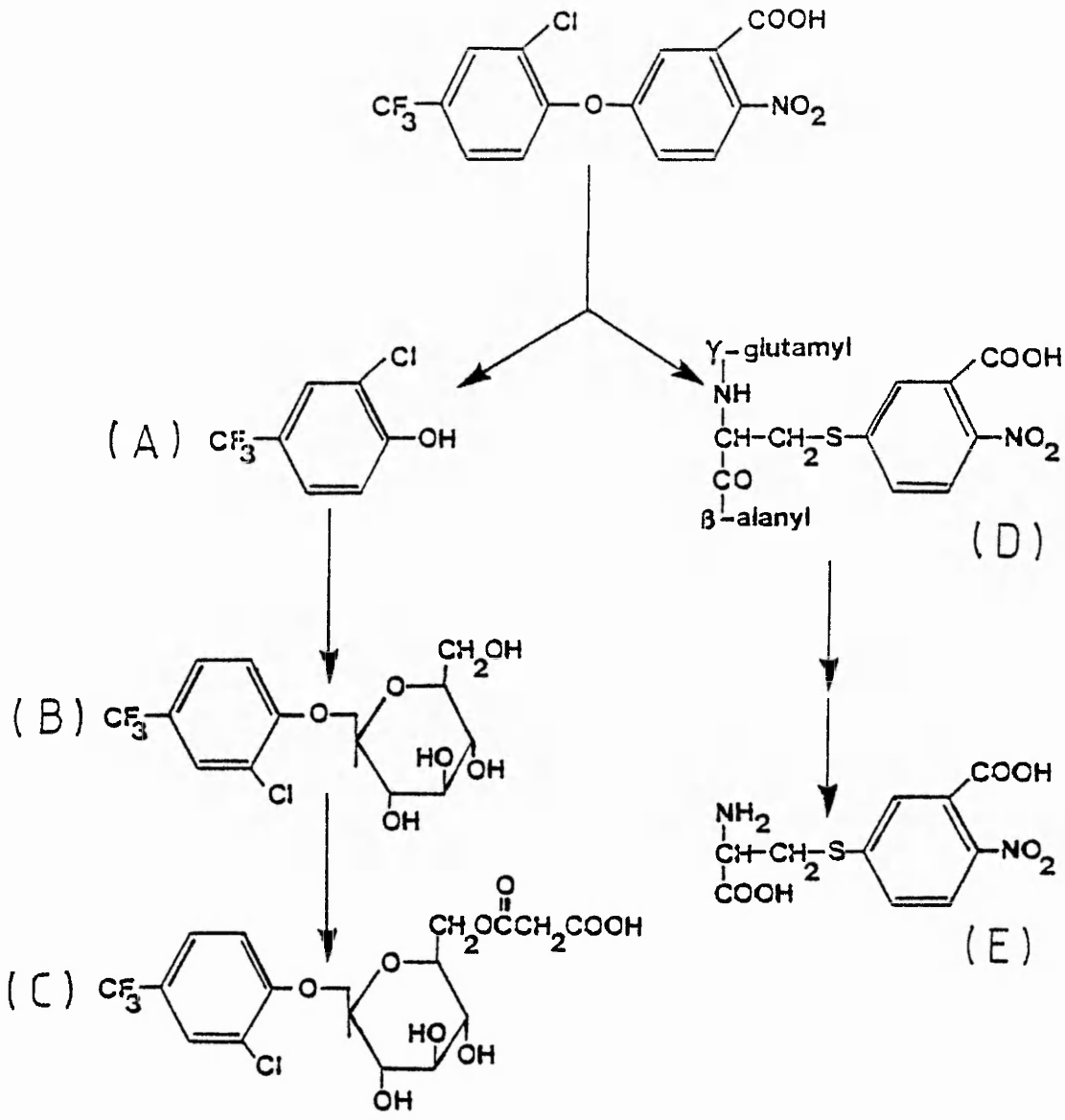


Figure 3. Metabolism of acifluorfen in soybeans (after Frear et al, 1983). Following cleavage of the ether bond, the halogenated aryl residue (A) is glucosylated (B) and may be further metabolised to the malonyl -β- D-glucoside (C). The nitroaryl residue becomes conjugated with homoglutathione (D) which is metabolised to a cysteinyl derivative (E).

1.4 A NOTE ON PLANT SPECIES USED IN THIS STUDY.

As an intended target weed for M&B 39279, Galium aparine L. was chosen as the major species to be used as experimenal material in this study. It was anticipated that by investigating M&B 39279 action in a target species, more meaningful results would be obtained than if a species such as tobacco, spinach, or a unicellular green alga, (the standard tools of many plant physiologists), were to be used. The more commonly used plants offer a range of advantages to the experimenter in terms of ease of growth, having a well studied physiology and the availability of established techniques suited to these plants. Data alluding to the mode of action of a herbicide collated from experiments using these species, would however, although valuable be ultimately questionable in their relevance to the situation of herbicide - weed interaction. Furthermore, the literature to date surrounding the mode of action of DPE - type herbicides, the group of compounds which the action of M&B 39279 closely resembles, has been dominated by reports of experiments involving cucumber seedlings (see papers of Orr, Hess, Duke, Kenyon and Halling for examples) and unicellular geen algae (see papers of Lambert, Boger, Ensminger and Hess). Only a few papers have been published involving weed species (eg. Gorske & Hopen, 1978; Ensminger and Hess, 1985b and Lydon and Duke, 1988). There exists, then, a need for information regarding the activity of DPE - type herbicides in a wider range of species than have been previously employed.

Galium aparine, commonly known as cleavers (Fig. 4), is an annual dicotyledonous plant, inhabiting hedgerows and fields almost worldwide (Holm et al, 1976). Although reported as an occasional

Figure 4. Galium aparine in a ripening wheat crop.

agricultural weed in a number of regions of the world such as Canada (Moore, 1975) and Japan (Noda et al, 1965), G.aparine is most important as a major weed of European crops. In a recent survey of the main cereal growing regions of southern England, Chancellor and Froud - Williams (1985) revealed G.aparine as the second most frequently occurring dicotyledonous weed. Workers at Long Ashton Research Station in England have furthermore classified G.aparine as the most competitive weed in U.K. winter wheat (Cussens, 1986). In cereals, G.aparine is a very damaging weed since it can seriously reduce yields even at moderate densities of infestation, reducing seed heads per unit area, average grain weight and grain number per seed head (Peters, 1984). Losses such as these often result from lodging, a consequence of the climbing, straggly growth habit of G.aparine's setiferous, branched stems which can reach up to 2m in length, entangling large clumps of cereal tillers (Froud - Williams, 1985).

It is evident from the review and experiments of Moore (1975), that there has been some confusion between G.aparine and the morphologically similar Galium spurium in the literature. this has occurred due to the natural variability within the species' giving rise to plants phenotypically intermediate of the two species. Moore, (1975) and literature cited therein, refer to a number of intraspecific taxa of both G.aparine and G.spurium. Furthermore, G.aparine exists in several levels of ploidy, the hexaploid (2n = 66) occurring most commonly (Moore, 1975), the tetraploid (2n = 44) less so and the chromosome number varying between 42, 44, 66, and 68 (Tutin et al, 1976). It is possible that this variability has contributed to the ability of G.aparine to becme a successful weed.

A second weed species, namely Viola arvensis Murr., (Fig. 5) was incorporated into several experiments in this study in order to

support or question data obtained from G.aparine - based eperiments. Unfortunately, restraints of time precluded inclusion of V.arvensis into all investigations.

Figure 5. Viola arvensis in a ripening wheat crop.

V.arvensis (common name, field pansy) is an annual dicotyledonous plant found throughout the Americas and Eurasia and is a major weed of european agriculture. Chancellor and Froud - Williams, (1984) described V.arvensis as the most frequently occurring dicotyledonous weed in U.K. winter wheat.

C H A P T E R 2

CHARACTERISATION OF HERBICIDE SYMPTOM DEVELOPMENT

2.1 INTRODUCTION

As M&B 39279 was a new and largely uncharacterised herbicide at the start of this study, it was considered necessary to undertake an initial study of the appearance and development of phytotoxic symptoms on the plant species chosen for study. Aquisition of information such as dose - response and time course of symptom development was needed to enable the design of further experiments. These initial experiments are described in this chapter. In addition to observing the effects of herbicide treatment on plant morphology and growth, the effects of such treatment on levels of certain major metabolites, i.e. proteins, free amino acids and reducing sugars was followed to identify any general effects on primary metabolism and therefore, possibly provide some indication as to the target site of M&B 39279.It has been mentioned that it was thought that M&B 39279 may have a similar mode of action to acifluorfen (section 1.2). Although no direct effects of DPE's on primary metabolism had been shown in the literature, this approach was thought to be justified in that (a) even though M&B 39279 appeared to have similar herbicidal properties, there may have been some undiscovered differences between them and (b) the literature surrounding the DPE mode of action was confusing, containing numerous controversies such as the role of photosynthesis and the role of light in DPE action. It was felt therefore, that in this analysis of M&B 39279 (and acifluorfen), the herbicidal properties of these compounds should be investigated as broadly as possible.

2.2 METHODS AND MATERIALS

2.2.1 GROWTH AND HUSBANDRY OF PLANT MATERIAL

Viola arvensis seed was sown onto peat - based potting compost (J. Arthur Bowers) in seed trays and covered with just enough compost to cover the seeds. Seedlings bearing 2 to 3 true leaves were transplanted into 3 inch pots (3 seedlings per pot) and grown on for use as required for various experiments. These conditions had previously been established in this laboratory by Sanders (1984). Galium aparine seed was sown in seed trays as described for V.arvensis. Weed seeds were obtained from Herbiseed, the Nurseries, Billingbear Park, Wokingham, Berkshire RG11 5RY. All plants were grown in a glasshouse with lighting supplemented by 400W high pressure sodium lamps providing from 200-400µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) in a 14 hour daylength. The temperature was maintained as close as possible to between 20 to 25 C.

Insect pests were controlled by general glasshouse hygiene and by use of insecticides when necessary. For control of aphids, the glasshouse was fumigated with pyrethroid and gamma-HCH smokes and for control of sciarid flies, soil drenches of diflubenzuron were applied. These were the only troublesome pests encountered and no adverse effects of the pesticides on the plants were noticed. Plants were not treated and / or analysed immediately following insecticide treatments thus minimising the risk of influence of insecticides on herbicide action.

The above conditions and practices are applicable throughout this thesis.

2.2.2 VISUAL CHARACTERISATION OF HERBICIDE SYMPTOMS AND EFFECTS OF HERBICIDES ON PLANT GROWTH

Galium aparine (growth stage: 2 expanded whorls of leaves, 3rd emerging) and Viola arvensis (growth stage: 5 to 6 expanded leaves, 7th to 8th emerging) plants were glasshouse grown as described above in 3 inch pots (3 plants per pot). These were sprayed using an experimental pot sprayer calibrated to deliver the equivalent of 200ℓ ha^{-1}. M&B 39279 and acifluorfen were dissolved in 5% (w/v) aqueous acetone. Herbicides were applied at doses equivalent to 6.25g, 25g and 50g ha^{-1} and control plants were sprayed with 5% (w/v) aqueous acetone. Two pots (six plants) were sprayed per treatment. Sprayed plants were returned to the glasshouse and observed at 1, 3, 7, 10 and 14 days after spraying and any symptoms of injury to plants noted.

This experiment was repeated but instead of visual assessments being made, plants were cut at soil level and the aerial parts weighed. These plants were then dried in an oven overnight at 90°C to constant weight. Fresh and dry weights were thereby recorded.

2.2.3 DETERMINATION OF CHANGES IN MAJOR METABOLITES IN M&B 39279 AND ACIFLUORFEN TREATED PLANTS

2.2.3.1 Treatment of Plants

G.aparine and V.arvensis plants were grown and sprayed with M&B 39279 and acifluorfen at rates equivalent to 25 and 50g ha^{-1} as described in section 2.2.2. Four plants, grown in two 3 inch pots were used per treatment.

2.2.3.2 Assay of Major Metabolites

The aerial parts of treated plants were harvested at 1, 3, 7, 10 and 14 days after spraying, weighed and subjected to the following fractionation procedure:

Plants were ground thoroughly in a chilled mortar with roughly 8ml of cold (4°C) 0.1M Tricine - NaOH buffer, pH 7.5. The homogenate was then centrifuged at 1000 x g for 5 minutes and the supernatant (A) reserved. The pellet was resuspended in 2.5ml of 1.0M NaOH and heated in a water bath at 70°C for 1 hour. Following centrifugation at 300 x g for 1 minute, the resulting supernatant was assayed for protein. This fraction was referred to as the insoluble fraction.

Supernatant (A) was made up to 10.0ml with Tricine - NaOH buffer, pH 7.5. Part of this solution was assayed for free amino acids and reducing sugars. Another 1.0ml of this solution was centrifuged in a bench 'microcentrifuge' (11 000 r.p.m.) for 2.5 minutes and the resulting supernatant assayed for protein. This was referred to as the soluble fraction. The pellet was resuspended in 0.1ml of 1.0M NaOH, heated to 70°C for 1 hour, diluted to 1.0 ml with distilled water and assayed for protein. This was referred to as the microsomal fraction.

Coomassie Blue Assay for Protein

The protein assay reagent was prepared as follows: 100mg of BDH 'Electran' Page Blue G90 (colour index number 42655, BDH Ltd., Broom Rd, Poole, Dorset, U.K.) was dissolved overnight with stirring in 50ml of 95% (v/v) aqueous ethanol. To this was added 100ml of 85% phosphoric acid (BDH) and the whole made up to 1000ml with distilled water. The reagent was stored in darkness, diluted by 50% with distilled water and filtered immediately prior to use.

For protein assays, 0.1ml of sample was mixed with 5.0ml of diluted Coomassie blue reagent. After 15 minutes the absorbance of the solution was read using a spectrophotometer at a wavelength of 595nm against a suitable blank. Protein - dye complex light absorbance readings were converted to protein concentration in the test solution by using a regression coefficient derived from standard bovine serum albumin solutions. It is worth noting that the dye used in this assay is marketed under a variety of names by different suppliers.

Ninhydrin - Hydrazine Assay for Amino Acids

Ninhydrin reagent was prepared as follows: 3.3g of ninhydrin, 250ml of 2-methoxyethanol, 50ml of 4M acetate buffer (250g sodium acetate (trihydrate) dissolved in water with heating and when cool mixed with 50ml glacial acetic acid and made up to 500ml) and 15ml of glacial acetic acid were mixed together and diluted to 500ml with distilled water and stored in darkness. Hydrazine reagent was prepared by dissolving 0.13g hydrazine sulphate in 500ml water and one drop of concentrated sulpuric acid was added.

For assay of amino acids, 1.0ml of sample was mixed with 1.2ml of ninhydrin reagent and 0.8ml of hydrazine reagent. This was heated in a boiling water bath for 15 minutes then allowed to cool. Three ml of 50% (v/v) aqueous ethanol was then added, the whole left for 10 minutes and the absorbance of the solution read in a spectrophotometer at a wavelength of 570nm against a suitable blank. Absorbance readings were converted to amino acid concentration by using a regression coefficient derived from a calibration graph constructed from glycine standard solutions.

Nelson's Assay For Reducing Sugars

"Nelson's A" reagent was prepared by dissolving 12.5g anhydrous sodium carbonate in 350ml water. To this was added 12.5g potassium sodium tartrate, 10.0g sodium bicarbonate and 100g sodium sulphate. The solution was diluted to 500ml. "Nelson's B" reagent was prepared by dissolving 7.5g copper sulphate pentahydrate in 50ml water and adding 1 drop of concentrated sulphuric acid.

For assay of reducing sugars, 1ml of sample was mixed with 1ml of Nelson's reagent (12.5ml Nelson's A + 0.5ml Nelson's B, freshly mixed) and heated in a boiling water bath for 20 mins. When cooled, 1ml of arsenomolybdate reagent (commercial product, BDH Ltd) was added with 7.0ml water and the whole left for 5 minutes prior to reading absorbance of the solution at 540nm. Absorption readings were converted to reducing sugar concentration by using a regression coefficient derived from a calibration graph constructed from glucose standards.

2.3 RESULTS

2.3.1 VISUAL CHARACTERISATION OF HERBICIDE SYMPTOMS

2.3.1.1 General Comments

Development of the toxicity symptoms of M&B 39279 and acifluorfen on G.aparine and V.arvensis were morphologically and chronologically very similar. As the visual appearance of these symptoms for both compounds were indistinguishable, they will be described below collectively to avoid repetition. Although the progression of symptom development was similar in both species and developed over a similar time scale, V.arvensis had a greater ability to recover from M&B 39279 / acifluorfen damage than G.aparine (compare Figs. 8 and 9 with Figs. 14 and 15). Also see below).

2.3.1.2 Galium aparine Sprayed with M&B 39279 and Acifluorfen

Initial symptoms of herbicide damage were apparent within one day after treatment (Fig. 6) and were manifest as water soaked (bruise - like) areas and considerable loss of turgor on leaflets at all doses with both herbicides. Symptoms at one day after treatment were restricted to only the youngest leaf whorls.

By 3 days after spraying, a dose - response of symptoms was apparent as was cessation of growth (Fig. 7). Patches of tissue collapse and loss of turgor were seen on most leaf whorls, though these were more severe on young leaflets, cotyledons remaining symptomless. Leaf desiccation and wilting of stem tips was also apparent.

Figure 6. G.aparine treated with (left to right) 0, 6.25, 25 and 50g ha^{-1} equivalent of acifluorfen, 1 day after spraying. Arrows indicate water - soaked patches.

Figure 7. G.aparine treated with (left to right) 0, 6.25, 25 and 50g ha^{-1} equivalent of acifluorfen, 3 days after spraying.

Figure 8. G.aparine treated with (left to right) 0, 6.25, 25 and 50g ha $^{-1}$ equivalent of acifluorfen, 7 days after spraying.

Figure 9. G.aparine treated with (left to right) 0, 6.25, 25, and 50g ha^{-1} equivalent of acifluorfen, 14 days after spraying.

Figure 10. G.aparine treated with (left to right) 0 6.25, 25, and 50g ha^{-1} equivalent M&B 39279, 7 days after spraying.

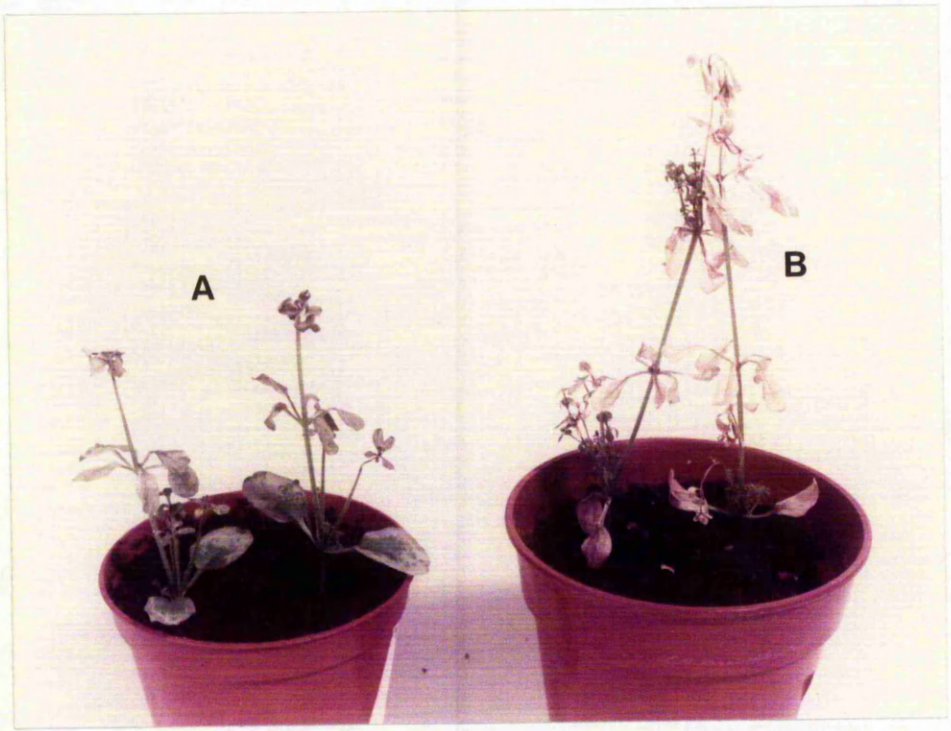


Figure 11. G.aparine treated with 50g ha^{-1} equivalent M&B 39279, (A) 14 days and (B) 28 days after spraying.

By 7 days after spraying, patches of tissue collapse were apparent on all leaf whorls at 25 and 50g ha^{-1} and most whorls at 6.25g ha^{-1} (Fig. 8). Many such patches had become necrotic (i.e. dry and pale brown). Some recovery from herbicide damage (i.e. regrowth) was also noted, particularly at 6.25g ha^{-1}. This was due to growth of axillary buds in the cotyledonary nodes as all main growing tips had been killed. No leaves bearing substantial toxicity lesions were seen to recover.

After 14 days following spraying, tissue collapse and necrotic and wilted patches were dominant on all plants, little tissue remaining healthy (Figs. 9 and 10). The growth of axillary shoots had progressed only slowly.

2.3.1.3 Viola arvensis Sprayed with M&B 39279 and Acifluorfen

Herbicide toxicity symptoms were apparent as water - soaked patches on leaves within one day after spraying. Only the young, rapidly expanding leaves were affected.

By 3 days after spraying, water - soaked patches were apparent on most leaves which were becoming desiccated.

After 7 days following treatment, many of the water soaked and desiccated areas were becoming necrotic and a dose - response in growth rate could be seen (Figs. 12 and 14). Some recovery from herbicide damage was also visible in the form of new leaf growth, particularly at 6.25g ha^{-1}.

By 14 days after spraying, plants treated with 6.25g ha^{-1} M&B 39279 / acifluorfen had grown to almost the same size as the control plants (Figs. 13 and 15). Recovery was much slower at 25 and 50g ha^{-1}.

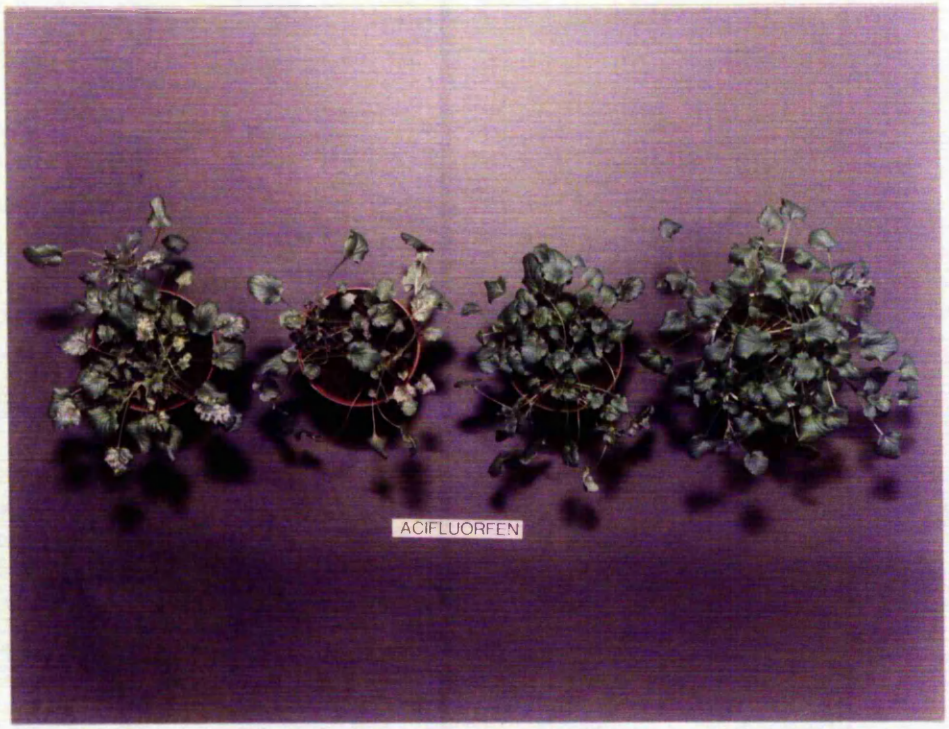


Figure 12. V.arvensis treated with (left to right) 50, 25, 6.25 and 0g ha^{-1} equivalent acifluorfen, 7 days after spraying.

Figure 13. V.arvensis treated with (left to right) 0, 6.25, 25, and 50g ha^{-1} equivalent acifluorfen, 14 days after spraying.

Figure 14. V.arvensis treated with (left to right) 50, 25, 6.25, and 0g ha^{-1} eqivalent M&B 39279, 7 days after spraying. Arrows indicate necrotic patches.

Figure 15. V.arvensis treated with (left to right) 0, 6.25, 25, and 50g ha $^{-1}$ equivalent M&B 39279, 14 days after spraying.

2.3.2 CHANGES IN GROWTH AND LEVELS OF MAJOR METABOLITES IN M&B 39279 AND ACIFLUORFEN - TREATED PLANTS

2.3.2.1 Changes in plant growth

Figs. 16 - 19 clearly reveal a difference in growth kinetics over the period studied between G.aparine and V.arvensis. A quantitative similarity in the effects of M&B 39279 / acifluorfen on plant dry weight and a clear dose - response of the two species to the applied herbicides were also noted. It was also apparent that the equivalent of 50g ha^{-1} was a near lethal dose of herbicide in both species, particularly in G.aparine. Some regrowth of treated plants even at 50g ha^{-1} was nonetheless evident and was reflected in fresh weights, dry weights and levels of proteins, amino acids and reducing sugars. This was due however, to new growth rather than recovery of injured tissue (also see section 2.3.1.2).

Figs. 20 - 23 show a dramatic decrease in the fresh weight / dry weight ratio in treated plants, reflecting rapid desiccation of damaged foliage following tissue collapse. The fresh weight / dry weight ratio of G.aparine sprayed at the equivalent of 50 g ha^{-1} M&B 39279 / acifluorfen decreased to approximately 60% of the control value and that of V.arvensis to 80% of the control, this suggesting a greater resistance to the effects of these compounds than G.aparine.

Changes in fresh weight were qualitatively very similar to changes in dry weight and are not shown.

As stated above, treatment of plants with M&B 39279 / acifluorfen resulted in rapid desiccation. It would therefore, have been

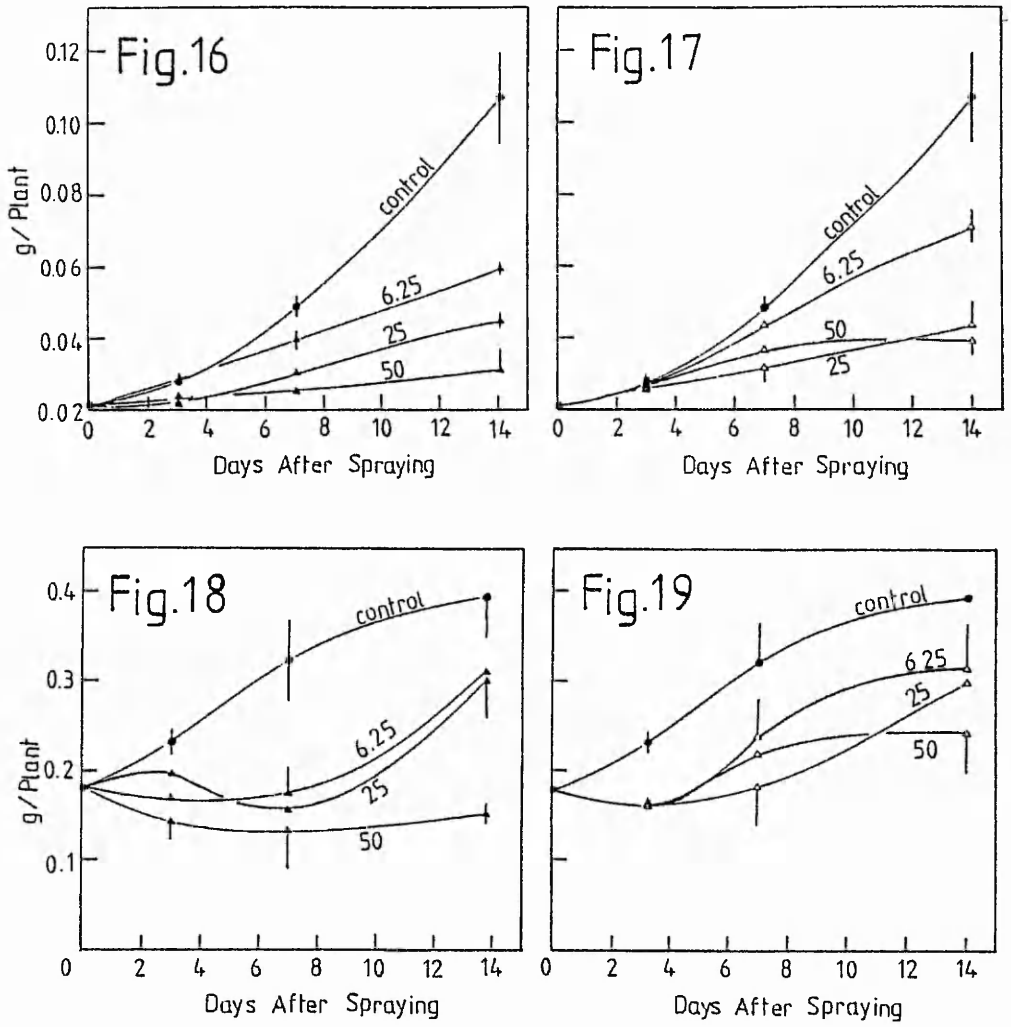
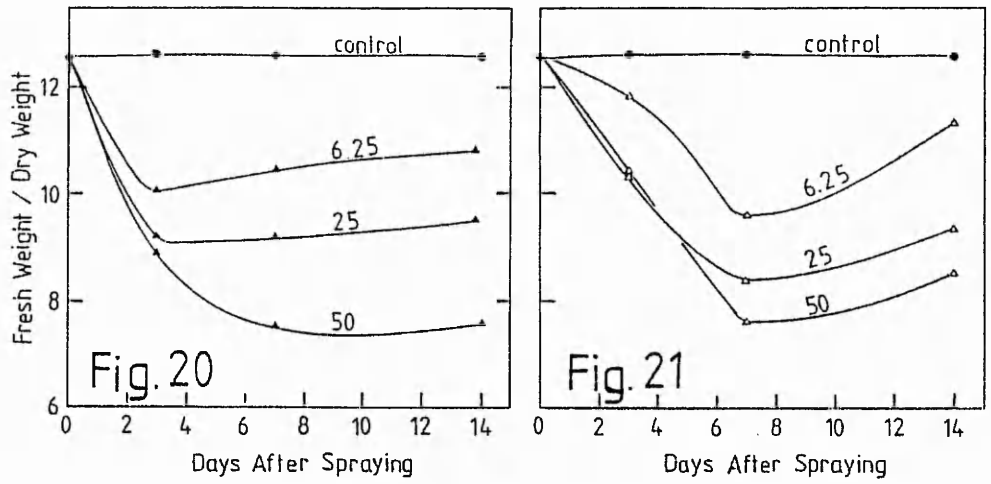


Figure 16. Changes in dry weight of G.aparine following treatment with 0 (control), 6.25, 25 and 50g ha^{-1} equivalent of M&B 39279.

Figure 17. Changes in dry weight of G.aparine following treatment with 0 (control), 6.25, 25 and 50g ha^{-1} equivalent acifluorfen.

Figure 18. Changes in dry weight of V.avensis following treatment with 0 (control), 6.25, 25 and 50g ha^{-1} equivalent M&B 39279.

Figure 19. Changes in dry weight of V.arvensis following treatment with 0 (control), 6.25, 25, and 50 g ha^{-1} equivalent acifluorfen.



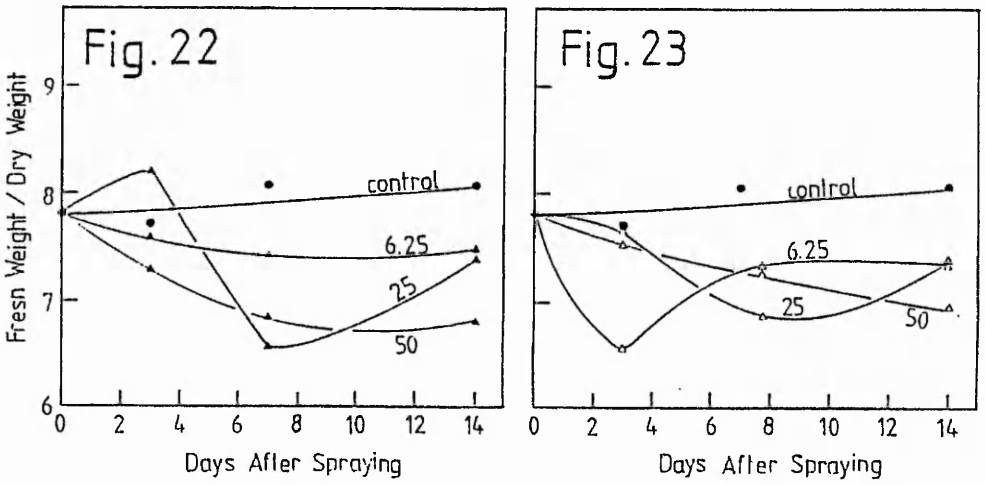


Figure 20. Changes in fresh weight / dry weight ratio of G.aparine following treatment with 0 (control), 6.25, 25 and 50g ha^{-1} equivalent M&B 39279.

Figure 21. Changes in fresh weight / dry weight ratio of G.aparine following treatment with 0 (control), 6.25, 25, and 50g ha^{-1} equivalent acifluorfen.

Figure 22. Changes in fresh weight / dry weight ratio of V.arvensis following treatment with 0 (control), 6.25, 25, and 50g ha^{-1} equivalent M&B 39279.

Figure 23. Changes in fresh weight / dry weight ratio of V.arvensis following treatment with 0 (control), 6.25, 25 and 50g ha^{-1} equivalent acifluorfen.

inappropriate to express changes in metabolite levels as grammes of metabolite per gramme fresh weight as the fresh weight itself changed during symptom development. As an alternative, data were expressed on a gramme per plant basis. For comparison, fresh weights of plants used for analysis were shown on graphs.

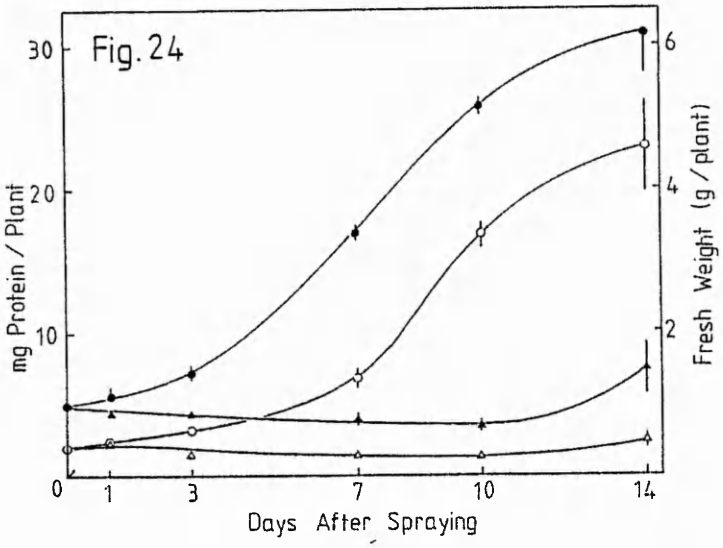
2.3.2.2 Changes in levels of major metabolites

The soluble protein fraction (which would have been enriched in many cytosolic, chloroplastic and vacuolar proteins) in both G.aparine and V.arvensis closely followed changes in fresh weight in both herbicide treated and control plants over the observed period (Figs. 24 - 27). The insoluble protein fraction (consisting largely of cell wall proteins, large protein complexes and integral membrane proteins) changed in a similar pattern to changes in fresh weight in both herbicide treated and untreated control plants (Figs. 28 - 31). Similarly, the microsomal fraction proteins (mitochondrial, peroxisomal and endomembrane proteins) changed in a similar pattern to changes in fresh weight in both herbicide treated and control plants.

Changes in free amino acid content of control plants closely followed chnges in fresh weight. In both M&B 39279 and acifluorfen treated plants however, there was a noticeable increase in free amino acids not accompanied by fresh weight changes reaching a peak around 7 days after spraying, then declining before a slight recovery around 14 days after spraying. This transient increase was far more noticeable in G.aparine than V.arvensis (Figs. 36 - 39).

Changes in reducing sugar levels in control plants followed closely changes in fresh weight. Changes of reducing sugar levels in M&B 39279 and acifluorfen treated plants however, showed a transient increase with a maximum around 7 days after spraying in G.aparine and between

3 and 7 days after spraying in V.arvensis (Figs. 40 - 43).



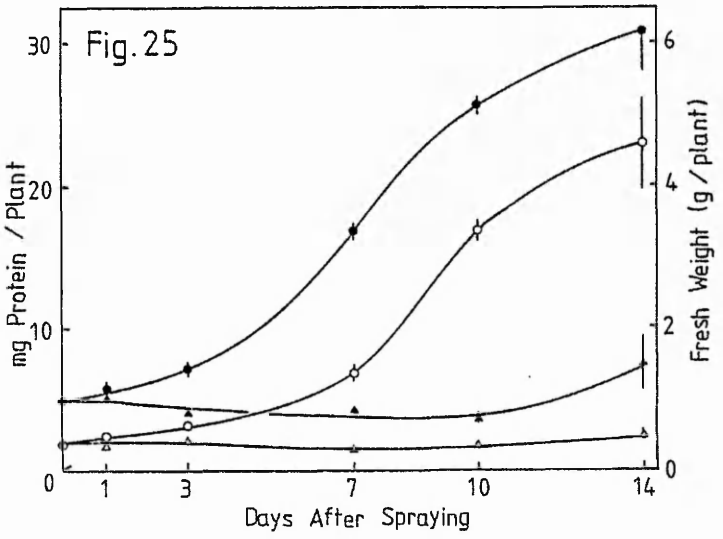
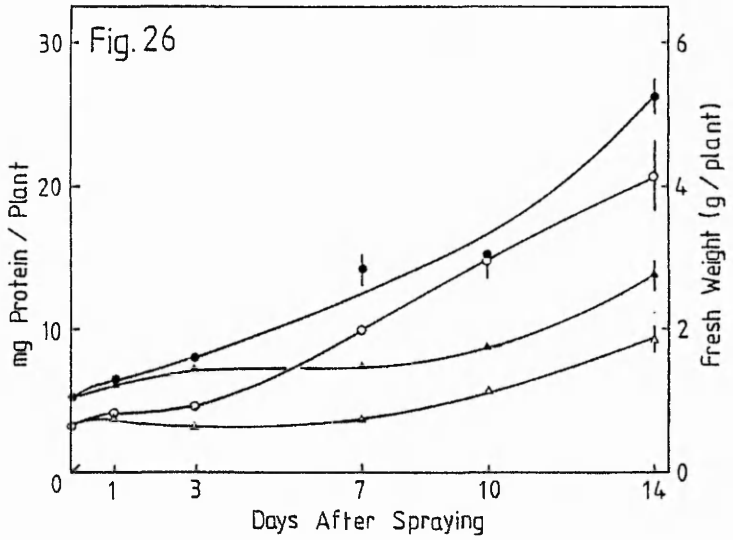


Figure 24. Changes in soluble protein content of G.aparine sprayed with 50g ha^{-1} equivalent M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = soluble protein.

Figure 25. Changes in soluble protein content of G.aparine sprayed with 50g ha^{-1} equivalent acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = soluble protein.

For figures 24 to 43 inclusive, data are means of 4 replicates and vertical bars are 2x standard error.



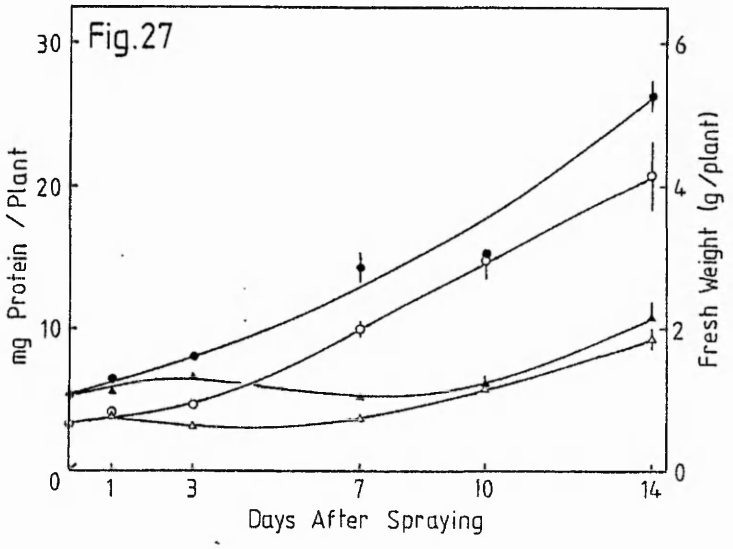
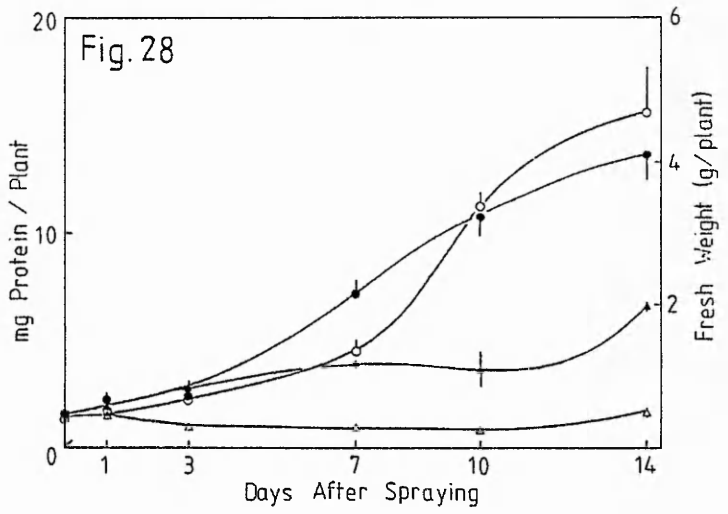


Figure 26. Changes in soluble protein content of V.arvensis sprayed with 50g ha^{-1} equivalent M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = soluble protein.

Figure 27. Changes in soluble protein content of V.arvensis sprayed with 50g ha^{-1} equivalent acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = soluble protein.



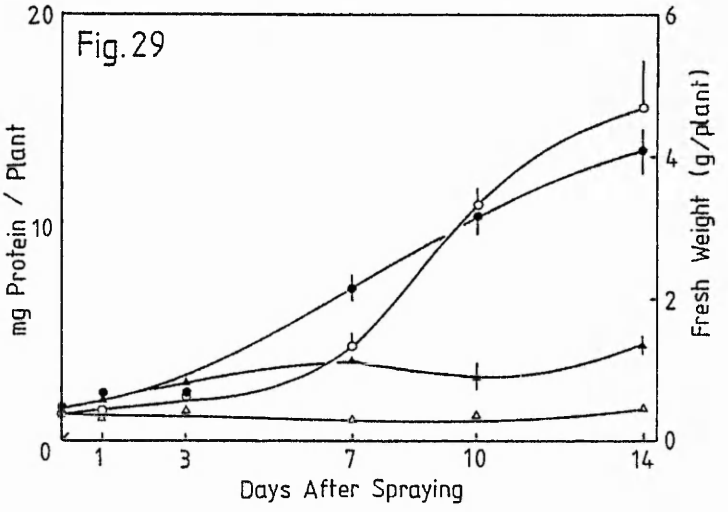
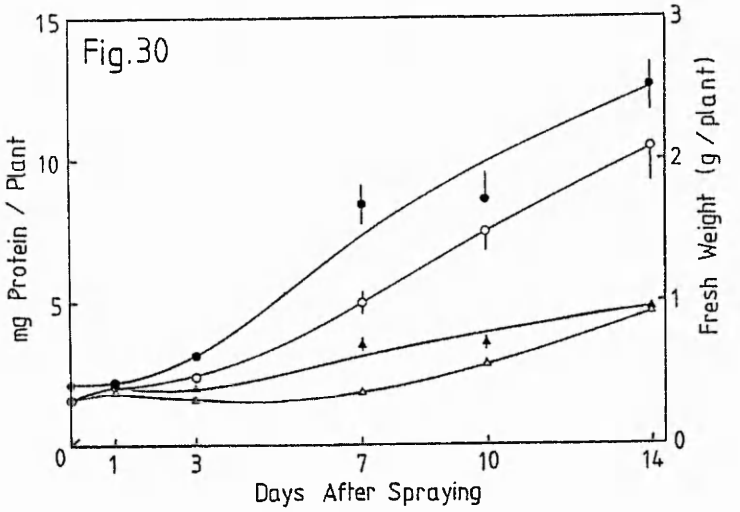


Figure 28. Changes in insoluble protein content of G.aparine sprayed with 50g ha^{-1} equivalent M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = insoluble protein.

Figure 29. Changes in insoluble protein content of G.aparine sprayed with 50g ha^{-1} equivalent acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = insoluble protein.



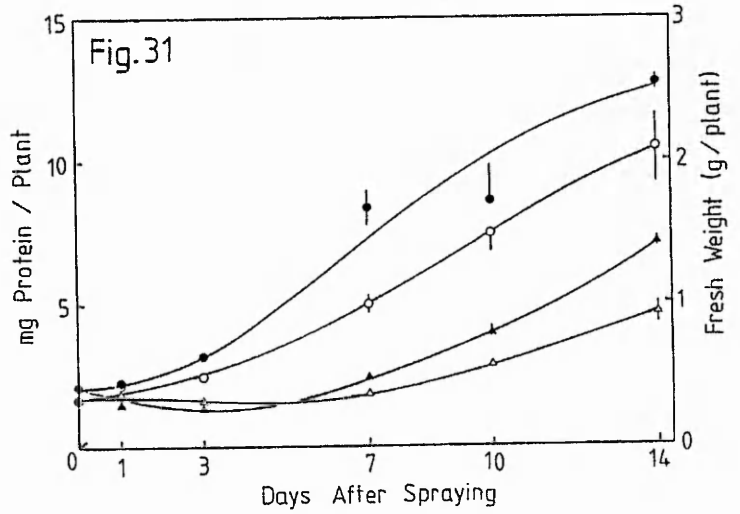
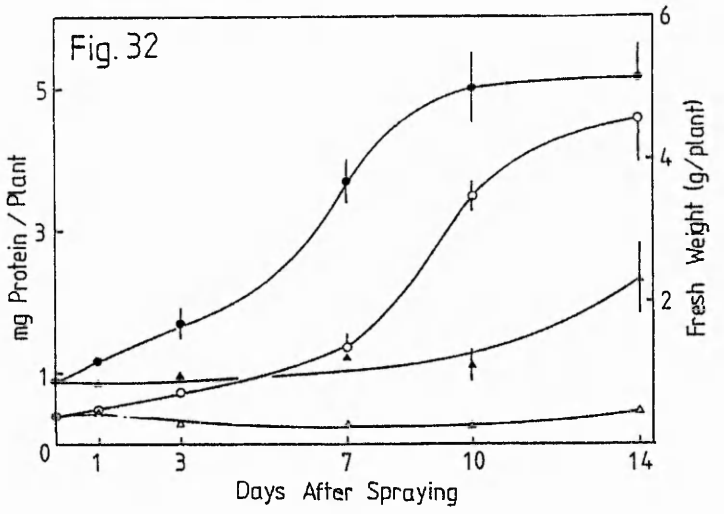


Figure 30. Changes in insoluble protein content of V.arvensis sprayed with 50g ha^{-1} equivalent M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = insoluble protein.

Figure 31. Changes in insoluble protein content of V.arvensis sprayed with 50g ha^{-1} equivalent acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = insoluble protein.



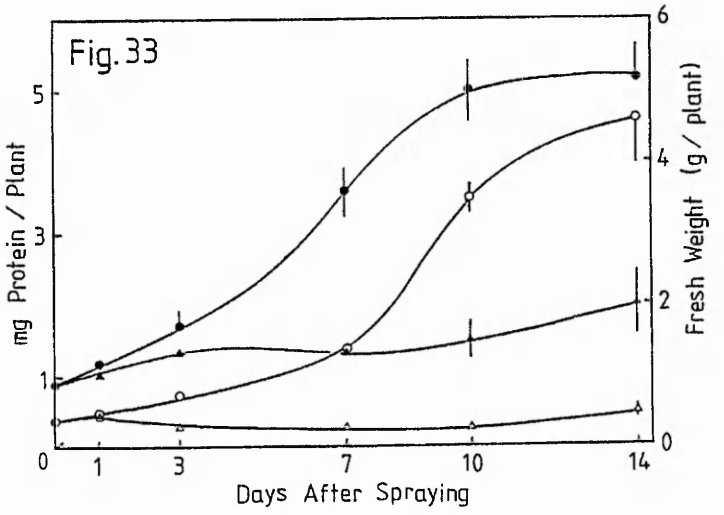
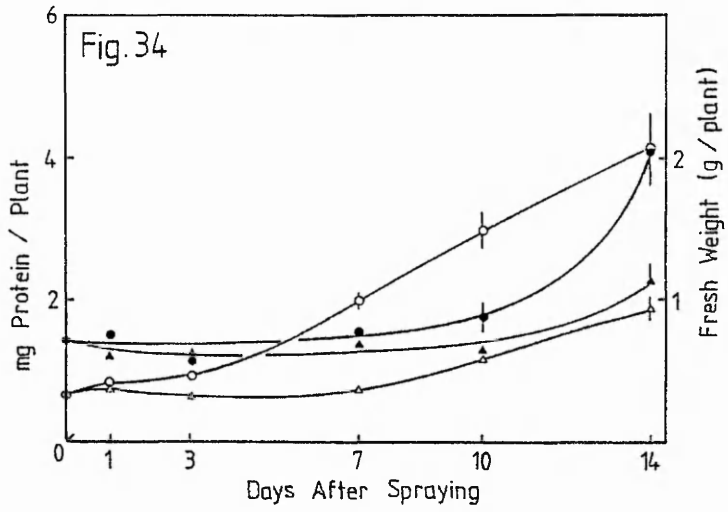


Figure 32. Changes in microsomal protein content of G.aparine sprayed with 50g ha^{-1} equivalent M&B 39279 compared with fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = microsomal protein.

Figure 33. Changes in microsomal protein content of Gaparine sprayed with 50g ha^{-1} equivalent acifluorfen compared with fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = microsomal protein.



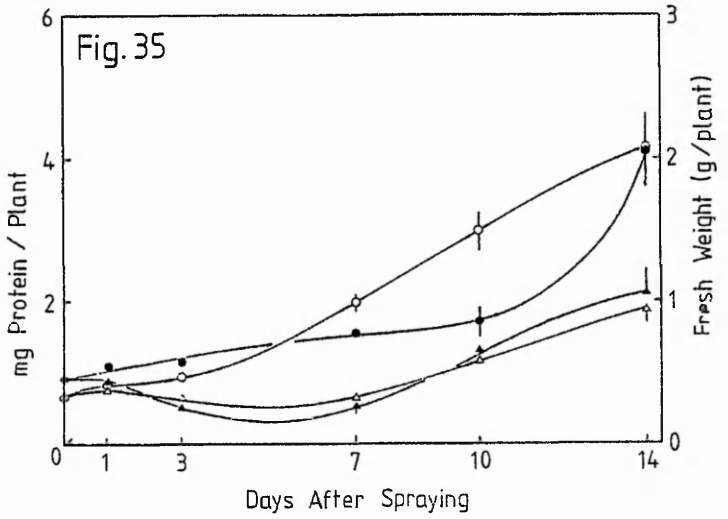
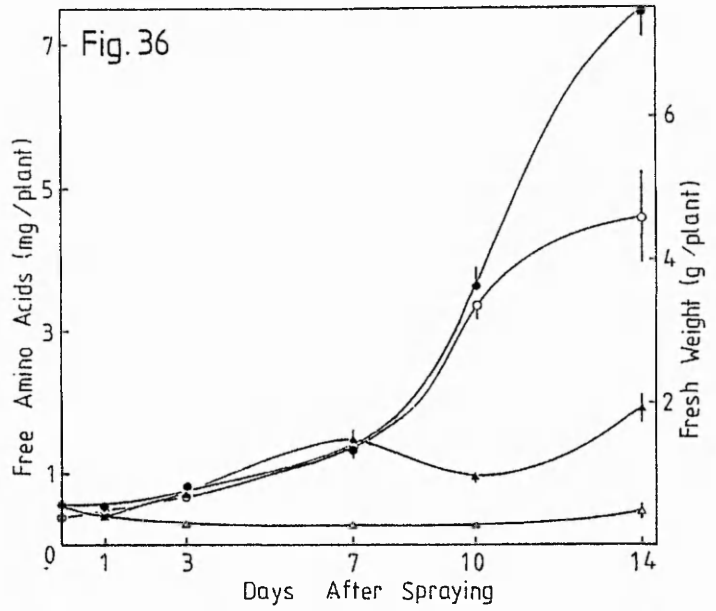


Figure 35. Changes in microsomal protein content of V.arvensis sprayed with 50g ha^{-1} equivalent acifluorfen compared with fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = microsomal protein.

Figure 34. Changes in microsomal protein content of V.arvensis sprayed with 50g ha^{-1} equivalent M&B 39279 compared with fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, Closed symbols = microsomal protein.



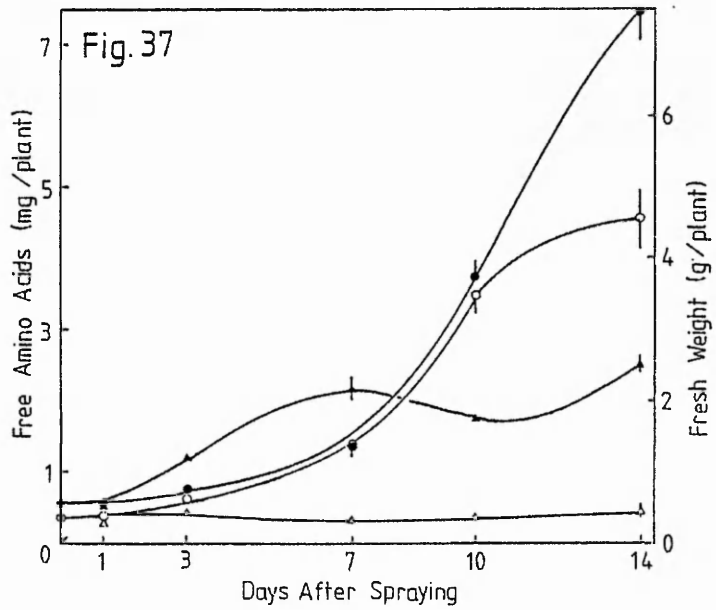
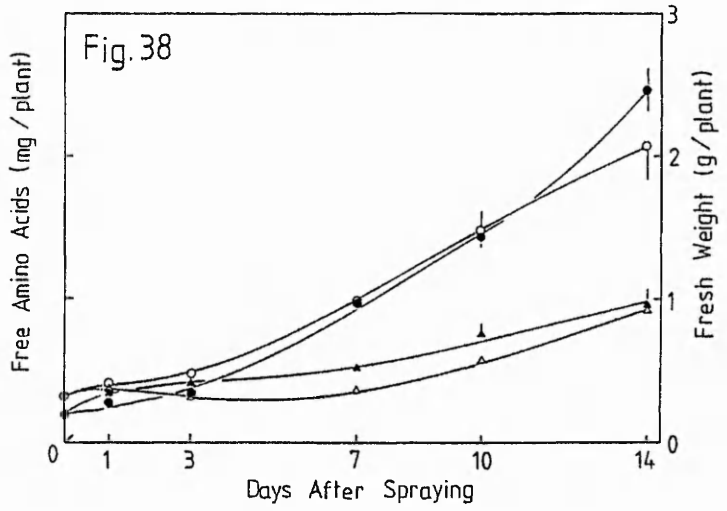


Figure 36. Changes in free amino acid content of G.aparine sprayed with 50g ha^{-1} M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = amino acids.

Figure 37. Changes in free amino acid content of G.aparine sprayed with 50g ha^{-1} acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = amino acids.



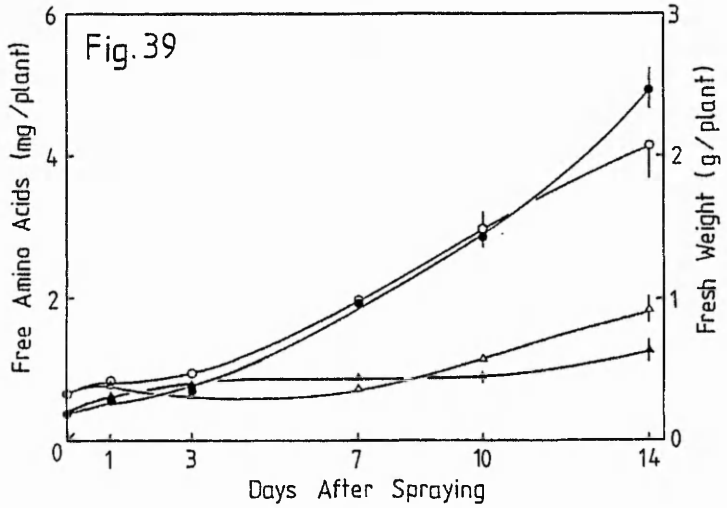
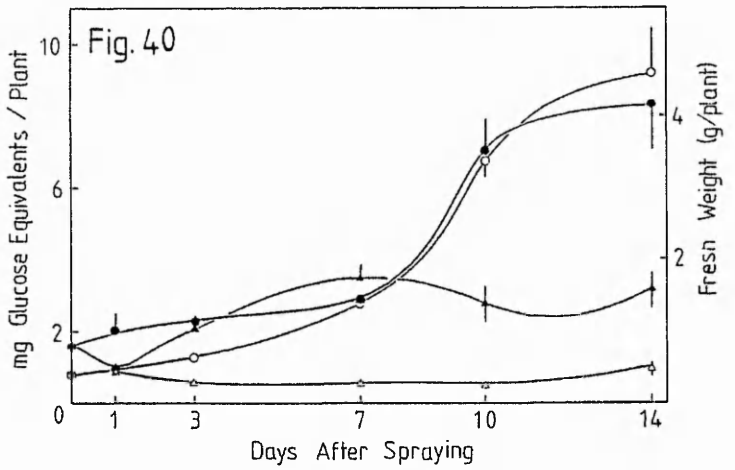


Figure 38. Changes in free amino acid content of V.arvensis sprayed with 50 g ha^{-1} M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = amino acids.

Figure 39. Changes in free amino acid content of V.arvensis sprayed with 50g ha^{-1} acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = amino acids.



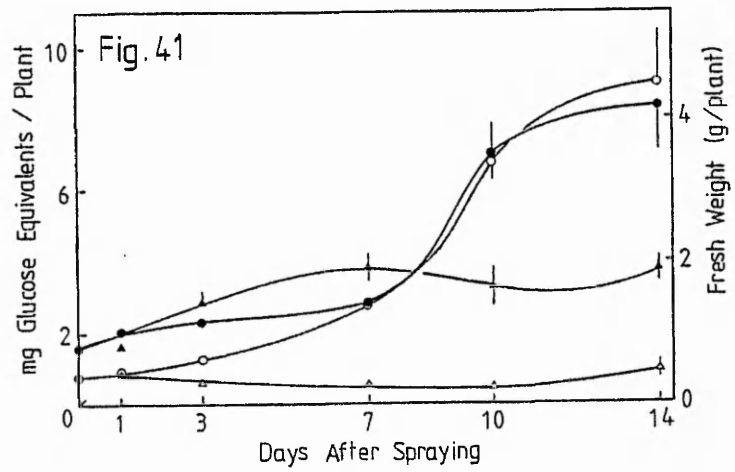
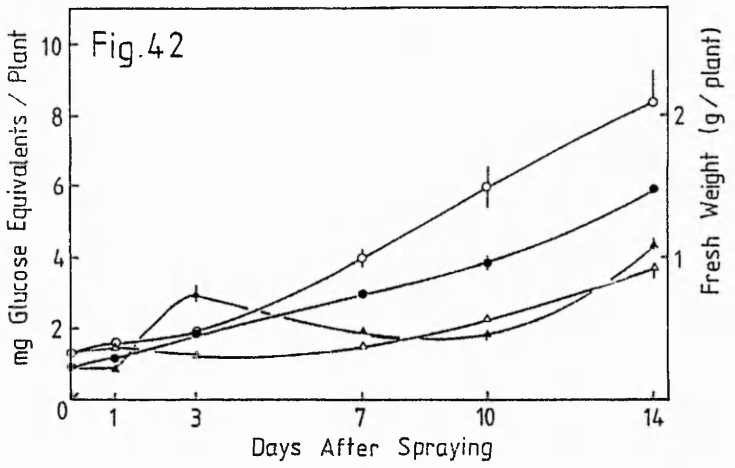


Figure 40. Changes in reducing sugar content of G.aparine sprayed with 50g ha^{-1} M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = reducing sugars.

Figure 41. Changes in reducing sugar content of G.aparine sprayed with 50g ha^{-1} acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = reducing sugars.



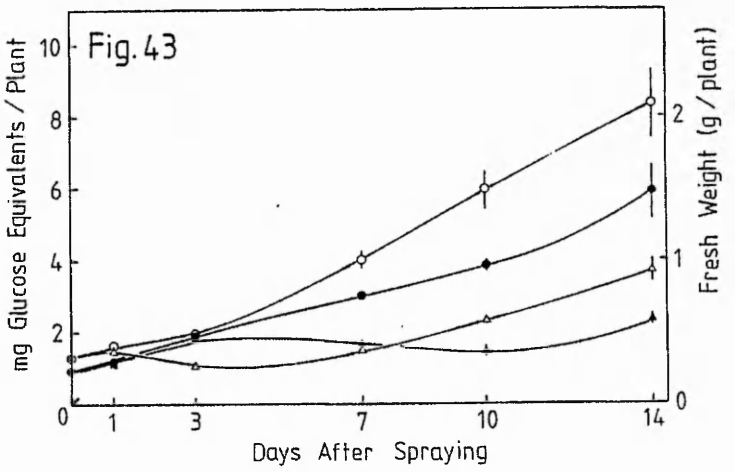


Figure 42. Changes in reducing sugar content of V.arvensis sprayed with 50g ha^{-1} M&B 39279 compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbol = fresh weight, closed symbols = reducing sugars.

Figure 43. Changes in reducing sugar content of V.arvensis sprayed with 50g ha^{-1} acifluorfen compared with changes in fresh weight. (● ○) = control, (▲ △) = herbicide treated. Open symbols = fresh weight, closed symbols = reducing sugars.

2.4 DISCUSSION

Visual observation of symptom development following administration of M&B 39279 / aciflorfen to G.aparine and V.arvensis revealed pathologic lesions typical of rapidly acting, contact herbicides. These symptoms were, in both species, always seen to develop initially and far more rapidly on young leaves. This may suggest that the herbicides were acting upon or dependent upon an anabolic system (having a higher turnover in young leaves than older tissues). Alternatively, this effect could be due to more rapid uptake of herbicide through the thinner and less well developed cuticle / immature cell walls of young leaves, this being the major barrier to herbicides reaching their target within the plant.

The accessibility of the sensitive young parts of the plants in conjunction with the contact nature of M&B 39279 / acifluorfen may have been responsible for the apparent greater tolerance of V.arvensis than G.aparine to these compounds (i.e. more rapid recovery following spraying). The spray droplets would not have reached the sensitive young growing point at the centre of the V.arvensis rosette - shaped plants, this being shielded by numerous petiolar bracts and overlapping young leaves. Conversely, the erect, open nature of G.aparine rendered all leaves, the apical growing tip and most axillary buds accessible to spray droplets. This is seen in Fig. 11B where growth of axillary buds following spraying is very limited and clearly bears symptoms of herbicide damage.

Observation of changes in major metabolite pools revealed changes in three protein fractions, free amino acids and reducing sugars which were merely typical of severely injured / dying plants. The transient increase in free amino acid and reducing sugar pools most likely

represented breakdown of proteins and polysaccharides respectively. Since no unexpected changes in protein levels were observed in this study, it was decided not to measure changes in nucleic acid levels in herbicide treated plants as originally intended. It is unlikely that changes in RNA / DNA levels in treated plants would have had no effect on protein / amino acid levels, as seen in this study. Furthermore, tissue extracts used in this study were unsuitable for detection of nucleic acids. Observation of nucleic acids would therefore, have necessitated a separate experiment using different plants, an exercise which was not deemed worthwhile.

It is clear from the data described in this chapter that it is unlikely that either M&B 39279 or acifluorfen exerts any rapid and / or significant effect upon primary metabolism in G.aparine or V.arvensis. The effects of these herbicides are therefore, either an unidentified interference with secondary metabolism or their herbicidal effects are independant of metabolism.

It is seen from the data described in this chapter that M&B 39 279 behaves both quantitatively and qualitativey in an indistinguishable manner to the DPE herbicide acifluorfen and so the choice of acifluorfen to parallel M&B 39 279 activity in these experiments appears to have been vindicated.

CHAPTER 3

ULTRASTRUCTURAL EFFECTS OF M&B 39279 AND ACIFLUORFEN

3.1 INTRODUCTION

A number of publications have reported on ultrastructural effects of DPE's in plants. These have been described and discussed previously in section 1.3.2, to which the reader is referred. Considering these reports, with the exception of that of Bowyer et al, (1987b), all other papers have described development of DPE - type herbicide induced ultrastructural changes in a single species, and indeed a single tissue, namely Cucumis sativus (cucumber) cotyledons. In addition to the need to document the ultrastructural effects of M&B 39279, there also exists, therefore, a need to characterise the ultrastructural effects of DPE's in other species' and in greater detail than previously reported. In view of these gaps in the literature and to reveal subcellular effects of M&B 39279, the aims of experiments reported on in this chapter were to attempt to construct a chronological sequence of ultrastructural changes following administration of M&B 39279 / acifluorfen to G. aparine and / or V. arvensis.

During this experiment, the initial approach was to study ultrastructural changes in leaves of G. aparine and V. arvensis sprayed with M&B 39279 and acifluorfen. The results from this work were, however, somewhat variable and difficult to interpret in a chronological sense. Only a limited number of micrographs from this work are therefore, shown (Plates 1,2 and 3). A second approach was more successful and involved administration of herbicides by floating excised G. aparine leaflets on herbicide solutions under closely controlled conditions. To substantiate ultrastructural observations, leaf photosynthesis, chlorophyll content and membrane integrity were also followed in leaves maintained under identical conditions.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL

Galium aparine grown as described in Chapter 2 were used when plants had two whorls of true leaves with a third expanding. Viola arvensis, plants grown as in chapter 2, bore 5-6 leaves.

3.2.2 HERBICIDE TREATMENT

For ultrastructural studies of sprayed plants, G.aparine and V.arvensis plants were sprayed with M&B 39279 and acifluorfen in 5% (v/v) aqueous acetone at a rate equivalent to 50g ha^{-1} / 200ℓ^{-1} ha^{-1} as described in Chapter 2 and maintained under glasshouse conditions following spraying.

For treatment of excised leaves, the constraints of time permitted only G.aparine to be used. Leaves were cut from the second whorl and floated abaxial surface down on 100μM aqueous solutions of M&B 39279 and acifluorfen (10 leaves on 10ml of solution) in 50mm diameter petri dishes. All solutions, including control treatments, contained 1% (v/v) aqueous acetone. The petri dishes were maintained at 25°C by floating on a water bath with water circulating to and from the bath via a pump, thermostat and heat exchanger. The petri dishes were also maintained under constant light conditions (50μmol m^{-2} sec^{-1} PPFD) provided by a bank of 'natural white' fluorescent tube lamps (G.E.C.). Light was excluded from dark treatments by aluminium foil. These conditions were employed in all experiments in this chapter, except for that described in the above paragraph.

3.2.3 LEAF PHOTOSYNTHETIC CAPABILITY AND CHLOROPHYLL CONTENT

After 3, 5, 15, 20, 25 and 30 hours light and after 30 hours darkness, the photosynthetic capability of the excised G.aparine leaves was assessed using a Hansatech LD2 gas - phase oxygen electrode (Hansatech Ltd, Paxman Rd, Hardwick Industrial Estate, Kings Lynn, U.K.). This apparatus was maintained at 25°C by thermostatically regulated water circulating through the instrument and illuminated with a tungsten - halogen projector lamp supplying 500 $\mu mol^{-2}\ s^{-1}$ PPFD to the leaves within the electrode. Photosynthetic capability was determined under this light intensity as it was found to permit a high rate of photosynthesis (Fig. 45) but avoiding the problem of heating which may have occurred had a higher light intensity been used.

The LD2 oxygen electrode has been described in detail by Delieu and Walker (1983) and incorporates a Clarke - type oxygen electrode but allows measurement of gas phase oxygen in a temperature - controlled chamber enclosing a leaf disc / small excised leaves. Carbon dioxide is provided by a small quantity of 1M sodium carbonate / bicarbonate buffer, pH 9.0. This generates an atmosphere of approximately 5% carbon dioxide in the electrode chamber, allowing sustained photosynthesis measurements to be made in the closed electrode chamber which would otherwise be rapidly depleted of carbon dioxide and overcoming the stomatal barrier to diffusion of carbon dioxide.

The electrode accommodated five G.aparine leaves and measurements were repeated four times in two experiments. The electrode was calibrated as described by Delieu and Walker (1983), flushing the electrode leaf chamber with nitrogen to obtain a zero oxygen signal and determining the chamber gas space volume by introducing a known volume of air into the sealed electrode chamber and thereby increasing

the partial pressure of oxygen in the chamber. Electrode output was monitored on a chart recorder. Following measurement of photosynthesis, leaves were ground using a mortar and pestle into 80% (v/v) aqueous acetone and their chlorophyll content determined using the absorption coefficients of Arnon (1949).

3.2.4 MEMBRANE INTEGRITY

Electrolyte leakage (i.e. an increase in bathing solution conductivity) and generation of thiobarbituric acid - reacting material (i.e. malondialdehyde, (MDA), a breakdown product of lipid peroxidation; see Fig. 44; Mead,(1976) and Pryor (1978)), provided measureable parameters broadly indicating membrane integrity. Conductivity of bathing solutions was measured after 5, 15, 20, 25 and 30 hours light and after 30 hours darkness using a PTI-18 digital conductivity meter (FSA Ltd, Loughborough, U.K.) coupled with a CC005 'flow - cell (Canterbury Scientific, Canterbury, U.K.) which allowed sampling, measurement and return of a small volume of liquid (approximately 3ml) from and back to the petri dishes. In a separate experiment, MDA was measured as follows: After 5, 15, 20 25 and 30 hours light and after 30 hours darkness, a 2ml sample of leaf bathing solution was mixed with an equal volume of 0.5% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid and this mixture heated in a boiling water bath for 25 minutes. The mixture was then cooled, centrifuged at 2500 x g for 5 minutes and absorbance of the supernatant determined at 532nm. A millimolar extinction coefficient of 155 mM cm^{-1} was used to estimate the MDA concentration in solution (Slater, 1984). Initial attempts to measure MDA generation within treated leaves were abandoned due to high absorbance readings at 532nm following thiobarbiturate reaction with extracts of untreated

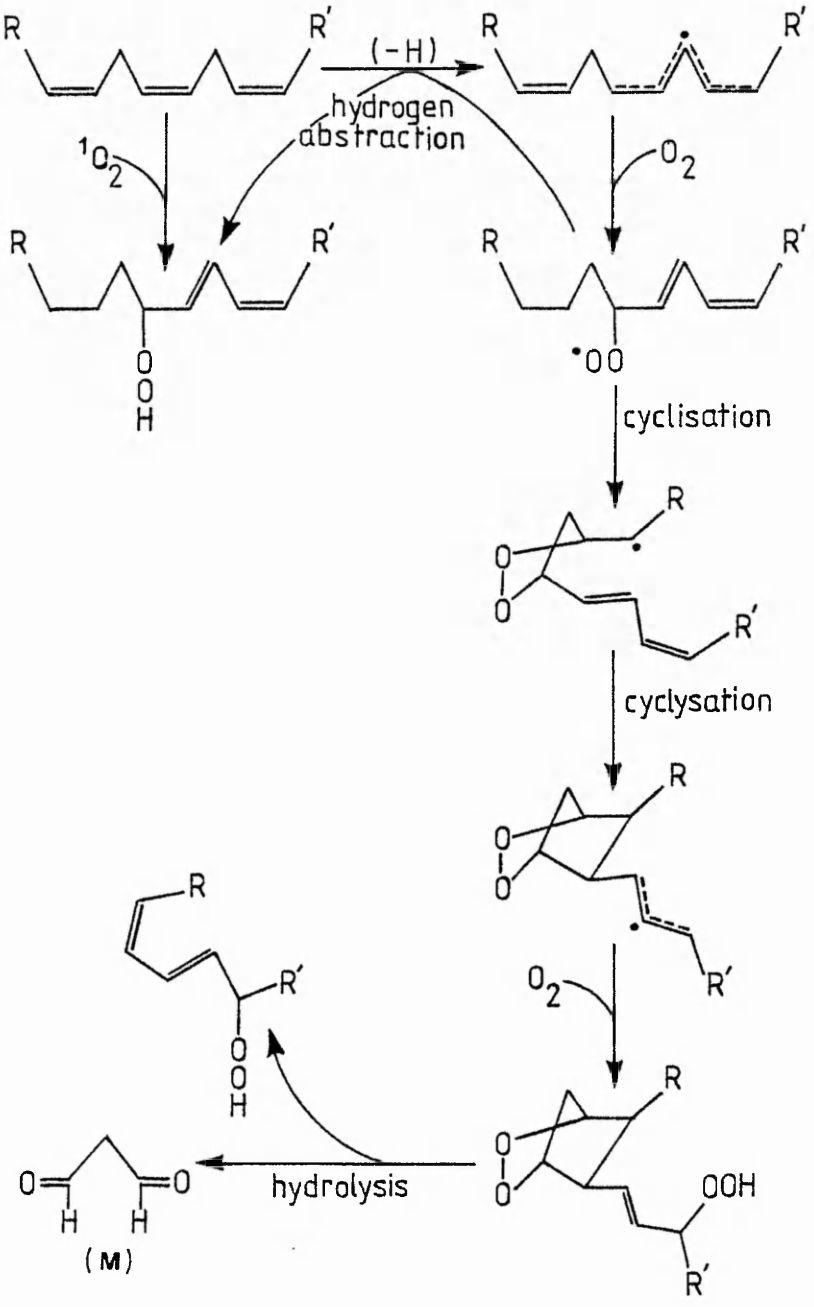


Figure 44. Sequence of events leading to formation of malondialdehyde (M), lipoyl hydroperoxides and endoperoxides from a polyunsaturated fatty acid. R and R' are the carboxyl and alkyl ends of the fatty acid molecule. Based on a scheme by Pryor, 1978.

leaves, indicating the presence of large amounts of interfering substances in G.aparine leaf tissue, the presence of such compounds commonly limiting the usefulness of this technique (Slater, 1984). Indirect measurement of MDA, i.e. within the leaf bathing medium was therefore necessary.

3.2.5 ELECTRON MICRSCOPY

Sprayed plants were sampled at 4, 12, 18, 24, 48, 72 hours and 1 week after spraying. G.aparine leaves floating on herbicide solutions were sampled after 3, 5, 15, 20, 25 and 30 hours in light and after 30 hours in darkness. Approximately $2mm^2$ pieces of leaf were cut from a standardised portion of the leaves, about a third of the length along the leaf from the petiolar end. Samples were fixed in 3% (v/v) glutaraldehyde in 50mM phosphate buffer, pH 7.0, for 30 minutes, washed 2 x 5 minutes in phosphate buffer followed by post fixation in 2% (w/v) osmium tetroxide in 50mM phosphate buffer, pH 7.0 for 2 hours. Samples were then stained for 30 minutes in 0.5% (w/v) aqueous uranyl acetate prior to dehydration with an acetone series (30, 50, 75, 95% (v/v) aqueous acetone, 5 minutes in each, then 2 x 30 minutes in 100% acetone previously dried over anhydrous sodium sulphate). Following dehydration, samples were transferred to 25% Spurr's resin (Spurr, 1969) in acetone overnight then 2 hours in each of 50% and 100% resin before curing at 70°C for 24 hours. Ultrathin sections were cut using a Reichert OMU-2 ultramicrotome, mounted on uncoated copper grids and stained with lead citrate (Reynolds, 1963) for 15 minutes. Grids were observed using an AEI EM6G transmission electron microscope. It should be noted that the above method, used for processing excised leaves, was a modification of that originally used for preparation of samples from sprayed plants.

Sprayed plant samples were processed in an identical manner except that uranyl acetate was not applied pre - dehydration but grids were floated on 0.5% (w/v) uranyl acetate for 20 minutes prior to lead staining. This method, however, stained mitochondrial and tonoplast membranes poorly and so was not used for later experiments. A minimum of 3 grids from each of 4 resin blocks were examined per treatment. Micrographs presented are representative of observed phenomena.

3.3 RESULTS

3.3.1 GENERAL COMMENTS

The most apparent underlying trend throughout the results of experiments described in this chapter was the qualitative and quantitative similarity of the effects of M&B 39279 and acifluorfen. As in Chapter 2 therefore, data from both of these compounds will be described and discussed collectively.

3.3.2 PHOTOSYNTHETIC CAPABILITY AND CHLOROPHYLL CONTENT

After a small initial increase, the photosynthetic capability of light incubated control leaves remained constant throughout the experiment (Fig. 46). The chlorophyll content of this tissue also remained constant over the 30 hour period. The photosynthetic capability of illuminated herbicide treated leaves diminished almost immediately whereas treated leaf chlorophyll content remained constant until after 15 hours incubation in light when a rapid decrease was noted. No changes in photosynthetic capability nor chlorophyll content were however, observed in dark incubated herbicide treated or control tissues.

3.3.3 MEMBRANE INTEGRITY

No noticeable increases in bathing solution conductivity nor MDA leakage occurred with illuminated control leaves (Figs. 48 and 49). Leakage of electrolytes from illuminated, herbicide treated leaves was first detectable after 15 hours incubation after which the conductivity of the bathing medium rose sharply (Fig. 48). Elevated levels of MDA were found in the bathing solutions of herbicide

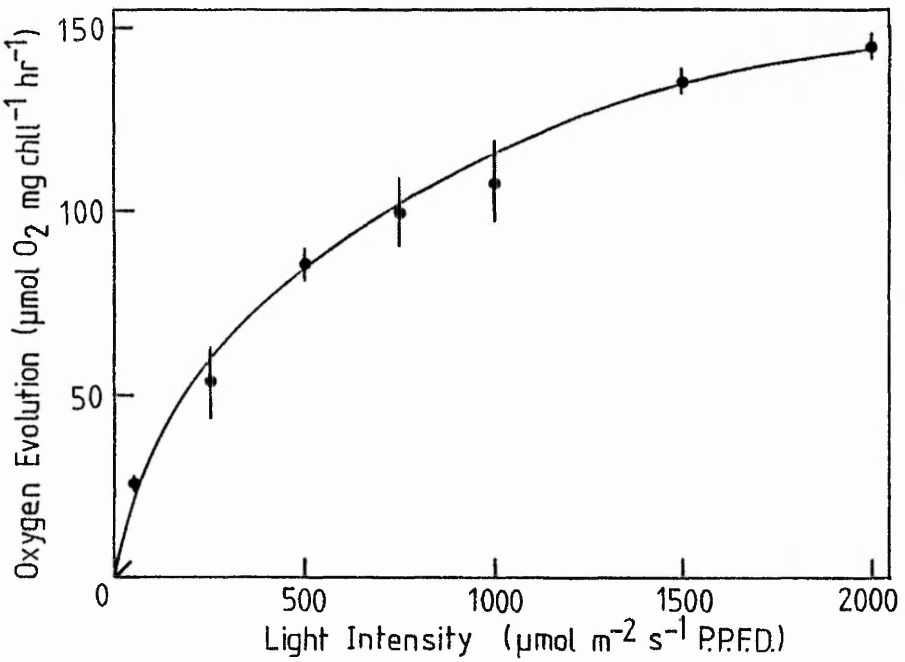


Figure 45. CO_2 - dependent oxygen evolution from excised G.aparine leaflets as a function of light intensity. Data are means of 4 replicates. Vertical bars are 2x standard error.

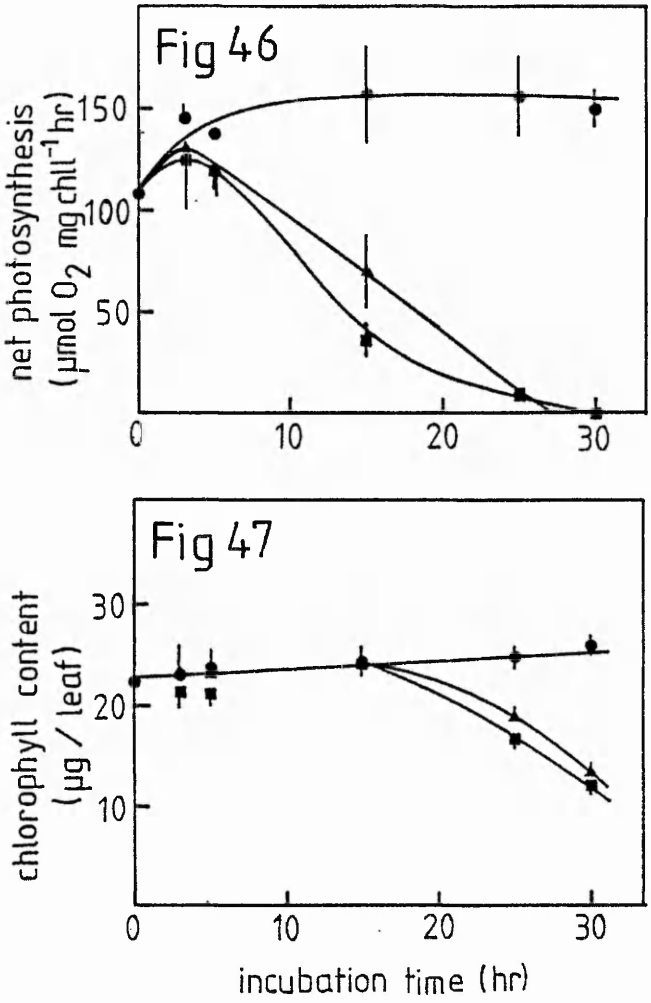


Figure 46. Changes in photosynthetic capability of G.aparine leaflets floating on 100µM M&B 39279 (▲——▲), 100µM acifluorfen (■——■), and water (●——●) under 50µmol m^{-2} s^{-1} PPFD. Vertical bars are 2x standard error.

Figure 47. Changes in chlorophyll content of G.aparine leaves floating on 100µM M&B 39279 (▲——▲), 100µM acifluorfen (■——■), and water (●——●). Vertical bars are 2x standard error.

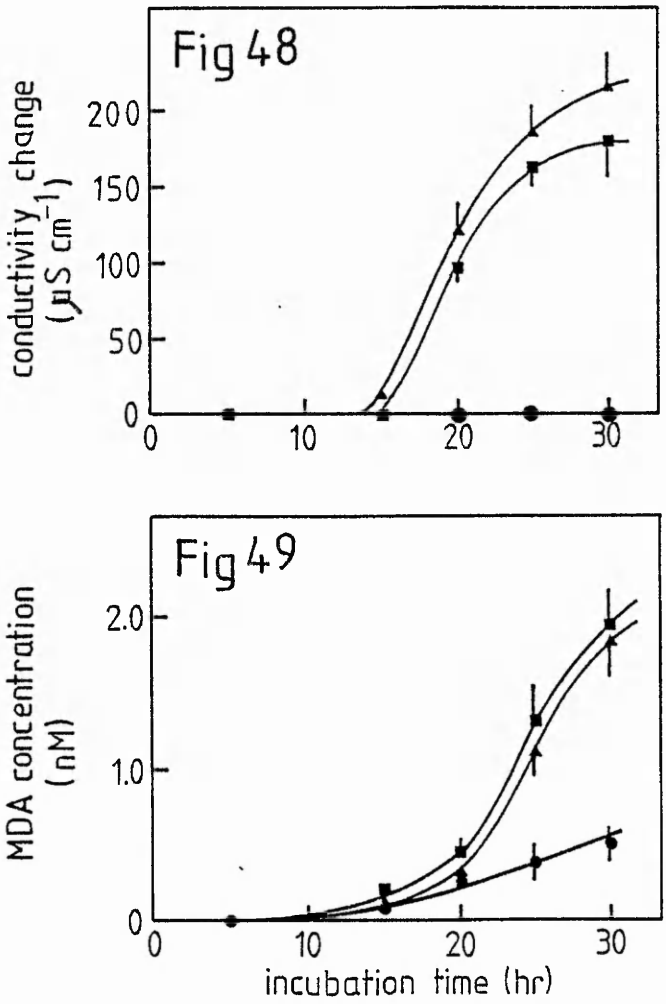


Figure 48. Electrolyte leakage from G.aparine leaflets floating on 100μM M&B 39279 (▲—▲), 100μM acifluorfen (■—■), and water (●—●). Vertical bars are 2x stadard error. Control bathing solution conductivity never exceeded 5.6 μS cm^{-1}. Conductivity change = μS cm^{-1} (test sample) - μS cm^{-1} (herbicide solution in water).

Figure 49. Malondialdehyde (MDA) leakage from G.aparine leaflets floating on 100μM M&B 39279 (▲—▲), 100μM acifluorfen (■—■), and water (●—●). Vertical bars are 2x standard error.

treated, illuminated leaves after 20 hours light (Fig. 49). Dark incubated herbicide treated and control leaf samples yielded bathing solution conductivity changes / MDA leakage in a manner indistinguishable from illuminated controls.

3.3.4 ELECTRON MICROSCOPY

3.3.4.1 Sprayed Plants

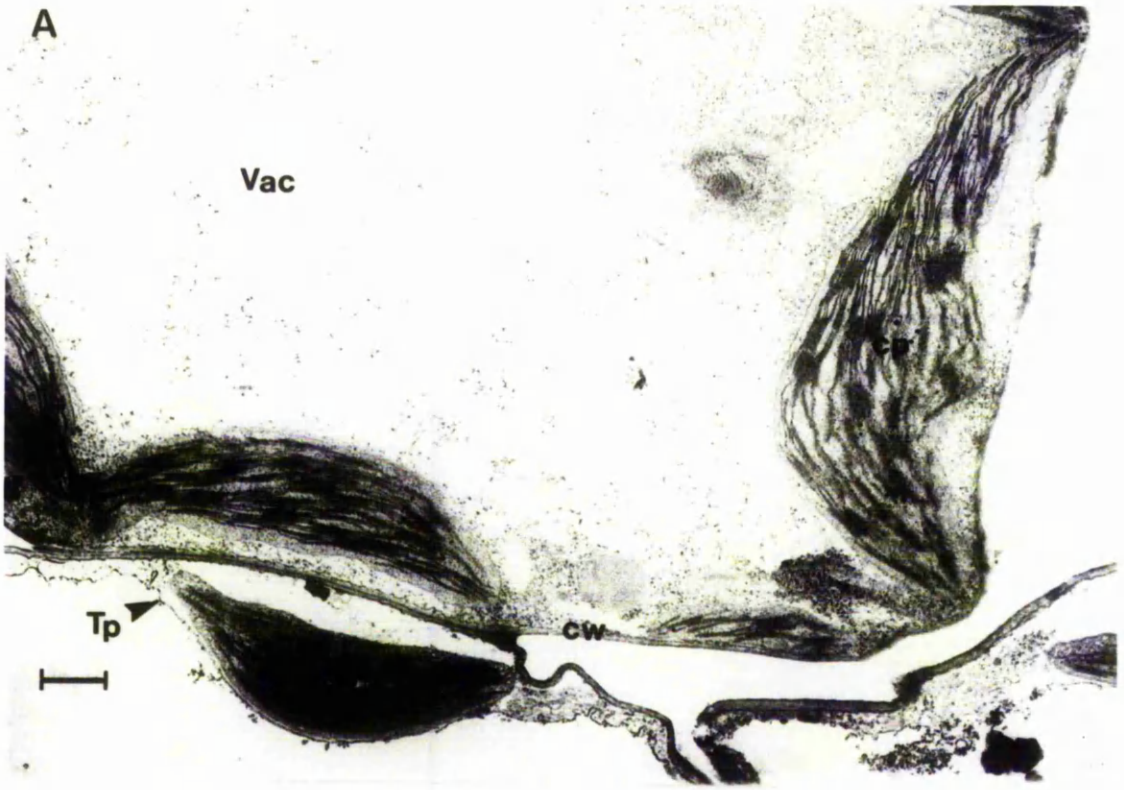
Ultrastructural analysis of herbicide sprayed plants was difficult owing to the variability of toxicity symptoms in leaves of such plants at any given time. This, although confusing did however, probably testify to the contact nature of DPE-type action, the spray droplets creating many tiny concentration gradients of herbicide within treated leaves. Further confusion was generated by the poor preservation / staining of mitochondrial and tonoplast membranes in sprayed material as mentioned in section 3.2.5 (see Plate 1). Although it was not possible to construct a chronological sequence of ultrastructural toxicity symptoms from this data, the following were however, noted. Firstly, in both G.aparine and V.arvensis, the apparently initial symptom of M&B 39279 / acifluorfen toxicity was the appearance of large vesicles within the chloroplasts (Plate 2A). Such vesicles were observed in almost all herbicide treated cells which had a good degree of cytoplasmic integrity. Secondly, following vesicle development in chloroplasts, tonoplast lysis, cytoplasmic vesiculisation and deposits of electron dense material at the periphery of chloroplasts appeared, though the sequence of these events was unclear (Plate 3A). Thirdly, chloroplast thylakoids appeared resistant to herbicide induced degeneration (Plates 2 and 3).

PLATE 1

(A) Untreated G.aparine leaf cell and (B) Untreated V.arvensis leaf cell showing large central vacuole and parietal cytoplasm containing typical ellipsoid chloroplasts.

Legend to Plates

cp = chloroplast, cpe = chloroplast envelope, CW = cell wall, eom = electron opaque material, ev = evagination, G = golgi body, iv = invagination, m = mitochondrion, mb = microbody, pg = plastoglobulus, pm = plasmalemma, sg = starch grain, Tp = tonoplast, v = vesicle, Vac = vacuole. Bar = 1 μm, all plates.



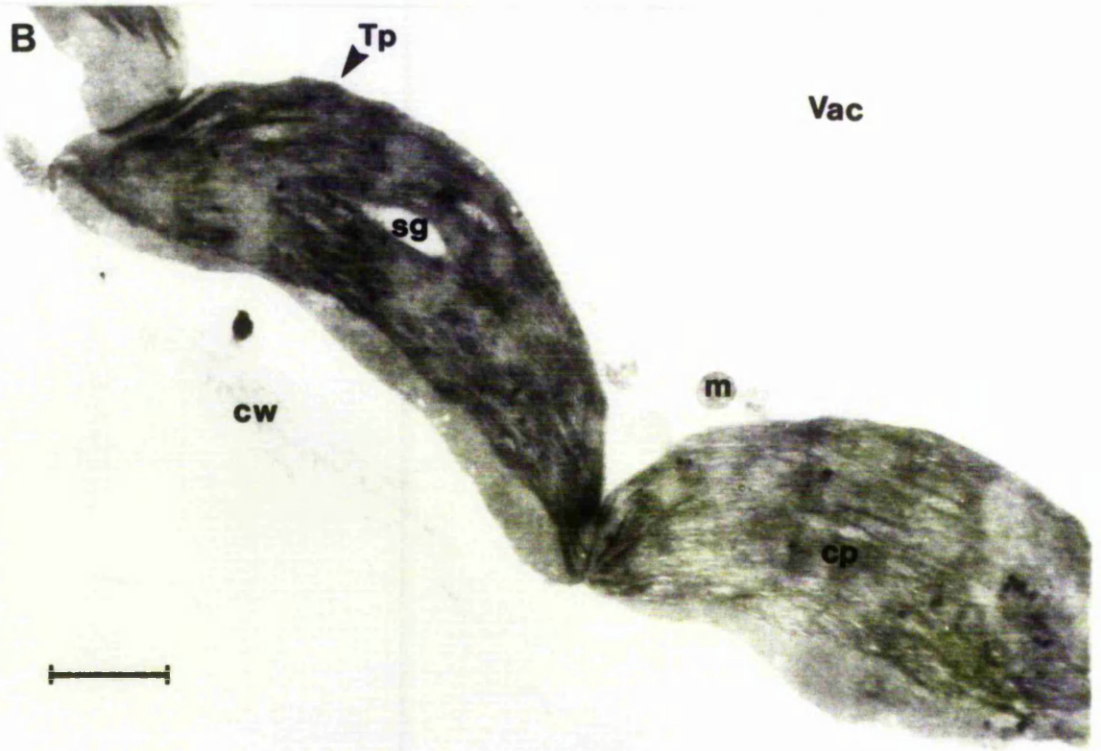
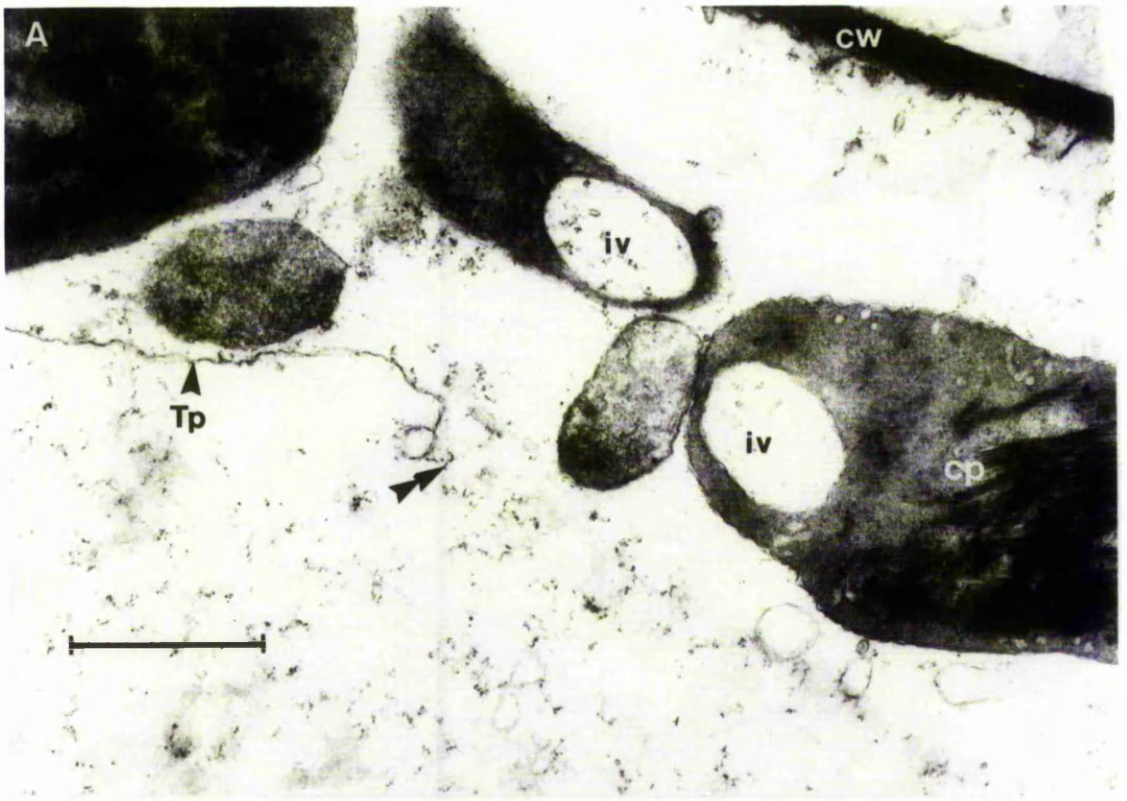


PLATE 1

Plate 2

V.arvensis leaf cells 24 hours after spraying with 25 gha^{-1} M&B 39279 (A) Note the tonoplast is fragmented (double arrow) and the presence of invaginations inside chloroplasts (B) The upper left hand cell displays a partially disrupted plasmalemma, an absence of the tonoplast and cytoplasm. Numerous vesicles are also present. Note integrity of chloroplasts in this cell.



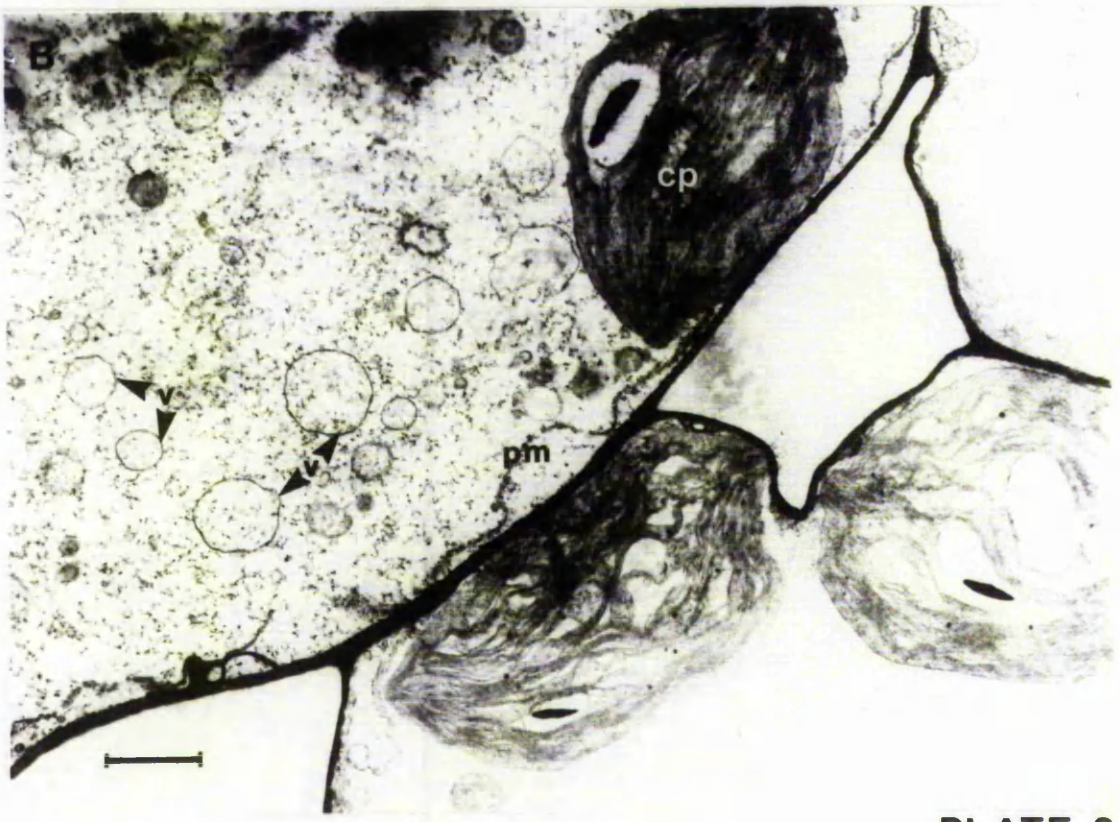
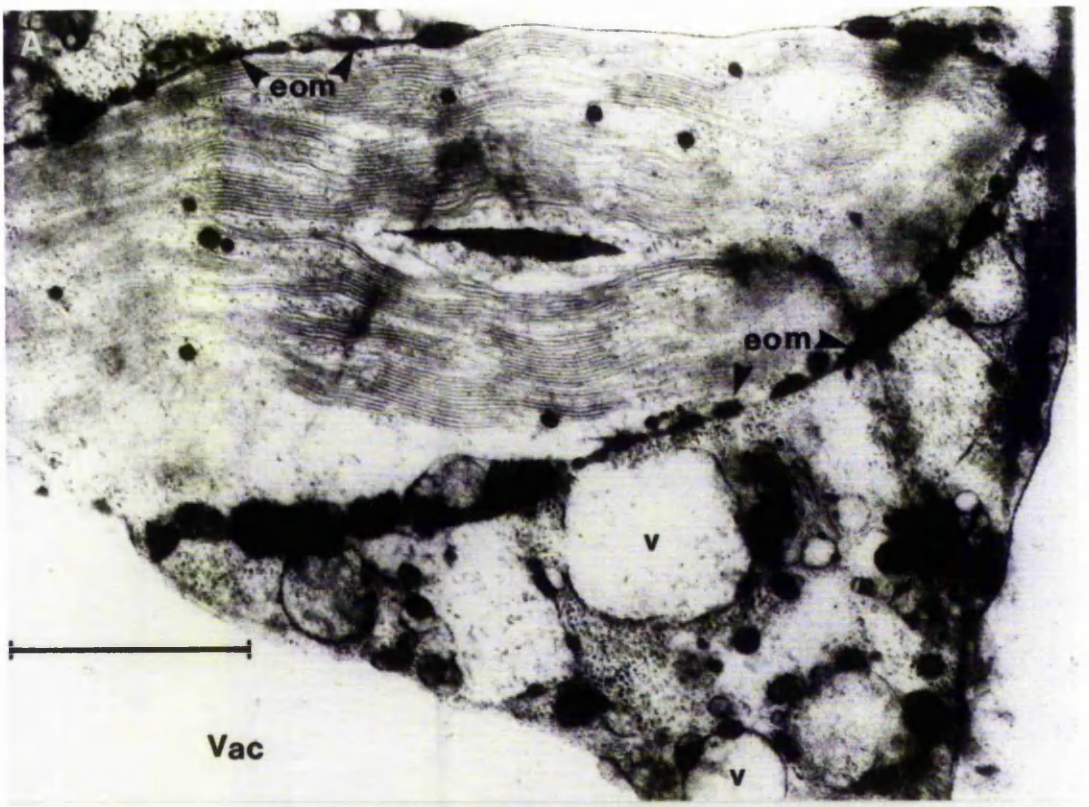


PLATE 2

Plate 3

G.aparine leaf cells (A) 48 hours and (B) 1 week after spraying with 25g ha^{-1} M&B 39279. Note in (A) the electron opaque material outlining the chloroplast and the adhering cytoplasm. In (B) note the relatively intact thylakoids in the two remaining chloroplasts. The remainder of this cell is filled with electron opaque material and numerous membraneous vesicles.



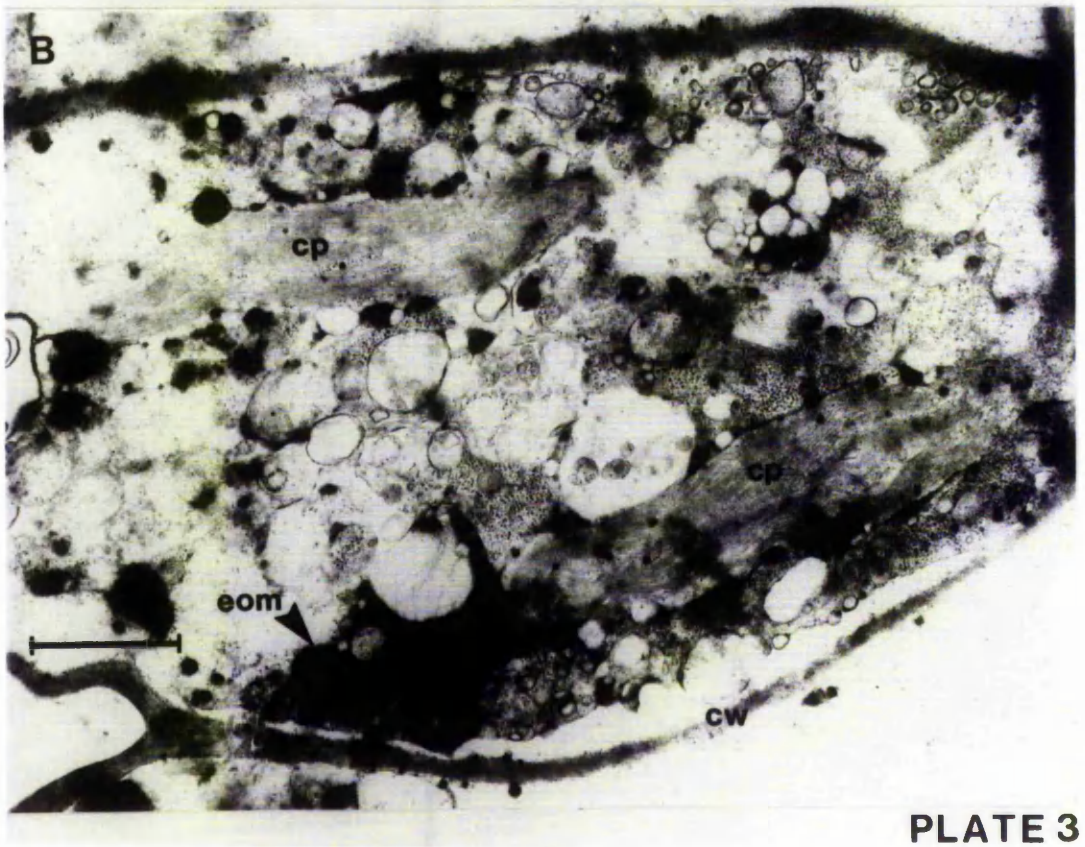


PLATE 3

3.3.4.2 Excised Leaves Floating on Herbicide Solutions

The poor staining of tonoplasts and mitochondria seen in sprayed plants stained post sectioning with uranyl acetate was not observed in excised leaves stained prior to dehydration. Swanson et al (1973a) reported on a study of lipid extraction in glutaraldehyde - fixed Nicotiana tabacum leaf tissue and found that the tonoplast and to a lesser degree, mitochondria were the most susceptible organelles to disruption due to lipid extraction by acetone. It is possible that in the species used in this study, glutaraldehyde and osmium tetroxide - fixed tonoplasts were somewhat labile during dehydration (lipid extraction) and that pre - dehydration treatment with uranyl acetate, helped to stabilise these membranes. In addition to better and more even staining of cells in the excised leaf system, the more rigorously controlled conditions of light, temperature, humidity and herbicide application greatly reduced variability of toxicity symptom appearance in treated tissues. This enabled a chronological sequence of ultrastructural changes to be established.

Cell ultrastructure of control tissues after a 3 hour incubation in light (Plate 4A) was similar to that of leaves freshly cut from G.aparine plants (not shown). Control cell ultrastructure remained largely unchanged throughout the experiment, except that by 30 hours incubation in light (Plate 4B) and darkness (Plate 11A) some chloroplasts became slightly irregular in shape and showed a slight increase in the number of plastoglobuli. These changes may represent the intial stage of senescence (Butler and Simon, 1971). Gut and Matile (1988) however, published evidence suggesting that galactolipids in senescing (dark incubated) leaves could be metabolised for gluconeogenesis derived energy. The increased number of plastoglobuli observed in leaves incubated for long dark periods in

this study may therefore represent diversion of thylakoid lipids for this purpose. Some cells in dark incubated leaves (control and herbicide treated) also showed altered alignment of thylakoids (Plate 11B). Neither acifluorfen nor M&B 39279 treated tissue showed any ultrastructural symptoms of toxicity after 30 hours in darkness (Plate 11), confirming, alongside the physiological data reported above, the absolute light dependency of these herbicides.

Ultrastructural alterations were noted in treated tissue after a 3 hour light incubation (Plate 5). Many chloroplasts were swollen and invaginations / evaginations of the chloroplast envelope were common. A number of cells also showed cytoplasmic irregularities (Plate 5B), which may represent swollen endoplasmic reticulum cisternae.

After 5 hours light, further vesiculisation of chloroplast envelopes was observed in treated tissue (Plate 6), the envelope membranes apparently yielding many intraplastid and cytoplasmic vesicles. The tonoplast of many cells was also disturbed.

After 15 hours illumination, the tonoplast had lysed in a large proportion of herbicide treated cells, yielding a mass of vesicles as cytoplasmic and vacuolar constituents mixed (Plate 7). Chloroplasts, although swollen, remained intact and contained a scarcely altered thylakoid system and prominent starch grains. Mitochondria appeared unaltered in such cells and the plasmalemma although intact, appeared to be shrinking from the cell wall. Those cells which still possessed an intact tonoplast after 15 hours light displayed numerous cytoplasmic and vacuolar membraneous vesicles (similar to Plate 3A).

By 20 hours, the only remaining recognisable structures in many treated cells were chloroplasts, these being distorted in shape and often having discontinuities in the envelope membranes (Plate 8). Electron dense material, probably representing lipids from degraded

membranes, was associated with many vesicles and defunct organelles.

After 25 - 30 hours incubation in light, treated cells showed a range of symptoms characterised by vesiculated remains of cytoplasm and an abundance of free vesicles, many bearing electron dense globules (Plates 9 and 10). The remains of chloroplasts were however, discernible, these possessing degenerating thylakoid systems. Particularly conspicuous were the highly modified chloroplast envelope membranes which consisted of little more than contiguous vesicles and electron opaque globules. Many chloroplasts bearing such effete envelopes were fused together (Plate 10).

Changes in ultrastructure of nuclei are not shown in plates I - II since they were observed comparatively infrequently in the sections due to the highly vacuolated nature of the mature leaf cells. Nuclei however, appeared largely unchanged in treated tissues and degraded on a similar time scale as mitochondria.

Plate 4

G.aparine leaf cells after (A) 3 hours and (B) 30 hours floating on 1% acetone in light. Note integrity of parietal cytoplasm in both micrographs and the increased number of plastoglobuli in (B).

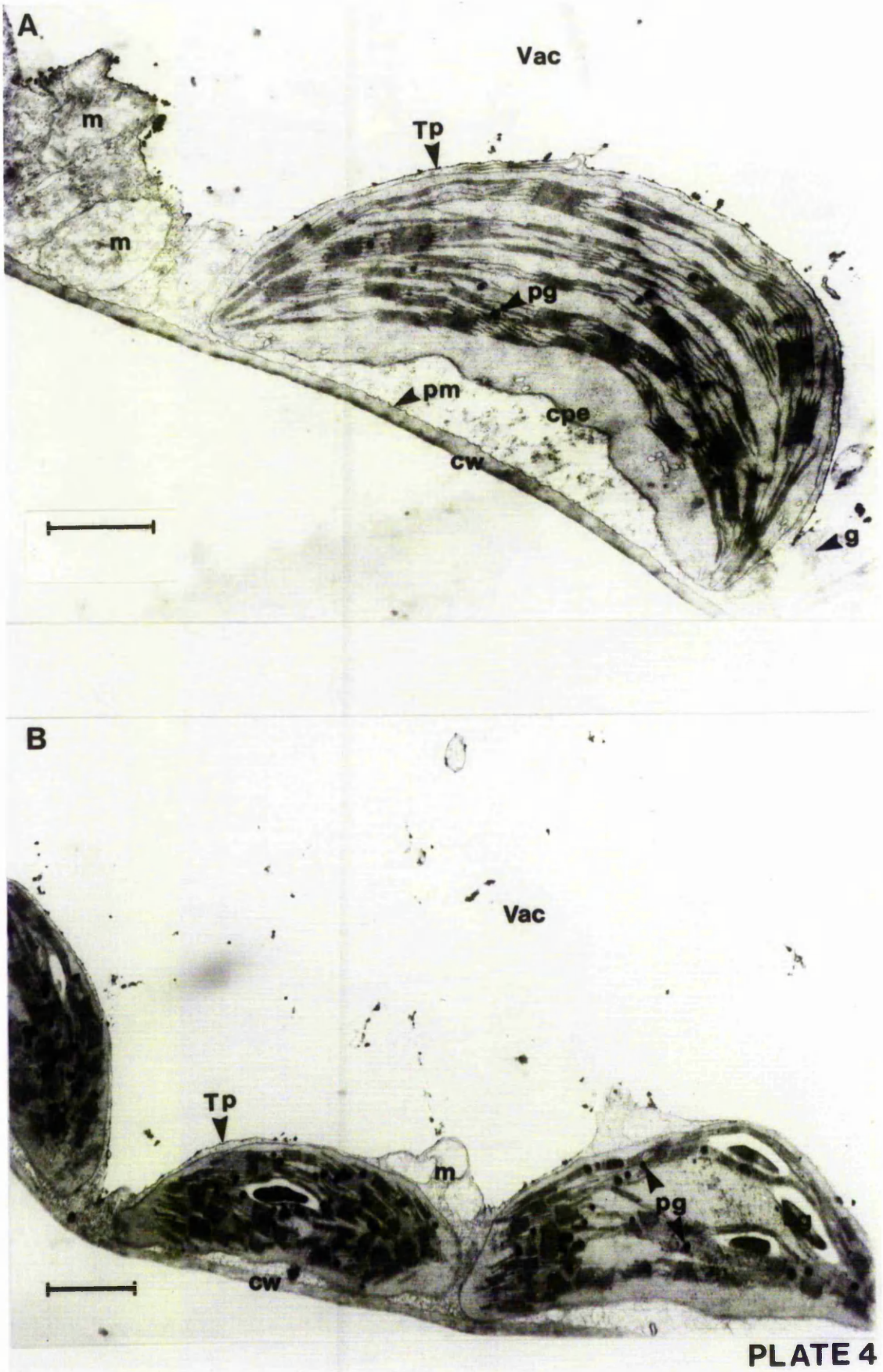


PLATE 4

Plate 5

G.aparine leaf cells floating on (A) 10^{-4}M M&B 39279 and (B) 10^{-4}M acifluorfen for 3 hours in light. Note swollen chloroplasts (large arrows) in (A) and invaginations / evaginations of chloroplast envelopes and cytoplasmic disturbance (double arrow) in (B).

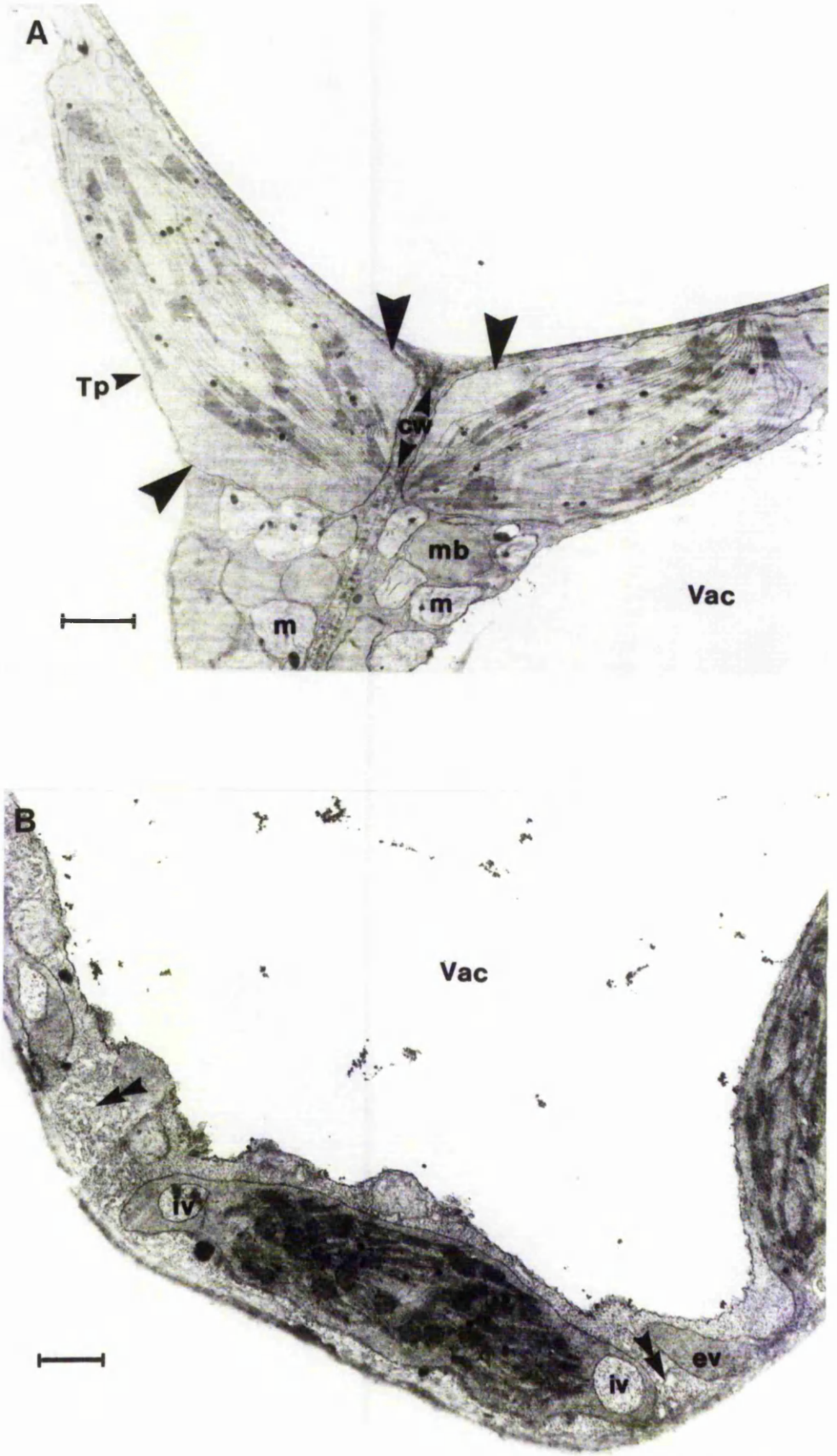


PLATE 5

Plate 6

G.aparine leaf cells floating on (A) 10^{-4}M M&B 39279 and (B) 10^{-4}M acifluorfen after 5 hours in light. (A) Note vesiculisation of chloroplast envelopes (small arrows) and disrupted tonoplast (large arrow). (B) Note invaginations of chloroplast envelopes.

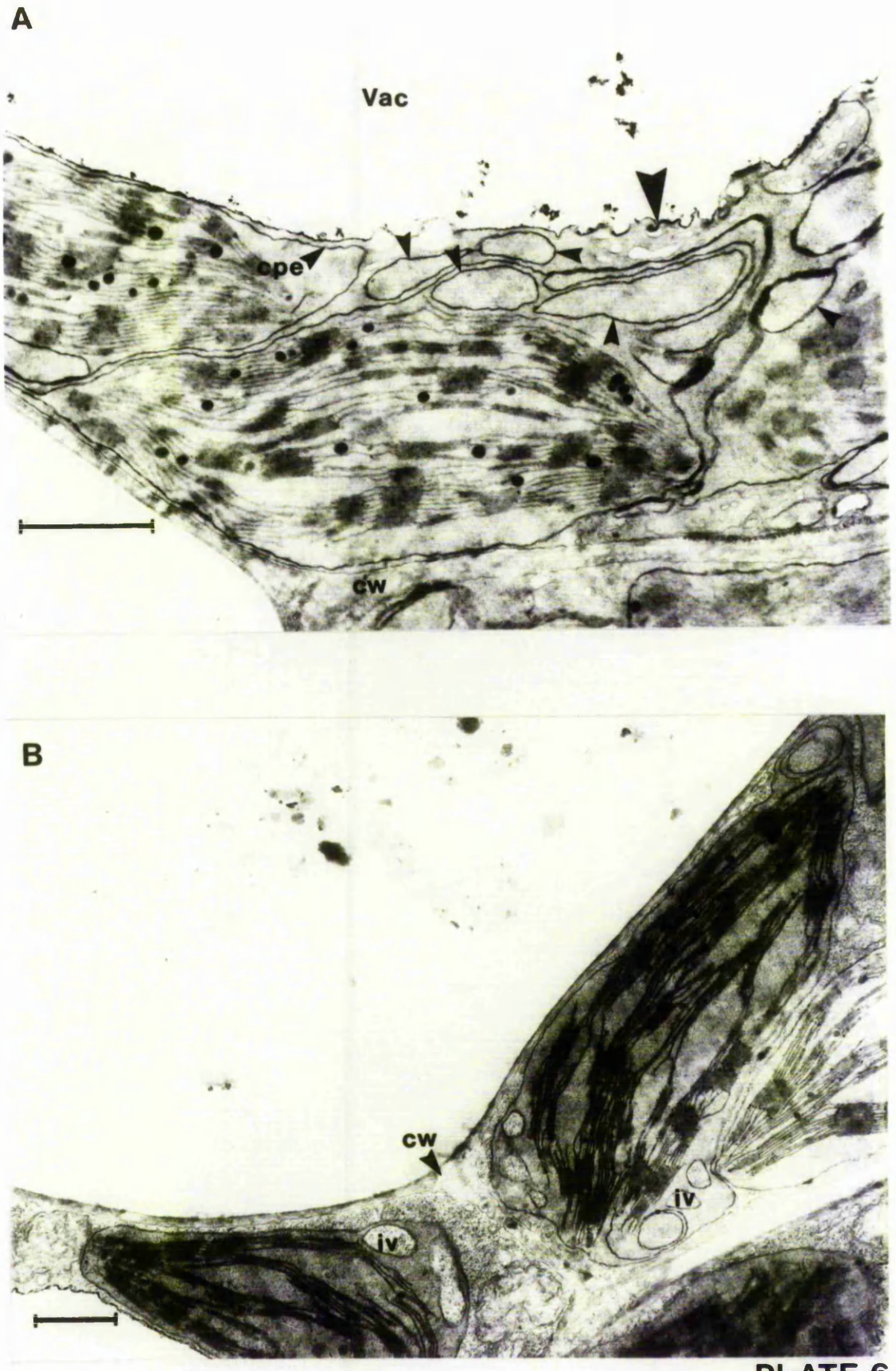
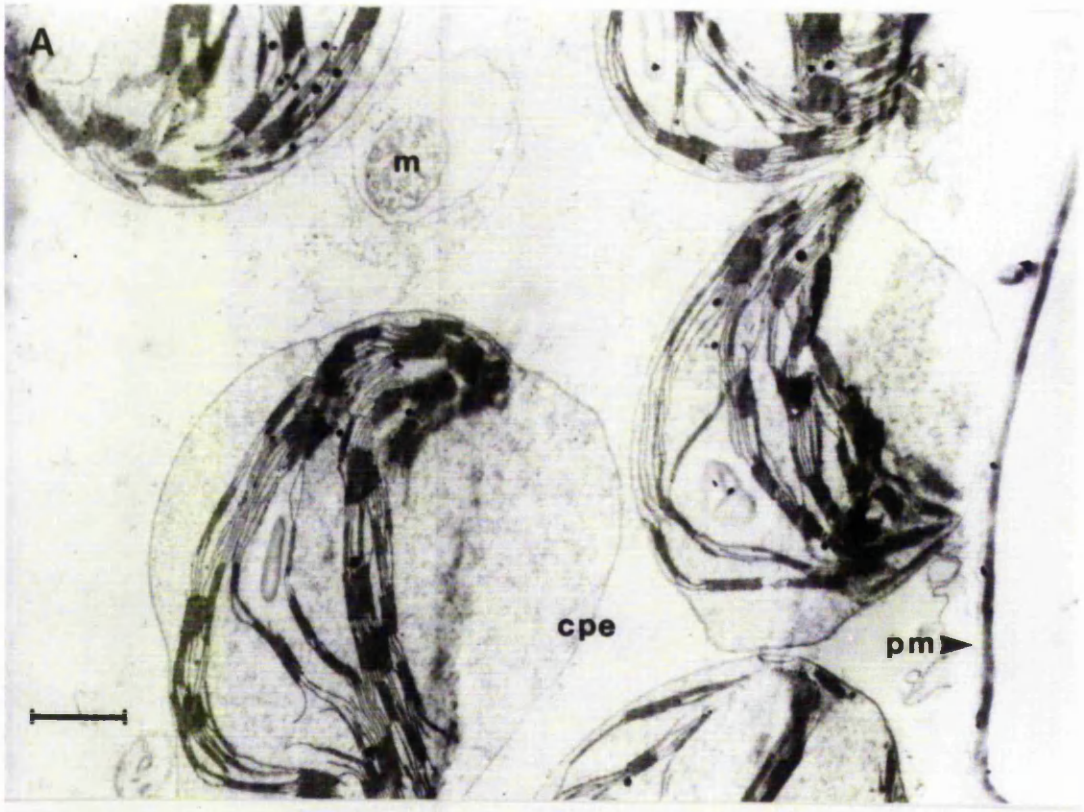


PLATE 6

Plate 7

G.aparine leaf cells floating on (A) 10^{-4}M M&B 39279 and (B) 10^{-4}M acifluorfen after 15 hours in light. Note lack of tonoplast, swollen chloroplasts, and integrity of thylakoids.



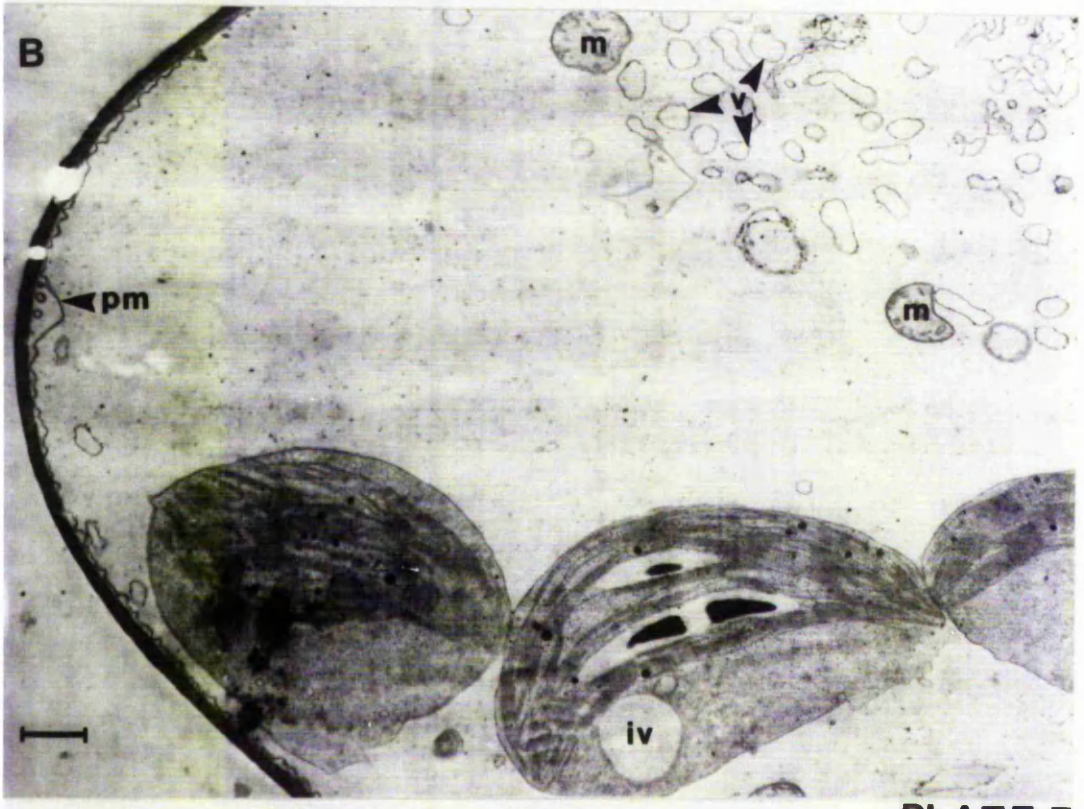


PLATE 7

Plate 8

G.aparine leaf cells floating on (A) 10^{-4}M M&B 39279 and (B) 10^{-4}M acifluorfen after 20 hours. (A) Note lack of chloroplast envelopes and electron opaque globules (arrows). (B) Note disruption of plasmalemma, particularly around plasmodesmata (arrows) and disrupted mitochondria (double arrows).

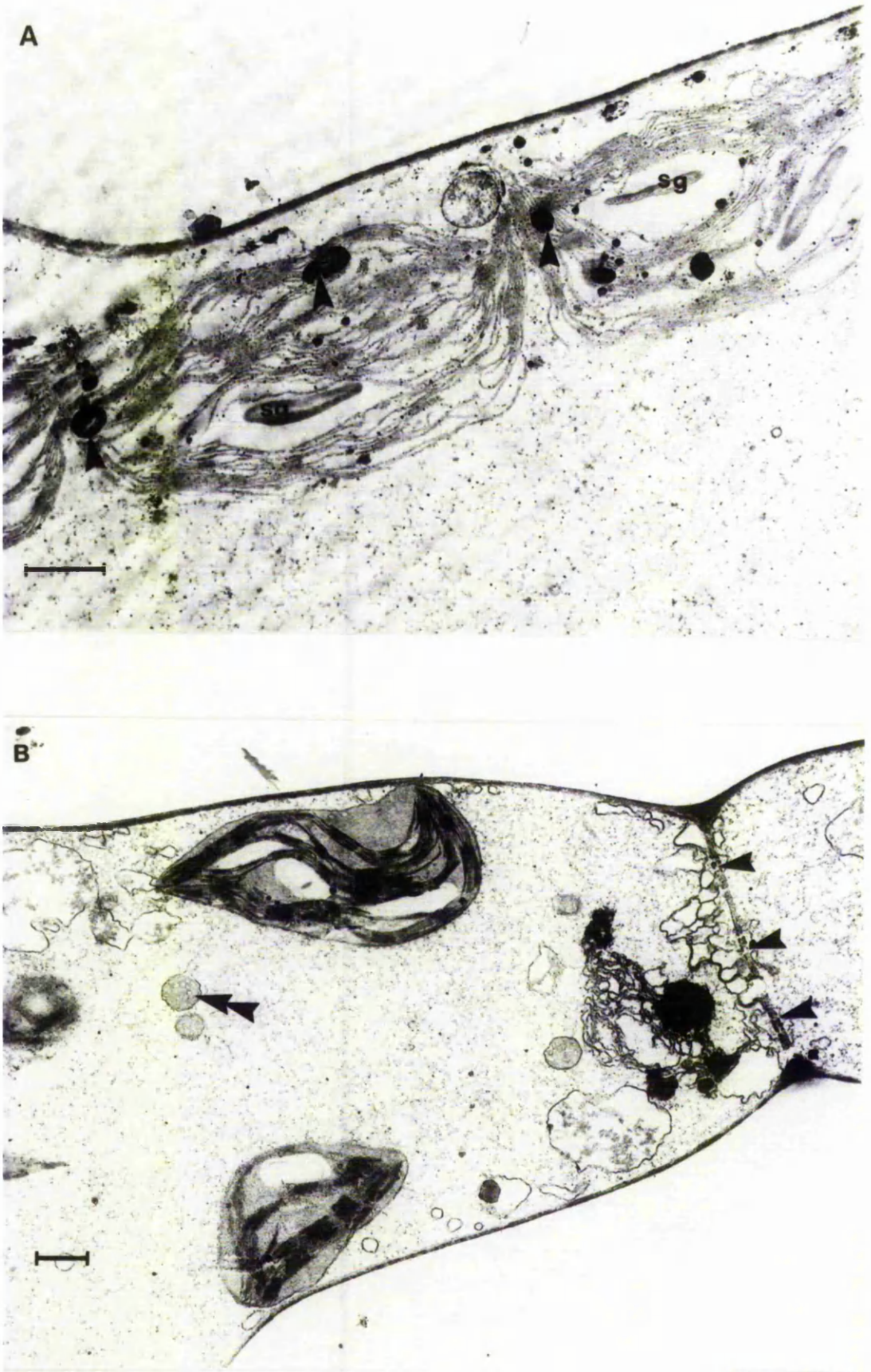


PLATE 8

Plate ,9

G.aparine leaf cells floating on (A) 10^{-4}M M&B 39279 and (B) 10^{-4}M acifluorfen after 25 hours light. (A) the entire cytoplasm is a mass of membraneous vesicles and electron opaque material. (B) Note lack of tonoplast, plasmalemma, disrupted mitochondria (arrows) and the swollen chloroplast (★) which has lost its stromal contents. Note relative integrity of thylakoids.

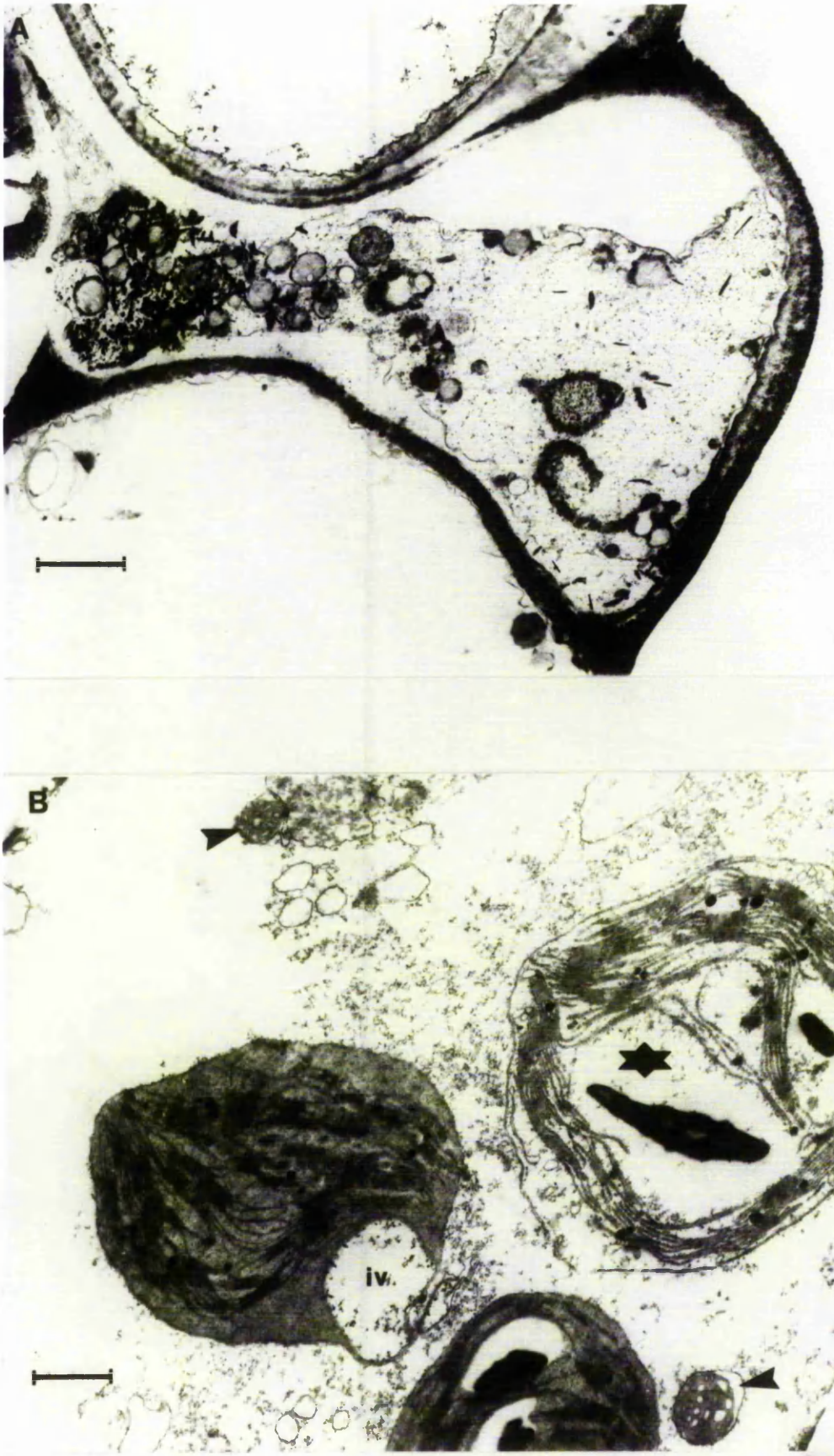


PLATE 9

Plate 10

G.aparine leaf cells floating on (A) 10^{-4}M M&B 39279 and (B) 10^{-4}M acifluorfen after 30 hours. (A) Note structure measuring approximately twice the long axis of normal chloroplasts and so likely to be two choroplasts fused between the large arrows. Note vesiculated chloroplast envelopes and thylakoid integrity. (B) Aggregate of fused chloroplasts and electron opaque material. Note thylakoid integrity.

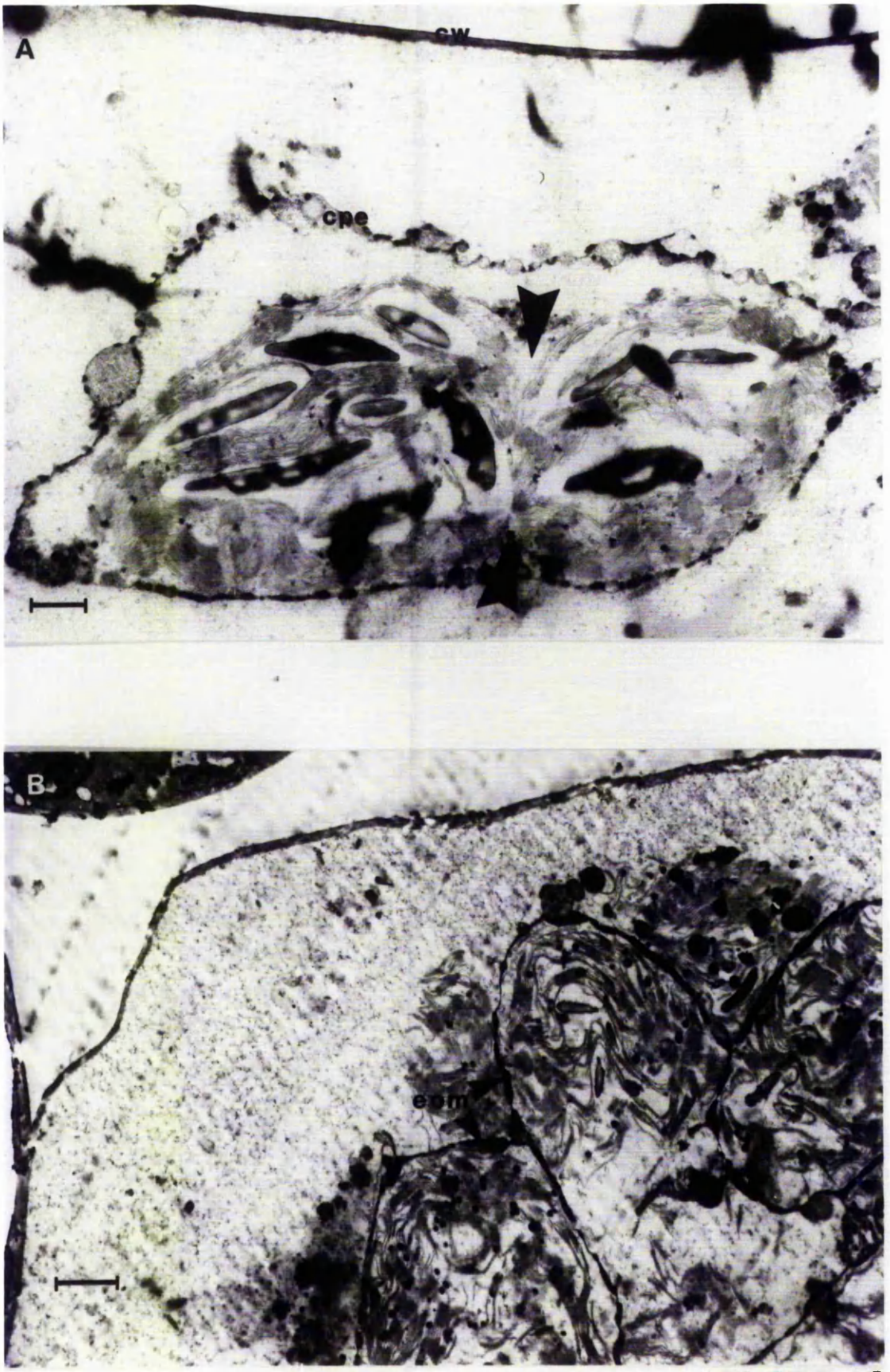


PLATE 10

Plate 11

G.aparine leaf cells floating on (A) 1% acetone (B) 10^{-4}M M&B 39279 (C) 10^{-4}M acifluorfen after 30 hours darkness. Note integrity of cell contents and alignment of thylakoids in (B).

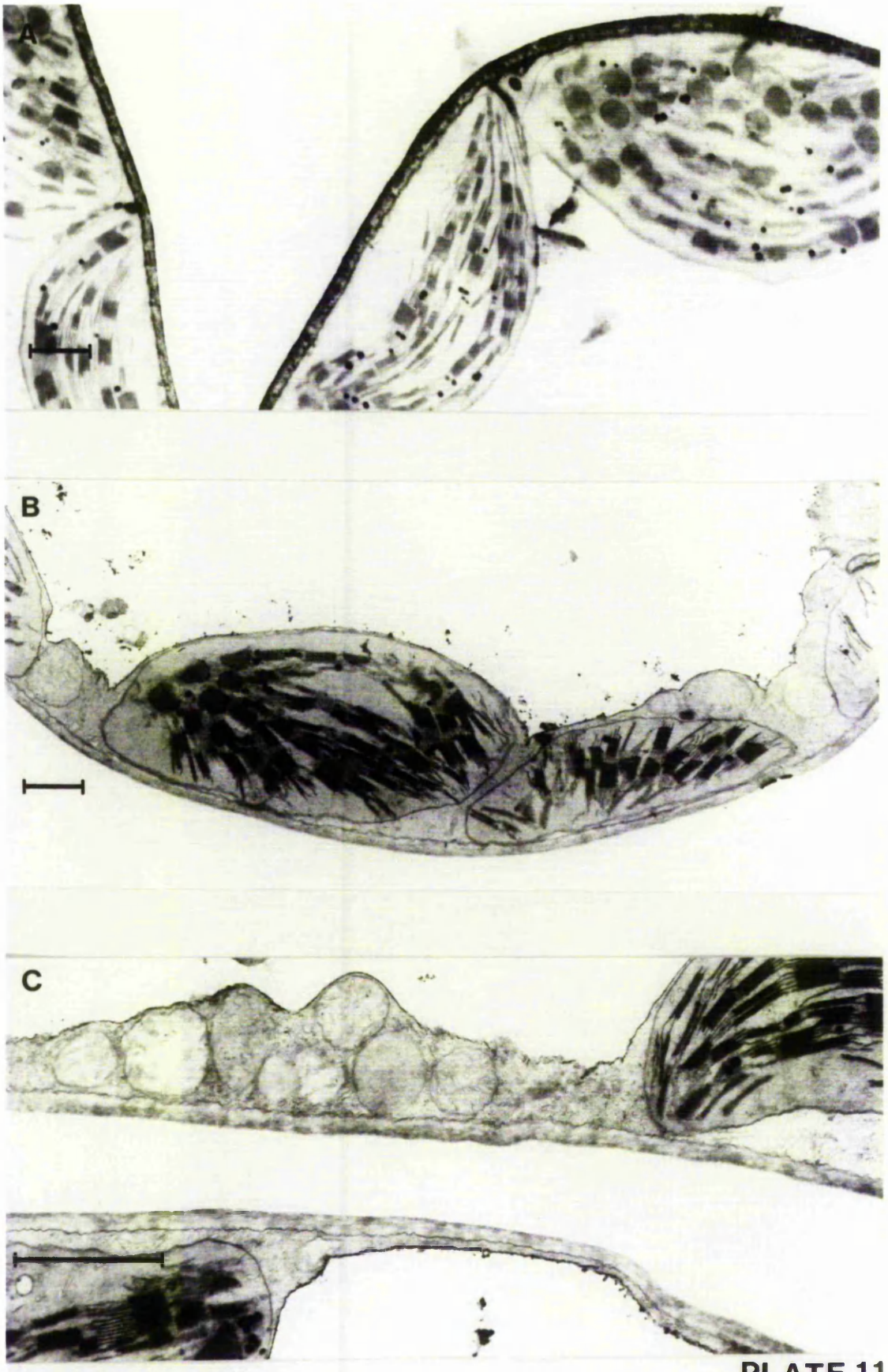


PLATE 11

3.4 DISCUSSION

The elucidation of herbicide - induced ultrastructural events with a view to locating a primary or initial site of action has an unavoidable limitation, namely that changes in subcellular morphology only show the result of the herbicides primary action which may be spatially separated from the actual site of causation of such changes. Nevertheless, if supported by relevant physiological measurements as in this study, ultrastructural observatons can be a useful addition to a study of herbicidal mode of action. It is also appropriate when examining a sequence of subcellular changes induced by a herbicide to consider the sequence of cytopathological changes which take place in a senescing cell in order to differentiate specifically herbicide effects and those which are typical of a moribund cell. Although variable between species and tissues, a generalised pattern of senescence is as follows: (1) A loss of free ribosomes (2) degeneration of chloroplast stroma (3) an increase in plastoglobuli (4) degeneration of thylakoids (5) mitochondrial alterations (6) swellings and vesiculisation of endoplasmic reticulum and pinching off vesicles into the vacuole (7) Tonoplast lysis followed by general destruction of cytoplasm and (8) plasmalemma lysis (Butler and Simon, 1971).

From the described ultrastructural observations, I propose that the following sequence of subcellular events occurs after administration of M&B 39279 / acifluorfen to G.aparine leaves in light under the described conditions: (1) By 3 hours incubation, swelling / distortion of chloroplast morphology (2) By 3 - 5 hours, evaginations and invaginations of chloroplast envelopes, some cytoplasmic disturbance

(swelling of endoplasmic reticulum?) and tonoplast perturbation (3) By about 15 hours, tonoplast disruption, lysis and / or cytoplasmic vesicle development (4) between 20 and 30 hours, a progressive degeneration of membranes and organelles is observed, yielding vesicles and osmiophilic globules. Chloroplasts develop discontinuous / highly modified envelope membranes containing large amounts of osmiophilic material. These membranes frequently coalesce. Thylakoid membranes and starch grains persist as the last cytoplasmic structures to undergo destruction with these herbicides. This sequence of ultrastructural changes was consistent with that described by Kenyon et al (1985, see section 1.3.3), and was totally dependent on the presence of light for its development (Plate 11 and section 2.3).

Since it was found that the speed of action of acifluorfen and M&B 39279 was dependent on light intensity (see Chapter 4), the conditions of low light intensity employed in this study (50μmol m^{-2} s^{-1} PPFD) were chosen to allow a relatively slow progression of the sequence of ultrastructural changes, thus enabling discrimination between events occurring early in the sequence and those arising at a later time. The use of a high light intensity would have rapidly yielded a difficult to interpret mixture of 'early' and 'late' symptoms (e.g. as in the micrographs of Orr and Hess, 1982b). Although in this study excised leaves were incubated under conditions of relatively low light intensity, these conditions would have been sufficient to support photosynthesis at approximately 25μmol O_2 mg $chl1^{-1}$ hr^{-1} (Fig. 45), roughly a third of the rate which would have been supported by the glasshouse conditions under which plants were grown (glasshouse light intensity was approximately 200 - 400μmol m^{-2} s^{-1} PPFD, and see Fig. 45). The leaf incubation light intensity was therefore, not unrealistically low. The validity

of the sequence of events proposed above was further qualified by the similar symptoms which were observed in whole G.aparine and V.arvensis plants sprayed with M&B 39279 / acifluorfen.

The above described sequence of ultrastructural events was accompanied by an immediate and rapid decline in photosynthetic capability (Fig.46). The onset of eletrolyte / malondialdehyde leakage and chlorophyll loss corresponded chronologically with tonoplast lysis and disruption of the plasmalemma (Figs. 47, 48 and 49). These results support the idea of DPE -type herbicide action via a peroxidative loss of membrane integrity.

Rapid swelling and morphological distortion of chloroplasts, disruption of envelope membranes prior to any other visible ultrastructural changes in treated tissues plus a rapid and concurrent decline in photosynthetic competence indicates that the chloroplast is a likely primary target for M&B 39279 / acifluorfen. Furthermore, since chloroplast envelope membranes displayed such rapid and dramatic changes in morphology which preceeded other ultrastructural changes in this study, it is not unreasonable to suggest that that chloroplast envelopes contain the entity with which DPE - type herbicides interact and initiate their toxic effect. The rapid decline in photosynthetic capability could easily be explained in terms of damaged chloroplast envelopes, these membranes being responsible for regulating transport of materials in and out of the chloroplast, notably inorganic phosphate / photosynthates and so play a pivotal role in maintaining the environment for photosynthesis within the chloroplast. Indeed, some 20 -25 % of envelope polypeptides are involved in this function (Douce and Joyard, 1979 and Douce et al, 1984). Invaginations and evaginations of chloroplast envelopes were the most prominent initial symptoms of M&B 39279 damage in G.aparine and V.arvensis sprayed

plants and G.aparine excised leaves. Such membrane perturbation would undoubtedly be accompanied by changes in membrane fluidity and osmotic properties. Similar alterations to chloroplast envelopes have been reported as a result of oxidative damage to leaf cells by ozone (Swanson et al, 1973b). It is possible, therefore, that lipid peroxidation as a result of DPE - type herbicide action (see section 1.3.6) could generate similar structures. Chloroplast envelopes would indeed be susceptible to lipid peroxidation as they contain a far higher lipid : protein ratio than any other cellular membrane (Douce and Joyard, 1979) and the majority of these lipids are galactolipids which contain the polyunsaturated fatty acid linolenic acid (polyunsaturated lipids are very susceptible to and propagate lipid peroxidation, see Porter, 1984; Elstner, 1987 and Thompson et al, 1987). The prenyl quinones, xanthophylls and carotenes present within the envelope and the antioxidative systems present in the stroma (see Salin, 1987) would however, temporarily check the destruction of these membranes. The apparent susceptibility of chloroplast envelopes to lipid peroxidation owing to their high unsaturated lipid content could also provide another equally plausible explanation for the observed morphological / physiological changes, in that envelope perturbation may infact be the result of generation of peroxidative species at adjacent subcellular sites such as the chloroplast stroma or the endoplasmic reticulum. Apparent swellings of endoplasmic reticulum was occasionally noted as an early symptom of M&B 39279 / acifluorfen toxicity (Plate 5). Although it was difficult to observe these structures in this study, the potential involvement of endoplasmic membranes could be further studied by cytochemical staining of tissues with zinc iodide - osmium tetroxide (Harris, 1978) and observing thick sections by high voltage electron microscopy. Alternatively, herbicide

induced changes to endomembranes could be observed in living cells utilising the fluorochrome Auramine-O which enables observation of these structures by fluorescence light microscopy (Harris and Gates, 1984).

As indicated in section 1.3.7, a role or necessity for chloroplast electron transport in DPE activity has been proposed by several workers, suggesting the thylakoid network as a possible primary site of action. It is seen from the results of this study however, that a remarkable degree of integrity is maintained by the thylakoid membranes during M&B 39279 / acifluorfen induced toxicity. This could be explained perhaps, in that although thylakoids are rich in unsaturated galactolipids, they are also heavily protected against photo-oxidative reactions as the electron transfer system predisposes these structures to such damage during normal metabolism (see Elstner, 1987). Such protection may have preserved thylakoid integrity during M&B 39279 / acifluorfen activity. The bipyridyl herbicides which are thought to act by diverting electrons from the electron transfer chain and thereby initiating lipid peroxidation, however, cause fairly rapid morphological distortions of the thylakoid system in a number of species (Harris and Dodge, 1972; Dodge and Lawes, 1974 and Harvey and Fraser, 1980) and so the evidence does not support direct involvement of thylakoids in M&B 39279 / acifluorfen action by such a mechanism. Further evidence to support chlroplast envelope rather than thylakoid mediated DPE action is provided in that etiolated plant tissues are susceptible to DPE herbicides. The chloroplast and etioplast envelope are morphologically and cytochemically similar in electron microscopy (Carde et al, 1982) and have similar chemical compositions (Douce and Joyard, 1979). This is not so with thylakoids

/ prolamellar bodies.

Although not precluding other subcellular structures, the results described in this chapter therefore, appear to support a model of DPE - type action such as that proposed by Duke and Kenyon (1987) which involved chloroplast envelope - situated photoreceptors with which the herbicides interact.

C H A P T E R 4

THE INVOLVEMENT OF LIGHT IN M&B 39279 AND ACIFLUORFEN ACTION

4.1 INTRODUCTION

The previous chapter demonstrated in visual, microscopical and physiological terms, that M&B 39279 and acifluorfen appear to be totally reliant upon light for ther activity. This has also been shown in the literature for other DPE - type compounds as described in section 1.3.4. Clearly therefore, light energy drives the chemical processes which in the presence of these herbicides, leads to cellular destruction and plant death. As revealed in Chapter 1 however, the literature regarding the exact role of light has been confusing, much of this confusion arising from advocacy or otherwise of the involvement of photosynthesis in the mechanism of action of DPE's (see Duke and Kenyon, 1987).

In view of such controversy in the literature and the data described in Chapters 2 and 3 of this thesis, a number of studies were carried out to explore the nature of the light dependency of M&B 39279 in qualitative and quantitative terms and to infer ideas based on the information gained and available literature as to possible systems which may be involved in the lethal process. Experiments investigating DPE - type herbicide light dependancy in this study may be divided into four approaches: (1) ultrastructural investigations, described in Chapter 3 ; (2) investigations into effects of varying light intensity on herbicide activity; (3) investigations into effects of varying light quality and (4) investigating interactions of photosynthetic systems and M&B 39279 and acifluorfen.

4.2 MATERIALS AND METHODS

4.2.1 GENERAL COMMENTS

To investigate the effects of varying light intensity and light quality on herbicidal activity, apparatus designed to regulate the light and thermal environment of excised leaves floating on herbicide solutions was employed. This apparatus was similar to that used for incubating leaves for ultrastructural studies but also incorporated a water heat filter to avoid excessive heating effects of the higher light intensities used in some experiments described in this chapter. The apparatus is illustrated schematically in Fig. 50.

4.2.2 INCUBATION CONDITIONS AND GENERAL TECHNIQUES

G.aparine plants were grown under glasshouse conditions as described in Chapter 2. Leaflets were taken from the second whorl of leaves when the diameter of this was 45 - 48mm and that of the third whorl, 5 - 10mm. Ten leaflets were floated, abaxial surface down on either 100μM M&B 39279, 100uM acifluorfen or 1% (v/v) aqueous acetone solutions in 5cm diameter Petri dishes. Petri dishes were placed into a perspex box with the inner surface painted matt black to exclude stray light. This box was placed into the water bath of the apparatus described in Fig. 50. Four dishes per treatment in each of six experiments were used. The temperature of the circulating water was maintained at 25°C. Light was provided by a SON-T 400W high - pressure sodium lamp (THORN-EMI) and heat produced by this lamp attenuated by passing the light through 2.5cm of cooled water and 0.5mm of clear perspex. Light intensities of 375, 750, 1000 and 1500μmol m^{-2} s^{-1} PPFD were achieved by raising and lowering the lamp.

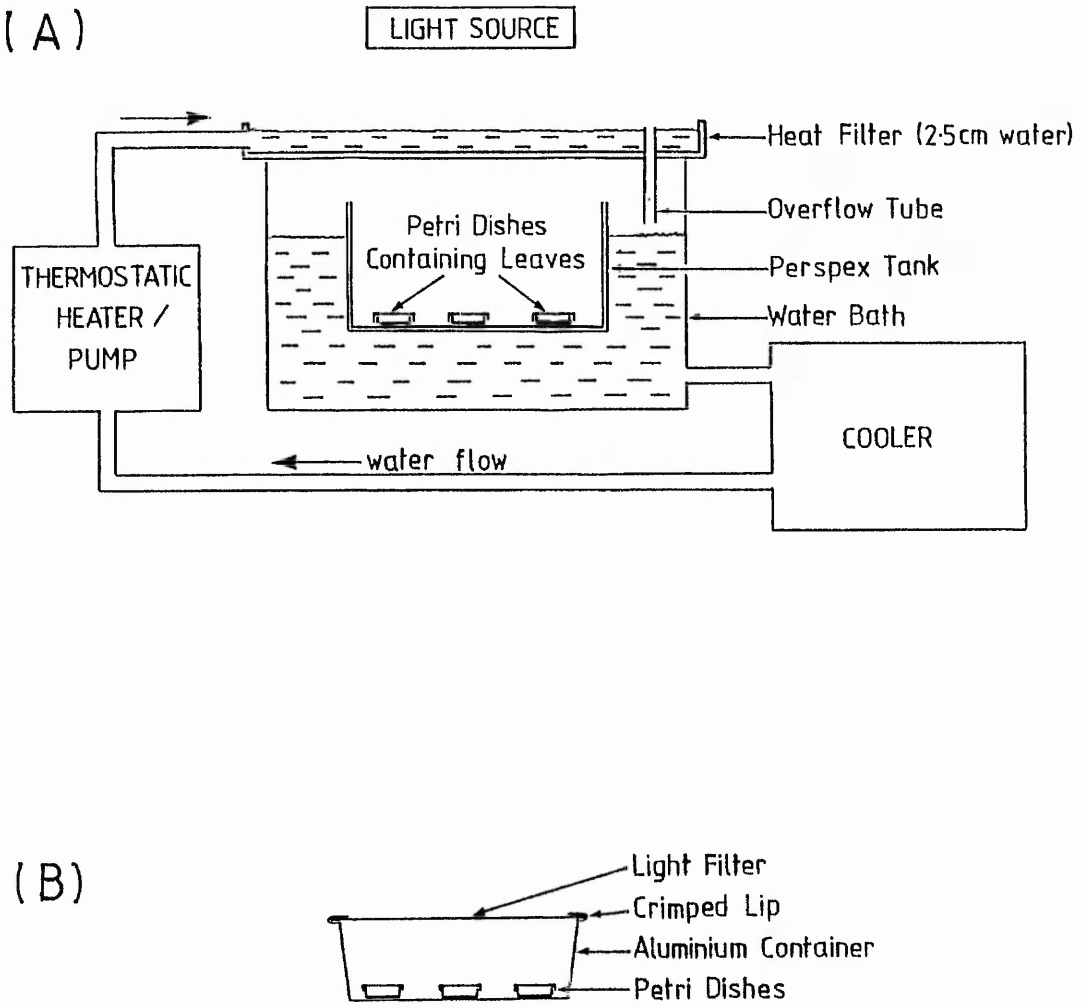


Figure 50. Schematic representation of controlled environment chamber used for incubation of leaves during experiments investigating effects of light on herbicide activity. (A) depicts the general apparatus and (B) depicts the chamber which replaced the perspex box for inclusion of colour filters.

Herbicidal damage to incubated leaves was assessed by measuring leaf chlorophyll content and changes in conductivity of the bathing medium as previously described in sections 3.2.3 and 3.2.4. Bathing solution conductivity and leaf chlorophyll content were measured after an incubation period of 24 hours.

In addition to investigating the effect of varying light intensity on herbicidal activity, a time course of electrolyte leakage and chlorophyll loss at 750μmol m^{-2} s^{-1} PPFD was undertaken, incubating leaflets as above and measuring these parameters after 5, 10, 15, 20 and 25 hours.

4.2.3 INVESTIGATIONS INTO LIGHT QUALITY EFFECTS ON M&B 39279 AND ACIFLUORFEN ACTIVITY

Three investigations into light quality effects on M&B 39279 / acifluorfen activity, the first introducing colour light filters into the controlled environment chamber. The second and third investigations followed reports in the literature that acifluorfen interferes with blue light - dependant biosystems (see section 1.3.5). One investigation involved introducing M&B 39279 and acifluorfen into a bioassay for phototropism, a blue light - dependant phenomenon (see Senger, 1980). A second system, in experiments performed by K.J. Nichols and Dr A.H. Cobb of Trent Polytechnic in the laboratory of Dr C.M. Willmer, University of Stirling, U.K. and at Trent Polytechnic, involved applying the herbicides to stomatal guard cells, stomatal movements also being blue light - dependant processes (see Willmer, 1983).

In order to measure the effects of varying light quality on M&B 39279 / acifluorfen activity, leaflets of G.aparine were incubated in the controlled environment apparatus as above in section 4.2.2 but

with the following alterations: Leaflets were incubated for 48 hours at 25°C in either blue, green or red light. These light environments were achieved by placing the Petri dishes in aluminium foil containers of the type used by 'fast food' retailers and with a sheet of colour filter crimped tightly into the fold over lip of the containers to exclude stray light (Fig.50 B). The light source was a bank of fluorescent tube lamps, red, green and blue light being provided by the following light source / colour filter combinations based on the recommendations of Smith (1975): For blue light, 20W 'natural white' fluorescent lamps (GEC) plus 'Cinemoid' no. 19A blue filter (Rank Strand). For green light, 20W 'cool white' fluorescent tubes (Thorn EMI) plus 'Supergel' no. 90 green filter (Rosco, Upper Ground, London, SE1 9PQ, U.K.). For red light, 20W 'Deluxe Natural' fluorescent tubes (Thorn EMI) plus 'Cinemoid' no. 14 red filter (Rank Strand). The spectral properties if these lamps and filters are shown in Figs. 51 and 52. The light intensity of red, green and blue light was maintained at 8μmol m^{-2} s^{-1} PPFD. The experiment was repeated at 15μmol m^{-2} s^{-1} PFD with red and blue light alone as this intensity could not be achieved with the green filter. In view of the low light intensities used in these experiments, the water heat filter was not used (Fig 50).

The phototropism bioassay was conducted as follows. Pisum sativum cv. Feltham First were dark grown in moistened vermiculite within seed trays. When the etiolated seedlings were approximately 5cm tall, M&B 39279 / acifluorfen solutions ($5x10^{-8}$M, 10^{-6}M, 10^{-5}M, $5x10^{-5}$M, 10^{-4}M and $5x10^{-3}$M concentrations in 2% acetone) were applied as droplets using a small artists brush to the leaf initials and epicotyl hook immediately adjacent. Plants were then exposed to unilateral blue light provided by a 'natural white'

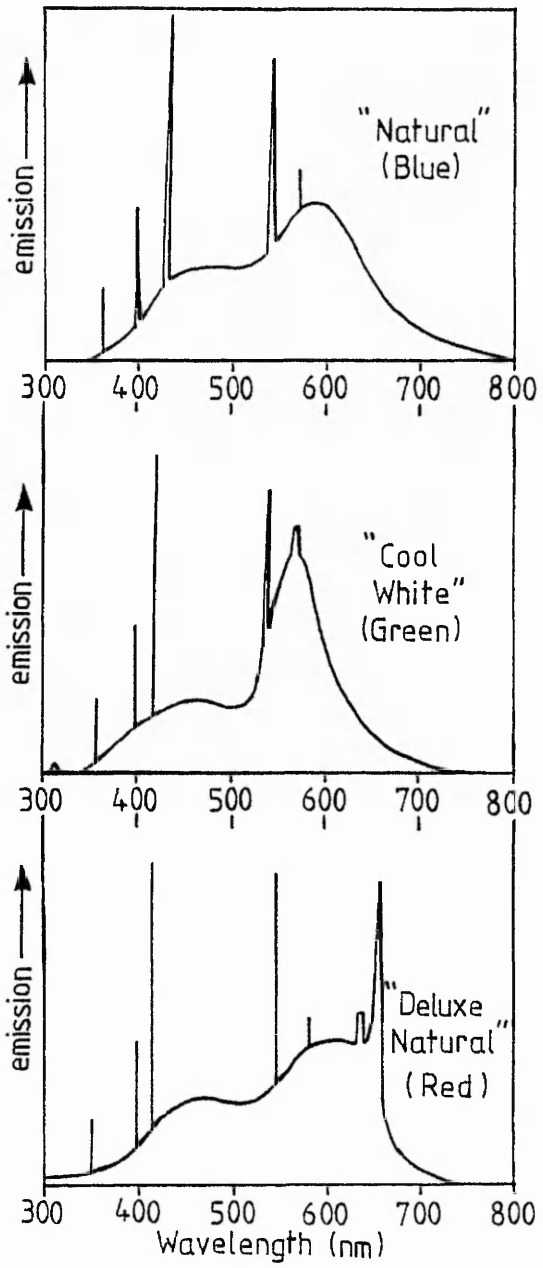


Figure 51. Emission spectra of fluorescent tubes used for blue, green and red light sources. Re - drawn from technical data.

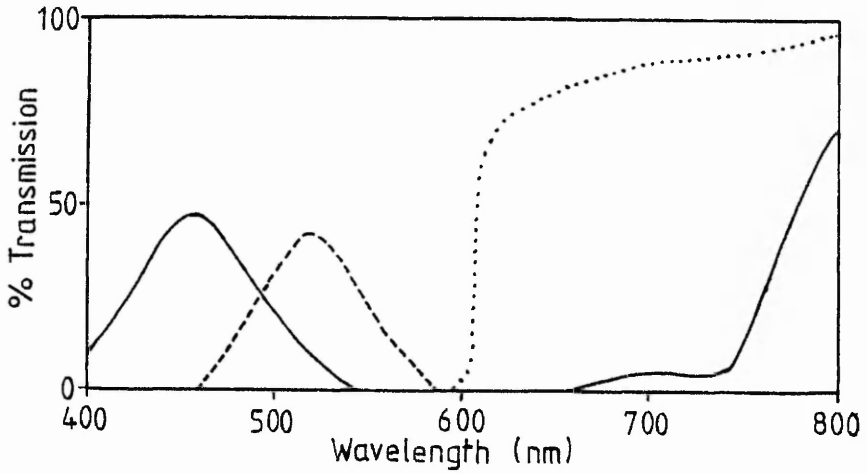


Figure 52. Transmission properties of colour filters used for blue (——), green (- - - - -) and red (·····) light sources.

fluorescent tube (GEC) filtered by one layer of 'Cinemoid' no. 19A filter (Rank Strand) at an intensity of 0.2 - 0.4μmol m^{-2} s^{-1} PPFD (difference in irradiation across seed trays) for 18 hours at 15 - 18°C. The angle of curvature was measured for 50 seedlings per treatment in each of two experiments (i.e. 2 seed trays per treatment).

Two experiments were performed testing the effects of M&B 39279 on blue light - induced stomatal opening (Nichols and Cobb, unpublished). Firstly, abaxial epidermal peels of G.aparine bearing closed stomata were floated on a solution containing 0.1mM calcium chloride, 10mM potassium chloride and 10μM M&B 39279 through which was bubbled carbon dioxide - free air. Peels were then exposed to 17μmol m^{-2} s^{-1} PPFD blue light. These conditions are conducive to stomatal opening. Pre - treatment stomatal closure was achieved by incubating epidermal peels for one hour in darkness on a solution containing 0.1mM calcium chloride through which was bubbled air containing carbon dioxide.

In a second experiment, M&B 39279 was added to a final concentration of 100μM to a suspension of Commelina communis stomatal guard cell protoplasts and blue light - induced protoplast swelling observed (17μmol m^{-2} s^{-1} PPFD blue light). In a process analogous to stomatal opening, guard cell protoplasts swell as a result of potassium ion uptake in response to blue light (see Fitzsimons and Weyers, 1986 and MacRobbie, 1988).

4.2.4 INTERACTIONS OF PHOTOSYNTHETIC SYSTEMS WITH M&B 39279 AND ACIFLUORFEN

4.2.4.1 General Comments

In view of the confusing nature of the literature described in section 1.3.7 regarding the involvement of or necessity for active thylakoidal photosynthetic electron transfer in full development of DPE - type herbicide induced symptoms, a number of experiments were conducted firstly to show whether or not thylakoidal electron transfer was necessary for M&B 39279 / acifluorfen action and secondly, whether these herbicides had the capability to interfere with the chloroplast electron transfer chain.

4.2.4.2 Necessity of photosynthetic Electron Transfer for M&B 39279 and Acifluorfen Activity

To show necessity of photosynthetic electron transfer for M&B 39279 / acifluorfen action, leaflets of G.aparine (from plants as described in section 4.2.2) were floated on 100μM DCMU (3-(3'4'-dichlorophenyl) -1,1-dimethyl urea) in 2% (v/v) aqueous ethanol for 20 hours in darkness and then transferred to 10ml of 100μM M&B 39279, 100μM acifluorfen, 10μM paraquat (methyl viologen) or 1% (v/v) aqueous acetone (controls) in 5mm diameter Petri dishes. Paraquat was included as a control to indicate the degree to which electron transfer had been arrested by the inhibitor DCMU as the action of paraquat is known to largely depend upon photosynthetic electron transfer for its activity (Dodge, 1971). Five leaflets were incubated per Petri dish and three dishes per treatment used in each of five experiments. These were exposed to 750 μmol m^{-2} s^{-1} PPFD high - pressure sodium light for 25 hours at 25°C in the

apparatus described in Fig. 50. Herbicidal injury was determined by electrolyte leakage and chlorophyll loss as described in sections 3.2.3 and 3.2.4.

4.2.4.3 Interactions of M&B 39279 and Acifluorfen with the Electron Transfer Chain

'Type E' chloroplast fragments (as defined by Hall 1972) were used in the experiments described in this section. These require the addition of of an oxidant to permit electron transfer reactions. The experiments designed to demonstrate M&B 39279 / acifluorfen effects on photosynthetic electron transfer employed two such oxidants in two separate systems. Potassium ferricyanide was used to allow a Hill reaction (Hill, 1937), electrons being transferred from water to ferricyanide ions via both photosystems I and II. Paraquat was employed in another electron transfer system involving only photosystem I, the redox chain being blocked at photosystem II with DCMU. Electrons were supplied in this instance to photosystem I by ascorbate / reduced DCPIP (2,6-dichlorophenolindophenol), paraquat being reduced by photosystem I and in turn, paraquat being oxidised by oxygen (such reactions are commonly referred to as Mehler reactions, (see Mehler, 1951). A third system employing isolated thylakoids was an attempt to assess any activity of M&B 39279 / acifluorfen in photophosphorylation inhibition, using the ferricyanide Hill reaction under conditions which permitted phosphorylation.

The above thylakoid reactions were followed by measuring oxygen evolution (Hill reactions) and uptake (Mehler reaction) in a Clarke - type oxygen electrode (Hansatech DW2 electrode, Hansatech Ltd, Kings Lynn, U.K.) operating at 25°C. The electrode was calibrated by depleting oxygen from 2ml of water in the electrode well by adding

crystals of sodium dithionite, thereby generating a zero oxygen signal. Electrode output was monitored on a chart recorder.

'Type E' chloroplast fragments were prepared from G.aparine and V.arvensis leaves by the following method. Chilled leaves from whorl 2 of G.aparine (whorl diameter was roughly 45 - 48mm) and from leaves 3 - 6 of V.arvensis (plants bearing 6 expanded leaves were macerated into very cold (2 - 3°C) maceration buffer (50mM Tricine, 0.3M NaCl, 3mM $MgCl_2$, 0.01% (w/v) bovine serum albumin, 0.1% (w/v) polyvinyl pyrrolidone MWt = 40 000, final pH = 7.5, adjusted with NaOH) using an 'Ultra Turrax' homogeniser operating for about 20 seconds, using 8.0ml of maceration buffer per 1.0g of leaves. The macerate was filtered through eight layers of muslin and centrifuged in a refridgerated centrifuge (MSE 'Chilspin', FSA Ltd, Loughborough, U.K.) at 1000xg for one minute. The resulting supernatant was then centrifuged at 3000xg for ten minutes (MSE 'Chilspin') and the pellet, consisting of isolated thylakoids and chloroplasts, was resuspended in 5mM Tricine, 2mM disodium ethylene diaminotetraacetic acid (EDTA), 0.1M sucrose, 3mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, pH = 8.0, adjusted with NaOH. One ml of this solution was added per 1.0g of leaves originally used and the suspension was stored on ice until required. Thylakoid preparations were adjusted to a concentration of 200µg chlorophyll / ml (chlorophyll estimated as by Arnon, 1949) with resuspension buffer and used as soon as possible, and never more than 2 hours after preparation.

Reaction mixtures for thylakoid - mediated reactions were:

(1)Ferricyanide Hill Reaction

The electrode well contained 0.2ml of 0.3M Tricine - NaOH, pH 8.0, 0.1ml of 10mM potassium ferricyanide, 0.2ml M&B 39279 / acifluorfen

solution, 1.0ml of water and 0.5ml thylakoids containing 200µg chlorophyll / ml. Final concentrations were 30mM Tricine, 0.5mM ferricyanide and thylakoids equivalent to 50µg chlorophyll / ml.

(2) Paraquat - mediated Mehler reaction

The electrode well contained 0.2ml of 0.3M Tricine - NaOH, pH 8.0, 0.1ml of 1mM DCPIP, 0.2ml of 10mM iso-ascorbate (sodium salt), 0.1ml of 0.2mM paraquat, 50µl 0f 1mM monuron (CMU, 3(4'-chlorophenyl)-1,1-dimethyl urea) dissolved in methanol, 0.2ml of M&B 39279 / acifluorfen solution , 0.65ml water, 0.5ml thylakoid preparation. Final concentrations were 30mM Tricine, 50µM DCPIP, 1mM iso-ascorbate, 10µM paraquat, 50µM monuron and thylakoids equivalent to 50µg chlorophyll / ml.

(3) Phosphorylating Hill Reaction

The electrode well contained 0.2ml of 0.3M Tricine - NaOH, pH 8.0, 0.1ml of10mM potassium ferricyanide, 0.1ml of 10mM adenosine diphosphate (ADP), 0.1ml of 100mM dipotassium phosphate trihydrate, 0.2ml M&B 39279 / acifluorfen solution 0.8ml water and 0.5ml thylakoid preparation. Final concentrations were 30mM Tricine, 0.5mM ferricyanide, 0.5mM ADP, 5mM phosphate and thylakoids equivalent to 50µg chlorophyll / ml.

M&B 39279 / acifluorfen solutions contained 0.1mM or 1.0mM herbicides giving final concentrations of 10µM and 100µM. These solutions also contained 5% (v/v) acetone. Final solvent concentrations were therefore, 0.5% for the Hill reactions and 3.0% for the Mehler reaction (0.5% acetone + 2.5% methanol in which monuron was dissolved). Equivalent concentrations of solvents were included in controls.

Prior to the above experiments, a test of ferricyanide Hill reaction was performed adding the uncoupling agent ammonium chloride to a final

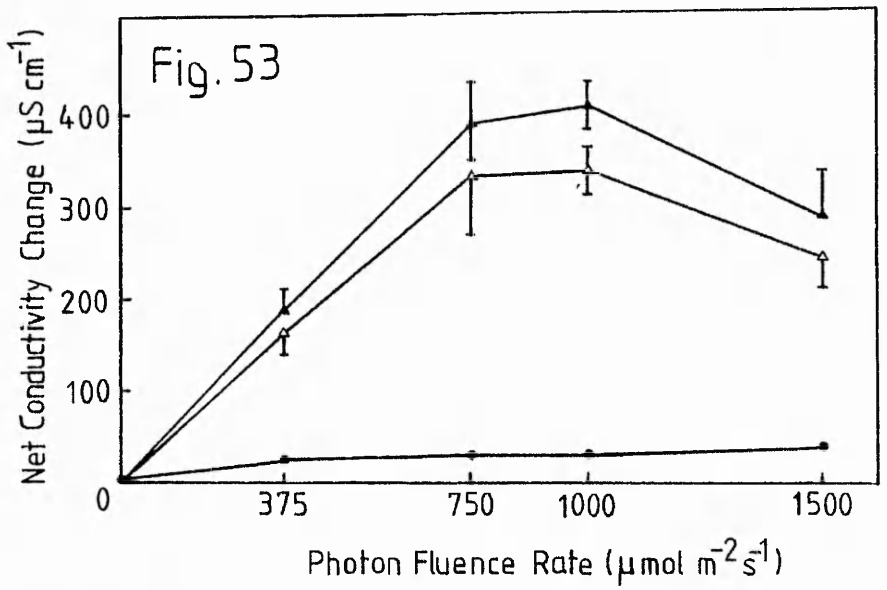
concentration of 1mM to assess tightness of coupling of each preparation, thereby giving an indication of thylakoid integrity. All preparations demonstrated at least a doubling in the rate of oxygen evolution after ammonium uncoupling.

Each treatment was repeated four times in four experiments.

4.3 RESULTS

4.3.1 EFFECTS OF LIGHT INTENSITY UPON M&B 39279 AND ACIFLUORFEN ACTIVITY

The influence of light intensity upon activity of M&B 39279 and acifluorfen and time courses of electrolyte leakage and chlorophyll loss are shown in Figs. 53-56. Over the period assessed, increasing light intensity resulted in increased electrolyte leakage from leaves floating on M&B 39279 or acifluorfen and increased chlorophyll loss up to a light intensity of 750-1000μmol m^{-2} s^{-1} PPFD. After incubation, water soaking and necrotic (bleaching) symptoms were observed on herbicide treated but not control leaflets. These were similar to symptoms observed on sprayed plants and increased in severity with increasing light intensity. Though not statistically significant, herbicidal effectiveness was approximately 30% less at 1500μmol m^{-2} s-1 PPFD than at 1000μmol m^{-2} s^{-1} PPFD (Figs 53 and 54).



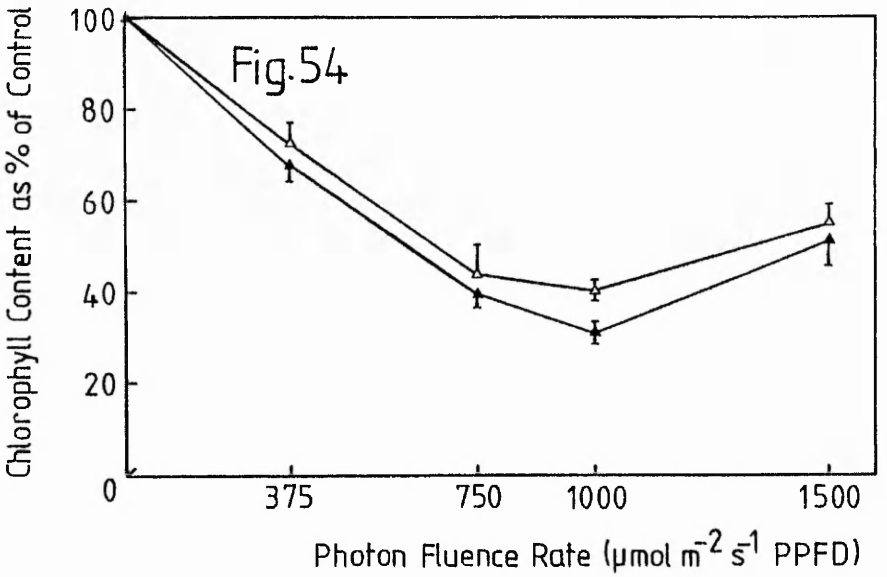
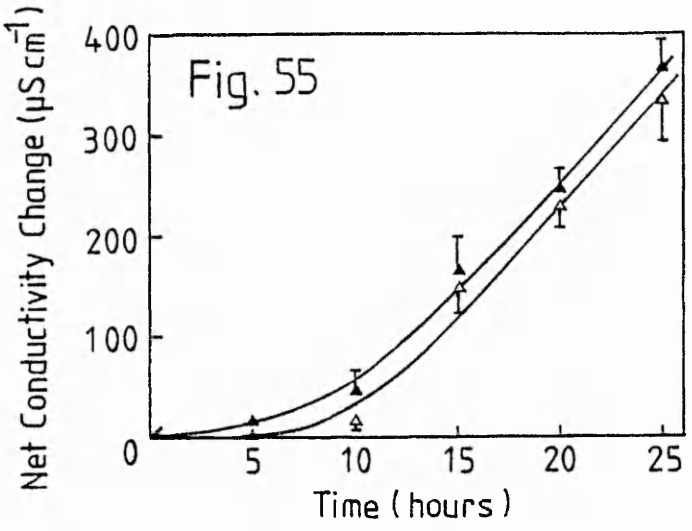


Figure 53. Electrolyte leakage from G.aparine excised leaflets floating on 100µM M&B 39279 (▲——▲), 100µM acifluorfen (△——△), and water (●——●) as a function of light intensity. Vertical bars are 2x standard error. Conductivity change = µS cm^{-1} (test sample) - µS cm^{-1} (herbicide solution in water). Control data are absolute conductivity values

Figure 54. Chlorophyll loss from G.aparine excised leaflets floating on 100µM M&B 39279 (▲——▲), 100µM acifluorfen (△——△), and water (●——●) as a function of light intensity. Vertical bars are 2x standard error.



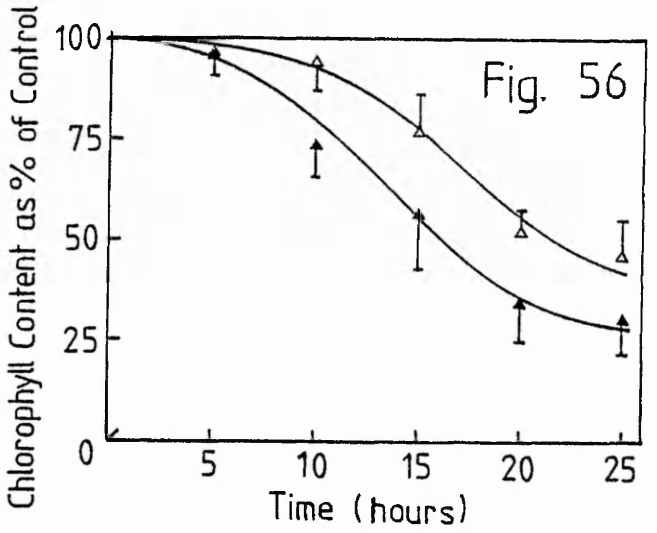


Figure 55. Time course of electrolyte leakage from G.aparine leaflets floating on 100μM M&B 39279 (▲—▲), 100μM acifluorfen (△—△) and water (●——●) under 750 μmol m^{-2} s^{-1} PPFD. Data are means of 4 replicates. Vertical bars are 2x standard error. Conductivity change - μS cm^{-1} (test sample) - μS cm^{-1} (herbicide solution in water). Control data are absolute conductivity values.

Figure 56. Time course of chlorophyll loss from G.aparine leaflets floating on 100μM M&B 39279 (▲——▲), 100uM acifluorfen (△——△) and water (●——●) under 750μmol m^{-2} s^{-1} PPFD. Data are means of 4 replicates. Vertical bars are 2x standard error.

4.3.2 THE INFLUENCE OF LIGHT QUALITY UPON M&B 39279 AND ACIFLUORFEN ACTIVITY

4.3.2.1 Effects of Varying Spectral Environment on M&B 39279 and Acifluorfen Activity.

Figs. 57 and 58 clearly demonstrate that blue light is considerably more effective in mediating M&B 39279 herbicidal activity than is red light, though red light is by no means ineffective. Although red light showed little ability to mediate M&B 39279 induced toxity at 8μmol m^{-2} s^{-1} PPFD, red light was effective in inducing electrolyte leakage and chlorophyll loss at a photon fluence rate of 15μmol m^{-2} s^{-1} PPFD (Fig. 58). Following the 48 hours incubation under blue and red light, herbicide treated leaflets showed water soaked but not necrotic or bleached patches. Green light-incubated and control leaflets bore no toxicity symptoms and no changes in bathing medium conductivity and leaf chloropyll content were obsered in these treatments. Additionally, some leaflets incubated under 15μmol m^{-2} s^{-1} PPFD red light demonstrated increased production of anthocyanins. This was, however, never seen in blue light incubated leaves, nor in red light incubated leaves at 8μmol m^{-2} s^{-1} PPFD.

Fig.57

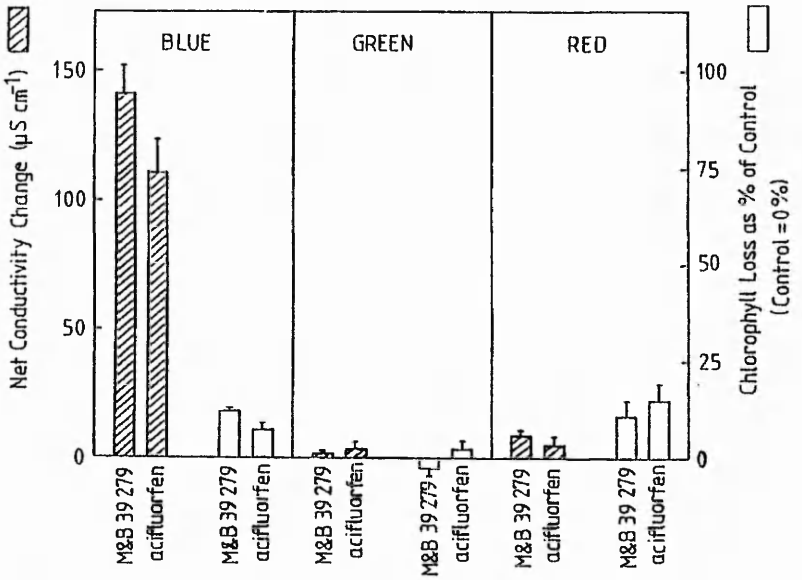


Fig.58

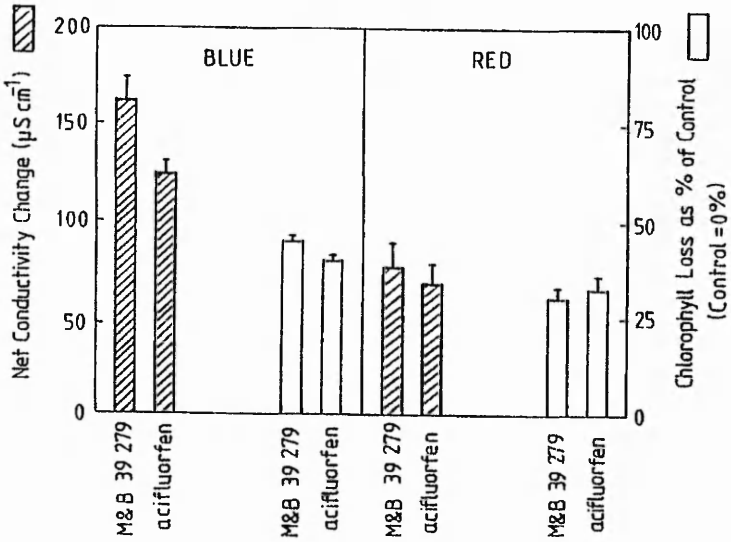


Figure 57. Electrolyte leakage (▨) and chlorophyll loss (□) from G.aparine leaflets floating on 100µM M&B 39279, 100µM acifluorfen and water (control) under blue, green and red light, all 8µmol m^{-2} s^{-1} PPFD, for 48 hours. Data are means of 6 replicates. Vertical bars are standard errors.

Figure 58. Electrolyte leakage (▨) and chlorophyll loss (□) from G.aparine leaflets floating on 100µM M&B 39279, 100µM acifluorfen and water (control) under blue and red light, both 15µmol m^{-2} s^{-1} PPFD fo 48 hours. Data are means of 6 replicates. Vertical bars are standard errors.

4.3.2.2 Effects of M&B 39279 and Acifluorfen in Blue Light Mediated Phototropism in Etiolated Pea Seedlings

Both M&B 39279 and acifluorfen inhibited blue light-induced phototropic curvature in etiolated P.sativum seedlings increasingly for herbicide concentrations between 5×10^{-8} and 5×10^{-4} M with maximal inhibition of approximately 10-25% between 5×10^{-4} and 1×10^{-4} M M&B 39279 under the described conditions (Fig. 59). M&B 39279 showed greatly reduced inhibition at 5×10^{-3}M and acifluorfen at this concentration had very little or no inhibitory effect.

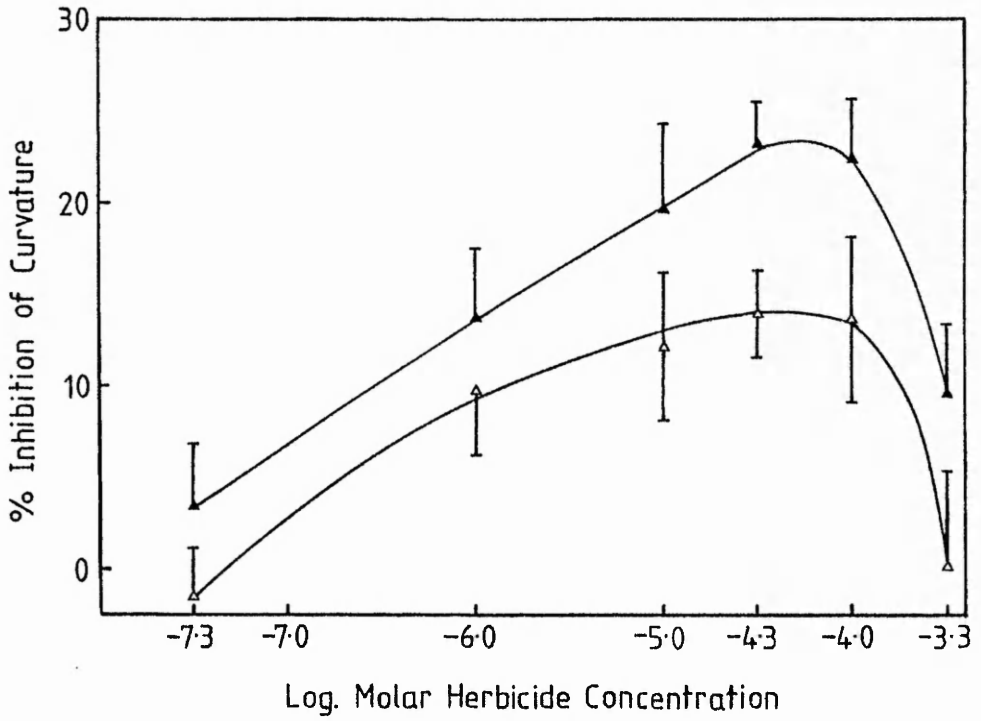
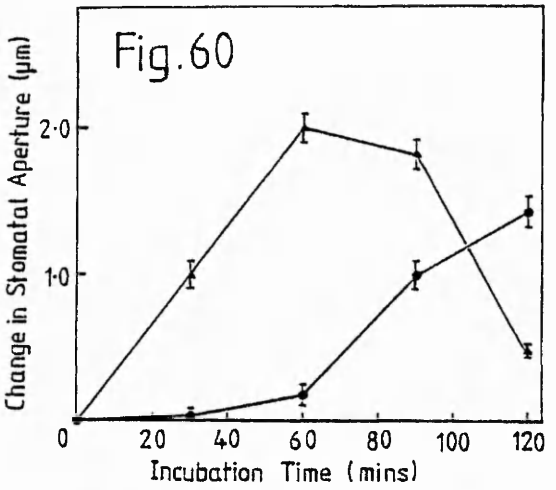


Figure 59 Inhibition of blue light - dependent curvature of Pisum sativum etiolated seedlings by M&B 39279 (▲——▲) and acifluorfen (△——△) at various concentrations.

4.3.2.3 Effects of M&B 39279 and Acifluorfen on Blue Light Induced Stomatal Opening

As shown in Fig. 60, 10μM M&B 39279 caused a remarkable stimulation of blue-light induced stomatal opening in G.aparine epidermal peels during the first 60 minutes of incubation, stomatal aperture of M&B 39279 - treated epidermal peels being approximately ten times that of control stomata after 60 minutes. This was however, only a transient effect and after 90 minutes, M&B 39279 treated stomatal aperture was rapidly reduced. Commelina communis stomatal guard cell protoplasts similarly showed a stimulation of protoplast swelling (analogous to stomatal opening) effected by 100μM M&B 39279 followed by protoplast shrinkage (analogous to stomatal closure) (Fig. 61).

Results in this section (4.3.2.3) are unpublished data of K.J. Nichols and A.H. Cobb, Trent Polytechnic.



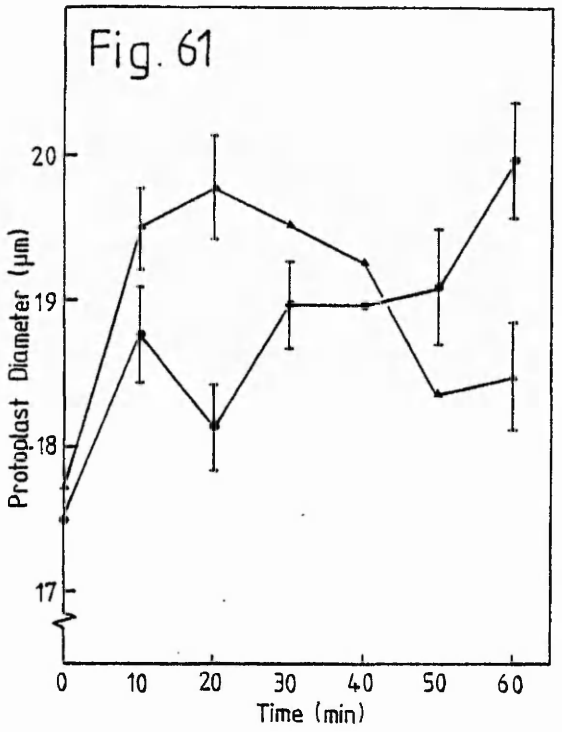


Figure 60. Effect of 10µM M&B 39279 (▲——▲) upon blue light - dependent opening of stomata in G.aparine epidermal peels under 17µmol m^{-2} s^{-1} PPFD blue light. (●——●) = control. Vertical bars are 2x standard error. Data are of Nichols and Cobb, Trent Polytechnic (unpublished).

Figure 61. Effect of 100µM M&B 39279 on blue light - dependent Commelina communis guard cell protoplast swelling under 17µmol m^{-2} s^{-1} PPFD blue light. (●——●) = control. Vertical bars are 2x standard error. Data are of Nichols and Cobb, Trent Polytechnic (unpublished).

4.3.3 INTERACTIONS OF PHOTOSYNTHETIC SYSTEMS WITH M&B 39279 AND ACIFLUORFEN

4.3.3.1 Necessity of Photosynthetic Electron Transfer for M&B 39279 and Acifluorfen Activity

Tables 3 and 4 show that the DCMU pretreatment of excised G.aparine leaflets only partially inhibited photosynthetic electron transfer since complete inhibition would have reduced paraquat activity to a much greater extent. Nevertheless, DCMU inhibited both M&B 39279 and acifluorfen activity to a similar level to which paraquat activity was inhibited. Since paraquat is known to directly depend upon photosynthesis electron transfer for activity, it may appear that in the incubation system used, photosynthetic electron transfer was involved in herbicidal symptom expression.

Treatment	Conducivity Change ($\mu S\ cm^{-1}$)	Percentage of Effect of Herbicide without DCMU
M&B 39279	126.5 ± 9.5	100
Acifluorfen	100.9 ± 12.8	100
Paraquat	130.0 ± 29.0	100
M&B 39279 + DCMU	81.6 ± 15.3	65
Acifluorfen + DCMU	84.9 ± 11.9	84
Paraquat + DCMU	88.3 ± 37.1	68

TABLE 3 Effect of DCMU (0.1 mM) upon M&B 39279 and acifluorfen (both 0.1mM) and paraquat (0.01mM) - induced electrolyte leakage from excised G.aparine leaflets. Conductivity change = μS cm-1 (sample) - μS cm-1 (herbicide solution in water)

Treatment	Chlorophyll Content (mg/ 5 leaflets)	Percentage of Control
Control	2.59 ± 0.09	100.0
M&B 39279	1.34 ± 0.13	56.9
Acifluorfen	1.54 ± 0.18	59.8
Paraquat	1.24 ± 0.15	48.2
M&B 39279 + DCMU	1.81 ± 0.09	76.6
Acifluorfen + DCMU	1.81 ± 0.15	70.5
Paraquat + DCMU	1.80 ± 0.14	76.5

TABLE 4 Effect of DCMU (0.1mM) upon M&B 39279 and acifluorfen (both 0.1mM) and paraquat (0.01mM) on chlorophyll content of G.aparine leaflets.

4.3.3.2 Interactions of M&B 39279 and Acifluorfen with the Photosynthetic Electron Transfer Chain

Tables 5 and 6 clearly show that in the thylakoid system used neither M&B 39279 nor acifluorfen at 10 and 100μM inhibit ferricyanide / paraquat - mediated photosynthetic electron transfer from water through photosystem II and photosystem I to the point with which paraquat (methyl viologen) interacts. It is seen in table 7 however, that both M&B 39279 and acifluorfen inhibit photosynthetic phosphorylation - stimulated electron transfer in 'Type E' thylakoid fragments by around 10% at 100 μM but not all at 10 μM. The photosynthetic control ratio of 2.04 (rate of electron transfer under phosphorylating conditions: basal rate of electron transfer) exhibited by the thylakoid preparations used was as expected for ' Type E' chloroplast fragments (Hall, 1972).

Numerous workers have described inhibition of photosynthetic electron transfer at I_{50} concentrations of 10^{-5}M or greater (see section 1.3.7). Even though the use of the term I_{50} in quantifiying inhibition of biological systems is somewhat vague, (see Naqui, 1983) the data herein do not indicate any such inhibitory property of M&B 39279 or acifluorfen even at 10^{-4}M concentration.

Basal Rate	100
Uncoupled Rate	267 ± 28
10^{-4}M M&B 39279	95 ± 6
10^{-5}M M&B 39279	100 ± 7
10^{-4}M Acifluorfen	101 ± 7
10^{-5}M Acifluorfen	99 ± 9

TABLE 5. Effect of M&B 39279 and Acifluorfen on the Ferricyanide Hill Reaction in 'Type E' Thylakoid Fragments isolated from G.aparine. Data are Oxygen Evolution Rates as a percentage of the Basal Rate. Basal Rate for the Hill Reaction was 24 ± 2 μmol O_2 mg chll^{-1} hr^{-1}.

Basal Rate	100
Uncoupled Rate	167 ± 14
10^{-4}M M&B 39279	101 ± 6
10^{-5}M M&B 39279	103 ± 8
10^{-4}M Acifluorfen	103 ± 8
10^{-5}M Acifluorfen	102 ± 7

TABLE 6. Effect of M&B 39279 and acifluorfen on a D.C.P.I.P. / paraquat - supported Mehler Reaction in 'Type E' thylakoid fragments isolated from G.aparine.

Data are oxygen evolution rates as a percentage of the basal rate. The basal rate for the Mehler reaction = 34 ± 3 μmol O_2 mg chll^{-1} hr^{-1}.

Control	100
Basal Rate (no ADP + Pi)	49 ± 3
100 µM M&B 39279	87 ± 5
10 µM M&B 39279	97 ± 5
100 µM acifluorfen	89 ± 11
10 µM acifluorfen	96 ± 4

TABLE 7. Effect of M&B 39279 and acifluorfen on energy transfer in 'Type E' thylakoid fragments isolated from G.aparine.

Data are rates of oxygen evolution as a percentage of control (phosphorylating) rate. The control yielded a mean of 64 ± 4 µmol O_2 $mgchl^{-1}$ hr^{-1}.

4.4 DISCUSSION

Figs. 53-56 confirm the light-dependancy of M&B 39279 and acifluorfen shown previously in Chapter 3. It is interesting that at 1500μmol m^{-2} s^{-1} PPFD that electrolyte leakage and chlorophyll loss from herbicide treated leaves was less than at 750-1000μmol m^{-2} s-1 PPFD. This may suggest that the mechanism of action of M&B 39279 / acifluorfen is dependant upon a system which is partially inhibited at very high light intensities. This is a likely possibility since G.aparine would never be exposed to light at an intensity equivalent to 1500μmol m^{-2} s^{-1} PPFD high-pressure sodium light in its natural environment nor under the glasshouse conditions under which plants were grown. It is conceivable therefore, that photoprotective systems within the plant would be unable to cope with such an intensity of light.

The data shown in Figs. 57 and 58 confirm the published action spectra for acifluorfen of Matsunaka (1969b), Ensminger and Hess (1985a) and for LS82-556 (Matringe and Scalla, 1987a) showing a major peak of DPE - type herbicide activity in blue wavelengths and a minor peak in red wavelengths. The implications for constitutive blue light photoreceptors which could feasibly be involved in the herbicidal process and could account for the observed spectra are described in section 1.3.5. The most likely candidates are chlorophylls or their precursors. In addition to confirming certain published action spectra, Figs 57. and 58 demonstrate that although the rate of M&B 39279 / acifluorfen reduced damage to G.aparine leaves is dependant upon light intensity (Figs. 53, 54, 57 and 58), only small photon fluence rates are necessary for their activity.

That increased biosynthesis of anthocyanins was found in some leaves incubated under 15μmol m^{-2} s^{-1} PPFD red light but not 8μmol m^{-2} s^{-1} could give cause for questioning the quantitative validity of the data shown in Fig. 58. Although photoprotective anthocyanin synthesis is believed to be under the control of the red light-absorbing pigment phytochrome, Mohr and Drumm-Herrel (1983) showed that in several species, there appeared to be a co-action between blue/uv light and red light in establishing increased phytochrome-dependant anthocyanin synthesis. They furthermore showed tht 10μM acifluorfen stimulated anthocyanin synthesis in tomato (Lycopersicon esculentum Mill.) seedlings exposed to blue light. Red light alone did not produce this effect with acifluorfen. The finding of increased anthocyanin synthesis under 15μmol m^{-2} s^{-1} PPFD in Fig. 58 may therefore indicate that at this intensity, (but not at 8μmol m^{-2} s^{-1}) the light filter did not remove all of the blue regions of the spectrum and so part of the symptoms observed were blue - light induced, exaggerating the effectiveness of red light in this system. Conversely, there is no reason to suspect that a blue light - far red light co-action system for anthocyanin synthesis occurs in G.aparine. No such effect was observed in G.aparine leaves incubated in blue light at 8 or 15μmol m^{-2} $^{-2}$ PPFD (and a small amount of far red, see Fig. 52) and Mohr and Drumm - Herrel failed to show such a system for mustard (Sinapis alba) and wheat (Triticum aestivum) seedlings. Furthermore, the acifluorfen stimulation of anthocyanin synthesis in tomato seedlings observed by Mohr and Drumm - Herrel (1983) was prevented by growing seedlings in the presence of the carotenoid synthesis inhibitor difunon, a treatment which did not affect the normal blue light induced

anthocyanin synthesis. This shows that bulk carotenoids are not involved in the blue light photoreceptor / transduction system in tomato (Mohr and Drumm - Herrel, 1983) and that acifluorfen was not interacting with this system. Since difunon inhibited the acifluorfen effect in this system as normally observed in DPE - type herbicide action (see section 1.3.5), it seems likely that acifluorfen was acting in its normal herbicidal mode in Mohr and Drumm - Herrel's system (15 hour exposure to low intensity blue light) and that the stimulation of anthocyanin synthesis was a stress response rather than a specific effect on the blue light photoreception - transduction pathway.

The finding that M&B 39279 and acifluorfen inhibited blue light - induced phototropic curvature in peas between approximately 10^{-7} to 5 x 10^{-3}M with a maximum inhibition at a concentration of approximately 5 x 10^{-4}M (Fig. 59) appears to contradict a number of reports in the literature. Leong and Briggs (1981a, 1982) for example, reported that acifluorfen (and other DPE herbicides) stimulated a blue light-induced absorbance change in membrane preparations derived from etiolated corn (Zea Mays L.) and oat (Avena Sativa L.) seedlings, and correspondingly inhibited dark re-oxidation of a cytochrome in this preparation. This membrane preparation has been proposed as containing a blue light photoreceptor (a flavocytochrome). Acifluorfen also stimulated blue light-induced phototropism in etiolated A.sativa seedlings. Two other reports describing interference of acifluorfen with blue light-dependant systems have been presented, by Mohr and Drumm-Herrel (1983) and by Gaba and Gressel (1987). The data of Mohr and Drumm - Herrel have been discussed above. Gaba and Gressel (1987) investigated

acifluorfen-induced stimulation of blue light-dependant sporulation of Trichoderma harzianum. The acifluorfen - induced stimulation was however, identical whether acifluorfen was administered to the fungus at the same time as a blue light pulse or whether it was administered in the dark 30 minutes after a blue light pulse, thus showing that the herbicide was not acting directly upon the blue light receptor. Although working on three different blue-light dependent systems (and possibly more than one photoreceptor - see Briggs and Iino 1983), with different levels of interaction of acifluorfen with these systems, Leong and Briggs (1981a, 1982), Mohr and Drumm-Herrel (1983) and Gaba & Gressel (1987) all demonstrated stimulation of blue-light dependant plant systems by DPEs. Additionally as shown in Figs. 60 and 61, M&B 39279 transiently effected a large stimulation of blue light-induced stomatal opening in G.aparine epidermal peels and protoplast swelling in Commelina communis guard cell protoplasts in this study. As the epidermal peels were exposed to 17μmol m^{-2} s^{-1} PPFD for 120 minutes and guard cell protoplasts to 15μmol m^{-2} s^{-1} PPFD for 60 minutes it is likely that the eventual reversal of stimulation of stomatal opening / protoplast swellings seen after 60 minutes in epidermal peels and 20 minutes with guard cell protoplasts (Figs. 60 and 61) was due to onset of herbicidal damage. Figs. 57 and 58 indeed show that photon fluence rates of 15-17μmol m^{-2} s^{-1} PPFD are adequate for DPE-type herbicidal activity.

In view of the above information , it is somewhat surprising that M&B 39279 and acifluorfen apparently inhibited rather than stimulated blue-light induced phototropism in P.sativum (Fig.59), especially since it has been reported that P.sativum seedlings display nearly identical phototropic responses to Zea mays (Baskin,1985) and so

stimulation of phototropism as described by Leong & Briggs (1981a) would have been expected. The system used in this study was, however, rather crude in comparison with that used in Brigg's labaratory. In this study, whole seed trays of seedlings were exposed to 2-4μmol m^{-2} s^{-1} PPFD light for 18 hours. Briggs exposed single rows of seedlings to a 10 second pulse of 0.01μmol m^{-2} s^{-1} PPFD light. More important than this, it was shown in this study that the conditions under which the P.sativum seedlings were exposed to blue light were sufficient to allow herbicidal activity of DPE-type compounds (Figs.57 and 58). Furthermore a chemical analogue having several fold greater herbicidal activity than M&B 39279 was applied to the P.sativum phototropism assay (supplied by Rhone-Poulenc Agriculture, designated M&B 39535, structure confidential) and necrotic, water - soaked lesions were seen on leaf initials after the 18 hour incubation period. There is therefore, a strong possibility that M&B 39279 and acifluorfen were acting herbicidally in the P.sativum assay and that inhibition of epicotyl curvature merely reflected general growth inhibition rather than a specific effect on phototropism. This explanation is further plausible in the light of recent publications suggesting that DPE-type compounds interfere with chlorophyll biosynthesis (which at least part of the pathway would be very active in de-etiolating seedlings), generating photosensitizing tetrapyrolles (see section 1.3.5), and data in this study showing the effectiveness of blue light for M&B 39279 and acifluorfen activity. The lack of inhibition by 5 x 10^{-3}M M&B 39279 and acifluorfen is not easily explained in these terms beyond regard to the large standard errors of the means shown on Fig. 59 for these values.

More work is clearly needed to clarify the interaction of DPE-type

compounds with the Pisum bioassay used in this study, and indeed to further explore the relevance of the data of Leong and Briggs (1981a and b, 1982) who found different results using a crude A.sativa coleoptile membrane preparation and a Triton X-100 solubilized extract of this preparation (Leong and Briggs, 1981a and b, 1982. The relevance of such further work in a study of the DPE-type herbicidal mode of action is however, questionable. It is likely that DPE - type herbicides have numerous minor effects upon plant metabolism which are of little consequence compared with the major mechanism of action. Interference with constitutive blue light photoreceptors in plants may be such a minor effect even though blue light is very efficient in DPE action. Interaction of these compounds with the blue light receptor itself, which may be a flavin-cytochrome complex (Leong & Briggs 1981b), is nevertheless interesting.

Duke and Kenyon (1987) listed almost equal numbers of publications presenting evidence for and against the involvement of photosynthesis in the activity of DPE-type herbicides, using several different experimental systems and several other papers could be added to their list eg. Tissut et al (1987), Haworth and Hess (1988) and Nurit et al (1988). This disagreement in the literature has also been described in section 1.3.7 of this thesis. One area of contention has been the effectiveness of substituted urea and triazine herbicides in suppressing DPE-type activity. Kunert et al (1985) and Bowyer et al (1987c, 1989) attempted to unify these apparently conflicting data by suggesting that urea and triazine herbicides reduce oxygen availability (via inhibiting photosystem II mediated water oxidation) thereby retarding lipid peroxidation. The data shown in this chapter reveal that DCMU successfully suppressed M&B 39279 and acifluorfen - induced chlorophyll loss and electrolyte leakage from

excised G.aparine leaves. In view of the conflicting literature surrounding this phenomenon, it is not at all clear whether this suggests a direct role for photosynthetic electron transfer in DPE - type activity or not. DCMU inhibition could for example, suppress a photosynthetic electron transfer - dependant system upon which DPE-type herbicide action depends. More confusing is that substituted urea / triazine herbicide supression of DPE-type activity has been reports on DPE-mediated inhibition of photosynthetic electron transfer. As indicated in section 1.3.7, DPE-type herbicides generally inhibit photosynthetic electron transport with 50% inhibitory concentrations (I_{50}'s) of approximately on greater than 10mM and often much greater than this concentration. Some workers have shown 50% inhibition of pset with DPE herbicide concentrations of less than 10mM (Moreland et al 1970), though as stated earlier, it must be borne in mind that I_{50} concentrations are variables indicating effectiveness of inhibitors upons systems, their value depending on variable parameters in the experimental system, rather than constants such as 'Ki' values which describe interactions of enzymes and inhibitors in a more precise way (see Naqui, 1983). Data from this study (Tables 6, 7) support previous proposals that DPE - type herbicides are poor inhibitors of photosynthetic electron transfer. Data in table 6 do not however, concur with those of Haworth and Hess (1988) who showed oxyfluorfen to stimulate DCPIP / paraquat supported Mehler reaction. The small (approximately 10%) retardation of electron transfer under phosphorylating conditions in this study (table 7) by 100μM but not 10μM acifluorfen and M&B 39279 occurred at concentrations likely to be too high to be of great physiological significance. The inhibition of photophosphorylation by DPE-type herbicides has been shown and

described several times previously (see section 1.3.7). Of particular interest are the few studies which have investigated inhibitory properties of DPE - type herbicides upon whole chain photosynthetic electron transfer systems, that is electron transfer from water to NADP rather than Hill or Mehler - type reactions. Gillham and Dodge (1987b) in agreement with data shown in Tables 5 and 6 showed that acifluorfen and oxyfluorfen were not inhibitory on either ferricyanide or paraquat dependant electron transport at concentrations between 1 and 50μM in 'Type E' Pisum thylakoid preparations. They additionally showed however, that oxyfluorfen and acifluorfen could reduce the rate of ferredoxin-dependant NADP reduction with 'Type E' chloroplasts plus exogenous ferredoxin and ferredoxin-NADP reductase and that these herbicides interfered with (stimulated) an in vitro system consisting of ferredoxin and ferredoxin-NADP reductase mediated oxidation of NADPH in the absence and presence of thylakoid membranes. These authors suggested from this work that DPE compounds may be reduced by ferredoxin, thereby initiating lipid peroxidation in a system similar to that described by Draper and Casida (1985) (see section 1.3.6). Contradicting this data, Alscher and Strick (1984) showed that electron transport from water to ferricyanide and water to ferredoxin/NADP were similar with respect to acifluorfen inhibition (200-300μM acifluorfen effected only a small reduction in electron transport) using 'Type D' spinach thylakoid preparations. This questions the relevance of the data of Gillham and Dodge who showed only a 30-40% reduction of the rate of Pisum thylakoid NADP reduction with 50μM oxyfluorfen and acifluorfen.

It is apparent that there has been and remains a great deal of controversy and contradiction surrounding interaction of DPE-type compounds and photosynthetic electron transport. Such contradictions

may have resulted from different research groups using slightly different experimental systems and result from variations in the very artificial *in vitro* thylakoid preparations used both in this study and most other reports on DPE - photosynthetic electron transfer chain interactions. DPE - type herbicides may have conceivably interacted with some thylakoid preparations in ways which are not encountered in an *in vivo* situation. Such interactions would have yielded convincing but misleading information. In spite of the considerable research efforts involving thylakoid systems, to date there has been no complete evidence put forward to suggest beyond reasonable doubt that DPE - type herbicides have a mode of action primarily or entirely dependant on thylakoid electron transfer.

CHAPTER 5

EFFECTS OF M&B 39279 AND ACIFLUORFEN ON PROTOPLAST STRUCTURAL AND METABOLIC INTEGRITY

5.1 INTRODUCTION

Previous chapters have described experiments investigating properties of herbicidal effects of M&B 39279 and acifluorfen on gross metabolism and physical appearance of whole plants, various physiological and ultrastructural effects and relationships with different light regimes in excised leaves. Characterisation of these herbicides was then attempted on a cellular level since experiments on whole organs or intact plants can be difficult to interpret owing to their high level of organisation and difficulty in manipulating such complex structures. This was achieved using protoplasts isolated from G.aparine.

Protoplasts have proved to be an extremely valuable tool in plant sciences and freed of problems associated with the cell wall, have enabled significant advances in many areas of plant biology, most notably in in vitro propagation, genetic transformation and manipulation, plant virology and those facets of the plant cell associated with the plasmalemma such as the cytoskeleton, cell surface receptors, cell wall synthesis, coated pits and endocytosis (eg. Fowke et al 1983). Comprehensive reviews discussing uses of plant protoplasts are widely available (eg Galun, 1981 and Pilet, 1985) and their use in herbicide mode of action studies has been discussed by Pallett et al (1986). There have been no reports, however, of preparation and use of G.aparine protoplasts in the literature however and so it was necessary in this study to devise techniques for this purpose. Once methods for satisfactory preparation of G.aparine protoplasts had been achieved, the effects of M&B 39279 and acifluorfen upon various physiological parameters were assessed.

5.2.1 PREPARATION OF PROTOPLASTS

The following procedure for preparation of protoplasts was found to be successful. Using fine forceps, the abaxial epidermis was peeled from G.aparine leaves and floated abaxial surface down on a plasmolysing medium (0.5M mannitol, 0.1% (w/v) polyvinyl pyrrolidone, (MWt = 40 000), 1mM $CaCl_2$ and 10mM 2- (N-morpholino) ethanesulphonic acid (MES) - KOH buffer, pH 5.5) until sufficient leaf material had been peeled to fill a 10cm diameter petri dish in a single layer. The plasmolysing medium was then replaced with an enzyme medium containing 1% (w/v) Cellulysin (Calbiochem), 0.05% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical) and 0.25% (w/v) bovine serum albumin, dissolved in plasmolysing medium and the pH corrected to 5.5 with aqueous KOH. Following incubation at 25°C under 50μmol m^{-2} s^{-1} PPFD provided by 'natural white' fluorescent tubes (GEC) for 1.5 hours, the resulting protoplast suspension was passed through two nylon filters (1.0mm and 0.2mm mesh), centrifuged at 100 x g for 2 minutes and the pellet resuspended in a storage medium (0.4M mannitol, 1mM $CaCl_2$, 20mM MES-KOH, pH 6.0). This crude protoplast preparation (Fig. 64) was purified by overlaying the suspension onto a discontinuous density gradient of 'Percoll' (Pharmacia) in iso-osmotic / iso-acidic storage medium (this consisted of 2 ml layers of 35, 30 and 25% (v/v) Percoll in 10ml centruge tubes). Gradients were centrifuged at 150 x g for 10 minutes. Protoplasts were collected from the 0/25% Percoll and the 25/30% Percoll interfaces diluted four fold with storage medium, re-centrifuged at100 x g for 2 minutes and resuspended in storage medium. The protoplast density in the suspension was then estimated

by counting in a haemacytometer and adjusted to 5 x 10^5 protoplasts per ml and stored at 25°C under 50μmol m^{-2} s^{-1} PPFD without shaking prior to use. Protoplast preparations were used within 4-5 hours following purification.

Leaf material for above protoplast preparations was taken from the second whorl of leaves having a 45-48mm diameter. Initially, protoplasts were prepared from whorls of diameter 35, 40, 45 and 50mm. All four gave good and similar yields of protoplasts with mean protoplast diameters of 34.3, 35.6, 35.1 and 35.4 respectively (n=25), of which 96-98% of protoplasts sequestered the vital stain neutral red in their vacuoles, and similar proportions excluded Evans Blue. Neutral red was dissolved in protoplast storage medium to a concentration of 0.1% (w/v). This was mixed with the protoplast suspension to give a final concentration of 0.01% neutral red. Evans blue was dissolved to a concentration of 0.04% (w/v) in protoplast storage medium. One drop of this solution was mixed with 1 drop of protoplast suspension. Both neutral red and Evans blue stained preparations were observed after 5 mins. Although both of these stains were used to indicate integrity of protoplast preparations during initial work to devise protoplast isolation techniques, they were not used further since it appeared that the only necessary criterion for neutral red uptake / Evans blue exclusion was an intact membrane. Isolated vacuoles had apparently identical properties to intact protoplasts with respect to these dyes (see Figs. 62 and 63). Since lysed protoplasts cannot be observed in a suspension, Evans blue and neutral red merely indicate the proportion of intact protoplasts possessing leaky membrane and give no indication of the proportion of protoplasts which have lysed. Counts of protoplast density were therefore used to determine the proportion of intact protoplasts in

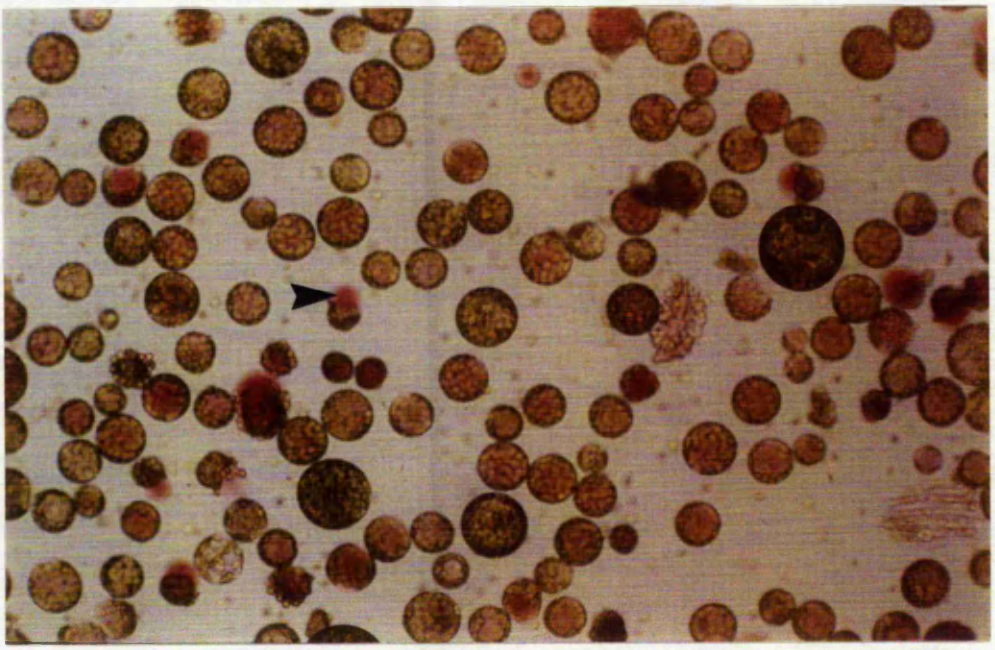


Figure 62. G.aparine protoplasts stained with neutral red. the dye is sequestered in the vacuole which is shown well in lysed protoplasts (arrow).

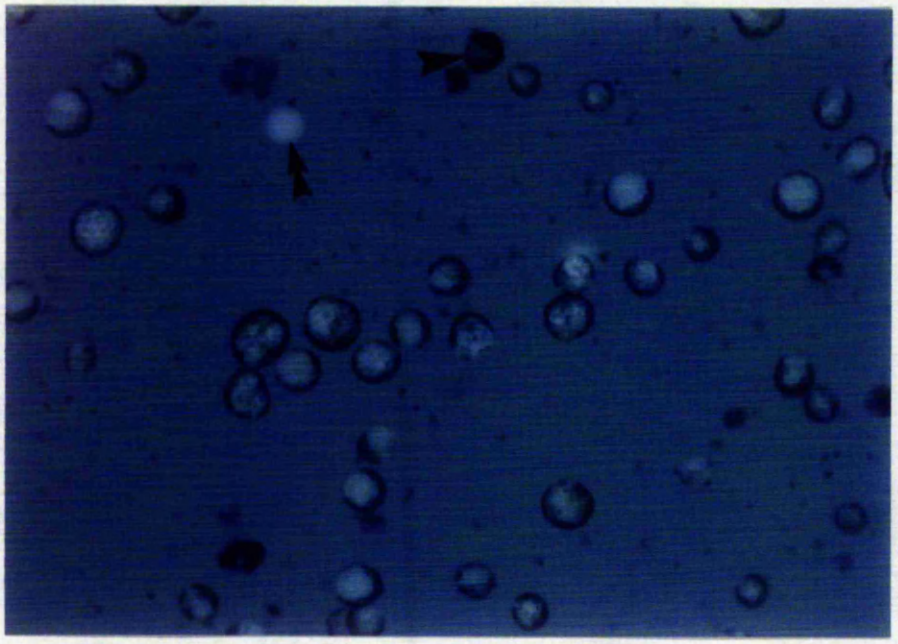
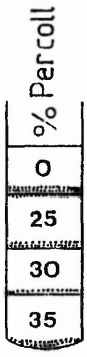


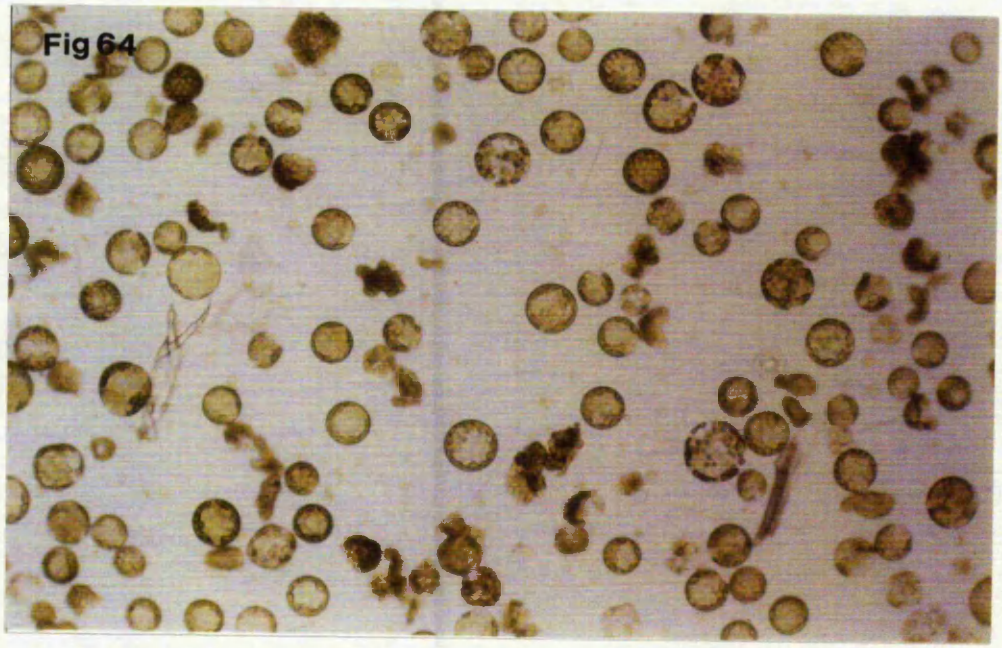
Figure 63. G.aparine protoplasts stained with Evans blue. The dye is excluded from intact protoplasts. Non - viable protoplasts (arrow) stain deep blue. Note that intact vacuoles from lysed protoplasts (double arrow) also exclude Evans blue.

suspensions. The successful purification of protoplasts on a 25/30/35% Percoll discontinuous gradient was achieved by trial and error, varying the concentrations of Percoll. A previous attempt to purify G.aparine protoplasts by floatation in a discontinuous gradient of 0.5M sucrose / 0.4M sucrose + 0.1 M mannitol / 0.5M mannitol (using 0.5M mannitol storage medium) as described by Edwards et al (1978) was unsuccessful, large numbers of protoplasts sedimenting out into the pellet upon centrifugation. The 25/30/35% Percoll gradient resolved 3 bands of protoplasts from the crude enzyme digest, the major band being at the 0/25% Percoll interface, and minor bands and the other interfaces. Properties of these bands are described in table 8 and it is seen that properties of protoplasts which banded at the 0/25 and the 25/30% Percoll interfaces were of similar size and displayed similar photosynthetic capabilities. Protoplasts were therefore collected from these bands and pooled together (see Fig 65) for use in experiments. The pellet, although containing some intact protoplasts, was largely composed of vascular debris and broken protoplasts (Fig. 66).



Band	% of Total Chlorophyll	Protoplast Diameter (μm)	Net Photosynthesis
1	48.2	33.7	88.3
2	22.5	34.6	86.1
3	12.3	n/a	n/a
pellet	17.0	n/a	n/a

Table 8. Properties of bands of protoplasts resolved on the 0/25/30/35% Percoll stepped density gradient as described in the text. Net photosynthesis data are μmol O_2 evolved / mg chll / hour. n/a = not assessed.



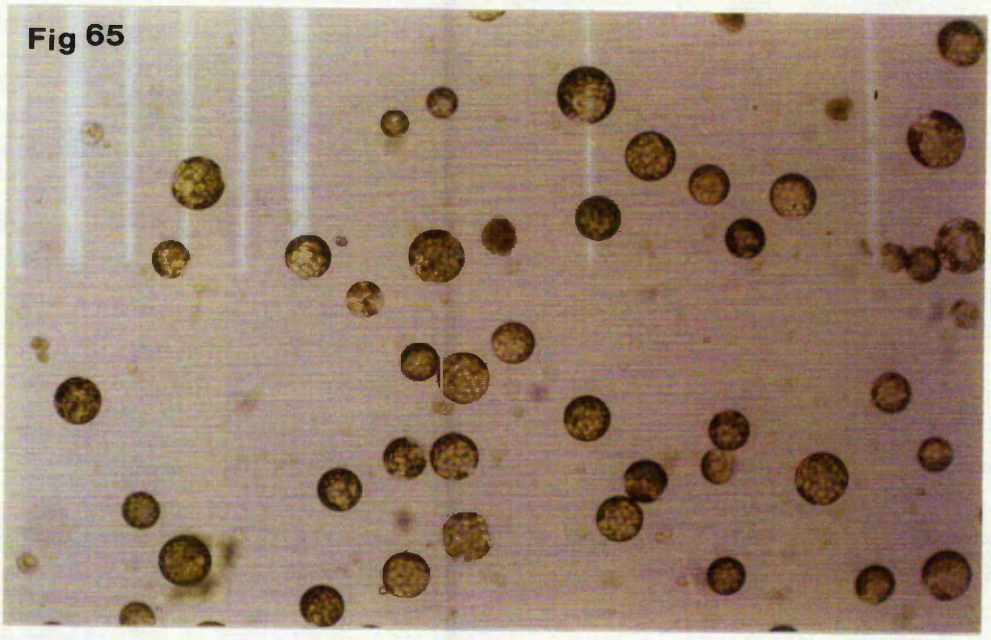


Figure 64. Crude protoplast digest showing vascular debris and clumps of lysed protoplasts.

Figure 65. Pure protoplast preparation following density gradient centrifugation.

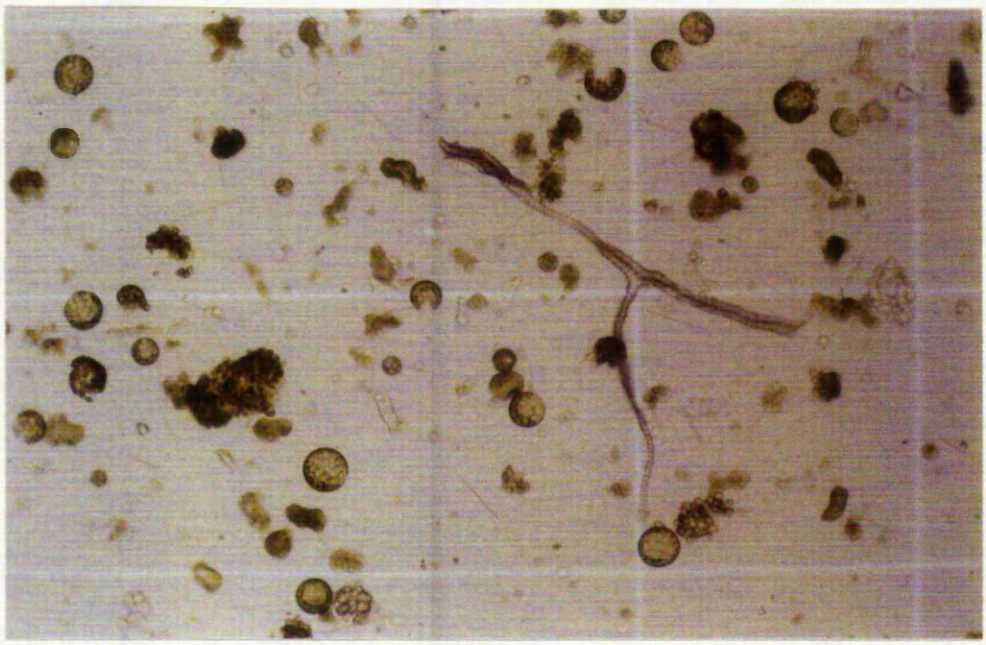


Figure 66. Pellet from Percoll density gradient showing xylem vessels, cell debris and a few intact protoplasts.

5.2.2 MEASURING EFFECTS OF M&B 39279 AND ACIFLUORFEN ON PROTOPLASTS

5.2.2.1 Incubation Conditions

Aliquots of protoplast suspension (2ml) were incubated in oxygen electrode wells (DW1 oxygen electrode, Hansatech Ltd, Kings Lynn, U.K.). Electrodes were operated at 25°C and illuminated by a tungsten-halogen projector lamp supplying 300μmol m^{-2} s^{-1} PPFD. 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 sufficiently to avoid excessive protoplast breakage, whilst maintaining a suspension and allowing an adequate electrode response to changes in oxygen concentration. Under these conditions, protoplast intactness remained stable for over 2 hours (see Fig 67). M&B 39279 and acifluorfen were added from stock solutions in acetone. Final solvent concentration in the incubation medium was 1.25% (v/v). These conditions were employed for all experiments in this chapter except for detection of lipid peroxidation- derived hydrocarbons.

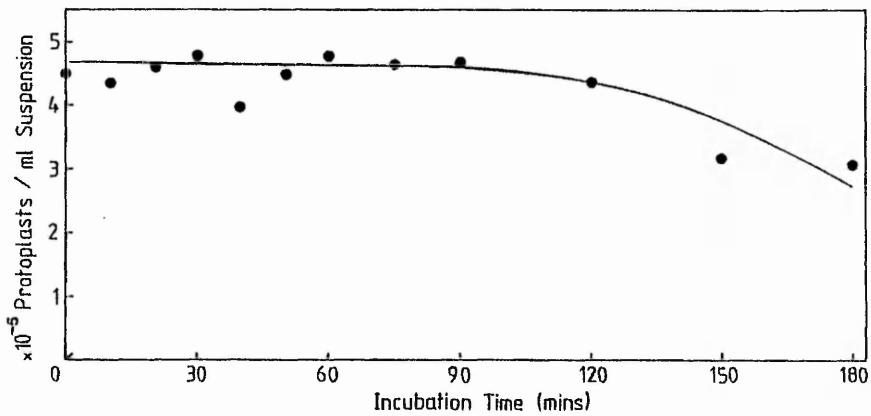


Figure 67. Protoplast intactness in oxygen electrode wells as described for incubation of protoplasts with herbicides.

5.2.2.2 Protoplast Intactness

Intactness was recorded as protoplast number / ml of suspension as a percentage of the original protoplast density. Counts of protoplasts were made on two haemacytometer grids per sample (treatment) in each of six experiments.

5.2.2.3 Effects of M&B39279 and Acifluorfen on Protoplast Membrane Integrity

Membrane integrity was assessed by (a) conductimetric monitoring of electrolyte leakage in the protoplast bathing medium and (b) gas chromatographic detection of hydrocarbons evolved by the protoplast suspension.

Leakage of electrolytes from protoplasts was measured using a PTI-18 digital conductivity meter (section 3.2.4). Protoplasts, incubated with or without the presence of M&B 39279 or acifluorfen as described in section 5.2.2.1 were allowed to sediment out of suspension in the electrode well by switching off the stirrer and the supernatant transferred to the conductivity electrode. This method prevented damage to protoplasts which would have occurred by centrifuging the suspension.

Detecting generation of short-chain hydrocarbons such as ethane and propane which result from degradation of lipid hydroperoxides is a well established means of monitoring lipid peroxidation in a variety of plant systems eg. Sandmann and Boger, (1982); Dodge and Gillham, 1986; Kunert and Boger, (1981); Dhindsa et al (1981). Evolution of ethane, the most commonly evolved hydrocarbon resulting from peroxidation of membrane lipids in higher plants, was detected by incubating protoplast suspensions in gas-tight vials and analysing the headspace gas. Protoplast suspensions (5ml aliquots) were placed in 20

ml glass vials sealed with a gas - tight teflon and neoprene septum (Pierce). The vials were submerged under 1cm water in a water bath thermostatically operated at 25oC and illuminated by a 400 W high - pressure sodium lamp. Light was filtered through 6 layers of muslin to achieve an intensity of 500μmol m^{-2} s^{-1} PPFD at the glass vials. It was found that shaking the vials, even at 1 cycle per second, caused a rapid loss of intact protoplasts (protoplast density was reduced from 5 x 10^5 to 2 x 10^5/ml over 3 hours). Protoplast density remained relatively stable for 5 hours however, if vials were slowly inverted several times every 15-20 minutes, the method finally adopted (protoplast density changed from 5 x 10^5 to 4.8 x 10^5/ml after 4 hours and to 4.6 x 10^5/ml after 5 hours). Protoplasts were incubated in the presence of 500 and 750μM M&B 39279 or acifluorfen added from stock solutions in acetone. Final solvent concentration was 1.25% v/v. 1ml samples of the gas headspace were taken from the vials every 30 minutes up to 5 hours using a 1ml gas-tight microsyringe (Hamilton) and hydrocarbon gases in the samples resolved using a Perkin - Elmer Sigma 3B gas chromatograph in conjunction with a Perkin - Elmer Porapak Q column (60 - 100 mesh) operating isothermically at 100oC. Output from the electron capture detector was monitored using a computing integrator (Perkin - Elmer LCI-100). The chromatograph was calibrated using authentic gas standards obtained from Alltech Associates, Carnforth, U.K. Standards were of ethane, pentane and a mixture of short chain alkanes.

5.2.2.4 Effects of M&B 39279 and Acifluorfen on Protoplast Photosynthesis

Two experiments were conducted investigating effects of M&B 39279

and acifluorfen on protoplast photosynthesis, investigating both rapid and slow effects. To assay photosynthesis, 1.5ml samples of protoplast suspension were centrifuged at 100 x g for 2 minutes and the pellet resuspended in 1.5ml of a solution containing 0.4M mannitol, 5mM $CaCl_2$, 25mM $NaHCO_3$ and 50mM Tricine - KOH, pH 7.6. This suspension was placed in an oxygen electrode well operating at 25°C and following equilibration for 3 minutes in darkness, protoplasts were exposed to 500μmol m^{-2} s^{-1} PPFD tungsten - halogen light and oxygen evolution monitored on a chart recorder. Following assay of photosynthesis, the chlorophyll content of the protoplast supension was estimated according to Arnon, (1949) to permit expression of photsynthetic rates on a chlorophyll basis. The electrode was calibrated using sodium dithionite as outlined in section 4.2.

To observe immediate effects of M&B 39279 acifluorfen on photosynthesis, the herbicides were added from stock solutions in acetone gining final concentrations of 100, 200, 300, 400 and 500μM prior to the 3 minutes dark incubation and photosynthesis measured as rate of oxygen evolution over the initial 3 minutes. Final solvent concentration never exceeded 1.25 % (v/v). For comparison, paraquat (final concentration of 50μm) was included in this experiment. Assays were repeated six times per treatment.

To assess slow effects of M&B 39279 and acifluorfen on photosynthesis, protoplasts were incubated in the presence of 0, 375, 500, and 750μM M&B 39279 and acifluorfen as described in section 5.2.3 and 1.5ml samples were removed from the electrode well, centrifuged, resuspended in Tricine - buffered bicarbonate medium and photosynthesis assayed as above. Samples were assayed after 30, 45, 60, 90 and 120 minutes incubation with 250μM M&B 39279 or acifluorfen,

after 15, 30 and 60 minutes incubation with 500μM M&B 39279 and after 5, 10, 15 and 30 minutes incubation with 750μM M&B 39279. Assays were repeated six times per treatment.

5.2.2.5 Vital Staining of Protoplasts with Fluorescein Diacetate

Intracellular hydrolysis of fluorescein diacetate to fluorescein (which fluoresces yellow - green under blue excitation) was exploited as a measure of protoplast physiological integrity (Widholm, 1972). Fluorescein diacetate hydrolysis depends on enzymatic esterase activity and so only viable cells convert fluorescein diacetate to the free acid which diffuses across membranes slowly, and so remains largely trapped within the cytoplasm. In this study, a technique was devised to assess not only the capability of protoplasts to hydrolyse fluorescein diacetate but also to reflect the rate at which such hydrolysis occurred. In order to achieve this, samples of protoplasts incubated in the presence of 0, 375, 500 and 750μM M&B 39279 or acifluorfen were taken after 0, 15, 30, 45, 60, 90, and 120 minutes incubation and equal volumes (15μl) of protoplast suspension and freshly made 0.01% (w/v) fluorescein diacetate (in protoplast storage medium containing 1% (v/v) acetone) were mixed together on a haemacytometer. Protoplasts were then counted under diascopic white light and exactly two minutes following addition of fluorescein diacetate, the number of protoplasts which fluoresced yellow under blue epifluoresence illumination (fluorescein fluorescence) were noted, discounting those which fluoresced red (chlorophyll fluorescence). This was performed using a Leitz Dialux 20EB microscope equipped with an episcopic mercury vapour lamp light source and Leitz dichroic mirror, excitation and barrier filters suitable for

concurrently observing fluorescein and chlorophyll fluorescence. Since it was found that after exactly two minutes, healthy protoplasts hydrolysed just enough fluorescein disacetate to mask chlorophyll fluorescence (ie. for protoplasts to appear yellow rather than red), any injury to protoplasts which reduced endogenous esterase activity was, therefore, revealed in that protoplasts were seen as orange or red rather than yellow under epifluorescence illumination (see Figs. 68-71). It was therefore, critical to count cells as rapidly as possible at exactly 2 minutes after adding fluorescein diacetate. For each sample, four microscope fields of view (each equivalent to 0.628µl of suspension) were assessed in each of six experiments.

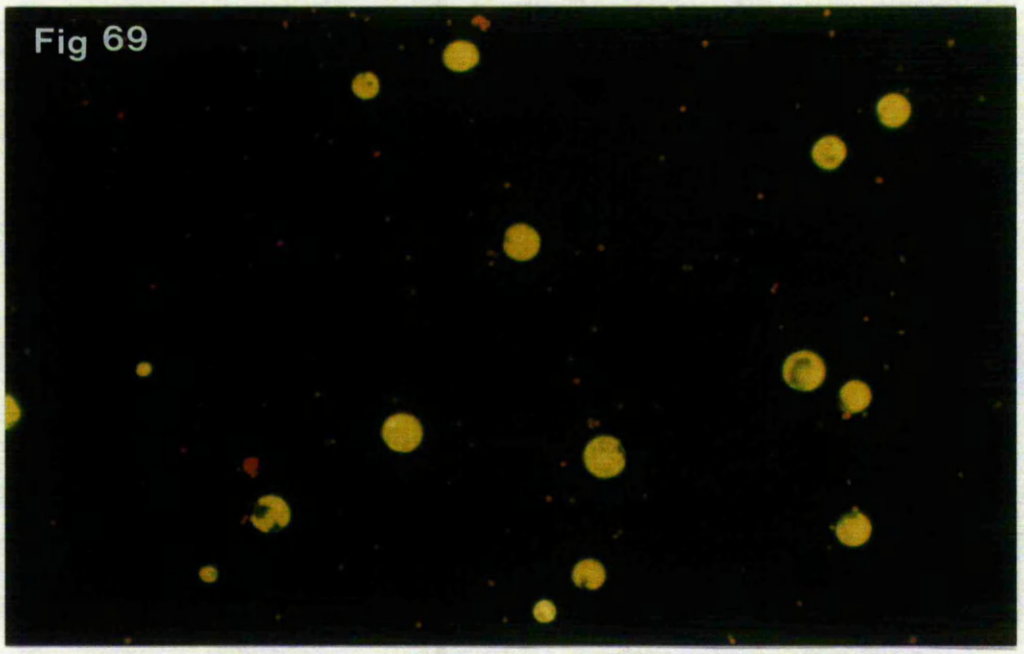
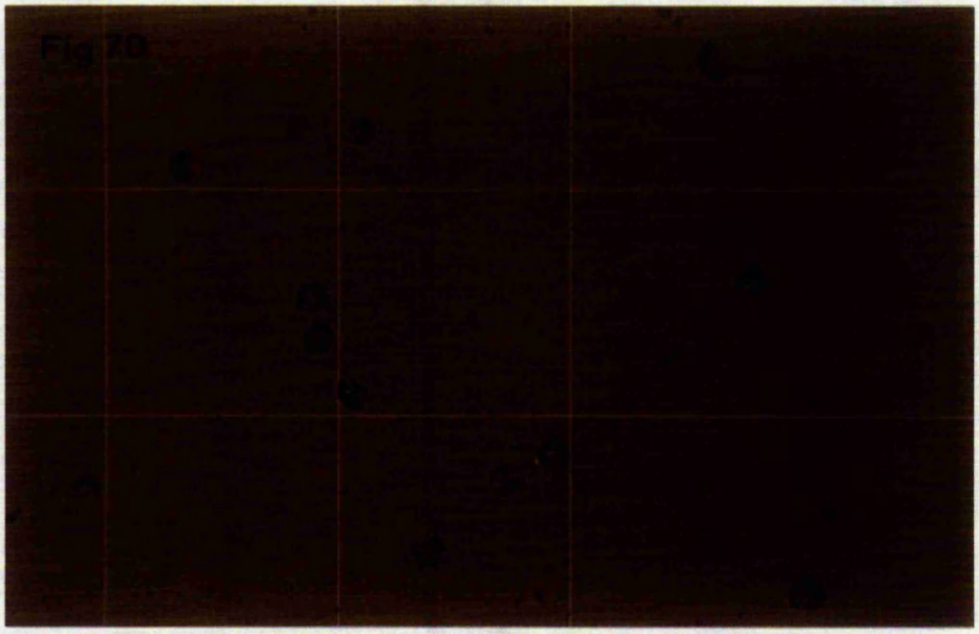


Figure 68. Diascopic illumination of control protoplasts stained with fluorescein diacetate.

Figure 69. Epifluorescence illumination of the protoplasts shown in Fig. 68. All protoplasts fluoresce yellow - green.



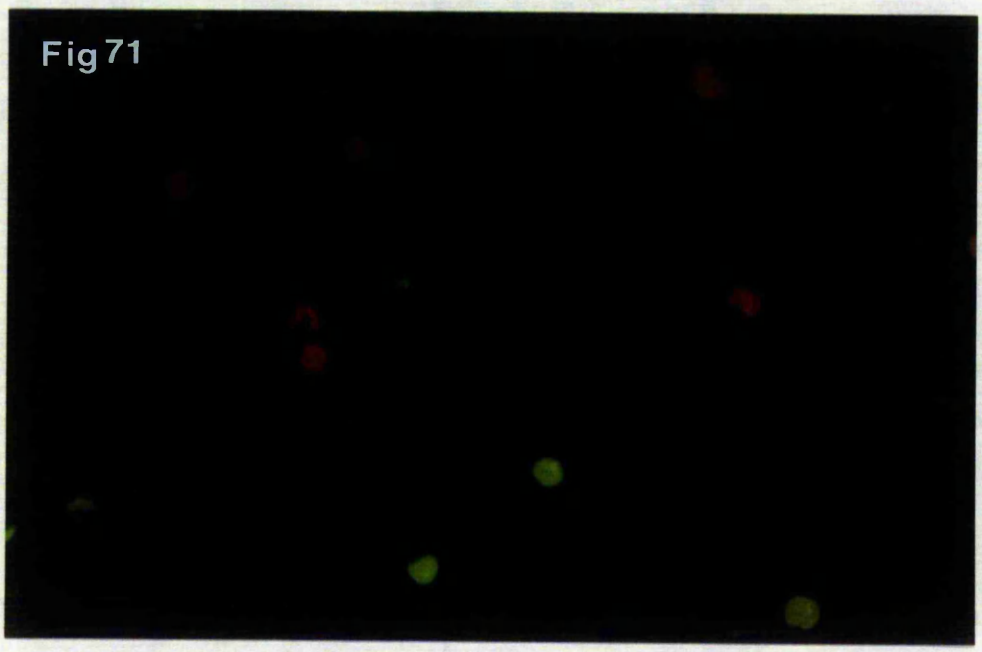


Figure 70. Diascopic illumination of M&B 39279 (500µM) treated protoplasts stained with fluorescein diacetate.

Figure 71. Epifluorescence illumination of the protoplasts shown in Fig. 70. Only 4 out of the 11 apparently intact protoplasts fluoresce yellow - green. The remaining 7 appear orange - red due to herbicide induced reduction of protoplast esterase activity.

5.3 RESULTS

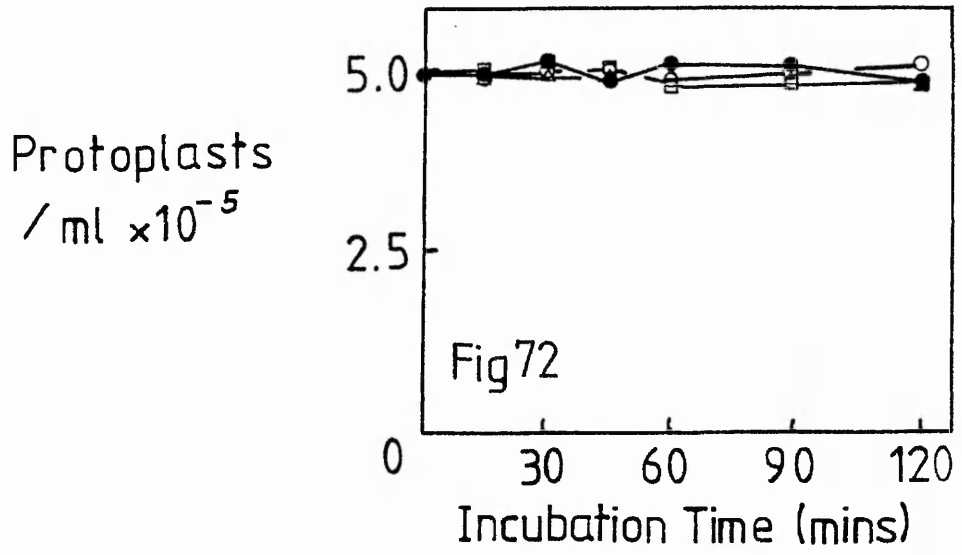
5.3.1 EFFECTS OF M&B 39279 AND ACIFLUORFEN ON PROTOPLASTS

5.3.1.1 Protoplast Intactness

Neither M&B 39279 nor acifluorfen significantly decreased protoplast intactness as determined by cell counting over the 2 hour duration of the experiment (Figs 72 and 73) at 375, 500 and 750μM concentrations. This was also reflected in measurements of bathing solution conductivity, which also remained stable over the duration of the experiment (Figs. 74 and 75).

5.3.1.2 Membrane Integrity

As reported above and in Figs. 74 and 75, protoplast bathing solution conductivity did not change to any measurable extent in either M&B 39279, acifluorfen, or control treatments over the 2 hour incubation period. Similarly, generation of either ethane or propane, which would have been indicative of large scale lipid peroxidation, was not observed over a 5 hour incubation period under the same conditions, even when the density of protoplasts in the suspension was doubled to 1×10^6 / ml in an attempt to detect small quantities of evolved hydrocarbons. After assay as described in section 5.2.2.5, protoplasts incubated for detection of hydrocarbons in the presence of M&B 39279 and acifluorfen at 375, 500 and 750μM were shown to be photosynthetically incompetent after 5 hours.



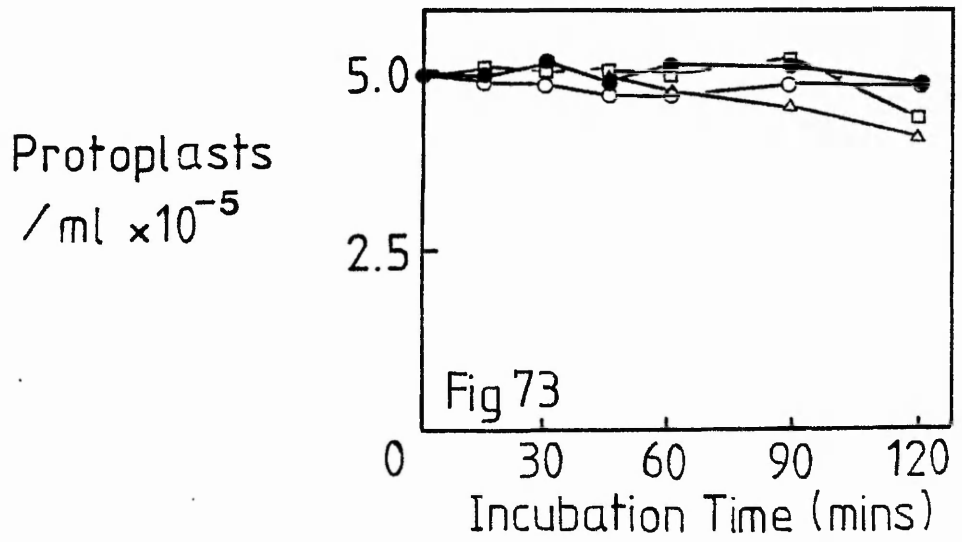
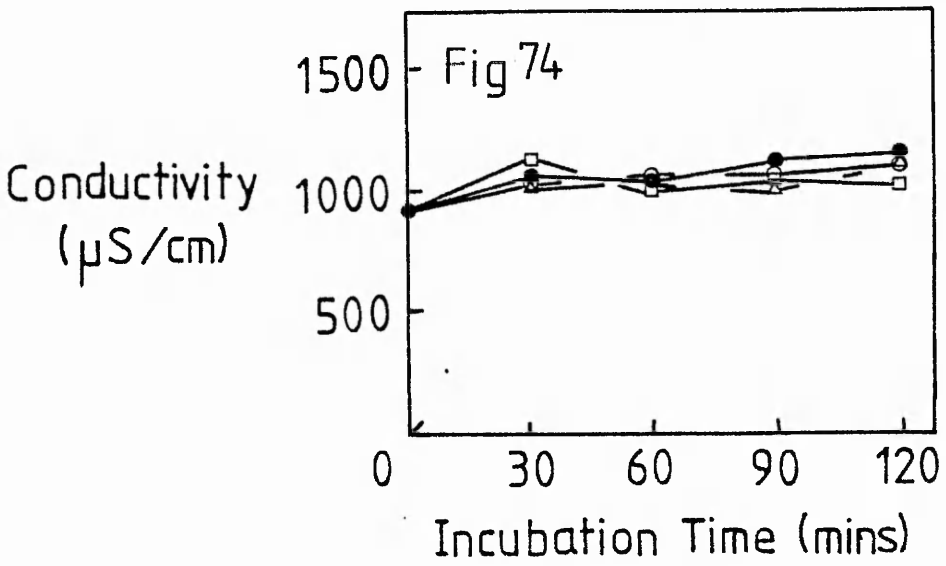


Figure 72. Density of G.aparine protoplast suspensions in the presence of 0 (●——●), 375 (○——○), 500 (□——□) and 750µM (△——△) M&B 39279. Data are means of 6 replicates. Vertical bars are 2x standard error.

Figure 73. Density of G.aparine protoplast suspensions in the presence of 0 (●——●), 375 (○——○), 500 (□——□) and 750µM (△——△) acifluorfen. Data are means of 6 replicates. Vertical bars are 2x standard error.



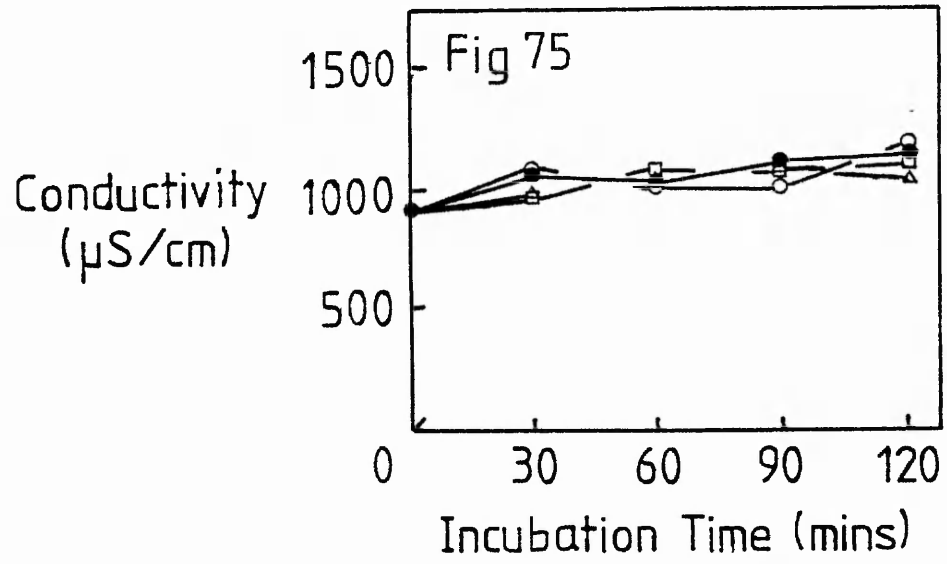
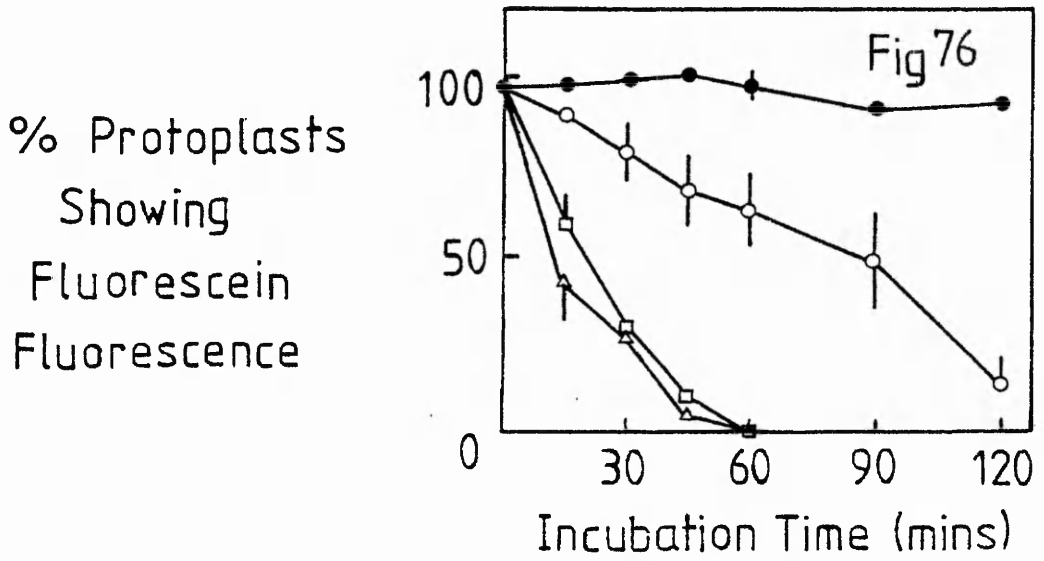


Figure 74. Electrolyte leakage from G.aparine protoplasts in the presence of 0 (●——●), 375 (○——○), 500 (□——□) and 750 μM (△——△) M&B 39279. Conductivity change = μS cm^{-1} (test sample) - μS cm^{-1} (storage buffer). Data are means of 6 replicates. Vertical bars are 2x standard error.

Figure 75. Electrolyte leakage from G.aparine protoplasts in the presence of 0 (●——●), 375 (○——○), 500 (□——□) and 750μM (△——△) acifluorfen. Conductivity change = μS cm^{-1} (test sample) - μS cm^{-1} (storage buffer). Data are means of 6 replicates. Vertical bars are 2x standard error.

5.3.1.3 Fluorescein Diacetate (FDA) Hydrolysis

The rate of FDA hydrolysis was rapidly reduced by both M&B 39279 and acifluorfen at 375, 500 and 750μM concentrations as shown by decreasing numbers of protoplasts displaying fluorescein fluorescence after 2 minutes incubation with fluorescein diacetate (Figs. 76 and 77).



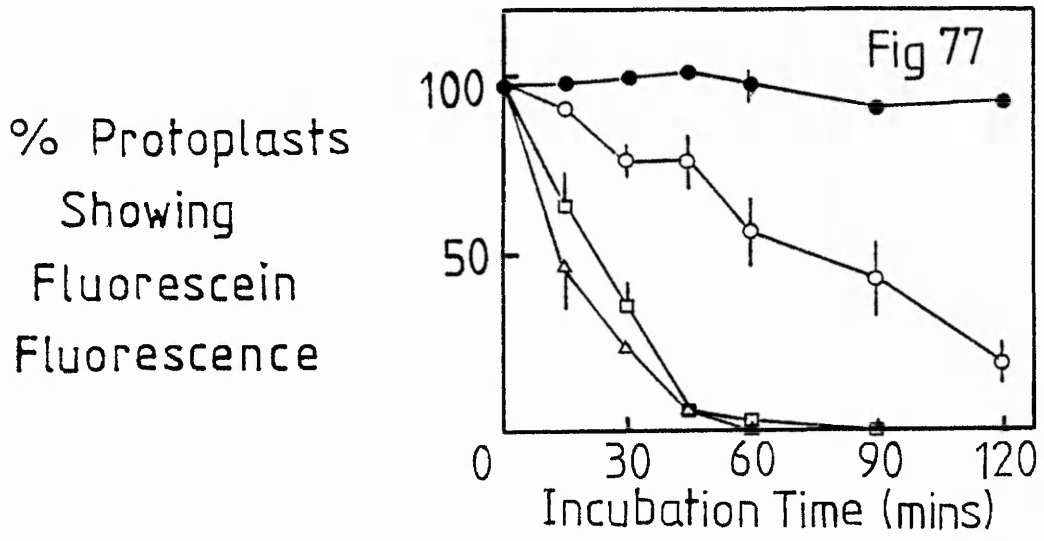


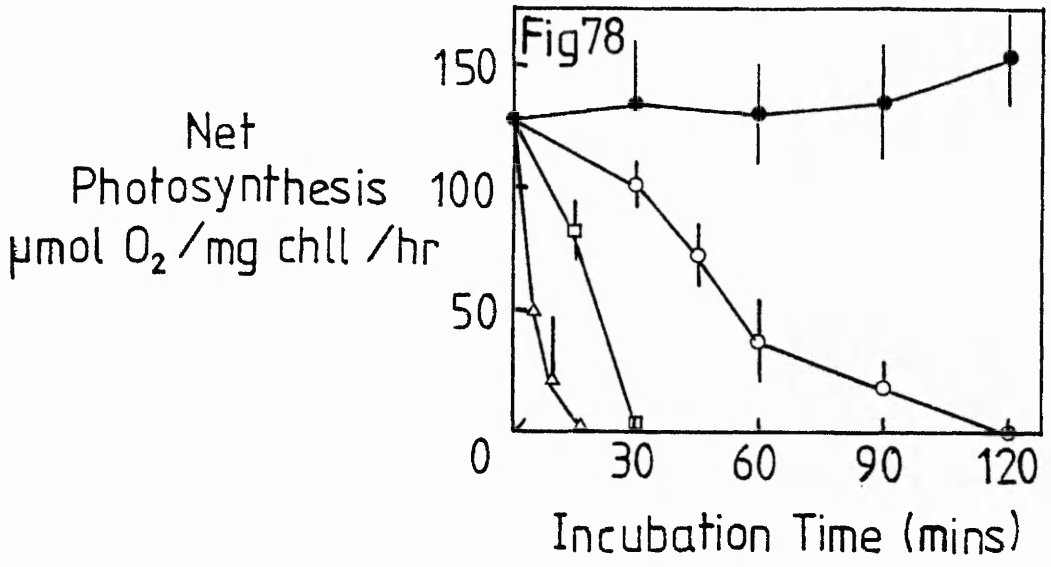
Figure 76. Effect of 0 (●——●), 375 (○——○), 500 (□——□) and 750µM (△——△) M&B 39279 on fluorescein diacetate hydrolysis by G.aparine protoplasts. Data are means of 6 replicates. Vertical bars are 2x standard error.

Figure 77. Effect of 0 (●——●), 375 (○——○), 500 (□——□) and 750µM (△——△) acifluorfen on fluorescein diacetate hydrolysis by G.aparine protoplasts. Data are means of 6 replicates. Vertical bars are 2x standard error.

5.3.1.4 Photosynthetic Capability

Photosynthetic capability of M&B 39279 and acifluorfen treated protoplasts declined rapidly at all three herbicide concentrations (Figs 78 and 79). Photosynthetic capability was in fact the most sensitive parameter assessed, the FDA test yielding data on a similar timescale, although repeatedly slightly more delayed.

Neither M&B 39279 nor acifluorfen demonstrated any significant immediate effects upon photosynthesis when added in darkness 3 minutes prior to assay (table 9) at concentrations between 100 and 500μM, although there was a trend towards a slight suppression of photosynthesis with M&B 39279 and acifluorfen treated protoplasts (mean photosynthetic rates were consistantly 5-10% less than the controls, see table 9). In contrast, 50μM paraquat reduced bicarbonate - dependant oxygen evolution by 68.6% when added in darkness 3 minutes prior to assay.



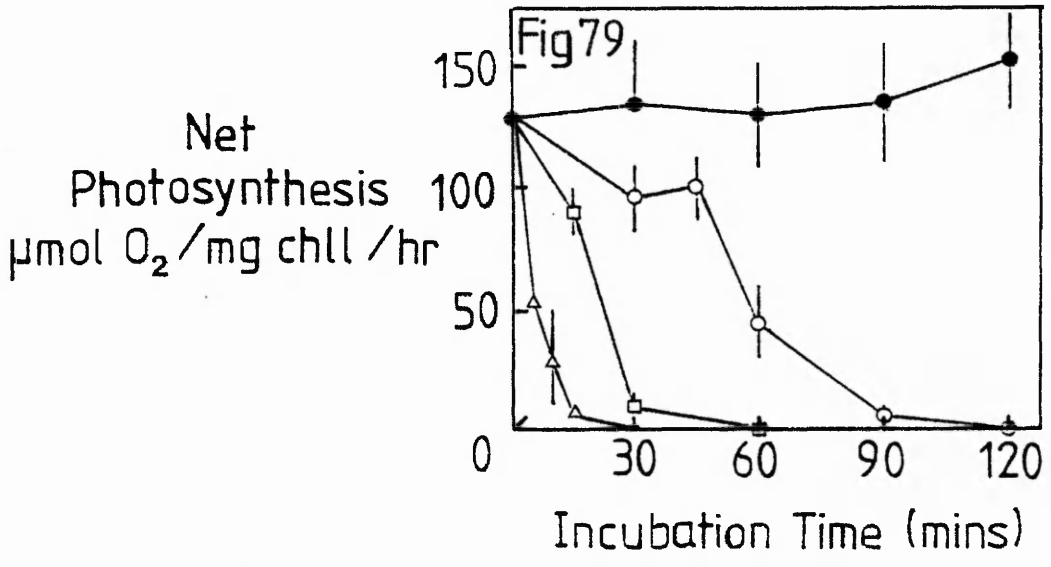


Figure 78. Effect of 0 (●——●), 375 (○——○), 500 (□——□) and 750µM (△——△) M&B 39279 on G.aparine protoplast photosynthesis. Data are means of 6 replicates. Vertical bars are 2x standard error.

Figure 79. Effect of 0 (●——●), 375 (○——○), 500 (□——□) and 750 µM (△——△) acifluorfen on G.aparine protoplast photosynthesis. Data are means of 6 replicates. Vertical bars = 2x standard error.

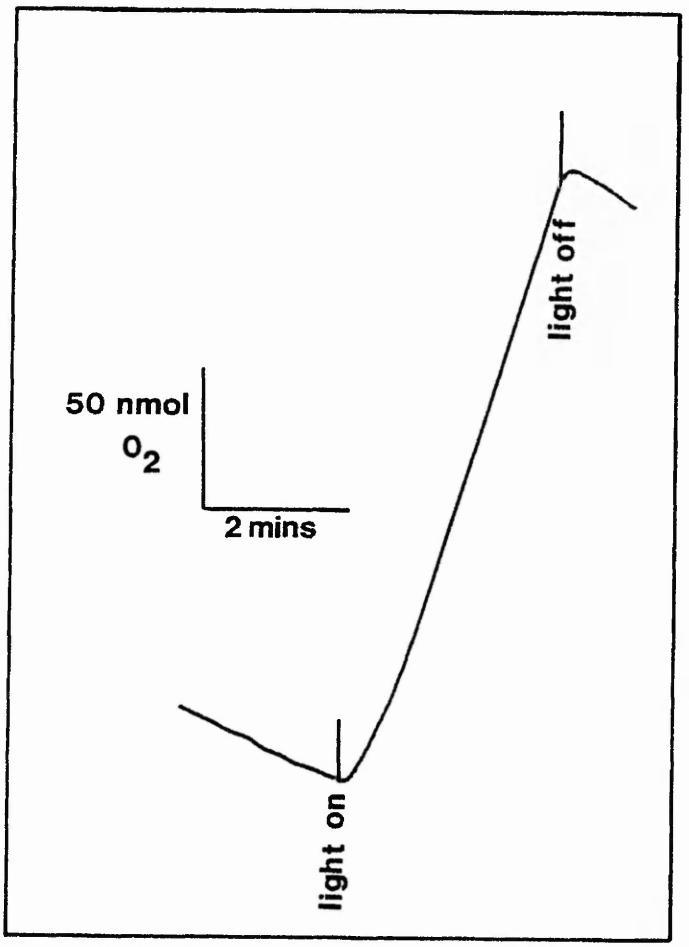


Figure 80. Copy of typical chart recorder trace of oxygen electrode output reflecting bicarbonate - dependent oxygen evolution by photosynthesising G.aparine protoplasts. Rate of oxygen evolution is equivalent to a net photosynthetic rate of 110.4 μmol O_2 mg $chll^{-1}$ $hour^{-1}$.

Herbicide		Gross Photosynthetic Rate (μmol O_2 / mg chll / hour)	% of Control
Control		81.05 ± 5.76	100.0
M6B 39279	100µM	79.21 ± 4.98	97.8
	200µM	78.44 ± 5.62	96.8
	300µM	80.11 ± 5.90	98.8
	400µM	77.05 ± 4.95	95.1
	500µM	74.18 ± 4.16	91.5
Acifluorfen	100µM	76.28 ± 5.48	94.1
	200µM	77.15 ± 4.16	95.2
	300µM	78.10 ± 5.92	94.0
	400µM	74.93 ± 4.51	92.5
	500µM	74.07 ± 8.98	91.4
Paraquat	50µM	31.4 ± 1.60	38.7

TABLE 9. Rapid effects of M&B 39279 and acifluorfen on G.aparine protoplast photosynthesis.

5.4 DISCUSSION

That M&B 39279 and acifluorfen did not immediately significantly reduce G.aparine protoplast photosynthesis at concentrations up to ten times that of paraquat causing a 68% decrease under identical conditions (table 9) provides a greater insight into photosynthesis - DPE - type herbicide interaction than have studies in previous chapters (sections 3.3.2, 4.3.3, Fig. 45 and tables 3 to 7). Since photosynthetic capability of protoplasts exposed to both light and M&B 39279 or acifluorfen decreased rapidly (completely inhibited by about 15 minutes for 750μM, 30 minutes for 500μM and about 2 hours for 375μM M&B 39279 , see Figs. 78 and 79), and yet these herbicides did not have an immediate effect on photosynthetic capability (Table 9), it can be suggested that a short lag period is necessary for M&B 39279 and acifluorfen mediated photosynthesis inhibition. In contrast, paraquat required no such lag period prior to activity (table 9). These results provide further evidence to suggest that the photosynthetic apparatus does not play an immediate or large part in DPE-type activity, and that a yet unidentified event(s) occur prior to photosynthesis inhibition. Tissut et al (1987) similarly showed that LS 82-556, a compound having DPE-type activity (Matringe et al, 1986) did not have any instantaneous effect upon spinach and cucumber cotyledon 'Type A' isolated chloroplast photosynthesis at concentrations up to 200μM and inhibited photosynthesis in cucumber cotyledon fragments at least 50 times more slowly that did an equivalent paraquat concentration. The fluorescein diacetate test described in section 5.2.2.4 proved to be a sensitive technique reflecting physiological integrity of herbicide treated protoplasts,

the method being almost as sensitive as measuring photosynthesis as an indicator of herbicidal toxicity (compare Figs. 76, 77, 78 and 79). Since enzymes capable of hydrolysing fluorescein diacetate are present in most, if not all cell compartments, loss of ability to hydrolyse fluorescein diacetate may reflect a loss of cellular compartmentation leading to enzyme inactivation. A leaking plasmalemma would also cause a decrease of fluorescein diacetate hydrolysis or fluorescein free acid retention but since no increase in conductivity of the protoplast bathing medium was detected (Figs. 74 and 75) this possibility can be discounted. Evolution of short chain hydrocarbons from protoplasts incubated with M&B 39279 and acifluorfen was not detected under the conditions described. As lipid peroxidation has been indirectly detected previously for M&B 39279 and acifluorfen (as generation of malondialdehyde, see section 3.3.3) and shown by many other workers in numerous experiments to be strongly implicated as a major mechanism the mode of action of DPE herbicides (see section 1.3.6), this result was perhaps, surprising. Failure to detect ethane or other short chain hydrocarbons in this system using methods fully competent to do so does not however, necessarily prove that lipid peroxidation had not been initiated in the protoplast suspension. Generation of short chain hydrocarbons (mainly ethane, propane and ethylene in higher plants) is only a small consequence of lipid peroxidation and is a result of lipid hydroperoxide degradation, probably via an alkoxy radical intermediate (Evans et al, 1967; Tappel, 1980) and so necessarily follows after hydroperoxide formation. Lipid peroxidation also results in the generation of numerous aldehydes (including malondialdehyde, see Fig. 45), ketones and alcohols formed via fission of hydroperoxides. These aldehydes react readily with amines, degrading lipids (eg. phosphatidyl

ethanolamine) and also can cause intramolecular crosslinking and disruption of hydrogen bonding and thus disrupt tertiary structure in proteins and nucleic acids. Lipid polymers and inactivated enzymes and nucleic acids result. Lipid peroxidation could have, therefore, been initiated in the protoplasts and only yielded small quantities of ethane / pentane which were below the limits of detection in the described protoplast system over the assessed duration or simply that evolution of hydrocarbons in this system does not occur until comparatively late in the sequence of toxic events. It has infact been previously reported that hydrocarbon generation in plant cells exposed to peroxidising conditions may be only detected after several hours. Lambert et al, (1987) did not detect a burst in hydrocarbon generation until after about 5 hours using Scenedesmus cells in the presence of 1μM oxyfluorfen. In the presence of 10μM oxyfluorfen, Bumilleriopsis cells did not exhibit hydrocarbon generation until 20 hour after the start of incubation. The difference between the species was correlated with differences in endogenous levels of antioxidants. Is is likely that in G.aparine protoplasts, endogenous antioxidative activity, (see Elstner, 1987) was, in part, checking the progress of ethane / pentane - generating lipid peroxidation though this was insufficient protection to maintain photosynthetic activity. It is very likely that ethane and pentane evolution would have been detectable at a later time but unfortunately, the protoplasts did not remain at a high level of intactness for more than 5 hours. It is most likely that ethane would be evolved in far greater amounts than pentane since ethane results from linolenic acid peroxidation this fatty acid being a major constituent of chloroplasts where toxicity appears to be initiated (Chapter 3). A possible alternative cause of slow lipid peroxidation

in the protoplast system could have been that since the protoplast storage buffer did not contain any bicarbonate to enhance photosynthetic activity, by a process analogous to inhibition of photosynthesis and M&B 39279 / acifluorfen activity by diuron (shown in Chapter 4), M&B 39279 / acifluorfen activity and therefore, lipid peroxidation and ethane evolution, may have been retarded by a lack of protoplast photosynthetic activity (and therefore, general metabolic activity). The conditions under which protoplasts were incubated were, however, clearly adequate to allow considerable loss of plastidic and cytoplasmic integrity as revealed by reduced esterase and photosynthetic activity in herbicide treated protoplasts. This demonstrated that evolution of short chain alkanes was not a sensitive indicator of DPE - type herbicide damage in the protoplast system used. Constraints of time prevented a more detailed examination of lipid peroxidation in the protoplast system. Numerous methods have however, been described for detection and quantitation of lipid hydroperoxides and their degradation products (see Gray, 1978 and Tappel, 1980) which could be used to further characterise peroxidation in the protoplast system. Heath and Tappel (1976) used glutathione peroxidase to catalyse oxidation of glutathione by hydroperoxides. This was coupled with glutathione reductase - catalysed NADPH oxidation, the latter being followed spectrophotometrically at 340nm. Fletcher, et al (1973) and Tappel (1980) described methods for detecting fluorescent conjugated Schiff base condensates having the general structure R-N=CH-CH=CH-NH-R' which arise from reactions between aldehyde fission products of hydroperoxides and cell constituents. More recently, Ebermann and Couperus (1987) detected hydroperoxides in a triethylenetetramine-Fe^{3+} - catalysed reaction with homovanillic

acid, which yielded a fluorescent product.

The data in this chapter support the following account of herbicide - induced events. Following a short lag period lipid peroxidation is initiated at a site within the chloroplast adjacent to the chloroplast envelope where initial manifestations of membrane damage were noted in Chapter 3. Damage to the envelope membranes effects a loss of intraplastid metabolic regulation and a rapid cessation of photosynthesis occurs (as in Figs. 78 and 79). As lipid peroxidation and loss of compartmental integrity progresses out from the chloroplasts, the rate of FDA hydrolysis decreases, at some time after photosynthesis inhibition. It must be borne in mind that the FDA test as used in this study reveals only a slowing and not an abolition of FDA hydrolysis (Figs. 76 and 77) and over a similar time scale, protoplasts still retained considerable esterase activity at the part of the incubation period when photosynthesis had been almost completely inhibited.

CHAPTER 6

GENERAL DISCUSSION

6.1 SUMMARY OF FINDINGS ON THE MODE OF ACTION OF M&B 39279 AND ACIFLUORFEN.

Above all other findings in this study, it is clear that in all experiments carried out, without exception, M&B 39279 has qualitatively indistinguishable and quantitively very similar properties to the nitrodiphenyl ether herbicide acifluorfen. The proposal that the mechanisms of herbicidal action of M&B 39279 and acifluorfen are the same has been substantiated beyond reasonable doubt. The following paragraphs summarise findings made during the course of this study in the context of literature published elsewhere and summarised in chapter 1.

Both M&B 39279 and acifluorfen are light - dependant, rapid - acting, contact herbicides which caused leaf tissue collapse and necrosis of G.aparine and V.arvensis (see Chapter 2). These toxicity symptoms were not accompanied by any changes in gross amino acid, reducing sugar or soluble, insoluble or microsomal protein pools beyond those expected of moribund plants. The active wavelengths for herbicidal activity appear to lie in the blue and to a much lesser extent, red regions of the spectrum and only low light intensities were necessary for activity with these compounds ie. less than 5umol m^{-2} s^{-1} PPFD (see Chapter 4). This, plus the fact that the presence of chorophylls are not necessary for DPE-type activity (see sections 1.3.5 and 1.3.7) implied that neither photosynthesis nor chloroplast electron transport are necessary for their action. Activity of M&B 39279 and acifluorfen was nevertheless saturable with respect to light intensity (see Chapter 4) and the chloroplast electron transport inhibitor diuron suppressed their activity (see Chapter 4). M&B 39279 and acifluorfen were additionally shown to have

no effect at 10-100uM concentrations on photosystem I - mediated or photosystem I and II - mediated electron transfer and only a small effect on non - cyclic photophosphorylation (see Chapter 4).

M&B 39279 and acifluorfen initiated development of invaginations and evaginations of the chloroplast envelope as an initial symptom of damage on an ultrastructural level in G.aparine. Vesiculisation of cytoplasm, tonoplast lysis and plasmalemma degradation followed, alongside development of numerous osmiophilic bodies in the cell which were particularly associated with chloroplast envelopes. Damaged chloroplasts furthermore, often fused together late in toxicity development. Thylakoid membranes generally disrupted late in the sequence of subcellular changes (see Chapter 3). These changes were accompanied by a rapid decline in photosynthetic capability and a later leakage of electrolytes, loss of chlorophyll and generation of malondialdehyde (see Chapter 3) suggesting that damage occurs via peroxidative destruction of membrane lipids. Although a rapid decline in photosynthesis was the most sensitive response of excised G.aparine leaves and protoplasts to M&B 39279 and acifluorfen, photosynthesis was not inhibited immediately as it was with paraquat in G.aparine protoplasts, but only after a short time lag (see Chapter 5). Both photosynthesis and a decline in the rate of fluorescein diacetate hydrolysis preceeded evolution of short chain hydrocarbons by at least four hours in G.aparine protoplasts (see Chapter 5).

6.2 APPRAISAL OF METHODS AND TECHNIQUES USED IN THIS STUDY.

The approach to investigating the mode of action of M&B 39279 undertaken in this study is clearly one of several which would have yielded similar and different information disclosing the herbicide's mode of action. The experimenter could have, as discussed in section 1.4, using better understood species than G.aparine. The reasons for the choice of G.aparine as the main experimental material has already been discussed (section 1.4). Alternatively, fewer experimental systems could have been used throughout this study, such as an isolated / cultured cell system. This would have had the advantage of being able to relate various sets of data to each other, these data being obtained under similar conditions. The task of this study was not, however, only to elucidate the mode of action of M&B 39279 but to characterise its toxic effects in a relevant target weed species. As species relevant to the potential use of the herbicide had been chosen, it was only congruous with the theme of this study to characterise effects of the compound on a subcellular, tissue and whole plant basis so that effects on say, the protoplast or thylakoid experimental systems could be related to microscopic examinations and excised leaf systems and these in turn to whole plant studies. In addition to this, as M&B 39279 was a novel herbicidal compound, it was necessary to characterise its effects on plants as broadly as possible, there being no published information on its activity available. Although it was suspected that M&B 39279 acted in a similar way to DPE herbicides, it was necessary to use acifluorfen as a parallel to M&B 39279 in all experiments, enabling the extrapolation of DPE - based literature to this novel and uncharacterised compound.

Perhaps the most obvious criticism of the work in this study was the regular use of relatively high (10^{-5}M, 10^{-4}M) herbicide concentrations. These concentrations were used in general to obtain rapid responses to herbicides. This approach can, however, lead to drawing conclusions based upon artefactual evidence ie. effects of herbicides occurring at concentrations of herbicide which would never occur *in vitro*. As long as the experimenter is conscious of such artefacts, the use of (moderately) high herbicide concentrations can be useful in indicating herbicidal effects. Such effects must, however, be further investigated using low herbicide concentrations.

6.3 PORPHYRIN BIOSYNTHESIS AS A TARGET FOR DPE - TYPE HERBICIDES.

It has been shown in sections 1.3.5, 1.3.6 and 1.3.7 that numerous reports published over the last few years have presented convincing evidence suggesting interactions between DPE - type herbicides and porphyrin biosynthesis as being a possible and likely mechanism of action for these compounds. Most of these reports have presented evidence suggesting that DPE - type herbicides induce a rapid accumulation of photosensitising porphyrins, mainly protoporhyrin IX, which leads to generation of toxic oxygen species (singlet oxygen) and so initiates peroxidation of membrane lipids (Kawakubo et al, 1979; Matringe and Scalla 1987b, 1988a and b; Kouji et al, 1988; Lydon and Duke, 1988; Witkowski and Halling, 1988; Bowyer et al, 1989; Duke et al, 1989; Matringe et al 1989a and b). Previous attempts at presenting a hypothesis for the mechanisms of action of DPE - type compounds have suggested that carotenoids / carotenoid - containing complexes serve as a light acceptor, this being based on observations that plant tissues devoid of cartenoids are insensitive to DPE - type herbicides (see section 1.3.5), though Matringe and Scalla (1987b) showed herbicidal damage induced by DPE - type compounds in cells essentially devoid of carotenoids. Suggestions that carotenoids, as photoreceptors, are directly involved in DPE - type toxicity have been therefore, based on theoretical and circumstantial evidence (eg. Orr and Hess, 1982a). No such involvement has been shown empirically. The most commonly proposed alternative to DPE - type herbicides interacting with carotenoid photoreceptors has been that nitro - diphenylethers are themselves converted to nitro free radicals via photosynthetic electron transfer (see section

1.3.5). However,no convincing *in vivo* evidence has ever been shown to support such a hypothesis and the concept does not explain the mode of action of non - nitro DPE's nor other DPE - type compounds which lack nitro or nitroso substituents, nor does it explain ultrastructural data from DPE - treated plants, nor the kinetics of photosynthesis inhibition by DPE - type compounds (see section 5.4 and Sato *et al*, 1987a), ie. that there is a short lag period prior to DPE - type inhibition. In contrast to these hypotheses, interference with porphyrin biosynthesis leading to accumulation of photosensitising tetrapyrroles as a theory has increasingly been supported by direct experimental evidence and provides convincing explantations for long - unanswered questions regarding the mode of action of DPE - type compounds. Furthermore, this theory could provide explanations for almost all of the data published regarding DPE - type herbicides and for data presented within this thesis.

Before further discussion of porphyrin biosynthesis as a mode of action for DPE - type herbicides, it would be appropriate to outline this biochemical pathway. Plant tetrapyrroles are prosthetic groups for a large number of proteins within the plant cell. Tetrapyrroles form part of the chlorophylls, phaeophytin and cytochromes of chloroplasts, the cytochromes of mitochondria, the catalase, cytochromes and peroxidases of the golgi - endoplasmic reticulum - lysosome system and the apoplastic peroxidases and halogenoperoxidases. The chromophore of phytochrome and possibly part of the chromophore of "cryptochrome" is a tetrapyrrole. It is seen therefore, that porphyrin / tetrapyrrole biosynthesis is involved in a wide variety of metabolic activity.

Plants, unlike animals, have two pathways for porphyrin

biosynthesis. The extrachloroplastic pathway begins with a condensation and decarboxylation of glycine and succinyl coenzyme A to generate 5-amino laevulinic acid, the precursor of all tetrapyrroles. The plastidic pathway however, generates glutamate-1-semialdehyde from glutamate via a transfer - RNA bound intermediate (Schon et al, 1986). Glutamate-1-semialdehyde is then transaminated to form 5-amino laevulinic acid. Figure 82 outlines the subsequent conversion of 5-aminolaevulinic to a macrocyclic tetrapyrrole structure via the straight chain intermediate porphobilinogen. Protoporphyrin IX is the common precursor of all porphyrins.

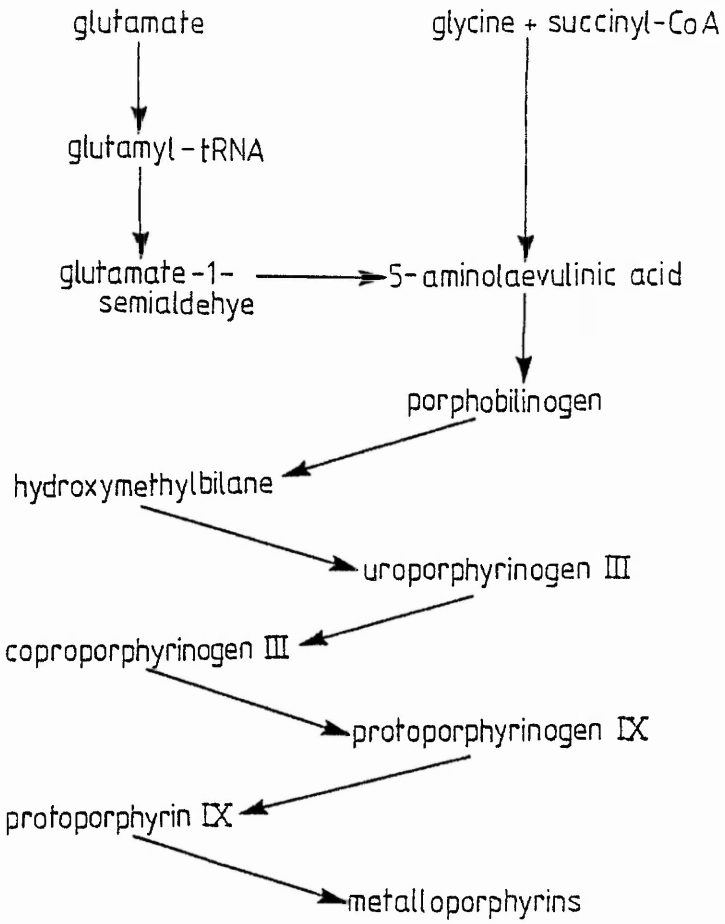


Figure 82. An outline of the porphyrin biosynthetic pathway.

It appears that synthesis of chlorophylls in higher plants is a complex process having a multi-branched pathway. Chlorophyll synthesis has been and is continuing to be studied by C.A.Rebeiz and his co-workers at the University of Illinois, U.S.A., who have published upwards of seventy papers on the subject. Recent reviews have been published by Rebeiz et al (1983, 1987) and Castelfranco and Beale (1983). Chlorophyll a synthesis is currently perceived to proceed via a six - branched pathway having numerous interconnections between these pathways. Ten variants of chlorophyll a are synthesised via these pathways, the intermediates varying in the composition of side chains attached to the porphyrin macrocycle. These pathways do not, however, all operate simultaneously and with equal magnitude. Different species synthesise different chlorophyll a types (Rebeiz, (1987), characterised four groupings of plants on the basis of chlorophyll synthetic routes) and different pathways operate with different fluxes according to photoperiod length. This chemical and metabolic heterogeneity is thought to be necessary to control the correct orientation of chlorophylls in photosynthetic membranes. Of greater relevance to DPE - type herbicide activity however, is that at least six variants of protoporphyrin IX have been found in plants, ie. the 2-monovinyl and divinyl forms of the 7-fatty acyl-6-methyl ester, the 7-alkyl ester and free acid forms of protoporphyrin IX. Figure 81 depicts the 2-monovinyl protoporphyrin IX free acid.

As stated in section 2.4, the observation that M&B 39279 and acifluorfen, in both G.aparine and V.arvensis, induce symptoms far more quickly on young, rapidly expanding leaves than on older, fully expanded leaves implied that a biosynthetic process may be involved in

the mode of action of these herbicides. Clearly, interference with porphyrin biosynthesis is compatible with this finding, biosynthetic systems being far more active in young rather than mature leaves. Similarly, reports in the literature that de-etiolating plants are as sensitive and occasionally more sensitive to DPE - type herbicides than green plants (see section 1.3.5) is in agreement with porphyrin biosynthesis as a target for these herbicides. Chlorophyll biosynthesis would obviously be operating very rapidly in de-etiolating plants. The results of investigations in Chapter 4 of this thesis regarding the involvement of light in M&B 39279 and acifluorfen activity are also congruent with porphyrin synthesis interaction as a mode of action. It was shown that light is required for herbicidal action (photo - excitation of porphyrins followed by generation of activated oxygen is a possible explanation for this) but only low light intensities were necessary (sufficient to mediate photodynamic reactions catalysed by porphyrins). It was reported in Chapter 4 that with respect to light intensity, herbicidal injury was saturable and was slightly suppressed at very high light intensities and suggested that this could also be explained in terms of increasing metabolic activity and turnover of chlorophyll at increasing light intensities, and reduced availability of metabolites and reducing power / nucleotide phosphates for biosynthesis following photoinhibition at very high light intensities. This may also explain the suppressing effects of diuron on activity of M&B 39279 / acifluorfen (Chapter 4 and section 1.3.7).

The relative activities of blue, green and red light in mediating DPE - type herbicide action reported in Chapter 4 and elsewhere (see section 1.3.5) can be readily considered as consistant with generation of photosensitising protoporphyrins IX as a mode of action. The

spectral absorption properties of protoporphyrins IX and protoporphyrin IX dimethylester are summarised in table 10 and Fig. 81. Copro- and uro- porphyrinogen have similar absorption characteristics. It is seen then that the absorption properties of protoporphyrin IX could account for M&B 39279 and acifluorfen activity (see Chapter 4). The published action spectra for these herbicides (Ensminger and Hess, 1985a ; Matringe and Scalla, 1987a; Sato et al, 1987b and Gaba et al, 1988) provide, however, a somewhat confused picture of light wavelength - DPE - type herbicide activity relationships. In general agreement though, is that blue light is most capable of mediating DPE - type activity. Yellow, green and and red wavelengths are active to varying degrees depending on the system used to establish an action spectrum.

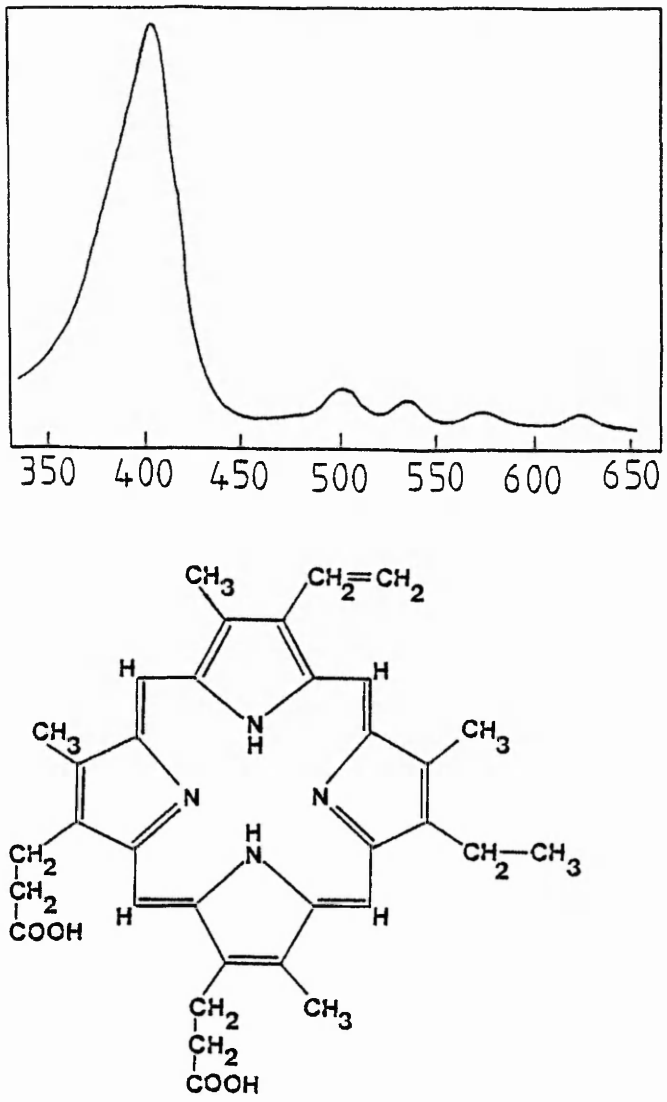


Figure 81. Absorption spectrum and chemical structure of protoporphyrin IX. The absorption spectrum is essentially that of Duggan and Gassman (1974) derived from a commercial preparation dissolved in acetone : methanol (4 : 1 v/v).

Solvent	Soret Peak	Visible Maxima	Reference
ether	405	501, 535, 577, 632	1
80% acetone	403	503, 538, 575, 630	1
20% methanol*	403	503, 537, 574, 630	1
chloroform**	407	505, 541, 575, 630	2

TABLE 10. Absorption maxima of Protoporphyrin IX. Reference 1= Duggan and Gassman (1974); 2 = Wijesekera and Dolphin (1985). * = in acetone; ** = protoporphyrin IX dimethylester. Data are in nm.

It is however, difficult to draw valid conclusions from the published spectra due to the variation between them. Action spectra should, additionally, be viewed with some caution since illumination of plants with monochromatic or near - monochromatic light sources is very artificial and will not provide clear information regarding interactions of different photoreceptors having different absorption properties in a system. It appears that light-absorbing chlorophylls could play a role in the DPE-type mode of action, this partially accounting for the large peaks of activity in red wavelengths reported by Ensminger and Hess (1985a) and Sato et al (1987b). The peak found by Sato et al (1987b), for S-23142 activity at 650nm for example was greatly reduced in magnitude if far - red grown plants were used for construction of an action spectrum rather than green plants. It is also worth noting that 400nm wavelength light (the absorption maximum of protoporphyrin IX) was more active in etiolated than green cucumber cotyledons in this system.

Although it is not disputed that lipid peroxidation is the mechanism by which plants are killed in DPE - type herbicide activity, the process which initiates peroxidation has not been studied in detail (see section 1.3.6). Lipid peroxidation is initiated and proceeds via a radical process. Photosensitiser induced peroxidation can be induced either by a "type I" or "type II" oxidative process. In type I processes, the sensitizer in its excited state reacts with a substrate to generate radicals or radical ions by hydrogen or electron transfer. Reaction of these products with oxygen initiates peroxidation. In a type II process the excited sensitiser interacts by energy transfer directly with ground state oxygen to generate singlet oxygen and thereby relaxing the sensitiser (Foote, 1985;

Spikes, 1989). Singlet oxygen is well known to be a potent initiator of lipid peroxidation. Porphyrins, particularly non-iron porphyrins, are known to be potent type II photosensitisers (Dolphin, 1978; Kessel, 1985; Rebeiz et al, 1987). Numerous porphyrins, notably "haematoporphyrin derivative" are widely used as phototherapeutic agents in treatment of certain human carcinomas by virtue of this property (see Kessel, 1985). It would be expected therefore, that porphyrin - mediated DPE - type action would be initiated via a type II mechanism. As suggested in section 1.3.6 however, this has not been conclusively demonstrated in intact tissues. Haworth and Hess (1988), nonetheless showed generation of singlet oxygen by low concentrations of oxyfluorfen in chloroplast preparations. Numerous attempts to demonstrate a mechanism via type I processes have however, not been successful in establishing a general mechanism for DPE - type herbicides (see section 1.3.6). More work is clearly needed to establish whether singlet oxygen generation is strongly implicated in DPE - type activity. Methods for the demonstration of singlet oxygen in photodynamic reactions have been described by Foote (1987) and by Khan (1987), detecting reaction products which are characteristic of singlet oxygen - mediated oxygen (eg. conversion of cholesterol to 5-hydroperoxycholesterol); by using inhibitors such as carotenes and DABCO (1,4-diazabicyclooctane) which specifically quench singlet oxygen; by near - infra red spectroscopy (Khan, 1987) and by demonstrating stimulation of singlet oxygen - mediated damage by using deuterium oxide rather than water as a solvent (eg. Knox and Dodge, 1985b). Singlet oxygen has a many fold longer lifetime in deuterium oxide than in water. These techniques could be employed to further characterise the peroxidation activity of M&B 39279. It is difficult to say how well ulstrastuctural investigations described in this

thesis and elsewhere (see Chapter 3) correlate with porphyrin biosynthesis as a target for DPE-type herbicides. As stated in Chapter 3, electron micrographs reveal only the results of a herbicide action which may be distant from the origin of the herbicide's effects. However, results from studies of acifluorfen and M&B 39279 described in Chapter 3 reveal the initial effects of these compounds in G.aparine to be perturbation of chloroplast envelopes, closely followed by disruption of endoplasmic reticulum and tonoplast lysis. The major sites of porphyrin biosynthesis in plant cells are within plastids for chlorophyll biosynthesis and within mitonchondria for generation of cytochromes. Since it was found that chloroplast envelopes were disrupted more rapidly than were mitochondria in this study, it is tempting to suggest that the plastid porphyrin pathway is more susceptible to DPE - type herbicide action than the mitochondrial or microsomal pathways. The idea of DPE - type herbicidal action beginning in chloroplasts also seems to be supported by evidence published elsewhere showing carotenoids to be essential for the mode of action of DPE-type compounds (see section 1.3.7). Ulstrastructural data in this study were supported by and similar to those of Kenyon et al (1985) who reported disruption of chloroplast envelopes as the initial microscopic symptom of acifluorfen - induced toxicity.

An important collection of evidence supporting the involvement of porphyrin biosynthesis in DPE - type action has been provided by the inhibition of herbicidal activity by inhibitors of tetrapyrrole biosynthesis and stimulation of herbicidal activity by stimulators of the pathway. Lydon and Duke (1988) and Witkowski and Halling (1988) reported almost complete inhibition of acifluorfen toxicity and Duke et al (1989) of oxadiazon toxicity by 0.33-0.5mM gabaculine (3-amino-2,3-dihydrobenzoic acid), a compound which inhibits

glutamate-1-semialdehyde aminotransferase and 5-amino laevulinic acid synthetase, enzymes which catalyse the synthesis of 5-aminolaevulinic acid by the glutamate and glycine pathways repectively (see Fig. 82) Lydon and Duke (1988) and Matringe and Scalla (1988), similarly showed dramatic reduction in acifluorfen activity, and Duke et al (1989) of oxadiazon activity, by 1mM 4,6-dioxoheptanoic acid which inhibits porphobilinogen biosynthesis catalysed by 5-aminolaevulinic acid dehydratase. Although both gabaculine and 4,6-dioxoheptanoic acid have been reported to have effects on plant metabolism other than inhibition of porphyrin biosynthesis (see Duke et al, 1989), the latter appears to be their only common biochemical property. Inhibition of DPE - type herbicides by both of these compounds therefore, strongly seems to implicate porphyrin synthesis as a herbicidal mode of action. Witkowski and Halling (1988) additionally showed 15mM laevulinic acid (which inhibits 5-amino-laevulinic acid synthesis) to inhibit acifluorfen activity and Lydon and Duke (1988) showed that 5-aminolaevulinic acid and the chelator 2,2'-dipyridyl stimulated acifluorfen induced toxicity.

The evidence discussed in this chapter make it seem almost inconceivable that the large increase in protoporphyrin IX found in plants treated with DPE - type compounds plays anything but a major role in the mode of action of DPE - type herbicides. If it is accepted that protoporphyrin IX is the primary photosensitiser in this system and the initiator of lipid peroxidation, it is of interest to discover which part of the porphyrin biosynthetic pathway is inhibited or stimulated in order to generate excessive quantities of protoporphyrin IX. Naturally, some proposals have been put forward suggesting that DPE - type herbicides inhibit metal chelatases which

catalyse conversion of protoporphyrin IX to magnesium protoporphyrin and heme. Lydon and Duke, (1988), Witkowski and Halling, (1988); Duke *et al*, (1989) and Kouji *et al* (1988) however, explored this possibility and found that a range of DPE - type compounds did not inhibit either magnesium chelatase or ferrochelatase. These authors consequently proposed that DPE - type herbicides act by stimulating 5-amino laevulinic acid synthesis (Rebeiz *et al* 1984, 1987 and 1988 had previously shown that an excess of 5-aminolaevulinic acid is lethal to plants). Matringe *et al* (1989a and b) similarly demonstrated that chelatases are not inhibited by DPE's at low concentrations using acifluorfen-methyl, LS-82340 (same as acifluorfen - methyl, but with the nitro substituent replaced with chlorine) and RH5348 (same as acifluorfen but the trifluoromethyl substituent is on position 5 rather than position 4 of the phenyl ring). Acifluorfen-methyl and LS-82340 have similar herbicidal activity and RH5348 has a lower herbicidal activity than these compounds. These herbicides and the DPE - type compounds oxadiazon, LS-82556 and M&B 39279 (as investigated in this thesis) were however, shown by these authors to be very potent inhibitors of protoporphyrinogen oxidase, the enzyme which catalyses the synthesis of protoporphyrin IX. These herbicides inhibited protoporphyrin oxidase at nanomolar concentrations. The enzymes from corn (*Zea mays*) etioplasts, potato (*Solanum tuberosum*) mitochondria, mouse (*Mus musculus*) liver mitochondria and yeast (*Saccharomyces cerevisiae*) were tested and were inhibited by the different herbicides by different amounts. Inhibition of protoporphyrinogen oxidase would result in an accumulation of its substrate, protoporphyrinogen IX. Matringe *et al* (1989a and b) proposed that much of this excess protoporphrinogen IX may be converted to protoporphyrin IX by non - enzymatic means.

Such non - enzymatic oxidation has infact been problematic in *in vitro* studies when attempting to assay protoporphyrinogen oxidase. Camadro *et al* (1982) furthermore demonstrated that non-enzymatic oxidation of protoporphyrinogen is greatly enhanced by the presence of phospholipids. It is therefore, conceivable that accumulation of protoporphrinogen following inhibition of its oxidase could be followed by non - enzymatic oxidation in a phospholipid environment ie. within membranes. Matringe *et al* (1989a and b) suggested that the protoporphyrin IX generated by this process diffused out of reach of magnesium chelatase activity and initiated singlet oxygen generation. The proposal of Matringe *et al* (1989a and b) seems therefore, quite plausible as a mechanism of action of DPE - type herbicides. These findings have interesting implications as to the subcellular location of DPE - type herbicide action. Smith and Rebeiz (1979) demonstrated the synthesis of protoporphyrin IX by stromal sub - chloroplast fractions. Protoporphyrin IX became subsequently membrane bound, as were magnesium protoporphyrin IX, 'longer wavelength metalloporphyrins' and protochlorophyllide. Magnesium chelatase has indeed been demonstrated to exist as being bound to the chloroplast envelope (Fuesler *et al*, 1984). Protoporphyrinogen oxidase is known to be strongly bound to the inner envelope membrane in eukaryotic mitochondria and to the plasmalemma of bacteria (Camadro *et al*, 1982). It seems, likely therefore, that plastidic protoporphyrinogen oxidase is membrane bound and probably to the inner chloroplast envelope membrane. It may also be true that although non enzymatic oxidation of protoporphyrinogen IX may occur readily in the absence of this enzyme, *in vivo* oxidation of protoporphyrinogen IX is accomplished by protoporphyrinogen oxidase to ensure that protoporphyrin IX remains membrane bound for chelation with metal ions

and is not free to initiate photodynamic reactions.

6.4 CONCLUSIONS ON THE MODE OF ACTION OF M&B 39279 AND ACIFLUORFEN

Following the preceeding discussion and based on data published elsewhere, I propose that the herbicides M&B 39279 and acifluorfen share a common mode of herbicidal action and that this action is based on interference with tetrapyrrole biosynthesis leading to a lethal accumulation of protoporphyrin IX. On the basis of this, DPE - type herbicides could henceforth be referred to generically as 'porphyrin biosynthesis inhibitor herbicides.'

Since both herbicides have very similar herbicidal properties and both are strong inhibitors of protoporphyrinogen oxidase (Matringe et al, 1989a and b), the following sequence of events can be envisaged following administration of either herbicide to susceptible plants. Initially, chloroplast envelope - bound protoporphyrinogen oxidase is inhibited by the herbicide causing an accumulation of protoporphyrinogen IX. Protoporphyrinogen IX then diffuses into the chloroplast envelope membrane and in this lipid rich environment is oxidised non enzymatically to protoporphyrin IX. Protoporphyrin IX diffuses both out of the chloroplast into the cytosol and back into the chloroplast stroma. As much of this pigment is now distant from its normal membrane - bound site (Smith and Rebeiz, 1979) and associated metal chelatases, protoporphyrin IX becomes excited by incident light and is quenched by oxygen, generating singlet oxygen and thereby initiating lipid peroxidation. The high lipid : protein ratio of the chloroplast envelope both encourages non - enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX and singlet oxygen - mediated lipid peroxidation. The magnesium chelatase activity is thus rapidly lost as the chloroplast envelope membranes are

irreversibly damaged as indicated by the electron micrographs in Chapter 4. This allows further accumulation of protoporphyrin IX. Additionally, the lack of metalloporphyrins may itself stimulate the porphyrin synthesis pathway (metalloporphyrins are known to have regulatory roles in 5-amino laevulinic acid biosynthesis) and so exacerbating protoporphyrin IX toxicity. Lastly, as chloroplast envelope integrity is lost, photosynthesis is arrested and thylakoid chlorophylls themselves become photosensitising as the function of electron transfer is lost.

If the interference of porphyrin biosynthesis is upheld as the primary mode of action of DPE - type herbicides, certain long held dogmas regarding DPE - type activity, which have been the source of much misinterpretation of experimental data in the past will be refuted. It has, for example, been long held that carotenoids play a central role and may be the photoreceptor involved in DPE type action. It now seems that the only role for carotenoids in DPE - type activity in that they are a necessary component of normal plastid structure and function. Additionally, since biosynthesis of protoporphyrin IX occurs independantly of light, it can now be suggested that light itself is not involved in the DPE - type mechanism of action, ie. the herbicide - plant interaction. DPE - type herbicides have been shown to induce protoporphyrin IX accumulation in darkness (e.g. Sandmann et al, 1984; Matringe and Scalla, 1988 and Witkowski and Halling, 1988). Light is, however essential to the mode of action of these herbicides but only for translation of herbicidal effects into a toxic process, i.e. initiation of lipid peroxidation via a protoporphyrin IX - mediated type II photodynamic process.

Note added in proof.

Strong inhibition of protoporphyrinogen oxidase by acifluorfen - methyl and proposal of this as the mode of action of this herbicide has been described by D.A. Witkowski and B.P. Halling in a paper entitled "Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl", to be published in Plant Physiology, August 1989. I thank Dr Witkowski for a pre-print of this article.

R E F E R E N C E S

ALSCHER,R. and C. STRICK (1984) Diphenyl - ether - chloroplast interactions. Pesticide Biochemistry and Physiology 21 248-255

ARNON,D.I., (1949) Copper enzymes in isolated chloroplasts: Polyphenoloxidase in Beta vulgaris. Plant Physiology 24 1-15.

BASKIN,T. (1985) Report in: Carnegie Institution of Washington Yearbook 84. Carnegie Institution of Washington, Stanford, California, U.S.A. p44.

BOGER,P. (1984) Multiple modes of action of diphenyl ethers. Zeitschrift fur Naturforschung 39c 468-475.

BOWYER,J.R., P.CAMILLERI and S.A.LEE (1987a) Use of barley mutants to probe the role of photosynthetic electron transport in the mode of action of nitrodiphenyl ether herbicides. In: Progress in Photosynthesis Research. J.Biggens (Ed). Martinus Nijhoff Publishers. 3 819-822.

BOWYER,J.R., B.J.SMITH, P.CAMILLERI and S.A.LEE (1987b) Mode of Action studies on nitrodiphenyl ether herbicides: 1. Use of barley mutants to probe the role of photosynthetic electron transport. Plant Physiology 83 613-620.

BOWYER,J.R., B.J.HALLAHAN, S.A.LEE and P.CAMILLERI (1987c) The role of photosynthetic electron transport in the mode of action of

nitrodiphenyl ether herbicides. Proceedings of British Crop Protection Conference - Weeds 1987 989-996.

BOWYER,J.R., B.J.HALLAHAN, P.CAMILLERI and J.HOWARD (1989) Mode of action studies on nitrodiphenyl ether herbicides II. The role of photosynthetic electron transport in Scenedesmus obliquus. Plant Physiology 89 674-680.

BRIGGS,W.R., and M.IINO (1983) Blue light-absorbing photoreception in plants. Philosophical Transactions of the Royal Society of London B303 347-359.

BUGG,M.W., J.WHITMARSH, C.E.RIECK and W.S.COHEN (1980) Inhibition of photosynthetic electron transport by diphenylether herbicides. Plant Physiology 65 47-50.

BUTLER,R.D., and E.W.SIMON (1971) Ultrastructural aspects of senescence in plants. Advances in Gerontological Research 3 73-129

CARDE,J.P., J.JOYARD and R.DOUCE (1982) Electron microscopic studies of envelope membranes from spinach plastids. Biology of the Cell 44 315-324.

CASTELFRANCO,P.A., and S.I. BEALE (1983) Chlorophyll biosynthesis: Recent advances and areas of current interest. Annual Review of Plant Physiology 34 241-278

CHANCELLOR,R.J., and R.J.FROUD-WILLIAMS (1984) A second survey of cereal weeds in central southern England. Weed Research 24 29-36.

CUSSANS,G. (1986) In: Long Ashton Research Station Annual Report for 1985, p26.

DELIEU,T.J., and D.A.WALKER (1983) Simultaneous measurement of oxygen evolution and chlorophyll fluorescence from leaf pieces. Plant Physiology 73 534-541.

DEVLIN,R.M., S.J.KARCZMARCZYK and I.I.ZBIEC (1983) Influence of norflurazon on the activation of substituted diphenylether herbicides by light. Weed Science 31 109-112.

DHINDSA,R.S., P.PLUMB-DHINDSA and T.A.THORPE (1981) Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany 32 93-101.

DODGE,A.D. (1971) The mode of action of the bipyridylium herbicides, paraquat and diquat. Endeavour 30 130-135.

DODGE,A.D., and D.J.GILLHAM (1986) Methods for assessing the action of light activated herbicides (i) The assessment of lipid peroxidation. Aspects of Applied Biology II 97-104.

DODGE,A.D., and J.P.KNOX (1986) Photosensitisers from plants. Pesticide Science 17 579-586.

DODGE,J.D., and G.B.LAWES (1974) Some effects of the herbicides diquat and morphamquat on the fine structure of leaf cells. Weed Research

14 45-49.

DOLPHIN,D. (Ed.) (1978) The Porphyrins. Vol2. Academic Press.

DOUCE,R., and J.JOYARD (1979) Structure and function of the plastid envelope. Advances in Botanical Research 7 1-116.

DOUCE,R., M.A.BLOCK, A.J.DORNE and J.JOYARD (1984) The plastid envelope membranes: Their structure, composition and role in chloroplast biogenesis. Chapter 1 in : Subcellular Biochemistry. Volume 10 pp.1-84 (Ed: D.B.Roodyn). Plenum Publishers.

DRABER,W., H.J.KNOPS and A.TREBST (1981) Mode of inhibition of photosynthesis electron transport by substituted diphenylethers. Zeitschrift fur Naturforschung 36c 848-852.

DRAPER,W.M., and J.E.CASIDA (1985) Nitroxide radical adducts of nitrodiphenyl ether herbicides and other nitroaryl pesticides with unsaturated cellular lipids. Journal of Agricultural and Food Chemistry 33 103-108.

DUGGAN,J., and M.GASSMAN (1974) Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. Plant Physiology 53 206-215.

DUKE,S.O., and W.H.KENYON (1986) Photosynthesis is not involved in the mechanism of action of acifluorfen in cucumber (Cucumis sativus L.). Plant Physiology 81 882-888.

DUKE,S.O., and W.H.KENYON (1987) A non-metabolic model of acifluorfen activity. Zeitschrift fur Naturforschung 42c 813-818.

DUKE,S.O., K.C.VAUGHN and R.L.MEEUSEN (1984) Mitochondrial involvement in the mode of action of acifluorfen. Pesticide Biochemistry and Physiology 21 368-376.

DUKE,S.O., J.LYDON and R.N.PAUL (1989) Oxadiazon activity is similar to that of p-nitro-diphenyl ether herbicides. Weed Science 37 152-160.

EASTIN,E.S. (1972) Fate of fluorodifen in susceptible cucumber seedlings. Weed Science 20 255-260

EBERMANN,R. and A.COUPERUS (1987) A non-enzymatic method for determination of hydrogen peroxide and organic peroxides. Analytical Biochemistry 165 414-419.

EDWARDS,G.E., S.P.ROBINSON, N.J.C.TYLER and D.A.WALKER (1978) Photosynthesis by isolated protoplasts, protoplast extracts and chloroplasts of wheat: Influence of orthophosphate, pyrophosphate and adenylates. Plant Physiology 62 313-319.

ENSMINGER,M.P. and F.D.HESS (1985a) Action spectrum of the activity of acifluorfen - methyl, a diphenyl ether herbicide, in Chlamydomonas eugametos. Plant Physiology 77 503-505.

ENSMINGER,M.P., and F.D.HESS (1985b) Photosynthesis involvement in the mechanism of action of diphenylether herbicides. Plant Physiology

78 (1) 46-50.

ENSMINGER,M.P., F.D.HESS and J.T.BAHR (1985) Nitro free radical formation of diphenyl ether herbicides is not necessary for their toxic action. Pesticide Biochemistry and Physiology 23 163-170.

ELSTNER,E.F. (1982) Oxygen activation and oxygen toxicity. Annual Review of Plant Physiology 33 73-96.

ELSTNER,E.F. (1987) Metabolism of activated oxygen species. Chapter 8 in :- The Biochemistry of Plants: a comprehensive treatise. Volume 11 (Ed: D.Davies). Academic Press pp253-315.

EVANS,C.D., G.R.LIST, A.DOLEV, D.G.McCONNELL and R.L.HOFFMANN (1967) Pentane from thermal decomposition of lipoxidase - derived products. Lipids 2 432-434.

FADAYOMI,O., and G.F.WARREN (1976) The light requirement for herbicidal activity of diphenylethers. Weed Science 24 (b) 598-600.

FEDTKE,C. (1983) Biochemistry and physiology of herbicide action. Springer - Verlag Publishers.

FINCKH,B.F., and K.J.KUNERT (1985) Vitamins C and E: An antioxidative system against herbicide-induced lipid peroxidation in higher plants. Journal of Agricultural and Food Chemistry 33 574-577.

FITZSIMONS,P.J., and J.D.B.WEYERS (1986) Potassium uptake by swelling Commelina communis guard cell protoplasts. Physiologia Plantarum

66 469-475.

FLETCHER,B.L., C.J.DILLARD and A.L.TAPPEL (1973) Measurement of fluorescent lipid peroxidation products in biological systems and tissues. Analytical Biochemistry 52 1-9.

FOOTE,C.S. (1987) Type I and type II mechanism of photodynamic action. In: Light Activated Pesticides. American Chemical Society Symposium Series No 339. (Eds: J.R.Heitz and K.R.Downum) pp22-38.

FOWKE,L.C., C.R.GRIFFING, B.G.MERSEY and P.van der VALK (1983) Protoplasts for studies of the plasma membrane and associated cell organelles. In:- 'Protoplasts 1983' (Proceedings of the 6th International Protoplast Symposium). Experientia supplementum 46 101-110.

FREAR,D.S., and H.R.SWANSON (1973) Metabolism of substituted diphenylether herbicides in plants. I Enzymatic cleavage of fluorodifen in peas. Pesticide Biochemistry and Physiology 3 473-482.

FREAR,D.S., H.R.SWANSON and E.R.MANSAGER (1983) Acifluorfen metabolism in soybean: Diphenylether bond cleavage and the formation of homoglutathione, cysteine and glucose conjugates. Pesticide Biochemistry and Physiology 20 299-310.

FROUD-WILLIAMS,R.J. (1985) The biology of cleavers (Galium aparine). Aspects of Applied Biology 9 189-195.

FRYER,J.D. and R.J.MAKEPEACE (Eds.) (1977) Weed Control Handbook Volume 1 : Principles. 6th Edition. Blackwell Scientific Publications.

FUESLER,T.P., Y.S.WONG and P.A.CASTELFRANCO (1984) Localisation of Mg-chelatase and Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase activities within isolated, developing cucumber chloroplasts. Plant Physiology 75 662-664.

GABA, V., and J.GRESSEL (1987) Acifluorfen enhancement of cryptochrome - modulated sporulation following an inductive light pulse. Plant Physiology 83 225-227.

GABA, V., N.COHEN, Y.SHAALTIEL, A.BEN-AMOTZ and J.GRESSEL (1988) Light-requiring acifluorfen action in the absence of bulk photosynthetic pigments. Pesticide Biochemistry and Physiology 31 1-12.

GALLAND, P., and H.SENGER (1988) The Role of Flavins as Photoreceptors. Journal of Photochemistry and Photobiology B:Biology 1 277-294.

GALUN, E., (1981) Plant protoplasts as physiological tools. Annual Review of Plant Physiology 32 237-266.

GODLEY, J.L., and L.M.KITCHEN (1986) Interaction of acifluorfen with fluazifop for annual grass control. Weed Science 34 936-941.

GILLHAM, D.J., and A.D.DODGE (1987a) Studies into the action of the

diphenyl ether herbicides acifluorfen and oxyfluorfen. Part I. Activation by light and oxygen in leaf tissue. Pesticide Science 19 19-24.

GILLHAM, D.J., and A.D.DODGE (1987b) Studies into the action of the diphenyl ether herbicides acifluorfen and oxyfluorfen. Part II: The interaction with photosynthetic electron transport reactions. Pesticide Science 19 25-34.

GORSKE, S.F., and H.J.HOPEN (1978) Effects of two diphenylether herbicides on common purslane (Portulaca oleracea). Weed Science 26 585-588.

GRAY, J.I. (1978) Measurement of lipid oxidation: A review. Journal of the American Oil Chemists Society 55 539-546.

GREGORY, R.P.F. (1989) Biochemistry of Photosynthesis. 3rd Edition. Wiley Publishers.

HALL, D.O. (1972) Nomenclature for isolated chloroplasts. Nature New Biology 235 125-126.

HALLING, B.P., and G.R.PETERS (1987) Influence of chloroplast development on the activation of the diphenylether herbicide acifluorfen-methyl. Plant Physiology 84 1114-1120.

HARRIS, N. (1978) Nuclear pore distribution and relation to adjacent cytoplasmic organelles in cotyledon cells of developing Vicia

faba. Planta 141 121-128.

HARRIS, N., and A.D.DODGE (1972) The effect of paraquat on flax cotyledon leaves: changes in fine structure. Planta 104 201-209.

HARRIS, N., and P.GATES (1984) Fluorescence microscopy of the endomembrane system of living plant cells. Plant, Cell and Environment 7 699-703.

HARVEY, B.M.R., and T.W.FRASER (1980) Paraquqat tolerant and susceptible rye grasses: effects of paraquat treatment on carbon dioxide uptake and structure of photosynthetic cells. Plant, Cell and Environment 3 107-117.

HAWORTH, P., and F.DAN HESS (1988) The generation Singlet Oxygen ($^1O_2$) by the nitrodiphenyl ether herbicide oxyfluorfen is independant of photosynthesis. Plant Physiology 86 672-676.

HAWTON, D., and E.H.STOBBE (1971) The fate of nitrofen in rape, redroot pigweed and green foxtail. Weed Science 19 555-558.

HEATH, R., and A.L.TAPPEL (1976) A new sensitive assay for the measurement of hydroperoxides. Analytical Biochemistry 76 184-191.

HIGGINS, J.M., T.WHITWELL, E.C.MURDOCK and J.E.TOLER (1988) Recovery of pitted morning glory (Ipomoea lacunosa) and ivyleaf morning glory (Ipomoea hederacea) following applications of acifluorfen, fomesafen and lactofen. Weed Science 36 345-353.

HILL, R. (1937) Oxygen evolved by isolated chloroplasts. Nature 139

881-882

HOAGLAND,R.E. D.J.HUNTER and M.L.SALIN (1986) Acifluorfen and oxidised ubiquinone in soybean mitochondria. Plant Cell Physiology 27 11-15

HOLM,L. J.V.PANCHO J.P.HERBERGER and D.L.PLUCKNETT (1976) A geographical atlas of world weeds. Wiley publishers.

HUCHZERMEYER,B (1982) Energy transfer inhibition induced by nitrofen. Zeitschrift fur Naturforschung 39c 787-792

HYAKUTAKE,H and ISHIZUKA (1975) Selective action of 2,4,6-trichloro -4'-nitro-diphenylether (CNP) on rice and barnyardgrass. Proceedings of the 5th Asian Pacific Weed Science Society Conference 192-197

K.KAWAKUBO, M.SHINDO and T.KONOTSUNE (1979) A mechanism of chlorosis caused by 1,3-dimethyl 1-4-(2,4-dichlorobenzoyl)-S-hydroxypyrazole, a herbicidal compound. Plant Physiology 64 774-779.

W.H.KENYON and S.O.DUKE (1985) Effects of acifluorfen on endogenous antioxidants and protective enzymes in cucumber (Cucumbis sativus) cotyledons. Plant Physiology 79 862-866.

W.H.KENYON, S.O.DUKE and K.C.VAUGHN (1985) Sequence of effects of acifluorfen on physiological and ultra-structural parameters in cucumber cotyledon discs. Pesticide Biochemistry 24 240-250.

W.H.KENYON, S.O.DUKE and R.N.PAUL (1988) Effects of temperature on the

activity of the p-Nitrosubstituted Diphenyl Ether herbicide Acifluorfen in cucumber (cucumis sativus L.). Pesticide Biochemistry and Physiology 30 57-66.

D.KESSEL (Ed.) (1985) Methods in porphyrin photosensitisation. Plenum publishers.

A.U.KHAN (1987) Identifying singlet oxygen in chemical, photochemical and enzymic reactions. In: Light Activated Pesticides. American Chemical Society Symposium Series No. 339 (Eds: J.R.Heitz and K.R.Downum) pp58-74.

G.M.KISHORE and D.M.SHAH (1988) Amino acid biosynthesis inhibitors as herbicides. Annual Review of Biochemistry 57 627-663.

J.P.KNOX and A.D.DODGE (1985a) Singlet Oxygen and Plants. Phytochemistry 24 (5) 889-896.

J.P.KNOX and A.D.DODGE (1985b) Isolation and activity of the photodynamic pigment hypericin. Plant, Cell and Environment 8 19-25.

T.KOMIVES and J.E.CASIDA (1983) Acifluorfen increases the leaf content of phytoalexins and stress metabolites in several crops. Journal of Agricultural and Food Chemistry 31 751-755.

A.W.T.KONINGS (1984) Lipid peroxidation in liposomes. Chapter 11 in :- Liposome Technology vol 1: Preparation of liposomes. G.Gregoriadis (Ed). C.R.C. Press pp139-161.

H.KOUJI, T.MASUDA and S.MATSUNAKA (1988) Action Mechanism of diphenyl ether herbicides: Light-dependant O_2 consumption in diphenyl ether-treated tobacco cell homogenate. Journal of Pesticide Science 13 495-499.

D.-J.KUNERT (1984) The diphenyl ether herbicide oxyfluorfen: A potent inducer of lipid peroxidation in higher plants. Zeitschrift fur Naturforschung. 39c 476-481.

K.J.KUNERT and P.BOGER (1981) The bleacing effect of the diphenyl ether oxyfluorfen. Weed Science 29 (2) 169-173.

K.J.KUNERT and P.BOGER (1984) The diphenyl ether herbicide oxyfluorfen: Action of antioxidants. Journal of Agricultural and Food Chemistry 32 725-728.

K.J.KUNERT, C.HOMRIGHAUSEN, J.BOHME and P.BOGER (1985) Oxyfluorfen and lipid peroxidation: Protein damage as a phytotoxic consequence. Weed Science 33 766-770.

R.LAMBERT, K.-J.KUNERT and P.BOGER (1979) On the phytotoxic mode of action of nitrofen. Pesticide Biochemistry and Physiology 11 267-274.

R.LAMBERT and P.BOGER (1981) Uber die wirkungswiese von nitrodiphenylathern. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 9 147-152.

R.LAMBERT, G.SANDMANN and P.BOGER (1983a) Correlation between structure and phytoxic activities of nitrodiphenyl ethers. Pesticide Biochemistry and Physiology 19 309-320.

R.LAMBERT, G.SANDMANN and P.BOGER (1983b) Mode of action of nitrodiphenylethers affecting pigments and membrane integrity. In: Pesticide Chemistry! Human Welfare and the Environment. 3 Mode of Action, Metabolism and Toxicology (Eds. S.Matsunaka, D.H.Hudson and S.D.Murphy) 79-102 Pergamon Press, Oxford.

R.LAMBERT, P.M.H.KRONECK and P.BOGER (1984) Radical formation and peroxidative activity of phytotoxic diphenylethers. Zeitschrift fur Naturforschung 39c 486-491.

R.LAMBERT, G.SANDMANN and P.BOGER (1987). Binding and peroxidative action of oxyfluorfen in sensitive and tolerant algal species. Zeitschrift fur Naturforschung 42c 819-823.

G.R.LEATHER and C.L.FOY (1978) Differential absorption and distribution as a basis for the selectivity of bifenox. Weed Science

26 76-81.

S.D.LEE and L.R.OLIVER (1982) Efficacy of acifluorfen on broadleaf weeds. Times and methods of application. Weed Science 30 520-526.

T.-Y.LEONG and W.R.BRIGGS (1981a) Effect of diphenyl ethers on the blue-light-induced absorbance change, phototropism and geotropism in etiolated corn and oat seedlings. Carnegie Institution of Washington Yearbook 80. Carnegie Institution of Washington, Stanford, California, U.S.A. 94-96.

LEONG,T.-Y. and W.R.BRIGGS (1981b) Partial purification and characterisation of a blue-light sensitive cytochrome-flavin complex from corn membranes. Plant Physiology 67 1042-1046

LEONG,T.-Y. and BRIGGS (1982) Evidence from studies with acifluorfen for participation of a flavin-cytochrome complex in blue light photoreception for phototropism of oat coleoptiles. Plant Physiology 70 875-881

LYDON,J and S.O.DUKE (1988) Porphyrin synthesis is required for photobleaching activity of the p-nitrosubstituted diphenylether herbicides. Pesticide Biochemistry and Physiology 31 74-83

MACROBBIE,E.A.C. (1988) Stomatal Guard Cells. In: Solute Transport in Plant Cells and Tissues. (Eds. D.A.BAKER and J.L.HALL) Longman Publishers 453-497.

MANGEOT,B.L., C.E.RIECK and J.P.DOWNS (1977) Weed control in soybeans with RH-6201. Proceedings of the Southern Weed Science Society of America 30 50.

MATRINGE,M., J.L.DUFOUR, J.LHERMINIER and R.SCALLA (1986) Characterisation of the Mode of Action of the Experimental Herbicide LS 82-556 [(S)-3-N-(methyl benzyl) carbamoyl-5-propionyl-2, 6-lutidine]. Pesticide Biochemistry and Physiology 26 150-159.

MATRINGE,M., and R.SCALLA (1987a) Induction of tetrapyrrole accumulation by diphenylether-type herbicides. Proceedings 1987 British Crop Protection Conference - Weeds 981-988.

MATRINGE,M., and R.SCALLA (1987b) Photoreceptors and respiratory electron flow involvement in the activity of acifluorfen-methyl and LS 82-556 on non-chlorophyllous soybean cells. Pesticide Biochemistry and Physiology 27 267-274.

MATRINGE,M., and R.SCALLA (1988a) Studies on the mode of action of acifluorfen-methyl in non-chlorophyllous soybean cells. Plant Physiology 86 619-622.

MATRINGE,M., and R.SCALLA (1988b) Effects of acifluorfen-methyl on cucumber cotyledons. Porphyrin accumulation. Pesticide Biochemistry

and Physiology 32 164-172.

MATRINGE,M., J.M.CAMADRO, P.LABBE and R.SCALLA (1989a) Protoporphyrinogen oxidase as a molecular target for diphenylether herbicides. Biochemical Journal 260 231-235.

MATRINGE,M., J.M.CAMADRO, P.LABBE and R.SCALLA (1989b) Protoporphyrinogen oxidase inhibition by three peroxidising herbicides: oxadiazon, LS 82-556 and M&B 39279. FEBS Letters 245 35-38.

MATSUI,T, T.KONOTSUNE and M.ISHIDA (1982) Structure-activity relationships of herbicidal pyrazole derivatives. In: Pesticide Chemistry: Human Welfare and the Environment (Eds. J.Miyamoto, P.C.Kearney, P.Doyle and T.Fujita) 1 327-332 Pergamon Press.

MATSUNAKA,S. (1969a) Acceptor of light energy in photoactivation of diphenylether herbicides. Journal of Agriculture and Food Chemistry 17 (2) 171-175.

MATSUNAKA,S. (1969b) Activation and inactivation of herbicides by higher plants. Residue Reviews 25 45-58.

MEAD,J.F. (1976) Free radical mechanisms of lipid damage and the consequences for cellular membranes. In: Free Radicals in Biology Vol.1 (Ed.W.A.Pryor) 51-68. Academic Press.

MEHLER,A.H. (1951) Studies on Reactions of Illuminated Chloroplasts. I. Mechanisms of the reduction of oxygen and other Hill reagants.

Archives of Biochemistry and Biophysics 33 65-77.

MOHR,A., and H.DRUMM-HERREL (1983) Coaction between phytochrome and blue/UV light in anthocyanin synthesis in seedlings. Physiologia Plantarum 58 408-414.

MOORE,R.J. (1975) The Galium aparine complex in Canada. Canadian Journal of Botany 53 877-893.

MORELAND,D.E., W.J.BLACKMAN, H.G.TODD and F.S.FARMER (1970) Effects of diphenylether herbicides on reactions of mitochondria and chloroplasts. Weed Science 18 636-642.

MORELAND,D.E., and W.P.NOVITZKY (1987) Interferance by herbicides with the transmembrane potential of thylakoids. Zeitschrift fur Naturforschung 42c 718-726.

NAQUI,A. (1983) What does I_{50} mean? Biochemical Journal 215 429-430.

NODA,K., K.IBARAKI, S.EGUCHI, and K.OZAWA (1965) Studies on ecological characteristics of the annual weed, cleaver, and its chemical control on drained winter paddy fields for wheat plants in temperate Japan. Bulletin of the Kyurka Agricultural Experimental Station XI 384 345-373.

NURIT,F., P.RAVANEL, and M.TISSUT (1988) The photodependant effect of LS 82556 and acifluorfen in cucumber cotyledon pieces: The possible indirect involvement of photosynthesis. Pesticide Biochemistry and Physiology 31

67-73.

OAKES,R.L., W.F.MEGGIT and D.PENNER (1981) Translocation and metabolism of ^{14}C-RH-8817 in soybean and jimsonweed (Datura stramonium var. Tatula (L.) Torr.) from shoot application and foliar vapour interception. Abstracts of the Weed Science Society of America Meeting 1981, 108.

ORR,G.L., and F.D.HESS (1981a) Characterisation of herbicidal injury by acifluorfen-methyl in excised cucumber (Cucumis sativus L.) cotyledons. Pesticide Biochemistry and Physiology 16 171-178.

ORR,G.L., and F.D.HESS (1981b) Mode of action of acifluorfen-methyl: II. Characterisation of herbicidal activity. Abstracts of the Weed Science Society of America Meeting 1981, 115.

ORR,G.L. and F.D.HESS (1982a) Proposed site(s) of action of new diphenyl ether herbicides. American Chemical Society Symposium Series No. 181 131-152.

ORR,G.L., and F.D.HESS (1982b) Mechanism of action of the diphenylether herbicide acifluorfen-methyl in excised cucumber (Cucumbis sativus) cotyledons. Plant Physiology 69 502-507.

PALLETT,K.E., R.T.REES, P.J.FITZSIMONS and A.H.COBB (1986) The isolation of mesophyll cells and protoplasts for use in herbicide research. Aspects of Applied Biology 11 139-148.

PEREIRA,J.F., W.E.SPLITTSTOESSER and H.J.HOPEN (1971) Response of

plant tissues to nitrogen. Weed Science 19 662-666.

PILET,P.E. (Ed.) (1985) The physiological properties of plant protoplasts. Springer-Verlag Publishers.

POLLAK,T., and G.CRABTREE (1976) Effect of light intensity and quality on toxicity of fluorodifen to green bean and soybean seedlings. Weed Science 24 571-573.

PORTER,N.A. (1984) Chemistry of Lipid Peroxidation. Methods in Enzymology 105 273-282 (Ed. L.Packer) Academic Press.

PRITCHARD,M.K., G.F.WARREN and DILLEY, R.A. (1980) Site of action of oxyflourfen. Weed Science 28 640-645

PRYOR,W.A. (1978) The formation of free radicals and the consequences of their reactions in vivo. Photochemistry and Photobiology 28 787-801.

RATNER,M. (1989) Crop biotech. '89 : Research efforts are market driven. Biotechnology 7 337-341

REBEIZ,C.A., S.M.WU, M.KUHADJA, H.DANIELL and E.J.PERKINS (1983) Chlorophyll a biosynthetic routes and chlorophyll a chemical heterogeneity in plants. Molecular and Cellular Biochemistry 57 92-125.

REBEIZ,C.A., A.MONTAZER-ZOUHOOR, H.J.HOPEN and S.M.WU (1984) Photodynamic herbicides: Concept and phenomenology. Enzyme and

Microbial Technology 6 390-401.

REBEIZ,C.A, A.MONTAZER-ZHOUHOOR, J.M.MAYASICH, B.C.TRIPATHY, S.M.WU and C.C.REBEIZ (1987) Photodynamic herbicides and chorophyll biosynthesis modulators. In: Light Activated Pesticides. American Chemical Society Symposium Series No 339 (Ed. J.R.Heitz and K.R.Downum) pp 295-328.

REBEIZ,C.A., A.MONTAZER-ZHOUHOOR, J.M.MAYASICH, B.C.TRIPATHY, S.M.WU and C.C.REBEIZ (1988) Photodynamic herbicides. Recent devolopments and molecular basis of selectivity. Critical Reviews in Plant Sciences 6 (4) 385-436. C.R.C. Press.

REYNOLDS,E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology 17 208-212

RICHARDSON,W.G. (1984) Phytoxicity and Selectivity of Aromatic Ethers. Presentation at the Society for Chemical Industry (London) Symposium on aromatic ethers, March 13th, 1984.

RIDLEY,S.M. (1983) Interaction of chloroplasts with inhibitors: Effects of two diphenylethers, fomesafen and nitrofluorfen on electron transport and some comparisons with dibromothymoquinone, diuron and paraquat. Plant Physiology 72 461-468.

RITTER,R.L., and H.D.COBLE (1981) Penetration, translocation and metabolism of acifluorfen in soybean (Glycine max), common ragweed (Ambrosia artemisiifolia) and common coekleburr (Xanthium

pensylvanicum). Weed Science 29 474-480.

SALIN,M.L. (1987) Toxic species and protective systems of the chloroplast. Physiologia Plantarum 72 681-689.

SANDERS,G.E. (1984) Studies into the Differential acitivity of the hydroxybenzonitrile herbicides on various weeds species. PhD. Thesis, Trent Polytechnic, Nottingham, U.K.

SANDMANN,G., and P.BOGER (1982) Volatile hydrocarbons from photosynthetic membranes containing different fatty acids. Lipids 17 35-41.

SANDMANN,G, H.RECK and P.BOGER (1984) Herbicidal mode of action on chlorophyll formation. Journal of Agriculture and food Chemistry 32 868-872.

SATO,R., E.NAGANO, H.OSHIO and K.KAMOSHITA (1987a) Diphenylether-like physiological and biochemical actions of S-23142, a novel N-phenylimide herbicide. Pesticide Biochemistry and Physiology 28 194-200.

SATO,R., E.NAGANO, H.OSHIO, K.KAMOSHITA and M.FURUYA (1987b) Wavelength effect on the action of a N-phenylimide S-23142 and a diphenylether acifluorfen-ethyl in cotyledons of cucumber (Cucumis sativus L.) seedlings. Plant Physiology 85 1146-1150.

SCHON,A., G.KRUPP, S.GOUGH, S.BERRY-LOWE, C.G.KANNANGARA and D.SOLL (1986) The RNA required in the first step of chlorophyll biosynthesis

is a chloroplast glutamate tRNA. Nature 322 281-284.

SENGER,H. (Ed.) (1980) The Blue Light Syndrome. Springer-Verlag Publishers.

SHIMABUKURO,R.H., G.L.LAMOUREUX, H.R.SWANSON, W.C.WLASH, L.E.STAFFORD and D.S.FREAR (1973) Metabolism of substituted diphenylether herbicides in plants. II Identification of a new fluorodifen metabolite, S-(2-nitro-4-trifluoromethylphenyl) glutathione in Peanut. Pesticide Biochemistry and Physiology 3 483-487

SHIMOTORI,H. and S.KUWATSUKA (1978) Absorption, translocation and metabolism of CNP , a diphenyl ether herbicide, and its amino derivative in rice plants. Journal of Pesticide Science 3 267-275.

SLATER,T.F. (1984) Overview of methods used for detecting lipid peroxidation. Methods in Enzymology 105 283-293 (Ed. L.Parker) Academic Press.

SMITH,B.B., and C.A.REBEIZ (1979) Chloroplast biogenesis XXIV. Intrachloroplastic localisation of the biosynthesis and accumulation of protoporphyrin IX, magnesium protoporphyrin monoester and longer wavelength metalloporphyrins during greening. Plant Physiology 63 227-231.

SMITH,H. (1975) Phytochrome and Photomorphogenesis. McGraw - Hill Publishers.

SPIKES,J.D., (1989) Photosensitisation. In: The Science of

Photobiology. (Ed. K.C.Smith) 2nd Edition. Plenum Press.

SPURR,A.R. (1969) A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research 26 31-43.

SWANSON,E.S., W.W.THOMSON, and J.B.MUDD (1973a) The effect of lipid extraction on electron - microscopic images of plant membranes. Canadian Journal of Botany 51 (1) 1221-1230.

SWANSON,E.S., W.W.THOMSON, and J.B.MUDD (1973b) The effect of ozone on leaf cell membranes. Canadian Journal of Botany 51 (1) 1213-1220.

TAPPEL,A.L. (1980) Measurement of and protection from in vitro lipid peroxidation. In: Free Radicals in Biology Vol 4. (Ed. W.A.Pryor) 1-47. Academic Press.

TISSUT,M., P.RAVANEL, F.NURIT, C.DESLANDRES, J.BOURGUIGNON (1987) Effects of LS 82556 on Thylakoid activities and photosynthesis. A comparison with paraquat and acifluorfen. Pesticide Biochemistry and Physiology 29 209-216.

TAKAHASHI,N., and R.P.MASON (1987) Identification of free radicals formed from nitrodiphenyl ethers by irradiation in solution. Journal of Pesticide Science 12 745-748.

TERAOKA,T., G.SANDMANN, P.BOGER and K.WAKABAYASHI (1987) Effect of cyclic imide herbicides on pigment formation in plants. Journal of Pesticide Science 12 499-504.

THOMPSON,J.E, R.L.LEGGE and R.F.BARBER (1987) The role of free radicals in senescence and wounding. New Phytologist 105 317-344.

TUTIN,T.G., V.H.HEYWOOD, N.A.BURGES, D.M.MOORE, D.H.VALENTINE, S.M.WALTERS and D.A.WEBB (1976) 'Flora Europaea'. Cambridge University Press.

UPHAM,B.L., and H.C.HATZIOS (1987) Potential involvement of allioxyl and hydroxyl radicals in the peroxidative reaction of oxyfluorfen. Pesticide Biochemistry and Physiology 28 248-256.

VAN DEN BERG,G., and J.TIPKER (1982) Quantitative structure - activity relationships of the inhibition of photosynthetic electron flow by stubstituted diphenylethers. Pesticide Science 13 29-38.

VANSTONE,D.E., and E.H.STOBBE (1979) Light requirement of the diphenylether herbicide oxyfluorfen. Weed Science 27 88-91.

WAKABAYASHI,K., K.MATSUYA, T.JIKIHARA, S.SUZUKI and S.MATSUNAKA (1978) Abstracts IV International Congress of Pesticide Chemistry. I.U.P.A.C. Abstracts 4 655.

WAKABAYASHI,K., K.MATSUYA, T.TERAOKA, G.SANDMANN and P.BOGER (1986) Effect of Cyclic Imide herbicides on chlorophyll formation in higher plants. Journal of Pesticide Science 11 635-640.

WAKABAYASHI,K., G.SANDMANN, H.OHTA and P.BOGER (1988) Peroxidising herbicides: Comparison of dark and light effects. Journal of Pesticide Science 13 461-471.

WETTLAUFFER,S.H., R.ALSCHER and C.STRICK (1985) Chloroplast-diphenyl ether interactions II. Plant Physiology 78 215-220.

WIDHOLM,J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47 189-194.

WIJESEKERA,T.P., and D.DOLPHIN (1985) Some preparations and properties of porphyrins. In: Methods In Porphyrin Photosensitisation. (Ed. D.Kessel) Plenum Press. pp 229-266.

WILLMER,C.M. (1983) Stomata. Longman Publishers.

WITKOWSKI,D.A., and B.P.HALLING (1988) Accumulation of photodynamic tetrapyrroles induced by acifluorfen-methyl. Plant Physiology 87 632-637.

WORTHING,C.R. (Ed.) (1987) 'The Pesticide Manual' 8th Edition. The British Crop Protection Council.

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

P. M. DERRICK, A. H. COBB

Department of Life Sciences, Trent Polytechnic, Clifton Lane, Nottingham NG11 8NS, UK

K.E. PALLETT

May and Baker Agrochemicals, Fyfield Road, Ongar, Essex, CM5 0HW, UK

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/m^2/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

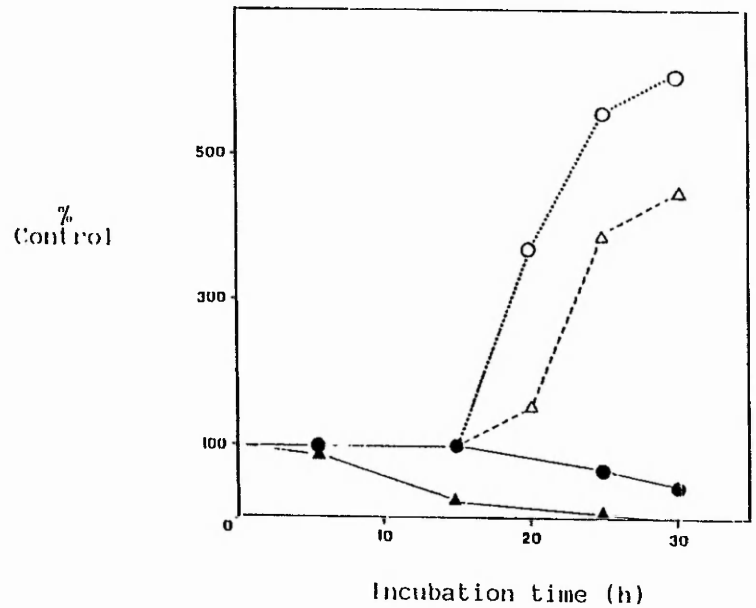


Fig. 1. The effect of 100µM acifluorfen on leaf photosynthesis (▲——▲), chlorophyll content (●——●), electrolyte leakage (O····O) and MDA formation (Δ- - -Δ) in excised G. aparine leaves at 25°C and 50 µmoles/m²/s PPFD. Data are expressed as a percentage of control values, which were constant throughout 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/m^2/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

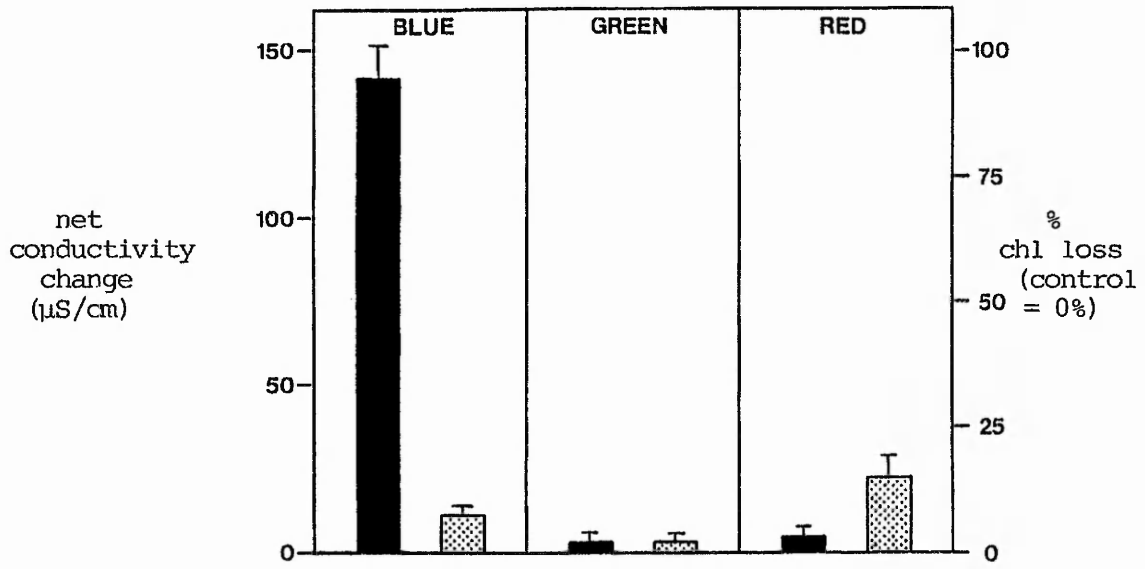


Fig. 2 The leakage of electrolytes (■) and loss of chlorophyll (⊡) from excised G. aparine leaves incubated for 48h with 100 μM acifluorfen under 8 μmoles/m^2/s PPFD blue, red and green light. Bars represent S.E.'s.

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

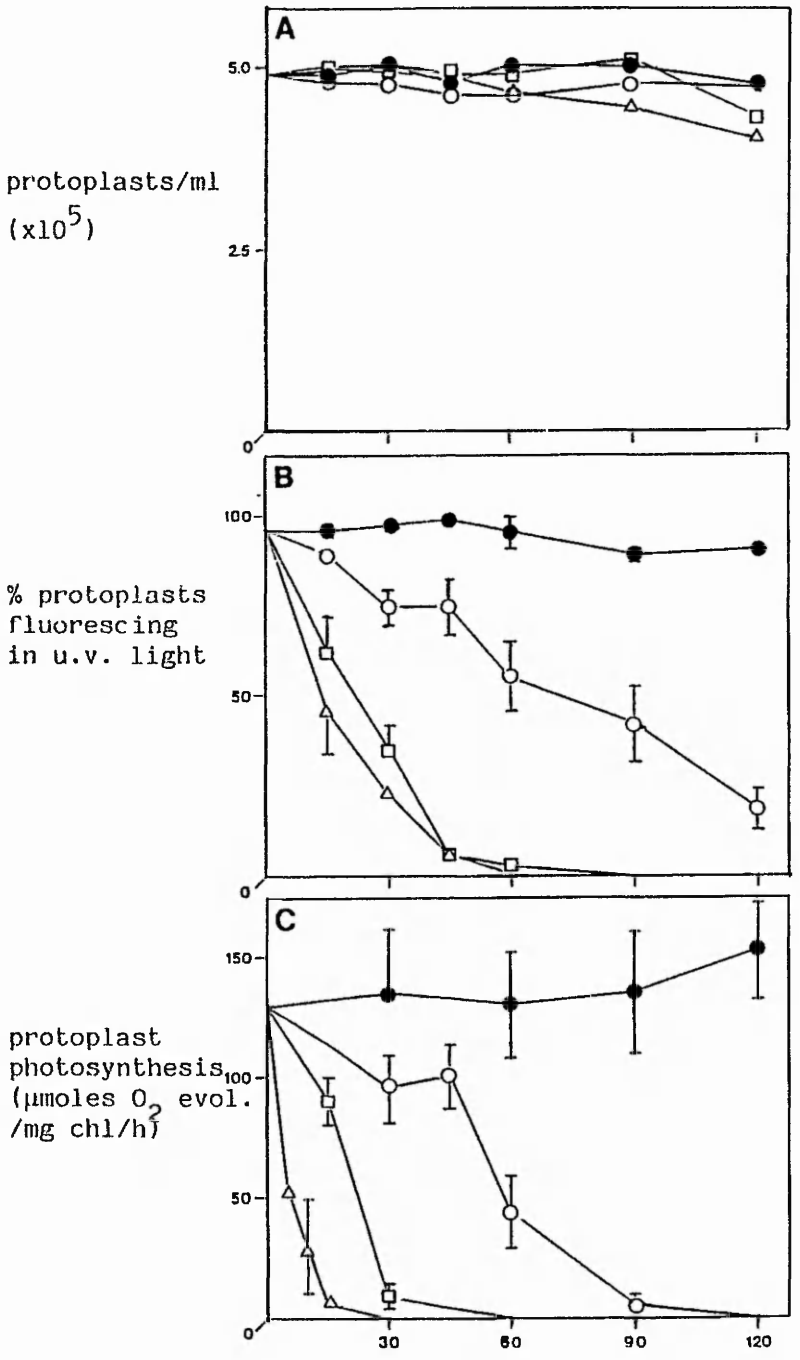


Fig. 3. The effect of 0 (●—●), 375 (○—○), 500 (□—□) and 750 (△—△) µM acifluorfen on protoplast intactness (A), protoplast viability (B) and protoplast photosynthesis (C). Bars represent S.E.'s.

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

Acknowledgement is given to May and Baker Agrochemicals for full financial support of this work.

REFERENCES

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts Polyphenoloxidase in Beta vulgaris. Plant Physiology 24, 1-15.

Bowyer, J. R.; Smith, B.J.; Camilleri, P., Lee, S.A. (1987). Mode of action studies on nitrodiphenyl ether herbicides I. The use of barley mutants to probe the role of photosynthetic electron transport. Plant Physiology 83, 613-620.

Derrick, P.M. (1987) An investigation into the mode of action of the herbicide M & B 39 279. Ph.D. Thesis, Trent Polytechnic

Derrick, P.M., Cobb, A.H.; Pallett, K.E. (1988) Ultrastructural effects of acifluorfen and the experimental herbicide M & B 39279 in Galium aparine (in preparation).

Douce, R.; Joyard, J. (1979) Structure and function of the plastid envelope. In: Advances in Botanical Reseach 7, Woolhouse, H.W. (Ed.) Academic Press pp1-116.

Duke, S.O.; Kenyon, W.H. (1986) Photosynthesis is not involved in the mechanism of action of acifluorfen in cucumber (Cucumis sativus L.) Plant Physiology 81, 882 - 888.

Duke, S.O.; Kenyon, W.H. (1987) A non-metabolic model of acifluorfen Activity. Zeitschrift fur Naturforschung 42c, 813 - 818.

Ensminger, M.P.; Hess, F.D. (1985) Photosynthesis involvement in the mechanism of action of diphenyl ether herbicides. Plant Physiology 78, 46-50.

Gillham, D. J.; Evans, M.W., Dodge, A.D. (1985) Oxyfluorfen activation by photosynthetic electron transport. Proceedings 1985 British Crop Protection Conference-Weeds 3, 1196 - 1201.

Harvey, B.M. R.; Fraser, T.W. (1980) Paraquat tolerant and susceptible perennial ryegrasses: effects of paraquat treatment on carbon dioxide uptake and ultrastructure of photosynthetic cells. Plant, Cell and Environment 3, 107 - 117.

Heath, R.L.: Packer, L. (1968) Photoperoxidation in isolated chloroplasts 1. Kinetics and stoichiometry of fatty acid peroxidation. Archives Biochemistry and Physiology 125, 189.

Kenyon, W.H.; Duke, S.O.; Vaughn, K.C. (1985) Sequence of effects of acifluorfen on physiological and ultrastructural parameters in cucumber cotyledon discs. Pesticide Biochemistry and Physiology 24, 240 - 250.

Matringe, M.; Dufour, J.L.; Lherminier, J.; Scalla, R. (1986) Characterisation of the mode of action of the experimental herbicide LS 82-556 [(S)3-N-(methyl benzyl) carbamoyl - S - propionyl - 2,6-lutidine]. Pesticide Biochemistry and Physiology 27, 267-274.

Orr, G.L.; Hess, F.D. (1982) Proposed site(s) of action of new diphenylether herbicides. In: Biochemical Responses induced by herbicides D.E. Moreland; J.B. St. John and F.D. Hess (Eds) American Chemical Society Symposium Series No. 181 Washington D.C. pp 131-152.

Widholm, J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47 (4), 189-194.

PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 32, 153–163 (1988)

Ultrastructural Effects of the Diphenyl Ether Herbicide Acifluorfen and the Experimental Herbicide M&B 39279

P. M. DERRICK,[1] A. H. COBB, AND K. E. PALLETT*

*Herbicide Research Group, Department of Life Sciences, Trent Polytechnic, Clifton Lane, Nottingham NG11 8NS, United Kingdom and *Rhone-Poulenc Agriculture, Fyfield Road, Ongar, Essex CM5 OHW, United Kingdom*

Received April 5, 1988; accepted June 28, 1988

The sequence of the herbicidal effects of acifluorfen and the novel herbicide M&B 39279 (5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazole) on ultrastructure in excised *Galium aparine* L. leaves has been examined in detail and supported by physiological measurements. Plant responses to acifluorfen and M&B 39279 were indistinguishable, the latter compound therefore being deemed to share a common mode of action with nitrodiphenyl ethers. After 3 hr in 50 μmol m^{-2} sec^{-1} photosynthetic photon flux density light, chloroplast swelling and invagination/evagination of chloroplast envelopes was observed in the presence of 100 μM acifluorfen and M&B 39279. Tonoplast lysis and/or development of cytoplasmic vesicles occurred by 15 hr whilst plasmalemma disruption, membraneous vesicle formation, and abundant electron opaque material were apparent between 20 and 30 hr. Chloroplasts, often fused together with highly modified envelope membranes, were the last identifiable cell structures remaining. Thylakoids were notably persistant, retaining integrity for about 20 hr. Under identical conditions, leaf photosynthetic capability diminished immediately following herbicide treatment. Leaf chlorophyll content remained constant for over 15 hr, after which a decline was accompanied by leakage of electrolytes and malondialdehyde from treated leaves. These results indicate that the diphenyl ether-type mode of action is initiated at the chloroplast envelope, but is independent of photosynthesis and involves lipid peroxidation.

INTRODUCTION

The herbicides commonly referred to as diphenyl ethers (DPEs)[2] are a group of rapidly acting, selective herbicides. Although most DPEs are of the *p*-nitro type (e.g., acifluorfen), a number of compounds which lack the phenoxybenzene structure, such as LS 82-556 (1, 2) and the cyclic imide herbicides (3, 4), appear to share the DPE mode of action (1–4). These compounds may also, therefore, be included in the DPE herbicide category. It is generally held that DPE-induced plant injury results from a light-dependent peroxidative destruction of cell membranes (5–8), although the mechanism by which these herbicides initiate lipid peroxidation remains obscure.

Electron microscopy has been used as an aid in probing the mode of action of DPEs in several studies (1, 5, 8–12). The paper of Kenyon *et al.* (8), however, is the only moderately detailed sequential analysis of DPE-induced ultrastructural changes published to date. These authors demonstrated that in cucumber (*Cucumis sativus*) cotyledons, the earliest perceptible damage involved the plasma membrane, tonoplast, and chloroplast envelope. This and allied physiological data led these authors to suggest that the plastid envelope may be a primary target of acifluorfen.

In order to further clarify the work of Kenyon *et al.* and others (1, 5, 9–12), this paper reports ultrastructural observations from a study designed to reveal the first subcellular structures to undergo DPE-

[1] To whom correspondence should be addressed at present address: Virology Division, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK.

[2] Abbreviations used: DPE(s), diphenyl ether(s); M&B 39279, 5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazole; PPFD, photosynthetic photon flux density; MDA, malondialdehyde.

0048-3575/88 $3.00

mediated morphological change. To achieve this, excised leaves of *Galium aparine* L., an important European agricultural weed (13), were maintained under conditions which allowed comparatively slow development of phytotoxic symptoms, thus enabling early ultrastructural changes to be discerned. To support and substantiate ultrastructural observations, basic physiological properties of treated tissues were monitored throughout the experiment. Additionally, this paper introduces the experimental herbicide M&B 39279 (5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazole) and we compare its effects with those of acifluorfen.

MATERIALS AND METHODS

Treatment of plant tissue. *G. aparine* plants were glasshouse grown at 20–25°C under 400-W high-pressure sodium lamps giving a 14-hr day length. Plants bearing two whorls of true leaves and a developing third whorl were used for experiments. Leaves were cut from the second whorl and floated abaxial surface down on 100 μ*M* aqueous solutions of acifluorfen and M&B 39279 (10 leaves on 10 ml of solution) in 50-mm-diameter petri dishes. All solutions, including control treatments, contained 1% (v/v) acetone. The petri dishes were incubated for up to 30 hr at 25°C under 50 μmol m^{-2} sec^{-1} PPFD provided by a bank of "natural white" fluorescent tubes (GEC). Light was excluded from dark treatments with aluminium foil.

Electron microscopy. After 3, 5, 15, 20, 25, and 30 hr in light and after 30 hr in darkness, 2-mm^2 pieces of leaf were cut from a standardised portion of the leaves, about a third of the length along the leaf from the petiolar end. Samples were fixed in 3% (v/v) glutaraldehyde in 50 m*M* phosphate buffer, pH 7.0, for 30 min followed by post-fixation in 2% (w/v) osmium tetroxide in 50 m*M* phosphate buffer, pH 7.0, for 2 hr. Samples were then stained for 30 min in 0.5% (w/v) uranyl acetate prior to dehydration with an acetone series and embedding in Spurrs resin (14). Ultrathin sections (50–90 nm thick) were cut using a Reichert OMU-2 ultra-microtome, mounted on copper grids and stained with lead citrate (15) for 15 min before observation with an AEI EM6G transmission electron microscope. A minimum of three grids from each of three resin blocks were examined per treatment.

Whole leaf photosynthesis and chlorophyll content. After 3, 5, 15, 20, 25, and 30 hr light and after 30 hr darkness, the photosynthetic capability of the excised leaves was assessed using an Hansatech LD2 oxygen electrode (Hansatech Ltd., Kings Lynn, UK) illuminated with a tungsten–halogen projector lamp supplying 500 μmol m^{-2} sec^{-1} PPFD to the leaves. This electrode system incorporates a Clarke-type oxygen electrode but allows measurement of gas-phase oxygen in a chamber enclosing an excised leaf (see (16)). The chamber accommodated five *G. aparine* leaves and measurements were repeated four times in two experiments. Leaf pigments were extracted into 80% (v/v) aqueous acetone and chlorophyll content was determined by the method of Arnon (17).

Membrane integrity. Electrolyte leakage (i.e., an increase in bathing solution conductivity) and generation of thiobarbituric acid reacting material (i.e., malondialdehyde (MDA), a breakdown product of lipid peroxidation), provided measurable parameters broadly indicating membrane integrity. Conductivity was measured after 5, 15, 20, 25, and 30 hr light and after 30 hr darkness using a PTI-19 conductivity meter (FSA Ltd., Loughborough, UK) coupled with a CCOO5 flow cell (Canterbury Scientific, Canterbury, UK) which allowed sampling, measurement, and return of a small volume of liquid from and to the petri dishes. It was not possible to measure MDA within *G. aparine* leaves due to the presence of interfering substances (see (18)). Leakage of MDA into the bathing medium was thus quantified as an indicator of lipid peroxidation. Thiobarbituric acid re-

acting material (i.e., MDA) was measured as follows: A 2-ml sample of bathing solution was mixed with an equal volume of 0.5% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid and this mixture was heated in a water bath for 25 min. The mixture was then cooled, centrifuged at 2500*g* for 5 min, and absorbance of the supernatant determined at 532 nm. A millimolar extinction coefficient of 155 m*M* cm^{-1} was used to estimate the MDA concentration in solution (18).

RESULTS

As with other studies (8, 19, 20), establishing a sequence of ultrastructural changes was complicated by the variability of response to herbicides within treated tissues due to the natural variability of cells within a leaf and variations in the internal distribution of herbicide. Sampling from a standardised part of the leaf and examination of an adequate number of grids (see Materials and Methods) minimised this problem.

Similarity of Acifluorfen- and M&B 39279-Mediated Phytotoxicity

The physiological and ultrastructural symptoms resulting from M&B 39279 and acifluorfen treatment were qualitatively and quantitatively very similar (Figs. 1–5). From this and other data (21) it was concluded that M&B 39279 and acifluorfen share a common mode of action. From a physiological standpoint, therefore, M&B 39279 can be regarded as a DPE-type herbicide. In view of this, for brevity and ease of discussion, results from M&B 39279 and acifluorfen treatments are described and discussed collectively.

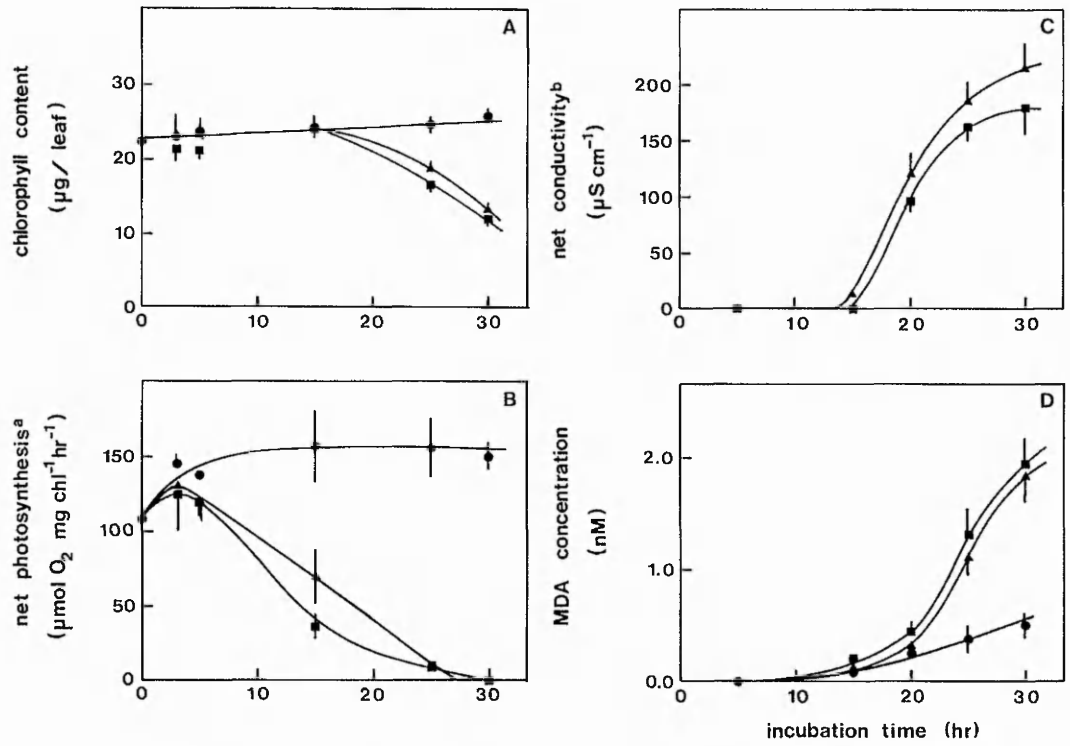


FIG. 1. *Changes in* (*A*) chlorophyll content of, (*B*) *photosynthetic capability of,* (*C*) *electrolyte leakage from, and* (*D*) *MDA release from* G. aparine *leaflets floating on water* (●), *100* μM *acifluorfen* (■), *and 100* μM *M&B 39279* (▲) *under 50* μ*mol* m^{-2}/sec^{-1} *PPFD for up to 30 hr.* [a]*Photosynthesis was measured at 500* μ*mol* m^{-2}/sec^{-1} *PPFD, a near-saturating light intensity for* G. aparine (*21*). [b]*Net conductivity* = μ*S* cm^{-1} (*test solution*) − μ*S* cm^{-1} (*100* μM *herbicide in distilled water*). *Control bathing solution conductivity never exceeded 5.6* μ*S* cm^{-1}. *Bars are standard errors.*

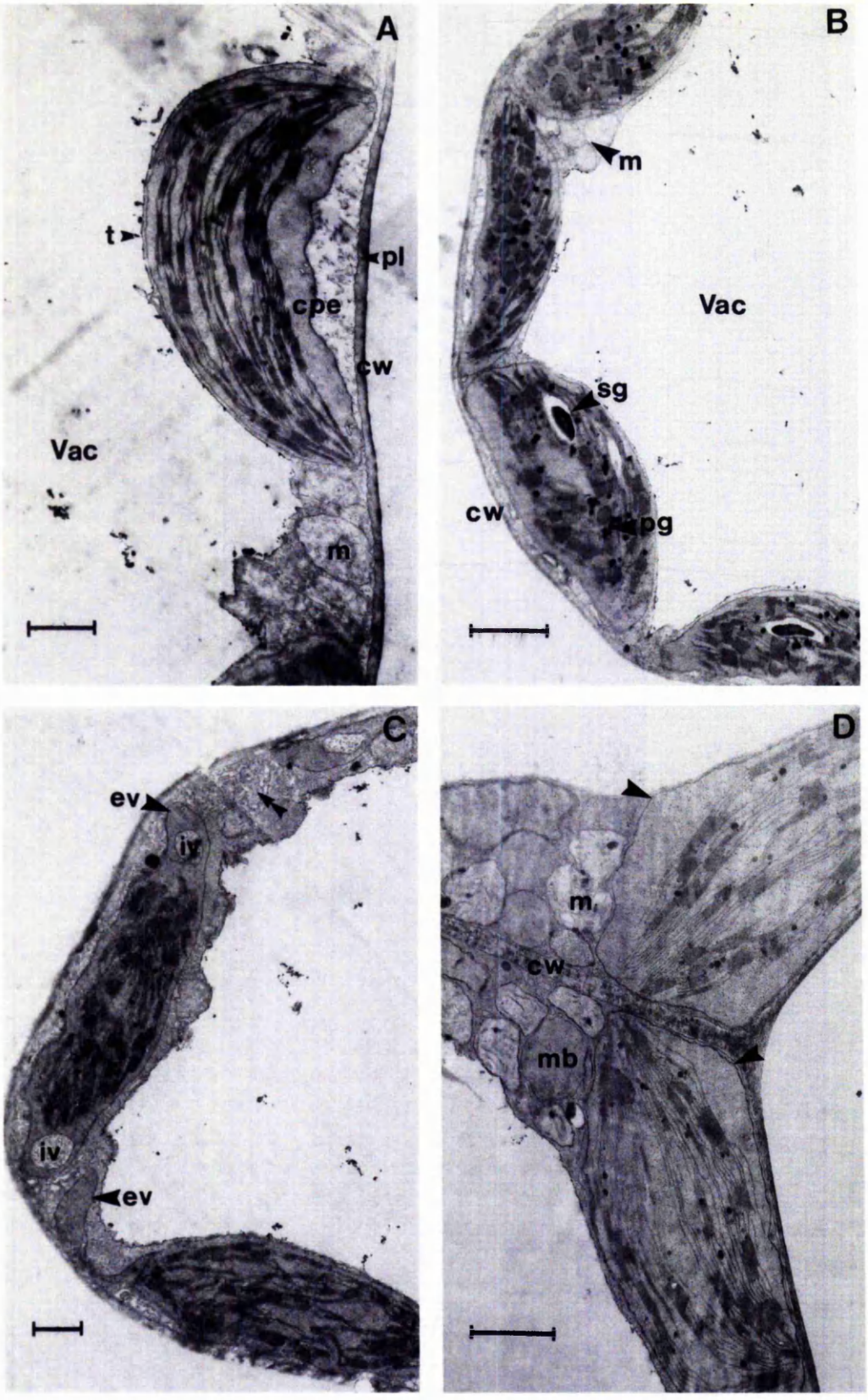


FIG. 2. *(A) Characteristic cell from untreated tissue after 3 hr light. cpe, chloroplast envelope; cw, cell wall; m, mitochondrion; p, plasmalemma; t, tonoplast; Vac, vacuole. Bar = 1 μm (Figs. 2–5). (B) Untreated tissue after 30 hr light. pg, plastoglobulus; sg, starch grain. (C) Acifluorfen-treated tissue after 3 hr light. Note invaginations (iv) and evaginations (ev) of chloroplast envelopes. Cytoplasmic disturbance (double arrows) is seen in the cell shown. (D) M&B 39279-treated tissue after 3 hr light. Note swollen chloroplasts (arrowed); mb, microbody.*

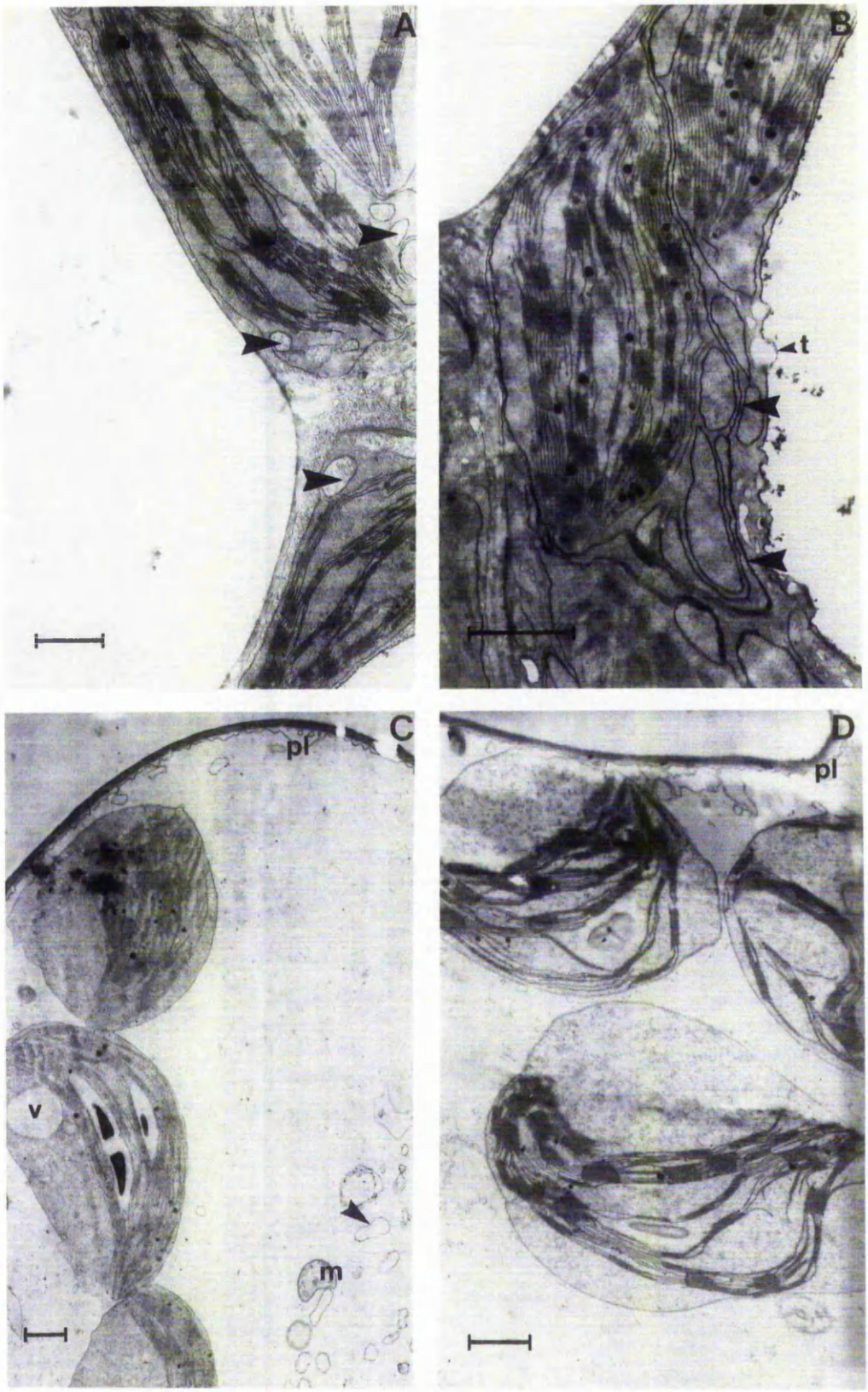


FIG. 3. *(A) Acifluorfen-treated tissue after 5 hr light. Note distorted chloroplast envelopes/membrane invaginations (arrowed). (B) M&B 39279-treated tissue after 5 hr light. Note distorted chloroplast envelopes (arrowed). (C) Acifluorfen-treated tissue after 15 hr light. Note apparent lack of tonoplast, vesicles (v) in swollen chloroplasts, and presence of membraneous vesicles (arrowed). (D) M&B 39279-treated tissue after 15 hr light.*

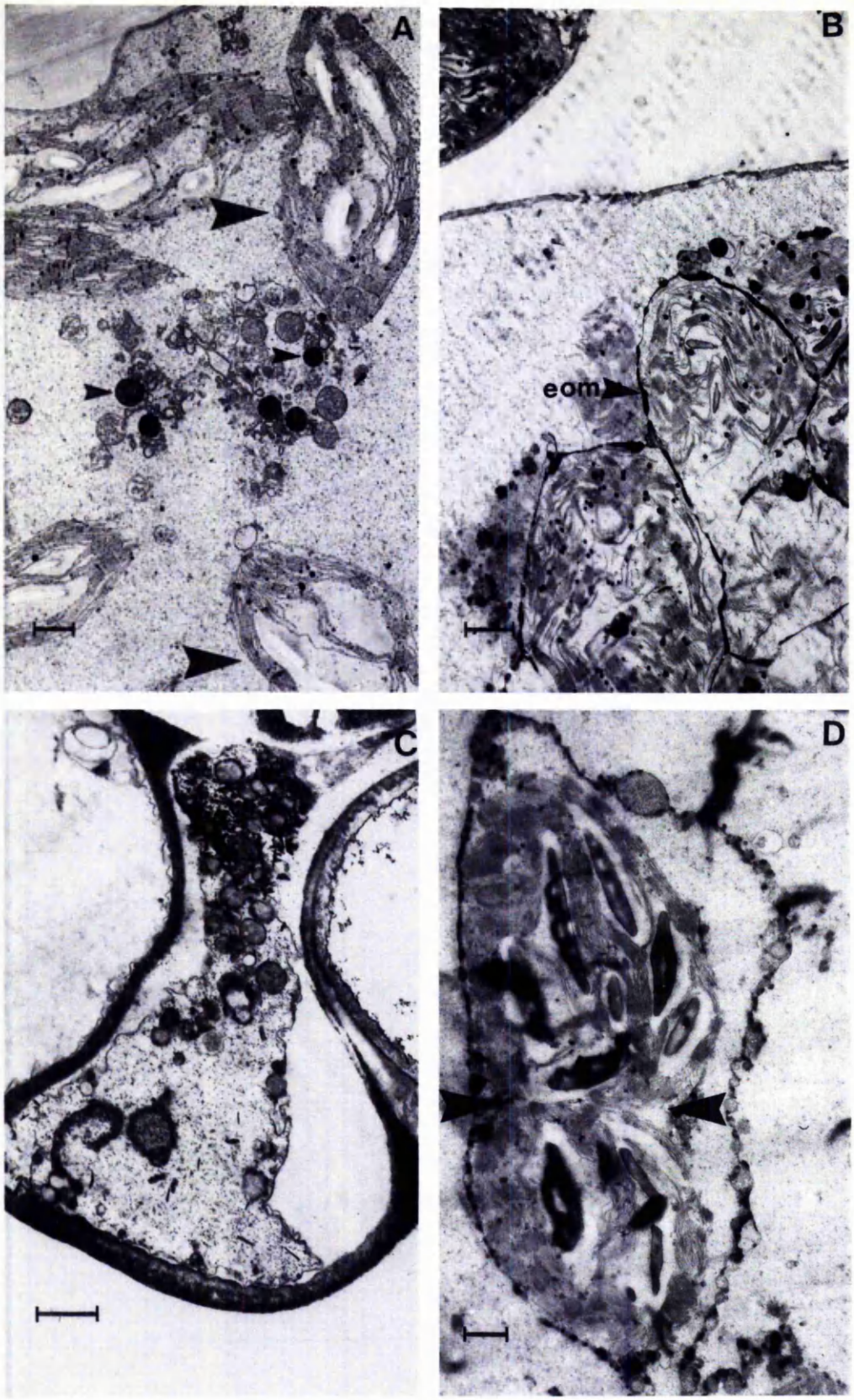


FIG. 4. *(A) Acifluorfen-treated tissue after 20 hr light. Note discontinuous chloroplast envelopes (large arrows), membraneous vesicles, and electron opaque globules (small arrows). (B) Acifluorfen-treated tissue after 25 hr light. Note fused chloroplast remains, disrupted thylakoids, and electron opaque material (eom) associated with defunct chloroplast envelopes. (C) M&B 39279-treated tissue after 25 hr light. The cytoplasm is reduced to a mass of vesicles and electron opaque debris. (D) M&B 39279-treated tissue after 30 hr light, showing a structure measuring roughly twice the long axis of most* G. aparine *chloroplasts and so likely to be two fused chloroplasts (arrows indicate position of apparent fusion). Note modified (fused) envelope membranes.*

Chlorophyll Content and Photosynthetic Capability

After a small initial increase, the photosynthetic capability of light-incubated control tissue remained constant throughout the duration of the experiment. The chlorophyll content of this tissue also remained constant over the 30-hr period. The photosynthetic capability of treated leaves decreased almost immediately whereas treated leaf chlorophyll content remained constant until after 15 hr incubation in light (Figs. 1A and 1B). No changes in photosynthetic capability nor chlorophyll content were observed in dark incubated treated or control tissues (data not shown).

Membrane Integrity

No significant increases in bathing solution conductivity or MDA leakage were observed in illuminated control tissue (Figs. 1C and 1D) nor in dark incubated control and treated tissue (not shown). Leakage of electrolytes from treated tissue was first detectable after 15 hr incubation in light after which the conductivity of the bathing solution rose sharply (Fig. 1C). Elevated levels of MDA were found in the bathing solution of treated tissue after 20 hr of light (Fig. 1D).

Ultrastructure

Cell ultrastructure of control tissues after a 3-hr incubation in light (Fig. 2A) was similar to that of leaves freshly cut from *G. aparine* plants (not shown). Control cell ultrastructure remained largely unchanged throughout the experiment, except that by 30 hr incubation in light (Fig. 2B) and darkness (Fig. 5A) some chloroplasts became slightly irregular in shape and showed a slight increase in the number of plastoglobuli. These changes may represent the initial stage of aging (22), although this was not reflected in measurements of photosynthetic competence. An increase in the number of plastoglobuli could alternatively reflect a partially redundant photosynthetic apparatus, the incubation light intensity being much lower than that under which the plants were grown. Some dark incubated cells (both control and treated) also showed altered alignment of thylakoids within chloroplasts (Fig. 5C). Neither acifluorfen- nor M&B 39279-treated tissue showed any ultrastructural symptoms of toxicity after 30 hr in darkness (Figs. 5B and 5C), confirming, alongside the physiological data reported above, the light dependency of these herbicides.

Ultrastructural alterations were noted in treated tissue after a 3-hr light incubation (Figs. 2C and 2D). Many chloroplasts were swollen and invaginations and evaginations of the chloroplast envelope were common. A few cells also showed cytoplasmic irregularities (Fig. 2C).

After 5 hr light, further vesiculisation of chloroplast envelopes was observed in treated tissue (Figs. 3A and 3B), the envelope membranes apparently yielding many intraplastid and cytoplasmic vesicles. The tonoplast of many cells was also disturbed.

After 15 hr illumination, the tonoplast had lysed in a large proportion of treated cells, yielding a mass of vesicles (Figs. 3C and 3D). Chloroplasts, although swollen, remained intact and contained a scarcely altered thylakoid system and prominent starch grains. Mitochondria appeared largely unaltered in such cells and the plasmalemma, although intact, appeared to be shrinking from the cell wall. Those cells which still possessed an intact tonoplast after 15 hr light displayed numerous cytoplasmic and some vacuolar membraneous vesicles (not shown but similar to DPE-induced symptoms detected in barley by Bowyer *et al.* (12)).

By 20 hr, the only remaining recognisable structures in many treated cells were chloroplasts, these being distorted and often having discontinuities in the envelope membranes (Fig. 4A). Electron opaque ma-

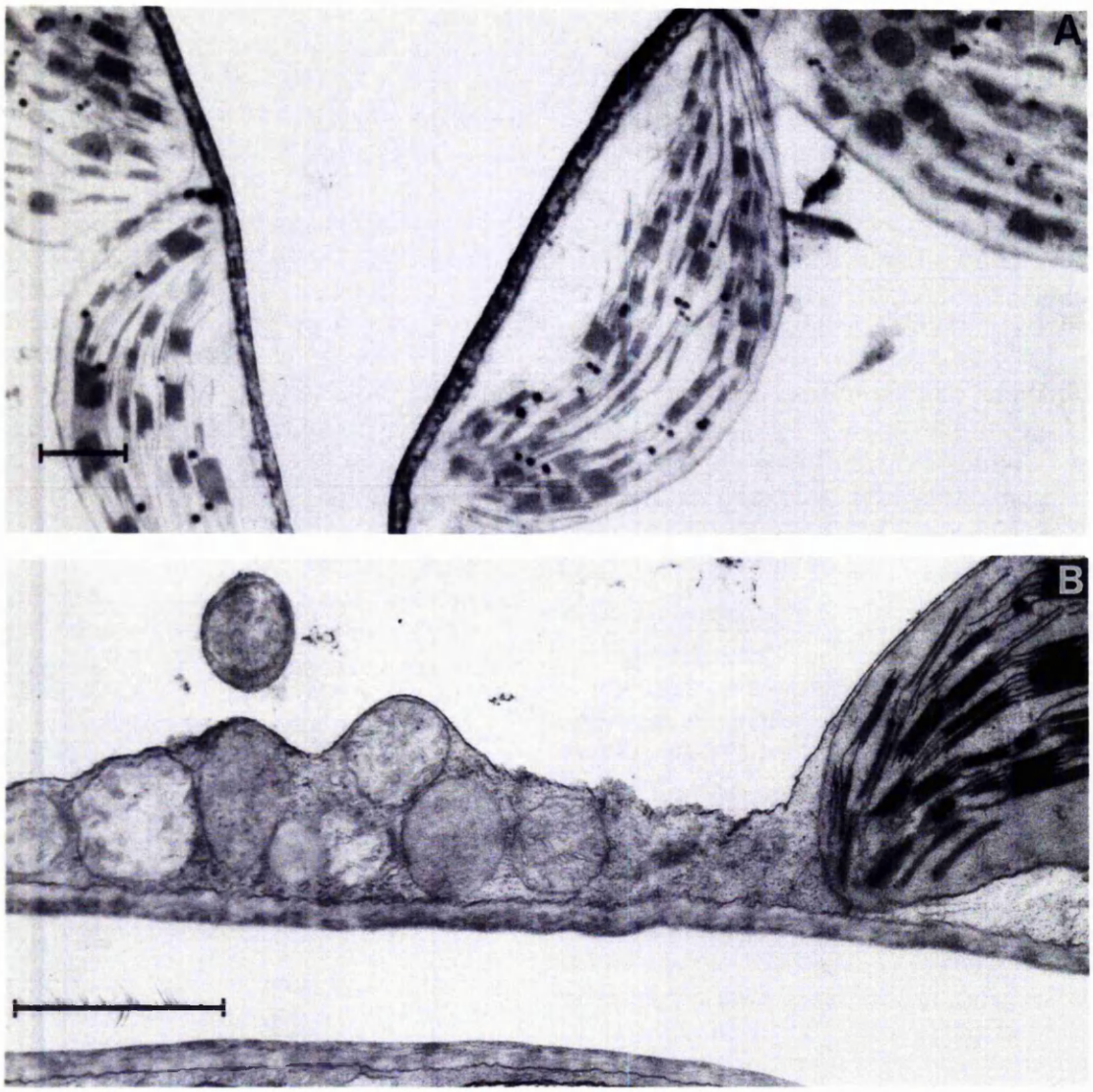


FIG. 5. *(A) Control tissue after 30 hr in darkness. (B) Acifluorfen-treated tissue after 30 hr in darkness. Note integrity of cytoplasmic contents.*

terial, probably representing lipids from degraded membrane systems, was associated with many vesicles and defunct organelles.

After 25–30 hr incubation, treated cells showed a range of symptoms characterised by vesiculated remains of cytoplasm and an abundance of free vesicles, many bearing electron opaque globules (Figs. 4B–4D). The remains of chloroplasts were discernible, these possessing degenerating thylakoid systems. Particularly conspicuous were the highly modified chloroplast envelope membranes which consisted of little more than contiguous vesicles and electron opaque globules. Many chloroplasts bearing such effete envelopes were fused together (Figs. 4B and 4D).

DISCUSSION

The elucidation of a sequence of herbicide-induced ultrastructural events with a view to locating a primary or initial site of action has an unavoidable limitation, namely that changes in subcellular morphology only show the result of the herbicides primary action, which may be spacially separated from the actual site of such action. Nevertheless, if accompanied by

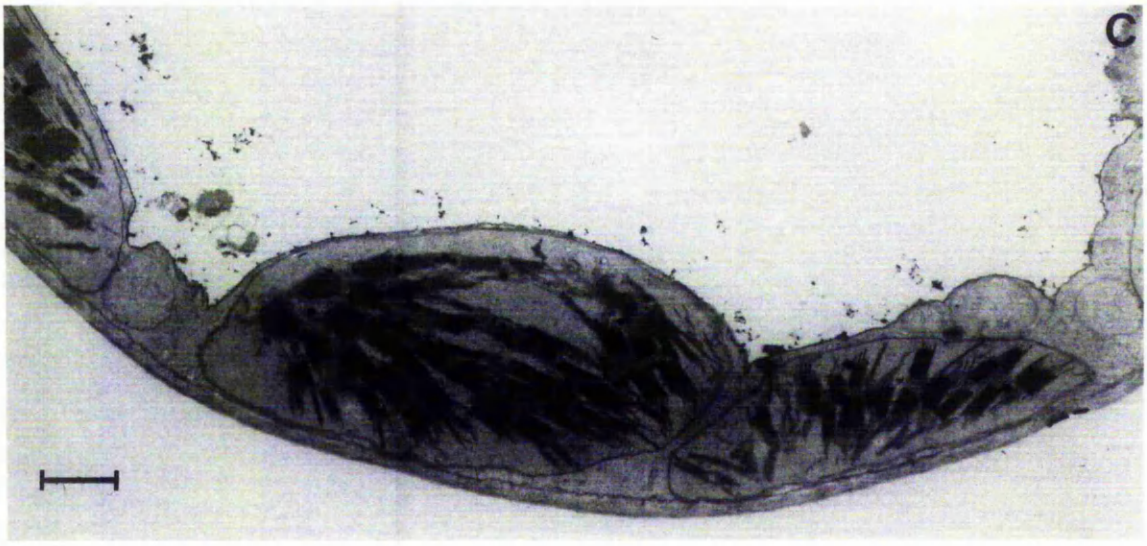


FIG. 5C. *M&B 39279-treated tissue after 30 hr in darkness. Note alignment of thylakoids.*

relevant physiological measurements, ultrastructural observations can be a useful addition to mode of action studies.

From our ultrastructural observations, we propose that the following sequence of cytoplasmic events occurs after administration of acifluorfen/M&B 39279 to *G. aparine* leaves in light under the described conditions: (1) by 3 hr incubation swelling/distortion of chloroplasts; (2) by 3–5 hr, evagination and invagination of chloroplast envelopes, some cytoplasmic disturbance (swelling of endoplasmic reticulum?), and tonoplast perturbation; (3) by about 15 hr, tonoplast disruption, lysis, and/or cytoplasmic vesicle development; (4) between 20 and 30 hr, a progressive degeneration of membranes and organelles is observed, yielding vesicles and osmiophilic globules. Chloroplasts develop discontinuous/highly modified envelope membranes containing large amounts of osmiophilic material. These membranes frequently coalesce. Thylakoid membranes and starch grains persist as the last recognisable cytoplasmic structures to undergo destruction with these herbicides.

Since the speed of action of acifluorfen and M&B 39279 is dependent on light intensity (21), the conditions of low light intensity employed in this study were chosen to allow a relatively slow progression of the sequence of ultrastructural changes, thus enabling discrimination between events occurring early in the sequence and those arising at a later time. The use of a high light intensity would have rapidly yielded a difficult to interpret mixture of "early" and "late" symptoms (e.g., (5)), due to tissue variations (see Results). Although in this study excised leaves were incubated under conditions of relatively low light intensity, these conditions were sufficient to support photosynthesis at approximately 25 μmol O_2 mg chll^{-1} hr^{-1} (21), roughly a third of the rate which would have been supported by the glasshouse conditions under which the plants were grown. A similar sequence of ultrastructural events was in fact observed with acifluorfen/M&B 39279-sprayed *G. aparine* and *Viola arvensis* Murr. plants maintained under glasshouse conditions (21).

The sequence of events described was accompanied by a rapid decline in photosynthetic capability. As expected, the onset of electrolyte/MDA leakage corresponded chronologically with tonoplast lysis and plasmalemma disruption. The detection of MDA in the bathing solution further supports the idea of a peroxidative mechanism for DPE-type herbicides (see (1, 4–12)).

Rapid swelling of chloroplasts, disruption of envelope membranes, and the rapid and concurrent decline in photosynthetic competence indicates that the chloroplast is a likely primary target for acifluorfen and M&B 39279. Since thylakoid membranes, however, persisted largely unchanged until a late stage in the development of phytotoxicity, it seems unlikely that they play a direct role in DPE-type toxicity. The hypothesis of Gillham *et al.* (23), suggesting that *p*-nitro DPEs are reduced by photosynthetic electron transport either directly or indirectly, after which they initiate peroxidative damage is, therefore, not supported by our data as a general mode of action for DPE-type compounds. Even though thylakoids are protected from damage by large amounts of antioxidants, it is likely that a mechanism of peroxidation initiated at the thylakoid itself would yield fairly rapid damage to these structures, as in bipyridyl herbicide activity (20, 24, 25). Further evidence against direct thylakoid involvement in DPE-type activity is provided by the sensitivity of etiolated tissue to these compounds (e.g., (5, 11)).

Since chloroplast envelope membranes showed such rapid and dramatic changes in morphology in this study, which preceeded all other ultrastructural changes, our results suggest that these membranes may contain the primary target of DPE-type herbicides. It is likely that the disruptions of the envelope, as in Figs. 3A and 3B, are in fact responsible for the observed rapid decline in photosynthesis as these membranes regulate metabolite transport, notably photosynthate/inorganic phosphate transport, between the chloroplast stroma and the cytoplasm (26) and, in so doing, have a central role in regulating photosynthetic metabolism. The changes in membrane permeability and/or fluidity which would no doubt accompany the observed changes in envelope morphology would almost certainly arrest photosynthesis.

As also demonstrated in this paper, the absolute requirement of light for DPE-type activity has been widely reported (see (5) and literature cited therein), the evidence of Pereira *et al.* (27) being an exception. The major remaining questions regarding DPE mode of action studies concern, however, the identity of the photoreceptive system necessary for activity and the nature of its interaction with DPE molecules. Blue and, to a lesser extent, red light have been implicated as the most effective regions of the spectrum in generating DPE-induced phytotoxicity (28–30) with carotenoids (4, 5, 9, 31–34), flavins (5), and chlorophyll precursors (30, 35) being suggested as possible photoreceptors involved in this process. Albeit hypothetical and speculative, Duke and Kenyon (35) presented a model for DPE action involving herbicide interaction with a putative carotenoprotein in the plastid envelope which initiates lipid peroxidation. Although not precluding other cell compartments, our data support such a model and provide compelling evidence implicating the involvement of the chloroplast envelope in the initial stages of DPE activity, substantiating and further expanding previous ultrastructual reports.

ACKNOWLEDGMENTS

We acknowledge Rhone-Poulenc Agriculture (formerly May and Baker Agrochemicals) for full financial support of this study. We also thank Mr. D. Lacey for developing and printing the micrographs.

REFERENCES

1. M. Matringe, J. L. Dufour, J. Lherminier, and R. Scalla, Characterization of the mode of action of the experimental herbicide LS 82-556 [(*S*)-3-*N*-(methylbenzyl)carbamoyl-5-propionyl-2,6-lutidine], *Pestic. Biochem. Physiol.* **26,** 50 (1986).
2. M. Matringe and R. Scalla, Photoreceptors and respiratory electron flow involvement in the activity of acifluorfen-methyl and LS 82-556 on nonchlorophyllous soybean cells, *Pestic. Biochem. Physiol.* 27, 267 (1987).
3. K. Wakabayashi, K. Matsuya, T. Jikihara, S. Suzuki, and S. Matsunaka, Mechanism of action of cyclic imide herbicides, *IV Int. Congr. Pestic. Chem. (IUPAC) Abstr.* IV, 655 (1978).
4. C. Fedtke, "Biochemistry and Physiology of Her-

bicide Action," p. 92, Springer-Verlag, Heidelberg/New York, 1982.

5. G. L. Orr and F. D. Hess, Proposed site(s) of action of new diphenyl ether herbicides, *Amer. Chem. Soc. Symp. Ser.* **181**, 131 (1982).
6. K. J. Kunert, The diphenyl ether oxyfluorfen: A potent inducer of lipid peroxidation in higher plants, *Z. Naturforsch. C* **39**, 476 (1984).
7. R. Lambert, P. M. H. Kroneck, and P. Boger, Radical formation and peroxidative activity of phytotoxic diphenyl ethers, *Z. Naturforsch. C* **39**, 486 (1984).
8. W. H. Kenyon, S. O. Duke, and K. C. Vaughn, Sequence of effects of acifluorfen on physiological and ultrastructural parameters in cucumber cotyledon discs, *Pestic. Biochem. Physiol.* **24**, 240 (1985).
9. G. L. Orr and F. D. Hess, Mechanism of action of the diphenyl ether herbicide acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons, *Plant Physiol.* **69**, 502 (1982).
10. S. O. Duke, K. C. Vaughn, and R. L. Meeusen, Mitochondrial involvement in the mode of action of acifluorfen, *Pestic. Biochem. Physiol.* **21**, 368 (1984).
11. S. O. Duke and W. H. Kenyon, Photosynthesis is not involved in the mechanism of action of acifluorfen in cucumber (*Cucumis sativus* L.), *Plant Physiol.* **81**, 882 (1986).
12. J. R. Bowyer, B. J. Smith, P. Camilleri, and S. A. Lee, Mode of action studies on nitrodiphenyl ether herbicides. 1. Use of barley mutants to probe the role of photosynthetic electron transport, *Plant Physiol.* **83**, 613 (1987).
13. R. J. Froud-Williams, The biology of cleavers (*Galium aparine*), *Aspects Appl. Biol.* **9**, 185 (1985).
14. A. R. Spurr, A low viscosity epoxy resin embedding medium for electron microscopy, *J. Ultrastruct. Res.* **26**, 31 (1969).
15. E. S. Reynolds, The use of lead citrate at high pH as an electron opaque stain in electron microscopy, *J. Cell. Biol.* **17**, 208 (1963).
16. T. J. Delieu and D. A. Walker, Simultaneous measurement of oxygen evolution and chlorophyll fluorescence from leaf pieces, *Plant Physiol.* **73**, 534 (1983).
17. D. I. Arnon, Copper enzymes in isolated chloroplasts: Poly-phenoloxidase in *Beta vulgaris*, *Plant Physiol.* **24**, 1 (1949).
18. T. F. Slater, Overview of methods used for detecting lipid peroxidation, *in* "Methods in Enzymology" (L. Packer, Ed.), Vol. 105, pp. 283–293, Academic Press, New York/London, 1984.
19. J. R. Baur, R. W. Bovey, P. S. Baur, and Z. El-Seify, Effects of paraquat on the ultrastructure of mesquite mesophyll calls, *Weed Res.* **9**, 81 (1969).
20. N. Harris and A. D. Dodge, The effect of paraquat on flax cotyledon leaves: Changes in fine structure, *Planta* **104**, 201 (1972).
21. P. M. Derrick, "An Investigation into the Mode of Action of the Herbicide M&B 39279," Ph.D. Thesis, Trent Polytechnic, 1988.
22. R. D. Butler and E. W. Simon, Ultrastructural aspects of senescence in plants, *Adv. Gerontol. Res.* **3**, 73 (1971).
23. D. J. Gillham, M. W. Evans, and A. D. Dodge, Oxyfluorfen activation by photosynthetic electron transport, *Proc. Brit. Crop Prot. Conf. Weeds*, 1195 (1985).
24. J. D. Dodge and G. B. Lawes, Some effects of the herbicides diquat and morphamquat on the fine structure of leaf cells, *Weed Res.* **14**, 45 (1974).
25. B. M. R. Harvey and T. W. Frazer, Paraquat tolerant and susceptible ryegrasses: Effects of paraquat treatment on carbon dioxide uptake and structure of photosynthetic cells, *Plant Cell. Environ.* **3**, 107 (1980).
26. R. Douce and J. Joyard, Structure and function of the plastid envelope, *Adv. Bot. Res.* **7**, 1 (1979).
27. J. F. Pereira, W. E. Splittstoesser, and H. J. Hopen, Response of plant tissues to nitrofen, *Weed Sci.* **19**, 662 (1971).
28. P. M. Derrick, A. H. Cobb, and K. E. Pallett, The effects of acifluorfen on membrane integrity in *Galium aparine* leaves and protoplasts, *Proc. Brit. Crop Prot. Conf. Weeds* **3**, 997 (1987).
29. M. P. Ensminger and F. D. Hess, Action spectrum of the activity of acifluorfen-methyl, a diphenyl ether herbicide, in *Chlamydomonas eugametos*, *Plant Physiol.* **77**, 503 (1985).
30. M. Matringe and R. Scalla, Induction of tetrapyrrole accumulation by diphenylether-type herbicides, *Proc. Brit. Crop Prot. Conf. Weeds* **3**, 981 (1987).
31. O. Fadayomi and G. F. Warren, The light requirements for diphenyl ethers, *Weed Sci.* **24**(6), 598 (1976).
32. M. K. Pritchard, F. G. Warren, and R. A. Dilley, Site of action of oxyfluorfen, *Weed Sci.* **28**, 640 (1980).
33. D. E. Vanstone and E. H. Stobbe, Light requirement of the diphenylether herbicide oxyfluorfen, *Weed Sci.* **27**, 88 (1979).
33. M. P. Ensminger and F. D. Hess, Photosynthesis involvement in the mechanism of action of diphenylether herbicides, *Plant Physiol.* **78**(1), 46 (1985).
35. S. O. Duke and W. H. Kenyon, A non-metabolic model of acifluorfen activity, *Z Naturforsch. C* **42**, 813 (1987).