STUDIES INTO THE DIFFERENTIAL ACTIVITY OF THE HYDROXYBENZONITRILE HERBICIDES IN VARIOUS WEED SPECIES

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ABSTRACT

The mechanism of action and selectivity of ioxynil and bromoxynil salts was investigated in Matricaria inodora, Stellaria media and Viola arvensis. The differential activity was characterized by monitoring symptoms such as chlorosis and necrosis, and by studying metabolic changes in sprayed plants. M. inodora, S. media and V. arvensis were susceptible, moderately susceptible and moderately resistant to ioxynil, and acutely susceptible, moderately resistant and resistant to bromoxynil respectively. Herbicide retention and leaf surface characteristics did not contribute to the differential response.

Greater than 80% of penetrated ^{14}C-herbicide remained in the treated leaves and contributed to chlorosis and necrosis. CO_2-fixation was rapidly inhibited and ultrastructural changes developed within 48 hours. In susceptible interactions ioxynil-induced thylakoid swelling and chloroplast disruption, typical of photooxidative damage. Such changes were less apparent with bromoxynil. Cellular disruption in M. inodora suggested an uncoupling and/or permeability action of bromoxynil. In V. arvensis, where a partial recovery of CO_2 fixation occurred, adaptive changes in chloroplast ultrastructure such as broader and higher granal stacks, were evident.

^{14}C-bromoxynil was more mobile than ^{14}C-ioxynil and significant inter-specific differences were found in the speed and direction of herbicide movement. A far greater proportion of translocated ^{14}C-ioxynil was recovered from the apex of susceptible M. inodora than that of moderately resistant V. arvensis. Bromoxynil appeared to be metabolized in V. arvensis and to a lesser extent S. media which may contribute towards reduced phytotoxicity in these species. A significant proportion of ^{14}C-hydroxybenzonitrile was undetected due to volatilization from the leaf surface.

Interspecific differences in herbicide activity were not apparent at the thylakoid level. Ioxynil was 3-4 times more effective as an inhibitory uncoupler in chloroplasts isolated from the three species. In radiolabelled displacement studies ioxynil partially replaced bound ^{14}C-bromoxynil, whereas bromoxynil did not replace bound ^{14}C-ioxynil, indicating stronger ioxynil binding.

DECLARATIONS

1. The observations in this study are, except where otherwise stated, entirely the work of the author.

2. The author has not received, and has not been registered for any other higher degree awarded by a University or the Council of National Academic Awards during the course of this work nor has any part of it been previously submitted in partial fulfilment for the award of a higher degree.

3. The author has attended conferences and programmes of study relevant to the present investigation.

4. Due acknowledgments have been made for the assistance given during the course of this work and in the presentation of the thesis on which it is based.

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PUBLICATIONS

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Studies into the different response of three weed species to the hydroxybenzonitriles.

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Physiological changes in Matricaria inodora following ioxynil and bromoxynil treatment.

Zeitschrift fur Naturforschung 39c: 505-509.

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In vitro activity and binding characteristics of the hydroxybenzonitriles in chloroplasts isolated from Matricaria inodora and Viola arvensis.

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Aspects of the investigations outlined in Chapters 2-4 are currently in preparation for future publication.

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CONTENTS

ABBREVIATIONS

ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
BSA	-	bovine serum albumin
Chl	-	chlorophyll
Cyt b_6-f	-	cytochrome b_6-f complex
DAD	-	diamino durene
DBMIB	-	dibromothymoquinone
DCPIP	-	dichlorophenol indophenol
DCMU	-	3-(3,4,-dichlorophenyl)-1,1-dimethylurea
DNA	-	deoxyribonucleic acid
DNOC	-	4,6-dinitro-o-cresol
dpm	-	disintegrations per minute
EDTA	-	ethylenediaminotetra acetic acid
FeCN	-	ferricyanide
GDA	-	gluteraldehyde
HBN	-	hydroxybenzonitrile
IRGA	-	infra red gas analysis
LDE	-	leaf disc electrode
LHCP-2	-	light harvesting chlorophyll a/b protein complex
MV	-	methyl viologen
$NADP^+$	-	nicotinamide adenine dinucleotide phosphate
P	-	plastocyanin
PMS	-	phenazine methosulphate
PSI	-	photosystem I
PSII	-	photosystem II
PQ	-	plastoquinone
PVP	-	polyvinylpyrrolidone
P_{680}	-	reaction centre chlorophyll of PSII
P_{700}	-	reaction centre chlorophyll of PSI
Q	-	primary acceptor of PSII
Q_B protein	-	herbicide binding protein
RNA	-	ribonucleic acid
SiMo	-	silicomolybdate
Tricine	-	-N-((trishydroxymethyl)-methyl) glycine
X	-	primary acceptor of PSI

CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION

Before the end of the nineteenth century weeds were controlled by ploughing, harrowing and crop rotation. At the turn of the century copper sulphate was introduced as the first selective herbicide in British agriculture. It's selectivity, was dependant upon differential wetting of morphologically contrasting species, i.e. a broadleaved dicotyledonous weed in a monocotyledonous crop. Other mineral salts were also used and in the 1930's substantial cereal acreage were annually sprayed with sulphuric acid. In 1932, the first examples of organic herbicides, dinitrophenols (DNOC) and cresols, were patented in France for the selective control of weeds in cereals.

However, the present era of chemical weed control began with the discovery of the herbicidal properties of chlorinated phenoxyacetic acids (e.g., 2,4-D, MCPA) in 1949. These compounds were found to selectively control the target species of charlock (Brassica arvensis) and other dicotyledonous weeds in wheat, oats and barley. The first carbomate, propham was synthesised at this time and thus MCPA, 2,4-D, DNOC and propham were the forerunners of the present array of selective herbicides. Since the 1940's, the number and variety of commercially available herbicides has increased exponentially.

By 1960 a large proportion of the cereal crop was being chemically treated for weed control. Important broadleaved weeds such as poppy (Papaver rhoeas), cornflower (Centaurea cyanus) and charlock (Brassica sinapis) were being eliminated. Dichloprop, mecoprop, dicamba, 2,3,6-TBA and ioxynil were introduced to broaden the spectrum of selective weed control. More and more broad-leaved weeds were controlled and gradually the "vacant" ecological niches were taken over by the grass weeds, wild oats (Avena fatua), blackgrass (Alopecurus myosuroides) and sterile brome (Bromus sterilis; Fletcher and Kirkwood 1982). Over the last decade, the continued use of substituted urea herbicides to control grass weeds has resulted in the emergence of hitherto unfamiliar weeds as serious pests, e.g. Persian speedwell (Veronica persica),

field pansy (Viola arvensis) and cleavers (Galium aparine; Makepeace 1982). This problem has resulted in a more sophisticated approach to weed control with the introduction of direct drilling, sequential applications, mixtures of herbicides, and a re-evaluation of the more established herbicides.

Selectivity between the crop and weed can now be achieved with pre-planting, pre-emergence and post-emergence applications of herbicides. Currently, the latter application technique is used for the majority of weed control, with the aerial shoots of both the crop and weed exposed to the herbicide. As already indicated, there is often a differential tolerance exhibited by different weed species, for example spring wild oats (Avena fatua) is adequately controlled by chlorfenprop-methyl whilst winter wild oat (Avena sterilis) is moderately resistant to this herbicide (Hack 1973). In general, the basis of selectivity may lie in a physical interaction between the leaf surface and droplet, differential penetration and movement of the herbicide, activation or deactivation within plant tissues or a biochemical interaction at the site of action.

The majority of the modern herbicides introduced during the last 30 years act upon the photosynthetic apparatus (Moreland 1980). These herbicides can be broadly classified as compounds which either interfere with electron transport, uncouple photophosphorylation, inhibit pigment biosynthesis or have multiple types of inhibition (Wright and Corbett 1979). Photosynthetic inhibition induces various physiological changes in the plant ranging from an adaptive response to decreased photosynthate production, through to the generation of toxic radicals with subsequent membrane damage (Fedtke 1982). Various other metabolic processes may also be directly or indirectly affected by photosynthetic inhibition.

In this chapter, factors affecting the selectivity of herbicides will be reviewed with particular reference to post-emergent foliar spray herbicides. In addition the biochemical mechanism of action and physiological consequences of photosynthetic inhibition will also be discussed. Finally the activity of the hydroxybenzonitrile herbicides will be reviewed in relation to the aims of this study.

1.2 FACTORS AFFECTING HERBICIDE SELECTIVITY

Differential response to herbicides has generally been assumed to result from differences in the morphological and physiological factors that affect the levels of herbicidal compound accumulating at the active site (Jensen 1982). Of these retention, uptake, translocation and particularly metabolism have repeatedly been shown to account for differential responses between species.

1.2.1 Retention

The enormous variety in leaf surface morphology and physiology provides ample scope for enhancing herbicide selectivity by exploiting differences in spray retention. This can be accomplished by utilizing the gross morphological differences between monocotyledons and dicotyledons, or more fundamentally by considering the physicochemical factors which govern leaf surface wettability. The surface, or epicuticular waxes consist of complex mixtures of various classes of aliphatic compounds (including alkanes, alkyl esters, primary and secondary alchohols, ketones and fatty acids) all of which contribute to the overall hydrophobic properties of the leaf surface (Fletcher and Kirkwood 1982).

A herbicide which is not normally considered selective, may elicit a differential response in morphologically contrasting species. For example dicotyledonous weeds with rosette habit may direct herbicidal sprays to meristematic tissue exposed at the growth point (Hassell 1982). In contrast, monocotyledonous plants often have vertical leaves which retain less spray, and may form a protective sheath around the meristem. Selectivity of the phenoxy acetic acids is partly based on this morphological difference. Similarly, greatest retention of asulam by Avena fatua occurs at the leaf axil relatively close to the meristem, whereas the highest retention on linseed plants occurs on the broad cotyledons where effect is minimal (Hibbitt 1969). In addition the selective action of bromoxynil octanoate was partially explained by retention differences between tolerant wheat and susceptible coast Fiddleneck (Schafer and Chilcote 1970a).

Clearly, accurate timing is necessary to maximise selectivity as gross morphology changes with growth stage e.g. retention by barley (Hordeum distichum) increases with age as the leaves become less vertical, conversely spray retention by horizontal mustard (Brassica alba) cotyledons become proportionately less important with growth of the main aerial shoots (Blackman, Bruce and Holly 1958).

Once intercepted, the wettability of the leaf surface then becomes important in selectivity. This property is governed by the same fundamental physico-chemical factors which govern the wetting of any solid surface, namely the nature of the exposed surface groups and the surface roughness (Holloway 1970). The wettability of the cuticle is usually quantified by measuring the angle of contact between the droplet and leaf surface. Surfaces high in alkane or ketone content tend to retain an easily displaced spherical droplet which has a high contact angle. In contrast, a liquid normally forms flattened droplets (therefore low contact angle) on epicuticular waxes rich in alcohols (Holloway 1969).

Spray retention can also be influenced by the topography of the leaf surface. For example, the shape and size of underlying epidermal cells, and presence or absence of veins contribute to differences in retention. Similarly the presence and pattern of trichomes can either aid wetting by capillary action (an 'open' pattern), or produce a highly water repellant surface typical of the Chrysanthemum genus (Holloway 1970). Surface topography is also influenced by the chrystalline or semi chrystalline nature of superficial wax deposits. In general flat deposits such as layers and crusts retain more spray solution than chrystalline deposits (Hall 1966). Variations in leaf surface characteristics will usually explain differences in spray retention between morphologically similar plant species.

Differences in leaf surface wettability can be further exploited by altering the physical characteristics of the spray droplet. Firstly, spray volume can be adjusted to utilize any

differences in the run-off point for crop and weed species. For example, maximum difference in retention between mustard, peas and barley occurred in the range 260 to 500 $l.ha^{-1}$ (Blackman, Bruce and Holly 1958). With each liquid:leaf surface interaction there is also a critical droplet diameter above which retention declines. This phenomena has been exploited for the control of common knotgrass (Polygonum aviculare) in broad beans by paraquat. In this case the critical diameter on the weed species was 250 μm compared to 400-500 μm on the crop (Douglas 1968). Recent experimentation by Taylor and Shaw(1982) has indicated that increased droplet speeds may provide an additional basis for selective herbicide action through differential retention.

Herbicide selectivity can also be enhanced by the addition of surface active agents to the spray solution. In general lowering of the surface tension reduces the volume retained by easily wetted leaf surfaces, and increases retention by more hydrophobic surfaces (Holly 1976). For example the hydrophobic qualities of Fat hen (Chenopodium album) leaves can be overcome by the addition of actipron at concentrations above 5 $ml.l^{-1}$ (Taylor, Davies and Cobb 1981).

Clearly, the differing wettability of leaf surfaces can make a major contribution towards herbicide selectivity. Spray retention is thus governed by a number of complex physico-chemical factors which can be manipulated to enhance activity against a target species. In general, a spray solution is formulated to achieve maximum retention on the target leaf surface in order to encourage herbicide uptake.

1.2.2 Uptake

Once retained by the leaf surface, a herbicide must penetrate through the cuticle in order to be transported to the site of action. This may be by direct absorption through lipoidal or aqueous components of the cuticle membrane (Sargent 1976), or by penetration through the stomatal pore. The role of stomata in herbicide uptake is somewhat controversial since penetration

of the pore can only occur if the droplets have a very low contact angle and a surface tension below 30 dyn.cm^{-1} (Schonherr and Bukovac 1972). Few commonly used surfactants can reduce the surface tension below this figure, and therefore the contribution of stomata to herbicide uptake is more likely to reflect preferential uptake through the thinner guard cell cuticle, than through the stomatal pore itself. Variations in cuticle thickness may influence uptake in other regions of the leaf surface for example the cuticle is frequently thinner over the main veins, and may possibly be penetrated by numerous ectodesmata (Fletcher and Kirkwood 1982). Thus the degree of herbicide uptake is directly affected by cuticle thickness, the amount and composition of embedded waxes; the extension of microfibrils into the cuticle; and the degree of hydration (Sargent 1976); and indirectly by the rate of uptake, translocation and metabolism within underlying tissues (Fletcher and Kirkwood 1982).

Spray solutions are generally formulated to achieve maximum retention on the target leaf surface in order to encourage increased herbicide uptake. However, the contribution of uptake to selectivity is variable and must be evaluated for each herbicide:plant interaction. As an example of this variation, two aspects of triazine selectivity will be considered. Firstly, cyanazine uptake was found to be 18x greater in susceptible Fall panicum (Panicum dichomotomiflorum) than in more tolerant corn (Zea mays) and largely contribute to the differential response of the 2 species (Kern, Meggitt and Penner 1975). In comparison, Jensen, Bandeen and Souza Machado (1977) found similar atrazine uptake by two contrasting biotypes of fathen (Chenopodium album). Selectivity in this case was related to differences at the biochemical site of action of the herbicide.

Studies into the uptake of 2,4-D by 6 contrasting species has indicated that whereas uptake was related to differences in wax quantity and composition, it could not be correlated to known field reactions to 2,4-D (Sargent and Blackman 1972). Indeed, corn (Zea mays) had the highest rate of penetration and was classified as the most resistant species.

For every example of differential uptake aiding herbicide selectivity (e.g. Davies, Drennan, Fryer and Holly 1968a; Pereira, Splittstoesser and Hopen 1971), there is another for which uptake plays little or no part (e.g. Fedtke and Smidt 1977; McIntosh, Robertson and Kirkwood 1981; Pallett 1983). Clearly the contribution of uptake to selectivity depends not only on the class and mode of action of the herbicide, but also on it's mobility once penetrated into the underlying tissues. As with retention, herbicide uptake is also highly dependant upon the physicochemical properties of the leaf surface. This parameter, and variations due to growth stage and environment, invalidate general assumptions on the relationship of uptake to selectivity.

1.2.3 Movement

The phytotoxic action of a herbicide is dependant upon its mobility subsequent to cuticle penetration and apoplast uptake. If it is confined to the apoplast, movement will occur in the direction of the transpiration stream towards the leaf tip. Such compounds tend to be root applied for maximum phytotoxic effect. In addition, there may also be penetration into the symplast resulting in either short or long-distance transport along the same routes as photosynthates. The speed, and type of mobility can contribute to selectivity by determining whether or not the herbicide reaches its major site of action.

The selective toxicity of dicamba for example, has been shown to be partly due to limited apoplastic movement in resistant wheat plants compared to symplastic mobility in Wild Buckwheat (Quimby and Nalewaja 1971). More frequently, selectivity is due to slower translocation or immobility of a herbicide in a resistant species. For example, Schafer and Chilcote (1970b) found that bromoxynil was more mobile in susceptible Coast Fiddleneck (Amsinckia intermedia) than resistant wheat (Triticum aestivum L., Var. Nuggines). Similarly, Robertson and Kirkwood (1970) described how MCPA and MCPB remained in the treated leaves of resistant Black Bindweed (Polygonum convolvulus) whereas both herbicides were extensively translocated by susceptible Fathen

(Chenopodium album). Many other examples of differential translocation have been reported in relation to selectivity of herbicides (Sargent 1976; Jensen 1982; Fletcher and Kirkwood 1982).

Translocation of some herbicides may be indirectly inhibited by their effect on the energy requiring steps known to be associated with phloem loading and long distance transport (Kirkwood 1976). Similarly inhibition of photosynthesis will limit photosynthate production, and consequently reduce the level of assimilate translocation. The so-called 'contact' herbicides are considered to inhibit their own translocation by rapid destruction of cell and organelle membranes with consequent disruption of all metabolic processes. Movement in this case is likely to be limited to diffusion and leakage from successively injured cells (Foy 1964). Furthermore, there may be fixation at metabolically non-active sites due to adsorption or binding to proteins or other macromolecules within leaf tissues (Fletcher and Kirkwood 1982). Selective conjugation will be discussed in Section 1.2.4.

The so-called translocated herbicides also accumulate in the treated leaf but it is the relatively low proportion which is translocated that causes phytotoxicity. This is particularly apparent with meristematically active compounds e.g. Difenzoquat, which is known to inhibit DNA synthesis. This herbicide was found to have greatest accumulation in the apex of the least tolerant winter wheat cultivar tested (Pallett 1983). Frequently the distinction between a contact and a translocated herbicide is ill-defined since there may be some translocation of contact herbicides which can contribute to their phytotoxic activity e.g. the hydroxybenzonitriles (Carpenter, Cottrell, De Silva, Heywood, Leeds, Rivett and Soundy 1964) and Nitrofen (Hawton and Stobbe 1971a & b).

Attempts have been made to identify the structural and physiological requirements for herbicide or pesticide transport. Christ (1978) suggested five interrelated physiological and physiochemical requirements for xenobiotic transport, a reduction in each could either prevent or reduce translocation. Four of

these pre-requisites have already been discussed (i.e. availability at source, penetration into plant tissues, transport to vascular tissue and non-interference with energy requiring processes), and there is no direct correlation between the fifth factor, water solubility and phloem transportability. Certain structural requirements for insecticide transport have been suggested by Crisp (1972) which may be relevant to herbicide movement. These include a heterocyclic ring with a hydrophilic/lipophilic balance, and a carboxyl group or functional groups which can form conjugates with sugars and amino acids. In more recent investigations with a plant growth regulator, the presence of a carboxyl group appeared to be the most important feature (Crisp and Look 1978). A number of translocated herbicides possess these structural characteristics e.g. the phenoxyalkanoics (Fletcher and Kirkwood 1982).

The translocation of a potential herbicide to its site of action is clearly related to the selectivity of the herbicide. However, examples exist where differential translocation plays only a minor role in selectivity (e.g. Pallett and Caseley 1980). Differing capacities of species to metabolize a herbicide can outweigh the effect of differential movement of the compound and may thus be a more important factor affecting selectivity.

1.2.4 Metabolism

As an abnormal molecule within plant tissues the mobility of a herbicide may be reduced by adsorption; reaction with certain plant constituents; or be subjected to enzymatic attack. Thus, as already discussed, a herbicide may not reach its site of action, or it may undergo changes leading to a non-toxic product. Conversely, the breakdown products might well exert toxic effects which are not exhibited by the original herbicide. A range of degradation mechanisms may therefore modify the herbicide molecule and lead to differences in phytotoxicity between one species and another.

Selectivity can frequently be related to differences in the rate of conjugation with endogenous metabolites. This

usually involves linkage of herbicides or their metabolites with sugars, amino acids or less commonly protein or lignin. For example, Mine, Miyakado and Matsunaka (1975) investigated the difference in tolerance between bentazon susceptible Cyperus serotinus and resistant rice. Recovery from photosynthetic inhibition in tolerant rice was related to a faster rate of bentazon conjugation with glucose. This conjugated product was not found in three other susceptible species. Similarly, metribuzin selectivity has also been related to glucose conjugation (Smith and Wilkinson 1974; Stephenson, Mcleod and Phatak 1976) and glutathione conjugation is important in the resistance of maize (Zea mays) to atrazine (Shimabukuro, Frear, Swanson and Walsh 1971).

In addition to deactivation by adsorption, a herbicide may be selectively detoxified by enzymatic reactions within the plant. Numerous types of reactions have been demonstrated including oxidation, decarboxylation, hydroxylation, hydrolysis, dealkylation, dehalogenation and ring cleavage (Ashton and Crafts 1981). Detoxification of the triazine herbicides appears to involve at least three different mechanisms (Wain and Smith 1976). For example, replacement of the substituent at carbon atom 2 by a hydroxyl group leads to complete detoxification of the triazine molecule. In addition, glutathione conjugation, and to a lesser extent N-dealkylation and non-enzymatic hydroxylation are responsible for the resistance of maize to atrazine (Shimabukuro, Frear, Swanson and Walsh 1971).

The selectivity of diclofop-methyl between wheat (Triticum aestivum) and wild oat (Avena fatua) has been postulated to involve different mechanisms in the two species. In wild oat, glycosyl ester conjugation was the main reaction, resulting in an inactive compound which may serve as a pool for diclofop, an active form of the herbicide (Donald and Shimabukuro 1980). The ability of wheat to irreversibly detoxify diclofop-methyl by aryl hydroxylation may be the primary selective factor between the two species (Shimabukuro, Walsh and Hoerauf 1979).

Side chain degradation, ring hydroxylation and conjugation have all been implicated in the metabolism of the phenoxyalkanoic acid herbicides (Pillmoor and Gaunt 1981). Differences in the rate of metabolic breakdown have been related to selectivity (Luckwill and Lloyd-Jones 1960; Montgomery, Chang and Freed 1971; Sanad and Muller 1972), although in other cases differential metabolism alone cannot fully explain tolerance inequalities (Fletcher and Steen 1971).

As indicated, detoxification processes generally involve a gradual cleavage of the ring substituents until the aromatic ring exists as a nontoxic compound. Differences in the rate and mechanism of these processes is a frequently reported basis for the selective action of herbicides, particularly when other explanations cannot be identified. Examples exist for all of the major classes of herbicide in addition to those discussed above (Wain and Smith, 1976; Ashton and Crafts 1981; Fletcher and Kirkwood 1982). In some instances, radiochemical analysis has indicated slow cleavage of the aromatic ring itself, although evidence for this is ambiguous (Frear and Shimabukuro 1970; Ashton and Crafts 1981), and its role in selective detoxification is likely to be minor.

The converse of selective detoxification, namely selective activation or 'lethal synthesis' was initially discovered by Synerholm and Zimmerman in 1947. Using bioassays and a homologous series of 2,4-dichlorophenoxy-alkanoic acids with side chains of various lengths, they demonstrated that only those with an even number of carbon atoms were degraded by β-oxidation to 2,4-D. In contrast, those with an odd number of carbon atoms were initially degraded to an unstable intermediate and finally converted to in active 2,4-dichlorophenol and CO_2. Further investigations by Wain and Coworkers revealed that the β-oxidase enzyme system was not universally present, for example annual nettle (Urtrica urens), Canada thistle (Cirsium arvense) and

charlock (Sinapis arvensis) could metabolize even number chain compounds to active 2,4-D, whilst only the acetic derivative was active against clover (Trifolium Pratense) or celery (Apium graveolens). This discovery led to the development of the selective herbicides MCPA and MCPB which are only 'activated' in susceptible weed species (Wain and Smith 1976).

A further example of lethal synthesis arises when the lethal moiety is a free radical. This occurs in the herbicidal activity of the bipyridilium herbicides which require light for their action. The bipyridilium ion is reduced by electrons from photosystem I and then subsequently forms a free radical which on autooxidation leads to the formation of hydrogen peroxide, superoxide radicals, hydroxyl radicals, and singlet oxygen, all of which are potentially phytotoxic. Selectivity of these contact herbicides is generally more likely to relate to differential retention rather than the mechanism of activation, although a paraquat resistant strain of Lolium perenne "Causeway" has been developed at the Northern Ireland plant breeding station. In the latter, tolerance is thought to relate to destruction of singlet oxygen by elevated concentrations of superoxide dismutase, and detoxification of the resulting hydrogen peroxide by increased levels of catalase and peroxidase, (Harper and Harvey, 1978; Faulkner, Lambe and Harvey 1980).

Selectivity of the herbicide Benzoylprop-ethyl depends on differential activation by hydrolysis and inactivation by conjugation (Jeffcoat and Harries 1973). The accumulation of the free acid (benzoylprop) in tolerant wheat (Triticum aestivum) appeared to be prevented by a high rate of conjugate formation, whereas the rate of free acid synthesis was more rapid in susceptible wild oats (Avena fatua). A number of similar herbicides are commercially available which also require conversion to the free acid for phytotoxic effect, e.g. flamprop-isopropyl (Jeffcoat and Harries 1975), chlorfenprop-methyl (Fedtke and Smidt 1977) and diclofop-methyl (Shimabukuro, Walsh and Hoerauf 1979).

Clearly the molecular structure of a herbicide is subject to enzymatic and non-enzymatic changes once penetrated into the plant. Most frequently this leads to an irreversible inactivation of the herbicide, although conjugation with endogenous metabolites may be reversible with subsequent release of the free herbicide. When subject to the same enzymatic processes some herbicides are degraded to a more phytotoxic compound. A combination of these processes are likely to occur to a greater or lesser extent with each plant:herbicide interaction, contributing to the intra- and inter-specific differences in tolerance noted in the field.

In the preceding discussion examples have been cited for which differences in retention, uptake, translocation or metabolism have been implicated as the primary reason for selectivity. In most cases, however, it is an interaction of all of these factors which determines the levels of herbicide reaching the site of action, and thus phytotoxicity. Recent investigations into triazine resistance have also revealed differences in the macromolecular structure of the electron transport chain which prevents herbicide binding to the site of action for these herbicides (Pfister, Radosevich and Arntzen 1979). Intra- and inter-specific selectivity is thus dependant on a number of physiological, morphological and physico-chemical factors.

1.3 PHOTOSYNTHETIC INHIBITOR HERBICIDES

Following uptake and movement to the site of action, the activity of a herbicide is also dependant on its mobility within the target cell. Presence in the cytoplasm may lead to interference with protein synthesis and lipid metabolism, whilst penetration of the mitochondrial membrane could lead to interference with respiratory electron transport and oxidative phosphorylation. However, greater than 50% of all herbicides are believed to enter the chloroplast and interact with the process of photosynthesis. This may be by a direct inhibition of electron transfer; uncoupling of photophosphorylation

thereby reducing the energy available for C reduction; or indirectly by interfering with pigment biosynthesis. Photosynthetic inhibition leads to concomitant changes in chloroplast ultrastructure and function, secondary effects on cell metabolism and ultimately to physiological changes in the whole plant.

1.3.1 The Photosynthetic Apparatus

The entire photosynthetic process takes place within disc-shaped organelles named chloroplasts. These vary in size from 3-10μm in length, and 1.5-3μm thick and are usually found around the outer perimeter of photosynthetic cells. There are considered to be three main structural regions of the chloroplast, i.e. a double membraned outer envelope, an amorphous stroma, and a highly organised internal lamellar structure, the thylakoid membranes (Barber 1976). In general, the light dependant reactions of photosynthesis are associated with the thylakoids whereas the dark reactions of CO_2 assimilation occur in the stroma.

In higher plant chloroplasts, the thylakoids are complex folded membrane systems consisting of stacked (granal) and unstacked (stromal) lamellae (Coombs and Greenwood, 1976), more recently classified as appressed and non-appressed membranes (Anderson 1982a; Barber 1983). The latter classification differentiates between regions where close membrane:membrane interaction occurs and those regions where the outer surface of the membranes are exposed to the stroma (end granal and stromal lamellae, Fig. 1.1). Evidence from electron microscopy (Gunning and Steer 1975), biochemical studies with thylakoid preparations (Barber and Chow 1979; Murphy and Woodrow 1983; Ramanujam and Bose 1983) and *in vivo* observations of thylakoid stacking (Lichtenthaler, Buschmann, Doell, Bach, Kozel, Meier and Ranmsdorf 1981) has revealed that thylakoid membrane organisation is not firmly fixed. Furthermore it is now possible to fractionate and separate appressed and non-appressed regions of the same continuous thylakoid membrane. Such studies have demonstrated considerable lateral asymmetries in protein distribution (Andersson and Anderson 1980; Anderson and Andersson 1982; Andersson and Hachnel 1982; Murphy and Woodrow 1983).

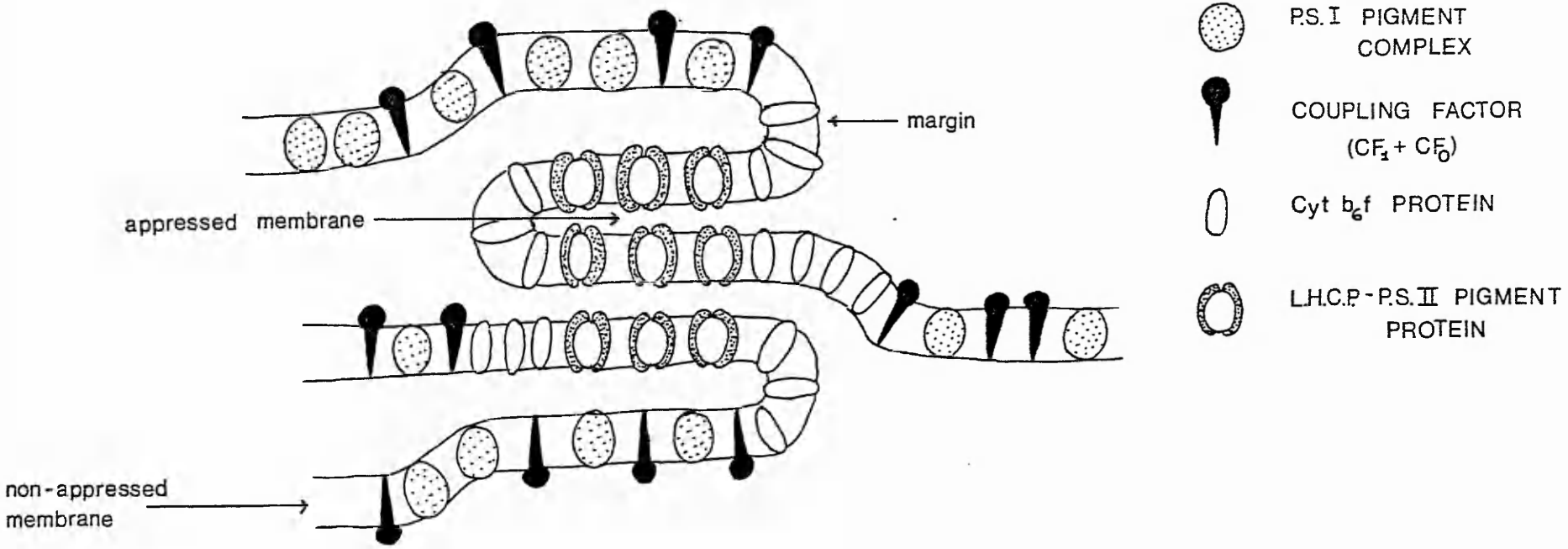


Figure 1.1: A model of the thylakoid membrane of higher plants suggesting the positioning of various intrinsic proteins in appressed and non-appressed membranes (after Barber 1983).

Approximately 50% of the thylakoid membrane is composed of acyl lipids which act as a support matrix and a fluid medium through which diffusion processes can occur (Barber 1983). Associated with this structural matrix are 5 multipeptide complexes which are concerned with light harvesting or NADP+ reduction and ATP synthesis (after Barber 1983):-

(i) Photosystem I complex (PSI): comprises P700, the antennae molecules of PSI, β-carotene, subunit III and Fe-S centres. This complex contains about 30% of the total chlorophyll, mainly chlorophyll a.

(ii) Photosystem 2 complex (PSII): comprising P680, antennae chlorophyll a molecules of PS2, β-carotene and oxygen evolving components including Manganese. This complex contains 10-15% of the total chlorophyll as chlorophyll a.

(iii) Light-harvesting chlorophyll a/b protein complex (LHCP-2): Closely associated with PSII containing 40-60% of the total chlorophyll with a chlorophyll a:b ratio of 1.5. The complex contains two major polypeptides, low β-carotene, and high xanthophyll content. Four separate LHCP-2 complexes are thought to aggregate with a single PS2 complex.

(iv) Cytochrome b_6-f complex: includes 2 molecules of cytochrome b_6 and one of cytochrome f complex.

(v) ATP synthetase (CF_0) complex: consists of a membrane scanning integral sector (CF_0) comprising 3 main polypeptides, and an externally located CF_1 factor comprising 5 polypeptides.

The molecular organisation of these intrinsic complexes is still under debate. Current theories (Anderson 1982a; Barber 1983; Murphy and Woodrow 1983) suggest that PS2 and LHCP-2 span the membrane and are confined to appressed regions of the grana partition, whilst the PSI and CF_0-CF_1 complexes are located in non-appressed membranes (Fig. 1.1). The cytochrome b_6-f complex is present in similar amounts, on a chlorophyll basis, in both membrane regions (Cox and Andersson 1981; Anderson 1982a + b).

There is every reason to believe that these complexes are in continuous lateral motion, especially in non-appressed membranes which are more fluid than appressed membranes (Ford, Chapman, Barber, Pendersen and Cox 1982). The thylakoid membrane is thus a fluid mosaic system in which fast and slow diffusional processes occur in order to facilitate and optimise electron transport processes.

The thylakoid membrane lamellae are retained in a hydrophilic proteinaceous continuum named the stroma. In C_3 plants the major stromal component is fraction-one protein of which more than 50% is associated with ribulose-bis-phosphate carboxylase (Coombs and Greenwood 1976). Embedded in the stroma are a number of discrete particles, the most common of which are starch grains (stored photosynthate) and plastoglobuli (stored lipids, Lichtenthaler 1968). Ribosomes are also frequently abundant and analytical studies have revealed the presence of RNA and DNA. Contained within the stroma are the enzyme systems required for carbon fixation, starch synthesis, fatty acid and protein synthesis, and for the incorporation of inorganic nitrogen, sulphur and phosphorous into organic compounds (McLilley 1983).

1.3.2 Photosynthetic Electron Transport

The reducing power required for C-reduction is generated by the light reaction of photosynthesis. Light energy is absorbed by light harvesting pigments and is transferred to the reaction centres of the two photosystems (PSI and PSII), where it is converted into chemical energy. The two photosystems co-operate via a series of oxidation-reduction reactions in linear electron transfer from $H_2O \longrightarrow NADP+$. The "Z-scheme" proposed by Hill and Bendall (1960) aligns the electron carriers according to oxidation/reduction potential (Figure 1.2). This scheme is widely accepted although more recent studies have shown that the fluid mosaic model of Singer and Nicolson (1972) is applicable to thylakoid membrane structure (e.g. Armond, Staehelin and Arntzen 1977; Andersson and Anderson 1980). The spatial arrangement of the electron carriers in relation to the function of the electron transport chain is thus the subject of current research (Haehnel 1984).

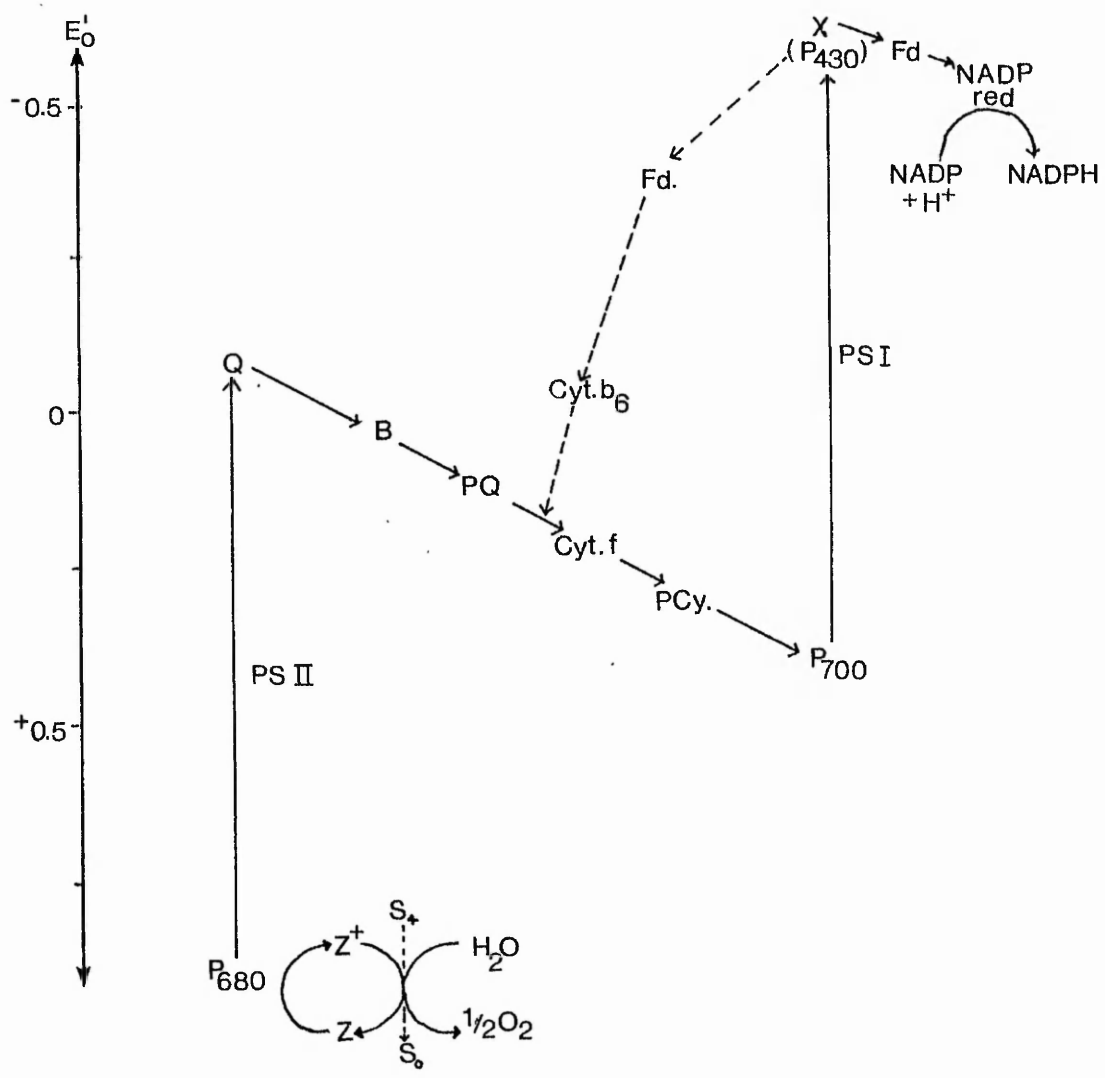


Figure 1.2: The photosynthetic electron transport system or "Z-scheme" roughly ordered by the standard redox potentials (E_o^1) of the individual redox carriers. The abbreviations used:- S (oxidoreducible water splitting enzyme), Z (primary electron donor to PSII), P680 (reaction centre chlorophyll of PSII), PSII (Photosystem II), Q (primary acceptor for PSII), B (secondary acceptor for PSII), PQ (plastoquinone), Cyt.f (cytochrome f), PCy (plastocyanin), P_{700} (reaction centre chlorophyll of PSI), PSI (photosystem I), X (electron acceptor for PSI), Fd (ferredoxin), NADP red (NADP reductase), cyt.b_6 (cytochrome b_6). Solid lines represent non-cyclic electron flow.

Electron transport is initiated by the absorption of light by the light harvesting pigment antennae which raise the reaction centre pigments to an excited singlet state (^{1}chl *). In this state, the chlorophyll is highly reactive and is a sufficiently powerful reducing agent to pass on an electron to a redox system (X or Q), and eventually to NADP+ (Fig. 1.3). Having donated an electron, the chlorophyll becomes a cationic free radical (chl+.) which is reduced back to ground state on receipt of an electron derived ultimately from water or plastocyanin.

The components of the electron transport system from water to NADP+ are organised into three supramolecular membrane-scanning complexes (PSI, PSII and cyt b_6-f, see section 1.3.1) which interact indirectly via small electron carriers. Plastoquinone links the PSII complex with the cyt b_6-f complex, and photocyanin links the cyt b_6-f complex with the PSI complex. Ferredoxin mediates electron transfer from PSI to the membrane bound ferredoxin-NADP+ reductase and possibly to the cyt b_6-f complex in cyclic electron transport (Haehnel 1984). Because of the known spatial arrangement of the major complexes, electron transport between PSII in the appressed membranes and PSI in the non-appressed thylakoids must involve mobile electron carriers (Velthuys 1980; Anderson 1981). Anderson (1982a) postulated that the additional presence of cyt b_6-f complex in the appressed membranes overcomes the problem of apparently slow plastoquinone diffusion within the lipid matrix. Indeed Millner and Barber (1984) have recently shown that there is sufficient time for plastoquinone to diffuse across the granal membrane within the half-life of electron donation to the cyt b_6-f complex. Electrons are believed to be transported from the cyt b_6-f complex of the appressed membrane to the PSI-complex of the granal margins and non-appressed membranes by plastocyanin. This carrier may either move rapidly whilst still attached to the membrane at the inner thylakoid surface, or may become detached from the membrane and diffuse into the intra-thylakoid space (Anderson 1982b). A scheme showing how the various components may interact to bring about electron transfer from H_2O to NADP+ is shown in figure 1.4.

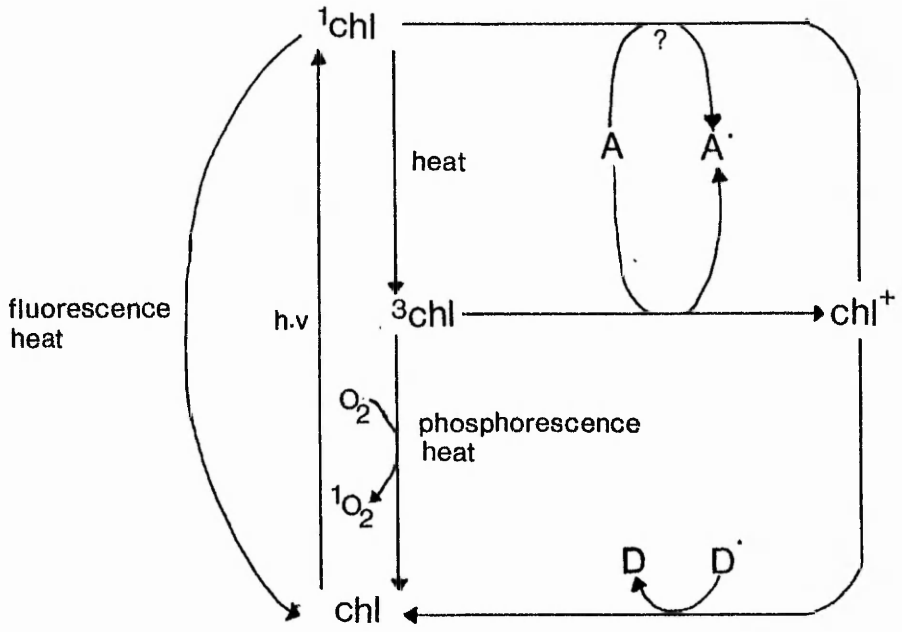


Figure 1.3: Excitation of chlorophyll by absorption of photons (h.v.) and subsequent possible reactions and transfers. Abbreviations used:- chl (ground state chlorophyll), ^{1}chl (singlet-state chlorophyll), ^{3}chl (triplet-state chlorophyll), chl$^+$ (excited chlorophyll), A (primary acceptor), A· (reduced form of A), D· (primary donor), D (oxidised form of donor) (Fedtke 1982).

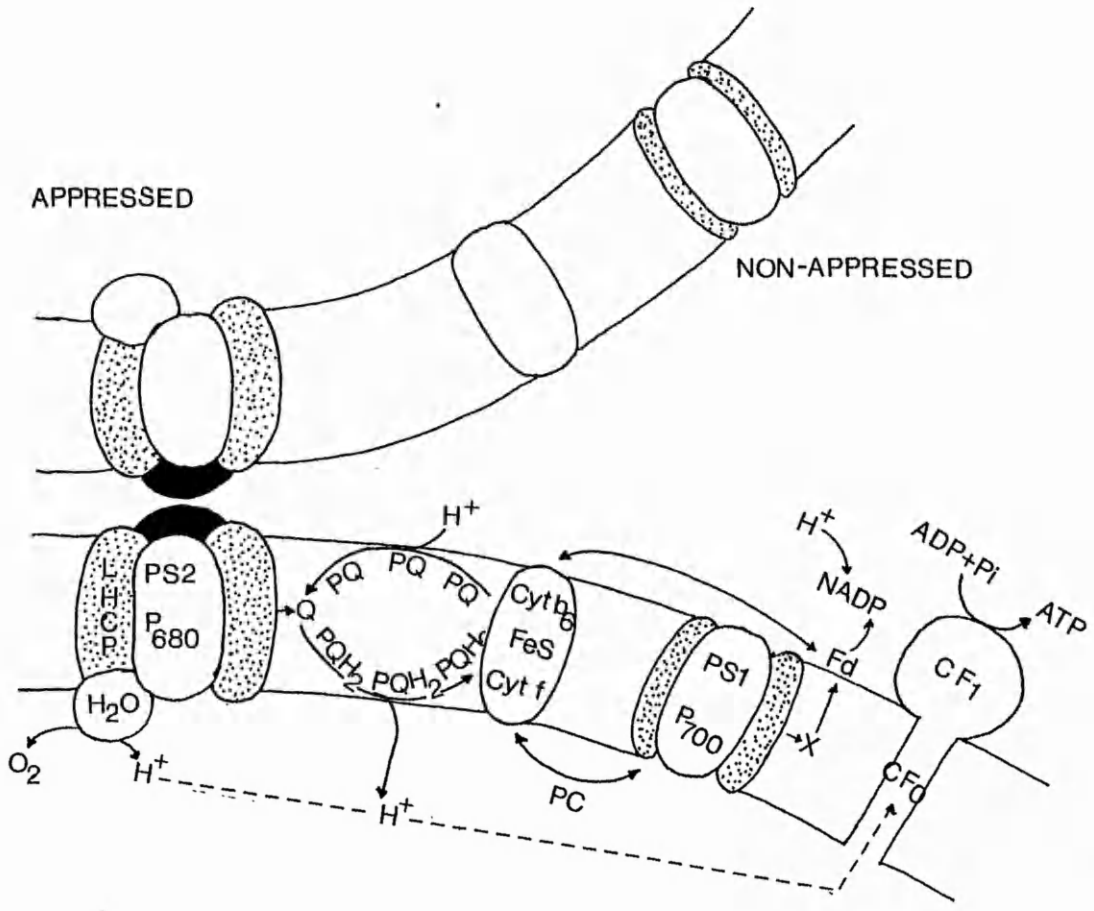


Figure 1.4: A scheme to show how the intrinsic and extrinsic thylakoid membrane components may interact together to bring about the transfer of electrons from H_2O to NADP. PQH_2 (plastoquinol), PC (plastocyanin) and Fd (ferredoxin) act as long-range diffusional electron transferring components. Other symbols: LHCP (light-harvesting chlorophyll a/b protein), PS2 (photosystem 2), PSI (photosystem 1), FeS (Rieske iron-sulphur protein), Cyt b_6 & Cyt f (cytochrome b_6-f complex), P_{680} (PS2 reaction centre), P_{700} (PSI reaction centre), X (primary PSI acceptor), Q (primary PS2 acceptor), CFo & CF (coupling factor complex). After Barber 1983).

Non-cyclic electron flow to NADP+ is coupled stoichiometrically to ATP formation. Localization of the coupling sites has proved difficult, although it is generally accepted that one coupling site exists between plastoquinone and the cytochrome b_6-f complex, and an additional site is associated with the oxygen evolving side of PSII (Trebst 1976). The mechanism by which photosynthetic electron transfer is coupled to photophosphorylation remains speculative. The most widely accepted theory is based upon the chemiosmotic hypothesis of Mitchell (1961). This suggests that electron transfer causes a vectoral movement of protons across the thylakoid membrane, setting up an electrochemical gradient or a "protonmotive force". The shuttle of two protons for every two electrons carried by the plastoquinone/plastoquinol redox system results in a pH decrease from about 7.5 to 4.0 across the thylakoid membrane (Fedtke 1982). The synthesis of ATP is catalysed by ATPase, a component of the CF_0-CF_1 complex. During the function of ATPase, protons are diffused back through the membrane following the prevailing proton gradient i.e. the pH gradient supplies the energy needed for the synthesis of ATP (Fedtke 1982). The exact mechanism by which protons are transported across the membrane remains unresolved, although several hypotheses have been suggested (e.g. Mitchell 1975; Velthuys 1979; Hammes 1983).

The coupling site associated with photolytic water oxidation was recognised when a plastoquinone antagonist, dibromothymoquinone (DBMIB) was introduced to block electron transfer from PSII to PSI. Electron flow through PSII to an artificial electron acceptor proceeded at very high rates and was coupled to ATP formation (Trebst 1974). The mechanism by which oxygen is evolved, and protons released is not fully understood. Current knowledge stems from the observations of Kok, Forbush and McGloin (1970) who showed that in short saturating flashes, the oxygen yields oscillate with a periodicity of four. They suggested that the water splitting enzyme system undergoes four oxidations (S_1-S_4) and only when the enzyme reaches S_4 does it

oxidize water causing oxygen evolution, and return to S_0. An additional intermediate "Z" is believed to be associated with the water splitting system. "Z" and "S" are likely to be metalloproteins, and their activity is dependant upon manganese which is considered to be loosely bound to the thylakoid membrane (Velthuys 1980).

The preceding discussion has concentrated on non-cyclic electron transport, however cyclic photophosphorylation can be induced in isolated chloroplasts by artificial compounds such as phenazine methosulphate (PMS). Cyclic photophosphorylation only involved PSI; electrons are believed to be channelled via ferredoxin to plastoquinone and ultimately back to PSI (see Fig. 1.2). Although the rate under normal conditions of CO_2 fixation appears to be very low (Halliwell 1978), the stimulatory effect of ferredoxin may indicate a possible physiological significance of cyclic electron flow (Trebst 1976). Arnon, Tsujimoto and Tang (1981) have recently suggested that cyclic electron flow around PSI may occur in the non-appressed thylakoids, independantly of PSII action. Cyclic photophosphorylation may possibly produce ATP for use in other synthetic processes within the chloroplast.

The photochemical reactions of chloroplasts can be studied by the addition of various artificial compounds to isolated chloroplast preparations. Ferricyanide (FeCN) and dichlorophenol indolphenol (DCPIP), when added to illuminated chloroplasts are reduced by PSII with the evolution of oxygen, in a reaction known universally as the Hill reaction. Similarly, compounds other than water can donate electrons to various components of the photosynthetic pathway and isolate certain sections of the transport chain. For example the ascorbate/DCPIP couple can be used to donate electrons prior to PSI, in combination with methyl viologen which acts as an electron acceptor at a position prior to ferredoxin. The addition of electron carriers such as pyocyanine or PMS results in an oxygen independant cyclic electron flow around PSI which is linked to photophosphorylation

(Izawa 1980). Thus the electron transport chain can be manipulated to study the partial reactions and the sites of action of photosynthetic inhibitor herbicides.

1.3.3 Inhibition of Chloroplast Photochemical Reactions

Herbicides that inhibit the photochemical reactions of chloroplasts have been routinely named Hill reaction inhibitors. For many years their action was evaluated under non-phosphorylating conditions, frequently with ferricyanide as the electron acceptor. More recent investigations of various parts of the electron transport pathway have revealed a greater knowledge of the differential action of Hill inhibitors. Consequently Moreland and Hilton (1976) separated herbicidal inhibitors of photochemical reactions into the following classes: (a) electron transport inhibitors, (b) uncouplers, (c) energy transfer inhibitors, (d) inhibitory uncouplers (Multiple types of inhibition), and (e) electron acceptors. The introduction of a new radiolabelled binding technique by Tischer and Strotmann (1977) has substantially improved our knowledge of electron transport inhibition, although the exact nature of the binding site(s) has not yet been fully elucidated. Current theories on the nature of the binding site will be discussed following a brief review of the classes of photosynthetic inhibitors.

(a) Electron Transport Inhibitors

Electron transport is inhibited when one or more of the intermediate electron transport carriers are removed or innactivated (Moreland 1980). The site of action of about half of the currently known herbicidal structures is closely associated with photosystem II (Fedtke 1982). Consequently reactions coupled to this photosystem are inhibited such as non-cyclic electron transport and pseudocyclic phosphorylation (Moreland 1980). Competitive binding studies have suggested that s-triazines, triazinones, pyridazinones, biscarbamate, ureas, uracils, acylanilides and thiadiazoles all interfere with a proteinaceous site between Q and PQ, known commonly as the DCMU site (Tischer and Strotmann 1977; Fedtke 1982).

The DCMU-type herbicides all have the structural elements N-C=X in common, where X signifies N or O (Trebst and Draber 1979). In addition, structurally different phenolic herbicides such as dinoseb, bromonitrothymol and ioxynil appear to bind to the same proteinaceous component but not at the same site (Reimer, Link and Trebst 1979; Oettmeier and Masson 1980). A further group of herbicides including dibromothymoquinone and trifluralin are plastoquinone antagonists and interfere with electron transport at the so-called DBMIB site (Izawa 1977; Robinson, Yocum, Ikuma and Hayashi 1977; Trebst, Reimer, Draber and Knops 1979). Interestingly trifluralin is believed to also inhibit the DCMU-site at high concentrations (Droppa, Horvath and Demeks (1981). In general, herbicides classified as electron transport inhibitors act in the concentration range 10^{-8} to 10^{-5}M, and are not strong inhibitors of oxidative phosphorylation. The additional activity of the dinitrophenols, dinitroanilines and benzonitriles against the latter process resulted in classification as inhibitory uncouplers (Moreland and Hilton 1976; Moreland 1980).

(b) Uncouplers

Uncoupling compounds dissociate electron transport from ATP formation thus inhibiting phosphorylation, but not electron transport. With the exception of perfluridone (Moreland 1980), these compounds are rarely specific to photophosphorylation and normally also uncouple oxidative phosphorylation in mitochondria. Wright, Baillie, Wright, Dowsett and Sharpe (1980) attempted to synthesise uncouplers of photophosphorylation from a series of aryloxyalkylamines. The most potent uncoupler, however was found to have a primary effect on carotenoid biosynthesis. The most important uncoupling herbicides also inhibit electron transport and are classified elsewhere.

As already discussed (section 1.3.2), the mechanism by which photophosphorylation is coupled to electron transport is most easily understood in terms of Mitchell's chemioosmotic theory. Oxidative phosphorylation in mitochondria is believed to be coupled by a similar mechanism (Elthon and Stewart 1983)

and it is generally accepted that uncoupling agents act in a similar manner in both organelles. Herbicidal uncoupling agents normally possess either a labile phenolic hydrogen or an acidic N-H group which can associate with H+ ions. These compounds dissolve into the lipid core of the membrane, diffuse across it and ionize to release H+ ions. Thus uncouplers act as protonophones and shuttle protons across the chloroplast or mitochondrial membrane equilibrating the H+ concentration on either side; and uncoupling subsequent ATP formation (Moreland and Novitzky 1984). A similar mechanism has been implicated for the uncoupling action of a substituted urea herbicide, D_5 (N-n-pentyl-N-methyl-N^1-(3,4-dichlorophenyl) urea). Gauvrit and Wilson (1983) established that whilst D_5 itself was not capable of transporting protons across the membrane, it did appear to catalyse the collapse of the H+ gradient by inducing a movement of protons across the mitochondrial membrane.

(c) Energy Transfer Inhibitors

Energy transfer inhibitors act directly on photophosphorylation and are considered to interfere with a more terminal step in phosphorylation than the one affected by uncouplers (Moreland 1980). Consequently they indirectly inhibit electron flow in otherwise coupled systems by removing the normal pathway for dissipating high energy intermediates. The 1,2,3-thiadiazoylphenyl ureas are the only herbicides currently associated with this mechanism of action in higher plants (Fletcher and Kirkwood 1982), although Lambert, Kunert and Boger (1979) have suggested that Nitrofen may be an energy transfer inhibitor in the chloroplasts of an algae Bumilleriopisis.

(d) Inhibitory Uncouplers

Compounds classified by Moreland and Hilton (1976) as inhibitory uncouplers have more than one site of action since they inhibit electron transport and uncouple oxidative and photophosphorylation. Herbicides which express this type of action include dinitroanilines, benzimidazoles, imidazoles, thiadiazoles and halogenated benzonitriles. The dinitrophenols are also classified in this group although their action is pH dependant

(Good and Izawa 1973). At pH 8.0 electron transport is inhibited whilst the uncoupling action is attributed to the protonated form present at pH 6.0. To a lesser extent, other inhibitory uncouplers may also exhibit pH dependant activity.

As previously shown, the mechanism of action of these compounds must involve an interaction with a proteinaceous component (the Q_B protein) and a lipoidal constituent of the thylakoid membrane. In <u>in vivo</u> studies, the uncoupling action of DNOC was the primary action of the herbicide whereas the converse was true for ioxynil (Van Oorschot 1974). Moreland (1980) considered it likely that more compounds classed as electron transport inhibitors may be classified as inhibitory uncouplers once assays for their activity were completed.

(e) <u>Electron Acceptors</u>

Compounds classified as electron acceptors are able to compete with some component of the electron transport pathway and undergo subsequent reduction (Moreland 1980). Certain bipyridiliums, e.g. diquat and paraquat can accept electrons in competition with X, the acceptor of PSI. This leads ultimately to the formation of highly phytotoxic superoxide ions. High herbicidal activity can only be achieved with bipyridiliums when the two pyridine rings assume a planar configuration. Other electron accepting compounds include te-triazines (Pilgram <u>et al</u> 1977), diquaternary salts of di-2-pyridyl ketone (Black and Summer 1970) and Metronidazole (Tetley and Bishop 1979).

1.3.4 <u>Interaction of Herbicides with the Q_B Protein</u>

Inhibition of electron transport by binding to a proteinaceous component between Q and PQ was initially confirmed by Renger (1976) following the development of a new technique for investigating the structural and functional organisation of photosystem II. Mild tryptic digestion of a thylakoid membrane preparation led to a decrease in Diuron sensitivity. It was postulated that the primary acceptor of PSII is covered by a surface exposed allosteric protein responsible for the regulation of electron transfer between Q and PQ to which herbicides can

bind. In subsequent years trypsinated chloroplast preparations were used to investigate the inhibitory action of many classes of photosynthetic herbicides and led to the establishment of two distinct groups of compounds which bind to the Q_B protein. Herbicides exhibiting the DCMU response, e.g. other ureas, triazines, triazinones and pyridazinones were classified as "DCMU-type" herbicides; and a second group for which the inhibitory action was either only partially reversed (Pallett and Dodge 1979) or even increased (Boger and Kunert 1979) depending on incubation conditions, were named "phenolic inhibitors" (e.g. Dinoseb and ioxynil).

The relationship between inhibitor binding by chloroplasts, and inhibition of photosynthetic electron transport was further elucidated by the introduction of competitive binding studies (Tischer and Strotmann 1977). Following the binding of a radiolabelled herbicide to the thylakoid membrane the likelihood of replacement by another unlabelled herbicide was investigated. By this replacement technique, Tischer and Strotmann confirmed that DCMU-type herbicides compete for the same binding site, and also established that there is one binding site per electron transport chain. Initial investigations with phenolic herbicides also suggested binding to the same site (Reimer, Link and Trebst 1979; Trebst 1979), however a detailed comparative study by Oettmeier and Masson (1980) revealed a non-competitive interaction between phenolic and urea herbicides. They concluded that the binding sites of both groups of inhibitors are not identical, although they are located on the same protein.

Identification of the molecular components involved in binding to the Q_B protein was originally restricted by problems involved in extracting the unstable Q_B:herbicide complex. In a theoretical study, Shipman (1981) concluded that the binding energy for metribuzin interaction was too small for covalent or hydrogen bonding to a protein. He suggested that polar components of the herbicide bind reversibly via weak coulombic interactions to either a protein salt bridge, or the terminus of an α-helix

on the Q_B protein. To overcome this problem, an azido-group was introduced onto a phenolic herbicide (Oettmeier, Masson and Johanningmeier 1980) and an s-triazine (Gardner 1980; Pfister, Steinback, Gardner and Arntzen 1981) which upon UV-irradiation with a thylakoid preparation binds covalently to the Q_B-protein. By introducing an additional radiolabel, ^{14}C-azido atrazine was found to bind to a 32-34 kDa protein (Pfister et al 1981) which could be removed by trypsin treatment (Steinback, McIntosh, Bogorad and Arntzen 1981). In contrast, Oettmeier, Masson and Johanningmeier (1982) established that phenolic herbicides bind to a 41 kDa protein which is probably part of the PSII reaction centre. These workers concluded that both the 32 and 41 kDa proteins must be located close together (fig. 1.5) and a conformational change in one due to herbicide binding must influence the other.

Further evidence for binding differences has been gained from investigations of triazine resistance in several weed biotypes. Thylakoids isolated from a resistant biotype were 60-3200x more tolerant of diuron, bromacil and s-triazine herbicides than those from a susceptible biotype of Common groundsel (Senecio vulgaris) (Radosevich, Steinback and Arntzen 1979). In addition, competition was absent between diuron and atrazine, and no specific atrazine binding was detected (Pfister, Radosevich and Arntzen 1979). However sensitivity to, and specific binding of, phenolic herbicides was enhanced in chloroplasts extracted from atrazine resistant redroot pigweed (Amaranthus retroflexis) (Oettmeier, Masson, Fedtke, Konze and Smidt 1982).

The molecular basis of this resistance has recently been identified (Hirschberg, Bleecker, Kyle, McIntosh and Arntzen 1984). These authors cloned the chloroplast gene that codes for the Q_B-protein in herbicide resistant and susceptible biotypes of Black Nightshade (Solanum nigrum). By DNA sequencing, a single base substitution was detected which results in an amino acid change (serine to glycine) in resistant plants. This mutation is exactly the same for herbicide resistant Amaranthus hybridus, and an atrazine resistant strain of Chlamydomonas

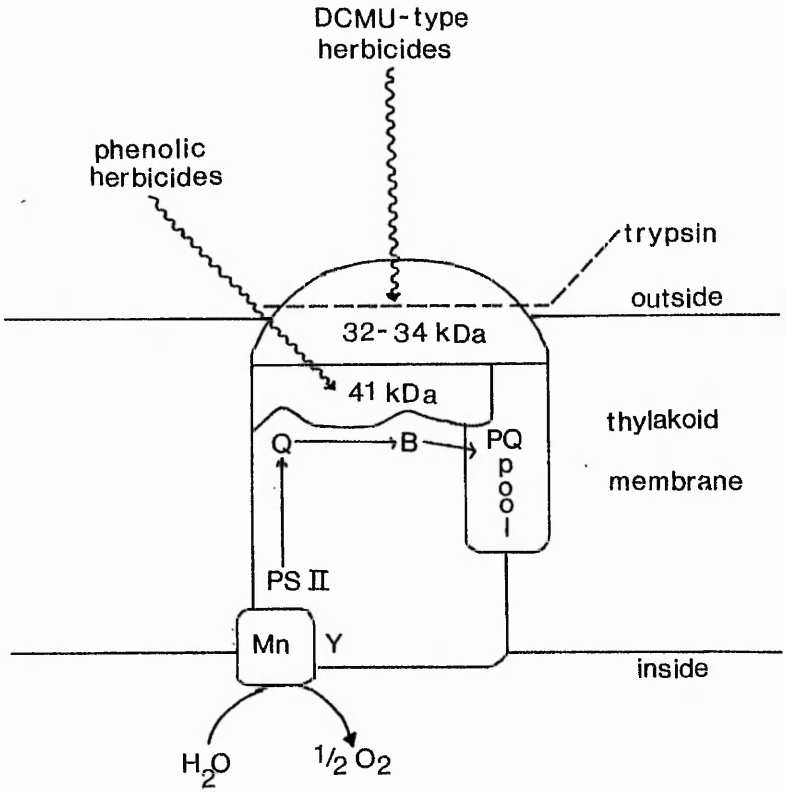


Figure 1.5: Model for the location of two herbicide-binding proteins (32-34 kDa, and 41 kDa), at the reducing side of PSII. Oettmeier, Masson & Johanningmeier (1982).

(Hirschberg and McIntosh 1983). Initial investigation of the site of covalent azido-atrazine attachment suggests binding to a site well removed from the mutation site which may lie close in situ, or may indirectly affect the tertiary structure of the Q_B-protein (Wolber and Steinback 1984).

Most of the current models on the functional mechanism of PSII herbicide action are based on competition studies between herbicides and synthetic quinone analogues (Velthuys 1981; Vermaas, Arntzen, Gu and Yu 1983; Oettmeier and Sol 1983; Vermaas, Renger and Arntzen 1984). Opinions are divided between firstly an inhibitor Q_B- competition model (Velthuys 1981; Urbach, Laasch and Schreiber 1984) based on competitive interactions between herbicides and Q_B- for binding to the 32 kDa protein; and secondly a multiple site model introduced by Vermaas, Renger and Arntzen 1984. The second model suggests the presence of multiple allosteric binding sites on the Q_B-protein such that binding at any one site causes a conformational change in the protein complex which limits the subsequent binding of unrelated inhibitors or the native plastoquinone. The binding interaction between structurally related herbicides is assumed to be truly competitive. This model is well supported by evidence from competitive binding studies and by the observation that ^{14}C-ioxynil and to a lesser extent ^{14}C-atrazine can still bind to the protein complex following the covalent binding of azido-quinone (Vermaas, Renger and Arntzen, 1984).

The current era in herbicide research can be considered to have started with the introduction of trypsin digestion by Renger in 1976. Since this time our knowledge of the interaction between a herbicide and the thylakoid membrane has increased exponentially. The most feasible model currently available is based on binding to a polypeptide complex consisting of at least a 32- and a 41 kDa protein. Allosteric changes in this protein complex may prevent electron transfer to plastoquinone, and thus inhibit non-cyclic electron transport. The primary structure of the 32 kDa protein has been established and further studies on

the entire herbicide binding complex are necessary before electron transport inhibition will be fully understood.

1.3.5 Toxicity after Photosystem II Inhibition

Early reports that Hill reaction inhibitors limited photosynthesis and caused a reduction in starch content led to the suggestion that herbicide treated plants were killed by "starvation". This theory was questioned when a lack of toxicity was found when treated plants were maintained in complete darkness. Various hypotheses were suggested including "light activation", the formation of free radicals by an interaction of the herbicide with light (Ashton 1963; Wain 1964), and injury by toxic components of the inhibited photosynthetic apparatus (Davis 1966). St anger and Appleby (1972) investigated pigment changes in DCMU treated chloroplasts and proposed that toxicity was due to an interaction between molecular oxygen and excited chlorophyll. Pigment changes were analysed following herbicide inhibition, and pigment destruction was found to occur in the order: β-carotene, chlorophyll a, xanthophylls and finally chlorophyll b. More recently, the early destruction of carotenoids has led to the suggestion that these pigments play an important photoprotective role in chloroplast function (Ridley 1977; Pallett and Dodge 1980a).

During uninhibited photosynthesis, oxygen is not only liberated by the water splitting enzyme system, but may also be partially reduced by the excited photosynthetic pigment system yielding highly reactive molecular species. This occurs if one electron in highly labile singlet state chlorophyll (^{1}chl) changes it's directional spin yielding triplet chlorophyll (^{3}chl). This reactive compound interacts with any molecular oxygen present in close proximity to yield singlet oxygen ($^1O_2$) and theoretically superoxide anion ($O_2.^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH.); all of which are potentially harmful (Fedtke 1982). In addition, "charge-transfer complexes" may form between excited chlorophyll and other molecules which may cause the singlet state to react in a similar manner.

Naturally occurring singlet oxygen is normally effectively quenched by β-carotene, and excess excitation energy may be omitted as fluorescence. Under conditions of electron transport inhibition, these protective mechanisms become overloaded and excessive amounts of singlet oxygen may eventually oxidize fatty acids, pigments and other membrane components (Fedtke 1982). In addition the excited chlorophyll molecules (chl* or chl+ after losing an electron) may directly initiate a chain reaction by abstracting an electron from unsaturated fatty acids, leading to lipid peroxidation. Furthermore, the intermediates of lipid peroxidation are also considered highly reactive (Feierabend and Winkelhusener 1982), contributing to general membrane disintegration. Thus inhibition of photosynthetic electron transport leads to a series of photooxidations which ultimately lead to chloroplast and cell destruction. Treated leaves exhibit chlorotic symptoms and plant death ensues.

It has also been suggested that toxicity may be linked to an inhibition of nitrite reduction leading to an accumulation of toxic nitrite in treated leaves (Klepper 1975, 1976). However, whereas DCMU and other photosynthetic inhibitors inhibit nitrite reduction *in vitro* (Neyra and Hageman 1974; Miflin 1974) an *in vivo* accumulation of nitrite in leaf tissue rarely occurs. Uncoupling agents do induce such an accumulation (e.g. ioxynil and dinoseb, Fedtke 1977), but this is more likely to be a direct result of uncoupling leading to higher levels of NADH and *in vivo* nitrate reductase activity, rather than an inhibition of nitrite reduction. Thus nitrite accumulation is unlikely to be a primary effect of photosynthetic inhibition, and phytotoxicity in plants is more likely to result from uncontrolled photooxidations.

1.3.6 Physiological changes associated with Photosynthetic Inhibition

All herbicides which inhibit electron transport *in vitro* also inhibit photosynthesis *in vivo* providing sufficient herbicide reaches the site of action. The primary effect (reduced ATP and NADPH formation) manifests itself immediately in decreased CO_2 uptake, especially under conditions of light limitation (Van

Oorschot 1974). The degree and speed of inhibition reflects the overall activity of a herbicide against a particular species. For example net photosynthesis was inhibited by 80% within 8 hours of atrazine treatment of a susceptible biotype of Redroot Pigweed (Amaranthus retroflexis \(West, Muzik and Witters 1976). In contrast, the photosynthetic rate of a resistant biotype was inhibited by 15% within one hour, and rapidly recovered to the control rate. Similarly recovery from metamitron inhibition in 8 different species varied between complete recovery within 4 hours (sugar beet) and no recovery within the 15 hour experimental period (Portulaca oleracea, Van Oorschot and Van Leeuwen 1979). Recovery may reflect the ability of resistant plants to metabolize the herbicide to non-inhibitory derivatives (Fletcher and Kirkwood 1982), or to adapt to ensuing low rates of photosynthesis (Fedtke 1973).

In susceptible species, overloading of the normal protective systems results in photo-oxidative injury of the chloroplast. Ultrastructural changes following atrazine treatment were reviewed by Anderson and Thomson (1973). A typical sequence is as follows: an initial swelling of intergranal thylakoids is followed by swelling of the whole thylakoid system and the entire chloroplast. Subsequently the tonoplast and chloroplast envelopes rupture resulting in total cellular disruption. These symptoms are also typical of other groups of photosynthetic inhibitor herbicides e.g. ureas (Pallett and Dodge 1980a + b), bromacil (Ashton, Gifford and Bisalputra 1963; Pallett 1978) and triazinones (Pallett and Dodge 1980b). In general, ultrastructural symptoms develop before chlorotic lesions are visible.

Sublethal applications of herbicides however, frequently result in 'adaptive' changes in the chloroplast of the type typically found when a "sun-plant" is moved to shade conditions (Fedtke 1982). Normally such chloroplasts exhibit broader and higher granal stacks which have a higher capacity for absorbing light (Lichtenthaler, Kuhn, Prenzel and Meyer 1982). Upto three LHCP-2 complexes may occur per electron transport chain resulting

in an increase in the overall chlorophyll content and a decrease in the a:b ratio (Lichtenthaler et al 1981, 1982; Meier and Lichtenthaler 1981). These 'adaptive' changes have been reported for sublethal concentrations of many groups of herbicides, for example methabenzthiazuron (Fedtke, Deichgraber and Schnept 1971), atrazine (Hiranpradit and Foy 1972), diuron (Boger and Schlue 1976) and bentazon (Lichtenthaler 1979). The granal stacking response has been observed within 3 days of treatment (Fedtke, pers. comm.).

The primary, secondary and tertiary effects of herbicides can be determined by monitoring the levels of primary metabolites within treated tissues. Inhibition of CO_2-fixation initially results in a decrease in the concentration of soluble reducing sugars (Fedtke 1972, 1974), followed by changes in the nitrogen metabolism of plants. There is frequently an increase in the soluble amino acid and soluble protein content, and a decrease in the total protein content suggesting autolysis of structural proteins (Pulver and Ries 1973; Fedtke 1972, 1973, 1974). An interaction may also exist between malate and nitrate levels e.g. Methabenzthiazuron stimulated nitrate formation and caused a decline in malate production in wheat tissue (Fedtke 1974). It has been suggested that a decrease in malate dehydrogenation limits the activity of nitrate reductase (Neyra and Hageman 1976).

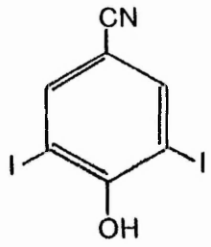
In conclusion, treatment of plants with photosynthetic inhibitor herbicides results in metabolic changes beyond those associated with photosynthesis. Rapid herbicide action is accompanied by wilting and necrosis resulting from photo-oxidative membrane destruction. In contrast, a resistant plant may exhibit a "greening effect" indicative of adaptive changes in chloroplast ultrastructure. Additional changes in metabolism follow photosynthetic inhibition.

1.4 THE HYDROXYBENZONITRILE HERBICIDES

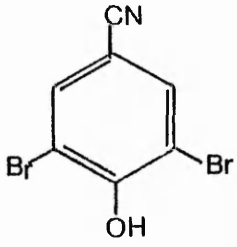
1.4.1 Introduction

The herbicidal activity of 3,5-diiodo- and 3,5-dibromo-4-hydroxybenzonitrile (ioxynil and bromoxynil respectively) was

discovered simultaneously in 1963 by Carpenter and Heywood of May and Baker Ltd., and R. L. Wain of Wye College (University of London). Extensive studies showed that both herbicides possessed



Ioxynil
3,5-diiodo-4-hydroxy-benzonitrile



Bromoxynil
3,5-dibromo-4-hydroxy-benzonitrile

post-emergent contact activity against a wide spectrum of broad-leaved weeds (Carpenter, Cottrell, DeSilva, Heywood, Leeds, Rivett and Soundy 1964). For example they are effective against young seedlings of members of the Polygonaceae (e.g. Polygonum convolvulus, P. aviculare and Fagopyrum tataricum); Compositae (e.g. Anthemis, Matricaria and Tripleurospermum spp.) and certain Boraginaceae (e.g. Amsinckia and Lithospermum spp., May and Baker Tech. Bulletin 1979).

Ioxynil is currently marketed by May and Baker Ltd., as the octanoate (Mate), and as a more commercially viable mixture with either bromoxynil (Oxytril c.m.), mecoprop (Brittox) or Isoproturon (Doublet and Twin-tak) which broaden the activity spectrum (May and Baker crop protection products 1983). The hydroxybenzonitriles are considered particularly useful for their activity against weeds which do not respond to the phenoxy herbicides (Ashton and Crafts 1981).

Ioxynil and bromoxynil are high melting point chrystalline solids which are virtually insoluble in water and sparingly soluble in most organic solvents. Aqueous solubility is enhanced

by preparation of the sodium salt of ioxynil (14% w/v) and potassium salt of bromoxynil (6.1% w/v), which can be applied to plants in an unformulated form. In contrast, the octanoate ester of each compound is virtually insoluble in water and needs to be formulated as a self-emulsifiable concentrate. Generally, the activity of the esters is slightly higher than that of the salts, although there is no difference in the weed spectrum.

In addition to selectivity between the graminaceous crop and dicotyledonous weeds, certain broad-leaved weeds exhibit a differential response to ioxynil and bromoxynil. For example, Stellaria media is moderately susceptible to ioxynil-Na, and resistant to bromoxynil-K whereas the reverse is true for Amsinckia intermedia (May and Baker technical bulletin 1979). Selectivity between crop and weed is largely based on differences in retention (Davies, Drennan, Fryer and Holly 1967; Schafer and Chilcote 1970a) plus small differences in uptake, translocation and metabolism, (Davies, Drennan, Fryer and Holly 1968a + b; Schafer and Chilcote 1970b). Somerville (1972) conducted an investigation of the differential activity of ioxynil and bromoxynil (salts and esters) against seven different species. He revealed that whereas differences in retention and penetration were important, other unidentified factors such as metabolism and fundamental enzyme system susceptibility were likely to contribute to the differential response. Thus the physiological basis for the differential action of the hydroxybenzonitrile herbicides remains unresolved.

Early experimentation established a dual mode of action for the hydroxybenzonitrile herbicides. Ioxynil and bromoxynil were found to inhibit the Hill reaction and uncouple photo- and oxidative-phosphorylation (Kerr and Wain 1964a, b). In more recent structure:activity investigations in vitro activity was found to depend on substitution, and decline in the order I > Br > Cl (Trebst, Reimer, Draber and Knops 1979). This differential activity may not be reflected in vivo since other factors

determine the amount of herbicide reaching the site of action (section 1.2).

1.4.2 Aims of the Investigation

A differential response between ioxynil-Na and bromoxynil-K is exhibited by three commercially important weeds of winter cereals. Stellaria media, which infests 77% of winter wheat acreage (Makepeace 1982), is adequately controlled by ioxynil-Na at young growth stages whereas bromoxynil-K has little effect. In contrast, bromoxynil-K is highly active against a weed considered difficult to kill by most herbicides, namely Matricaria inodora, whilst ioxynil-Na is less effective. The sole use of substituted ureas to control black-grass has recently led to the emergence of Viola arvensis as an increasingly important weed. As with other herbicides, the hydroxybenzonitriles are less effective against this species, although ioxynil-Na is the slightly more active compound (May and Baker technical bulletin 1979).

In an attempt to understand the basis of this differential activity, symptom development following hydroxybenzonitrile treatment will be investigated in Matricaria inodora, Stellaria media and Viola arvensis. This will involve a study of net photosynthesis, ultrastructural changes in leaf cells, and changes in primary metabolites following hydroxybenzonitrile treatment. Furthermore, hydroxybenzonitrile retention, uptake, translocation and metabolism will be compared in the three contrasting species. Finally selectivity at the site of action will be studied by a series of in vitro assays with chloroplast fragments isolated from the three weed species.

CHAPTER TWO: THE DEVELOPMENT OF HERBICIDE SYMPTOMS

2.1 INTRODUCTION

An investigation of the differential activity of the hydroxybenzonitriles must begin with a study of symptom development in selected weed species. Herbicide activity is commonly quantified by measuring the fresh and dry weight of aerial tissues at set intervals following treatment (e.g. Merritt 1983). However, the same reduction in weight may occur when plants are treated with either a meristematically active growth retardant or with a foliage contact herbicide. This problem can be overcome by a purely qualitative visual assessment key, or less subjectively by a study of appropriate metabolites which would be indicative of changes in primary metabolism.

The rapidity of herbicide action is reflected in the development of visual symptoms. Slow photosynthetic inhibition may produce light green to yellow tissue indicative of chlorophyll destruction, whereas rapid herbicide action is accompanied by wilting, necrosis, and subsequent plant death (Fedtke 1982). For example, the rate of chlorophyll destruction in hydroxybenzonitrile treated dwarf bean plants (Phaseolus vulgaris var. Canadian Wonder) declined in the order ioxynil > bromoxynil > chloroxynil (Zaki, Taylor and Wain 1967). Thus, the photooxidative breakdown of chlorophylls and other pigments (see section 3.5) can be assayed to quantify the development of visible symptoms following photosynthetic inhibition.

Phytotoxicity and selectivity are influenced by the environmental conditions before, during, and after herbicide treatment. Chlorophyll destruction is most pronounced when plants are maintained in low light intensities before application, and transferred to higher light intensities after treatment. Changes in light intensity, relative humidity, soil moisture, and air temperature have all been shown to influence the potency of the hydroxybenonitriles (Savory, Hibbit, and Catchpole 1975; Merritt 1983, 1984). Thus a study of the differential response of these herbicides necessitates the use of controlled environmental conditions wherever possible. In this way, reproducibility will be enhanced, and the influence of changes in environment excluded.

As with many other herbicides, the activity of the hydroxybenzonitriles against a particular weed species is also dependant upon growth stage. Studies on the minimum effective dose for a number of weed species gave particularly variable results with Chenopodium album, Stellaria media, and Polygonum species (Carpenter, Cottrell, De Silva, Heywood, Leeds, Rivett and Soundy 1964). This variability was directly correlated to differences in growth stage, and it was found that S. media became less susceptible to ioxynil as more leaves expanded. For this reason, 3 different growth stages of the experimental species were treated with ioxynil and bromoxynil to verify the reported response, and identify a suitable growth stage for further study.

Herbicide treatment of plants results in various physiological changes dependant upon the susceptibility of the species. In general, inhibition of CO_2 fixation manifests itself in an initial decrease in the concentration of soluble reducing sugars (Fedtke 1972, 1974). Partial photosynthetic inhibition has also been correlated with a stimulation of protein synthesis (Pulver & Ries 1973; Fedtke 1974) resulting in increases in the soluble protein and amino acid content. Other physiological changes have been observed (see section 1.3.6), particularly following the application of sublethal concentrations of herbicides (Ries 1976). By monitoring the levels of primary metabolites in treated plants it is thus possible to determine the metabolic status of herbicide treated plants, and compare the nature of phytotoxicity in different species.

2.2 MATERIALS AND METHODS

2.2.1 Plant growth conditions

S. media and M. inodora seeds were surface sown, in trays of J. Arthur Bowers seed and potting compost. A fine layer of compost (< 1mm) was sprinkled over the seeds and the tray maintained under greenhouse conditions with supplementary lighting during the winter months (plate 2.1). V. arvensis seeds were sown at a depth of 3 mm and grown under the same conditions. For spray experiments seedlings were transferred to 3 inch pots at least two weeks before treatment. In an initial experiment to determine

plate2.1 : plant growth conditions

the effect of growth stage on selectivity, plants were sprayed at the following growth stages:

TABLE 2.1: Initial growth stage at the time of treatment

	Growth stage		
	I	II	III
VIOLA ARVENSIS	1l + 1d	4l + 1d	5l + 1d + 1s
MATRICARIA INODORA	2l + 1d	4l + 1d	10l + 1d
STELLARIA MEDIA	2l + 2d	6l + 2d + 2s	12l + 2d + 8-10s

where l = fully developed leaves, d = developing leaves,
s = lateral shoots

In all subsequent experiments plants were treated when 6-8 leaves had developed on the main shoot of S. media, and 3-4 leaves had developed on V. arvensis and M. inodora.

For the investigations of symptom development reported in this chapter, plants were transferred to an environmental chamber (Fisons model 600G3, type ^{TTL}, 14 hour day 20°C, 10 hour night 14°C) for 24 hours before and after treatment, and then returned to greenhouse conditions for the remaining experimental period.

2.2.2 Preparation of ioxynil-Na and bromoxynil-K

20g each of ioxynil phenol (white solid) and bromoxynil phenol (cream solid) were dissolved in 100ml of distilled water. The resulting slurry was stirred continually and the pH monitored. Following the addition of 20ml of 1M NaOH to the ioxynil solution and 20ml of 1M KOH to the bromoxynil solution, the pH rose to approximately 12.0 and slowly returned to 7.0. Further 5ml aliquots of hydroxide solution were added until the rate of pH decrease had declined. The pH was returned to neutral by addition of dilute HCl, and the resulting suspension filtered under vacuum. The salt-containing filtrates were dried to chrystalline solids. Off-white ioxynil-Na and pale-brown bromoxynil-K chrystals were stored at room temperature and used as described.

2.2.3 Herbicide application

In all foliar spray experiments, aqueous solutions of ioxynil and bromoxynil (salts) were sprayed on test plants using a laboratory pot sprayer (Mardrive Marine Engineering Co. Ltd.) fitted with an 80^{o} t-jet nozzle. Herbicide solutions were placed in a sealed chamber which could be moved hydraulically across an overhead gan try to deliver an even spray mist over upto 12 x 3" plant pots. The speed of movement and spray chamber pressure could be adjusted in order to calibrate the sprayer. In this process, pre-weighed petri-dishes containing mineral oil (Sigma) were placed on inverted 3" plant pots and sprayed with water. The dishes were quickly re-weighed and the difference in weight, and surface area related to application rate in $l.ha^{-1}$. Plants were routinely sprayed at a rate equivalent to 200 $l.ha^{-1}$ and 30 p.s.i. (field conditions).

2.2.4 Visual assessment of symptoms

In initial experiments the development of symptoms was monitored visually and recorded using a visual assessment key devised by the author (Table 2.2). Each plant was assessed individually for necrotic and chlorotic symptoms, wilting and stunting; and an average figure for 6 replicate plants given in the format $N_2W_2S_1C_3$ which represents 21-40% of tissue necrotic, 21-40% of tissue wilted, 5-20% of tissue stunted, and 21-40% of tissue chlorotic.

TABLE 2.2: Visual Assessment Key

SCALE	N % of necrotic tissue	W % of wilted tissue	S stunting % of control height	C % of chlorotic tissue
0	0	0	96-100	0
1	1-20	1-20	80-95	0-10
2	21-40	21-40	60-79	11-20
3	41-60	41-60	40-59	21-40
4	61-80	61-80	29-39	40-75
5	81-95	81-95	0-19	75-95
6	96-100	96-100	dead	96-100

2.2.5 Fresh and dry weight analysis

The aerial shoots of treated plants were firstly weighed (fresh weight) and then placed in an oven in pre-weighed enamel crucibles. After 72 hours at 95°C the crucibles were re-weighed and the difference considered to be the dry weight of the plant tissue.

2.2.6 The effect of growth stage on selectivity

Six replicate plants per growth stage (Table 2.1) were sprayed with 0.56 kg a.i.ha^{-1} ioxynil or bromoxynil at a rate equivalent to 200 l.ha^{-1}. After 7 and 14 days each replicate was assessed for visual symptoms (Table 2.2, section 2.2.4) and analysed for fresh and dry weight (section 2.2.5).

2.2.7 The effect of application rate on symptom development

Plants were sprayed with ioxynil or bromoxynil at rates equivalent to 0.28, 0.56 (field rate), 1.12 and 2.24 kg a.i.ha^{-1}. The aerial shoots of four replicate plants per treatment were harvested 7, 14 and 28 days after spraying. At each harvest, plants were photographed, weighed, visually assessed for damage and chlorotic symptoms were quantified by chlorophyll analysis.

Analysis of chlorophyll content

Aerial shoots were ground with 5ml of 80% acetone using a pestle and mortar. A further 5ml were added and the resulting chlorophyllous liquor centrifuged at 3000 x g for five minutes. After this time the supernatant was retained, and the pellet ground with a further 5ml of 80% acetone until all chlorophyllous pigments were removed. Following re-centrifugation the two supernatants were added together and made upto a total volume of 25ml. The absorbance was measured at 645 and 663nm enabling the chlorophyll content and a:b ratio to be determined by the method of Arnon (1949).

2.2.8 Changes in key metabolites following ioxynil and bromoxynil treatment

Plants were harvested 7, 14, 21 and 28 days after field rate application (560 $g.ha^{-1}$, 2001.ha^{-1}) of ioxynil or bromoxynil. The 12 replicate plants were divided into two groups of 6 and assayed in the following manner.

GROUP 1 (total extracts)

Each treated plant was ground with 80% acetone until a bleached pellet was obtained (as section 2.2.7). The chlorophyllous supernatant was used to determine the chlorophyll content and retained for protein extraction. The pellet was resuspended in 5ml of 1M NaOH and incubated at 70°C for 1 hour to solubilize proteins. The proteins present in an aliquot of the chlorophyllous supernatant were precipitated by the addition of an equal volume of 1M $HClO_4$ and separated by centrifugation at 10,000 xg. for 10 minutes. The resulting pellet was resuspended in a small volume of 1M NaOH and incubated at 70°C for one hour. The protein content of each extract was determined by modifying the Hartree (1972) adaptation of the Lowry method to overcome the adverse effect of the NaOH present in the sample. The modification gives a linear response over the range 0-120 ug bovine serum albumin.

ADAPTATION OF HARTREE (1972) PROTEIN ASSAY

Reagent A (500 ml)

Potassium sodium tartrate	1g
Sodium carbonate	50g
0.444 M NaOH	250ml
Water	to 500ml

Reagent B (50ml)

Potassium sodium tartrate	1g
Copper sulphate	0.5g
1 M NaOH	45ml
Water	to 50ml

Reagent C (prepared freshly when required)

1 volume of Folin-Ciocaltcau reagent : 15 volumes of water.

Method:

A 0.25ml sample was diluted to 1ml with water and incubated at 50°C with 0.9ml of reagent A for 10 minutes. The sample was rapidly cooled to room temperature, mixed with 0.1ml of reagent B, and maintained at this temperature for a further 10 minutes. 3ml of reagent C were quickly added and the mixture again incubated for 10 minutes at 50°C. When cooled to room temperature the absorbance of the blue solution was measured at 650nm against a blank prepared in the same manner.

GROUP 2 (Water soluble extracts)

Each replicate plant was placed in a covered boiling tube with 20ml of 80% ethanol and incubated in a water bath for 10 minutes at 80°C. The bleached plant tissue was removed and the extract made upto a standard volume. 5ml aliquots were placed under nitrogen in a Tecam SC-3 sample concentrator at 40°C to remove the ethanol by evaporation. The remaining aqueous extract was made upto 10ml with water and assayed for reducing sugars by Nelson's method (Nelson 1944) and soluble proteins by the standard Hartree (1972) method. An aliquot of the ethanol extract was assayed for amino acids using Ninhydrin-hydrazine reagent (Yemm and Cocking 1950).

2.3 RESULTS

2.3.1 Plant growth conditions

Preliminary investigations were carried out to find optimum growth conditions for the three weed species. Highest germination rates occurred when S. media and M. inodora seeds were surface sown and V. arvensis seeds were covered with 3mm of compost (data not presented). Initially all seeds were purchased from B. & S. Weed Seed Suppliers, however extremely low S. media germination (< 0.5%) necessitated propagation of seed in the greenhouse. A small batch of seeds (supplied by The Weed Research Organisation) were grown to maturity, and seeds harvested after 7-8 weeks. Germination rates were found to reach 80% following a 2-4 week ripening period. V. arvensis seeds obtained during the summer, failed to germinate until the following December-January. This dormancy problem was overcome by a minimum 4 week refrigeration at $4^{o}C$ and by retaining sufficient seed from the previous season.

Following sowing, V. arvensis and M. inodora took 4-5 weeks to reach growth stage II (Table 2.1), whereas S. media grew more rapidly and reached the 6-8 leaved stage in around 3 weeks. V. arvensis and M. inodora leaves grew in rosette format and after 7-8 weeks a single flower spike grew from the apex (plates 2.2 and 2.4). In contrast, lateral shoots began to develop when 3-4 pairs of leaves were present on the main stem of S. media. These shoots grew rapidly in accordance with the ground cover habit of this species (plate 2.3).

2.3.2 The effect of growth stage on selectivity

M. inodora was clearly the most susceptible species studied (Tables 2.3 and 2.4). After 14 days, both herbicides were equipotent against the youngest growth stage whereas bromoxynil was more effective against stages II and III. In contrast, ioxynil was more effective than bromoxynil against all growth stages of V. arvensis and S. media. The youngest stage of V. arvensis was most susceptible to both herbicides, and the plants became increasingly resistant at later growth stages. This

Plate 2.2 Viola arvensis

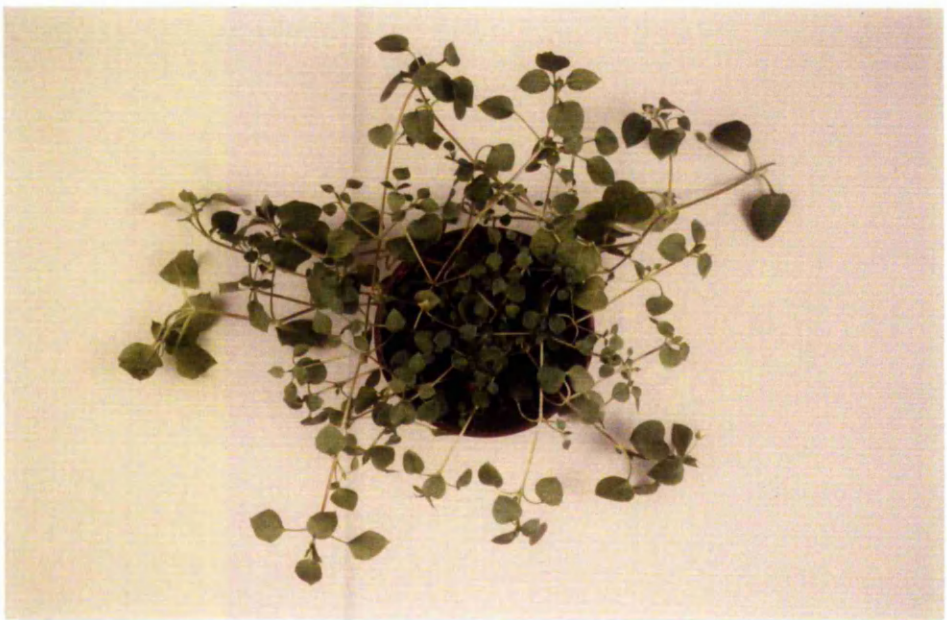


Plate 2.3 Stellaria media

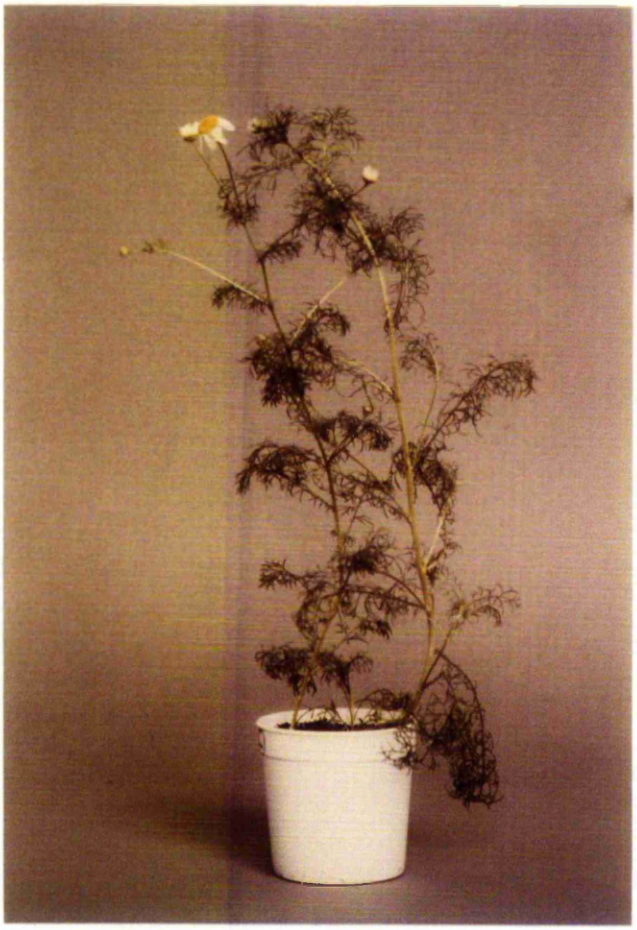


Plate 2.4 Matricaria inodora

TABLE 2.3: The effect of growth stage on symptom development: Visual assessment of symptoms 14 days after treatment

Viola arvensis

	Growth stage		
	I	II	III
Ioxynil	$N_2W_4S_3C_0$	$N_0W_2S_3C_0$	$N_0W_0S_1C_0$
Bromoxynil	$N_0W_1S_3C_0$	$N_0W_0S_0C_0$	$N_0W_0S_0C_0$

Matricaria inodora

	I	II	III
Ioxynil	$N_6W_6S_6C_6$	$N_4W_6S_4C_5$	$N_4W_6S_4C_4$
Bromoxynil	$N_6W_6S_6C_6$	$N_6W_6S_6C_5$	$N_3W_3S_4C_4$

Stellaria media

	I	II	III
Ioxynil	$N_1W_1S_3C_4$	$N_2W_2S_2C_5$	$N_2W_1S_1C_3$
Bromoxynil	$N_0W_0S_1C_2$	$N_0W_0S_1C_0$	$N_0W_0S_1C_0$

TABLE 2.4: The effect of growth stage on symptom development: Fresh weight of aerial foliage 14 days after treatment

Viola arvensis (Mean g/plant)

	Growth stage		
	I	II	III
Control	0.139	0.316	0.627
Ioxynil	0.008	0.075	0.584
Bromoxynil	0.048	0.323	1.169

Matricaria inodora (Mean g/plant)

	I	II	III
Control	0.132	0.315	1.631
Ioxynil	0.003	0.032	0.083
Bromoxynil	0.000	0.016	0.035

Stellaria media (Mean g/plant)

	I	II	III
Control	0.316	1.348	4.175
Ioxynil	0.037	0.509	1.701
Bromoxynil	0.151	0.651	1.832

expected pattern was less apparent for S. media. In this species, an inequality existed between symptoms assessed visually and the reduction in fresh weight following treatment. Ioxynil treatment of stage I and II plants reduced the fresh weight by 88 and 62% respectively even though chlorotic and necrotic symptoms were more marked in the older plants. This suggests some regrowth in older plants, whereas lateral shoot development was halted at the youngest growth stage.

In general, the hydroxybenzonitriles were most active against the youngest growth stages of all three species and least effective against growth stage III. Stage II plants exhibited the differential response implicated in the May and Baker technical bulletin (1979) in that ioxynil was more effective than bromoxynil against S. media and V. arvensis, whilst the converse was true for M. inodora. The degree of susceptibility declined in the order M. inodora > S. media > V. arvensis.

Symptom development in stage II plants is summarized in Table 2.5. The major symptoms of hydroxybenzonitrile action are thus necrosis in susceptible interactions and chlorosis and stunting in more resistant interactions. These features are illustrated in plates 2.5, 2.6 and 2.7 which represent stage II plants 14 days after treatment with field rate ioxynil and bromoxynil.

TABLE 2.5: Time course of symptom development in Stage II plants

IOXYNIL

Days after treatment	M. inodora	S. media	V. arvensis
2	None	None	None
4	Slight chlorosis of treated leaves	Chlorosis of treated leaves	Slight chlorosis of treated leaves
7	Chlorosis and some necrosis of treated and developing leaves	Chlorosis of treated leaves, limited chlorosis of developing leaves	Some chlorosis of treated leaves, limited chlorosis of developing leaves
14	Necrosis and wilting of treated leaves, chlorosis of developing leaves	Chlorosis and necrosis of treated leaves, rapid growth of lateral shoots	Some chlorosis of treated leaves developing shoots growing rapidly.

BROMOXYNIL

2	Slight chlorosis	None	None
4	Wilting of treated leaves, limited necrosis	None	None
7	All treated leaves completely necrotic, developing leaves chlorotic	Slight chlorosis of treated leaves	Slight chlorosis
14	Death of plant	No chlorotic symptoms, rapid growth of lateral shoots	No symptoms, regrowth evident

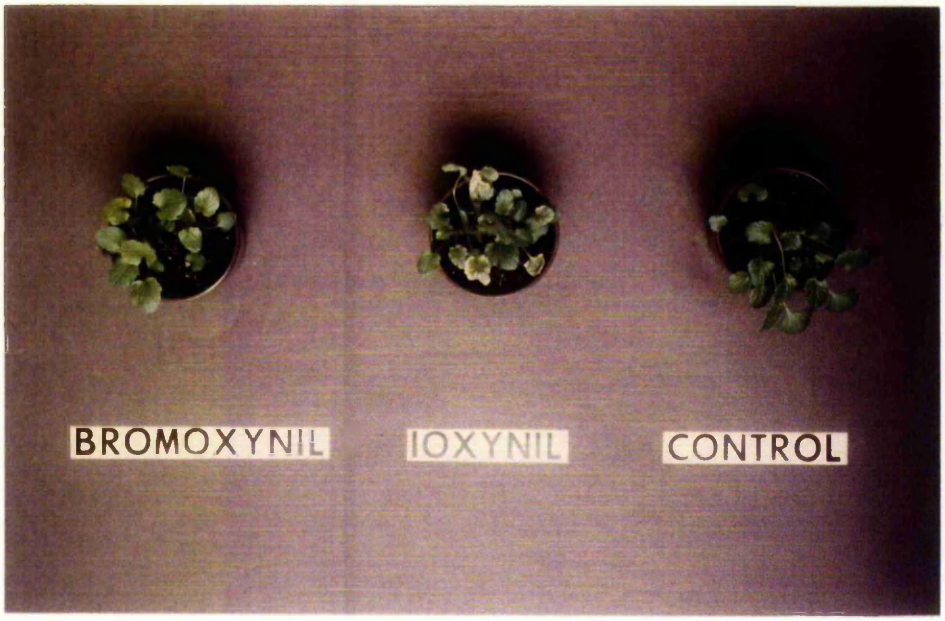


Plate 2.5: V.arvensis 14 days after treatment

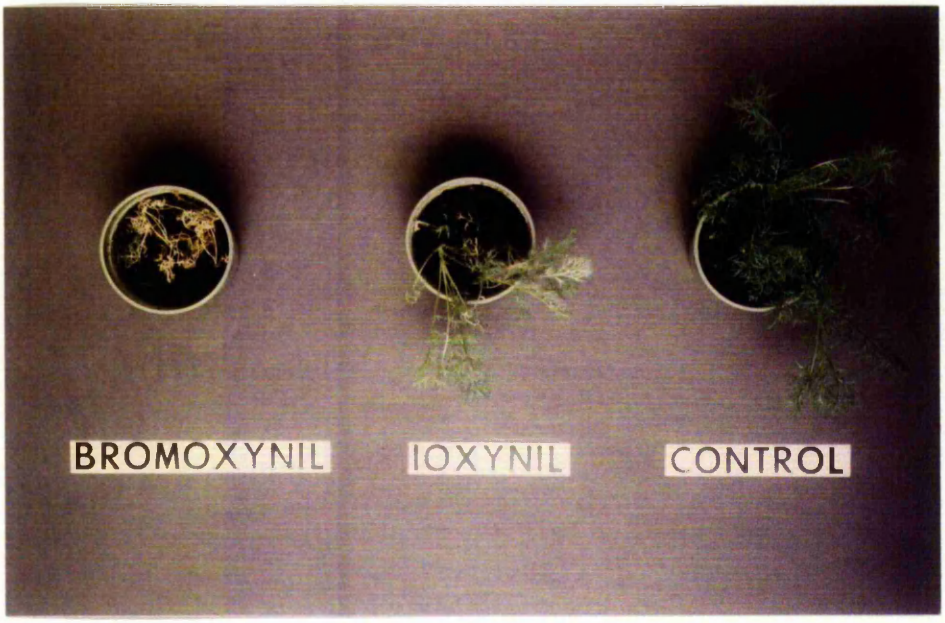


Plate 2.6: M.inodora 14 days after treatment

Plate 2.7:S.media 14 days after treatment

2.3.3 The effect of application rate on symptom development

As already indicated, hydroxybenzonitrile treatment of V. arvensis, S. media and M. inodora results in varying degrees of chlorosis. This symptom has been quantified in a dose response study by monitoring the chlorophyll content of treated tissue 7, 14 and 28 days after application. From Fig. 2.1 it is apparent that all concentrations of ioxynil and bromoxynil caused an initial reduction in fresh weight in the three experimental species. The overall activity against V. arvensis was most pronounced at the 14 day harvest, whereas S. media was more rapidly effected and exhibited some recovery within 14 days, particularly following 0.28 and 0.56 kg a.i.ha^{-1} treatments. The highest concentrations of ioxynil and bromoxynil caused complete death of M. inodora within 14 days of treatment.

All concentrations of ioxynil initially reduced the fresh weight and chlorophyll content of V. arvensis by a similar percentage, from which recovery was dependant on application rate. In this more resistant species, there was no significant difference between the 7 day chlorophyll content of control and treated plants when calculated as $mg.g^{-1}$ fresh weight (data not presented). Thus a reduction in growth appears to be the primary effect of hydroxybenzonitrile treatment. Ioxynil and bromoxynil were equally effective in reducing the fresh weight of V. arvensis during the first 14 days after treatment. Within a further 14 day period, bromoxynil treated plants exhibited more recovery than corresponding ioxynil treated plants. In this species, the chlorophyll content recovered to higher levels than fresh weight, for example 28 days after treatment with 0.56 kg. a.i.ha^{-1} ioxynil plants weighed 57.3% of control weight and contained 78.7% of control chlorophyll content. This may indicate either a "greening" effect of the herbicides, or delayed senescence in treated plants.

Older leaves of untreated S. media plants were senescent at the end of the experimental period, and the apparent stimulation in chlorophyll content (Fig. 2.2) reflected a delay in the onset of senescence. This sublethal effect was more pronounced in

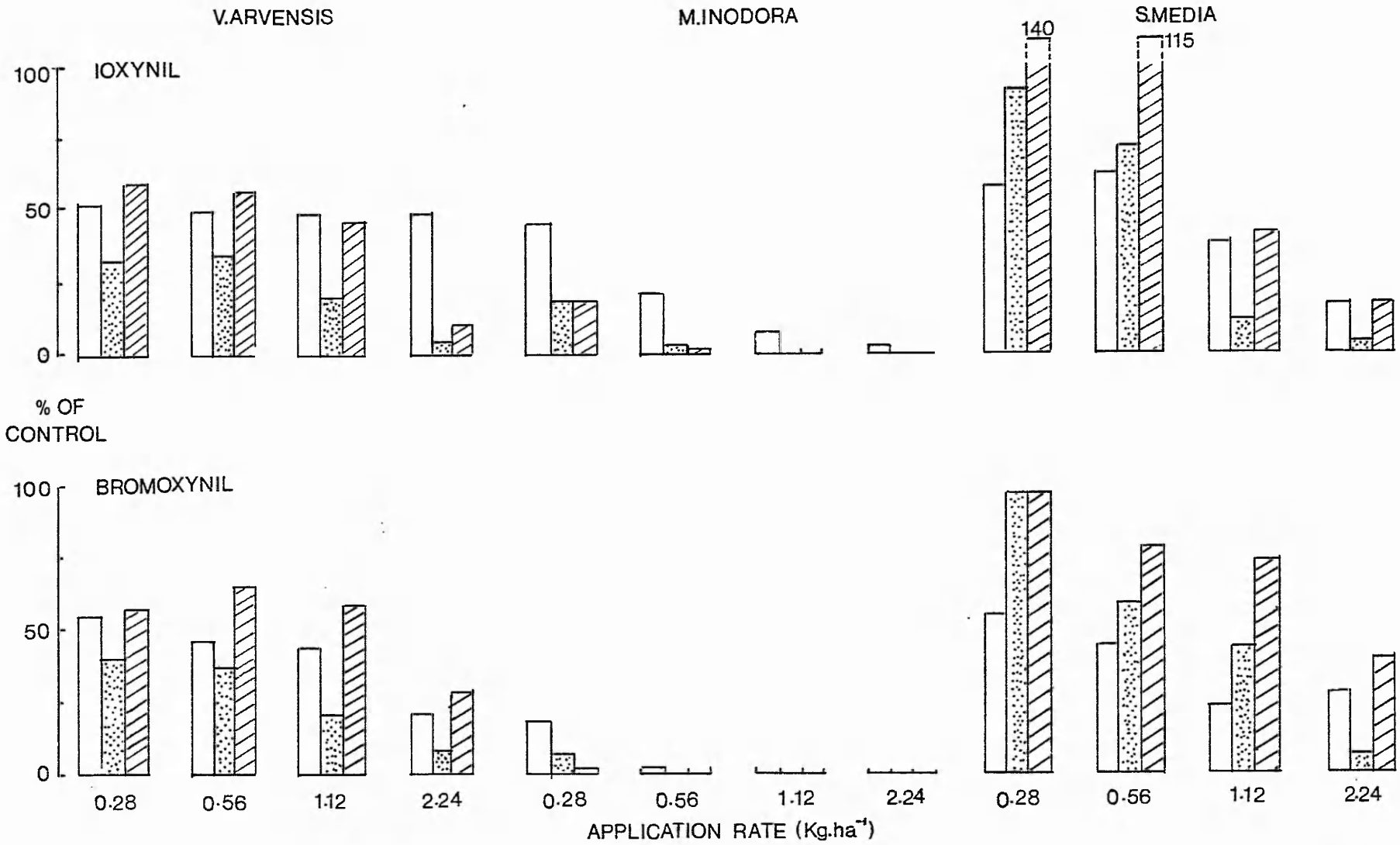


Figure 2.1: The fresh weight of plants 7 (□), 14 (▩) and 28 (▨) days after treatment with ioxynil or bromoxynil at rates equivalent to 0.28, 0.56 (field rate), 1.12 and 2.24 kg a.i.ha.$^{-1}$.

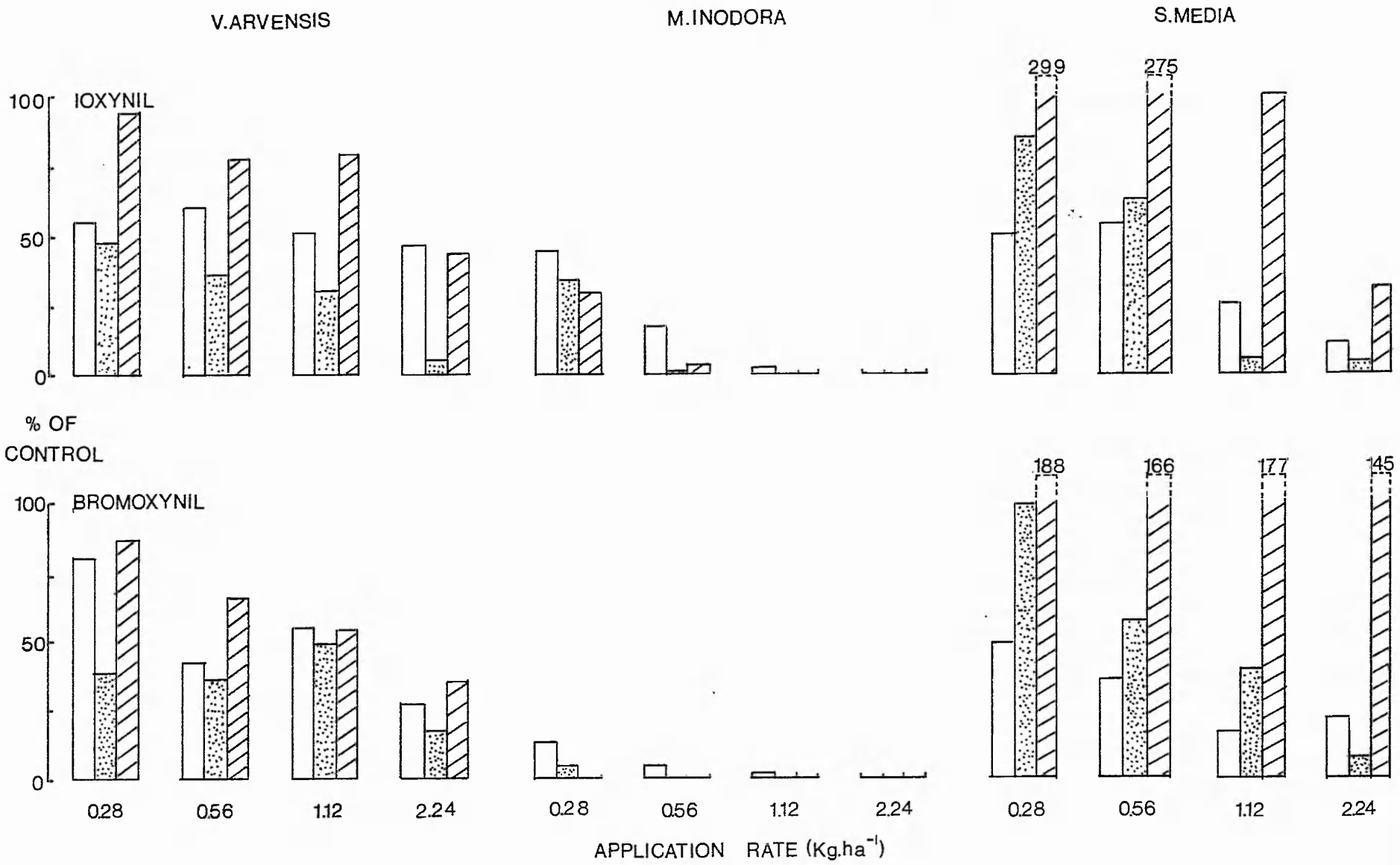


Figure 2.2: The chlorophyll content of plants 7 (☐), 14 (▩) and 28 (▨) days after treatment with ioxynil or bromoxynil at rates equivalent to 0.28, 0.56 (field rate), 1.12, and 2.24 kg a.i.ha.$^{-1}$.

S. media than V. arvensis, and reflects greater activity against this species. As with V. arvensis ioxynil was more effective than bromoxynil, particularly when sprayed at rates equivalent to 1.12 and 2.24 kg.a.i.ha^{-1}.

In contrast, all concentrations of bromoxynil were more active against M. inodora than ioxynil. The rapidity of action is reflected in the gross reduction in fresh weight. For example, field rate bromoxynil treated plants averaged only 14 mg after 7 days, compared to an initial weight of 210 mg. Treated tissue was thus severely desiccated and resembled oven dried leaves (see plate 2.6).

2.3.4 Changes in key metabolites following ioxynil and bromoxynil treatment

The differential response of ioxynil and bromoxynil was further characterized by a study of various metabolic parameters following herbicide treatment. V. arvensis and S. media data is represented in fig. 2.3 and 2.4 as percentage of control levels, having initially been calculated on a metabolite per g fresh weight basis. However, M. inodora data was calculated as the amount of metabolite per plant as the rapid desiccating action of the hydroxybenzonitriles in this species distorted estimations on a weight basis. For example, after 14 days, control plants weighed 1.416g and contained 19.29mg total protein.g^{-1}., whereas bromoxynil treated plants weighed only 0.034g and had a protein content equivalent to 211 mg.g^{-1}. Clearly this was not a stimulatory effect of the herbicide. The desiccating effect was much less prominent in S. media and V. arvensis, and comparisons will tentatively be made between figures 2.3, 2.4 and 2.6 taking into account the different method of calculation.

As previously indicated, changes in chlorophyll content exhibited the same trends as fresh weight following hydroxybenzonitrile treatment of M. inodora (fig. 2.1 and 2.2). Immediately after treatment there was a decline in the total protein content of aerial shoots with corresponding increases in soluble amino acids and proteins (ioxynil only). As the plants recovered from

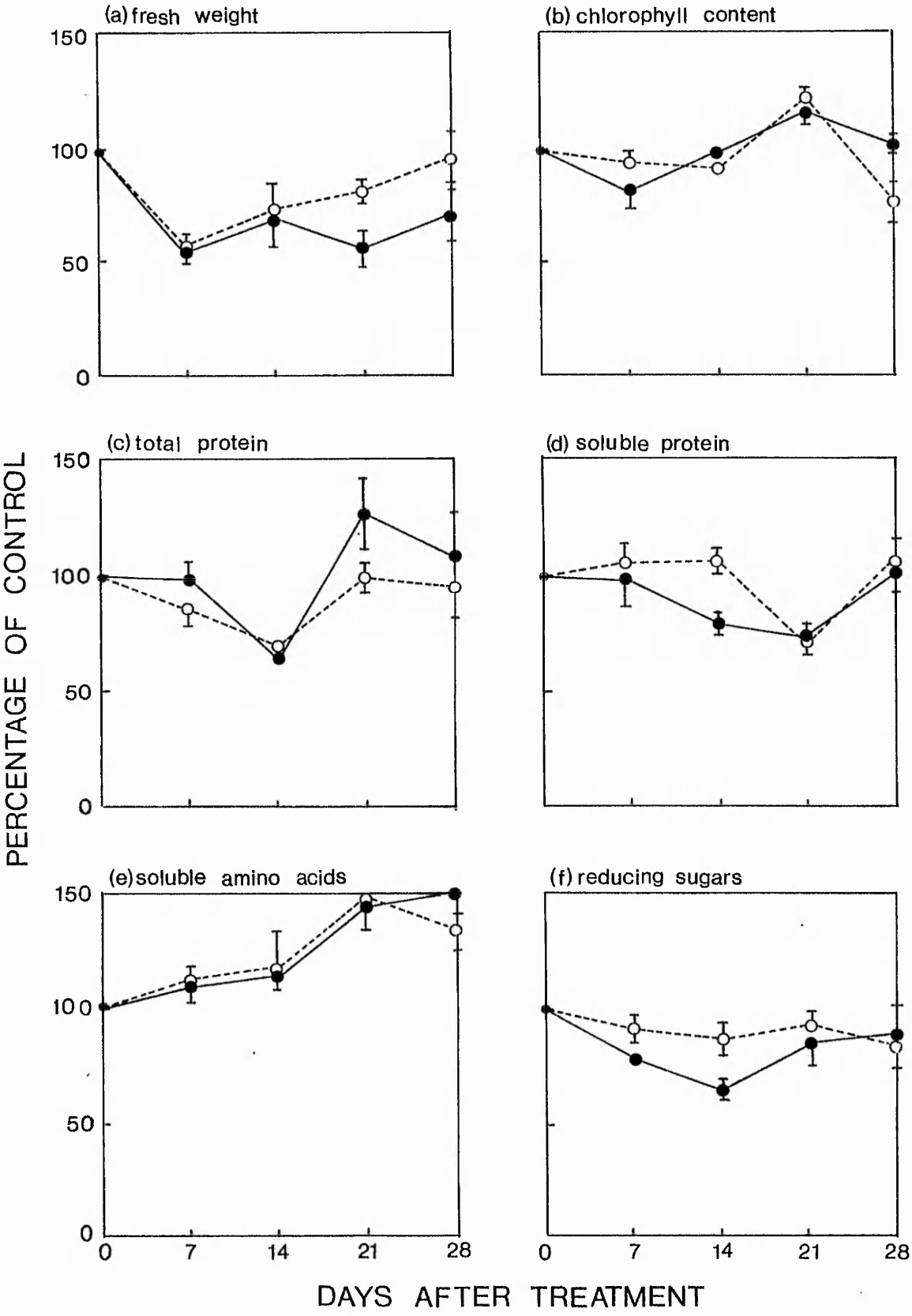


Figure 2.3: Changes in metabolic parameters after ioxynil (—●—) and bromoxynil (--o--) treatment of Viola arvensis. Bars = standard error.

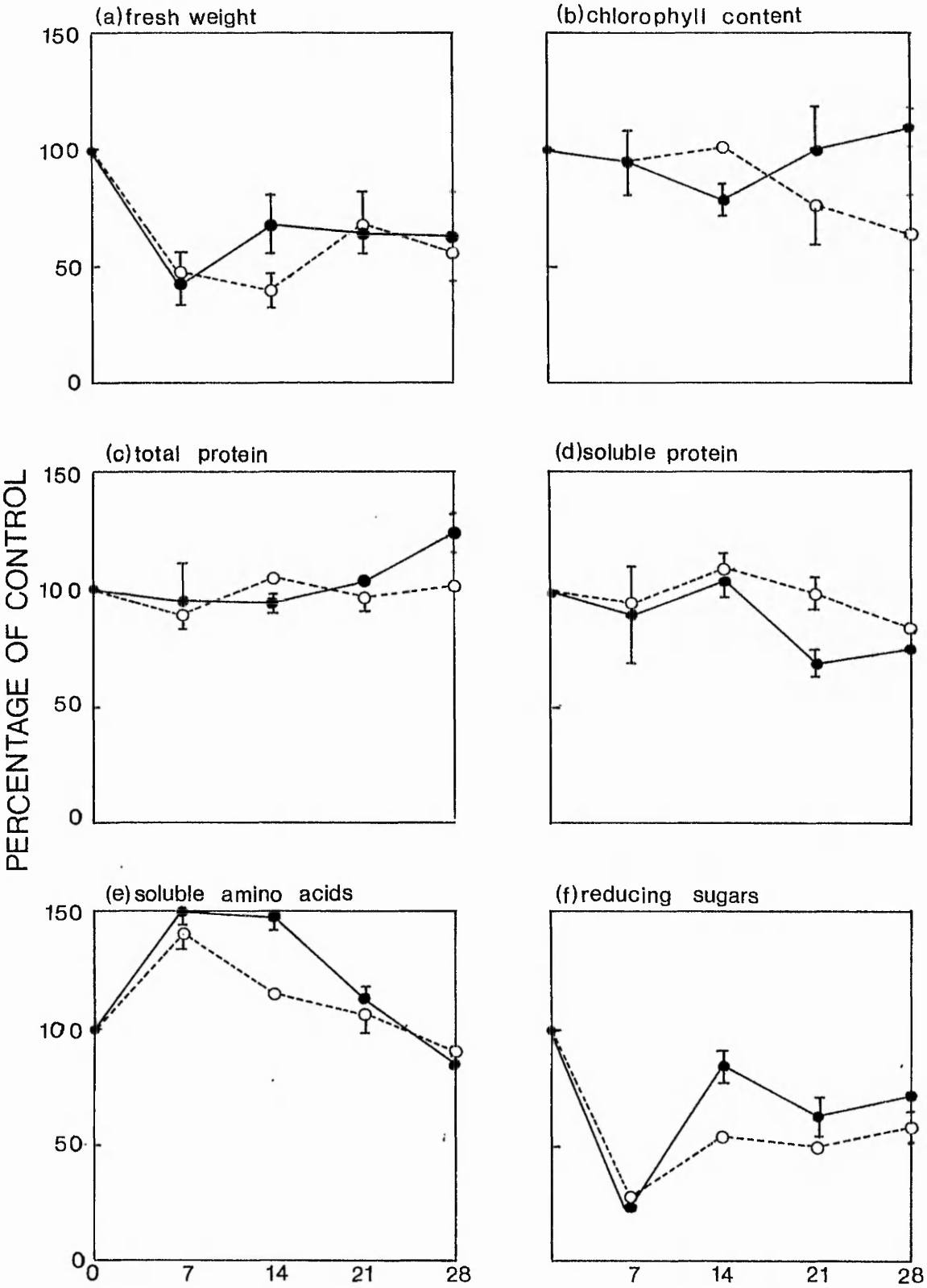


Figure 2.4: Changes in metabolic parameters after ioxynil (—●—) and bromoxynil (--○--) treatment of Stellaria media. Bars = standard error.

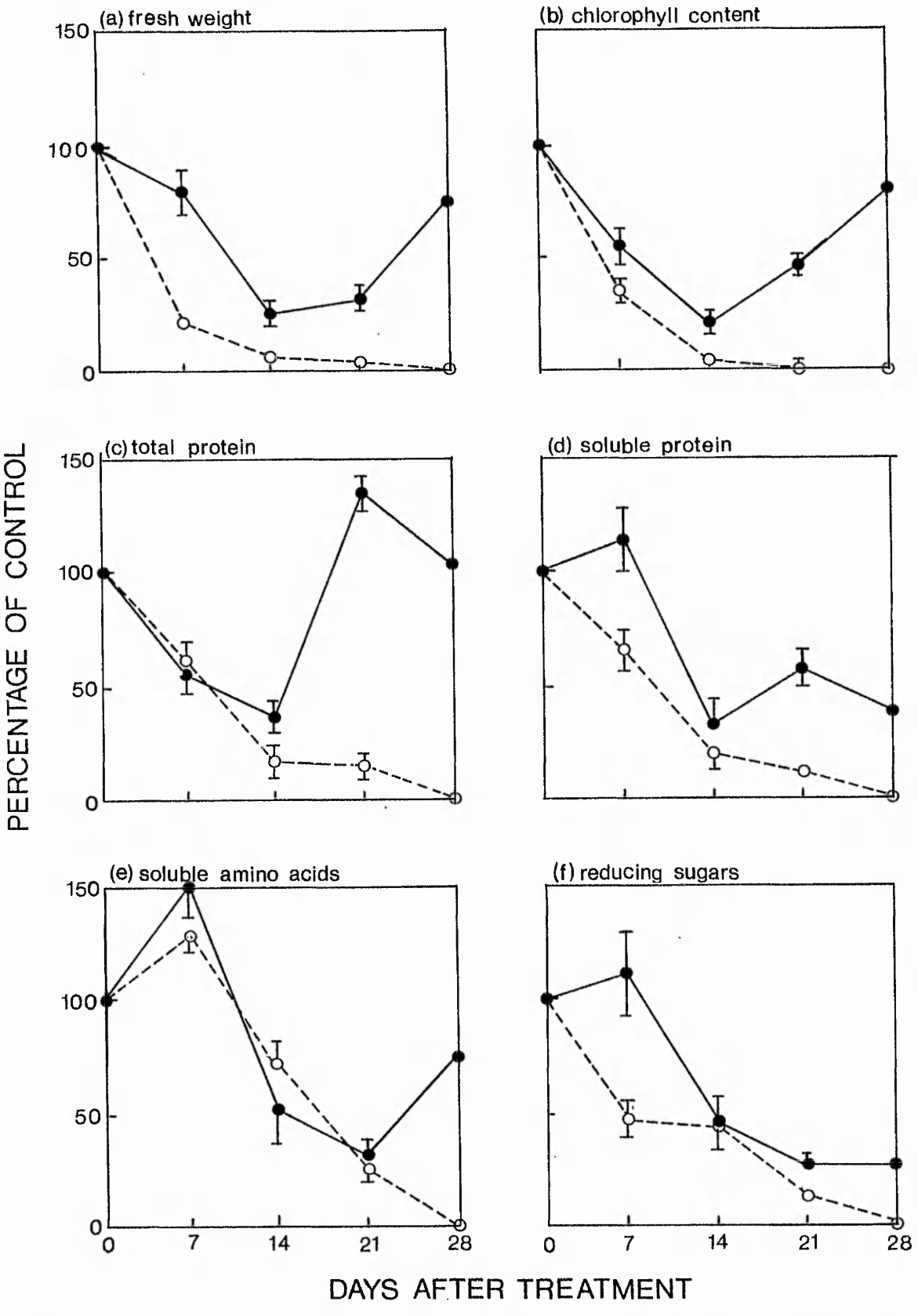


Figure 2.5: Changes in metabolic parameters after ioxynil (—●—) and bromoxynil (--o--) treatment of Matricaria inodora. Bars = standard error.

ioxynil application, the total protein content rapidly increased and amino acids, soluble proteins and reducing sugars declined. Seven days after bromoxynil treatment, all parameters investigated declined as plant death gradually occurred.

Intraspecific differences were less marked in V. arvensis and S. media. Both species exhibited a decline in fresh weight and limited recovery, although bromoxynil treated V. arvensis recovered to approximately the same weight as control plants. The increase in total protein and chlorophyll content following ioxynil treatment further substantiates the delayed senescence effect noted in the previous section. Unlike M. inodora, stimulation of soluble amino acid content did not correspond to a decline in total protein content. Bromoxynil had little effect on the reducing sugar content of V. arvensis, and any inhibitory action of ioxynil was reversed within 28 days. In contrast, reducing sugars rapidly declined to minimal levels in S. media, although recovery was apparent within 14 days.

To gain an impression of the effect of ioxynil and bromoxynil on growth rate, the changes in fresh weight have been recalculated as $mg.day^{-1}$ over each 7 day period (fig. 2.6). All treatments caused an initial reduction in growth rate and with the exception of bromoxynil treated M. inodora, the treated plants all recovered to a similar growth rate to control plants at the end of the experimental period. In each case, the recovery in growth rate corresponded to a gradual increase in the total protein content.

2.4 DISCUSSION

Inter and intra-specific differences have been noted in the response of three weed species to ioxynil and bromoxynil treatment. The observed wilting and necrosis of M. inodora are indicative of a rapid herbicide action in this species. In contrast, symptoms were limited to minor chlorosis of treated tissue in the most resistant herbicide plant interaction (bromoxynil: V. arvensis). Ioxynil and

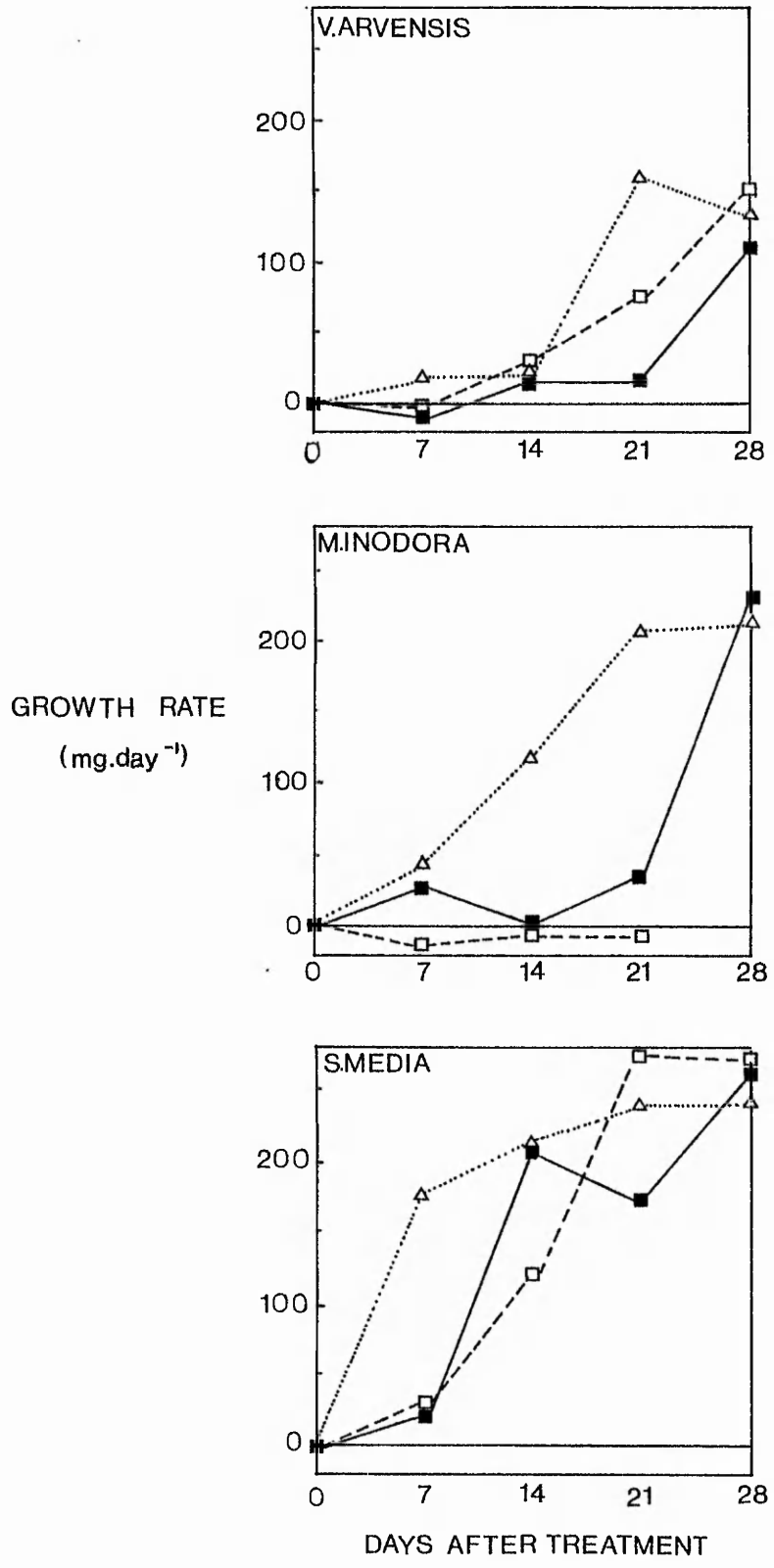


Figure 2.6: The effect of ioxynil (—■—) and bromoxynil (--□--) on the growth rate following treatment. · ·····△····· represents the growth rate of untreated plants.

bromoxynil are considered contact foliage herbicides with some translocated activity (Carpenter et al 1964). In this study, contact effects were prominant in all interactions, and the limited chlorosis of developing tissue indicated a translocated effect, particularly following ioxynil treatment (Table 2.5).

The herbicidal activity of the hydroxybenzonitriles has been shown to be dependant on growth stage, as resistance generally increased with age of plants. When older growth stages of S. media were sprayed, the treated leaves exhibited marked necrosis and chlorosis and stopped growing, whilst the lateral shoots continued to grow rapidly and take over from the main shoot. This rapid regrowth contributes to the resistance of this species to other herbicides (Fryer and Makepeace 1978). Growth stage I plants were sprayed prior to lateral shoot development and were not able to "grow through" the herbicidal effect. In similar studies Carpenter et al (1964) found that the LD_{50} for ioxynil doubled for every leaf which developed on S. media.

The increased susceptibility of V. arvensis, S. media and M. inodora at the early seedling stage, is a common feature of herbicide treated annual weeds (Aberg & Stecko 1976). For example, phytotoxicity of chlortoluron against velvet leaf (Abutilon theophrasti Medic.), jimson weed (Datura stramonium) and cocklebur (Xanthium pensylvanicum Wallr) decreased with stage of development (Carlson and Wax 1970). This effect may possibly relate to differences in wax formation and retention (Verity, Walker and Drennan 1981), a decrease in uptake (Sargent and Blackman 1972), or changes in the general physiological status of the plant (Aberg and Stecko 1976).

In an earlier investigation of the differential response of the hydroxybenzonitriles, Somerville (1972) quantified symptom development by a visual assessment key and fresh and dry weight analysis. The assessment key used in the present study was more detailed and covered the main symptoms of hydroxybenzonitrile action namely chlorosis, necrosis, wilting and reduction in growth. These methods of analysis were adequate for an investigation of the effect of growth stage on selectivity, but any visual assessment must be considered highly subjective. The development of chlorotic symptoms following hydroxybenzonitrile treatment is well established (Zaki, Taylor and Wain, 1967;

Davis, Drennan, Fryer and Holly, 1967; Schafer and Chilcote 1970; Somerville 1972) and thus measurement of chlorophyll content enables a direct effect of the herbicides to be monitored. Figs. 2.1 and 2.2 illustrate that trends in fresh weight and chlorophyll content were generally similar, and any differences which occurred could be related to senescence or a "greening effect" of the herbicide (section 1.3.6).

The effects of the hydroxybenzonitriles on key metabolic processes were studied at weekly intervals after treatment. However, earlier measurements (e.g. 2 and 4 days) would have enabled a distinction to be made between primary and secondary effects of the herbicides. Nevertheless the method used made it possible to detect physiological changes during the recovery phase of hydroxybenzonitrile action.

As a consequence of assumed photosynthetic inhibition, minimum levels of soluble reducing sugars were anticipated within 7 days of treatment. Kinetic studies with potato (Solanum tuberosum L.) and spring wheat (Triticum aestivum L. "Caribo") suggest this parameter is the first to be effected following metribuzin and methabenzthiazuron treatment of roots (Fedtke 1972, 1974). In the present study, S. media plants exhibited this response and earlier measurements of V. arvensis and M. inodora may have detected such a decline. Interestingly, levels of reducing sugars were least effected in the most resistant species (V. arvensis), and the difference between ioxynil and bromoxynil reflected the differential activity in this species.

Low concentrations of reducing sugars following treatment with photosynthetic inhibitor herbicides has been correlated with enhanced nitrogen metabolism (Fedtke 1973, 1974a & b; Pulver and Ries 1973). The stimulation of soluble amino acid content and to a lesser extent soluble proteins observed in V. arvensis and S. media plants, may be related to increased nitrate reduction (Fedtke 1972, 1973; Mann, Huckesby and Hewitt 1979) and/or an increase in RNA synthesis (Pulver and Ries 1973). The latter is less likely as the hydroxybenzonitriles are known to uncouple oxidative and photo-phosphorylation (Moreland 1980); leading to a decrease in ATP availability, and inhibition of RNA and protein synthesis (Moreland, Malhotra, Gruenhagen and Shokrah 1969; Chand and Roy 1981). The gradual decline in growth rate (Fig. 2.6)

and total protein content (Fig. 2.3 and 2.4) may relate to the uncoupling action of the herbicides, and only in the recovery phase may there be a stimulation of RNA synthesis. In contrast, the rapid decline in total protein content, and increase in soluble amino acids and protein content in susceptible M. inodora is more likely to result from photo-oxidative processes induced by photosynthetic inhibition. Peroxidation of membrane components may have released amino acids and proteins into the cytoplasm resulting in an increase in soluble content. It can thus be tentatively suggested that increases in soluble amino acid and protein levels in M. inodora and V. arvensis involve different mechanisms, and that S. media exhibits an intermediate response.

In conclusion, these preliminary investigations of symptom development have enabled the differential response to be quantified. Limitations of space prevented all treated plants from being maintained in a controlled environment chamber, and variations in response between experiments are most likely to reflect residual variations in greenhouse conditions. In an attempt to overcome environmental changes, comparisons of species and herbicides were always carried out simultaneously. After consideration of the preceeding experimentation, the activity of the hydroxybenzonitriles can be summarized as follows:-

	Ioxynil	Bromoxynil
V. arvensis	moderately resistant	resistant
S. media	moderately susceptible	moderately resistant
M. inodora	susceptible	acutely susceptible

S. media was only slightly less susceptible to bromoxynil than ioxynil, and did not exhibit the resistance to bromoxynil suggested in the May and Baker technical bulletin (1979). All other herbicide:plant interactions exhibited the expected response under greenhouse conditions.

CHAPTER THREE: THE RETENTION, UPTAKE AND MOVEMENT OF THE HYDROXYBENZONITRILES

3.1 INTRODUCTION

Differences in the rapidity and intensity of hydroxybenzonitrile symptom development (Chapter 2) implicate the possibility of differences in the amount of herbicide reaching the major site (or sites) of action. The factors determining this response (i.e. retention, uptake, movement and metabolism) were discussed in section 1.2, and have previously been investigated in relation to the selectivity of the hydroxybenzonitriles in some crop and weed species. For example bromoxynil selectivity between winter wheat (Triticum aestivum L. var. Nugains) and coast fiddleneck (Amsinckia intermedia Fisch & Mey) was mainly attributed to differential metabolic breakdown by the two species (Schafer and Chilcote 1970a & b). In a similar study, differential retention by morphologically different barley (Hordeum distichon L., cv. Proctor), pea (Pisum sativum L., cv. Big Ben) and white mustard (Sinapis alba L., English White) largely explained ioxynil selectivity (Davies, Drennan, Fryer and Holly 1967, 1968a & b). In addition, Somerville (1972) investigated the differential response of 7 weed and crop species to ioxynil and bromoxynil, and related selectivity to differences in uptake, metabolic breakdown, and other undefined factors.

The relative contribution of retention, uptake, movement and metabolism to the differential action of the hydroxybenzonitriles has thus previously been shown to vary according to the species investigated. In each study, no one factor was solely responsible for the differential action, and other frequently undefined factors were considered to be involved. It was therefore considered necessary to make a comprehensive study of the contribution of each factor to the activity of ioxynil and bromoxynil against S. media, V. arvensis and M. inodora.

The leaf surface provides the first site at which selectivity can occur. Differential retention may result from general differences in leaf morphology or more specifically from differences in the ultrastructure and composition of epicuticular waxes. Spray retention is usually determined by spraying a water-soluble dye, e.g. tartrazine,

onto sample plants, washing the dye from the foliage, and quantifying the concentration by spectrophotometry (Blackman, Bruce and Holly, 1958; Pallett and Caseley 1980; Verity, Walker and Drennan 1981; Pallett 1984). This gives an overall impression of spray retention, and experiments with microdroplets enable the angle of contact between the leaf surface and spray droplet to be determined (e.g. Taylor, Davies and Cobb, 1981). These parameters have been determined in the present study and were related where possible to qualitative differences in wax composition, and to scanning electron micrographs of appropriate leaf surfaces.

The physico -chemical characteristics of the epicuticular waxes may also influence the degree and rate of herbicide uptake. Various techniques are available for the study of herbicide uptake including cathodoluminescence in conjunction with scanning electron microscopy (e.g. Ong, Falk and Bayer, 1973); fluorescent tracer dye techniques (e.g. Dybing and Currier 1961; Merritt, 1980)
and most commonly used radio-labelled herbicide methods (e.g. Pallett and Caseley 1980; Devine, Bestman, Hall and Vanden Born 1984). Each method makes the general assumption that the reduction in herbicide recovery/fluorescence is due to uptake by the underlying leaf tissue. This assumption has been questioned in the present study, and the possibility of herbicide loss by volatility has been examined. Radiochemical methods have been used to investigate hydroxybenzonitrile uptake and movement within treated plants.

The hydroxybenzonitriles are generally considered to be contact herbicides with only limited translocated activity (Fletcher and Kirkwood 1982). Foy (1964) demonstrated slow acropetal and basipetal movement within the treated leaf and attributed ioxynil movement to diffusion and leakage from successively injured cells. Movement from the treated leaf has been reported (Somerville 1972; Davis, Drennan, Fryer and Holly, 1968a) and there is some evidence for greater mobility in susceptible species (Schafer and Chilcote 1970). Initial studies (Table 2.5) revealed the development of chlorotic lesions in newly developing tissue; and also spreading chlorosis of treated leaves indicative of contact and translocated effects in the experimental

species. These symptoms have been further investigated in the present chapter by radiochemical analysis of ioxynil and bromoxynil movement within and from the treated leaf.

As with many other groups of herbicides, hydroxybenzonitrile selectivity is frequently attributed to differences in herbicide metabolism (e.g. Somerville 1972). However, in a recent review of hydroxybenzonitrile metabolism, Frear (1976) pointed out that there is little detailed information on the isolation and identification of hydroxybenzonitrile metabolism and pathways remain largely speculative. From the evidence of Schafer and Chilcote (1970b), Somerville (1972) and Buckland, Collins and Pullin (1973) it seems likely that the nitrile group is hydrolysed to form benzamide and benzoic acid derivatives in some species. Dehalogenation has also been implicated in the phytotoxicity of the hydroxybenzonitriles (Zaki, Taylor and Wain 1967; Pallett 1978). A similar pathway has been identified for microbial degradation in soil cultures (Crouch and Pullin, 1974; Smith and Cullimore 1974; Hsu and Camper 1975a, b 1979). The significance of differential metabolic breakdown in the selectivity of the hydroxybenzonitriles against V. arvensis, M. inodora and S. media has been assessed in this study.

3.2 MATERIALS AND METHODS

3.2.1 Investigation of epicuticular wax ultrastructure by scanning electron microscopy

Adaxial and abaxial sections (approx. 2 mm^2) were carefully removed from the youngest fully developed leaves of each species, and mounted on 1 cm diameter aluminium stubs using double-sided adhesive tape. The stubs were coated with gold using a Polaron Mark II E.5000 sputter coater. Specimens were examined in a Cambridge Stereoscan 600 electron microscope operated at an H.T. voltage of 25 kV.

3.2.2 Tartrazine dye retention

The laboratory pot sprayer was used to apply a 5 $g.l^{-1}$ tartrazine dye solution to 6 replicate plants per species, at a rate equivalent to 200 $l.ha^{-1}$ and 30 p.s.i. When the spray solution had dried, the foliage of each plant was weighed and then rinsed for 20 seconds in 10 ml of distilled water. The absorbance

of the resulting dye solution was measured at 434 nm using a spectrophotometer, and the quantity of dye retained by the foliage calculated using a standard curve.

3.2.3 Measurement of contact angles

A strip of youngest fully developed leaf was carefully attached to a piece of double sided adhesive tape and fixed to a plastic cuvette (stage). Upto 3 x 0.5 ul droplets were placed on the leaf surface using a Burkard microsyringe. The image of the droplets was projected onto a screen, and the outline of the droplet recorded. The advancing contact angle was measured for 10 droplets of field rate herbicide on the adaxial and abaxial surface of V. arvensis, M. inodora and S. media leaves.

3.2.4 Extraction of epicuticular waxes

Epicuticular wax extracts were prepared from 2.5g of leaf tissue by washing individual leaves in chloroform for 15 seconds. The samples were filtered, evaporated to dryness using a rotary evaporator, and redissolved in chloroform to give a final concentration of 5 $mg.ml^{-1}$. 15 µl of each extract was spotted onto a 20 x 20 cm Kieselgel 60 plate (0.25 mm), and the plate ran in toluene for approximately 1 hour. After drying, the plate was sprayed with 40% sulphuric acid and incubated at 120°C for 30-40 minutes until brown spots had developed on a white background.

3.2.5 Radio-labelled herbicide techniques

In the following investigations (^{14}C-ring)-ioxynil-Na and (^{14}C-ring)-bromoxynil-K of specific activities 33.7 and 41.9 $\mu Ci.mg^{-1}$ respectively, were diluted with field rate herbicide to the desired concentration of dpm $.\mu l^{-1}$. Microdroplets were applied to leaf surfaces using a Burkard microdroplet applicator. Where appropriate, the total applied dose was determined by placing microdroplets on a leaf surface and immediately immersing the leaf in 5 ml of distilled water. The leaf was washed for 20 seconds, and a 1 ml aliquot of wash solution counted for radioactive content.

Assay for radioactivity

3.2.5.1 Aqueous solutions

1 ml aliquots were combined with 9 ml of BDH cocktail 'T' scintillation fluid (666 ml toluene, 334 ml Triton X-100, 5 g PPO , 0.15 g POPOP) and counted for emissions on a Packard liquid scintillation spectrophotometer (model 3330). The recorded counts per minute were corrected for quench using the channels ratio method (Williams and Wilson 1975) and converted to disintegrations per minute.

3.2.5.2 Leaf tissue

Individual tissue sections were immersed in 1 ml of 1 soluene 350:1 toluene. When completely solubilized (2-4 days), 0.5 ml of hydrogen peroxide (20 vols) was added to decolourize the chlorophyllous solution. One day later, the samples were mixed with 15 ml of acidified BDH scintillation cocktail T (14 ml scintillant:1 ml of 1M HCl), and assayed for radioactivity in the described manner.

3.2.6 Uptake of (^{14}C)-ioxynil and (^{14}C)-bromoxynil

5 x 0.2 µl droplets of field rate (^{14}C)-labelled herbicide (≈ 20,000 dpm) were placed on the youngest fully developed leaf (pair of leaves: S. media) of 8 replicate plants. The treated plants were maintained in the controlled environment chamber for the duration of the experiment. After 0, 1, 2, 4, 7 and 14 days each treated leaf was washed in 5 ml of distilled water for 20 seconds. 1 ml aliquots of the wash media were assayed for radioactivity as described (section 3.2.5). Uptake was estimated by subtracting the dpm of the wash solution from the total applied dose recovered after zero time.

3.2.7 Movement of (^{14}C) ioxynil and (^{14}C) bromoxynil

3.2.7.1 Movement within the treated leaf

5 x 0.2 µl droplets of ^{14}C-labelled herbicide (≈ 25,000 dpm) were placed across the centre of the adaxial surface of the youngest fully developed leaf, perpendicular to the mid vein (fig. 3.1). After 0, 1, 4, 7 and 14 days the treated leaves of 6 replicate plants were washed as described (section 3.2.6), and divided into 5 equal strips and the petiole using a disposable scalpel (fig. 3.1). Tissue segments were solubilized and assayed for radioactive content (section 3.2.5.2).

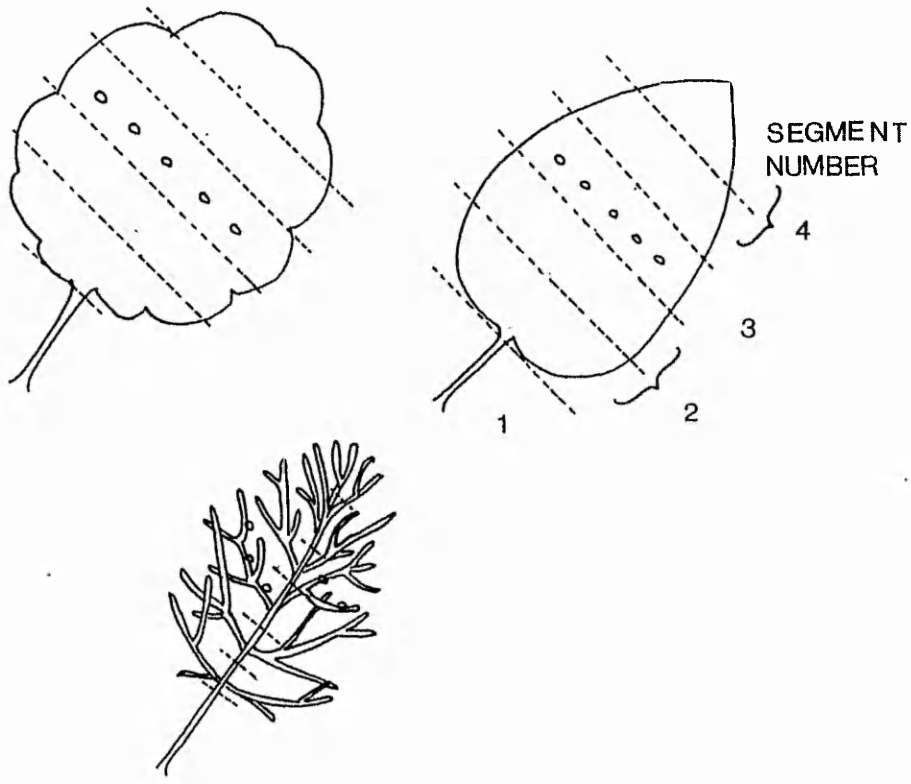


Figure 3.1: The division of treated leaves into segments.

3.2.7.2 Movement from the treated leaf

15 x 0.2 µl droplets of ^{14}C-labelled herbicide (≈ 100,000 dpm) were evenly spaced on the adaxial surface of the youngest fully developed leaves of V. arvensis, M. inodora and S. media. After 4 and 7 days, any remaining (^{14}C)-herbicide was washed from the treated leaf of 6 replicate plants (section 3.2.6). Each plant was then divided into the following sections: treated leaf, young and developing leaves, mature leaves and cotyledons, apex and lateral shoots, stem (if applicable) and roots. Tissue sections were solubilized and assayed for radioactivity as described above (section 3.2.5.2).

3.2.7.3 Movement in hydroponically grown plants

Growth of plants

Seeds of each species were surface sterilized by immersion in 2% sodium hypochlorite solution for 5 minutes. The seeds were then washed several times with sterile distilled water and allowed to germinate on moist filter paper in sterile petri dishes. After 3 days, germinating seeds were carefully placed onto the muslin raft of individual culture jars prepared as illustrated in fig. 3.2. The jars contained full strength Hoaglands solution (Blankendaal, Hodgson, Davis, Hoerauf and Shimabukuro 1972) which was topped up daily and completely replaced at 7 day intervals. Culture jars were maintained in the controlled environment chamber until the seedlings had reached the desired growth stage (4-6 weeks).

Treatment of plants

15 x 0.2 µl droplets of (^{14}C)-labelled herbicide (≈ 100,000 dpm) were evenly spaced on the adaxial surface of the youngest fully developed leaf of 6 replicate plants. The nutrient solution was sampled for radioactivity after 0, 1, 2, 3, 4 and 7 days (section 3.2.5.1).

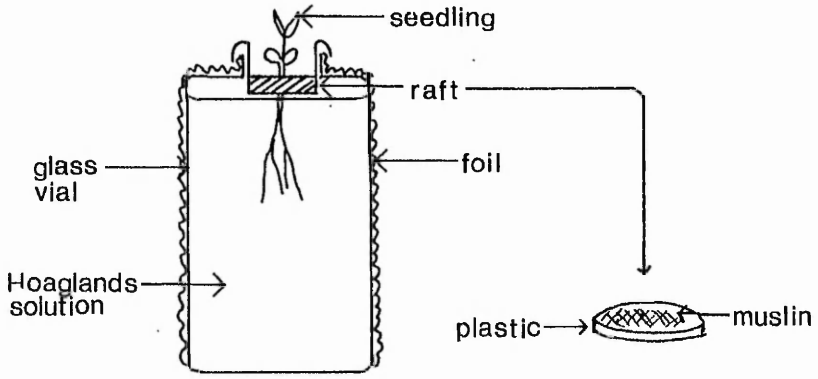


Figure 3.2: The culture system for hydroponically grown plants.

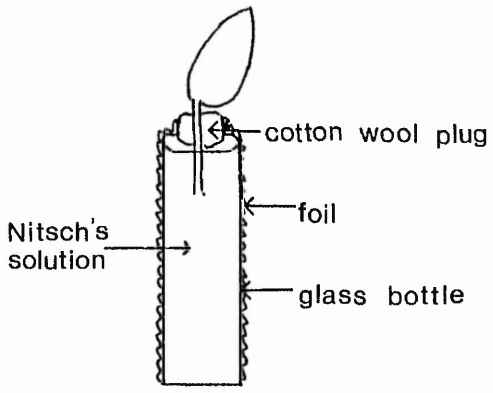


Figure 3.3: The explant system used in volatility experiments.

Seven days after treatment, any remaining (^{14}C)-herbicide was washed from the treated leaf, and each plant was divided into treated leaf, apex and developing leaves, other leaves, stem, and roots. The radioactivity in each section was determined in the described manner (section 3.2.5.2).

3.2.8 Herbicide volatility

The contribution of herbicide volatility to uptake and movement studies was assessed by a simultaneous investigation of herbicide uptake, recovery from an explant system, and recovery from an artificial model of the leaf surface.

3.2.8.1 Herbicide uptake

10 x 0.2 µl droplets of ^{14}C-labelled herbicide (≈ 15,000 dpm) were applied to the youngest fully developed leaf of 8 replicate plants. After 0, 2, 4, 7 and 14 days the uptake was calculated as the percentage activity not recovered from the leaf surface (section 3.2.6).

3.2.8.2 Explant system

Each explant (leaf and petiole) was placed in a dark glass vial containing 5 ml of ½ strength Nitsch's solution (fig. 3.3). 10 x 0.2 µl droplets of (^{14}C)-herbicide (≈ 15,000 dpm) were applied to the adaxial surface, and the vials were maintained in the controlled environment chamber for the duration of the experiment. After 0, 2, 4, 7 and 14 days 8 replicate explants per herbicide were harvested by washing in distilled water for 20 seconds, followed by solubilization of the leaf and petiole (section 3.2.5.2). The nutrient medium was made upto 5 ml and a 1 ml aliquot analysed for radioactivity (section 3.2.5.1).

3.2.8.3 Artificial leaf surfaces

Epicuticular waxes were extracted from the three weed species (section 3.2.4) and redissolved in

chloroform to a concentration of 2 mg.ml^{-1}. 20µl aliquots were placed on the surface of glass coverslips and allowed to dry. 24 hours later, 10 x 0.2 µl droplets of (^{14}C)-ioxynil or (^{14}C)-bromoxynil were applied to the wax extract of each coverslip. After 0, 2, 4, 7 and 14 days, 8 replicate artificial surfaces per treatment were harvested by initially washing each waxed surface in 5 ml of distilled water for 20 seconds. When dried, 200 µl of chloroform was repeatedly squirted over each coverslip to remove the wax extract. 1 ml of the aqueous wash and the total chloroform wash were each mixed with 9 ml of BDH scintillation cocktail 'T', and counted for radioactivity (section 3.2.5.1). As a precaution, each remaining coverslip was broken up, thoroughly shaken with scintillant and the scintillant assayed for radioactivity (section 3.2.1).

3.2.9 Hydroxybenzonitrile metabolism

24 plants of S. media and V. arvensis and 48 plants of M. inodora were sprayed with field rate ioxynil, bromoxynil or water. The treated plants were maintained in the controlled environment chamber and harvested after either 7 days (V. arvensis and S. media) or 4 and 7 days (M. inodora). The plants were divided into 4 groups of 6, weighed, and washed for 20 seconds in distilled water to remove unpenetrated herbicide. Each group of plants was dried with paper tissue, homogenized in 10 ml of chilled 0.3M $ZnSO_4$ and centrifuged at 3000 xg for 3 minutes. The supernatant was filtered to remove floating leaf debris, and the pellet was recentrifuged with a further 10 ml of zinc sulphate solution. After filtration, the supernatants were combined and shaken with 1 drop of 6N HCl and 15 ml of Analar ethyl acetate. On separation the lower aqueous extract was washed with a further 10 ml of ethyl acetate, and the two ethyl acetate extracts were combined. Each extract was then evaporated to dryness using a rotary evaporator at 40°C; and resuspended in 0.5 ml of ethyl acetate.

15 µl samples of extract were spotted onto 20 x 20 cm kieselgel 60 plates (0.25 mm) and ran against halogenated hydroxybenzonitrile derivatives supplied by May and Baker Ltd. (15 µl of 200 ppm solutions, prepared in methanol). Plates were ran in either 19 chloroform:1 acetic acid (Smith and Cullimore 1974; Hsu and Camper 1979) or 100 butanol:25 ethanol:28 water (Pallett 1978). Halogen containing compounds were detected using the F.F.C.A. reaction (Zappi 1967), which was prepared by mixing reagents A, B and C in a ratio 5A:5B:1C and diluting 50:50 with distilled water.

Reagent A: 2.7 g of $FeCl_3.6H_2O$ dissolved in 100 ml of 2N HCl.

Reagent B: 3.5 g of $K_3Fe(CN)_6$ dissolved in 100 ml distilled water.

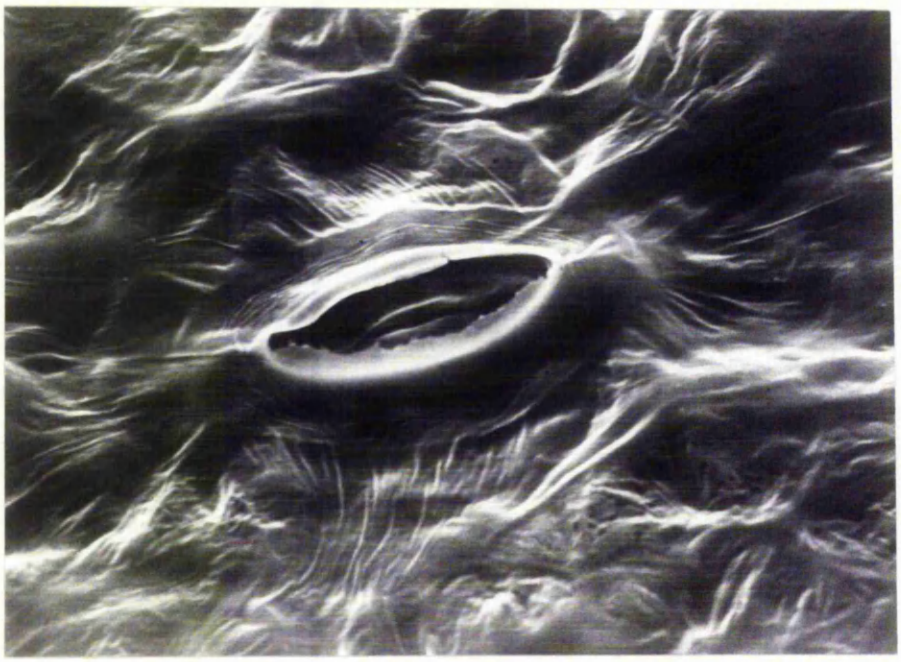
Reagent C: 5 g of $NaAsO_2$ dissolved in 30 ml of 1N NaOH at 0^oC and stirred vigorously with 65 ml 2N HCl. Any arsenious oxide precipitate formed was removed by filtration.

All reagents were stored in the dark. Freshly prepared F.F.C.A. reagent was sprayed onto dried TLC plates, and the plates were immediately covered with glass and placed in the dark for 15 minutes. Blue spots appeared on a yellow background where halogenated compounds were present.

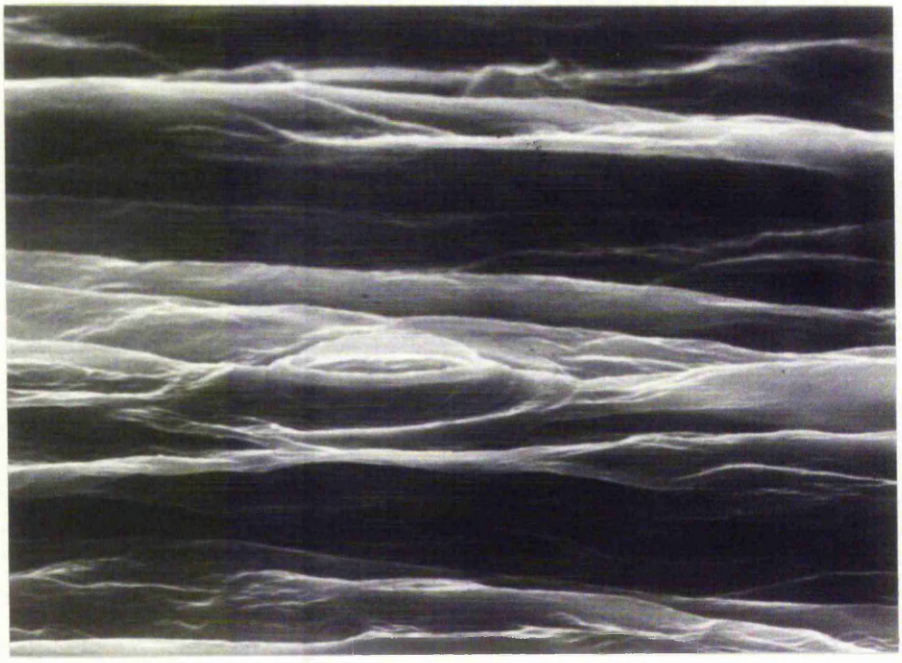
3.3 RESULTS

3.3.1 Leaf surface characteristics and spray retention

Abaxial and adaxial leaf surfaces were initially observed by scanning electron microscopy. There was no evidence of structured surface wax deposits in each species (plates 3.1a, b and c) confirming reported observations of S. media and M. matricoides (Verity, Walker and Drennan, 1981). The ridges observed on V. arvensis and S. media leaf surfaces (plates 3.1a and c) were more prominent after chloroform treatment and were therefore unlikely to be wax platelets. Similar parallel ridges have been observed on the leaf surfaces of some graminaceous species e.g. meadow fescue (Festuca pratensis) and Timothy Grass

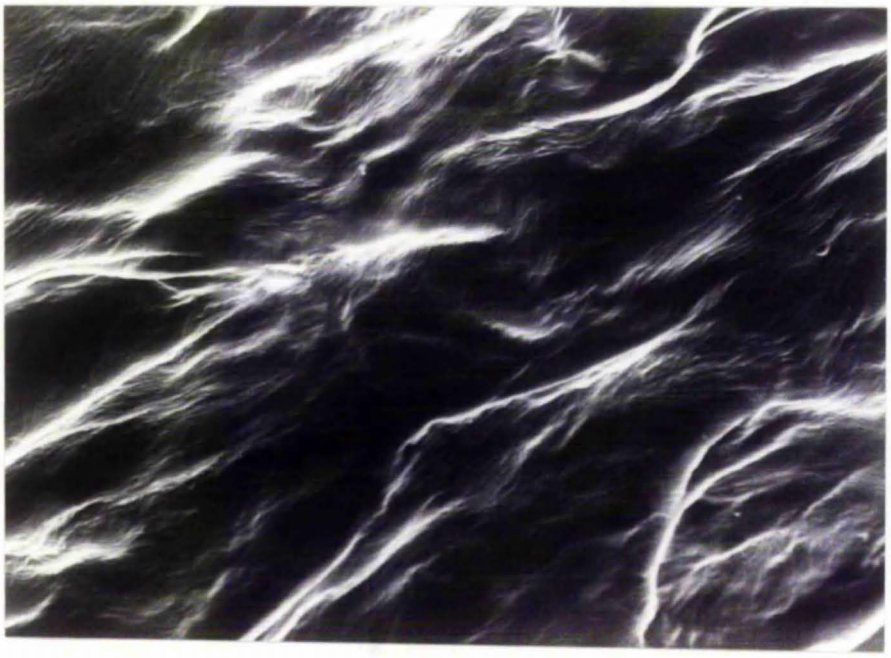


(a) V.arvensis x 2000



(b) M.inodora x1950

Plate 3.1: Adaxial leaf surface ultrastructure



(c) S.media x1900

(Phleum pratense) and were considered to represent the roughness of the underlying epidermal cells (Holloway 1970). Magnifications greater than 2000 times could not be achieved as the soft leaf tissue was subject to blistering under the beam of the scanning electron microscope. Attempts to overcome this problem by drying the leaf tissue prior to sputter coating proved unsuccessful. Other investigations in this laboratory have however revealed wax platelets at similar magnifications on Chenopodium album (Taylor, Davies and Cobb 1981), and Setaria viridis (Carr, unpublished).

Interspecific differences were obtained in the retention of aqueous solutions of tartrazine dye (Table 3.1). When the experimental growth stages were compared, susceptible M. inodora retained slightly more tartrazine dye per plant than V. arvensis and S. media. However, a more realistic interspecific comparison has been made on a weight basis, in which retention declined in the order V. arvensis > S. media > M. inodora, the converse of the order of relative susceptibility. The influences of the leaf surface on wettability was also investigated by measuring the angle of contact between the droplet and the leaf surface (Table 3.2). For each species, there was no significant difference between the contact angles of ioxynil and bromoxynil droplets. Contact angles were lower on S. media and M. inodora than V. arvensis, and were generally lower on the abaxial than adaxial surface.

Chloroform extraction of epicuticular waxes revealed a greater wax yield for V. arvensis (4 $mg.g^{-1}$ fresh weight) than M. inodora (2.9 $mg.g^{-1}$) and S. media (2.4 $mg.g^{-1}$). Differences were also obtained in the chemical composition of the extracted waxes (Fig. 3.4). Individual components were tentatively identified by comparison with the results of Taylor (1980). Primary alcohol and fatty acid content were similar in all 3 species, and S. media and V. arvensis were richer in hydrocarbons, esters and ketones than M. inodora. S. media waxes also contained probable aldehydes and secondary alcohol compounds which were absent in the other extracts.

TABLE 3.1: Tartrazine dye retention

	$\mu l.plant^{-1}$	$\mu l.g^{-1}$
V. arvensis	9.45 (± 1.00)	55.83 (± 2.34)
M. inodora	11.90 (± 0.90)	41.50 (± 2.51)
S. media	8.38 (± 0.57)	49.83 (± 1.89)

TABLE 3.2: Contact angles of ioxynil + bromoxynil droplets on adaxial + abaxial leaf surfaces

	Adaxial surface		Abaxial surface	
	Ioxynil	Bromoxynil	Ioxynil	Bromoxynil
V. arvensis	74.65 (± 2.22)	69.70 (± 4.48)	68.90 (± 1.57)	70.70 (± 2.28)
M. inodora	62.55 (± 2.18)	63.90 (± 3.45)	47.60 (± 2.28)	46.56 (± 2.52)
S. media	65.00 (± 3.28)	63.33 (± 2.88)	61.50 (± 0.71)	50.60 (± 3.35)

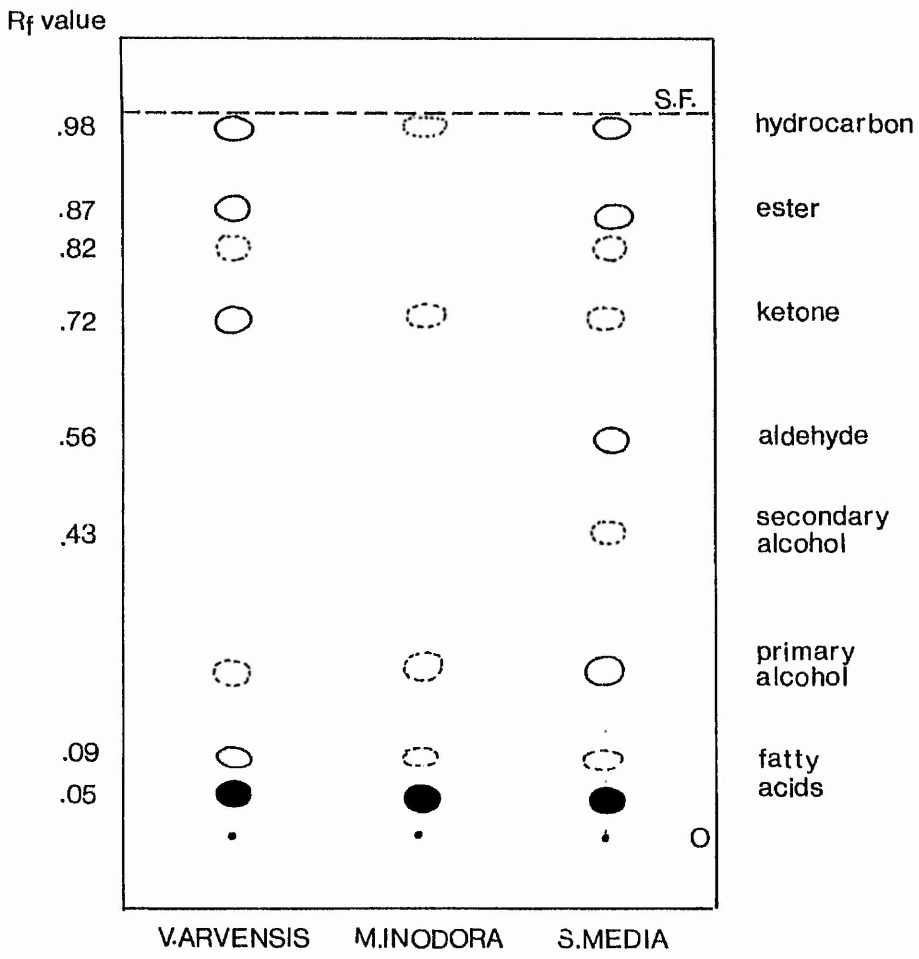


Figure 3.4: The separation of the epicuticular waxes of V. arvensis, M. inodora and S. media by thin layer chromatography.

3.3.2 Uptake and movement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil

Hydroxybenzonitrile uptake was initially estimated by subtracting the amount of (^{14}C)-herbicide recovered from the leaf surface, from the total applied radioactivity. This approach revealed similar (^{14}C)-bromoxynil uptake by all three species and approximately twice as much (^{14}C)-ioxynil uptake by V. arvensis (fig. 3.5). In general (^{14}C)-ioxynil and (^{14}C)-bromoxynil uptake was relatively slow, frequently reaching only 30-40% 14 days after treatment. In each species uptake was more rapid during the first 7 days (28-73% of (^{14}C)-ioxynil and 23-30% of (^{14}C)-bromoxynil) and declined between 7 and 14 days during which time only a further 11-16% of (^{14}C)-ioxynil and 5-12% of (^{14}C)-bromoxynil had penetrated.

Microdroplet (^{14}C)-ioxynil treatment of V. arvensis and S. media caused spreading chlorotic lesions to develop along the nearest veins (plates 3.2 a & c). This apparently slow herbicide movement was quantified by an initial experiment to determine the extent and direction of movement within the treated leaf. From Fig. 3.6 it can be seen that the highest proportion of (^{14}C)-ioxynil and (^{14}C)-bromoxynil was detected in the treated segment of V. arvensis leaves. The proportion of radioactivity detected in this segment increased at each harvest until 53.4% of the applied (^{14}C)-ioxynil and 7.5% of the (^{14}C)-bromoxynil was recovered after 14 days. After 1 day 2.26% of the applied (^{14}C)-ioxynil and 0.26% of the (^{14}C)-bromoxynil had moved out of the treated segment and was detected in all segments of the treated leaf. At the 4, 7 and 14 day harvests, consistantly more (^{14}C)-ioxynil and (^{14}C)-bromoxynil had moved in a basipetal rather than acropetal direction. For example after 14 days 5.25% of (^{14}C)-ioxynil was detected in segments 1 and 2 whereas only 1.66% was detected in segment 4. Throughout the experimental period there was a marked decline in the total radioactivity recovered with only 71.5% of the applied (^{14}C)-ioxynil and 73.9% of the (^{14}C)-bromoxynil detected in the treated leaf.

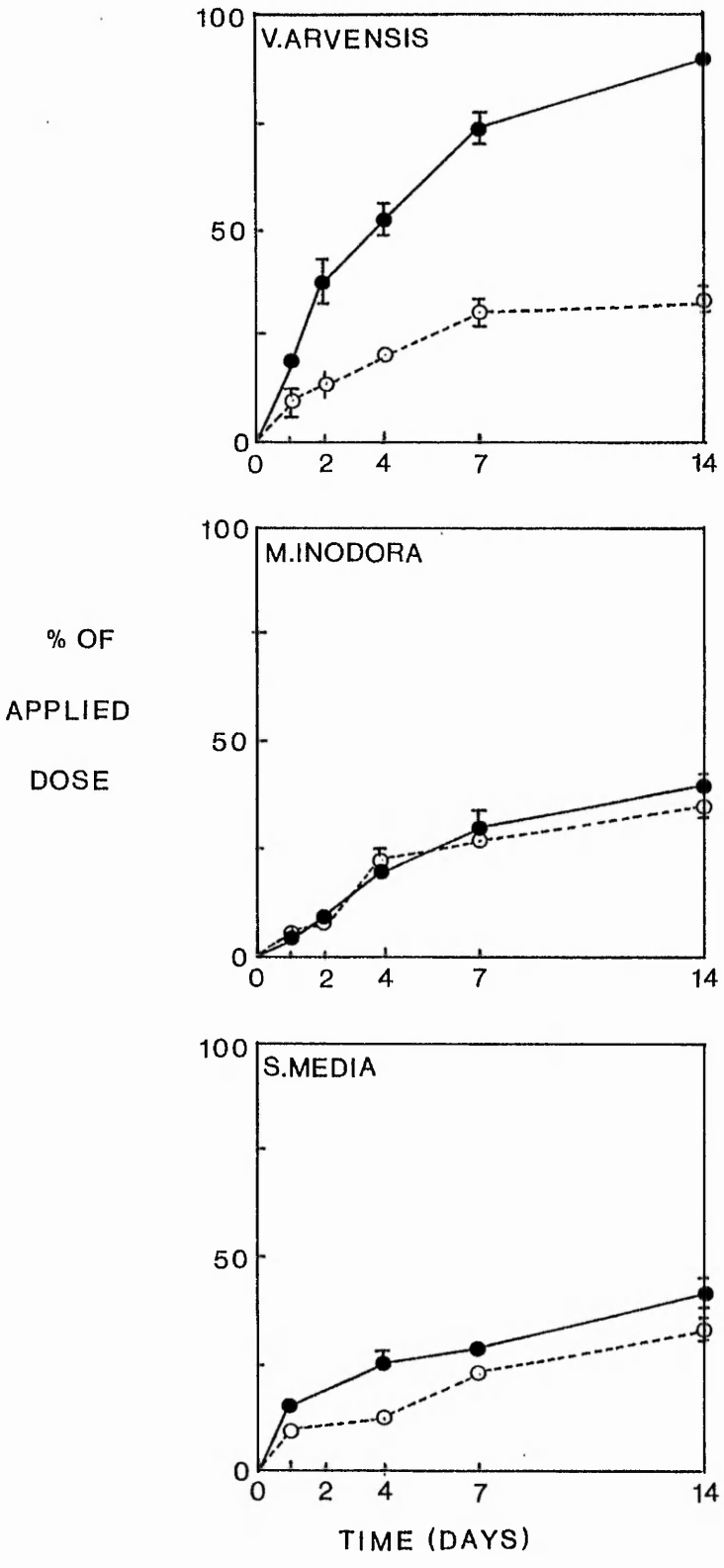
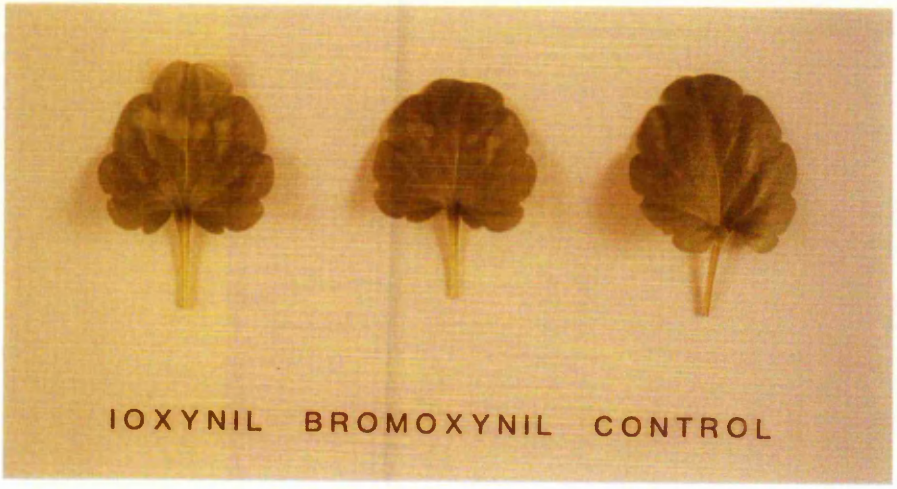
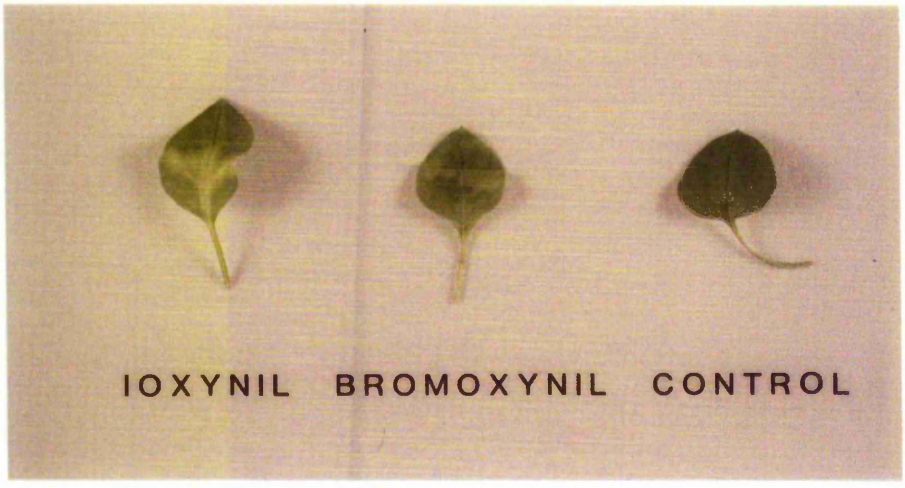


Figure 3.5: Uptake of (^{14}C)-ioxynil (—●—) and (^{14}C)-bromoxynil (--o--) by the youngest fully developed leaf. Bars = standard error.



(a) V.arvensis

(b) M. inodora



(c) S.media

Plate 3.2 : chlorotic symptoms 14 days after microdroplet treatment of leaves

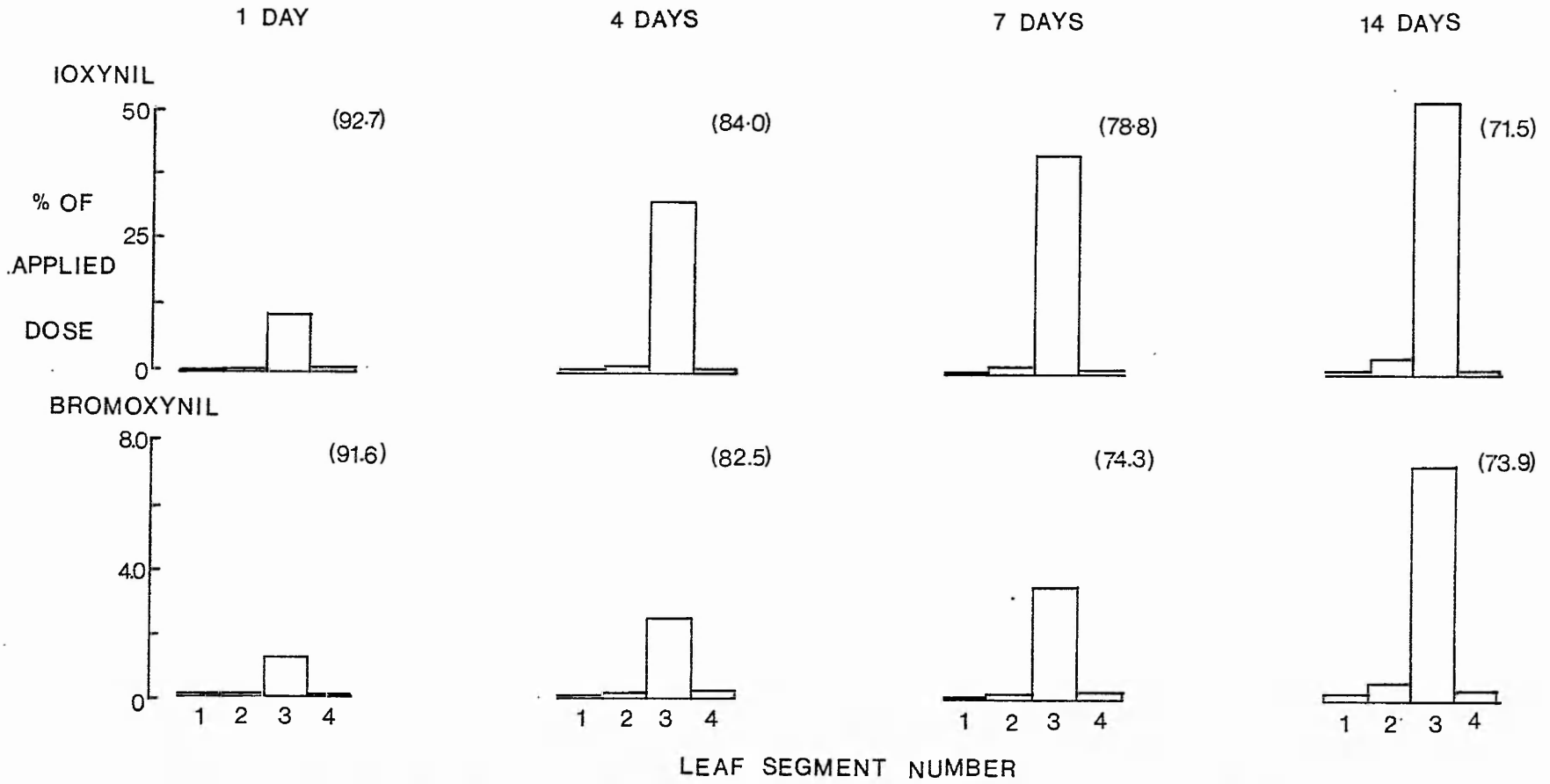


Figure 3.6: Movement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil within the treated leaf of Viola arvensis. (Figures in brackets represent the total radioactivity recovered at each harvest).

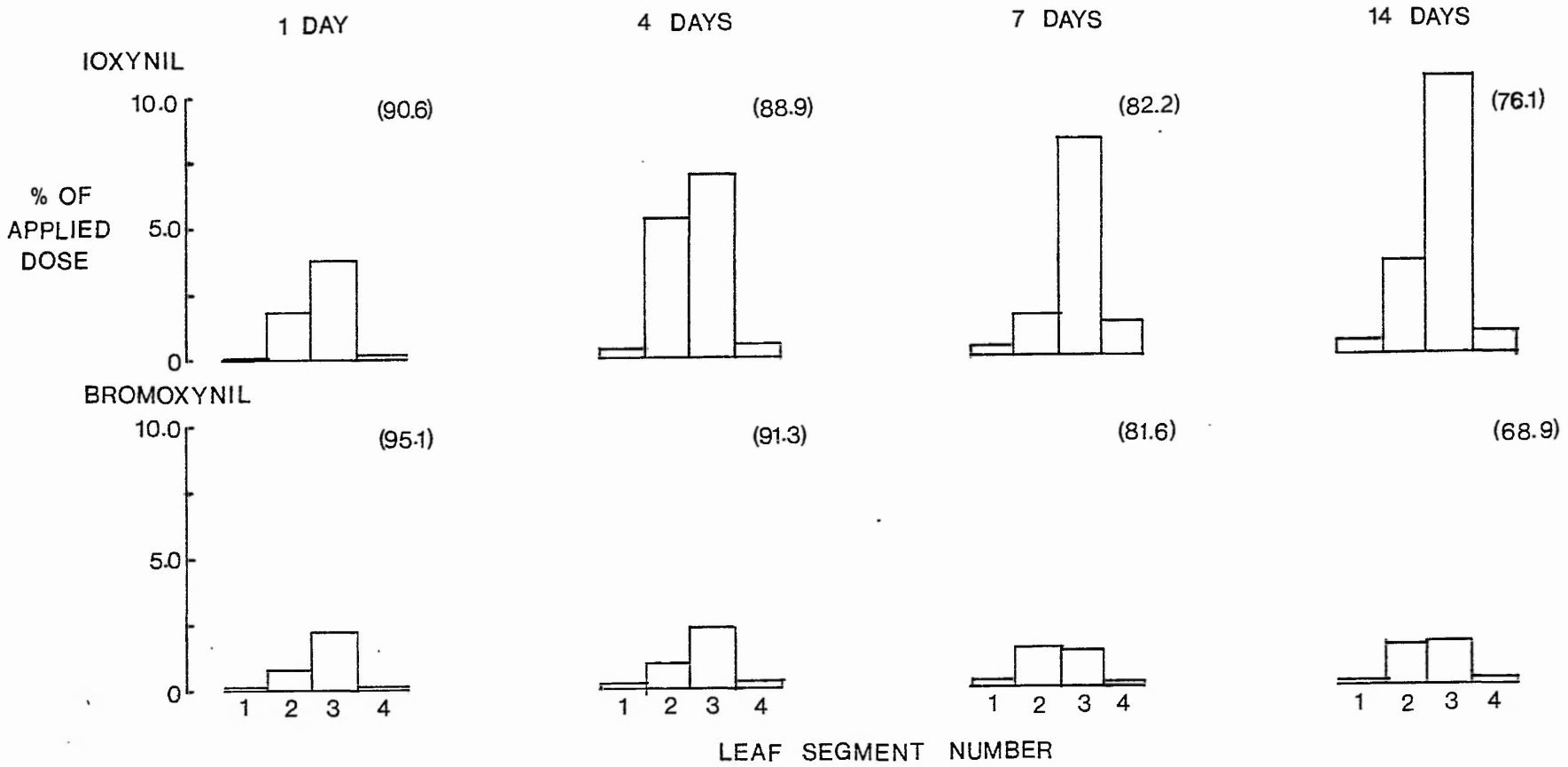


Figure 3.7: Movement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil within the treated leaf of Stellaria media. (Figures in brackets represent the total radioactivity recovered at each harvest).

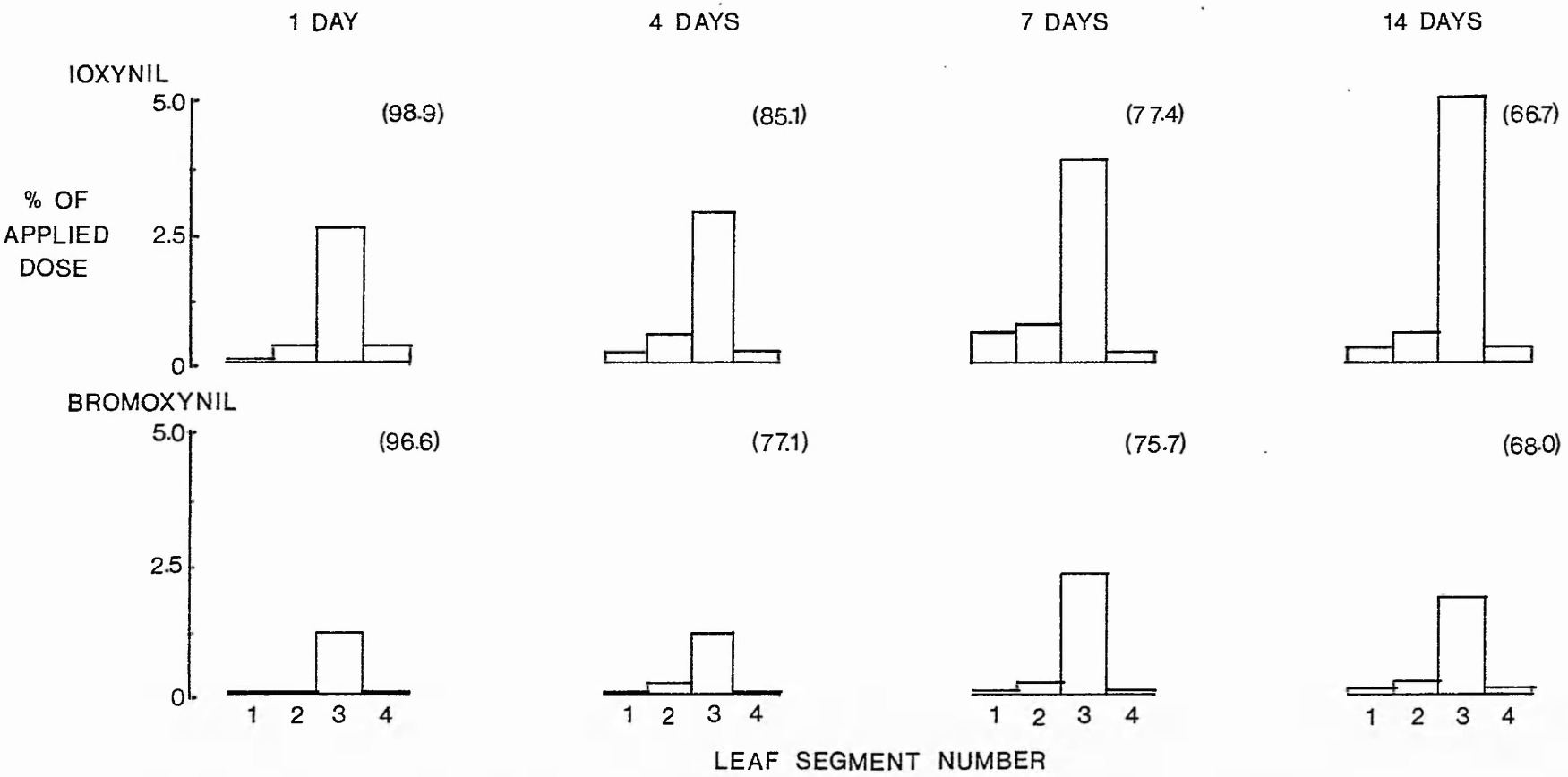


Figure 3.8: Movement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil within the treated leaf of Matricaria inodora. (Figures in brackets represent the total radioactivity recovered at each harvest).

Significantly less of either herbicide was detected in the treated segment of S. media (fig. 3.7) reflecting the lower hydroxybenzonitrile uptake in this species (fig. 3.5). The proportion of (^{14}C)-ioxynil recovered from the treated segment steadily increased to 10.62% after 14 days whereas the proportion of (^{14}C)-bromoxynil gradually declined from 2.10% after 4 days to 1.62% after 14 days. At the 7 and 14 day harvest a similar proportion of (^{14}C)-bromoxynil was also detected in segment 2 indicating some basipetal movement. Similarly a high proportion of (^{14}C)-ioxynil had moved in a basipetal direction in this species, although this may partially reflect the difficulty in cutting narrow segments from relatively small S. media leaves. As with V. arvensis there was a gradual decline in the total recovered herbicide with only 76.1% of the applied (^{14}C)-ioxynil and 68.9% of the applied (^{14}C)-bromoxynil detected at the 14 day harvest.

The lowest proportions of (^{14}C)-ioxynil and (^{14}C)-bromoxynil was detected in the treated segment of M. inodora (fig. 3.8). The proportion of (^{14}C)-ioxynil increased from 2.59% after 1 day to 5.02% after 14 days, whereas (^{14}C)-bromoxynil content reached a peak of 2.23% after 7 days and declined to 1.93% after 14 days. There was less movement from the treated segment of M. inodora than S. media although at each harvest proportionately more (^{14}C)-ioxynil and (^{14}C)-bromoxynil had moved in a basipetal rather than acropetal direction. The overall decline in total recovered radioactivity followed a similar trend to V. arvensis and S. media with 32.0% of the (^{14}C)-bromoxynil and 33.3% of the (^{14}C)-ioxynil undetected at the 14 day harvest. This decline in the recovery of radio-labelled herbicides necessitated a study of hydroxybenzonitrile movement within the whole plant, as the undetected herbicides may have moved out of the treated leaf.

The distribution of (^{14}C)-ioxynil and (^{14}C)-bromoxynil was investigated 4 and 7 days after treatment, corresponding to the period of most rapid symptom development (Table 2.5). Each

TABLE 3.3: Movement of ^{14}C-ioxynil and ^{14}C-bromoxynil 7 days after application to the youngest fully developed leaf

% of applied herbicide recovered from:-	IOXYNIL			BROMOXYNIL		
	VIOLA ARVENSIS	MATRICARIA INODORA	STELLARIA MEDIA	VIOLA ARVENSIS	MATRICARIA INODORA	STELLARIA MEDIA
treated leaf	16.562 (± 2.10)	7.486 (± 1.23)	10.940 (± 0.56)	3.547 (± 0.33)	1.344 (± 0.13)	4.462 (± 0.54)
apex + lateral shoots	0.101	0.613	0.025	0.198	0.095	0.172
developing leaves	0.001	0.056	0.131	0.005	0.036	0.081
mature leaves	0.000	0.006	0.042	0.005	0.021	0.036
stem	0.037	-	0.343	0.049	-	0.274
roots	0.074	0.103	0.057	0.077	0.040	0.074
aqueous wash	70.690 (± 3.346)	74.430 (± 3.39)	62.490 (± 10.96)	87.140 (± 0.02)	82.680 (± 3.88)	86.960 (± 1.75)
total recovered	84.150 (± 2.51)	80.600 (± 3.06)	74.040 (± 10.36)	91.430 (± 1.80)	84.920 (± 3.82)	92.260 (± 1.46)
"missing activity"	15.850	19.400	25.960	8.570	15.080	7.740

harvest yielded a similar trend in hydroxybenzonitrile movement and the 7 day data is presented in Table 3.3. Contrary to anticipated results almost all of the radioactivity was detected within the treated leaves of V. arvensis, M. inodora and S. media. In agreement with figures 3.6-3.8 upto 5-6 times more (^{14}C)-ioxynil than (^{14}C)-bromoxynil was detected within the treated leaf of each species. The lowest proportion of (^{14}C)-ioxynil (8.26%) and (^{14}C)-bromoxynil (1.54%) was recovered from the most susceptible plants (M. inodora). In this species however, (^{14}C)-ioxynil was more mobile with 0.613% detected in the apex and lateral shoots compared to only 0.101% in V. arvensis and 0.025% in S. media. A significant proportion of (^{14}C)-ioxynil (0.343%) and (^{14}C)-bromoxynil (0.274%) was detected in the stem of S. media. In addition, a relatively high proportion of mobile (^{14}C)-bromoxynil was detected within the apex and lateral shoots of each species.

When the total radioactivity recovered within the treated plants was added to that washed from the surface, 7.7 to 25.9% of the radio-labelled herbicides were unaccounted for. This proportion was considered too high to be ignored when compared with the total activity detected within the treated leaves. The possible fate of this "lost" activity will be discussed below.

3.3.3 Investigation of the undetected radio-labelled herbicides

There are various possible explanations for the apparent loss of radio-labelled herbicide from the test system. The (^{14}C ring)-labelled hydroxybenzonitriles may have been metabolically degraded into volatile derivatives including $^{14}CO_2$; exuded from the roots; photochemically degraded or volatized on the leaf surface; or lost through technical error.

The contribution of technical error was investigated by treating V. arvensis leaves with (^{14}C)-ioxynil and (^{14}C)-bromoxynil and immediately processing the tissue by the usual method (section 3.2.5.2). When compared to the total applied dose,

101.9 ± 0.78 and 100.6 ± 1.24 of (^{14}C)-ioxynil and (^{14}C)-bromoxynil respectively were detected in the processed samples. Clearly the herbicides were not forming volatile compounds through a reaction with either soluene-350, H_2O_2 or HCl. Errors in droplet application, tissue sampling and processing were unlikely to wholly account for losses in radioactivity, especially as consistently more ioxynil than bromoxynil remained undetected.

A further possibility is that resistant plants may selectively exude herbicides from their roots, for example cultured soybean plants have been shown to release atrazine, EPTC, and amiben into the culture medium (Moody, Kust and Buchholtz 1970). To test this hypothesis, the herbicides were applied to the youngest fully developed leaf of hydroponically grown plants, and the nutrient medium assayed at intervals for radioactivity. Table 3.4 shows that although upto 0.32% (V. arvensis:ioxynil) of the radio-labelled herbicide was detected in the nutrient medium, this did not account for the still considerable losses of (^{14}C)-ioxynil and (^{14}C)-bromoxynil. Hydroponically grown plants exhibited a similar distribution of (^{14}C)-hydroxybenzonitriles compared to soil grown plants (Table 3.3) although relatively more herbicide had penetrated. This may possibly relate to environmental differences in growth conditions, which may have reduced cuticle formation in growth chamber grown plants. Although hydroponically grown plants may have been physiologically different to soil grown plants, the data suggests that exudation through the roots is unlikely to be a major cause of the reduction in herbicide recovery.

Losses of the type described are normally ascribed to metabolic breakdown of the herbicide molecule, resulting in the release of $^{14}CO_2$ and other volatile (^{14}C)-compounds. The hydroxybenzonitriles used in the present study were ring-labelled, and thus ring fission would have to occur if metabolic breakdown is to explain the significant losses of (^{14}C)-ioxynil and (^{14}C)-bromoxynil. Numerous extraction and analytical procedures are

TABLE 3.4: Movement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil in hydroponically grown plants (7 days after treatment)

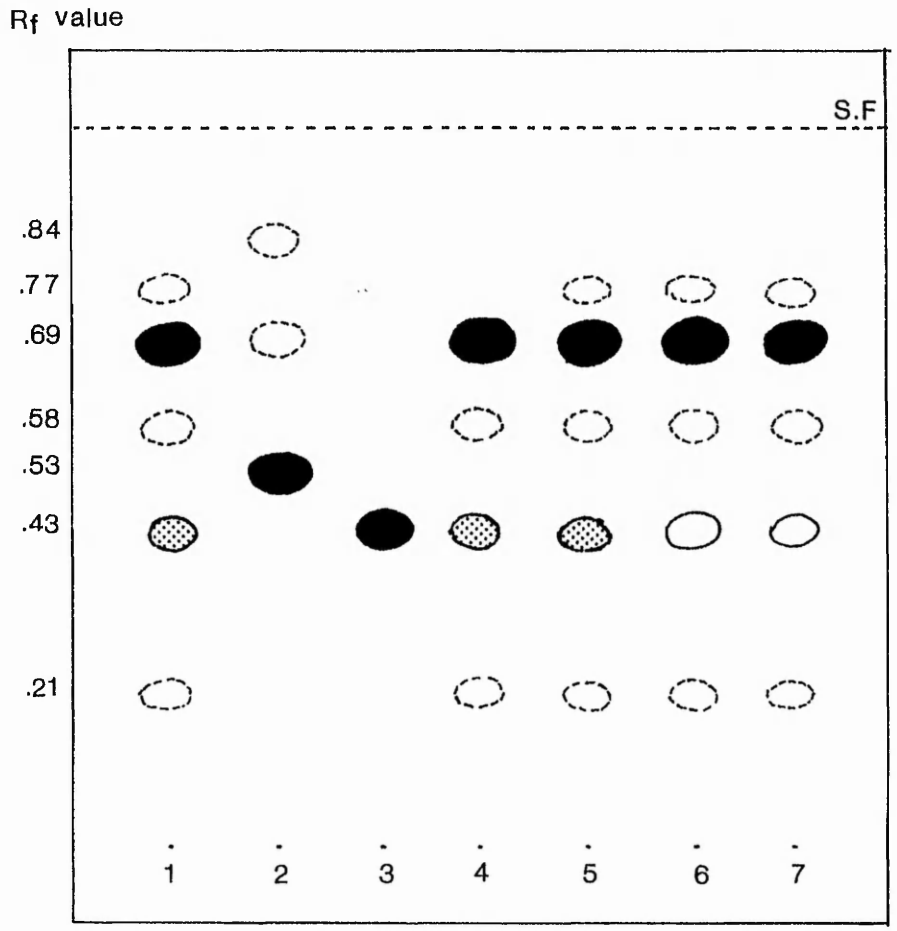
% of applied dose recovered from:-	IOXYNIL			BROMOXYNIL		
	VIOLA ARVENSIS	MATRICARIA INODORA	STELLARIA MEDIA	VIOLA ARVENSIS	MATRICARIA INODORA	STELLARIA MEDIA
treated leaf	18.15 (± 1.57)	10.36 (± 0.98)	18.91 (± 1.13)	3.46 (± 0.52)	2.28 (± 0.04)	5.12 (± 0.79)
apex + lateral shoots	0.114	0.344	0.000	0.112	0.333	0.020
other leaves	0.000	0.010	0.010	0.000	0.162	0.023
roots	0.107	0.270	0.246	0.107	0.335	0.256
nutrient medium	0.319	0.040	0.000	0.125	0.030	0.000
aqueous wash	50.13 (± 3.37)	70.21 (± 1.45)	66.45 (± 1.97)	78.45 (± 2.08)	74.21 (± 2.71)	73.84 (± 1.09)
total recovered	71.57 (± 2.60)	80.98 (± 0.73)	85.21 (± 2.37)	81.95 (± 2.06)	77.34 (± 2.76)	79.61 (± 1.17)
"missing activity"	28.43	19.02	14.79	18.05	22.70	20.39

documented for the hydroxybenzonitriles, and after consultation of the literature an extraction procedure based on Hsu and Camper (1975a, 1979) and Pallett (1978) was established. The efficiency of the extraction procedure was tested by addition of 30 µl of (^{14}C)-ioxynil (≈ 75,000 dpm) to 6 replicate leaf samples prior to centrifugation. 80.7% (± 2.4) was detected in the ethyl acetate extract, 3.7% (± 0.13) in the aqueous zinc sulphate extract (after separation) and 18.5% (± 0.57) remained in the pellet at the end of the centrifugation step. This method was used to determine qualitative changes in ioxynil and bromoxynil 4 days after treatment of M. inodora, and 7 days after treatment of V. arvensis, S. media and M. inodora. M. inodora was harvested at two separate times because of the rapidity of hydroxybenzonitrile action in this species.

Figures 3.9 and 3.10 represent typical thin layer chromatograms of extracts from ioxynil and bromoxynil treated plants, and appropriate standards (where available). Halogen containing compounds were identified using the F.F.C.A. reagent (Zappi 1967) and any spots which stained blue in untreated control extracts were omitted from the chromatograms shown in figures 3.9 and 3.10. A major feature of these chromatograms is the apparent impurity of ioxynil and bromoxynil. Five compounds were detected for ioxynil: a primary spot of Rf value 0.69 considered to be ioxynil, a secondary compound identified as 3-iodo-4-hydroxybenzonitrile (Rf = 0.43) and traces of 3 other unidentified compounds (Rf = 0.21, 0.58, 0.77). The impurity of ioxynil nullified detailed quantitative analysis of the plant extracts, although this data did suggest that there were no major changes in ioxynil structure. Similarly, free iodide was not detected when the extracts were ran in 100 Butanol:28 water:25 ethanol (after Pallett 1978).

Bromoxynil separated into a major compound of Rf value 0.59 considered to be bromoxynil, and a minor compound of Rf value 0.68. Interestingly, V. arvensis and to a lesser extent S. media appeared to metabolically change the proportions

Figure 3.9: Thin layer chromatogram of ioxynil treated plant extracts. Intensity of spots declined in the order ● > ◎ > ⬭ > ◌.



1 ioxynil-Na

2 3,5diiodo-4-hydroxy benzoic acid

3 3-iodo-4-hydroxybenzonitrile

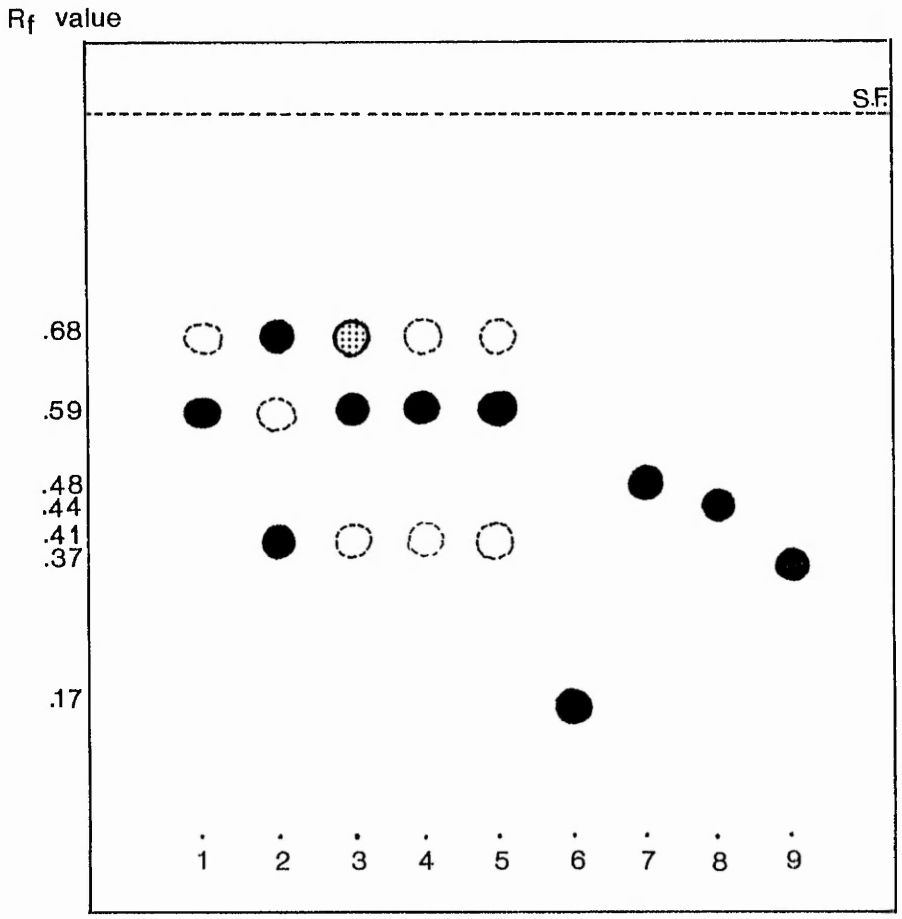
4 V.arvensis extract (7days)

5 S.media extract(7days)

6 M.inodora extract (4 days)

7 M.inodora extract (7 days)

Figure 3.10: Thin layer chromatogram of bromoxynil treated plants. Intensity of spots declined in the order ● > ◎ ≫ ○



1 bromoxynil-K

2 V.arvensis extract (7 days)

3 S.media extract (7 days)

4 M.inodora extract (4 days)

5 M.inodora extract (7 days)

6 3,5-dibromo-4-hydroxybenzamide

7 3,5-dibromo-4-hydroxybenzoic acid

8 3-bromo-4-hydroxybenzonitrile

9 3-bromo-4-hydroxybenzoic acid

of these two compounds, and lead to an additional unidentified compound of Rf value 0.37. Thus qualitative evidence was obtained for metabolic changes in the bromoxynil molecule. The compounds formed could not be identified by the standards provided, but may possibly be sugar or amino acid conjugates of bromoxynil or its metabolites.

The undetected radiolabelled herbicides may not necessarily have penetrated into the leaf tissues and could have been lost from the leaf surface. This possibility was investigated by comparing losses from artificial leaf surfaces, with an explant system and with the normal uptake method. From figure 3.11 it can be seen that losses of (^{14}C)-ioxynil and (^{14}C)-bromoxynil from wax treated coverslips were relatively similar with 10-15% of the radiolabel lost within 48 hours and a further 10-15% loss between the 2 and 14 day harvests. Losses from the explant system followed a similar trend with upto 26.6% of (^{14}C)-bromoxynil and 18.33% of (^{14}C)-ioxynil lost within 4 days of treatment of M. inodora explants. Losses were highest from explants of this species, whereas the recovery of (^{14}C)-ioxynil and (^{14}C)-bromoxynil from V. arvensis and S. media explants and artificial surfaces was relatively similar. At the 14 day harvest consistently more (^{14}C)-bromoxynil (33.1-43.0%) than (^{14}C)-ioxynil (16.4-28.3%) was not detected in the explant system. Interestingly, after 48 hours a similar proportion of (^{14}C)-ioxynil and (^{14}C)-bromoxynil was missing from the treated surface of whole plants and wax treated coverslips suggesting minimal uptake. Between 2 and 14 days the differences between the proportion of hydroxybenzonitriles recovered from artificial surfaces and whole plants were inconsistent and estimations of the actual uptake could not be made.

Detailed 7 day results are presented in Table 3.5. When compared with the whole plant situation (Table 3.3) there was consistently less (^{14}C)-ioxynil uptake by explants but comparatively similar (^{14}C)-bromoxynil uptake. Minimal levels of herbicide had moved out of V. arvensis and M. inodora explants, and

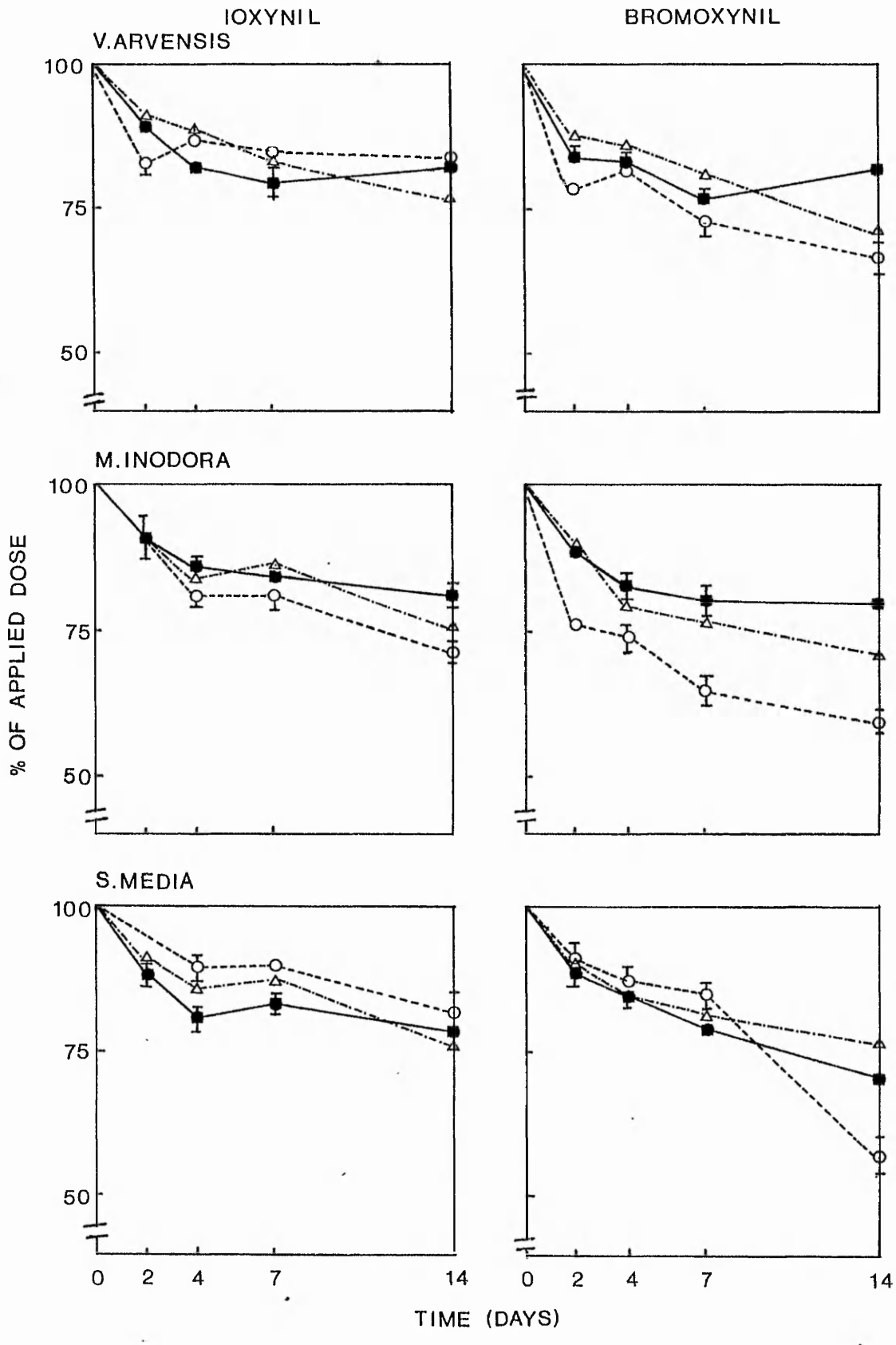


Figure 3.11: Recovery of (^{14}C)-ioxynil and (^{14}C)-bromoxynil from artificial surfaces (—■—), leaf surfaces (--△--) and an explant system (--○--).

TABLE 3.5: Recovery of ^{14}C-ioxynil and ^{14}C-bromoxynil from plants, explants and artificial surfaces 7 days after treatment (% of applied herbicide)

	IOXYNIL			BROMOXYNIL		
	VIOLA ARVENSIS	MATRICARIA INODORA	STELLARIA MEDIA	VIOLA ARVENSIS	MATRICARIA INODORA	STELLARIA MEDIA
PLANT						
% washed from treated leaf	82.81 (± 1.21)	79.53 (± 2.10)	86.90 (± 1.25)	81.38 (± 1.20)	72.66 (± 3.20)	81.06 (± 2.32)
% missing	17.19	20.47	13.10	18.17	27.34	18.94
EXPLANT						
% washed from treated leaf	77.50 (± 0.71)	72.58 (± 1.65)	78.61 (± 2.38)	69.52 (± 3.57)	61.77 (± 2.59)	66.65 (± 3.82)
% recovered in leaf and petiole	8.33 (± 0.66)	5.81 (± 0.54)	9.61 (± 1.4)	3.86 (± 0.61)	2.70 (± 0.38)	3.34 (± 0.71)
% recovered in nutrient medium	0.00	0.11	0.85	0.00	0.07	0.34
TOTAL MISSING	14.95(± 0.94)	18.21(± 1.54)	10.12(± 0.72)	26.90(± 3.04)	35.01(± 1.88)	24.37(± 0.97)
ARTIFICIAL SURFACE						
% washed from surface	62.51 (± 0.03)	72.53 (± 0.84)	66.93 (± 2.24)	73.94 (± 1.82)	80.50 (± 2.03	73.14 (± 0.78)
% retained on coverslip	17.61 (± 1.16)	11.70 (± 0.44)	15.31 (± 0.59)	3.44 (± 0.25)	2.82 (± 0.22)	6.60 (± 0.39)
TOTAL MISSING	20.33 (± 2.67)	15.69 (± 0.93)	17.32 (± 1.53)	22.68 (± 1.99)	20.32 (± 2.00)	20.58 (± 0.70)

the higher proportion of movement evident from S. media explants is most likely to reflect technical difficulties in retaining the short petiole in the nutrient medium. Table 3.5 also shows that although losses of (^{14}C)-ioxynil and (^{14}C)-bromoxynil were relatively similar from artificial surfaces, 2.3 to 5.1 times more (^{14}C)-ioxynil had adsorbed onto the wax treated coverslips than (^{14}C)-bromoxynil.

Artificial surfaces were also used to investigate the relative importance of light in inducing losses of hydroxybenzonitriles. Recovery of (^{14}C)-ioxynil in the light (66.95% ± 5.41 mean of 6, 14 days after treatment) were not significantly different to dark recoveries (65.58% ± 3.05). Therefore losses of (^{14}C)-ioxynil and (^{14}C)-bromoxynil were more likely to result from volatilization and non-photochemical breakdown on the leaf surface, rather than photochemical decomposition.

Attempts were made to trap volatile compounds by placing (^{14}C)-ioxynil treated waxed coverslips in a closed system. Air was pumped across the coverslips and through an aqueous (distilled water) and organic (acetone) trap. After 14 days, 68.17% (mean of 4) was retained on the coverslip, 0.18% collected in the aqueous trap, 0% in the organic trap and 2.72% washed from the connecting rubber tubing. Although attempts to trap (^{14}C)-ioxynil were unsuccessful, presence in the rubber tubing proves the release of volatile compounds. It is likely the remaining (^{14}C)-ioxynil either adsorbed onto and passed through the rubber tubing, or failed to be trapped by water or acetone. Attempts to use ethyl acetate as a trap (Hsu and Camper 1975) were unsuccessful as the ethyl acetate frequently evaporated to dryness. Acetone was less volatile, and the majority of possible breakdown products were soluble in this solvent.

Generally the difference between recovery from artificial surfaces and intact plants could not be interpreted as herbicide uptake. The wax treated coverslips did not therefore completely model the leaf surface, but it can be concluded that a significant proportion of the radiolabelled herbicides "disappear" from the leaf surface and do not penetrate the leaf cells.

3.4 DISCUSSION

S. media, V. arvensis and M. inodora are morphologically contrasting weed species (plates 2.2-2.4). The differing leaf angles and surface areas of each species affected the degree of spray interception, and may well have provided the major explanation for interspecific selectivity. However, on a weight basis, the most susceptible species M. inodora, retained the least quantity of aqueous dye solution (Table 3.1). Nevertheless, some evidence has been obtained to suggest that the M. inodora leaf surface was more readily wetted than that of V. arvensis. Contact angles were lower, and the reduced ketone and ester content of wax extracts has previously been equated with easily wetted surfaces (Fernandes 1963; Holloway 1970). Separate wax extraction from adaxial and abaxial surfaces would have qualified this assumption.

Positive differences in retention undoubtedly contribute to a greater or lesser degree to the selectivity of most herbicides, and the relative importance in hydroxybenzonitrile activity appears to depend on the margin of difference. For example, ioxynil selectivity between mustard (Sinapis alba L., c.v. English White), pea (Pisum sativum L., c.v. Big Ben) and barley (Hordeum distichon L. c.v. Proctor) was largely attributed to differential retention (Davies, Drennan, Fryer and Holly 1967, 1968b). In contrast however, coast fiddleneck (Amsinckia intermedia, Fisch and Mey) was estimated to be 30 times more susceptible than winter wheat (Triticum aestivum L. var. Nugaines) even when differences in spray retention and penetration were accounted for (Schafer and Chilcote 1970a & b). In the present study, differences in retention were not sufficient to explain the differential action, and other factors must clearly be involved.

To exert a phytotoxic effect, a herbicide must diffuse across the cuticle and penetrate into the underlying cells. This process is normally monitored by examining the disappearance of radiolabelled herbicides from the leaf surface (e.g. Somerville 1972; Schafer and Chilcote 1970b; Devine, Bestman, Hall and Vanden-Born 1984). Thus it is generally assumed that all herbicide missing from the leaf surface must have penetrated into the leaf. In the present study however,

large differences were found between the actual recovery from the treated plant, and the theoretical uptake. The significance of this will be discussed later, and meanwhile uptake will be considered to be the percentage of herbicide recovered within the treated plant (Table 3.6, whole plant translocation data). In each species there was greater ioxynil than bromoxynil uptake, and in the majority of interactions there was little or no further uptake between 4 and 7 days. The exceptions were ioxynil treatment of V. arvensis and bromoxynil treatment of S. media, each of which were classified as moderately resistant interactions in section 2.4. On this basis, greater bromoxynil uptake by V. arvensis was anticipated. With this exception it appears that in susceptible and moderately susceptible interactions the hydroxybenzonitriles must limit their own uptake possibly by interferring with the energy requiring processes of underlying cells.

In comparison with other herbicides (e.g. difenzoquat, Pallett 1983a & b; and isoproturon, McIntosh, Robertson and Kirkwood 1981), the rate of hydroxybenzonitrile uptake by S. media, V. arvensis and M. inodora was relatively slow. This could possibly have been enhanced by the addition of surfactants such as Tween 20 and Actipron which are known to increase the uptake of other herbicides (e.g. bentazon, Dunleavy 1983). Nevertheless sufficient herbicide was penetrating into each species to exert a phytotoxic effect, and selectivity appears to be independant of the rate of uptake by the three experimental species.

The observed difference between actual and theoretical hydroxybenzonitrile recovery from treated plants has been noted previously but not fully explained. Somerville 1972 considered losses of 21.6% ioxynil and 49.1% bromoxynil within 24 hours of S. media treatment to result from metabolic breakdown to $^{14}CO_2$. Little evidence was provided to substantiate the theory and radiolabelled degradation products were not found in treated tissues. Similarly 10-11% losses of bromoxynil within 4 days of treatment were noted, but not explained by Schafer and Chilcote 1970b.

In the present study, the losses were unlikely to result from either breakdown during tissue processing or from selective root exudation. Metabolic breakdown to $^{14}CO_2$ or volatile (^{14}C)-compounds would necessitate ring cleavage; and evidence from soil culture studies

strongly suggests that this occurs several stages after ioxynil breakdown to 3, 5-diiodo-4-hydroxybenzoic acid (Hsu and Camper 1979). As shown in fig. 3.9 there were no marked changes in the composition and intensity of iodonated compounds extracted from the three experimental species. In particular, no compounds corresponding to 3,5-diiodo-4-hydroxybenzoic acid were detected, and therefore it is unlikely that ring fission of the ioxynil molecule occurs. In contrast, some evidence of bromoxynil breakdown was obtained from V. arvensis and possibly S. media extracts. The halogenated compounds could not be identified by the standards provided even though these represented the most probable breakdown products. The compound of Rf-value 0.37 extracted from V. arvensis and in trace quantities from S. media and M. inodora may possibly represent a halogenated conjugate, whereas the compound of Rf-value 0.69 is more likely to be a direct breakdown product of bromoxynil as it is present in smaller quantities in bromoxynil solutions. Either or both compounds may be less phytotoxic and it can be tentatively suggested that the ability of V. arvensis to metabolically change bromoxynil may contribute to the resistance of this species. Furthermore metabolic breakdown may explain why there was no increase in (^{14}C)-bromoxynil uptake between 4 and 7 days after treatment of V. arvensis. However, this is highly speculative as little or no formal evidence has been obtained.

Metabolic breakdown to volatile compounds is therefore unlikely to explain the observed losses of (^{14}C)-ioxynil, and may only partially explain losses of (^{14}C)-bromoxynil. A comparative study of residual radioactivity on various real and artificial leaf surfaces revealed a more probable explanation of the lost activity. It can be concluded from Fig. 3.11 that at least 10% of (^{14}C)-ioxynil and (^{14}C)-bromoxynil was lost from the leaf surface within 2 days of treatment. This light independant loss may initially occur during droplet evaporation followed by further volatilization of the herbicide residue.

Ioxynil and bromoxynil volatility on the leaf surface proved difficult to quantify. In a previous study, Foy (1964) measured (^{14}C)-ioxynil volatility by counting emissions from a metal planchette. No significant loss was detected after 30 hours at room temperature whereas rapid volatility losses (approaching 90-95% after 15 hours) occurred at

60°C. Herbicide volatility can also be measured by trapping the vapour phase of a herbicide solution and quantifying the trapped herbicide by gas chromatography (Grover 1975). A bioassay is commonly used in industry in which treated plants are placed with untreated sensitive plants in a wind tunnel; symptom development on the susceptible species indicates volatility problems. Each of these methods, whilst establishing the occurrence of volatility, fail to accurately quantify herbicide loss in relation to the treated leaf. In the present study it was necessary to mimic the leaf surface, with and without the influence of herbicide penetration. Wax coating of coverslips overcame initial problems of adsorption onto the glass; and the explant system (leaf & petiole) of Kirkwood (1978) was used as an alternative to hydroponically grown plants. Such leaves closely resembled the intact plant, and were relatively easily harvested to give a total radioactivity content.

Although it has not been possible to directly measure volatilized herbicide, considerable indirect evidence has been accumulated to substantiate the theory that the majority of undetected activity does not contribute to the phytotoxic action of the hydroxybenzonitriles. After consideration of this factor, the wholeplant movement data was reanalysed to determine the relative importance of movement in hydroxybenzonitrile selectivity. Table 3.7 shows the distribution of (^{14}C)-ioxynil and (^{14}C)-bromoxynil 4 and 7 days after treatment represented as the percentage of radioactivity recovered within the treated plant. There was a clear relationship between the mobility of the herbicide, and the ability of the plant to recover from herbicide treatment.

Although greater (^{14}C)-ioxynil uptake occurred in V. arvensis 98-99% remained within the treated leaf, and there was no significant movement to the apex and developing leaves. Proportionately more (^{14}C)-ioxynil moved from the treated leaves of S. media, and the largest mobile percentage was detected in the stem at each harvest. The lack of build up in the apex and laterals may explain the rapid regrowth of side shoots in this species. (^{14}C)-ioxynil was even more mobile in susceptible M. inodora in which upto 8.1% was concentrated in the apex, and presumably interfering with shoot development.

Generally less (^{14}C)-bromoxynil penetrated into the treated leaves, although the herbicide appeared more mobile in all three species than (^{14}C)-ioxynil. (^{14}C)-bromoxynil appeared particularly mobile in M. inodora and at the 4 day harvest 7.1% was present at the growing point and a further 8.1% had accumulated in the mature leaves. This additional mobility throughout the plant may contribute to the greater phytotoxicity of bromoxynil compared to ioxynil in this species. A similar percentage of (^{14}C)-bromoxynil had moved from the treated leaves of S. media but at each harvest a significant proportion was detected in the stem. (^{14}C)-bromoxynil was detected in similar proportions in the apex and laterals of S. media and V. arvensis, although these tissues showed no visible herbicide symptoms.

Evidence therefore suggests that there are two forms of hydroxybenzonitrile movement i.e. a contact effect in treated tissue, and a translocated effect involving movement to the growing points. Any movement within the treated leaf may be attributed to "diffusion and leakage from successively injured cells"(Foy 1964), probably caused by an interference with chloroplastic and mitochondrial processes (see Chapters 4 and 5). The hydroxybenzonitriles may also limit their own movement by interferring with the energy requiring processes believed to be involved in phloem loading and translocation (Sonovick, Geiger and Fellows, 1974).

The presence of (^{14}C)-ioxynil and (^{14}C)-bromoxynil in the apex and lateral shoots does however prove that a small but significant proportion of the herbicides are translocated along the same route as assimilates. This is in agreement with Foy 1964; Carpenter et al (1964); Davies et al (1963a); and Schafer and Chilcote (1970b) also found that bromoxynil was more mobile in susceptible coast fiddleneck (Amsinckia intermedia, Fisch and Mey) and resistant winter wheat (Triticum aestivum L., var. Nugaines).

The relative importance of these two forms of movement may largely contribute to the selectivity of the hydroxybenzonitriles. For example, this evidence suggests that ioxynil has a contact effect only against moderately resistant V. arvensis, whereas a combination of a contact effect and rapid translocated action may enhance the susceptibility of

M. inodora to bromoxynil. However, the herbicides may not be metabolically active at each of these sites, and in vivo and in vitro studies on the mode of action of ioxynil and bromoxynil were undertaken (Chapters 4 and 5).

TABLE 3.6: Uptake of ^{14}C-ioxynil and ^{14}C-bromoxynil: % of applied activity recovered within the treated plant

	IOXYNIL		BROMOXYNIL	
	4 days	7 days	4 days	7 days
V. arvensis	12.21	16.77	3.91	3.83
M. inodora	7.08	8.26	1.48	1.54
S. media	11.84	11.54	2.76	5.09

TABLE 3.7: Movement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil within soil grown plants: Data recalculated as the percentage recovered within the treated plant

	IOXYNIL					
	V. ARVENSIS		M. INODORA		S. MEDIA	
	4 days	7 days	4 days	7 days	4 days	7 days
Treated leaf	98.14	98.73	93.68	90.59	93.19	94.82
Apex + developing leaves	0.76	0.61	3.17	8.10	1.21	1.35
Other leaves	0.25	0.00	1.56	0.06	2.18	0.37
Stem	0.23	0.22	-	-	2.69	2.97
Roots	0.62	0.44	1.59	1.25	0.73	0.49
	BROMOXYNIL					
Treated leaf	89.49	91.39	82.13	87.50	82.51	87.51
Apex + developing leaves	5.55	5.30	7.11	8.53	3.22	4.96
Other leaves	0.97	0.04	8.05	1.37	3.91	0.71
Stem	2.25	1.26	-	-	9.09	5.37
Roots	1.74	2.01	2.71	2.60	1.27	1.45

CHAPTER FOUR: A PHYSIOLOGICAL STUDY OF PHOTOSYNTHETIC INHIBITION IN TWO CONTRASTING SPECIES

4.1 INTRODUCTION

The chlorotic and necrotic symptoms of hydroxybenzonitrile action are typical of photosynthetic inhibitor herbicides (section 1.3.6). Symptom development was most rapid in M. inodora, whereas ioxynil and bromoxynil were considerably less active against V. arvensis. This difference may partially reflect either slower photosynthetic inhibition in this species, or rapid recovery from an initial inhibition. Photosynthetic inhibition can be monitored in attached leaves by infra red gas analysis, or by placing detached leaves in a leaf disc electrode.

The infra red gas circuit was first introduced by Gaastra (1959) and adapted for herbicide study by Van Oorschot and Belksma (1961). These authors applied herbicides to the nutrient solution of hydroponically grown plants and were able to monitor CO_2 uptake before, during, and after herbicide treatment. In recent years, this group have used the system to investigate the influence of various herbicide treatments on photosynthesis, photorespiration, and transpiration (Van Oorschot 1974 ; 1980), and also between (van Oorschot & Van Leeuwen 1979) and within species (Van Oorschot and Van Leeuwen 1984).

In a review of photosynthetic inhibition, Moreland and Hilton (1976) distinguished between four hypothetical responses of plants to root absorbed herbicides. Readily absorbed and translocated compounds, such as s-triazines, phenyluneas, and uracils were likely to completely inhibit photosynthesis within 24 hours, whereas less readily absorbed herbicides may take longer to induce the same effect. Inhibitory uncouplers may also initially inhibit CO_2 uptake, although complete inhibition may not occur as the energy supply for active root uptake is interferred with. Finally, tolerant species may exhibit some inhibition followed by recovery to untreated rates. Similar responses may occur with foliar spray applications although for obvious reasons effects can only be monitored after treatment. In the present study, the short and long term effects of ioxynil and bromoxynil on CO_2 uptake have been determined using the IRGA circuit developed in this laboratory by Dunleavy (1983).

As previously described, electron transport inhibition leads to a reduction in the rate of CO_2 fixation (section 1.3.6), and the resulting changes in CO_2 uptake are normally monitored by infra red gas analysis. Measurement of the associated oxygen evolution has until recently been limited by problems of sensitivity and stomatal closure at high CO_2 concentrations (Delieu and Walker 1981). However, extension of the oxygen electrode principle led to the development of a leaf disc electrode for the measurement of gaseous oxygen evolution by leaf discs (Delieu and Walker 1981). The efficacy of this system for monitoring CO_2 dependent oxygen evolution by hydroxybenzonitrile treated leaves has been assessed in this study.

Associated with photosynthetic inhibition are a number of changes in cell ultrastructure. The general trends in symptom development were described in section 3.6 and are dependent upon the degree of photosynthetic inhibition. Complete inhibition leads to photooxidative destruction of all cellular membranes (Anderson and Thomson 1973; Pallett 1978; Pallett and Dodge 1980a) whereas partial inhibition may result in adaptive changes in chloroplast ultrastructure to maximise light interception and energy transfer (Fedtke, Deichgraber and Schnepf 1977; Lichtenthaler, Burkard, Grumbach and Meier 1980; Meier and Lichtenthaler 1981; Lichtenthaler, Kuhn, Prenzel and Meier 1982). Membrane disruption by ioxynil has been related to photooxidative and free radical processes (Pallett 1978; Pallett and Dodge 1980a) and to the uncoupling action of the herbicide (Moreland and Novitsky 1984).

In the present study, the development of ultrastructural symptoms will be related to pigment changes and photosynthetic inhibition in M. inodora and V. arvensis. This will further characterize the contrasting visual symptoms exhibited by these species. Problems with IRGA construction and sensitivity eliminated the possibility of monitoring CO_2 uptake by S. media (see section 4.3.2.); and as this species exhibited an intermediate response to ioxynil and bromoxynil, detailed investigations of the ultrastructural effects were not carried out.

4.2 MATERIALS AND METHODS

4.2.1 Calculation of leaf surface area

Twenty representative leaves were removed from each species and attached to an overhead projector acetate. The image was projected onto a screen and the outer perimeter of each leaf marked on a sheet of graph paper. The leaf shape was cut out, weighed, and the area determined by considering the weight of a known area of graph paper, and the magnification of the image. Various measurements of the leaf shapes were plotted against surface area until a linear relationship was established. For each species it was possible to determine the area from measurements of maximum width and length (fig. 4.1). Thus leaf surface area could be determined easily, and without damage to the leaf.

The following relationships were used to determine leaf surface area throughout this study:

V. arvensis

$$\text{Leaf surface area } (cm^2) = 2 \times \left(0.848 \left(\begin{matrix}\text{maximum} \\ \text{width}\end{matrix} \times \begin{matrix}\text{maximum} \\ \text{length}\end{matrix}\right) - 0.025\right)$$

(correlation coefficient = 0.993)

S. media

$$\text{Leaf surface area } (cm^2) = 2 \times \left(0.699 \left(\begin{matrix}\text{maximum} \\ \text{width}\end{matrix} \times \begin{matrix}\text{maximum} \\ \text{length}\end{matrix}\right) + 0.012\right)$$

(correlation coefficient = 0.996)

M. inodora

$$\text{Leaf surface area } (cm^2) = 2 \times \left(0.182 \left(\begin{matrix}\text{maximum} \\ \text{width}\end{matrix} \times \begin{matrix}\text{maximum} \\ \text{length}\end{matrix}\right) + 0.258\right)$$

(correlation coefficient = 0.862)

maximum length and width were measured in cm. Maximum length was measured from the base of the petiole to the tip of the leaf. The lower correlation coefficient for M. inodora reflects difficulties in accurately tracing and cutting out leaf images.

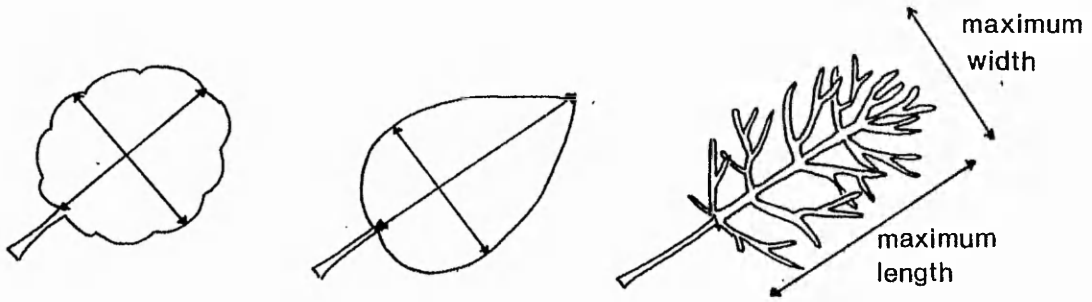


Figure 4.1: Calculation of leaf surface area: measurement of leaves

4.2.2 Measurement of oxygen evolution by leaf disc electrode

Plants were sprayed at 40 minute intervals with field rate ioxynil, distilled water, and bromoxynil. After 0, 1, 2, 4, 8, 16 and 24 hours, two fully expanded leaves were removed from each of 2 replicate plants and placed in the well of a Hansatech leaf disc electrode. The pre-assembled electrode contained an electrolyte of 1.0 M sodium bicarbonate/sodium carbonate in 50% saturated KCl, and Risler paper moistened with a bicarbonate/carbonate buffer (20 of 1M $NaHCO_3$:1 of 1M Na_2CO_3), to maintain a constant CO_2 concentration within the chamber. The reaction chamber was sealed, and the electrode calibrated with nitrogen by the method of Delieu & Walker (1981). After a dark incubation of 10 minutes, the reaction chamber was irradi ated with 750 µE. $m^{-2}.sec^{-1}$ supplied by a Griffin Halight projector. Twenty minutes later, the surface area of the 4 replicate leaves was determined (section 4.2.1), and the rate of oxygen evolution calculated in µmole O_2. $dm^{-2}.h^{-1}$. The experiment was repeated three times for V. arvensis and M. inodora.

4.2.3 Measurement of CO_2 uptake by infra red gas analysis (IRGA)

CO_2 uptake was measured by a G.P. instrument IRGA 120 in differential mode. The IRGA was attached by 5 mm polypropylene tubing to an auto analyser (ADC:WA161), which alternatively sampled air from the 6 assimilation chambers. Air pumped through the system from an outside resevoir was maintained at 25°C by passing through a controlled waterbath, and by a constant temperature water jacket surrounding the assimilation chamber. The chamber was evenly irradiated by a camrex solarcolour LGH PS/U sodium fluorescent tube (400 W) which could be adjusted to give light intensities in the range 0-1500 $\mu E.m^{-2}.s^{-1}$. Following an investigation of the effect of light intensity on net photosynthesis, the assimilation chamber was routinely irradiated with saturating light (750 $\mu E.m^{-2}.s^{-1}$). A heat shield consisting of a 3 cm deep perspex tray of circulating water was placed between the light source and the assimilation chamber and removed excess heat.

The assimilation chamber (fig. 4.2) was designed by the author to hold 5 replicate leaves and an empty reference chamber. The internal dimensions of the assimilation chamber were designed to allow fully developed leaves of V. arvensis and M. inodora to fit with only limited dead space.

Net photosynthesis was calculated as follows:- (After Gaastra 1959).

$$P.S. = Q \times \frac{1.96}{44} \times \frac{ppm}{2A} \quad \mu mole\ dm^{-2}.sec^{-1}$$

Q = air flow rate in $dm^3 s^{-1}$

2A = 2 x leaf surface area in dm^2

ppm = change in ppm

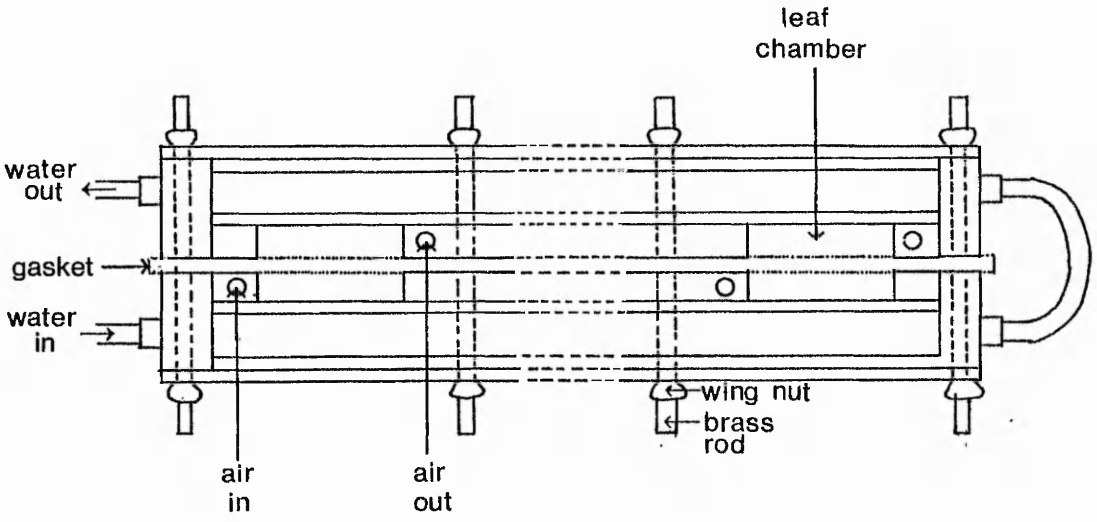


Figure 4.2: Plan of the infra-red gas analyser assimilation chamber. The assimilation chamber contained 6 individual leaf chambers of internal dimensions 3 x 3 x 1.5 cm.

Measurement of CO_2 uptake for 8 hours following treatment

At the beginning of each experimental period 3 plants were sprayed with herbicide whilst 2 were sprayed with distilled water as controls. When the spray droplets had dried, the youngest fully developed leaf of each plant was sealed into the assimilation chamber with non phytotoxic putty. The assimilation chamber was illuminated, and CO_2 uptake was measured for 5 minutes per chamber, at 30 minute intervals. Ioxynil and bromoxynil inhibition were monitored on alternate days as it was considered necessary to always include sufficient control leaves in the chamber during any experimental period. Six replicate plants per treatment were assayed.

Measurement of CO_2 uptake at intervals upto 14 days following treatment

Three groups of plants were sprayed with field rate herbicides or distilled water at 11.00, 13.30 and 16.00 hours. The youngest fully developed leaf of each plant was marked at the time of spraying and assayed for 2 hours (1 hour either side of spraytime), 1, 2, 3, 7, 11 and 14 days later. At the end of each time period CO_2 uptake was calculated as the mean of four recordings for each of 5 replicates. Plants were maintained in a controlled environment ($20^{\circ}C$ 14 hour day, $14^{\circ}C$ 10 hour night), for the duration of the experiment.

4.2.4 Electron Microscopy

Reagents

1. 0.1M phosphate buffer

   (a) Na_2HPO_4 $16.8g.l^{-1}$

   (b) NaH_2PO_4 $15.6g.l^{-1}$

   solutions (a) and (b) were mixed in a ratio of 3:2 until pH 7.0 and diluted to 0.05M for the washing of leaf samples.

2. 1% Soresens buffered gluteraldehyde (G.D.A.)

0.1M phosphate buffer	50ml
25% gluteraldehyde	12ml
Water	38ml

3. 2% $^{W}/_{v}$ Osmium tetroxide (OsO_4)

250mg of osmium tetroxide was dissolved in 12.5ml of 0.05M phosphate buffer and stored at 4°C in a stoppered glass bottle. (This reagent was prepared at least 48 hours before use to ensure all of the osmium tetroxide had dissolved).

4. Dehydration series

15%. 50% and 100% absolute alcohol.

5. Spurr's Low viscosity resin (Spurr, 1969).

Vinyl cyclohexane dioxide (Erl 4206)	10ml
Polypropylene glycol (Der 736)	6ml
Nonamyl sunninic anhydride (NSA)	26ml
Dimethylaminoethanol (5-1)	0.4ml

(prepared in advance, and stored in 20ml syringes at 4°C).

6. Uranyl acetate

A saturated solution was prepared in 70% ethanol.

7. Reynolds Lead citrate

1.33g of lead nitrate and 1.76g of sodium citrate were added to approximately 30ml of CO_2 free distilled water, and stirred for 30 minutes to ensure complete conversion of lead nitrate to lead citrate. After this time 8ml of 1M NaOH was added, and the solution made upto 50ml with CO_2 free distilled water.

Treatment of plants

V. arvensis and M. inodora plants were sprayed with field rate ioxynil, bromoxynil and water, and maintained in a controlled environment chamber (14 hour day 20°C, 10 hour night 14°C). Six

replicate plants were harvested for each treatment after 0, 2, 4 and 7 days.

Preparation of blocks

Approximately 10 1mm squares were quickly cut from the youngest fully developed leaf of 6 replicate plants, and immediately placed in a specimen tube containing G.D.A. When all sections were cut, the samples were placed under a low vacuum for 5 minutes to aid penetration of fixative. The samples were then incubated on a rotary shaker for 30 minutes at room temperature.

Following GDA fixation, the samples were washed three times with phosphate buffer, ensuring complete removal of GDA, before post fixing with OsO_4 for 1 hour on the rotary shaker. The samples were then washed with two 5 minute changes of phosphate buffer and one change of distilled water prior to the dehydration sequence. This sequence consisted of a 5 minute wash in 15% absolute alcohol, 50% absolute alcohol, and three 5 minute washes in 100% absolute alcohol.

The samples were incubated overnight in spurrs low viscosity resin on the rotator to allow impregnation of the sample. Four samples per treatment were then placed at the tips of flat embedding moulds and each mould was filled with fresh resin from a syringe. The moulds were incubated at 60°C for 72 hours until the resin was hard.

Ultramicrotomy

The resin blocks were trimmed and sectioned in a ultramicrotome. Sections were cut with a glass knife and floated onto sterile distilled water. After stretching with chloroform the ultra-thin sections were mounted on 400 mesh copper grids.

Staining of sections

Droplets of uranyl acetate were placed on parafilm in a glass petri dish. Each copper grid was washed in distilled water, dried on filter paper, inverted, and placed in a droplet of stain. After 30 minutes each grid was washed in 0.02M NaOH and placed in lead citrate stain which had been set up in the same way as

uranyl acetate. Four pellets of sodium hydroxide were added to the petri dish ensuring no reaction with CO_2 forming lead carbonate. The grids were then washed with 0.02M NaOH and distilled water, dried and stored in grid boxes.

Inspection of grids

The grids were inspected in a AEI 802 electron microscope. Electron micrographs were recorded on "Kodak 4489" electron microscope film, and printed on Ilford Ilfospeed photographic paper.

4.2.5 Chlorophyll analysis

Nine replicate plants per treatment were sprayed with field rate ioxynil, bromoxynil or water. After 7 days the aerial shoots were harvested, weighed and analysed for chlorophyll content and a:b ratio (see section 2.2.7).

4.2.6 Determination of the number of grana per stack

Electron micrographs were prepared of 10 typical chloroplasts from each 7 day treatment of V. arvensis. The number of grana per stack was counted for 5 granal stacks from each chloroplast and the mean number for 50 stacks calculated.

4.3 RESULTS

4.3.1 The effect of ioxynil and bromoxynil on oxygen evolution by M. inodora and V. arvensis

Measurement of oxygen evolution by detached leaves gives an indication of the photosynthetic status of the whole plant. Control leaves of V. arvensis and M. inodora exhibited a mean photosynthetic rate of 62.4 ± 4.67 and 143.68 ± 9.18 µmole O_2. $dm^{-2}hr^{-1}$ respectively. However, within each experiment, control rates fluctuated between 90-213 (M. inodora) and 22-95 µmole O_2. $dm^{-2}.hr^{-1}$ (V. arvensis). Respiration rates (i.e. O_2 uptake in the dark) exhibited similar variations i.e. mean control rates of O_2 uptake were 32.69 ± 3.01 (M. inodora) and 26.34 ± 6.82 (V. arvensis). These fluctuations in the control rate of oxygen evolution and uptake did not necessarily correlate with changes in treated leaves, and thus only general trends can be established using this technique.

Ioxynil and bromoxynil had little effect on oxygen evolution for the first 8 hours after treatment of V. arvensis (fig. 4.3). O_2 evolution declined rapidly in treated plants between the 8 and 16 hour measurements, and both herbicides were completely inhibitory within 24 hours of application. There was generally more inhibition of oxygen evolution during the first 8 hours after treatment of M. inodora. After 16 and 24 hours oxygen evolution was completely inhibited by bromoxynil, whereas ioxynil was less inhibitory. In each species, ioxynil and bromoxynil had little detectable effect on oxygen uptake (data not presented).

4.3.2 The effect of ioxynil and bromoxynil on CO_2 uptake by intact plants

The assimilation chamber was designed to minimise dead space and allow air to circulate freely around the system. Sensitivity was enhanced by reducing the air flow rate through the system thus maximising the difference in CO_2 concentration between the reference and sample leaf chambers. Fluctuations in ambient CO_2 concentrations caused residual variation in recordings and were partially overcome by drawing air from a partly sealed 50 l reservoir placed outside the laboratory. The same assimilation chamber could be used for similarly sized V. arvensis and M. inodora leaves whilst a much smaller chamber was necessary for S. media leaves. The relative changes in CO_2 concentration were too small in the latter to accurately monitor inhibition of CO_2 assimilation. Sensitivity of the GP instruments IRGA could not be improved and thus it was not possible to use this system for S. media. Similarly accurate measurements of dark respiration could not be made.

When grown under the described environmental conditions, net CO_2 assimilation in M. inodora and V. arvensis was found to saturate at a photon flux density of about 750 $\mu E.m^{-2}.s^{-1}$ (fig. 4.4). At this level, control rates of net photosynthesis were relatively constant averaging 72.9 $\pm$ 1.37 $nmole.dm^{-2}.hr$ (V. arvensis) and 101.8 $\pm$ 2.55 (M. inodora). There was also no

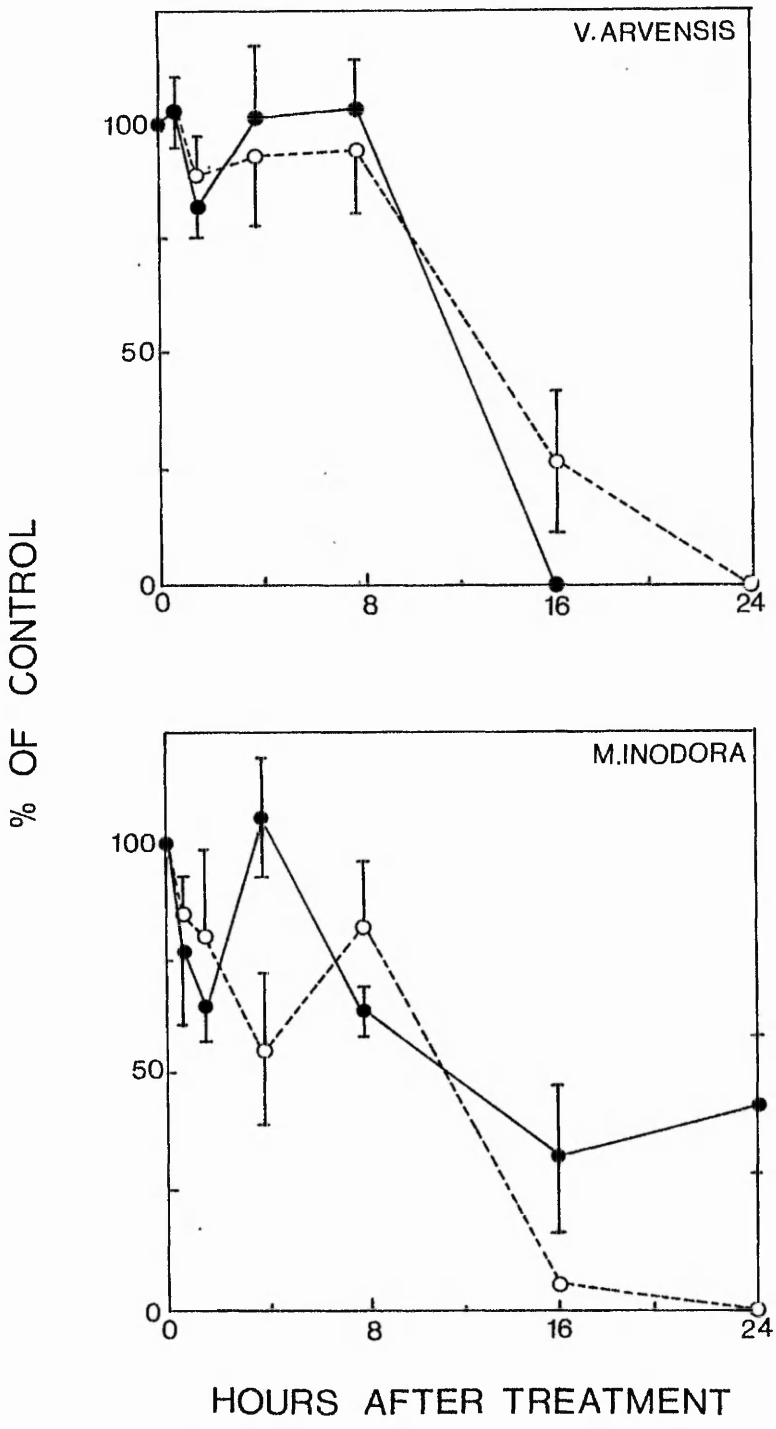


Figure 4.3: The effect of ioxynil (—●—) and bromoxynil (--o--) on oxygen evolution by V. arvensis and M. inodora. bars = standard errors.

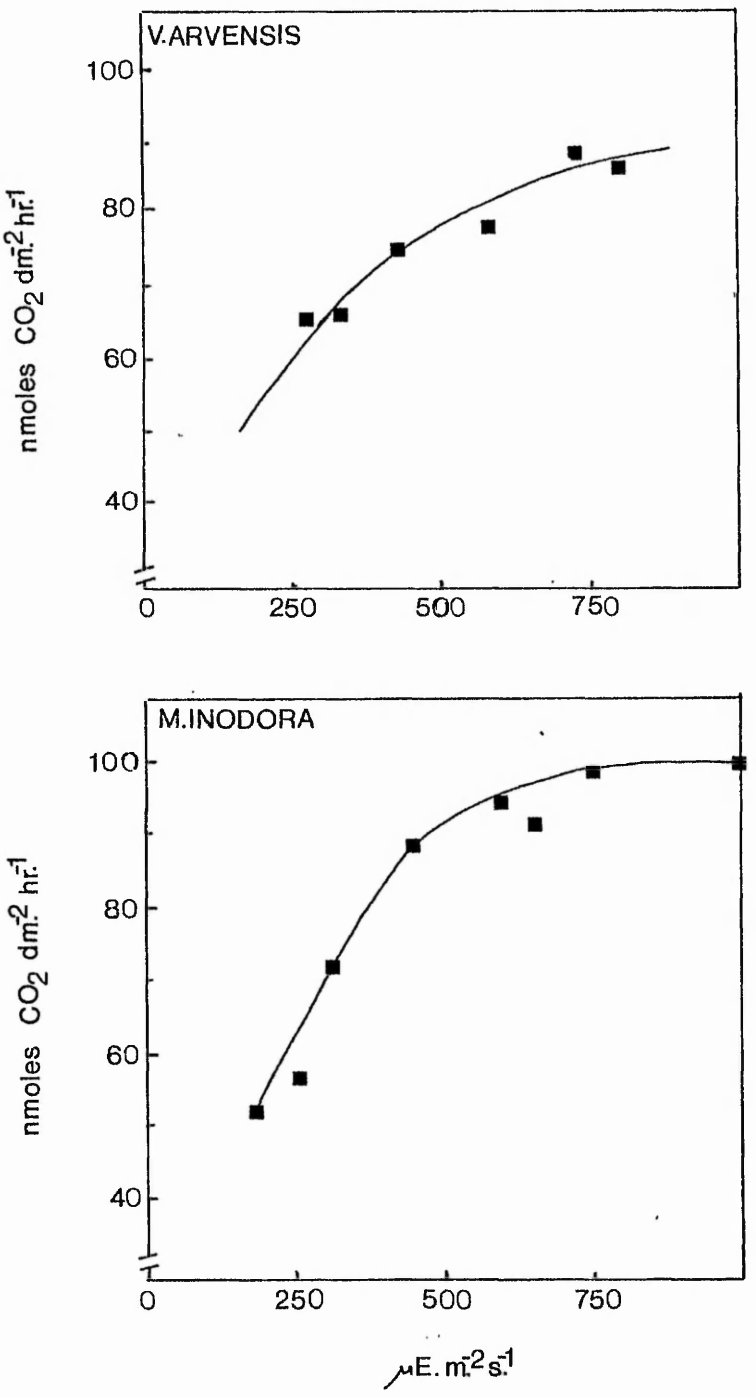


Figure 4.4: The effect of light intensity on CO_2 uptake by V. arvensis and M. inodora

significant decline in control rates over either the 8 hour or 14 day experimental period.

When plants were maintained in saturating light immediately following treatment, bromoxynil was found to be a more rapid inhibitor of CO_2 assimilation in both species (fig. 4.5). Under these conditions, bromoxynil inhibited CO_2 uptake by 90% within 7 hours of treatment whereas ioxynil caused only 40% inhibition. This difference was independant of the overall activity of the hydroxybenzonitriles in V. arvensis and M. inodora.

In a longer experiment, plants were maintained in the controlled environment chamber after treatment. Under the lower light intensities of the cabinet ($79 \pm 0.76\ \mu E.m^{-2}.s^{-1}$), CO_2 assimilation by both species was inhibited by 65-75% within 24 hours (fig. 4.6). Four days after treatment, ioxynil and bromoxynil completely inhibited net photosynthesis in treated M. inodora leaves. In contrast, CO_2 uptake by V. arvensis treated leaves recovered to approximately 35% of the control rate following bromoxynil treatment and 15% of the control rate after ioxynil treatment. These much reduced rates were maintained for the duration of the experiment.

4.3.3 The Ultrastructure of hydroxybenzonitrile treated leaves

Plates 4.1-4.24 are representative micrographs of mesophyll cells from treated and untreated M. inodora, V. arvensis and S. media. Each micrograph was chosen to illustrate typical ultrastructural symptoms at the treatment stage indicated in the legend. The sequence of symptom development in V. arvensis and M. inodora is illustrated in plates 4.1-4.21, and plates 4.22-4.24 represent typical 7 day symptoms in S. media.

There were marked similarities in the ultrastructure of untreated leaves from each species. Mesophyll cells were highly vacuolated with chloroplasts and other cytoplasmic inclusions adjacent to the cell wall and maintained within the tonoplast (plates 4.1, 4.2, 4.12, 4.13 and 4.22). Chloroplasts were discoid

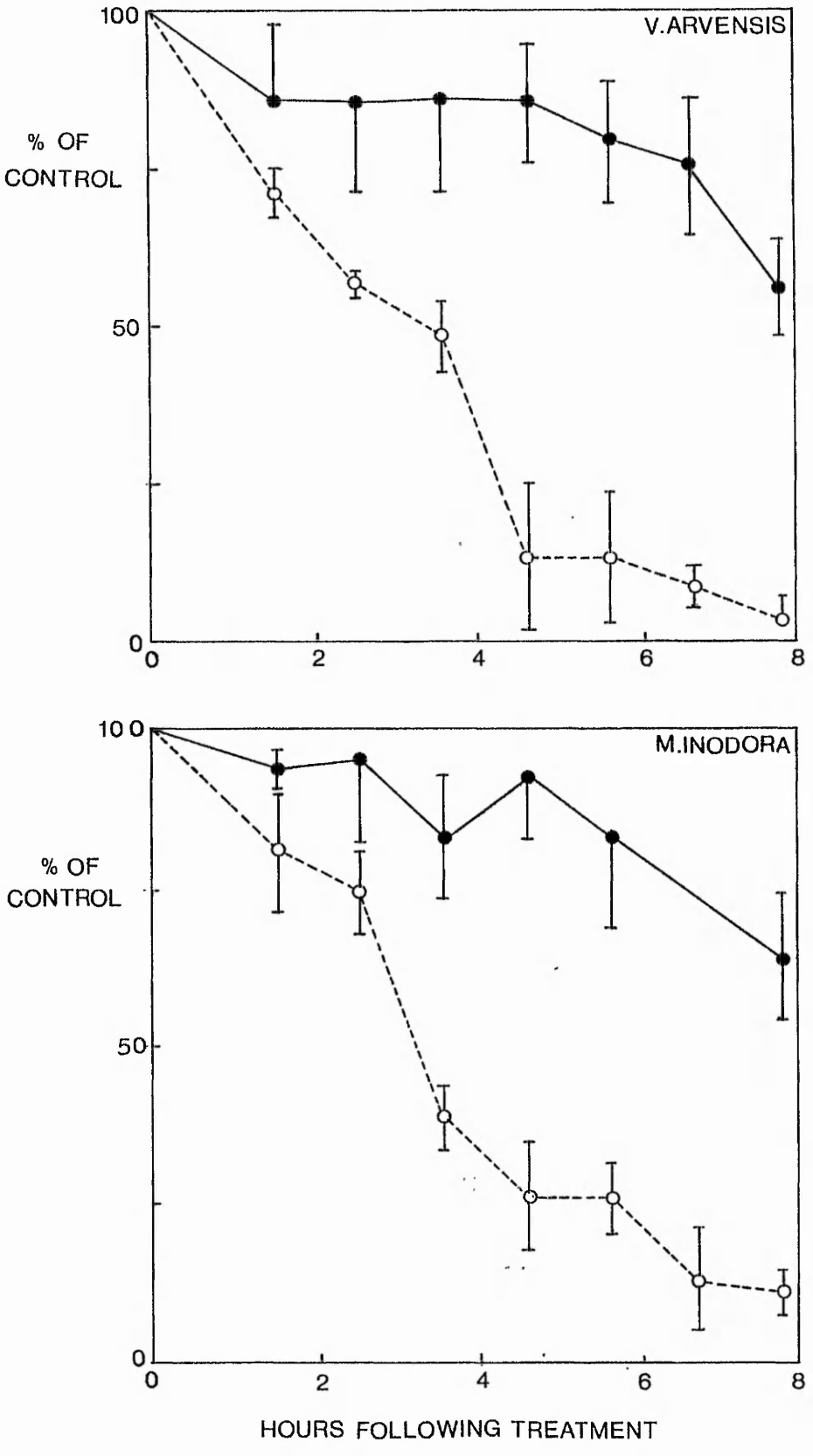


Figure 4.5: The effect of ioxynil (—●—) and bromoxynil (--o--) on CO_2 uptake during the first 8 hours after treatment. Bars = standard errors.

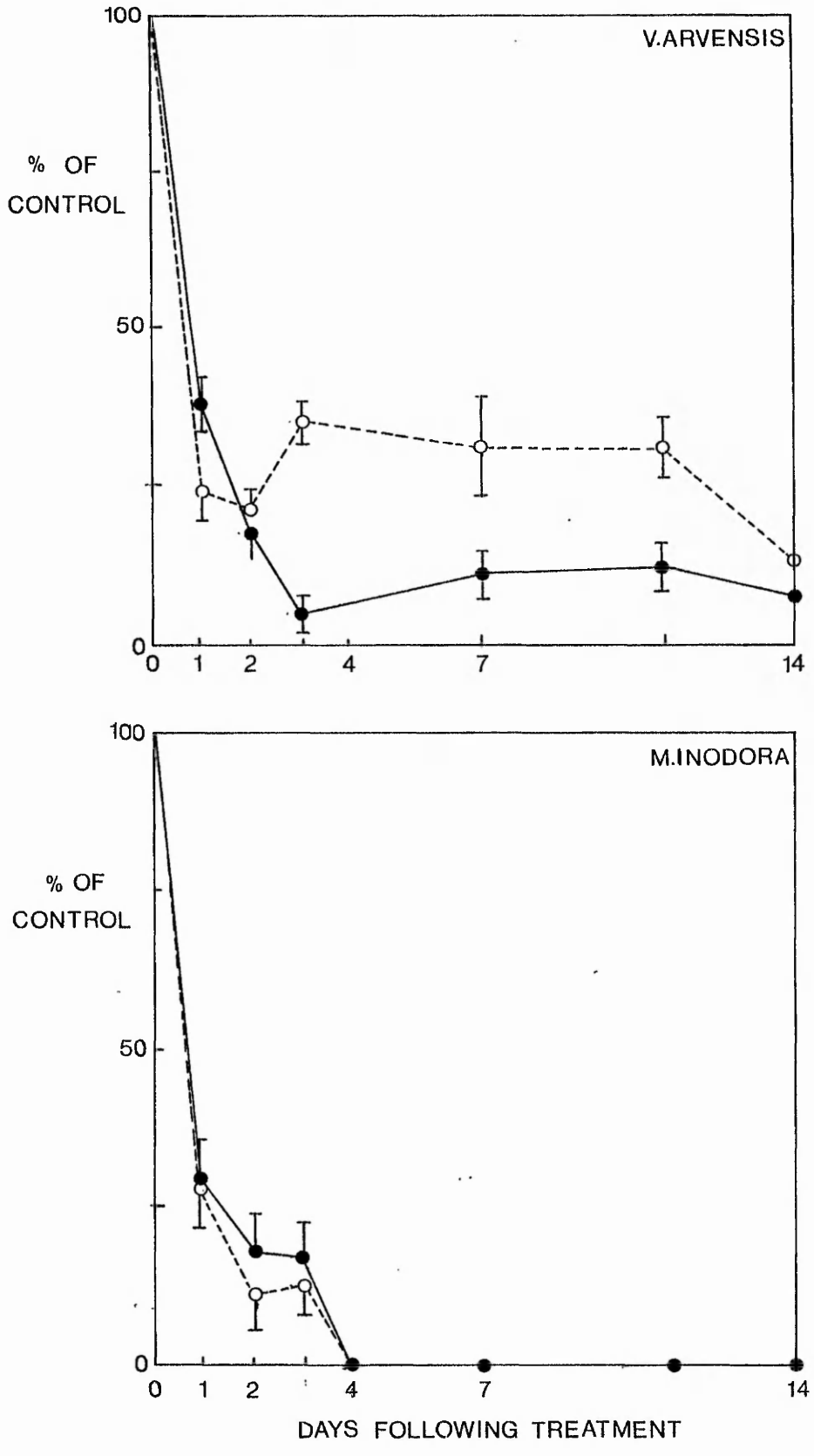


Figure 4.6: The effect of ioxynil (—●—) and bromoxynil (- -o- -) on CO_2 uptake by the youngest fully developed leaf of V. arvensis and M. inodora. Bars = standard errors.

and maintained within a double membraned chloroplast envelope (e.g. plate 4.2). The stroma contained ribosomes, plastoglobuli, starch grains, and intergranal and granal thylakoids. The peripheral reticulum was particularly apparent adjacent to the inner membrane of V. arvensis chloroplasts (plates 4.12, 4.13). Other cytoplasmic inclusions were associated with the chloroplasts such as endoplasmic reticulum and mitochondria in which cristae could be seen (e.g. plate 4.12). Control tissue was sampled after 0, 2, 4 and 7 days (V. arvensis and M. inodora) and there were no apparent changes in cell ultrastructure for the duration of the experiment.

Ioxynil treatment of M. inodora had little effect on the chloroplast within 48 hours, although fewer starch grains were noted than in control chloroplasts (plate 4.3). Similarly mitochondria and other cytoplasmic inclusions were also apparently unaffected. Ultrastructural changes were more obvious after 4 days, when chloroplasts were generally slightly swollen and intergranal and granal thylakoids were frequently vacuolated (plate 4.4). At this stage the tonoplast and plasmalemma were still intact and there were no obvious changes in other cytoplasmic inclusions. Ultrastructural symptoms developed rapidly, and considerable chloroplast and cellular disruption was evident after 7 days (plates 4.5 and 4.6). The thylakoid membranes had separated and swollen, and numerous membrane bound vesicles were present in the stroma. The chloroplast envelope remained intact whereas the tonoplast had ruptured and the plasmalemma appeared convoluted. In contrast to the swollen chloroplast, mitochondria were generally unaltered by ioxynil treatment.

In contrast, bromoxynil treated M. inodora leaves appeared to exhibit a different pattern of symptom development (plates 4.7-4.11). Within two days there was typically some swelling of the chloroplast and vacuolation of the intergranal thylakoids (plate 4.7). Other cytoplasmic inclusions were unaltered and the tonoplast was intact. Chloroplast swelling continued, and after a

further 48 hours the thylakoid system was generally bowed against the inner membrane of the chloroplast envelope (plate 4.8). The granal stacks were relatively intact although some vacuolation of the intergranal thylakoids was evident. At this stage, starch grains were completely absent in all observed chloroplasts and plastoglobuli were present within the stroma. Seven days after treatment, the tonoplast had ruptured and the plasmalemma had separated from the cell wall (plates 4.9-4.11). There was a complete loss of cellular integrity with cytoplasmic inclusions mixed with those of the tonoplast. In most cases the chloroplast envelope had ruptured and the chloroplasts were misshapen. The thylakoid system was relatively intact even when free within the cytoplasm, although the difference between granal and intergranal thylakoids was less defined (plates 4.10 and 4.11). Numerous plastoglobuli were associated with the thylakoid system. All membrane bound inclusions were swollen, and numerous small vacuoles were present within the mesophyll cells (plate 4.11). In some ultrathin sections (e.g. plate 4.11), the cell wall was convoluted reflecting the desiccated state of treated leaves.

Ultrastructural symptom development was also studied in V. arvensis which is more resistant to the hydroxybenzonitriles. Starch grains were generally absent from ioxynil treated leaves (plates 4.12-4.15) and chloroplasts exhibited fewer symptoms than ioxynil treatment of M. inodora. After two days, chloroplasts and other cytoplasmic inclusions were relatively unaffected by ioxynil (plate 4.14). Within 4 days chloroplasts were frequently slightly swollen compared to control chloroplasts (plate 4.15). Unlike, M. inodora chloroplasts, no vacuolation of thylakoids was observed and there was possibly an increase in the number of grana per stack. Swelling continued and after 7 days chloroplasts ranged in shape from discoid to spherical (plates 4.16 & 4.17). Granal stacks remained intact and the thylakoid system comprised a large volume of the chloroplast. Numerous plastoglobuli were present within the stroma. In addition there was no evidence of tonoplast rupture or plasmalemma effects and mitochondria and

other cytoplasmic inclusions were relatively unaffected.

Bromoxynil treated V. arvensis leaves exhibited few symptoms (see section 2.3), however changes were apparent in chloroplast ultrastructure. Within 2-4 days there was an obvious increase in the number of grana per stack (plates 4.18-4.19). Few starch grains were present, and the chloroplasts retained the usual discoid shape. No other changes in cell ultrastructure were observed. By 7 days, the increased stacking response was very prominent (plate 4.20) resulting in an apparent increase in the thylakoid volume. Chloroplasts were otherwise relatively unchanged, and other cell components also appeared unaffected (plate 4.21).

The increased stacking response was quantified by analysis of the number of grana in 50 granal stacks per treatment. Table 4.1 shows that there were approximately twice as many grana per stack in bromoxynil treated chloroplasts compared to control chloroplasts. Ioxynil treatment resulted in an intermediate response. There was also a small, but significant decline in the chlorophyll a:b ratio of treated leaves, with no change in chlorophyll content. In contrast, the decline in a:b ratio of M. inodora corresponded to a marked decrease in the total chlorophyll content.

For comparative purposes, ultrastructural symptoms were also investigated 7 days after treatment of S. media (plates 4.22, 4.23 and 4.24). Starch grains were very prominent in control chloroplasts (plate 4.22) but almost completely absent in all treated chloroplasts (plates 4.23 and 4.24). The tonoplast was frequently ruptured in ioxynil treated cells, and chloroplasts were swollen and occasionally disrupted. The thylakoid system was relatively intact and numerous plastoglobuli were present in the stroma. Fewer symptoms were evident in bromoxynil treated cells. Chloroplasts were discoid and contained much reduced starch grains (plate 4.24). There was possibly an increase in the width and height of some granal stacks although the effect

was much less defined than in bromoxynil treated V. arvensis chloroplasts. Plastoglobuli were present in the stroma. All other cell components appeared unaffected.

TABLE 4.1: Chloroplast and pigment characteristics 7 days after treatment of V. arvensis and M. inodora

	Mean Grana per stack	Total Chlorophyll content ($mg.g^{-1}$)	Chlorophyll a:b ratio
Viola arvensis			
Control	11.92 ± 0.48	1.659 ± 0.04	3.352 ± 0.05
Ioxynil	15.14 ± 0.85	1.646 ± 0.02	3.069 ± 0.03
Bromoxynil	22.40 ± 1.47	1.642 ± 0.04	3.043 ± 0.02
		($mg.plant^{-1}$)	
Matricaria inodora			
Control	-	0.677 ± 0.08	3.456 ± 0.08
Ioxynil	-	0.251 ± 0.05	2.984 ± 0.11
Bromoxynil	-	0.229 ± 0.04	2.936 ± 0.25

4.4 DISCUSSION

Throughout these experiments, data has been collected on the photosynthetic capacity of M. inodora and V. arvensis. The light saturation curves (fig. 4.4), and maximum photosynthetic rates are comparable with similar studies on other temperate weed species (Van Oorschot and Van Leeuwen 1979, 1984; Holt, Stemler and Radosevich 1981; Dunleavy 1983). This data strongly suggests that photosynthesis in M. inodora and V. arvensis follows a typical C_3 carbon reduction pathway. Ultrastructural investigations confirmed this theory, as chloroplasts from untreated leaves exhibited the usual features of C_3 plants (Coombs and Greenwood 1976). These included the presence of large starch grains, a low degree of granal stacking and a relatively high value for the chlorophyll a:b ratio, all of which are also indicative of "high light" or "sun" chloroplasts (Fedtke, Deichgraber and Schnepf

1977; Lichtenthaler, Buschman, Doell, Fletz, Bach, Kozel, Meier and Rahmsdorf 1981; Lichtenthaler, Kuhn, Prenzel and Meier 1982; Lichtenthaler 1983). Throughout these investigations the photosynthetic rates of M. inodora were generally higher than those obtained for V. arvensis.

Two different aspects of photosynthetic inhibition have been monitored in V. arvensis and M. inodora. Measurements of oxygen evolution by L.D.E. and CO_2 uptake by IRGA were variable, although general trends could be established. Variations in control rates may have been partially due to genetic variation between individual plants (Van Oorschot, pers. comm.) and also to electrical interference and instability in instrument sensitivity. It was particularly difficult to standardize CO_2 concentration within the L.D.E. reaction chamber and at the end of each incubation considerable water vapour had collected between the leaf surface and perspex lid. A lack of air circulation within the chamber may have greatly reduced accuracy. These problems were overcome with the somewhat more sophisticated IRGA system and variations were most likely to reflect differences in the CO_2 concentration of reference air. In general, measurements of CO_2 uptake by IRGA were more reliable than L.D.E. techniques and it was more appropriate to measure the photosynthetic activity of attached rather than detached leaves. However, measurements of both parameters presents a useful comparison, particularly when studying the effects of inhibitory uncoupling herbicides.

Oxygen evolution was generally inhibited more slowly than CO_2 uptake, particularly after bromoxynil treatment of the two species (fig. 4.3 and 4.5). Bromoxynil may possibly have initially uncoupled photo- (and presumably oxidative-) phosphorylation resulting in stimulation of electron transport and thus masking any inhibitory effect on oxygen evolution. However the resulting reduction in ATP and NADPH formation may have immediately limited CO_2 fixation causing the rapid decrease in CO_2 uptake noted in figure 4.5. Accurate measurement of respiration rates would have substantiated this theory. In previous studies ioxynil-induced stimulation of respiration corresponded to rapid inhibition of CO_2 uptake by flax cotyledons (Pallett 1978). The

relative contributions of photosynthetic and respiratory effects are difficult to assess, although Van Oorschot (1974) considered photosynthetic inhibition to be more important in the action of ioxynil against Phaseolus vulgaris L. c.v. Berna. In the present study, electron transport inhibition and uncoupling of photophosphorylation in chloroplast fragments are compared in Chapter 5.

Foliar spray applications of ioxynil rapidly inhibited CO_2 uptake by Phaseolus vulgaris L. c.v. Berna (Van Oorschot and Van Leeuwen 1974). In this study, intra and inter specific differences were noted in the response of V. arvensis and M. inodora to hydroxybenzonitrile treatment (figs. 4.5 and 4.6). Within two days, CO_2 uptake by M. inodora was reduced to 10-20% of control rates. At this stage, small changes in chloroplast ultrastructure were noted. Starch grains were less apparent indicating rapid metabolic breakdown as a consequence of low CO_2 fixation rates (Fedtke 1982). Similarly, the slight swelling of intergranal frets after bromoxynil treatment is commonly reported as a first symptom of PSII inhibition (Hill, Putala and Vengris 1968; Geronimo and Herr 1970; Anderson and Schaelling 1973; Pallett 1978). This effect was more pronounced within 4 days of ioxynil treatment and has been classified elsewhere as "moderate thylakoid swelling" (Hill, Putala and Vengris 1968). At this stage, both herbicides completely inhibited CO_2 fixation, and there were rapid changes in cell ultrastructure. The chloroplasts of bromoxynil treated leaves began to swell and there was an increased tendency for the thylakoids to be bowed against the chloroplast envelope. This phenomena is commonly reported (Geronimo and Herr 1970; Harris and Dodge 1972; Pallett and Dodge 1980), and may be due to changes in the osmotic potential of the cell vacuole (Dodge and Lawes 1974).

As treatment progressed, disruption of the chloroplast envelope, tonoplast and plasmalemma (plates 4.9, 4.10 and 4.11) indicated further permeability changes within treated cells (Anderson and Schaelling 1973). Changes in chloroplast ultrastructure may have been initiated by photosynthetic inhibition but the general disruption of all cellular membranes compared to the relatively intact thylakoid membranes suggests an additional mechanism is likely to be involved.

This is most probably related to the uncoupling action by bromoxynil as high concentrations of hydroxybenzonitriles are thought to alter the permeability and integrity of all cellular membranes by disrupting active transport (Moreland and Novitsky 1982, 1984).

Ultrastructural changes in ioxynil treated M. inodora leaves were more typical of anticipated photo-oxidative membrane destruction. Complete inhibition of CO_2 uptake may have led to the generation and accumulation of toxic species (e.g. singlet O_2, section 1.3.5) resulting in pigment breakdown, lipid peroxidation, and the thylakoid membrane disintegration apparent in plates 4.5 and 4.6. The total chlorophyll content decreased rapidly (Table 4.1), and the decline in chll a:b ratio may be due to more extensive photo-oxidative bleaching of chlorophyll a compared to chlorophyll b (Giannopolitis and Ayers 1978).

A similar degeneration of the thylakoid system was described when flax cotyledons were floated in solutions of ioxynil (Pallett 1978; Pallett and Dodge 1980). Changes in chloroplast ultrastructure were more rapid than in the present study probably due to enhanced ioxynil uptake by excised leaves. Ultrastructural damage was attributed to singlet O_2 induced photo-oxidation, and the toxic action of iodide and iodine liberated by the degradation of ioxynil within flax cotyledons. The observed changes in ultrastructure may also be related to the membrane permeability effect described above. In particular, the marked chloroplast deterioration during dark incubation with ioxynil may relate to the uncoupling action of the herbicide. Ultrastructural changes in susceptible M. inodora probably result from a combination of each of these processes.

In contrast, measurement of CO_2 uptake by V. arvensis revealed that neither ioxynil or bromoxynil were completely inhibitory in this species (fig. 4.6). Ultrastructural changes were less marked, and reflected the continuing photosynthetic ability of treated leaves. The most noticeable early feature was the general absence of starch grains within two days and a gradual increase in the number of grana per stack in bromoxynil treated chloroplasts. This effect was more prominent after 4 and 7 days, and the additional swelling of ioxynil treated chloroplasts reflected the greater inhibition of CO_2 uptake by this herbicide. Granal stacks were also frequently broader and higher

in bromoxynil treated S. media chloroplasts (plate 4.24), although the effect was less pronounced. It is therefore likely that photosynthesis was not completely inhibited in S. media following bromoxynil treatment.

Whereas control chloroplasts could be classified as "high light" or "sun" chloroplasts, bromoxynil treated leaves contained chloroplasts which exhibited typical features of low light or shade adaptation (Fedtke et al 1977; Lichtenthaler et al 1981, 1982; Lichtenthaler 1983). These included higher and broader granal stacks which increase the proportion of pigment antennae and LHCP complexes for light interception (see figure 1.1). Stacked membrane regions contain 75-80% of the chloroplast PSII units (Andersson and Anderson 1980) and have a chlorophyll a:b ratio of 2.1-2.3 (Melis and Brown 1980). The increase in granal stacking observed in this study corresponded to a decline in the chlorophyll a:b ratio of hydroxybenzonitrile treated leaves. Ultrastructural changes in bromoxynil treated V. arvensis thus appears to be an adaptation to lower photosynthetic rates induced by the herbicides.

This type of response has been reported for sublethal applications of other photosynthetic inhibitor herbicides e.g. methabenzthiazuron (Fedtke, Deichgraber and Scnepf 1977), diuron (Boger and Schlue 1976; Lichtenthaler, Burkard, Grumbach and Meier 1980), and atrazine (Hirandradit and Foy 1972). Most frequently this effect is investigated following the addition of herbicide to hydroponically grown plants. In this way, radish seedlings (Raphanus sativus L.) have been induced to form shade type chloroplasts in high light intensities by sublethal concentrations of bentazon (Lichtenthaler, Burkard, Grumbach and Meier 1980; Meier and Lichtenthaler, 1981; Lichtenthaler, Kuhn, Prenzel and Meier 1982). Few investigations have studied foliar spray herbicides, although a "greening effect" particularly on crop plants has been noted in the field (Fedtke 1982).

In conclusion, the development of ultrastructural symptoms in V. arvensis and M. inodora were related to changes in the photosynthetic capacity of treated leaves. Ultrastructural changes were most marked following complete inhibition of photosynthesis and probably resulted from photo-oxidative and possibly free radical processes. When the

photosynthetic capacity was retained at a residual level, there were adaptive changes in chloroplast ultrastructure to enhance light interception. In general, photosynthetic inhibition and ultrastructural symptoms reflected the overall activity of the hydroxybenzonitriles against M. inodora, V. arvensis and S. media.

Abbreviations used in plates 4.1 - 4.24

Ce	-	chloroplast envelope
Cs	-	intercellular space
Cw	-	cell wall
Cy	-	cytoplasm
G	-	granum
I	-	intergranal thylakoids
M	-	mitochondrion
Mv	-	membrane bound vesicles
N	-	nucleus
Pg	-	plastoglobuli
Pm	-	plasma membrane or plasmalemma
Pr	-	peripheral reticulum
R	-	ribosomes
Sr	-	stroma
Sg	-	starch grains
T	-	tonoplast
Ts	-	thylakoid membrane system
V	-	vacuole

Legends for Plates 4.1 - 4.11

Plates 4.1 - 4.11: Ultrastructural symptoms in M. inodora

Plate 4.1: Untreated mesophyll cell chloroplast. Magnification x 21,500.

Plate 4.2: Untreated mesophyll cell chloroplast. Magnification x 46,750.

Plate 4.3: Chloroplast 2 days after ioxynil treatment. Note: absence of starch grains. Magnification x 19,600.

Plate 4.4: Chloroplast 4 days after ioxynil treatment. Note: vacuolation of intergranal and granal thylakoids (arrowed). Magnification x 13,200.

Plate 4.5: Mesophyll cell 7 days after ioxynil treatment. Note: swelling of chloroplasts and thylakoid membranes, presence of membrane bound vesicles within the stroma, ruptured tonoplast (arrowed) and damaged plasmalemma. Magnification x 15,000.

Plate 4.6: Chloroplast 7 days after ioxynil treatment. Magnification x 23,100.

Plate 4.7: Mesophyll cell 2 days after bromoxynil treatment. Note: slight swelling of chloroplasts + intergranal thylakoids (arrowed). Magnification x 16,000.

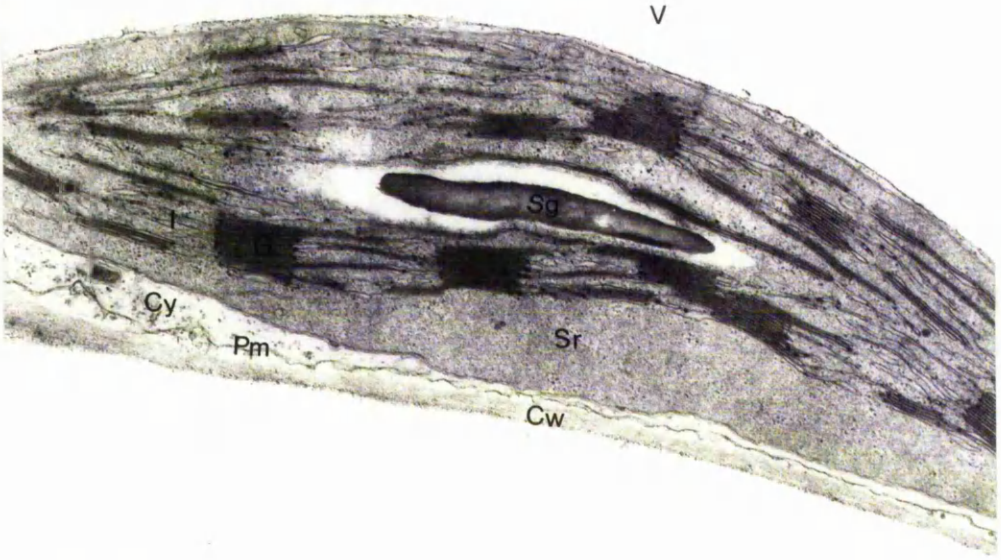
Plate 4.8: Chloroplast 4 days after bromoxynil treatment. Note: swollen chloroplast, thylakoid system bowed against chloroplast envelope (arrowed). Magnification x 22,600.

Plate 4.9: Mesophyll cell 7 days after bromoxynil treatment. Note: ruptured tonoplast, plasmalemma and chloroplast envelope; swollen chloroplasts and mitochondria; relatively intact thylakoid system containing numerous plastoglobuli. Magnification x 8,750.

Plate 4.10: Mesophyll cell chloroplast 7 days after bromoxynil treatment. Note: ruptured chloroplast envelope (arrowed). Magnification x 22,500.

Plate 4.11: Mesophyll cell 7 days after bromoxynil treatment. Note: convoluted cell wall, disruption of tonoplast and plasmalemma (arrowed). Magnification x 8,750.

4.1



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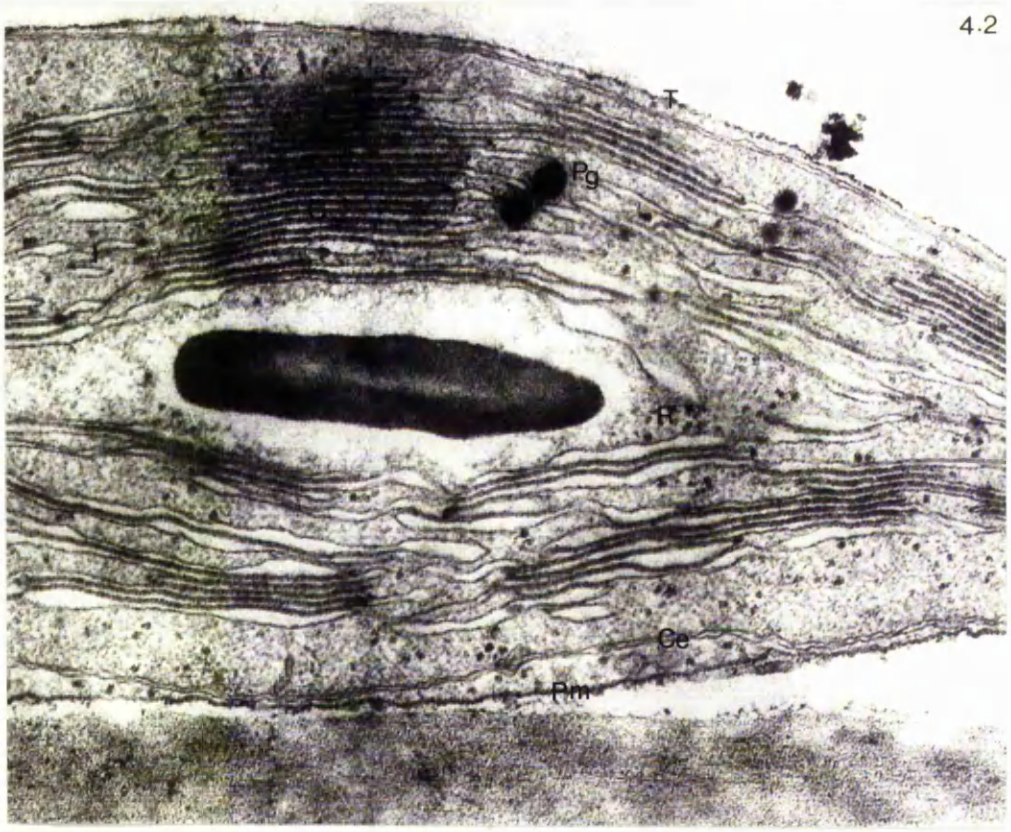

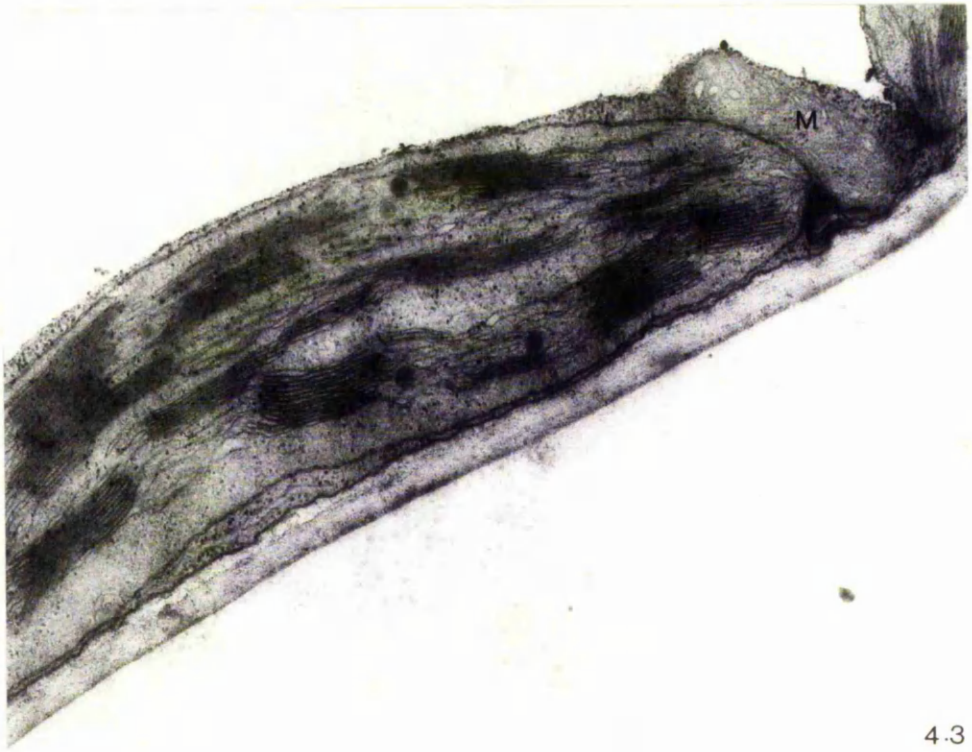

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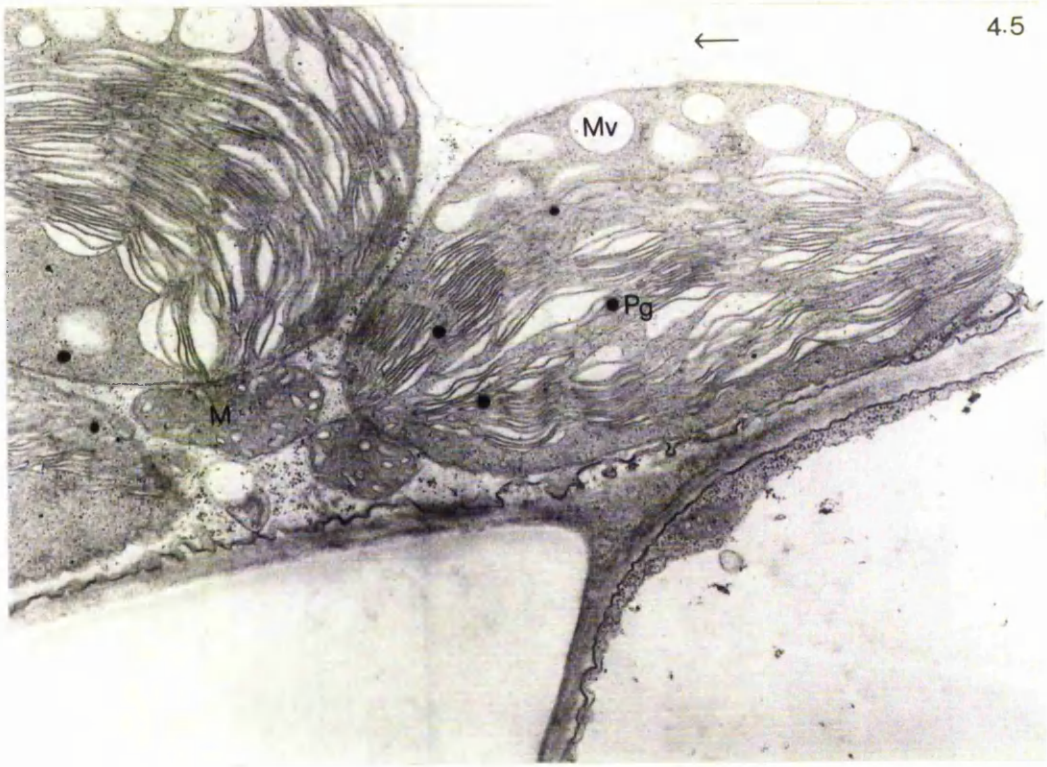

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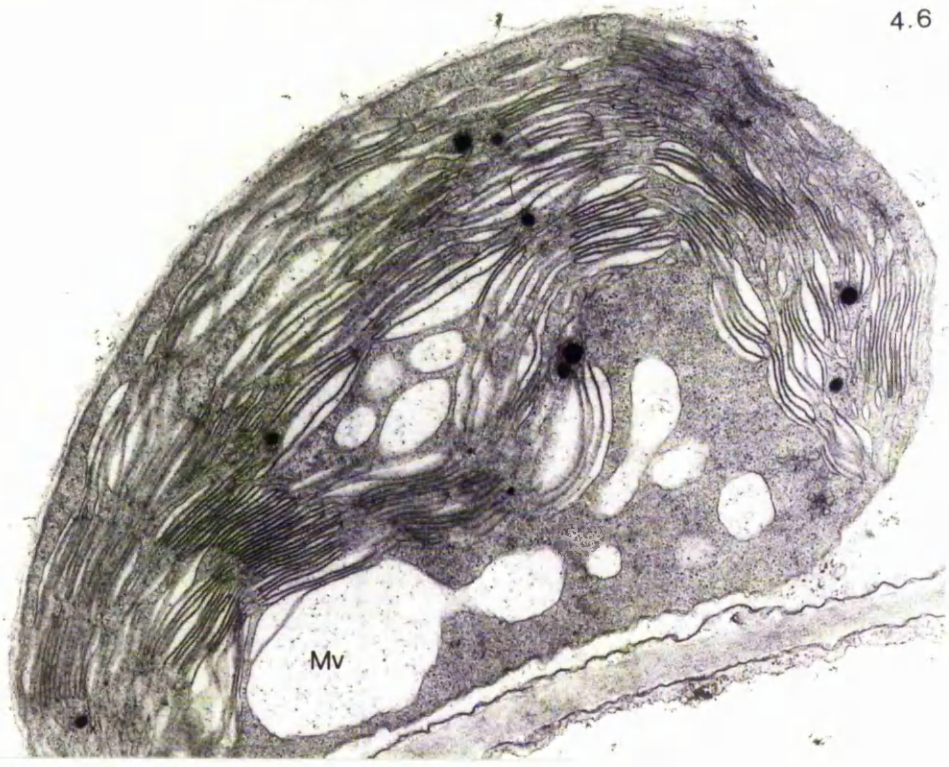

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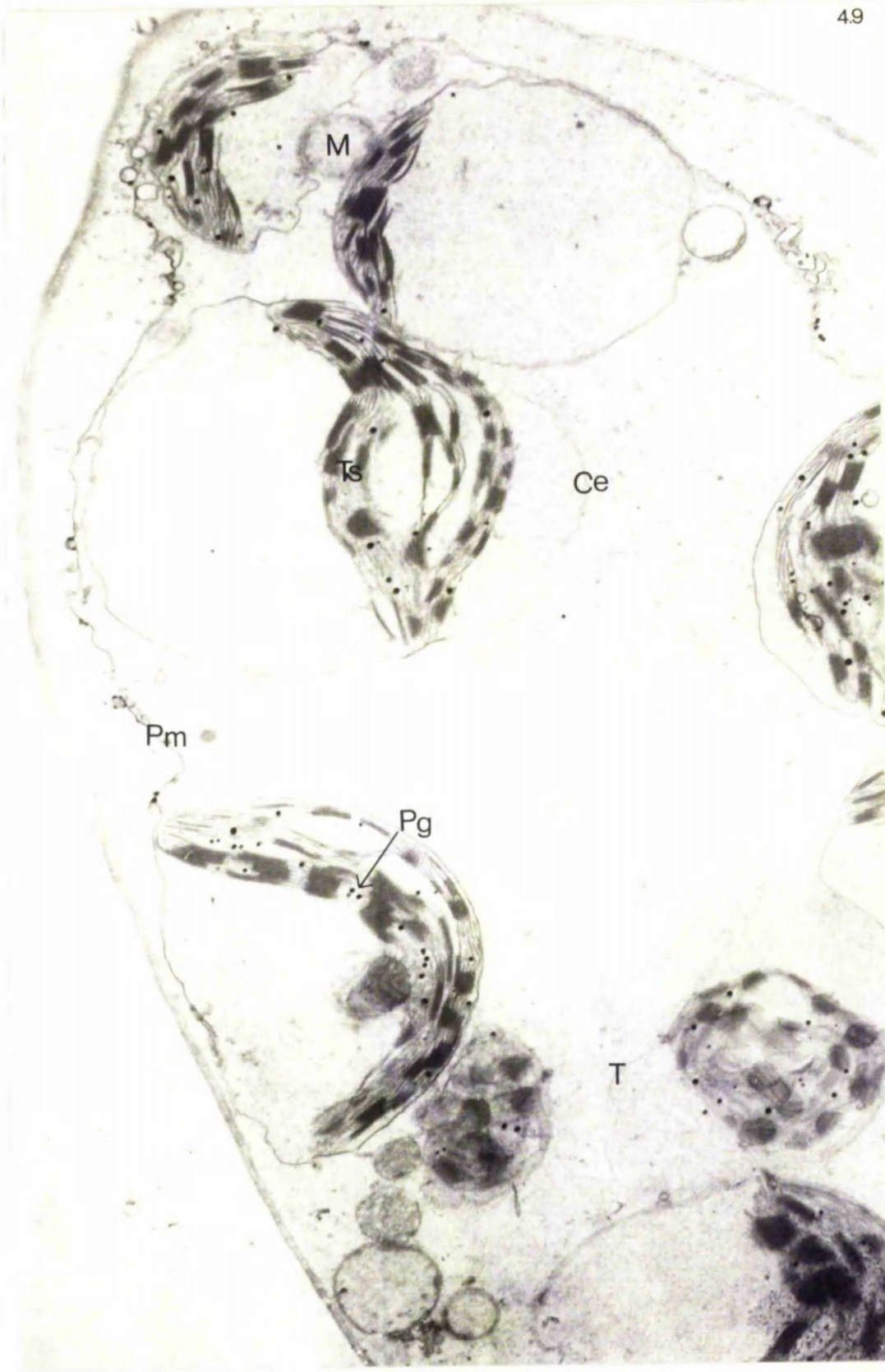
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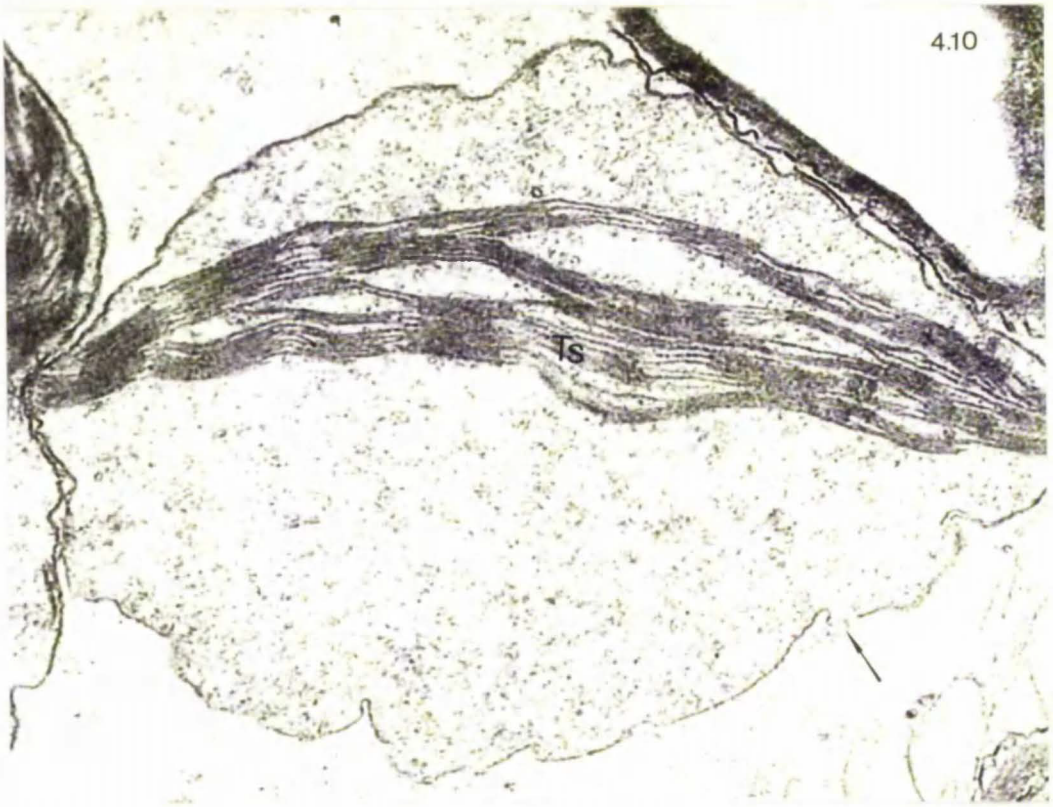
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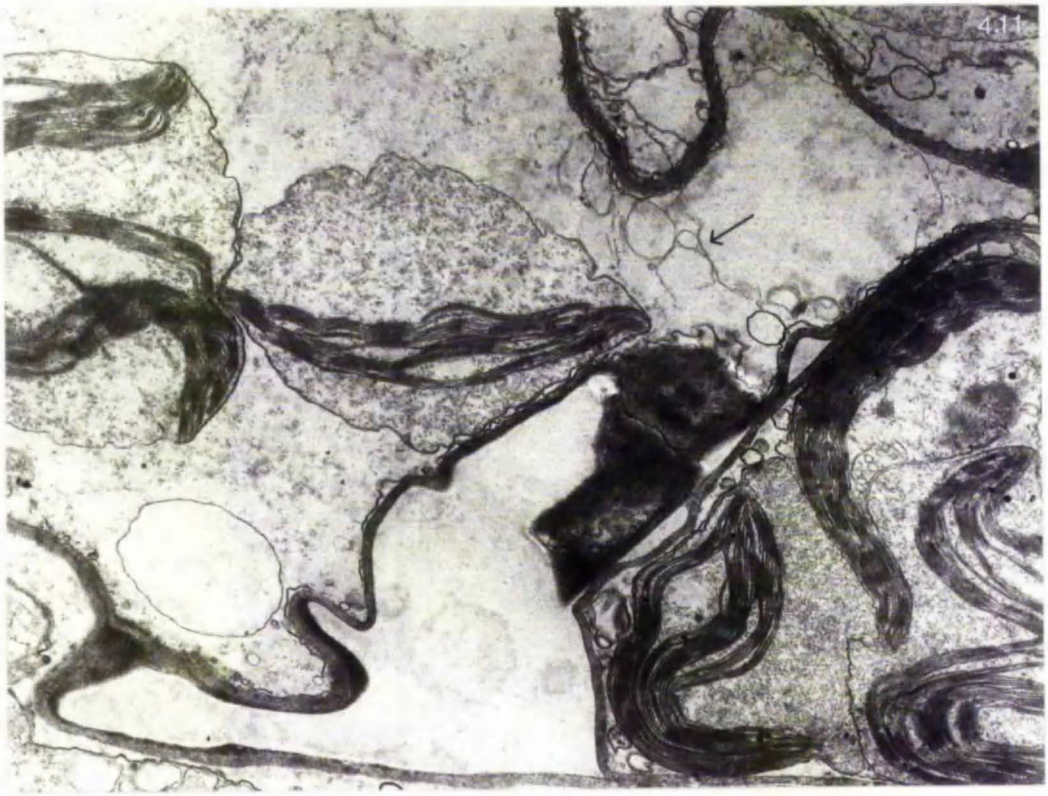

4.11

Legends for Plates 4.12 - 4.21

Plates 4.12 - 4.21: Ultrastructural symptoms in V. arvensis

Plate 4.12: Untreated mesophyll cell. Magnification x 8,400.

Plate 4.13: Untreated mesophyll cell chloroplast. Magnification x 31,700.

Plate 4.14: Mesophyll cell 2 days after ioxynil treatment. Note: absence of starch grains. Magnification x 14,100.

Plate 4.15: Mesophyll cell 4 days after ioxynil treatment. Note: slight swelling of chloroplasts (arrowed). Magnification x 9,400.

Plate 4.16: Mesophyll cell 7 days after ioxynil treatment. Note: swelling of chloroplasts, numerous plastoglobuli Magnification x 9, 100.

Plate 4.17: Chloroplast 7 days after ioxynil treatment. Note: swollen chloroplast, mitochondria appears normal. Magnification x 19,000.

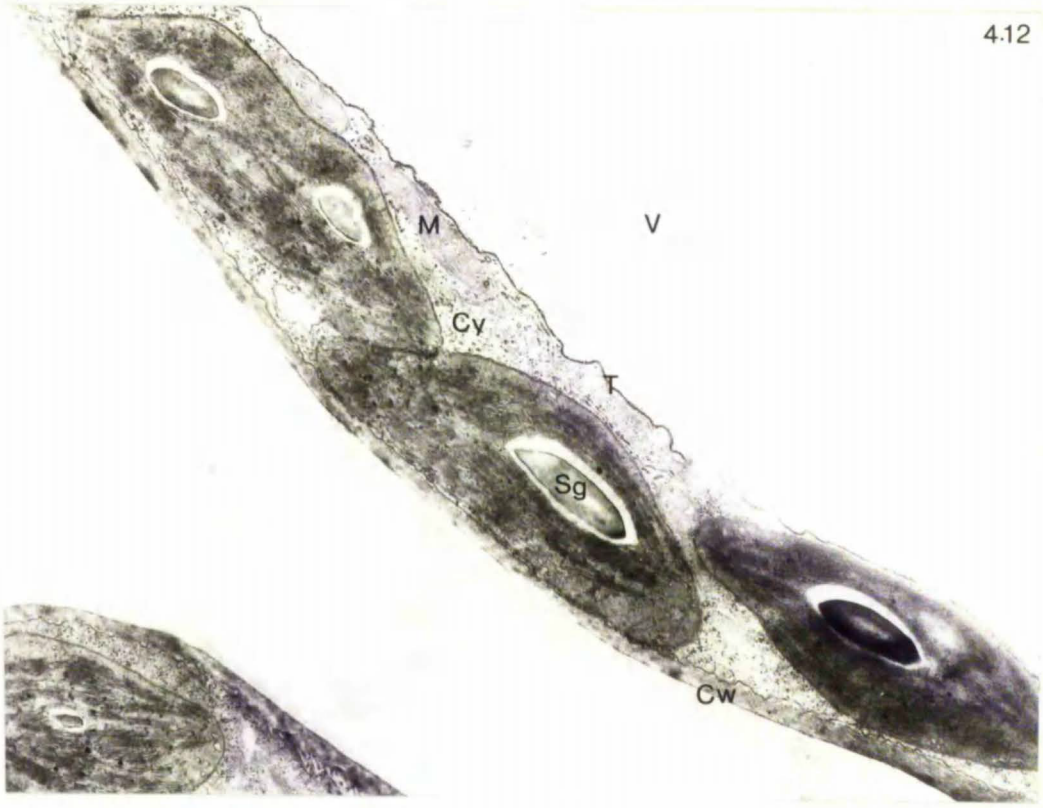
Plate 4.18: Chloroplast 2 days after bromoxynil treatment. Note: possible increase in number of grana per stack, (arrowed). Magnification x 36,000.

Plate 4.19: Mesophyll cell 4 days after bromoxynil treatment. Note: increase in granal stacking and dense cytoplasm. Magnification x 22,000.

Plate 4.20: Chloroplast 7 days after bromoxynil treatment. Note: large granal stacks. Magnification x 17,500.

Plate 4.21: Mesophyll cell 7 days after bromoxynil treatment. Note: increase in granal stacking, dense stroma and cytoplasm, otherwise normal cell. Magnification x 35,400.

4.12



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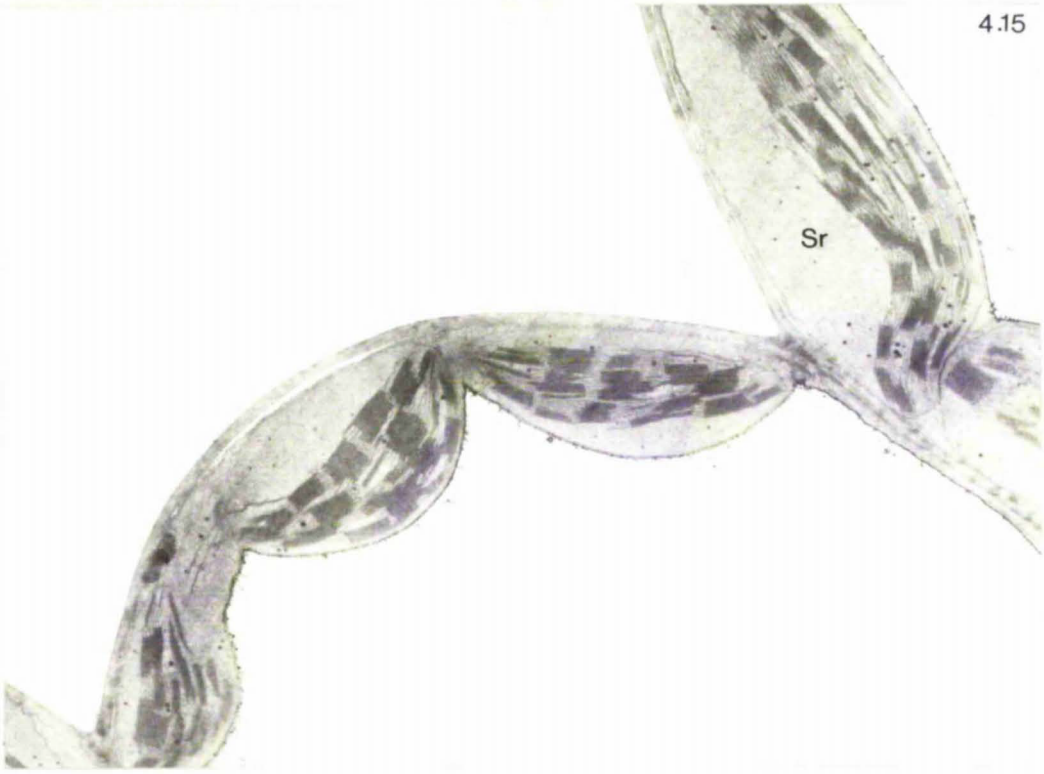
4.14



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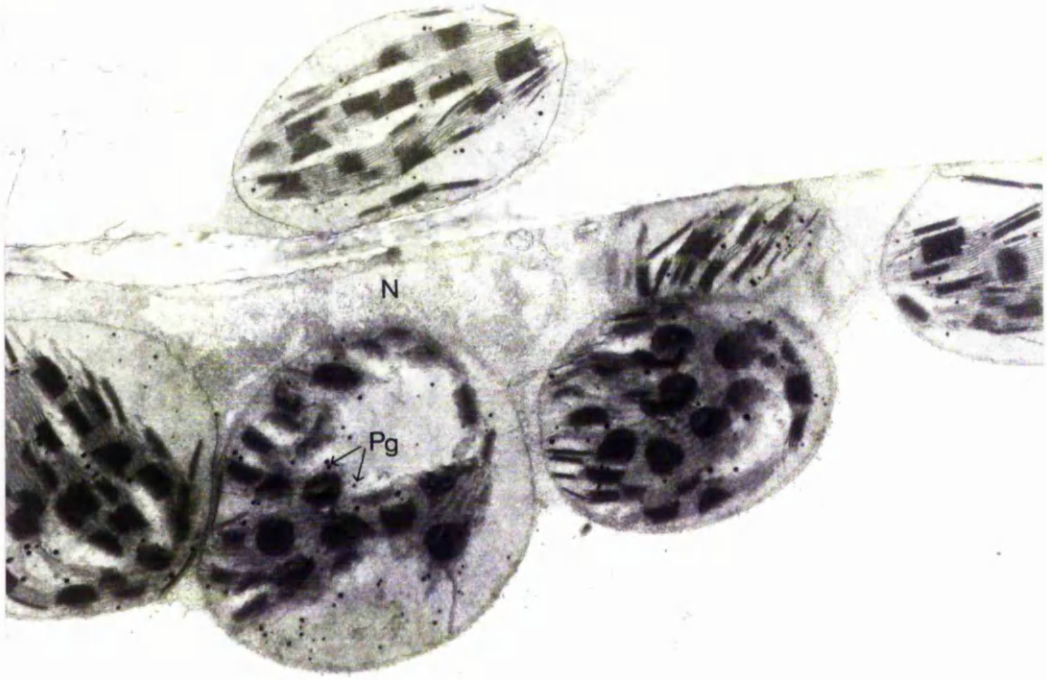

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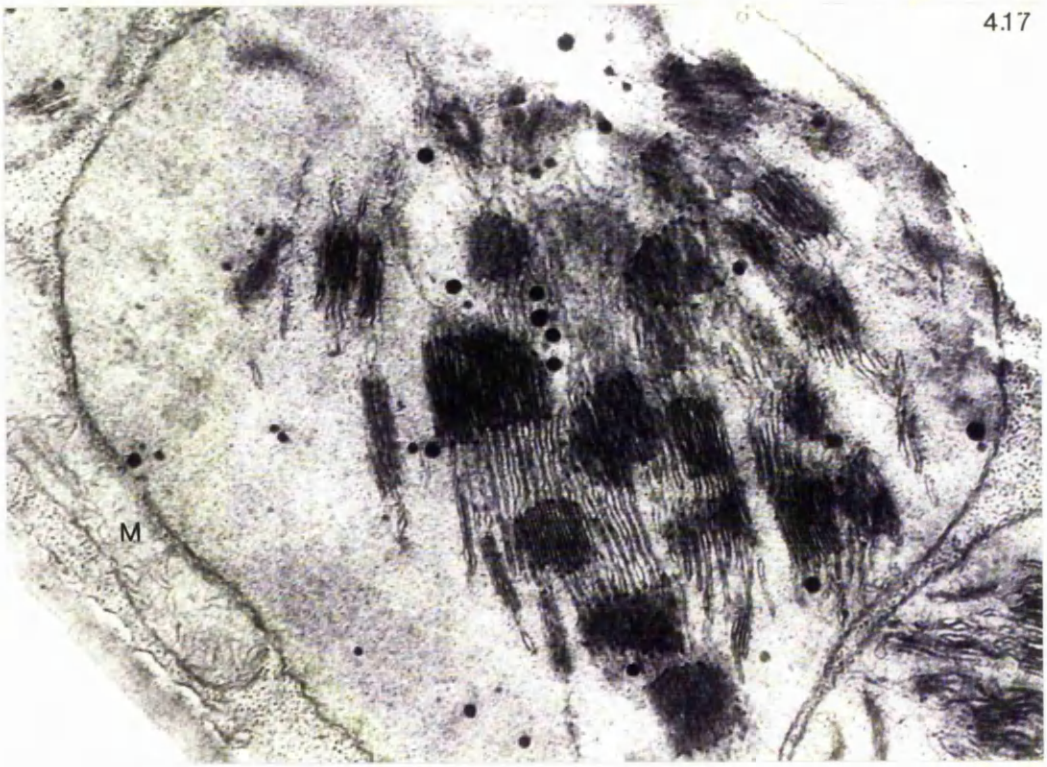

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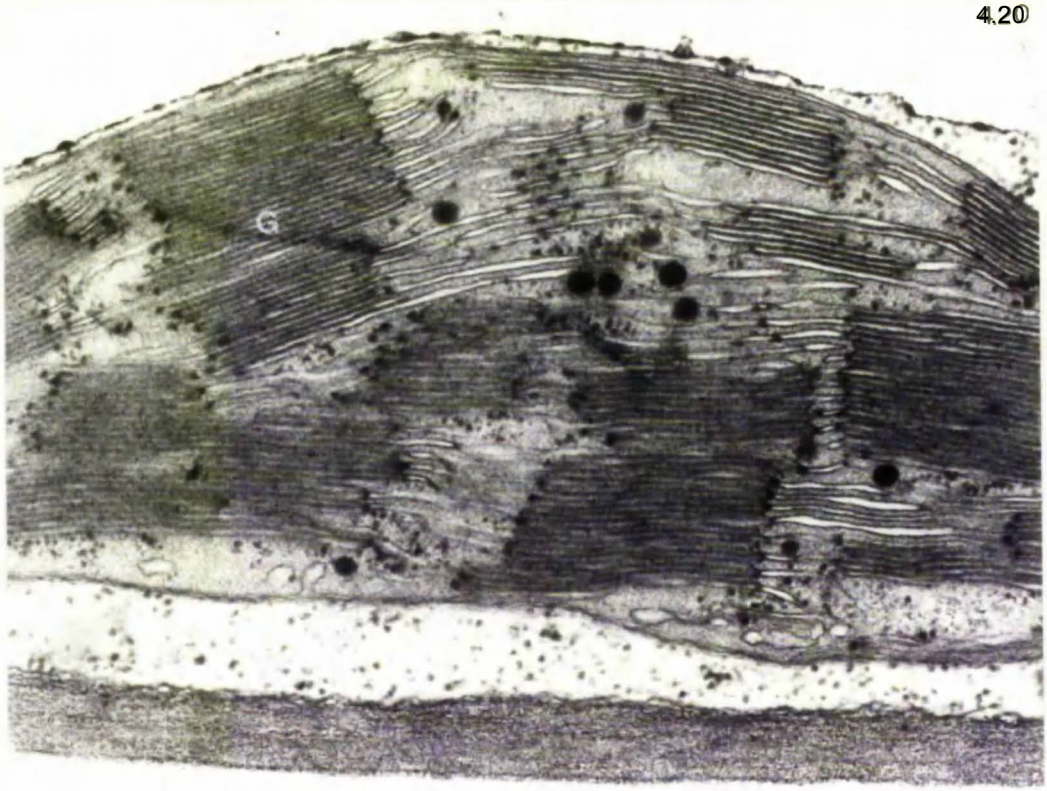
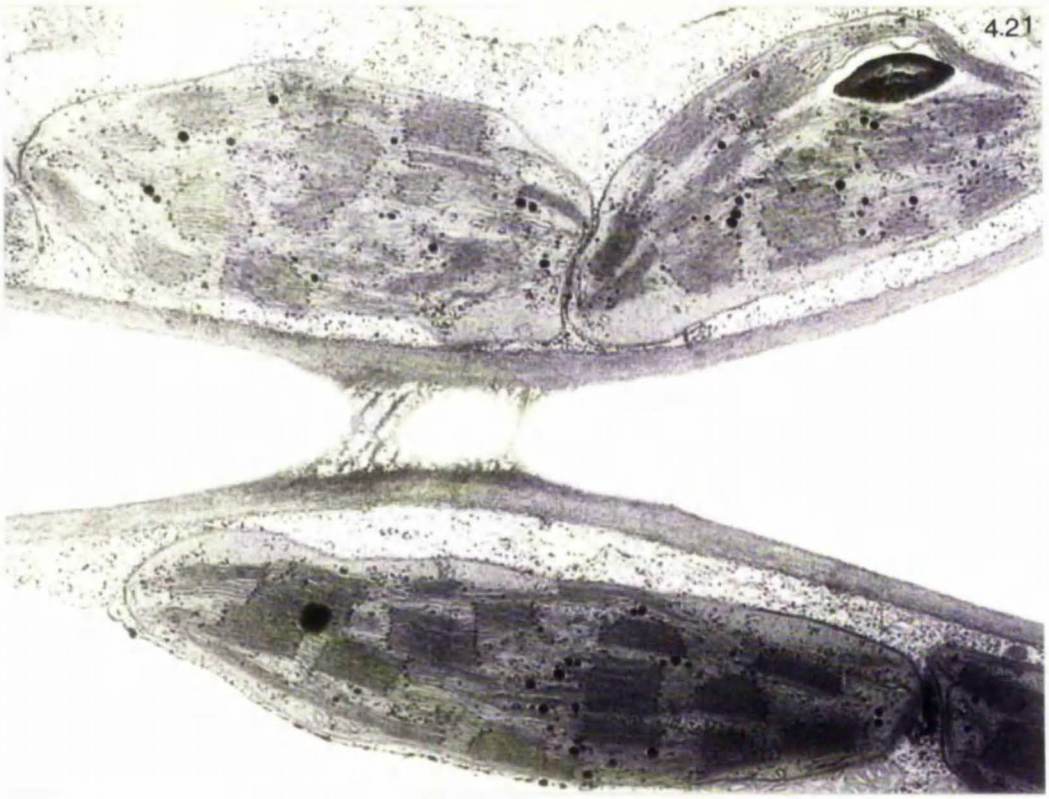
4.19

4.20





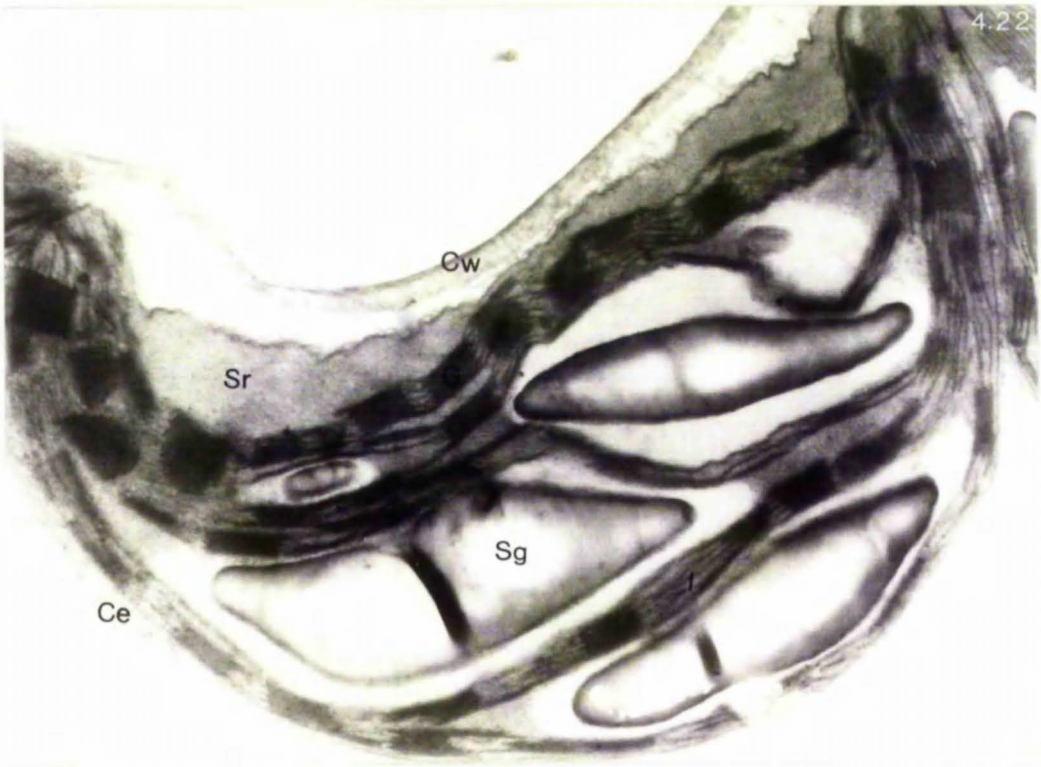
Legends for Plates 4.22 - 4.25

Plates 4.22 - 4.25: Ultrastructural symptoms in S. media

Plate 4.22: Untreated mesophyll cell chloroplast. Magnification x 22,000.

Plate 4.23: Mesophyll cell 7 days after ioxynil treatment. Note: absence of starch grains, numerous plastoglobuli, tonoplast ruptured (arrowed). Magnification x 21,000.

Plate 4.24: Mesophyll cell chloroplast 7 days after bromoxynil treatment. Note: much-reduced starch grains, some increase in granal stacking (arrowed). Magnification x 20,500.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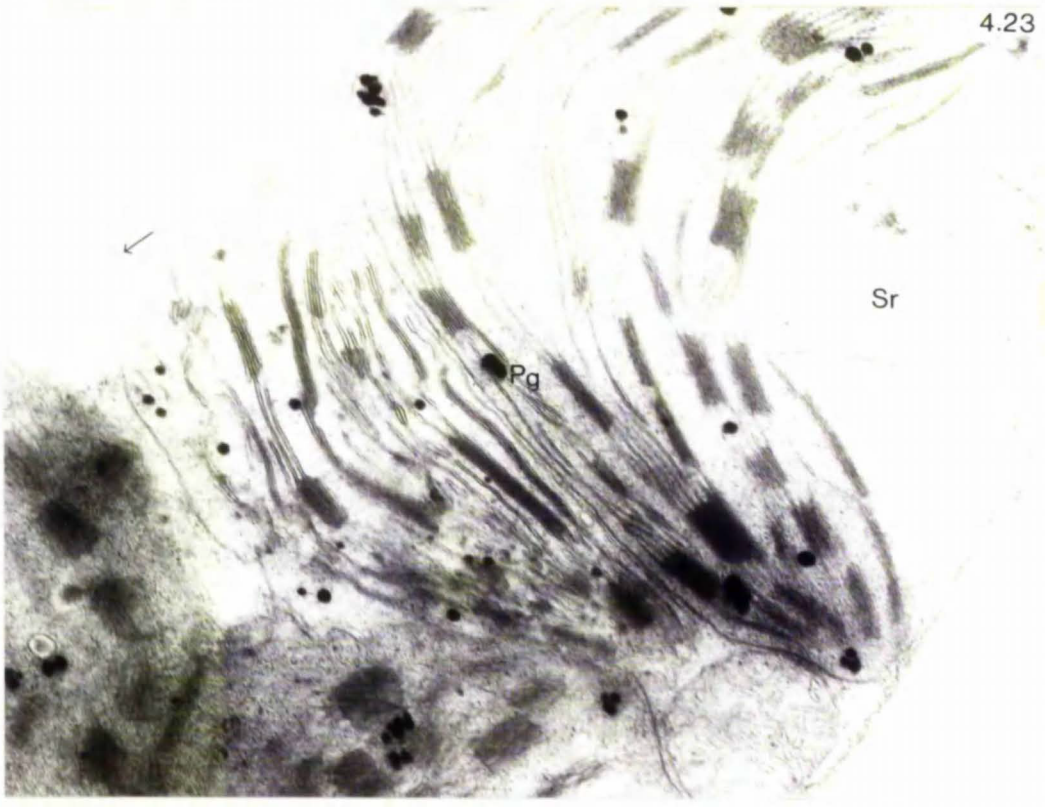
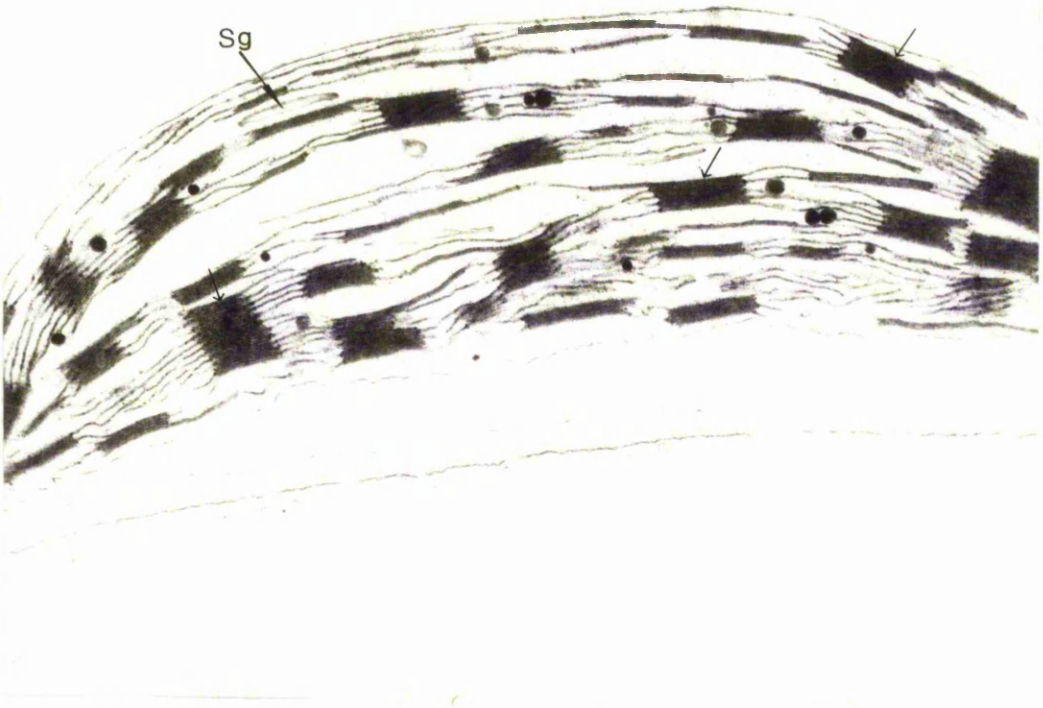
Sr
Sg
Ce


4.23
Sr
Pg

4.24



CHAPTER FIVE: THE INHIBITORY UNCOUPLING ACTION OF THE HYDROXYBENZONTRILES IN ISOLATED CHLOROPLASTS.

5.1 INTRODUCTION

It was established in Chapter 4 that the hydroxybenzonitriles rapidly inhibit CO_2 fixation in treated leaves. However, studies with intact plants give no indication of the mechanism by which phenolic herbicides inhibit photosynthesis. As inhibitory uncouplers, the hydroxybenzonitriles are believed to be active in both the chloroplast and mitochondrion (Moreland 1980), and thus subcellular preparations of these organelles are necessary to investigate the mechanism of action.

In vitro assays of the photosynthetic activity of herbicides usually involve chloroplasts isolated from standardized tissue such as peas and spinach (e.g. Belbachir, Matringe, Tissut and Chevallier 1980; Van Rensen and Vermaas 1981; Ridley and Horton 1984). Techniques are well documented and preparations ranging in quality from highly active intact class 'A' chloroplasts to class 'F' subchloroplast particles are possible (Reeves and Hall 1980). Attempts to isolate chloroplasts from other species have generally resulted in fragments of poor activity through either the presence of contaminating polyphenols, and polyphenol oxidases, or problems in tissue maceration (Reeves and Hall 1980). By modifying standard isolation procedures it has been possible to extract active type E chloroplast fragments from *M. inodora*, *S. media* and *V. arvensis*. These fragments were used to determine whether the inhibitory action reflected whole plant activity, or whether it depended on substitution and declined in the order I > Br > Cl as previously found with spinach thylakoids (Trebst, Reimer, Draber and Knops 1979). In this study two electron acceptors were used to polarographically measure electron transport inhibition in the region of photosystem II.

The uncoupling action of the hydroxybenzonitriles can also be monitored in isolated chloroplast preparations providing any effects on electron transport inhibition are excluded. Cyclic electron flow, coupled to ATP formation can be induced by the addition of artificial electron acceptors and donors such as phenazine methosulphate (P.M.S.) or diaminodurene (D.A.D., Trebst 1976). Cyclic photophosphorylation only

involves PSI and is thus insensitive to the inhibitory action of PSII inhibitors. The uncoupling action can also be monitored by studying the modified Mehler reaction (ascorbate/DCPIP $\longrightarrow$ Methyl viologen) which is stimulated when the rate limiting step of ADP phosphorylation is uncoupled from electron transport (Van Rensen and Hobe 1979).

The hydroxybenzonitriles have also been reported as uncouplers of oxidative phosphorylation in mitochondria (Ferrari and Moreland 1969). However, much of the earlier experimentation was carried out on rat liver mitochondria (Parker 1965) or on nonchlorophyllous tissue such as potato tubers (Ferrari and Moreland 1969), roots (Paton and Smith 1965, 1967) or cucumbers (Foy and Penner 1965), excluding chloroplastic effects. Extraction of active coupled mitochondria from chlorophyllous tissue has only recently reached perfection (Neuberger et al 1982; Nash and Wiskich 1983) and in each case yields per gram of tissue were very low. After consideration of these factors and the lack of measurable effect on whole plant respiration, (section 4.3), investigations were limited to uncoupling of photophosphorylation in chloroplasts. Interestingly, all previous investigations of uncoupling, regardless of source or assay procedure have shown that the activity of the hydroxybenzonitriles depends on substitution, and declines in the order I $>$ Br $>$ Cl (Parker 1965; Kerr and Wain 1964b; Ferrari and Moreland 1967, 1969).

Investigations into the interaction between herbicides and the thylakoid membrane have increased considerably over recent years (see section 1.3.4). Tischer and Strotmann (1977) introduced displacement techniques which have been used by other workers to investigate the nature of herbicide binding. In studying displacement of the phenolic herbicide dinoseb by different classes of herbicides, Oettmeier and Masson (1980) found that ioxynil and dinoseb compete for the same binding site. These compounds differ considerably in their ring substitution and have different binding constants. In this study, the replacement technique of Tischer and Strotmann has been used to investigate the effect of iodine and bromine substitution on binding to chloroplast fragments extracted from two species with contrasting in vivo activity.

5.2 MATERIALS AND METHODS

5.2.1 Chloroplast isolation procedure

The following extraction procedures were all carried out at 4°C. Chloroplast preparations were retained on ice until used.

5.2.1.1 VIOLA ARVENSIS and STELLARIA MEDIA type E fragments

Grinding medium

50 mM Tricine-sodium hydroxide buffer (pH 7.5)
300 mM Sodium chloride
3 mM Magnesium chloride
0.1 $mg.ml^{-1}$ Bovine serum albumin

Resuspension medium

5 mM Tricine-sodium hydroxide (pH 7.5)
100 mM Sucrose
3 mM Magnesium chloride
2 mM E.D.T.A.
1 $mg.ml^{-1}$ Bovine serum albumin

5g of leaf tissue were homogenised in 20ml of grinding medium using an ultraturrax at full speed for 20 seconds. After filtering through four layers of muslin, cell debris was removed by centrifugation at 1000g for one minute. The supernatant liquor was recentrifuged for 10 minutes at 3000g, and the chloroplast pellet resuspended in a small volume of resuspension medium.

5.2.1.2 MATRICARIA INODORA type E fragments

Grinding medium

50 mM Tricine-sodium hydroxide buffer (pH 7.5)
300 mM Sodium chloride
3 mM Magnesium chloride
2 mM E.D.T.A.
0.1 $mg.ml^{-1}$ Bovine serum albumin
1 $mg.ml^{-1}$ Polyvinylpyrrolidone

Resuspension medium (as for V. arvensis and M. inodora).

5g of leaf tissue was homogenised for 20 seconds in 20ml of grinding medium. The brei was filtered through 8 layers of muslin and centrifuged at 3000g for 1 minute. The pellet was immediately resuspended in a small volume of resuspension medium.

5.2.1.3 PISUM SATIVUM cv Meteor type C broken chloroplasts

Grinding medium

50 mM Tricine-NaOH pH 7.6
300 mM Sodium chloride
3 mM Magnesium chloride
0.1 $mg.ml^{-1}$ Bovine serum albumin

Resuspension medium

5 mM Tricine-NaOH buffer pH 7.0
100 mM Sucrose
3 mM Magnesium chloride
1 $mg.ml^{-1}$ Bovine serum albumin

2g of young expanding leaf tissue were homogenised in 20ml of grinding medium and filtered through 8 layers of muslin. After centrifugation at 3000g for 1 minute, the pellet was carefully resuspended in a small volume of resuspension medium.

5.2.2 Chlorophyll assay

100 µl of chloroplast preparation were mixed with 6-7ml of 80% acetone. After a 5 minute dark incubation ensuring complete extraction of the chlorophyll into the acetone the suspension was centrifuged at 3000g for 3 minutes. The supernatant was made up to 10 ml and assayed for chlorophyll content by the method of Arnon (1949).

5.2.3 Assays for electron transport inhibition

The reactions were carried out in a Hansatech oxygen electrode linked to a Bryans chart recorder. The reaction chamber routinely contained 3 ml of reaction mixture which was stirred constantly at 20°C. Irradiation of 3000 $\mu E.m^{-2}.sec^{-1}$ was provided by a Griffin Halight 300 projector. The electrode was calibrated using sodium dithionite before each experiment and the rate of oxygen evolution determined after a 3 minute period of illumination.

The reaction mixture consisted of the following:

Dichlorophenol indophenol reduction

0.3M Tricine-sodium hydroxide buffer pH 8.0	300 µl	
10mM Dichlorophenol indophenol (DCPIP):		
S. media preparation	40 µl	optimum concentration
M. inodora preparation	75 µl	optimum concentration
V. arvensis preparation	100 µl	optimum concentration
Chloroplasts equivalent to 100 µg chlorophyll		
Ioxynil/bromoxynil: variable concentrations		
Distilled water	to 3 ml	

Silicomolybdate reduction

0.3M Tricine-NaOH buffer pH 8.0	300 µl	
10mg.ml^{-1} Silicomolybdate (SiMo):		
S. media preparation	50 µl	optimum concentration added at illumination
M. inodora preparation	50 µl	optimum concentration added at illumination
V. arvensis preparation	75 µl	optimum concentration added at illumination
Chloroplasts equivalent to 100 µg chlorophyll		
Ioxynil/bromoxynil: variable concentrations		
Distilled water	to 3 ml	

5.2.4 Assays for the uncoupling of photophosphorylation

5.2.4.1 P.M.S. mediated cyclic phosphorylation

Reaction mixture

0.2M Tricine 20mM NaCl 10mM Na_2HPO_4	Adjusted to pH 8.0 with NaOH	... 1 ml
0.1M $MgCl_2$		200 µl
0.1M glucose		200 µl
10 $mg.ml^{-1}$ hexokinase		50 µl
4.75 $mg.ml^{-1}$ ADP-disodium salt		50 µl
1.5 $mg.ml^{-1}$ Phenazinmeth osulphate (PMS)		50 µl
10mM Monuron (in methanol)		30 µl
Chloroplasts equivalent to 100 µg		
Water to 3 ml		

The reaction mixture was placed in the reaction chamber of an O_2 electrode, stirred continually, at 20°C and irradiated with 3000 $uE.M^{-2}.sec^{-1}$ of light. A 0.5 ml aliquot was removed at two minute intervals for 10 minutes, immediately shaken with 50 ul of 10% Trichloroacetic acid (T.C.A.) and placed on ice to stop the reaction. Each aliquot was then centrifuged at 2,500 g for 5 minutes after which time 0.3 ml of the supernatant was removed and diluted to 3 ml for phosphate assay. A dark control was used to determine esterification of phosphate into ATP, and thus the difference between the light and dark control equalled the rate of phosphate incorporation.

Phosphate assay

10 ml of a stock solution of 10% ammonium molybdate in 10 N H_2SO_4 was daily diluted to approximately 75ml with water. 5g of ferrous sulphate were added, and when dissolved the solution was made up to 100 ml. 2 ml of this reagent was added to 3 ml of sample, mixed and the absorbance at 720 nm recorded after 10 minutes. All glassware was acid washed before use.

The level of coupling was determined before and after phosphate determination by measuring the $H_2O \rightarrow$ Ferricyanide Hill reaction, with and without the presence of the uncoupler NH_4Cl (1mM).

5.2.4.2 The ascorbate/DCPIP → methyl viologen modified Mehler reaction

This light dependent reaction was measured polarographically using an O_2 electrode and the same incubation conditions as section 5.2.3 . Chloroplast fragments (type E) were prepared as above although the second centrifugation step was reduced to five minutes for S. media and V. arvensis, to increase the level of coupling.

The reaction mixture consisted of:-

30mM Tricine-NaOH buffer pH 8.0
10mM sodium isoascorbate
25 µM monuron (in methanol)
10 µM DCPIP
100 µM methyl viologen
Chloroplasts equivalent to 50 µg chlorophyll
Water to 1.9 ml

100 µl of herbicide (corresponding to 0.1 → 500 µM) was added once a linear rate of O_2 uptake was established. Uncoupling was determined as the percentage increase in control rate. Chloroplast preparations which showed less than a doubled rate on addition of NH_4Cl (1mM) were discarded.

5.2.5 Binding and Displacement experiments

The following reaction mixture was incubated at 20°C in a darkened O_2 electrode reaction chamber. After 5 minutes a 1 ml aliquot

30mM tricine-NaOH pH 8.0
20 µl of radiolabelled herbicide
Chloroplasts equivalent to 100 ug chlorophyll
water to 2 ml

was removed and immediately placed in a microfuge for three minutes at maximum speed. The supernatant was carefully retained and a further 0.5 ml of water was added to the Ependorf tube with minimal disruption of the pellet. After a second centrifugation, the supernatants were combined and counted for radioactivity by mixing with 14 ml of Fisoflour 2 scintillant (see Section 3.2.5). The concentration (nmoles) of free unbound herbicide was calculated as:-

$$\frac{\text{dpm in supernatant}}{\text{total dpm applied}} \times \text{total concentration applied (nmoles)}$$

The nmoles bound was determined by subtracting nmoles free from the total nmoles applied. The specific activity of ^{14}C-(ring) ioxynil Na was 33.7 μCi.mg^{-1} ($\equiv$ 7.56 mM) and of ^{14}C-(ring) bromoxynil K was 41.9 μCi.mg^{-1} ($\equiv$ 7.58 mM).

In displacement experiments, the reaction mixture contained:

30mM Tricine-NaOH pH 8.0
30 μl of ^{14}C-labelled herbicide
Chloroplasts equivalent to 150 μg of chlorophyll
Water to 3 ml

A 1 ml aliquot was removed after 5 minutes and assayed as before. At this time 20 μl of unlabelled herbicide ($\equiv$ 30 nmoles) were added and after a further 5 minute dark incubation a 1 ml sample was removed and again centrifuged as above. If the unlabelled herbicide was replacing the labelled herbicide on the thylakoid membrane there would be an increase in the amount of radioactivity detected in the supernatant.

5.3 RESULTS

5.3.1 Isolation of active chloroplast preparations

There are almost as many different methods of chloroplast isolation as there are research groups in the field. Ideally chloroplast preparations should as closely as possible resemble the chloroplast *in vivo* and be isolated from metabolically competent tissue. In an attempt to standardize isolation procedures,

Reeves and Hall (1980) reviewed preparation techniques for various classes of chloroplasts. Highest rates of electron transport were obtained in type C broken chloroplasts prepared by resuspending intact chloroplasts (type A) in a hypotonic medium. This method is suitable for standard tissue such as spinach and peas, however problems in tissue maceration make it more difficult to initially extract intact chloroplasts from other species.

Tissue maceration is less critical when type E chloroplast fragments are isolated directly from leaves. Pallett and Dodge (1979) obtained chloroplast fragments from peas which were capable of coupled $H_2O \longrightarrow$ ferricyanide Hill reaction rates of 9.5-12.0 umole O_2.mg.Chl $^{-1}$.hour^{-1}. Their method involved a short slow centrifugation step to remove cell walls and vascular tissue, and a faster centrifugation lasting 10 minutes to pellet out remaining chloroplast fragments. This method was initially used to isolate type E fragments from Stellaria media. As can be seen from Fig. 5.1, the rate of DCPIP reduction declined after 20 minutes. Addition of a chelating agent, EDTA, to the resuspension medium improved the rate of electron transport by 50%, and slowed the decline in activity. Similar rates of DCPIP reduction occurred when chloroplast fragments were isolated from Viola arvensis using the same procedure (Fig. 5.2). In this case, the activity remained almost constant for greater than 45 minutes. When chloroplast fragments were isolated using only one centrifugation step, the rate of DCPIP reduction gradually declined in both species, probably reflecting the cruder preparation procedure.

Preparation of active chloroplast fragments from M. inodora proved more difficult as the standard method (Pallett and Dodge 1979) resulted in a preparation with virtually no activity. At the end of the 10 minute centrifugation stage the supernatant exhibited a brown colouration indicative of high levels of phenolic compounds (Reeves & Hall 1980). The brown pigments (polymerized quinones and phenolic compounds) are known to precipitate and/or inhibit many enzymes and subcellular organelles.

Legends for Figures 5.1- 5.4

The effect of changes in isolation procedure on the photochemical activity of chloroplast fragments isolated from Stellaria media, Viola arvensis and Matricaria inodora.

Figure 5.1: Stellaria media

□----□ Grinding medium with added EDTA, PVP, BSA and ascorbate, resuspension medium with added EDTA, 1 spin method.

▲----▲ Standard grinding and resuspension medium, 2 spin method.

■——■ Standard grinding medium, resuspension medium with added EDTA, 2 spin method.

Figure 5.2: Viola arvensis

■——■ Standard grinding medium, resuspension medium with added EDTA, 2 spin method.

□----□ Standard grinding medium, resuspension medium with added EDTA, 1 spin method.

Figure 5.3: Matricaria inodora

■——■ Grinding medium with added EDTA, PVP and BSA, resuspension medium with added EDTA, 1 spin method.

△----△ Grinding medium with added EDTA, PVP, BSA, and ascorbate, resuspension medium with added EDTA, 2 spin method.

▲----▲ Grinding medium with added EDTA, PVP, BSA, and ascorbate, resuspension medium with added EDTA, 1 spin method.

□----□ Standard grinding medium, resuspension medium with added EDTA and PVP, 2 spin method.

Figure 5.4: The effect of light intensity on DCPIP reduction by S. media chloroplast fragments.

Figure 5.1

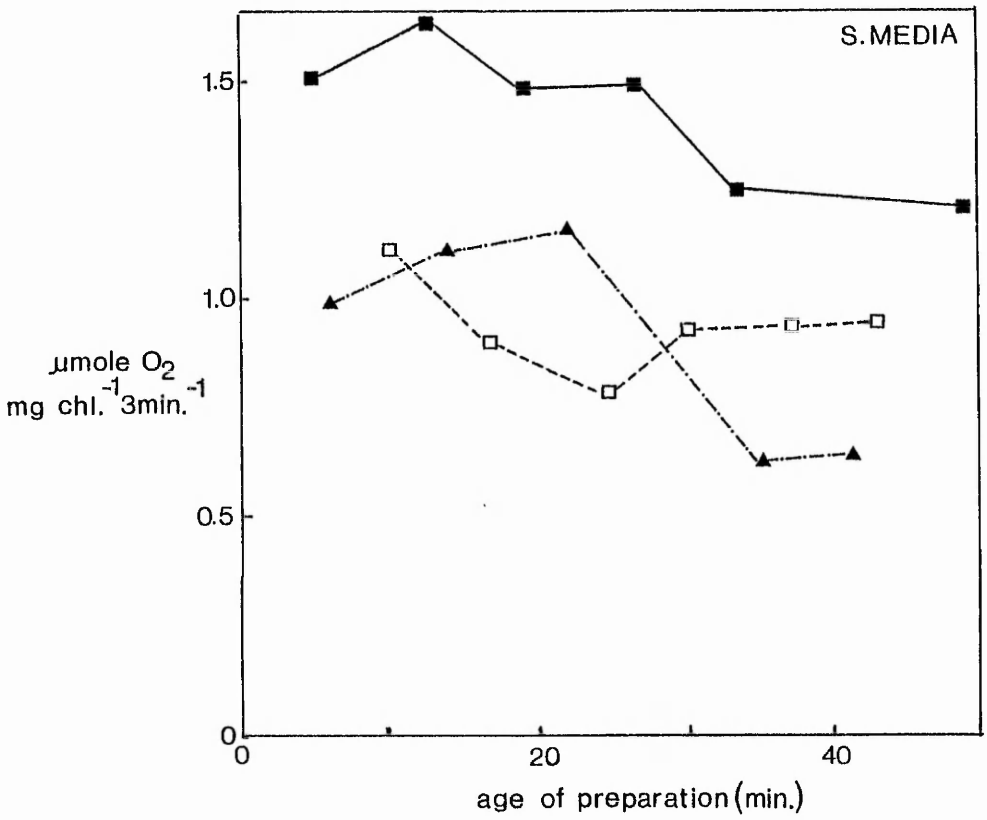


Figure 5.2

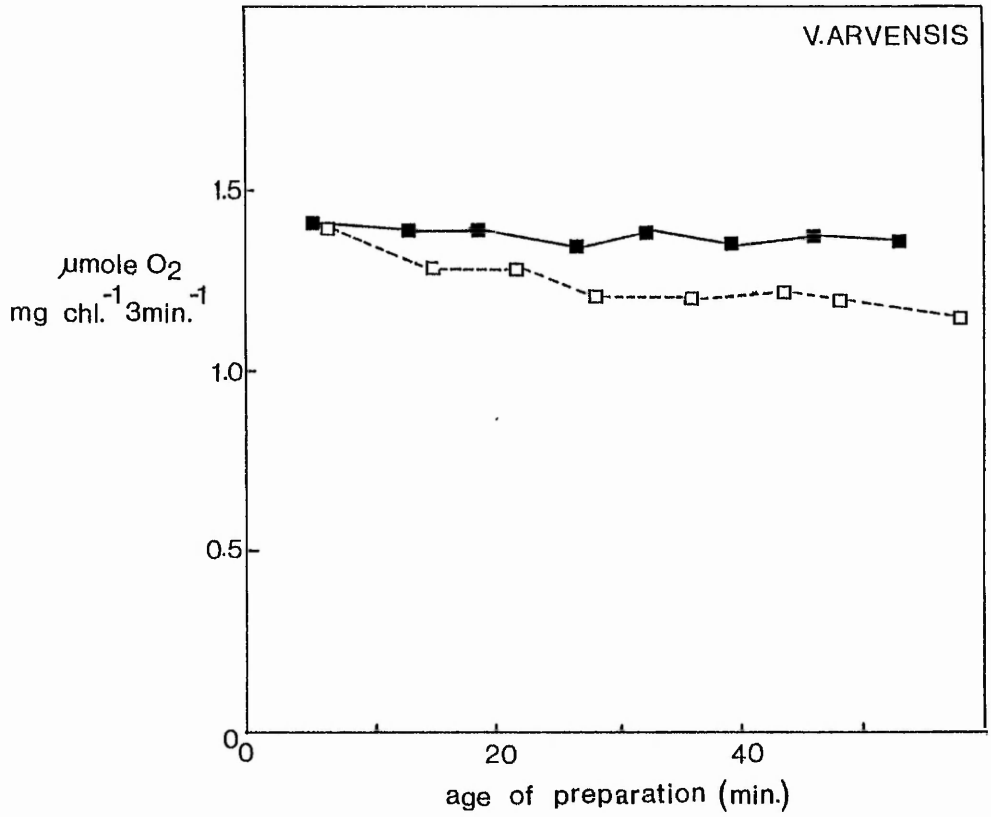


Figure 5.3

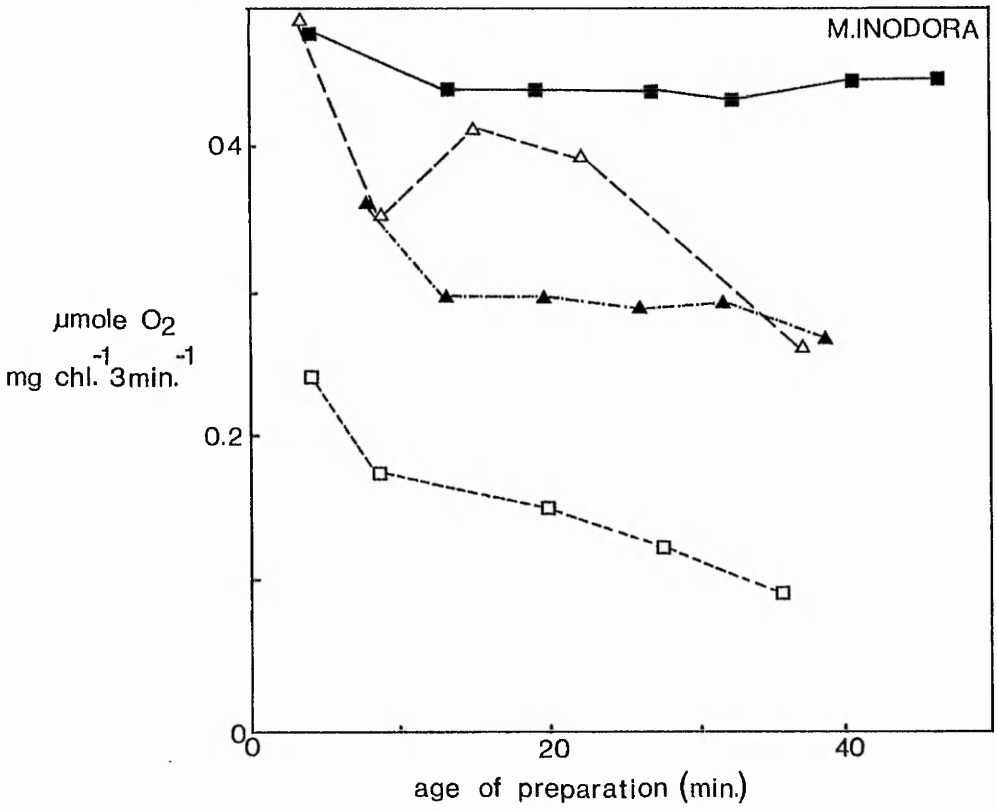
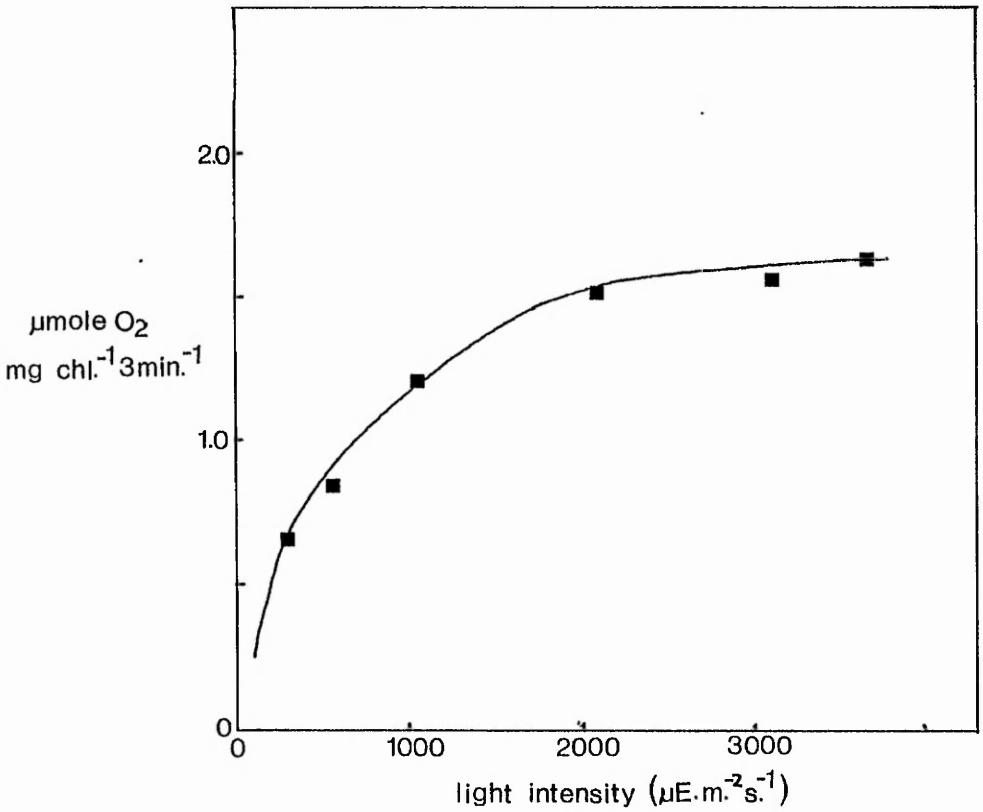


Figure 5.4



Standard tissue such as spinach and leguminous plants are relatively low in phenolic compounds (Baldry, Bucke and Coombs 1970.), and yield highly active chloroplast preparations. In an attempt to overcome the deleterious effects of phenolic contaminants in M. inodora preparations, additional BSA, polyvinylpyrrolidone (PVP) and ascorbate were added to the grinding and resuspension media. BSA and PVP are thought to prevent oxidation of phenolic compounds and subsequent enzyme inactivation (Baldry, Bucke and Coombs 1970), whilst ascorbate probably exerts a protective role by acting as a more general antioxidant (Leegood, Edwards and Walker 1982). Optimum levels of these additives were used to prevent any deleterious effects on the preparation itself.

Highest rates of DCPIP reduction were sustained when leaf tissue was macerated in a grinding medium containing additional EDTA, PVP and BSA, rapidly centrifuged at 3000g for one minute, and the pellet resuspended in a media containing 2mM EDTA (Fig. 5.3). The resulting chloroplast fragments were contaminated by cell debris and free chlorophyll which would explain apparently lower rates of oxygen evolution when calculated in μmole O_2.mg $Chll^{-1}$.hr^{-1}. As with S. media and V. arvensis rates were lower than those reported for spinach and peas (Reeves and Hall 1980), but were high enough to accurately measure electron transport inhibition, and stimulation of the modified Mehler reaction in photophosphorylation studies. Hill reaction rates were monitored at the beginning and end of a series of assays, and any decline in activity taken into consideration when the results were calculated. Generally, a chloroplast preparation was discarded after either 30 minutes (S. media) or 45 minutes (V. arvensis and M. inodora).

The photochemical reactions of chloroplasts are routinely monitored at saturating light intensities. Fig. 5.4 illustrates a light saturation curve for S. media fragments from which it was established that 3000 $\mu E.m^{-2}.sec^{-1}$ was a suitable level of irradiance. This intensity was used in all subsequent assays of light dependant reactions. Similarly, optimum levels of electron

acceptors were established for preparations from each species.

5.3.2 Electron Transport inhibition

Electron transport inhibition was monitored using two electron acceptors which act at dissimilar sites on the electron transport chain. The site of DCPIP reduction is pH dependent and under alkaline conditions, reduction of the ionized form is likely to occur in the region of PSI. In contrast, at acidic pH's the lipid soluble undissociated form predominates which can also intercept electrons from PSII, and uncouple photophosphorylation (Izawa 1980). Under the slightly alkaline conditions of the assay, DCPIP thus acts as a typical PSI electron acceptor although fragmentation of the chloroplast may allow some access to PSII. Inhibition of any stage of the intermediate transport chain would thus cause a decrease in the rate of DCPIP reduction and the rate of oxygen evolution. Increasing concentrations of ioxynil and bromoxynil resulted in sigmoidal inhibition curves (Fig. 5.5).

Silicomolybdate reduction was also investigated in order to specifically monitor the PSII activity of the hydroxybenzonitriles. This electron acceptor is believed to be reduced at 2 sites closely associated with the Q_B protein. The site of reduction has been found to be concentration dependant (Pallett and Dodge 1977) and at the levels used in these studies (0.5 to 0.75 mg/preparation) both sites of SiMo reduction were likely to be operative. The resulting biphasic curves (Fig. 5.6) may be due to differential inhibition of the two sites of SiMo acceptance.

In all three weed species ioxynil was a more effective inhibitor of electron transport than bromoxynil, irrespective of whole plant activity. 50% inhibition of DCPIP reduction required 2.6-4.5 x more bromoxynil than ioxynil, whereas inhibition of SiMo reduction occurred at lower concentrations, and ioxynil was even more inhibitory than bromoxynil (3.9-10.4 x) (Table 5.1). This difference may implicate an interaction between hydroxybenzonitrile binding sites, and SiMo acceptance sites.

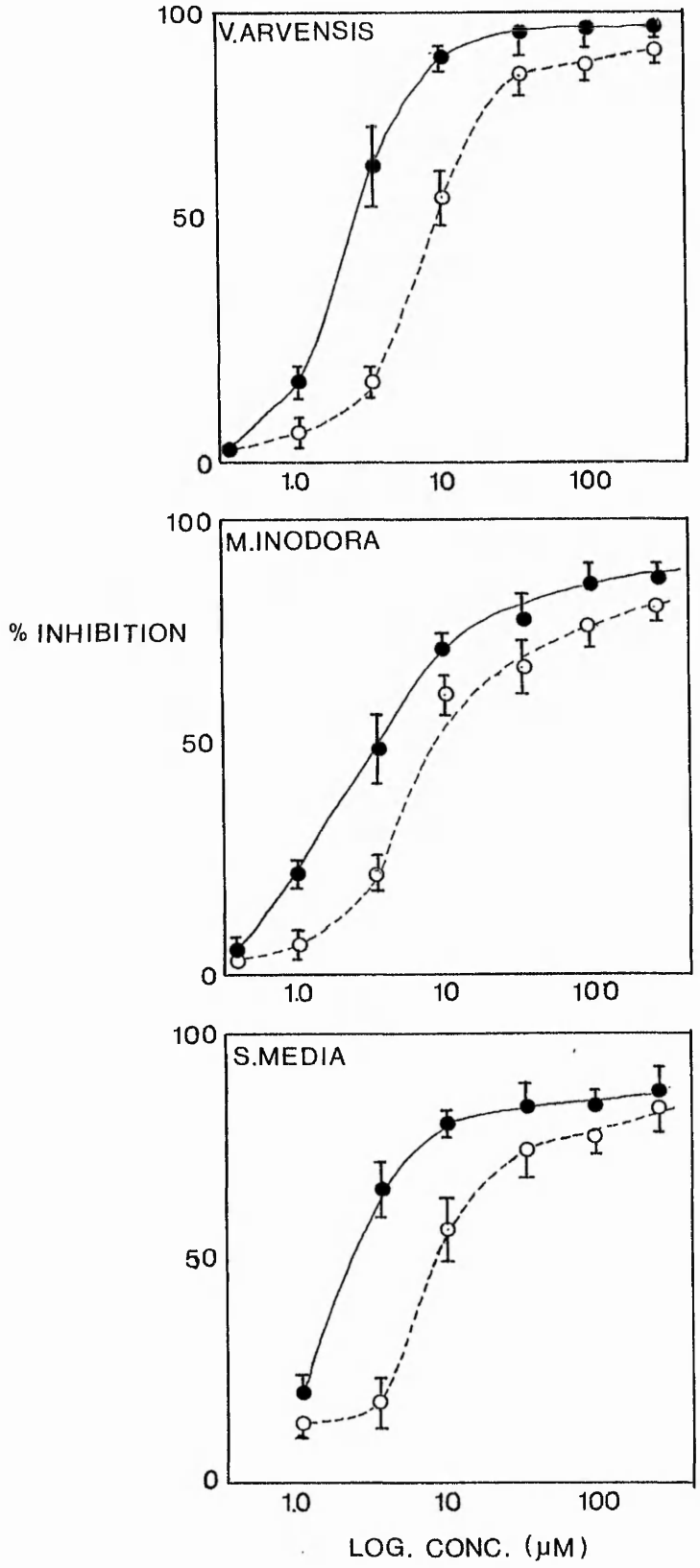


Figure 5.5: The effect of ioxynil (—●—) and bromoxynil (--o--) on DCPIP reduction by chloroplast fragments isolated from V. arvensis, S. media and M. inodora. Data is the mean of four replicates.

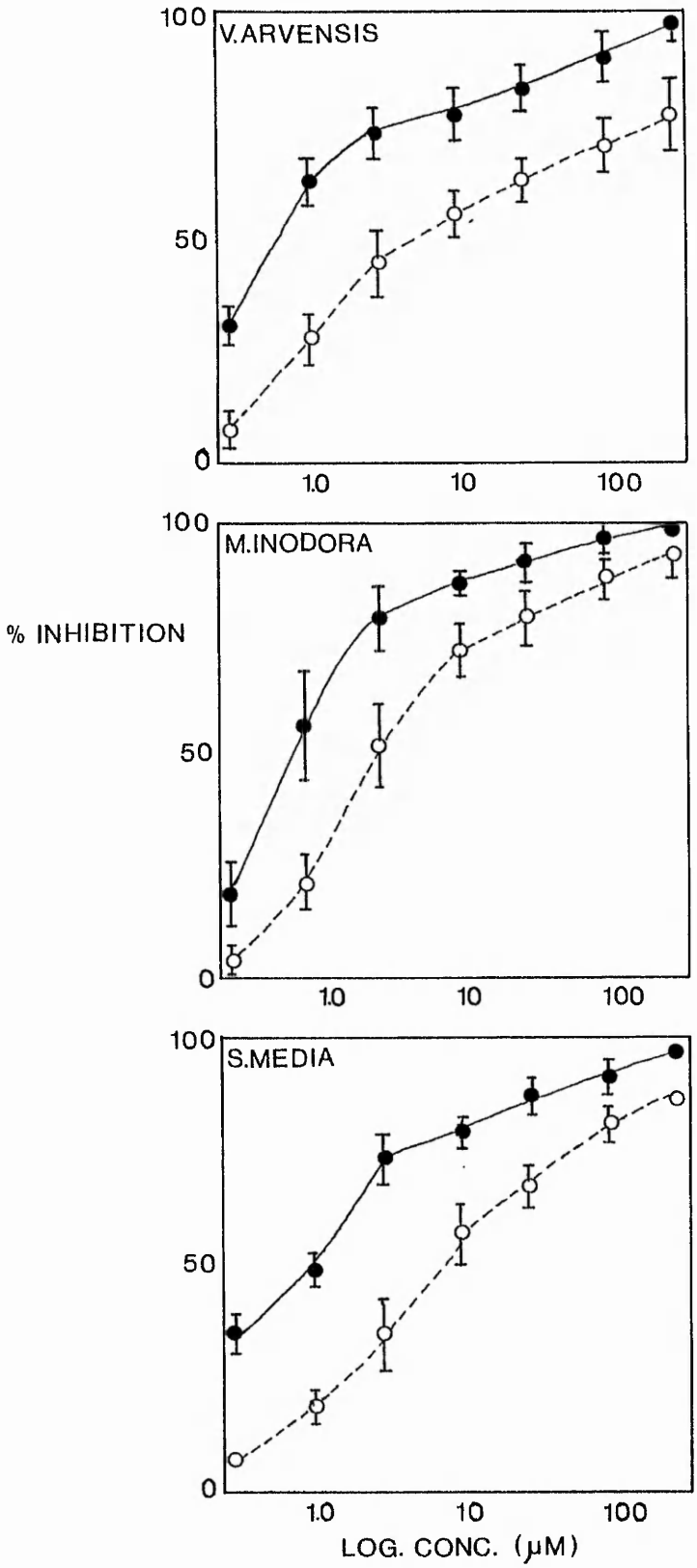


Figure 5.6: The effect of ioxynil (—●—) and bromoxynil (--○--) on silicomolybdate reduction by chloroplast fragments isolated from V. arvensis, S. media and M. inodora. Data is the mean of four replicates.

TABLE 5.1: The concentration of Herbicide necessary to give 50% inhibition of electron transport (I_{50}) with DCPIP and SiMo as electron acceptors

	I_{50} VALUE (μM)			
	IOXYNIL		BROMOXYNIL	
	DCPIP	SiMo	DCPIP	SiMo
V. arvensis	2.0	0.9	9.0	7.3
M. inodora	2.6	0.7	6.8	3.5
S. media	2.2	1.1	8.3	8.3

5.3.3 Uncoupling of photophosphorylation

Measurement of phosphate incorporation by P.M.S. mediated cyclic photophosphorylation indicated that broken chloroplasts (type C) isolated from young pea tissue were well coupled. Control chloroplasts were capable of phosphate incorporation rates averaging 174 μmoles phosphate. mg $chl^{-1}.hr^{-1}$. Attempts to isolate type C chloroplasts from the three experimental species were unsuccessful, and P.M.S. mediated cyclic phosphorylation in type E fragments yielded rates as low as 10 μm phosphate.$mg.chl^{-1}$. $hour^{-1}$. Further experimentation with pea chloroplasts revealed that the ascorbate/DCPIP → methyl viologen Mehler reaction was stimulated by ioxynil and bromoxynil in the same concentration range as cyclic photophosphorylation was reduced (Fig. 5.7). This reaction was considered by Van Rensen and Hobé (1979) to be a suitable assay for the uncoupling action of DNOC, and was thus investigated using type E chloroplast fragments isolated from V. arvensis, M. inodora and S. media. Control rates (μmole.mg. $chl^{-1}.hr^{-1}$) prior to the addition of uncoupling agents averaged 112.9 for Pisum sativum, 166.8 for V. arvensis, 82.1 for M.inodora and 185.7 for S. media.

Maximum stimulation of the Mehler reaction varied between 1½ times for S. media fragments to 6 times for peas giving an indication of the degree of coupling of each chloroplast preparation (Fig. 5.8). Isolation of active fragments from M. inodora

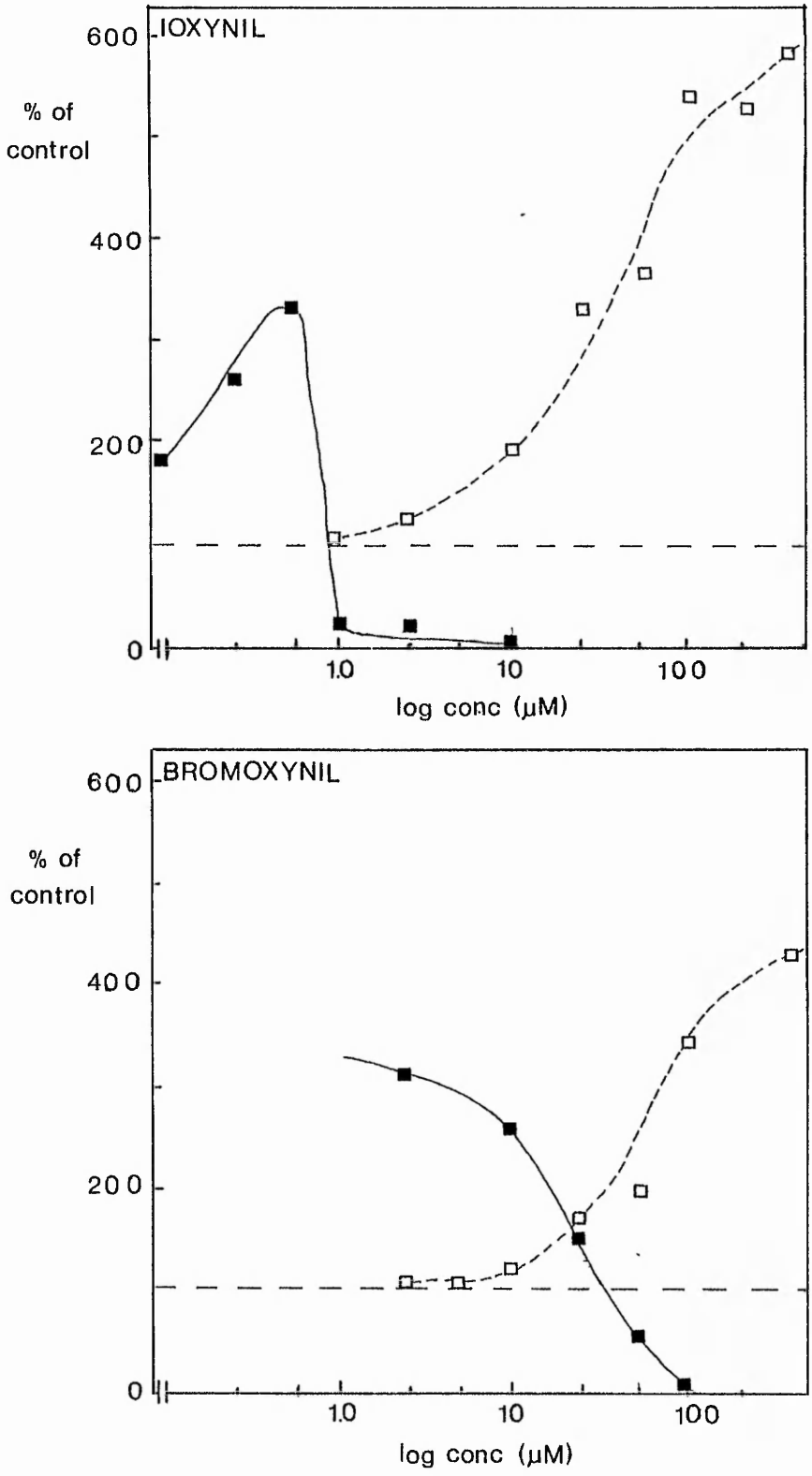


Figure 5.7: The effect of ioxynil and bromoxynil on the modified Mehler reaction (---□---) and PMS-mediated cyclic photophosphorylation (—■—) in pea chloroplasts (type C, Hall 1972). Data is the mean of four replicates.

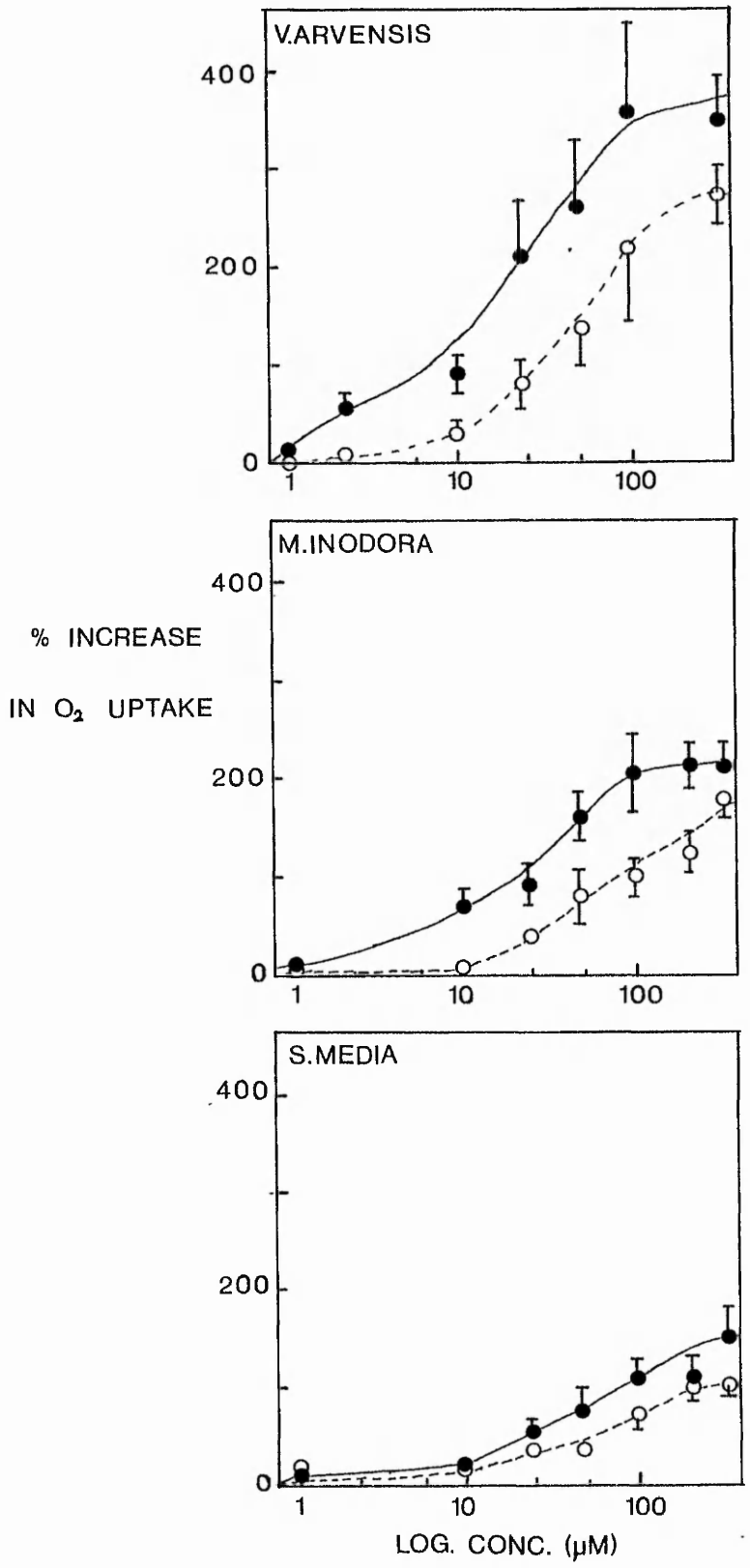


Figure 5.8: The effects of ioxynil (—●—) and bromoxynil (-·O·-) on the ascorbate/DCPIP Methyl Viologen modified Mehler reaction. Data is the mean of four replicates.

necessitated the inclusion of several chelating and antioxidizing agents which may have interferred with photophosphorylation in this species. Similarly the necessity for a crude preparation may have further uncoupled the chloroplasts. S. media fragments exhibited high control rates implying that the preparation was partially uncoupled before ioxynil and bromoxynil were added. Clearly interspecific comparisons are invalid although intra-specific analogies can be made.

As with electron transport inhibition, ioxynil was found to be a more effective uncoupler of the modified reaction than bromoxynil (Fig. 5.8). For comparison with the I_{50} value, the concentration required to stimulate the Mehler reaction by 50% of the maximum for a particular species, and that required to double the control rate (S_{100}, Moreland and Novitsky 1984) are tabulated below:

TABLE 5.2: Stimulation of the Mehler reaction by ioxynil and bromoxynil

	µM required to stimulate the Mehler reaction by 50% of maximum uncoupled rate		µM required to double the Mehler reaction rate, S_{100}	
	Ioxynil	Bromoxynil	Ioxynil	Bromoxynil
V. arvensis	25	76	10	40
M. inodora	29	130	19	95
S. media	85	185	95	180

Ioxynil was 2-5 x more effective than bromoxynil and in general these compounds uncoupled the Mehler reaction at concentrations approximately 10 fold higher than they inhibited the $H_2O \rightarrow$ DCPIP Hill reaction.

5.3.4 Binding Studies

Accurate separation of membrane bound herbicide from unbound herbicide was critical for the investigation of binding properties. The two fractions are usually separated by either

filtration through a micropore filter (Reimer, Link and Trebst 1979) or by a rapid high speed centrifugation (Oettmeier and Masson 1980; Pfister, Steinback, Gardner and Arntzen 1981). In this study, separation by microfuge was considered most practical as the use of disposable Ependorf tubes reduced the risk of contamination between samples. Incorporation of a second wash of the pellet reduced standard errors by 25% (from 2.09 to 1.57%, mean of 6 separations).

Previous studies of ioxynil binding have indicated a time lag of upto 5 minutes for maximum binding, and replacement of ^{14}C-Metribuzin (Reimer, Link and Trebst 1979). An initial time course experiment (Fig. 5.9) confirmed that ioxynil binding to thylakoids reached maximum after 5 minutes, and remained at the same level for a further 5 minutes. It was thus considered acceptable to sample binding and replacement experiments following 5 minute dark incubations. Binding to M. inodora thylakoids exhibited little change for upto 60 minutes after extraction (Fig. 5.10), and all subsequent assays were carried out within 45 minutes of isolation.

The binding of ^{14}C-ioxynil and ^{14}C-bromoxynil was initially investigated over the concentration range 0-15 μM which accounted for 0-95% inhibition of DCPIP reduction. Binding curves were slightly biphasic, and no significant difference was identified between the amount of ioxynil and bromoxynil bound at a given concentration (Fig. 5.11). Calculation of the inhibition (Ki) and binding (Kb) constants (Fig. 5.14 and 5.15) revealed a Ki/Kb ratio of approximately 1 for ioxynil binding and 3 for bromoxynil (Table 5.3) which implies that bromoxynil binds less specifically to the thylakoid preparation. Figure 5.11 also shows that there was approximately $1\frac{1}{2}$ x more herbicide binding to V. arvensis than M. inodora fragments which most probably reflects the difference in purity of each preparation. As confirmed by Table 5.4, V. arvensis fragments contained more binding sites on a chlorophyll basis than M. inodora fragments.

The radiolabelled herbicide displacement technique of Tischer and Strotmann 1977 was used in this study to investigate

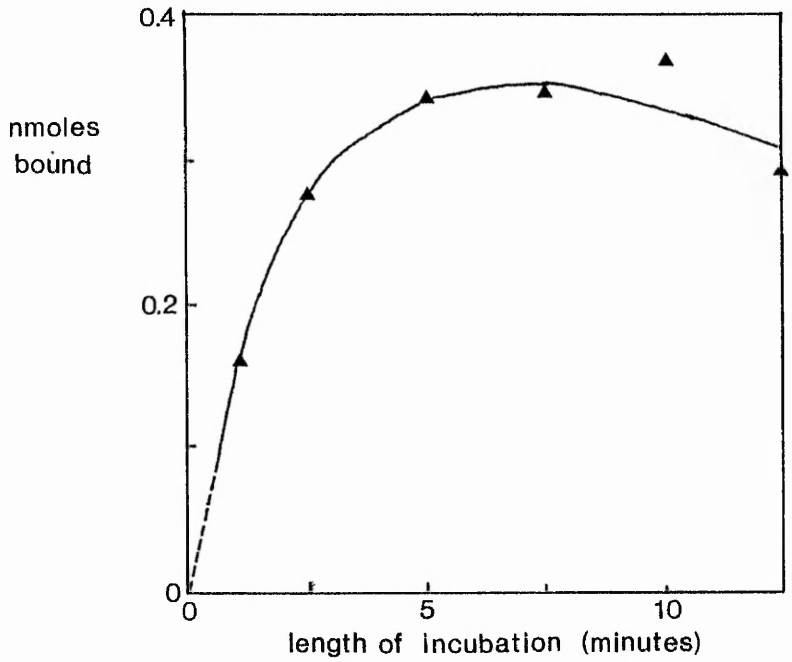


Figure 5.9: The binding of 4nmoles of (^{14}C)-ioxynil to a V. arvensis chloroplast fragments preparation. Data is the mean of 3 replicates.

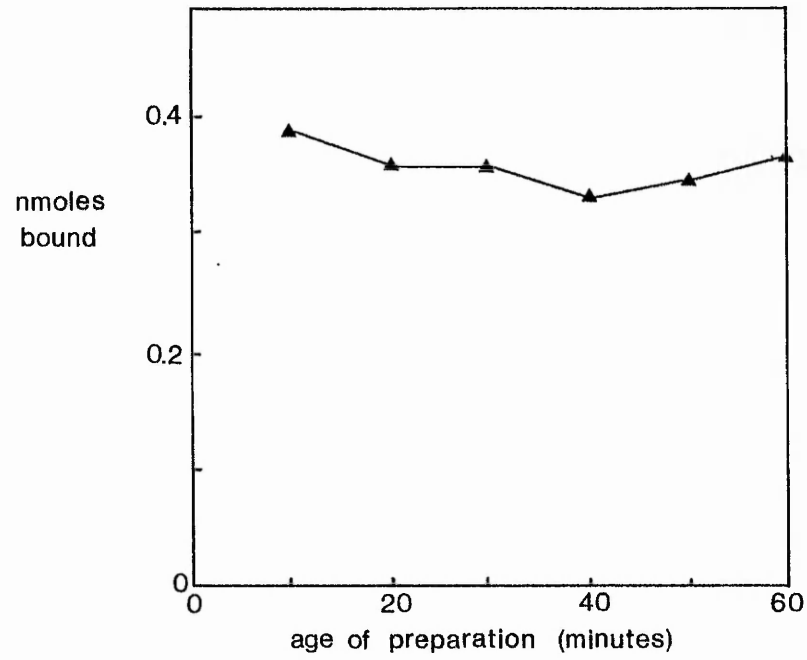


Figure 5.10: The effect of age of preparation on the binding of 4.0 nmoles of (^{14}C)-ioxynil to M. inodora chloroplast fragments. Data is the mean of 3 replicates.

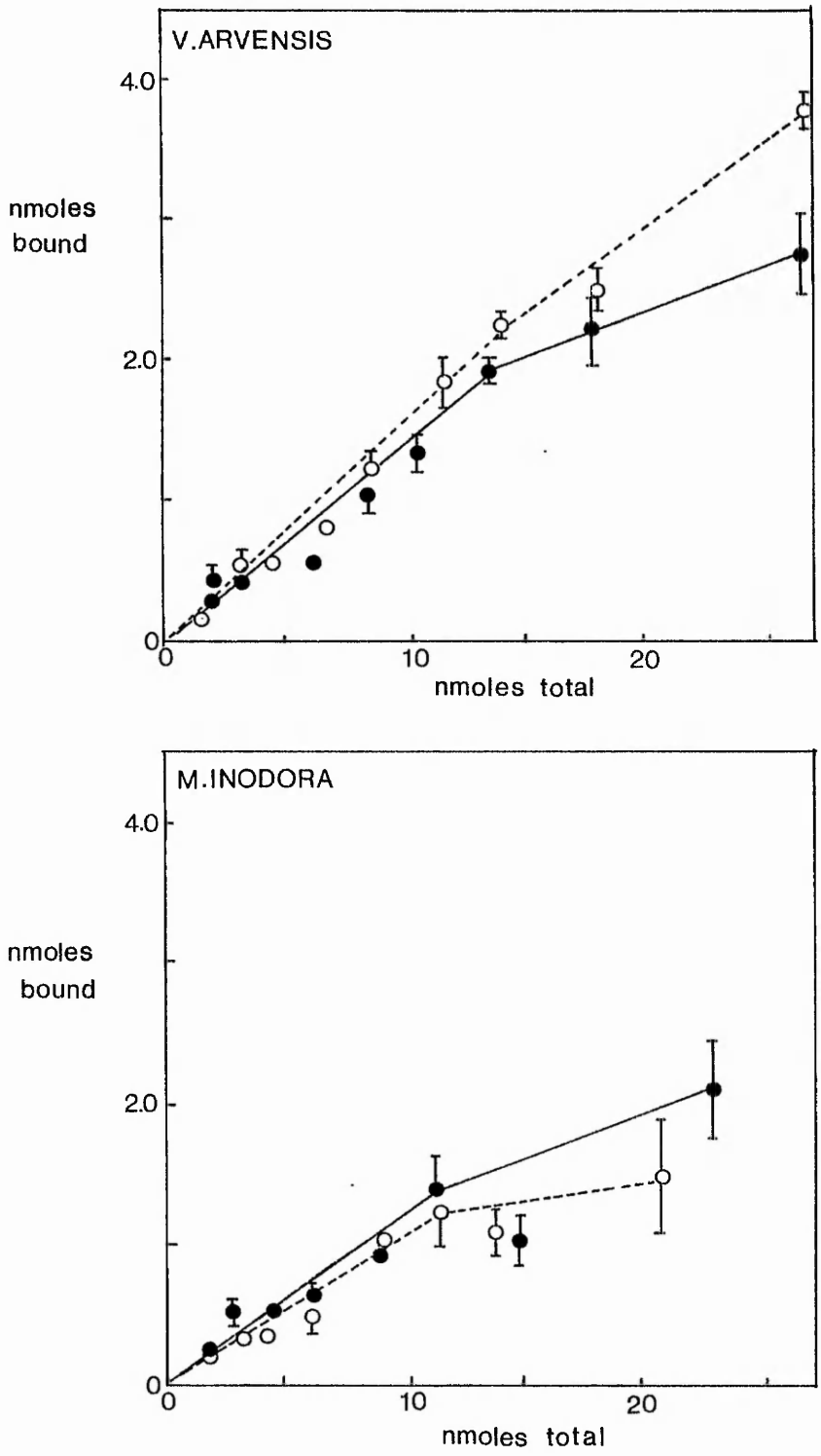


Figure 5.11: The binding of (^{14}C)-ioxynil (—●—) and (^{14}C)-bromoxynil (--o--) to chloroplast fragments isolated from V. arvensis and M. inodora. Data is the mean of six replicates.

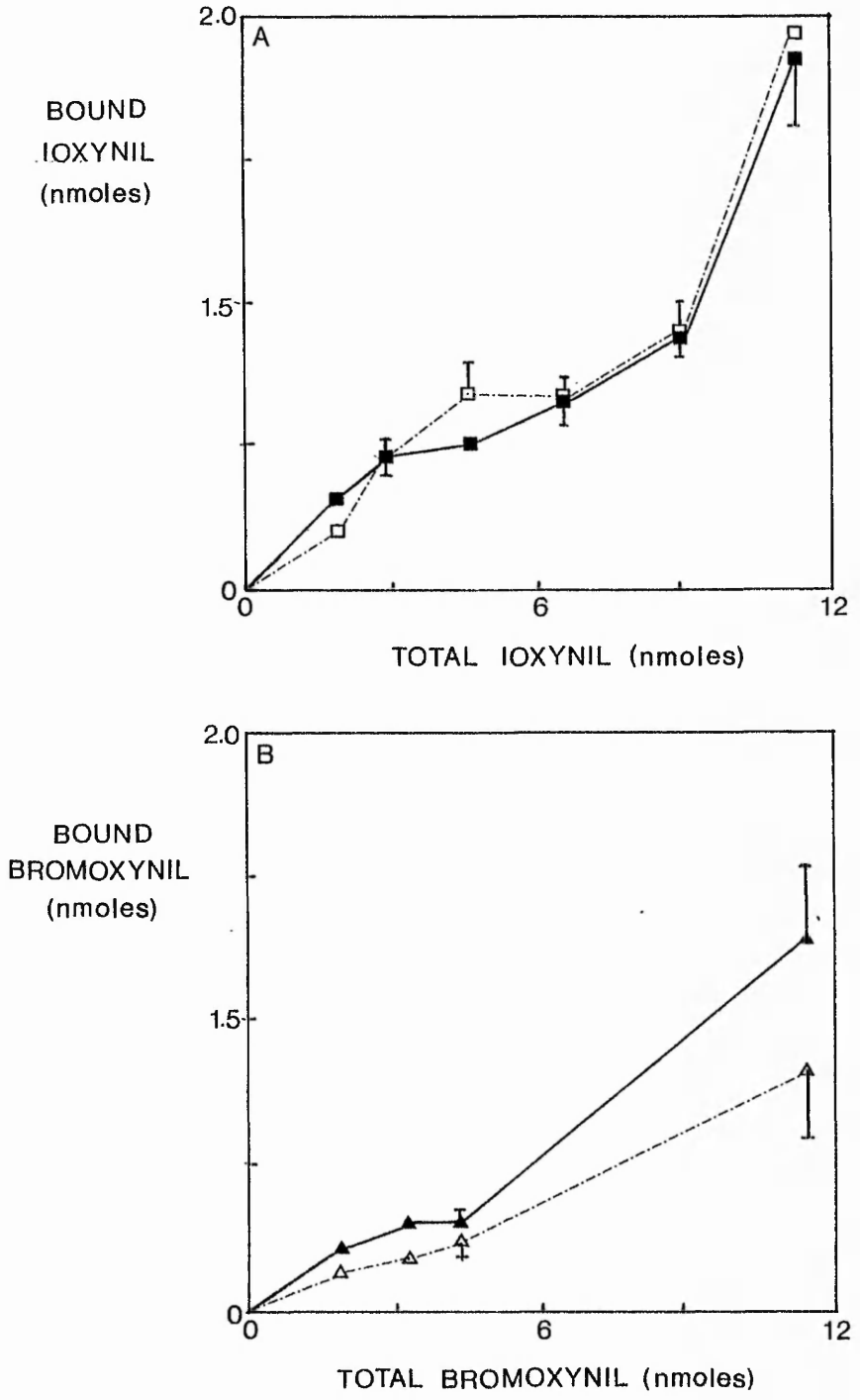


Figure 5.12: Binding of the hydroxybenzonitriles to M. inodora chloroplast fragments (A) (^{14}C)-ioxynil before (—■—) and after (--□--), the addition of 30nmoles bromoxynil, and (B) (^{14}C)-bromoxynil before (—▲—) and after (--△--) the addition of 30nmoles ioxynil. Data is the mean of five replicates.

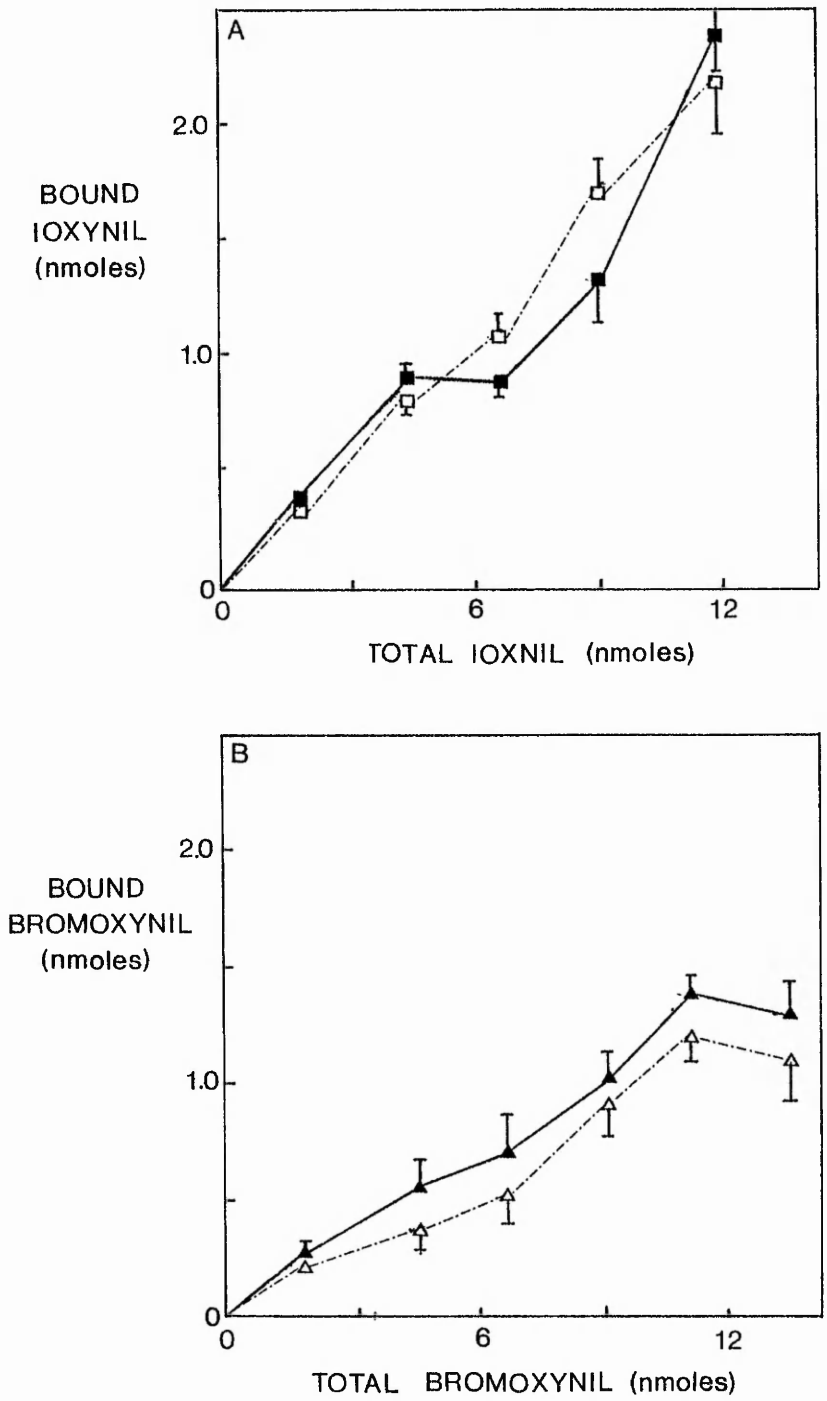


Figure 5.13: Binding of the hydroxybenzonitriles to V. arvensis chloroplasts (A) (^{14}C)-ioxynil before (—■—) and after (--□--) the addition of 30nmoles bromoxynil and (B) (^{14}C)-bromoxynil before (—▲—) and after (--△--) the addition of 30nmoles ioxynil.

the relative binding efficiency of ioxynil and bromoxynil. Concentrations of herbicide which correspond to the first phase of binding curves (2-12 nmoles) were incubated with chloroplast fragments isolated from the two species. Limitations on availability of radiolabelled herbicides meant that there was only sufficient radiolabel to investigate displacement from the thylakoid membrane by one concentration of unlabelled herbicide. A high concentration was chosen in order to maximise displacement, even though other workers tend to use lower concentrations (e.g. Tischer and Strotmann 1977; Oettmeier and Masson 1980).

This approach has revealed that once bound to V. arvensis and M. inodora thylakoids, ioxynil is not readily displaced by bromoxynil (Figs. 5.12 and 5.13). In contrast, about 35% of bound ^{14}C-bromoxynil was displaced from M. inodora thylakoids by ioxynil. A similar, although less pronounced displacement occurred with V. arvensis chloroplast fragments. The competitive nature of this interaction has been quantified by double reciprocal plots to determine changes in the binding constant (Kb) and the number of occupied binding sites per mg of chlorophyll, x (t), (Figs. 5.14 and 5.15). These plots show that there was clearly a competitive interaction between ioxynil and bromoxynil, once bromoxynil was bound to the thylakoid membrane (Figs. 5.16 and 5.17). The number of occupied binding sites remained unchanged whilst there was an increase in the binding constant (Table 5.4). Addition of bromoxynil to M. inodora chloroplast fragments had no significant effect on the number of binding sites occupied by ioxynil, or the binding constant; and the apparent increase in binding sites in V. arvensis fragments was not significant at the 5% level. Thus ioxynil appears to bind more strongly to the thylakoid membrane and is less easily displaced by bromoxynil.

TABLE 5.3: Comparison of the binding constant Kb and Inhibition constant, Ki (Data extrapolated from inhibition curves)

	Ki	Kb	Ki/Kb
Ioxynil			
V. arvensis	3.34×10^{-9}	3.217×10^{-9}	1.04
M. inodora	3.31×10^{-9}	3.680×10^{-9}	0.90
Bromoxynil			
V. arvensis	2.00×10^{-8}	6.272×10^{-9}	3.19
M. inodora	2.08×10^{-8}	5.854×10^{-9}	3.55

TABLE 5.4: Replacement of (^{14}C)-ioxynil and (^{14}C)-bromoxynil: Changes in the binding constant, Kb and the number of binding sites, x(t)

	Kb (M)	x(t) (nmoles. mg chl^{-1})
V. ARVENSIS		
^{14}C-ioxynil only	$3.217\ (\pm 0.045) \times 10^{-9}$	14.16 (±0.86)
^{14}C-ioxynil + bromoxynil	$4.996\ (\pm 0.179) \times 10^{-9}$	19.62 (±1.91)
^{14}C-bromoxynil only	$6.727\ (\pm 0.61) \times 10^{-9}$	15.29 (±1.68)
^{14}C-bromoxynil + ioxynil	$8.765\ (\pm 1.34) \times 10^{-9}$	14.79 (±0.32)
M. INODORA		
^{14}C-ioxynil only	$3.680\ (\pm 0.21) \times 10^{-9}$	11.682 (±0.46)
^{14}C-ioxynil + bromoxynil	$4.075\ (\pm 0.43) \times 10^{-9}$	11.516 (±0.77)
^{14}C-bromoxynil only	$5.854\ (\pm 0.31) \times 10^{-9}$	9.483 (±0.97)
^{14}C-bromoxynil + ioxynil	$9.184\ (\pm 0.67) \times 10^{-9}$	9.259 (±1.52)

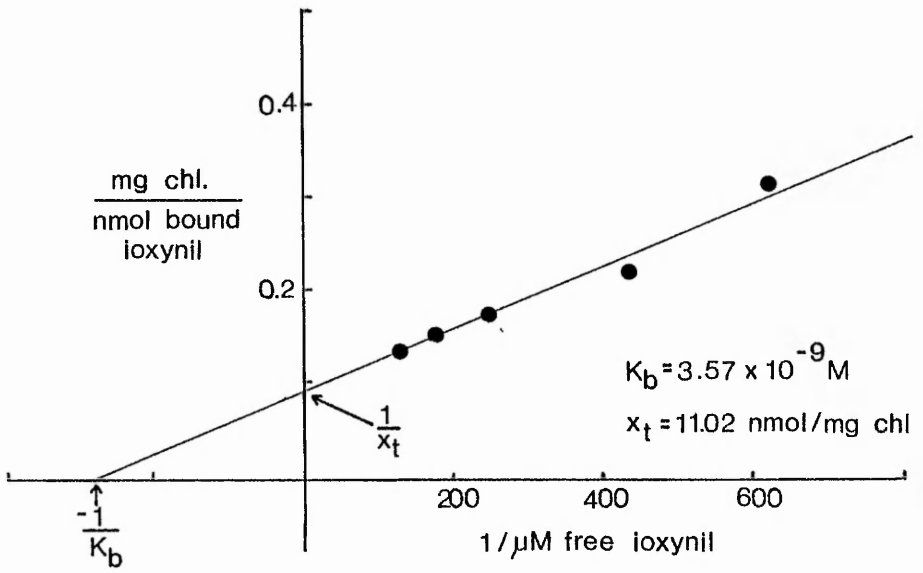


Figure 5.14: Double reciprocal plot of (^{14}C)-ioxynil binding to M. inodora chloroplast fragments. Determination of the binding constant, K_b, and the number of binding sites, x(t).

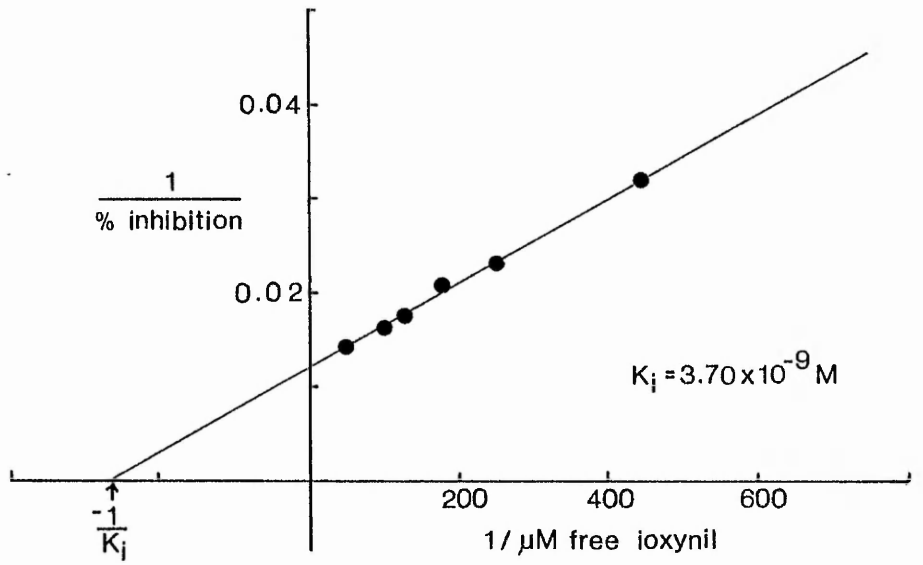


Figure 5.15: Double reciprocal plot of the inhibition of DCPIP reduction by ioxynil. Determination of the inhibition constant, Ki (data extrapolated from Inhibition curve).

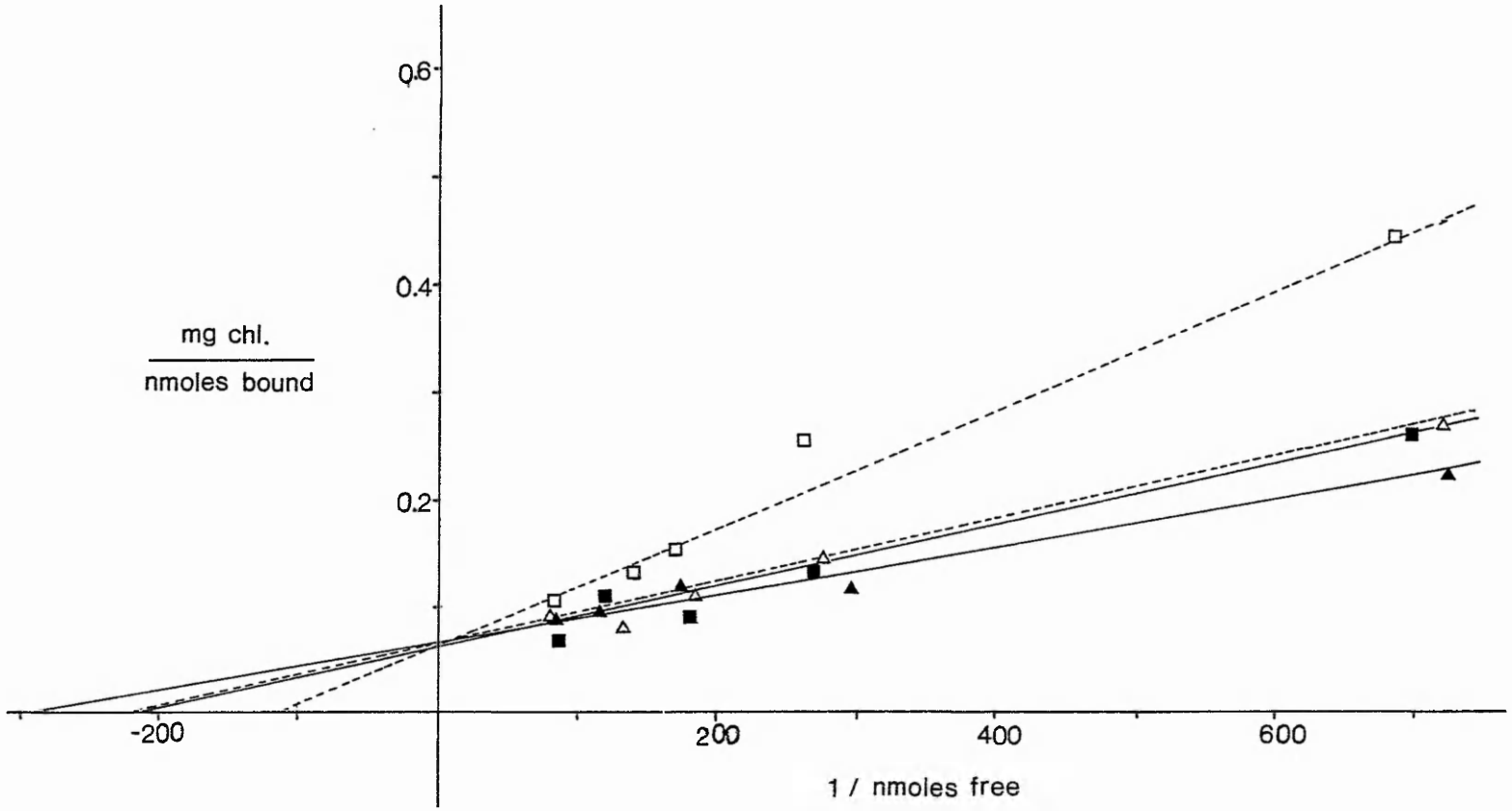


Figure 5.16: A double reciprocal plot for the binding of (^{14}C)-ioxynil alone (—▲—), (^{14}C)-bromoxynil alone (—■—), (^{14}C)-ioxynil plus bromoxynil (---△---) and (^{14}C)-bromoxynil plus ioxynil (--□--) in chloroplast fragments isolated from M. inodora.

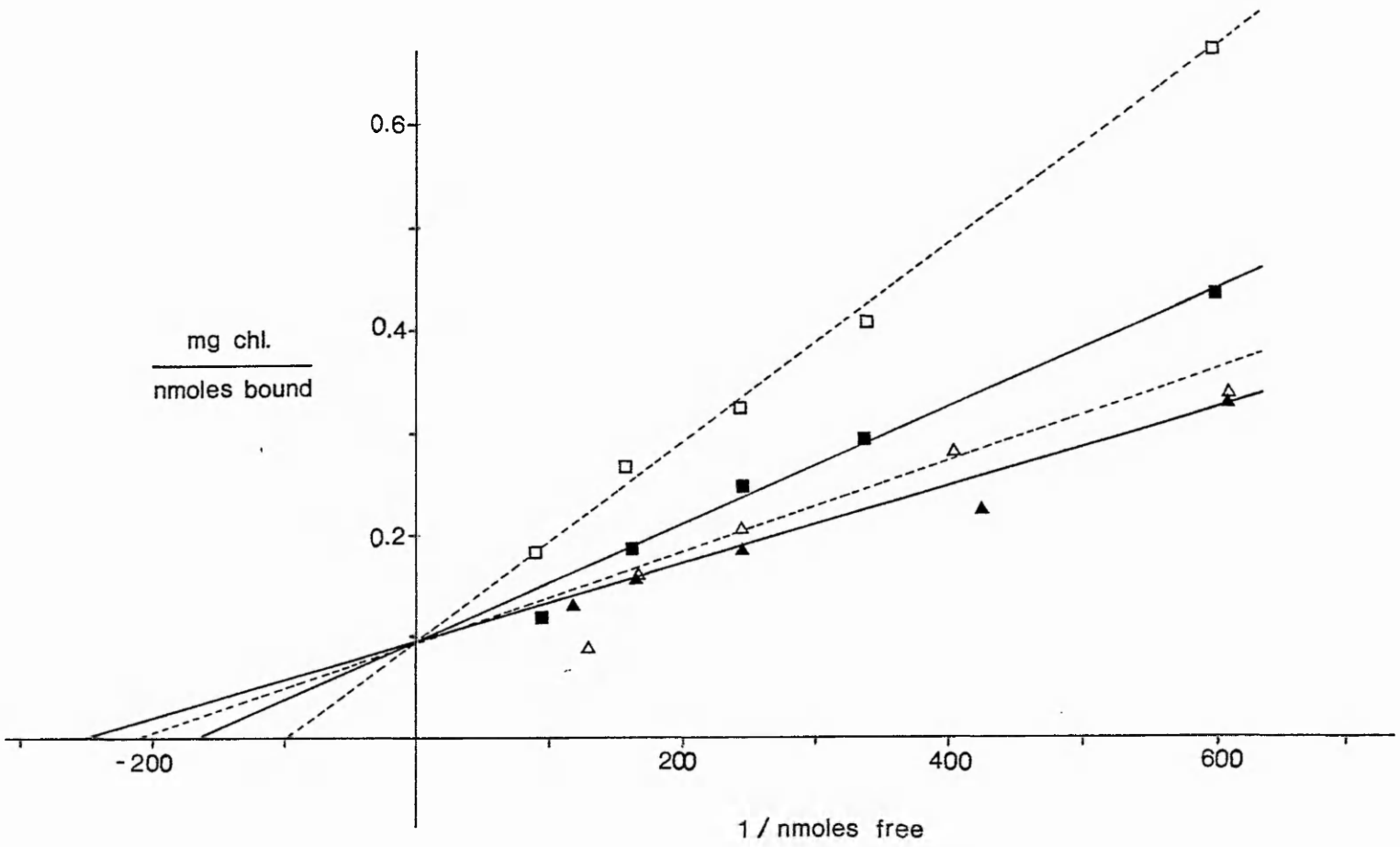


Figure 5.17: A Double reciprocal plot for the binding of (^{14}C)-ioxynil alone (—▲—), (^{14}C)-bromoxynil alone (—■—), (^{14}C)-ioxynil plus bromoxynil (--△--), and (^{14}C)-bromoxynil plus ioxynil (--□--) in chloroplast fragments isolated from V. arvensis.

5.4 DISCUSSION

Isolation of active chloroplast fragments from *M. inodora*, *V. arvensis* and *S. media* has enabled the mechanism of action of the hydroxybenzonitriles to be investigated. *In vitro* studies have confirmed that ioxynil and bromoxynil can uncouple photophosphorylation and inhibit electron transport and can justifiably be classified as inhibitory uncouplers (Moreland 1980). The mechanism of action thus involves an interaction with proteinaceous and/or lipoidal components of spatially separated regions of the thylakoid membrane.

Phenolic herbicides are considered to uncouple photophosphorylation by acting as protonophones and shuttling protons across the thylakoid membrane (Moreland and Novitzky 1982). Activity is dependant upon lipid solubility and the Pk of the molecule. When dissociated, the hydroxybenzonitriles form weak acids which are capable of reacting with protons in the described manner (Section 1.3.3). Ioxynil may be a more effective uncoupler because it has a lower Pk value than bromoxynil (3.96 compared to 4.08) and is therefore more readily dissociated to the acid form. Similar investigations with carbamate herbicides have revealed that uncoupling activity is dependant on the lipophilicity of the herbicide molecule (Moreland,and Novitzky 1982).

In a more recent study Moreland and Novitzky 1984 attributed the protonophone action to low concentrations of uncouplers. At higher concentrations these compounds were found to disrupt the permeability and integrity of all cellular membranes (see Chapter 4). Uncoupling ability was generally correlated to binding affinity for the Q_B protein, even though the two effects were considered independant. The results of the present study are in agreement with this generalization (Tables 5.2 and 5.3).

In this study uncoupling of photophosphorylation was assayed by monitoring stimulation of the Mehler reaction, and inhibition of P.M.S.-modiated cyclic photophosphorylation. Each assay was equally sensitive in pea chloroplasts, and the hydroxybenzonitriles generally uncoupled photophosphorylation at concentrations approximately 10 fold

higher than required to inhibit electron transport (Tables 5.1 and 5.2). Early investigations with pea chloroplasts and mitochondrial fragments showed a similar difference (Kerr and Wain 1964a, b). In contrast, Moreland and Novitzky (1984) compared various spinach chloroplast assays and found that I_{50} values for noncyclic electron transport and photophosphorylation were similar, whereas assays involving PSI were upto 1000 times less sensitive to ioxynil. Clearly an element of caution is necessary before *in vitro* results are extrapolated to the *in vivo* situation.

Inhibition of PSII-dependant reactions is believed to occur in appressed regions of the granal stacks. Electrophoretic studies of the PSII-core complex have revealed at least 5 polypeptide components: 47 KDa (chla binding PSII reaction centre protein), 43 KDa (light harvesting role), 32 KDa (herbicide binding or Q_B protein), 6 KDa (associated with cytochrome b_{559}) and a 34 KDa protein associated with the core complex (Satoh, Nakatani, Steinback, Watson and Arntzen 1983). A 41 KDa polypeptide has also been attributed to this complex, and implicated in herbicide binding (Oettmeier, Masson and Johanningmeier 1982). The primary and secondary electron accepting (Q_A + Q_B) plastoquinones are situated within this protein framework, and binding to the 32 KDa protein is proposed to block electron transfer between Q_A and Q_B (Vermaas and Govindjee 1981).

Hydroxybenzonitrile inhibition has been attributed to binding to both the 32 KDa Q_B protein and the 41 KDa reaction centre protein (Laarsch, Pfister and Urbach 1982; Oettmeier *et al* 1982). Oettmeier, Masson and Johanningmeier (1982) concluded from photoaffinity labelling and competitive binding studies that all phenolic herbicides block electron transfer by binding to a 41 KDa protein. However, the photoaffinity labelled compound also bound to 32, 25, 17 KDa proteins and low molecular weight compounds such as lipids and pigments. Fluorescence and luminescence studies revealed a more likely mechanism of action for the hydroxybenzonitriles (Pfister and Schreiber 1984). These compounds were thought to initially bind to the 32 KDa protein inhibiting electron transfer on the acceptor side of PSII, and additionally although less strongly to a 41 KDa protein associated with the PSII donor site. This

is in agreement with previous studies which have implicated a second binding site (Pallett and Dodge 1979), and the time requirement for maximal inhibition and binding (Reimer, Link and Trebst 1979).

Interaction of the hydroxybenzonitriles with the PSII complex was initially investigated in this study by a dose response curve for SiMo reduction (Fig. 5.6). This electron acceptor is believed to be reduced at or before Q when electron transfer is blocked by DCMU, and also prior to PQ when the full electron transport chain is operative (Giaquinta and Dilley 1975; Pallett and Dodge 1977). The biphasic nature of hydroxybenzonitrile inhibition suggests a mechanism which is concentration dependant. At low concentrations, the hydroxybenzonitriles probably bind preferentially to the surface exposed 32 KDa protein and inhibit SiMo reduction at PQ. Once the 32KDa binding sites are saturated by higher concentrations, the herbicide may additionally diffuse into the PSII-core complex and bind to the 41 KDa protein thus inhibiting SiMo reduction at Q (Fig. 5.18).

The situation may be further complicated by binding of SiMo to the Q_B protein. Controversial evidence suggests that herbicide inhibition is due to a decrease in the number of sites available for reduction (Boger 1982). However, independant investigations showed that SiMo reduction at Q was unaffected by depletion of the 32 KDa protein from Spirodela thylakoids (Vermaas and Govindjee 1981). In the present study, ioxynil and bromoxynil were incubated with chloroplast fragments prior to the addition of SiMo, and competition for binding sites on the Q_B protein may have decreased SiMo reduction near PQ. This could possibly explain why I_{50} values were lower for SiMo reduction than DCPIP reduction, and why concentrations below 1 uM significantly inhibited SiMo reduction.

In agreement with previous studies (Kerr and Wain 1964b; Trebst et al 1979) ioxynil was a more effective inhibitor than bromoxynil, regardless of in vivo activity (Figs. 5.5 and 5.6). This differential response was further investigated using the radiolabelled herbicides binding and displacement technique of Tischer and Strotmann 1977. Herbicide binding curves are generally biphasic indicating high affinity or specific binding at low concentrations, and low affinity or unspecific

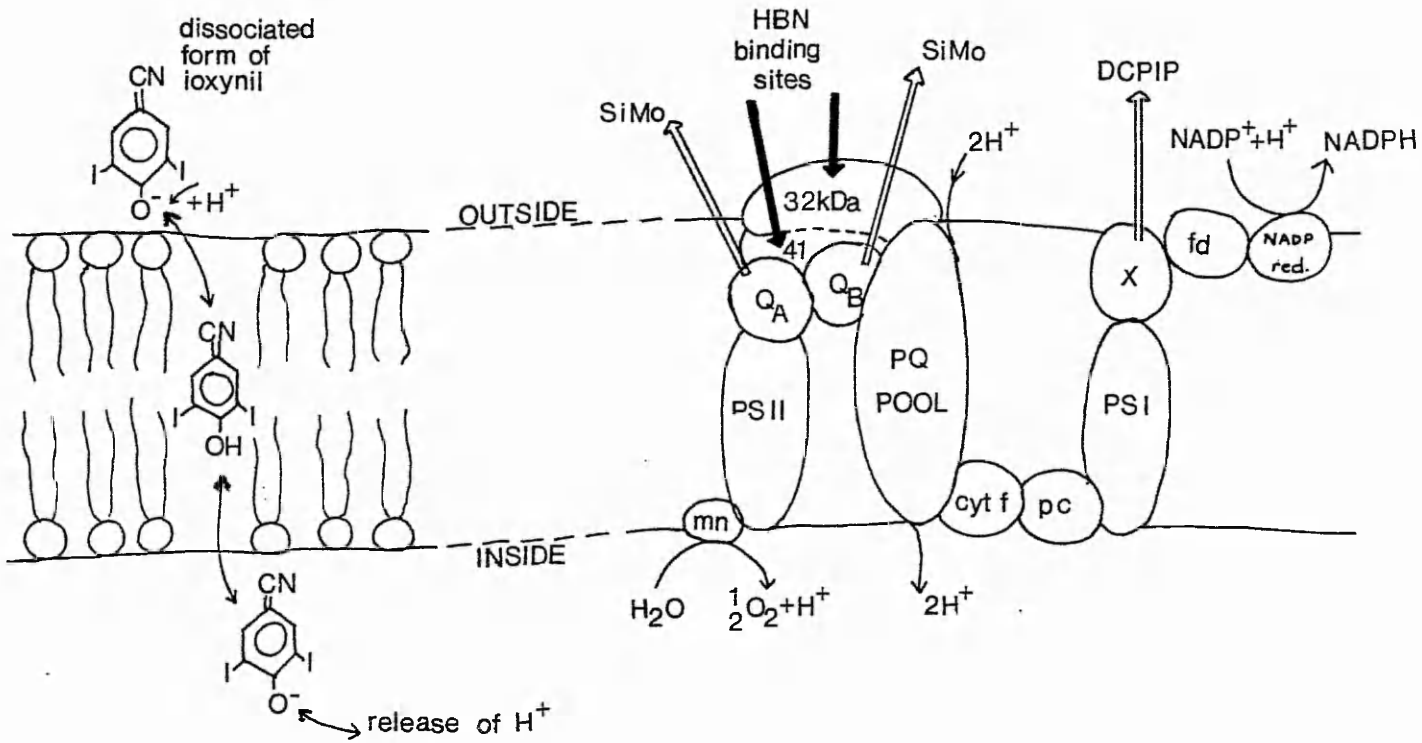


Figure 5.18: A scheme showing the proposed hydroxybenzonitrile binding sites and DCPIP and SiMo electron acceptance sites on the thylakoid membrane. A possible uncoupling mechanism is also included.

binding at higher concentrations (e.g. atrazine, Tischer and Strotmann 1977; dinoseb , Oettmeier and Masson 1980). In the present investigation phase changes were illdefined although proportionately less hydroxybenzonitrile binding occurred at concentrations above 12-16 nmoles (Fig. 5.11). Displacement experiments were performed for 2-12 nmoles of herbicide (equivalent to 1-6 μM) which accounted for 16-78% of ioxynil inhibition, and 6-45% of bromoxynil inhibition. Other workers have found ioxynil binding to be highly specific at concentrations below the I_{50} value (Laarsch et al 1981; Oettmeier et al 1982) and thus it was tentatively concluded that this concentration range represented specific binding to V. arvensis and M. inodora thylakoids.

Double reciprocal plots were used by Tischer and Strotmann 1977 to show that the specific binding constant K_b, was correlated to the inhibition constant Ki. Herbicide binding was considered totally specific when Ki and K_b exhibited a 1:1 relationship. When these constants were compared in the present study (Table 5.3) ioxynil binding was found to be approximately three times more specific than bromoxynil binding. This difference in specificity correlates directly with differences in the I_{50} values.

It has also been possible to determine the number of occupied binding sites (x_t) from double reciprocal plots (Fig.5.14 and Table 5.4). In comparison with other studies of PSII inhibitor binding (Tischer and Strotmann 1977; Oettmeier, Masson and Johanningmeier 1980; Pfister, Steinback, Gardner and Arntzen 1981), the x_t values for the hydroxybenzonitriles were relatively high suggesting unspecific binding. When recalculated, the xt values correspond to approximately one binding site per 110-130 chlorophyll molecules (M. inodora) and one site per 60-80 chlorophyll molecules (V. arvensis). DCMU-type inhibitors are frequently reported to bind to one site per 300-500 chlorophyll molecules, or approximately one site per electron transport chain (Tischer and Strotmann 1977; Pfister, Radosevich and Arntzen 1979) whereas one site per 415-540 chlorophyll molecules has been reported for phenol-type inhibitors (Laasch, Pfister and Urbach 1981). In the present study, the higher number of binding sites per electron transport chain may be further evidence for the involvement of 2 binding sites in hydroxybenzonitrile inhibition of PSII. Furthermore, uncoupling may occur in

the concentration range used, which may possibly involve binding to the coupling factor, or in the region of PSI (Barber 1983). Thus more than one binding site may be involved in the inhibitory uncoupling action of the hydroxybenzonitriles.

The degree of specificity also appears to affect the type of interaction between structurally dissimilar compounds. At high concentrations, Oettmeier et al 1982 found that ioxynil interacts non-competitively with DCMU-type herbicides, and competitively with other phenols providing non specific binding was taken into consideration. In contrast, Laarsch, Pfister and Urbach (1982) directly measured high specific binding and suggested that all herbicides interact competitively in the specific concentration range. In this study, ioxynil and bromoxynil were found to interact competitively in the concentration range 2-12 nmoles. As with other competitive interactions (e.g. Tischer and Strotmann 1977), the number of occupied binding sites remained unchanged, and the binding affinity increased once an unlabelled competitor was added to the reaction mixture. Interestingly, bromoxynil was more readily replaced by ioxynil, whereas only minimal amounts of ioxynil were displaced by bromoxynil. Thus replacement experiments further substantiate the theory that ioxynil is a more effective inhibitor because it binds more strongly to the thylakoid membrane.

In conclusion, in vitro studies have shown that interspecific differences in activity are overcome at the thylakoid level. In each species, ioxynil was a more effective inhibitory uncoupler than bromoxynil, and bound more strongly to the thylakoid membrane. Furthermore, these results also suggest that PSII inhibition involves hydroxybenzonitrile binding at two sites believed to be associated with the Q_B protein.

CHAPTER SIX: CONCLUDING DISCUSSION

In this investigation into the differential activity of the hydroxybenzonitriles, it has been necessary to study various aspects of the mode of action of this group of herbicides. As a result, considerable information has been gained which confirms and in some cases elaborates on previous observations of hydroxybenzonitrile action. The mechanism of phytotoxicity will be discussed initially, followed by a detailed analysis of the basis for differential activity.

6.1 The mechanism of action of the hydroxybenzonitrile herbicides

Early *in vitro* investigations revealed that the hydroxybenzonitriles are active in both the chloroplast and mitochondrion (Kerr and Wain 1964a + b; Paton and Smith 1965). More recently experiments with trypsin treated chloroplasts, photo-affinity labelled herbicides and competitive binding studies have pinpointed the site(s) of hydroxybenzonitrile electron transport inhibition (Pallett and Dodge 1979, 1980; Oettmeier and Masson 1980; Oettmeier, Masson and Johanningmeier 1982; Laarsch, Pfister and Urbach 1982). However the uncoupling sites remain somewhat speculative (Barber 1983). The inhibitory uncoupling action of the hydroxybenzonitriles promotes a complex mechanism involving an interaction of the two actions.

Ioxynil and bromoxynil are believed to inhibit electron transport by binding to a proteinaceous component of the LHCP-PSII complex (Vermaas and Govindjee 1981). Evidence suggests that all phenolic herbicides bind initially to a 32 kDa protein on the acceptor side of PSII, and at higher concentrations to an additional 41 kDa site on the donor side of PSII (Pfister and Schreiber 1984). The biphasic nature of SiMo inhibition curves obtained in the present study (Fig. 5.6) provides additional evidence for this theory. Furthermore, radiolabelled herbicide binding experiments have confirmed the presence of 2-3 binding sites per electron transport chain (Table 5.4). Hydroxybenzonitrile binding is believed to cause a conformational change in the protein complex (the Q_B protein) preventing electron transfer between Q_A and Q_B (Vermaas and Govindjee 1981; Vermaas, Renger and Arntzen 1984).

The net effect of hydroxybenzonitrile binding is thus to halt plastoquinone reduction, and prevent the normal dissipation of the light energy absorbed by chlorophyll, through electron transport. An excess of energy may be emitted as fluorescence and/or lead to the formation of highly activated oxygen species ($^1O_2$) and chlorophyll molecules (e.g. chl* and chl+, see section 1.3.5). $^1O_2$ is normally effectively quenched by the carotenoid pigments and other protective agents, but excessive amounts can prevent the regeneration of carotenes and α-tocopherol resulting in an overloading of the protective system. The unquenched $^1O_2$ then initiates a chain reaction leading to the photooxidative breakdown of pigments and membranes.

Previous studies on the mechanism of action of ioxynil have revealed the successive breakdown of carotene, xanthophyll and chlorophyll pigments in excised flax cotyledons (Pallett 1978). It is probable that a similar pattern of pigment destruction occurred in susceptible M. inodora, particularly as the development of chlorotic symptoms corresponded with complete photosynthetic inhibition (Table 2.5 and Fig. 4.6). In addition, ultrastructural studies revealed early swelling of granal and intergranal thylakoids (Plates 4.3, 4.4, 4.7 and 4.8); a symptom which is commonly attributed to PSII inhibition and $^1O_2$ formation (Hill, Putala and Vengris 1968; Geronimo and Herr 1970; Anderson and Schaelling 1970; Pallett 1978). Thylakoid membrane disintegration may also be indicative of $^1O_2$-induced peroxidation of membrane lipids such as linolenic acid (Fedtke 1982). Furthermore the intermediates of lipid peroxidation are also considered highly reactive (Feierabend and Winkelhusener 1982) and may have interacted with proteins, contributing to the decline in total protein content observed in some treated plants (Figs.2.3 and 2.5). Clearly, inhibition of electron transport leads to a series of chain reactions resulting in photooxidative damage within the chloroplast, and ultimately within the whole plant.

The mechanism of hydroxybenzonitrile action is further complicated by uncoupling of oxidative- and photo-phosphorylation (Kerr and Wain 1964b; Gromet-Elhanon 1968). In vitro assays have suggested that PSII inhibition is the primary action in chloroplasts (Tables 5.1 and 5.2), although other studies with isolated mitochondria have implicated the

converse for respiratory processes (Fedtke 1982). Uncoupling of either form of phosphorylation would have led to a reduction in the cellular ATP supply and affected all anabolic processes.

Ioxynil and bromoxynil are proposed to uncouple photophosphorylation by acting as protonophones and shuttling protons across the thylakoid membrane (Moreland and Novitzky 1982, 1984). The integrity of all cellular membranes may be similarly affected at higher concentrations (Moreland and Novitzky 1984), but permeability increases are more likely to relate to a decrease in energy supply. For example, the osmotic balance of the chloroplast is maintained by energy dependant active transport, and absence of ATP would promote a passive movement of cations and water into the chloroplast (Moreland and Hilton 1976) leading to swelling and envelope rupture of the type illustrated in plates 4.9-4.11 (bromoxynil treated M. inodora). Ioxynil has also been reported to inhibit energy dependent Ca^{2+} uptake by plant mitochondria (Hertel and Marmé 1983) and disturb all Ca^{2+} dependant processes such as microtubule formation (Nishida, Kumagai, Ohtsuki and Sakai 1979) and NAD+ kinase activity (Dieter and Marmé 1980).

A reduction in ATP synthesis would have had severe effects on all aspects of cell metabolism, since upto 90% of the ATP generated within plant cells is believed to be involved in macromolecular synthesis (Cherry 1976). Severe reductions in growth rate were observed within 7 days of treatment (Fig. 2.6) implying an effect on protein and other macromolecular synthesis. Previous studies have shown that effects of ioxynil and bromoxynil on RNA and protein synthesis are indirect, and are related to a reduction in energy availability (Mann, Jordan and Day 1965; Moreland, Malhotra, Gruehagen and Shokrah 1969; Moreland and Blackmon 1970; Gruehagen and Moreland 1971). Ioxynil has also been shown to act as a pre-prophase inhibitor during prolonged incubation of Vicia faba and Pisum sativum root meristems (Rost, Morrison and Sachs 1977).

The reduction in total protein content in susceptible interactions (Fig. 2.5) is likely to be a secondary effect of both electron transport inhibition and uncoupling of phosphorylation. Photo-oxidative breakdown of membrane components may have released proteins and amino

acids into the cytoplasm resulting in an increase in soluble content (Figs. 2.4 and 2.5). In addition, the uncoupling action of the hydroxybenzonitriles will have prevented further protein synthesis and therefore halted growth. Both mechanisms will have reduced the availability of ATP and NADPH for the carbon reduction cycle. This was immediately manifested in a decreased rate of CO_2 uptake (Fig. 4.5) and a subsequent decrease in reducing sugar content (Fig. 2.5). Carbohydrate mobilization followed, as depicted by the absence of starch grains in mesophyll chloroplasts (Plates 4.5 and 4.9).

In conclusion, the development of symptoms observed in this study implicate a complex mechanism of action for the hydroxybenzonitriles. Uncoupling and inhibition lead to a series of secondary and tertiary effects on cell metabolism and physiology. The interrelationships of these effects are summarized in figure 6.1 which illustrates a proposed scheme for the mechanism of hydroxybenzonitrile action. Differences in ultrastructural symptoms and *in vivo* photosynthesis suggest that inhibition may be the primary action for ioxynil, whereas uncoupling may be more important in bromoxynil phytotoxicity. The significance of this observation will be discussed below in relation to other aspects of the differential action.

6.2 The differential activity of the hydroxybenzonitriles

Ioxynil and bromoxynil have been shown to have a complex mechanism of action in susceptible species. Symptoms resulting from this mode of action include wilting, chlorosis and necrosis and are summarized in Table 2.5. This study has also shown that *V. arvensis* and *S. media* exhibit a degree of resistance, particularly to bromoxynil treatment. The overall susceptibility of the 3 experimental species declined in the order *M. inodora* > *S. media* > *V. arvensis*. Ioxynil was more active against *S. media* and *V. arvensis* whilst bromoxynil was the more active herbicide against *M. inodora*. The diversity of symptoms implicates a complex basis for selectivity and necessitates an overview of the preceding chapters.

The first site at which selectivity can occur is through the differential wetting of leaf surfaces. Some evidence suggests *M. inodora* may be more readily wetted by ioxynil and bromoxynil (Table 3.1) but

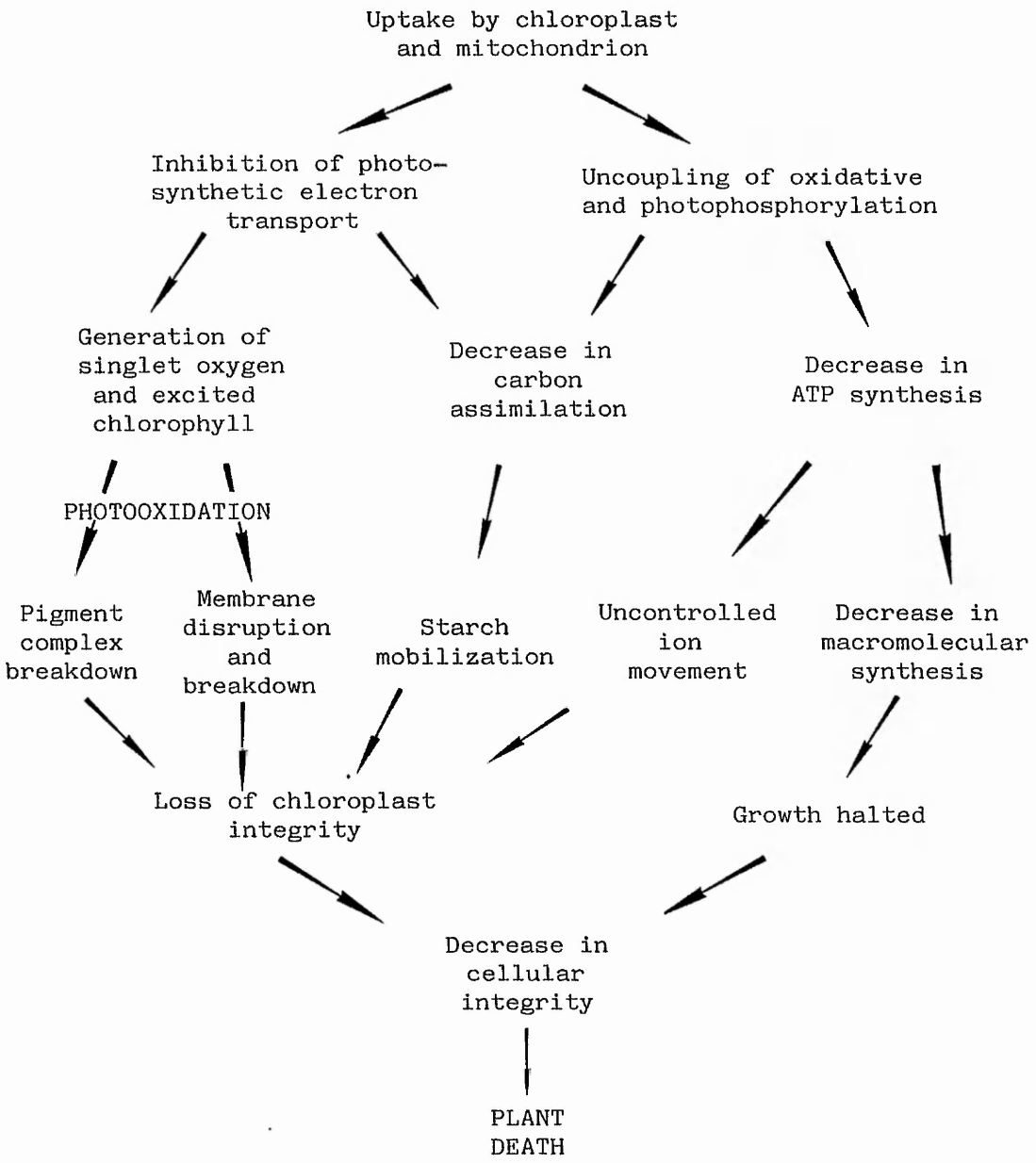


Figure 6.1: A proposed mechanism of hydroxybenzonitrile phytotoxicity

in general differential retention by the three experimental species did not contribute to interspecific differences in phytotoxicity (Table 2.5). Uptake by the underlying leaf cells is considered to involve four stages, i.e. sorption into the cuticle, movement across the cuticle, desorption into the apoplast and finally uptake by the epidermal cells (Fletcher and Kirkwood 1982). At one or more of these stages differential adsorption/desorption must occur as consistently more ioxynil than bromoxynil was detected within treated plants (Table 3.3). The converse was anticipated as bromoxynil has a higher Pk value than ioxynil (4.08 compared to 3.96) and is therefore more lipophilic and more likely to penetrate (e.g. benzoic acids,Bukovac, Sargent, Powell and Blackman 1971). Nevertheless the rapid inhibitory action of bromoxynil against CO_2 uptake (75-90% inhibition within 4 hours, figure 4.5) suggests that bromoxynil may initially penetrate more rapidly than ioxynil.

Further hydroxybenzonitrile uptake will depend upon the metabolic status of the underlying cells. Rapid inhibition of electron transport and photophosphorylation would cause a reduction in the quantity of ATP available for active cellular uptake of all substances, including herbicides. Sufficient ioxynil and bromoxynil had penetrated into M. inodora to completely inhibit CO_2 fixation within four days of treatment (Fig. 4.6). Marked changes in ultrastructure were apparent (plates 4.8-4.11), and generally the cells may have been metabolically incapable of further uptake. In more resistant interactions, CO_2-fixation was not completely inhibited (Fig. 4.6) and uptake continued resulting in greater ioxynil and bromoxynil uptake by the most resistant species (V. arvensis, Table 3.6). The degree of hydroxybenzonitrile uptake therefore appears to depend inversely upon the susceptibility of the underlying cells. Clearly, if a species is susceptible then a very low proportion of ioxynil or bromoxynil is necessary to exert a phytotoxic effect.

The development of phytotoxicity depends upon the fate of ioxynil and bromoxynil once penetrated into the epidermal cells. The herbicides are likely to follow a similar symplastic route to sugars, moving from

cell to cell via plasmodesmata towards the companion cells (Robards 1975). However, only a small proportion of the total penetrated hydroxybenzonitriles are translocated out of the treated leaf (1.3 to 12.5% after 7 days, Table 3.7), and therefore localized adsorption, or 'fixation' must occur along the short distance between cuticle and phloem.

The degree of fixation within the treated leaf varied between the three experimental species and declined in the order *V. arvensis* > *S. media* > *M. inodora*. This suggests physiological differences between the three species which prevent hydroxybenzonitrile movement in the most resistant species. There are several possible explanations of this phenomena. Firstly, ioxynil and bromoxynil may bind to, for example, protein, lipid or polysaccharide components of the cell, thus reducing movement to the site of action. Indeed, the unidentified bromonated compounds extracted from bromoxynil treated *V. arvensis* may possibly represent non-phytotoxic conjugates (Fig. 3.10). Changes in chloroplast ultrastructure were much less marked in this species implying that less herbicide was in contact with the thylakoid membrane system. Thus the higher proportion of penetrated herbicide must be present in a non-active form or be compartmentalized within the cell or cell spaces. Alternatively more fundamental differences in leaf morphology may explain translocation differences, particularly between *V. arvensis* and *M. inodora*. It is probable that there is a lower ratio of vascular:non-vascular tissue in the simple (oval and coarsely serrate) leaves of *V. arvensis* compared to the compound (finely and irregularly pinnatisect) *M. inodora* leaves, and thus more cell to cell movement is necessary before phloem uptake (for comparison see plates 3.2 a + b). Possibly each of these factors, plus other undefined factors interact to limit the long distance translocation of the hydroxybenzonitriles in *V. arvensis*.

In each species ioxynil was found to be proportionately less mobile than bromoxynil. *In vitro* studies have shown that ioxynil is more potent as an inhibitory uncoupler (Tables 5.1 + 5.2) and would be expected to more effectively inhibit photosynthesis and indirectly reduce the rate of assimilate and herbicide translocation. This may eventually occur *in vivo* in *V. arvensis* (Fig. 4.6), however bromoxynil

initially more rapidly inhibited photosynthesis in V. arvensis and M. inodora (Fig. 4.5), presumably reflecting the greater mobility of this herbicide. Thus although a reduction in assimilate production may eventually limit ioxynil translocation, other factors must be involved initially. Ioxynil may however have a direct influence on the energy requiring process of phloem loading, and assimilate translocation may thus be prevented via a similar mechanism to dinitrophenol action (Sovonick, Geiger and Fellows 1976). As the more effective uncoupler, ioxynil may more readily inhibit phloem loading than bromoxynil and therefore inhibit its own movement. Alternatively, if the chloroplast binding data (Table 5.4) can be extrapolated to include other proteins then it is probable that ioxynil may bind more strongly than bromoxynil to membrane and cytoplasmic proteins unconnected with the mechanism of action. The high number of chloroplast binding sites also suggests that the hydroxybenzonitriles may bind non-specifically to cell components other than the known thylakoid binding sites. Furthermore, the additional translocation of bromoxynil may simply reflect the formulation as a potassium salt. Localized addition of K^+ to leaves has been shown to increase assimilate translocation presumably by enhancing the K^+:H+ ion pump believed to be involved in phloem loading (Giaquinta 1980).

The increased mobility of bromoxynil may greatly enhance it's activity in acutely susceptible M. inodora. The herbicide is translocated rapidly throughout the plant with significant proportions collecting in the apex and developing leaves, and also the mature leaves. Ioxynil translocation is less rapid although within 7 days 8.1% of the penetrated herbicide was present in the apex and developing leaves (Table 3.7). Thus in susceptible M. inodora the phytotoxicity of the hydroxybenzonitriles appears to involve both translocated and contact activity. In contrast however, ioxynil has contact activity only in moderately resistant V. arvensis. S. media exhibits an intermediate response with the largest proportion of translocated herbicide detected within the stem. Thus there appears to be two different forms of hydroxybenzonitrile movement, and the relative proportion of each contributes to the differential activity in S. media, V. arvensis and M. inodora.

As already discussed, 87-99% of the penetrated hydroxybenzonitriles remain within the treated leaves and are responsible for the major phytotoxic action of the herbicides. *In vitro* studies have shown that the inhibitory uncoupling action is very similar in each species, with ioxynil the more effective compound. Ultrastructural changes were indeed more marked after ioxynil treatment, however the degree and speed of symptom development reflected the overall susceptibility of the species. Thus a form of selectivity is likely to occur within the mesophyll cells of treated plants.

In *M. inodora* mesophyll cells, sufficient ioxynil must have penetrated into the cell and through the chloroplast envelope to bind effectively to the thylakoid membranes, and completely inhibit CO_2-fixation within 4 days (Fig. 4.6). Thylakoid swelling and chloroplast disruption were typical of photooxidative processes and subsequent symptom development followed the pathway described above (section 6.1). Ultrastructural symptoms were less marked in *S. media* and *V. arvensis* mesophyll cells (Plates 4.14-4.17, 4.23) even though more ioxynil had penetrated into the treated leaves of these species (Table 3.6). This suggests that either the herbicide is being selectively excluded by the mesophyll cell, or that it is present in a non-active form in the chloroplast or cytoplasm. Infra-red gas analysis showed that the treated leaves of *V. arvensis* retain the capacity to photosynthesize (Fig. 4.6) and thus less photooxidation and subsequent membrane destruction occurs.

Ultrastructural symptoms were more diverse in bromoxynil treated leaves. Even though bromoxynil uptake by *M. inodora* was quickly inhibited, sufficient herbicide had penetrated to exert a rapid effect on cell metabolism (Figs. 2.5, 4.5 and 4.6), and to lead ultimately to plant death. The development of ultrastructural symptoms implicated a different mechanism to the photo-oxidative damage induced by ioxynil. Whereas the initial swelling of intergranal thylakoids may be induced by photosynthetic inhibition, the total disruption of all cellular membranes within 7 days (Plates 4.9-4.11) is more likely to reflect a permeability effect of bromoxynil (Moreland and Novitzky 1984). Although the thylakoid membranes remained relatively intact, they were unlikely to be functional as cell metabolism is considered to be irreversibly inhibited once the tonoplast has ruptured (Dodge 1971).

The lack of photo-oxidative damage may reflect discontinued bromoxynil uptake or alternatively may relate to the lower binding affinity of bromoxynil established in in vitro studies (Table 5.3).

In contrast to the rapid herbicide action in M. inodora, bromoxynil caused only limited chlorosis of V. arvensis leaves. The photosynthetic capacity was maintained at approximately 40% of the control rate and electron microscopy revealed no symptoms of photo-oxidative damage. Instead, the chloroplasts exhibited adaptive changes to the reduced photosynthetic rate (Plates 4.20 and 4.21). An increase in granal stacking and decrease in chlorophyll a:b ratio will have improved light interception by increasing the number of LHCP-PSII complexes (Lichtenthaler, Buschmann, Doll, Fietz, Bach, Kozel, Meier and Rahmsdorf 1981). By implication, cell metabolism is most probably fully functional since LHCP is encoded in the nucleus, synthesized in the cytoplasm, and transported into the chloroplast in precursor form (Bennett 1983). The increased proportion of appressed membranes may also relate to the uncoupling action of bromoxynil. A reduction in ATP levels would prevent phosphorylation of the LHCP-2 complex and maintain thylakoid membranes in an appressed state (Barber 1983). The mechanism of LHCP-2 phosphorylation is still unclear, however investigations with the uncoupler NH_4Cl suggest that LHCP-2 phosphorylation is only affected when the rate of CO_2 fixation is reduced (Allen 1984).

Thus incomplete inhibition of electron transport, and possibly uncoupling of photophosphorylation lead to adaptive changes in V. arvensis chloroplasts. The phytotoxicity of bromoxynil may be reduced in this species by either conjugation with other plant constituents or metabolic breakdown to less active compounds (Fig. 3.10). Similar deactivation may also occur in S. media and a limited ultrastructural investigation has revealed comparable but less defined adaptive changes in S. media chloroplasts (Plate 4.24). In contrast there was no evidence of bromoxynil breakdown/conjugation in M. inodora probably reflecting the rapid disruption of all metabolic processes in this species. Thus the capacity of individual species to deactivate bromoxynil may largely contribute to interspecific differences in phytotoxicity.

The differential activity of the hydroxybenzonitriles can also be correlated with the speed and direction of movement from the treated leaf. Ioxynil and bromoxynil were rapidly translocated to the apex of M. inodora. The susceptibility of this species may relate to the uncoupling action of the herbicides, i.e. limiting ATP availability for cell elongation and division. Mitosis, DNA, RNA and protein synthesis may have been rapidly inhibited (Moreland, Malhotra, Gruenhagen and Shokrah 1969; Gruehagen and Moreland 1971; Rost, Morrison and Sachs 1977; Chand and Roy 1981), preventing further development of leaf primordia and stem elongation. Visual evidence suggests that the rapid translocation of bromoxynil to the apex of M. inodora leads ultimately to the death of the apex and prevents regrowth and recovery of the treated plant. Incomplete inhibition by less mobile ioxynil eventually allows regrowth to occur (Figs. 2.5 and 2.6). A significant but lower proportion of bromoxynil was also translocated to the apices of S. media and V. arvensis (Table 3.7) possibly contributing to the initial decline in growth observed in these species (Fig. 2.6). Furthermore, a significant proportion of the hydroxybenzonitriles were detected within the stem of S. media where the herbicides were less likely to be metabolically active.

In conclusion, this study has revealed a complex basis for the differential activity of ioxynil and bromoxynil against M. inodora, S. media and V. arvensis. In vitro studies have shown that ioxynil binds more strongly to the thylakoid membrane and is more effective as an inhibitory uncoupler than bromoxynil. This in vitro similarity between the three species is overcome in the in vivo situation and therefore selectivity must occur before the herbicides bind to the thylakoid membrane. It is proposed that bromoxynil is selectively metabolized by V. arvensis and to a lesser extent S. media into less active compounds which reduce phytotoxicity in these species. A similar mechanism may prevent photo-oxidative damage within ioxynil treated V. arvensis, or alternatively ioxynil may be compartmentalized within the cytoplasm or apoplast. Interspecific differences in phytotoxicity also relate to the capacity of each individual species to translocate ioxynil and bromoxynil out of the treated leaves towards the growing points. Movement is most rapid and pronounced in susceptible M. inodora, and the

greater phytotoxicity of bromoxynil in this species most probably relates to rapid translocation to, and subsequent death of the apex. In contrast, contact effects only are important in moderately resistant V. arvensis. S. media was able to overcome initial contact and limited translocated effects because of the rapid growth of unaffected lateral shoots in this morphologically different species. Thus physiological differences between the three experimental species contribute to the selectivity of the hydroxybenzonitriles.

Throughout this study considerable information has been accumulated to quantify the differential response of M. inodora, S. media and V. arvensis to ioxynil and bromoxynil treatment. Various parameters have emerged which may explain the differential activity, however a complete understanding would require a more detailed study of the biochemistry and physiology of each individual weed species. It is also apparent that the complex interaction of primary secondary and tertiary responses which dictate hydroxybenzonitrile phytotoxicity within a particular species negate generalizations on the selectivity of these herbicides. It is this complex mechanism of action which is indirectly leading to increased usage of the hydroxybenzonitriles, particularly in herbicide mixtures. This is because of the recent development of resistance amongst many weed species following the extensive use of triazine herbicides in Maize crops (Bandeen, Stephenson and Cowett 1982). A single amino acid change in the Q_B-protein prevents DCMU-type herbicides from binding to the site of action (Hirschberg, Bleecker, Kyle, McIntosh and Arntzen 1984). This type of resistance is less likely to spontaneously develop to the hydroxybenzonitriles as several changes in the chloroplast and mitochondrion would have to occur. To maximise usage of these herbicides it is therefore advantageous to have an understanding of the mechanism of action and the basis for selectivity.

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Physiological Changes in *Matricaria inodora* Following Ioxynil and Bromoxynil Treatment

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Matricaria inodora, Photosynthesis, Ioxynil, Bromoxynil, Herbicide Symptoms

A range of biochemical and physiological changes were monitored in *Matricaria inodora* following field rate applications of ioxynil and bromoxynil. Bromoxynil showed greater phytotoxicity with decreased reducing sugars, amino acids and proteins occurring within 28 days of treatment, when plant death was apparent. After an initial decline these parameters increased as plants appear to recover from ioxynil treatment. CO_2 fixation was completely inhibited within 4 days in leaves treated with ioxynil and bromoxynil. Ultrastructural effects of both herbicides were similar with chloroplast swelling, decrease in starch grains and thylakoid disruption prior to cellular destruction. *In vitro* activity of the herbicides on isolated chloroplasts revealed ioxynil to be slightly more inhibitory than bromoxynil toward electron transport and approximately one hundred times more effective as an uncoupler of PMS cyclic photophosphorylation.

Introduction

Ioxynil and bromoxynil are post emergence contact herbicides which exhibit differential activity amongst certain weed species [1–3].

The hydroxybenzonitriles were classified by Moreland and Hilton [4] as inhibitory uncouplers because of their apparent ability to inhibit chloroplast electron transport and uncouple photophosphorylation, and oxidative phosphorylation in mitochondria. However, *in vitro* studies have indicated chloroplast electron transport to be 100–1000× more sensitive than plant mitochondria or cyclic photophosphorylation suggesting photosynthetic inhibition to be the primary site of action [5, 6].

Following the application of photosynthetic inhibitor herbicides various other metabolic processes may be directly or indirectly effected. For example, nitrate reductase activity was limited in wheat following methabenzthiazuron treatment [7], and simazine increased protein synthesis in barley [8].

In this report, the physiological basis for the development of symptoms is investigated in *Matricaria inodora*, a weed species which is very susceptible to bromoxynil and exhibits some recovery after ioxynil treatment.

Abbreviations: DCPIP, dichlorophenol indophenol; PMS, phenazine methosulfate.

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Materials and Methods

Treatment of plants

Plants were grown as previously reported [3] and used for experimentation when 3–4 leaves had developed. In all spray experiments, ioxynil-Na and bromoxynil-K salts were applied at a dose rate equivalent to the field rate (560 g a.i. ha^{-1}) using a hydraulic laboratory pot sprayer (Mardrive Marine Engineering Co. Ltd.) fitted with an 80° T-jet nozzle.

Assay of metabolic symptoms

7, 14, 21 and 28 days after treatment plants were harvested and fresh weight, total chlorophyll, reducing sugars, amino acids and proteins were extracted and determined by established methods [Sanders 1984, in preparation].

CO_2 fixation

Net photosynthesis was measured with an infrared CO_2 analyser (G.P. instruments Ltd.) in differential mode. Six replicate chambers were sampled automatically at 5 minute intervals by connection to an autoanalyzer (The Analytical Development Company Ltd.). For the duration of measurements the chambers were uniformly irradiated with saturating 750 μ $E.m^{-2} \cdot sec^{-1}$ light from a Camrex Solarcolour LGH, PS/U sodium fluorescent tube, and maintained at 25 °C by a constant temperature water jacket.

Electron microscopy

Representative samples of leaves four days after herbicide treatment were processed for transmission electron microscopy as reported by Pallett and Dodge [9].

Chloroplast isolation

Chloroplast fragments [Type E, 10] were isolated from *M. inodora* by modifying established techniques [11, 12] until optimum photochemical activity was maintained for 45 minutes. Assay conditions for photochemical activity using dichlorophenolindophenol (DCPIP) as electron acceptor were those reported previously [3].

The uncoupling activity of ioxynil and bromoxynil was investigated by monitoring PMS mediated cyclic photophosphorylation in isolated chloroplast fragments [13]. Initial experiments reported in this paper, were carried out using chloroplast fragments isolated from 10–14 day pea seedlings.

Results

3–4 days after foliar application of the hydroxybenzonitriles the first visual symptoms appeared as wilting and necrosis of treated leaves. Within 1 days of bromoxynil treatment, *M. inodora* exhibite complete necrosis whereas only the treated leave developed necrotic symptoms after ioxynil treat ment. In the latter, the apex and lateral shoots con tinued to grow reaching approximately 60% of th control height after 28 days.

This apparent recovery is reflected in the fres weight and chlorophyll content of treated foliag (Fig. 1a and b). Immediately following treatmen there was a decline in the total protein content o the aerial shoots, with corresponding increases i soluble amino acids and proteins (ioxynil only Fig. 1c, d and e). As the plant recovered fron ioxynil application, the total protein content rapidl increased and amino acids and soluble protein declined. 7 days after bromoxynil treatment al parameters investigated declined as plant deat gradually occurred.

Net photosynthesis was rapidly inhibited reach ing 80% inhibition within 48 hours (Fig. 2). N recovery of photosynthetic capacity was detecte and by the end of the experimental period, leave treated with both herbicides were totally necrotic Complete inhibition of CO_2 uptake within 4 day

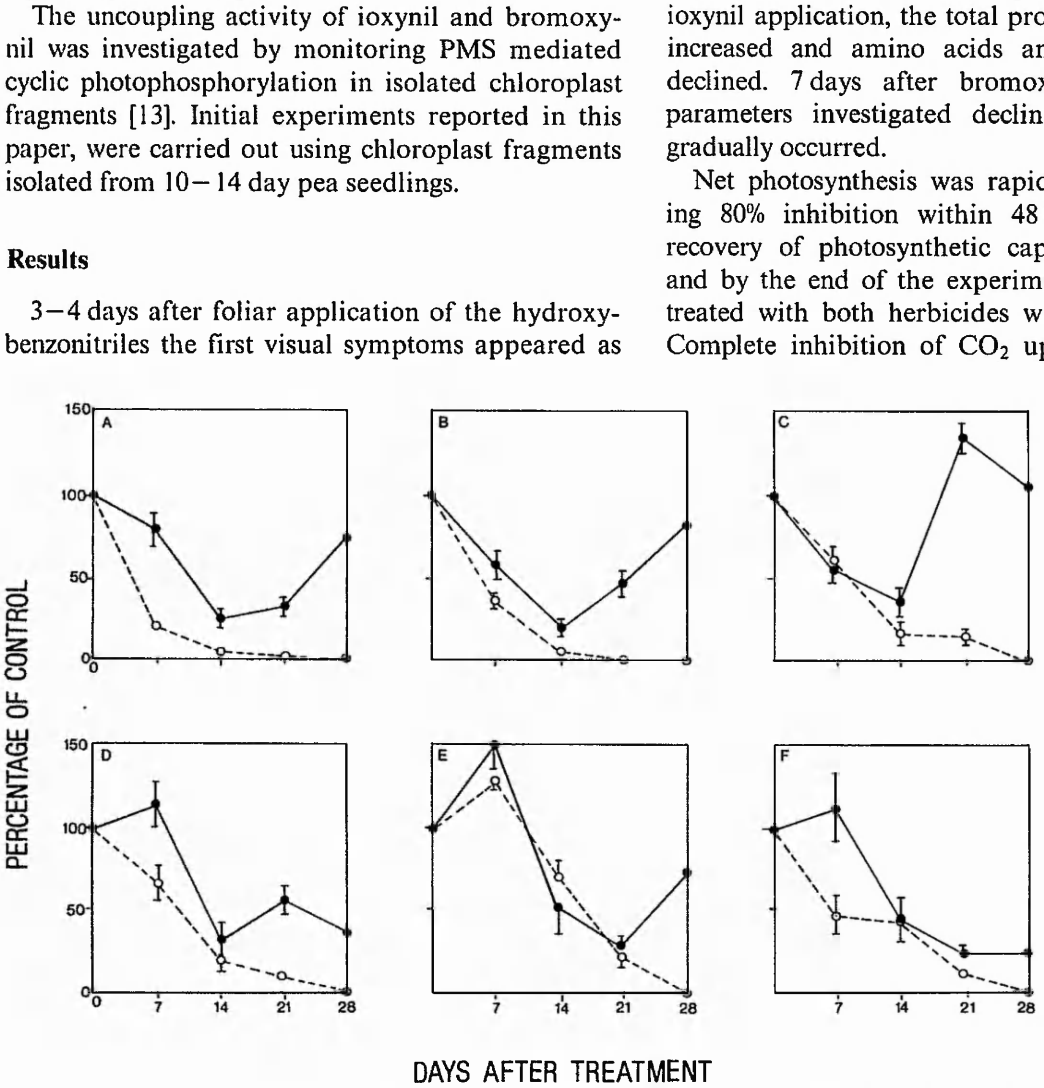


Fig. 1. Changes in metabolic parameters after ioxynil (—●—) and bromoxynil (––○––) treatment of *Matrica inodora*. (A) fresh weight, (B) chlorophyll content, (C) total proteins, (D) soluble proteins, (E) soluble amino and (F) ducing sugars. Each point is the mean of 6 replicates and standard error bars are included where they exceed the symb size.

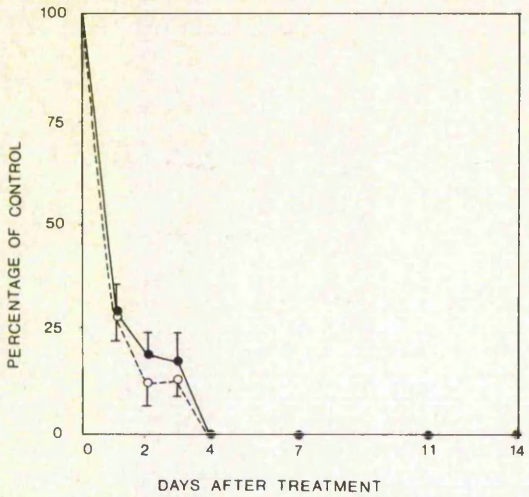


Fig. 2. Inhibition of CO_2 uptake by field rate ioxynil (—●—) and bromoxynil (––○––). Each point is the mean of 6 replicates with standard error bar.

was accompanied by ultrastructural changes in the chloroplast.

Plate A shows a typical disc shaped chloroplast from a control treatment. Both herbicides caused a progressive swelling of the chloroplast, decrease in starch and swelling of the thylakoid system. Plate B shows the early stages of chloroplast deterioration as a result of ioxynil treatment, and plate C reveals later deterioration with bromoxynil. The development of these ultrastructural symptoms appeared slightly more rapid with bromoxynil treatment, reflecting the development of visual symptoms.

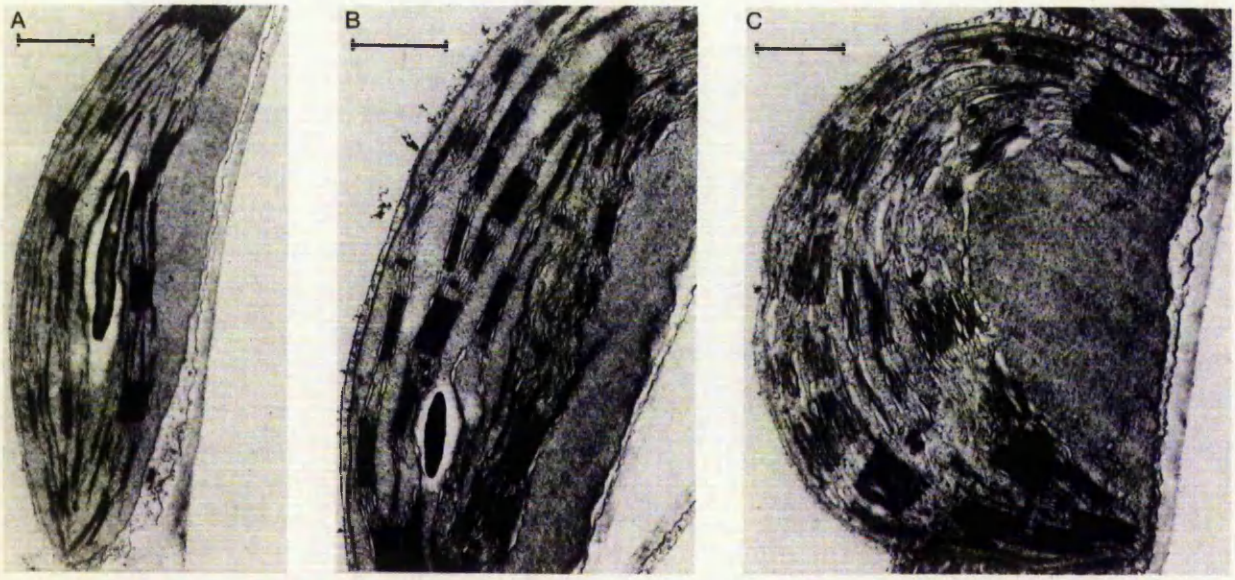
Table I shows ioxynil to be slightly more inhibitory than bromoxynil towards electron transport in chloroplasts isolated from *M. inodora*. Both herbicides uncouple PMS-mediated cyclic photophosphorylation in pea chloroplasts (Table II). Ioxynil

Table I. Inhibition of DCPIP reduction in chloroplasts isolated from *M. inodora* (each figure is the mean of 4 replicates).

	% of control	
Conc^n [μm]	Ioxynil	Bromoxynil
1.0	80.5	93.0
10.0	26.0	40.0
100.0	15.0	17.5

Table II. Inhibition of PMS-mediated photophosphorylation in isolated pea chloroplasts (each figure is the mean of 4 replicates).

	% of control	
Conc^n [μm]	Ioxynil	Bromoxynil
0.1	186.1	–
1.0	23.4	–
10.0	0.0	262.1
30.0	–	150.0
60.0	–	49.3
100.0	–	6.5



Plates A, B, C: Typical chloroplasts 4 days after treatment (A) control (× 12 200), (B) ioxynil (× 14 500) and (C) bromoxynil (× 13 100). Internal marker = 1 μm.

was approximately one hundred times more active than bromoxynil. At lower concentrations photophosphorylation was stimulated by both herbicides.

Discussion

The observed wilting and necrosis of treated foliage are indicative of a rapid herbicide action in this species. Within 4 days inhibition of CO_2 fixation was complete and chloroplasts had undergone marked ultrastructural changes. Chloroplast disruption has been proposed to result from an overloading of the protective carotenoid system inducing singlet oxygen and free radical formation as a result of electron transport inhibition [9, 14]. This, coupled with possible free radicals formed by degradation of ioxynil within the leaf [13, 15] may explain the rapid loss of turgidity in treated leaves.

Within 48 hours starch grains were less apparent in treated chloroplasts indicating rapid metabolic breakdown as a consequence of low CO_2 fixation rates. Inhibition of net photosynthesis was complete by 96 hours with both herbicides, at which time chloroplasts typically exhibited increased vacuolation of intergranal thylakoids and plastoglobuli content. There was also an increased tendancy for the thylakoids to be bowed against the chloroplast envelope. This phenomena is commonly reported [9, 16, 17] and was considered by Dodge and Lawes [18] to be the likely result of decreased osmotic potential of the cell vacuole.

As a consequence of photosynthetic inhibition minimal levels of reducing sugars were anticipated within seven days. Kinetic studies with potato and spring wheat suggest this parameter is the first to be effected following metribuzin and methabenzthiazuron treatment of roots [19, 20]. It is possible an earlier measurement would have detected such a decline. However, in bromoxynil treated plants, with the exception of soluble amino acids, all of the primary metabolites studied declined at a similar rate to fresh weight. During the recovery phase of ioxynil treatment, reducing sugar levels did not increase at the same rate as fresh weight implying a reduced photosynthetic rate newly developing foliage.

The interaction between total protein, soluble protein and soluble amino acids, particularly apparent after ioxynil treatment is commonly reported especially for more resistant herbicide:plant interactions [7, 8, 19, 20]. Fedtke [21] reports enhanced N_2-metabolism at sublethal herbicide concentrations and suggests increased nitrite formation may contribute to the toxic action of ioxynil. This interaction may also be explained by autolysis of structural proteins resulting in increased free amino acids and proteins, and possibly by selective inhibition of protein synthesis [22].

Therefore *in vivo* studies have shown that ioxynil and bromoxynil rapidly inhibit CO_2 uptake. The consequences are physiological changes in chloroplast ultrastructure and the development of necrotic tissue from which there is limited recovery from ioxynil treatment. *In vitro* studies, however have indicated that ioxynil is slightly more inhibitory than bromoxynil at the thylakoid level (Table I). A similar *in vitro* response occurred in *Stellaria media* and *Viola arvensis* which exhibit some resistance to the two herbicides [3]. This supports previous reports that electron transport inhibition by the hydroxybenzonitriles depends on substitution and decreases in the order I > Br > Cl [23].

The uncoupling action of these two herbicides on photophosphorylation may also contribute to phytotoxicity (Table II), particularly ioxynil, which uncouples at concentrations lower than those which inhibit electron transport. Inhibition of electron transport by ioxynil and bromoxynil was similar for chloroplasts isolated from *M. inodora* (Table I) and peas (data not presented). The marked stimulatory effect at low concentrations (Table II) is a common feature of many herbicides interfering with biochemical processes [21].

The greater susceptibility of *M. inodora* to bromoxynil cannot be explained by this *in vitro* data for photosynthetic interference. Other factors are likely to be involved such as penetration, translocation and metabolism of the herbicides, which are currently under investigation.

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STUDIES INTO THE DIFFERENT RESPONSE OF THREE WEED SPECIES TO THE HYDROXYBENZONITRILES

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Summary. The chlorophyll content of three weed species, Matricaria inodora, Stellaria media and Viola arvensis were compared after treatment with 0, 0.28, 0.56, 1.12 and 2.24 kg a.i./ha ioxynil and bromoxynil salts. Bromoxynil caused a greater chlorophyll loss than ioxynil with M. inodora, whereas ioxynil caused greater loss in S. media. The chlorophyll content of V. arvensis was similarly reduced with both herbicides. In chloroplasts isolated from the three species ioxynil was 2-3 times more inhibitory than bromoxynil towards electron transport. Sigmoidal type inhibition curves occurred with DCPIP (dichlorophenolindophenol) as electron acceptor. It is proposed that the two binding sites of the hydroxybenzonitriles and the two electron acceptor sites of SiMo (silicomolybdate) resulted in biphasic inhibition curves which occurred with this electron acceptor. The greater inhibition of electron transport by ioxynil may contribute to the response of S. media. However other factors are involved in the response of M. inodora and V. arvensis. Hydroxybenzonitriles, Matricaria inodora, Stellaria media, Viola arvensis, binding sites, electron transport inhibition.

INTRODUCTION

Ioxynil and bromoxynil are postemergence contact herbicides which control a wide range of annual broad-leaved weeds in cereals (Carpenter et al., 1964). The selective action of ioxynil and bromoxynil may be partly due to a greater herbicide degradation in barley and wheat (Davies et al., 1968; Shafer and Chilcote, 1970). Somerville (1972) reported differences in the retention and penetration of ioxynil and bromoxynil, however, this alone could not explain the differential susceptibility of certain weed species to these herbicides.

This paper reports initial data in which the biochemical and physiological effects of ioxynil and bromoxynil salts were compared on three important weed species, Matricaria inodora, Stellaria media and Viola arvenis.

METHODS AND MATERIALS

Plants were grown in seed compost (J. Arthur Bowers) in 3 inch pots (spray experiment) or seed trays (chloroplast isolation) in a greenhouse. Plants were used for experimentation when 6-8 leaves had developed on the main shoot of S. media and 3-4 leaves had developed on the main shoots of M. inodora and V. arvensis.

Plants were sprayed with ioxynil Na or bromoxynil K salts in an experimental sprayer at rates equivalent to 0.28, 0.56 (field rate), 1.12 and 2.24 kg a.i. ha^{-1} and a volume rate of 200 l ha^{-1}. Spray retention was determined using an aqueous solution of tartrazine (Pallett and Caseley, 1980). Four replicate plants were used for each treatment and harvested 7 and 14 days after spraying. Plants were weighed and chlorophyll determined by the method of Arnon (1949).

Chloroplast fragments (Type E, Hall, 1972) were isolated from the three species. The following isolation conditions were employed to maintain optimum photochemical activity for 45-60 min:

(a) S. media and V. arvensis 5g of leaf tissue was homogenised in 20ml of media containing 50mM Tricine-NaOH buffer (pH 7.5); 300mM NaCl; 3mM $MgCl_2$ and 0.01% bovine serum albumin (BSA). After filtering through 4 layers of muslin, cell debri was removed by centrifugation at 1000g for 1 min. The supernatant was recentrifuged at 3000g for 10 min and the chloroplast pellet resuspended in media containing 5mM Tricine -NaOH buffer (pH 7.5); 100mM sucrose; 3mM $MgCl_2$; 2mM EDTA and 0.1% BSA. All procedures were carried out at 4°C.

(b) M. inodora 5g of leaf tissue was homogenised in 20ml media containing 50mM Tricine-NaOH buffer (pH 7.5); 300mM NaCl; 3mM $MgCl_2$; 2mM EDTA; 0.01% BSA and 0.1% polyvinylpyrrolidone. After filtering through 8 layers of muslin and centrifugation at 3000g for 1 min the pellet was immediately resuspended in the above media.

Photosystem II activity was monitored by measuring O_2 evolution in an O_2 electrode (Hansatec Limited). The reaction chamber was maintained at 20°C and illuminated by a projector lamp giving 3000µE $m^{-2}s^{-1}$ at the electrode. The reaction medium contained 0.3ml 300mM Tricine NaOH buffer (pH 8.0); optimum concentrations of either, 10mM dichlorophenolindophenol (DCPIP) (M. inodora, 75µl; S. media, 40µl; V. arvensis, 100µl) or silicomolybdate (SiMo), (M. inodora, 50µl; S. media, 50µl; V. arvensis, 75µl); chloroplasts equivalent to 100µg chlorophyll; variable concentrations of pure ioxynil Na or bromoxynil K salts; and water to 3ml.

RESULTS

A major symptom of the hydroxybenzonitriles is chlorosis of treated foliage (Carpenter et al., 1964). This is shown in Figure 1, which presents the chlorophyll content of plants treated with ioxynil and bromoxynil. S. media appeared least affected by the two herbicides, whereas M. inodora showed considerable chlorophyll loss at the 0.28 kg a.i./ha doses. Chlorophyll loss was greatest with bromoxynil in M. inodora, compared with ioxynil. Ioxynil was more effective in S. media and both herbicides caused similar effects in V. arvensis. Similar responses to the two herbicides were apparent from fresh and dry weight values (data not presented).

The spray retention values for the three species were: M. inodora, 540.6µl/g Dry Wt. (7.9µl/plant); S. media, 645.6µl/g Dry Wt. (23.9µl/plant); and V. arvensis, 986.9µl/g Dry Wt. (18.0µl/plant). These values cannot explain the results shown in Figure 1.

The primary site of action of the hydroxybenzonitriles is well established as an inhibition of chloroplast electron transport (the so-called Hill reaction). This inhibition can be monitored with isolated chloroplasts using artificial electron acceptors such as DCPIP and SiMo. Figure 2 shows inhibition curves of ioxynil and bromoxynil salts with chloroplast fragments isolated from the three weed species and DCPIP and SiMo as electron acceptors. Ioxynil was more inhibitiory than bromoxynil in all three weed species. With DCPIP as electron acceptor the inhibition curves were sigmoidal, however with SiMo they were biphasic.

The I_{50} values calculated from Figure 2 are shown in Table 1. They are similar in the three species for ioxynil however the values for bromoxynil are lower for M. inodora, particularly with SiMo as electron acceptor.

DISCUSSION

The loss in chlorophyll following treatment with herbicides that inhibit electron transport is due to photoxidative processes (Pallett and Dodge, 1980). When electron transport is prevented, light energy absorbed by chlorophyll in the photosystems cannot drive electron transport and unless channelled elsewhere, chlorophyll destruction will occur. Carotenoids, particularly β-carotene, can dissipate some of this energy harmlessly, however when electron transport is inhibited the carotenoid system becomes overloaded and chlorophyll breakdown and toxic species, such as singlet oxygen are generated, which lead to chloroplast and subsequently cell

Fig. 1

The chlorophyll content of plants 7 (□) and 14 (▨) days after treatment with ioxynil Na or bromoxynil K salts at doses equivalent to 0, 0.28, 0.56, 1.12 and 2.24 kg a.i. /ha.

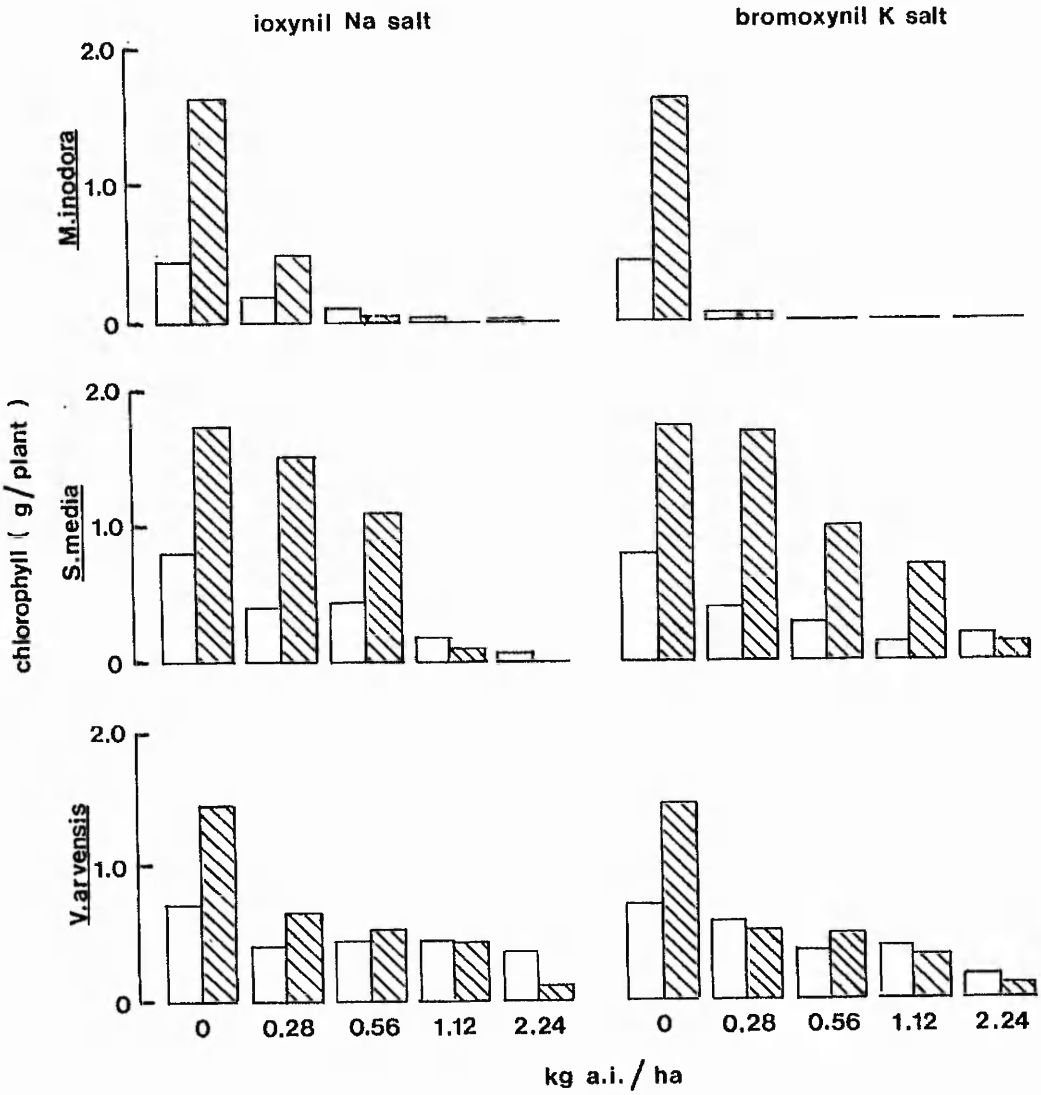


Table 1

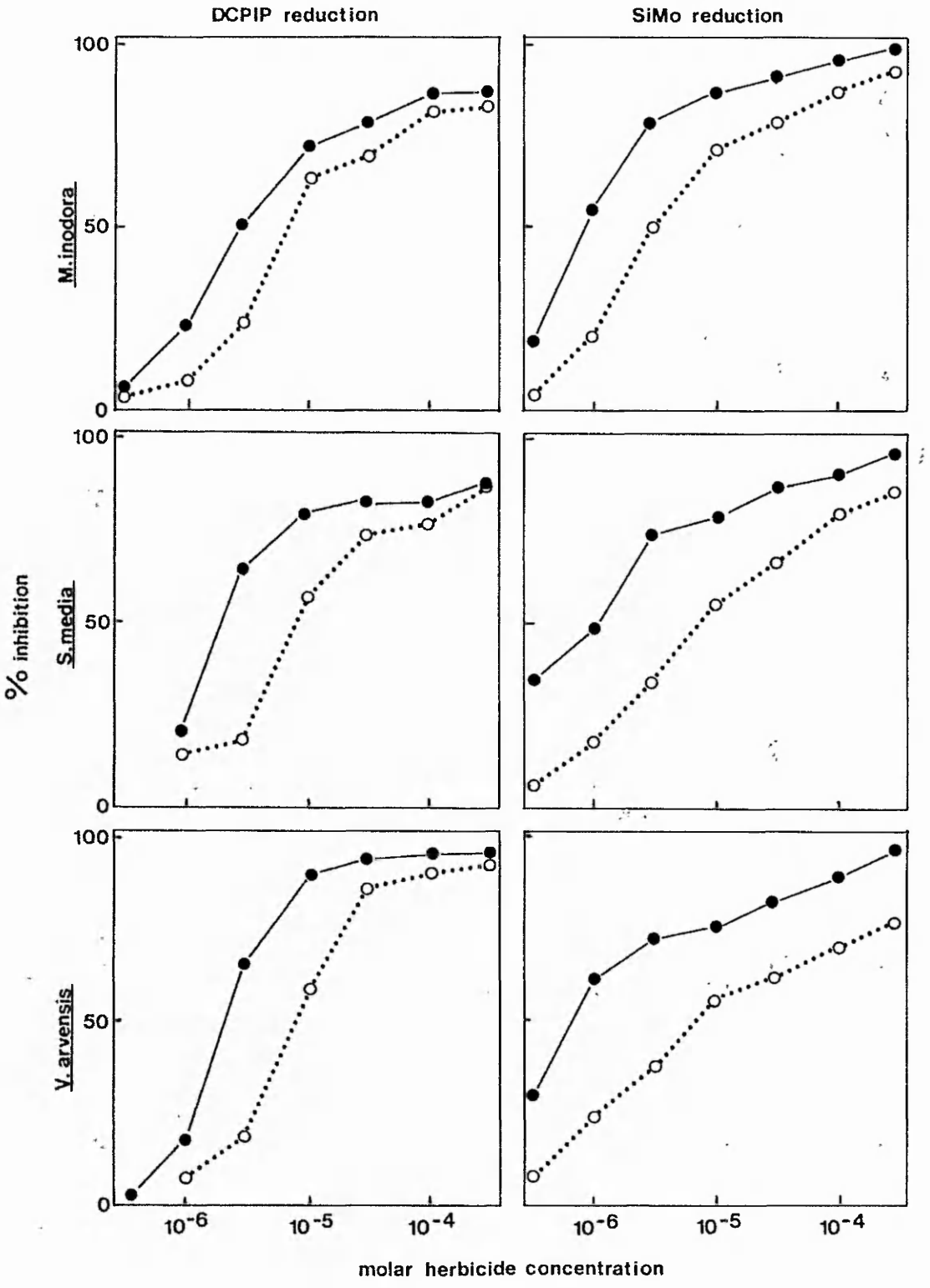
The concentration of herbicide necessary to give 50% inhibition of electron transport (I_{50}) with DCPIP and SiMo as electron acceptors

(determined from Figure 2)

	I_{50} value (μM)					
	Ioxynil			Bromoxynil		
	M. inodora	S. media	V. arvensis	M. inodora	S. media	V. arvensis
DCPIP	2.6	2.2	2.0	6.8	8.3	9.0
SiMo	0.9	1.1	0.7	3.5	8.3	7.3

Fig. 2

The effect of ioxynil Na (●) and bromoxynil K (○) on oxygen evolution from chloroplast fragments with DCPIP and SiMo as electron acceptors.



destruction. In addition the activity of ioxynil may be enhanced by free radicals produced by the breakdown of ioxynil in the leaf (Zaki et al., 1967; Pallett, 1978).

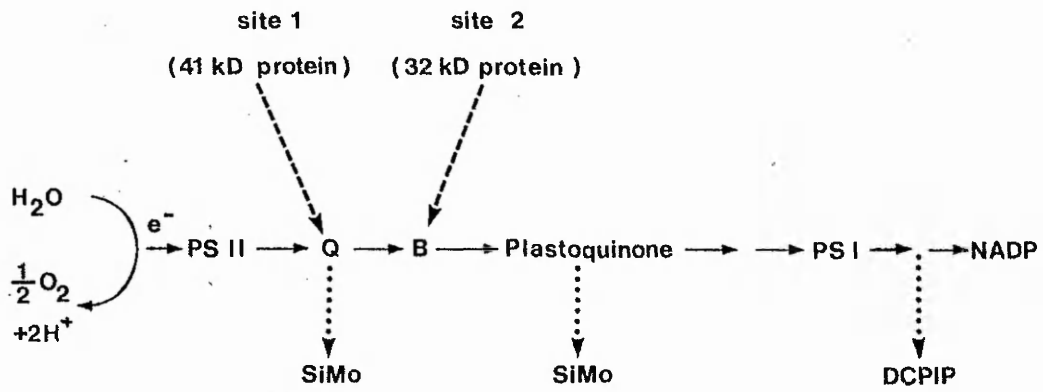
The degree of electron transport inhibition is obviously an important factor in the development of herbicide symptoms. The wide range of herbicides that inhibit electron transport vary considerably in their effectiveness as electron transport inhibitors (Moreland, 1969). Resistance that has developed in recent years, to the triazine class of herbicides is due to a greatly reduced ability to bind to, and inhibit electron transport (Pfister and Arntzen, 1979).

In all three weed species ioxynil was a more effective inhibitor of electron transport (Fig. 2 and Table 1). This supports previous reports that the inhibition of electron transport by the hydroxybenzonitriles depends on substitution and decreases in the order I>Br>Cl (Trebst et al., 1979).

The binding site for photosynthetic inhibitor herbicides is well established as a proteineous component situated between Q and plastoquinone. This site was believed to be common for all classes of inhibitor herbicides, however techniques, including trypsin treatment of isolated chloroplasts, binding studies and photoaffinity labelling have revealed two binding sites (Pallett and Dodge, 1979; Oettmeier et al., 1982) Site 1, a 41kD protein probably associated with Q and the PSII reaction centre which binds only phenolic type herbicides (hydroxybenzonitriles) and site 2, a 32-34 kD protein possibly associated with B which can bind the DCMU-type herbicides (ureas, triazines, uracils) and the phenolic type compounds (Fig. 3). Phenolic inhibitors

Fig. 3

A scheme showing the proposed herbicide binding sites and electron acceptor sites. For explanation see Text.



can displace DCMU-type inhibitors and vice versa and therefore the two sites are likely to be closely associated. It has been proposed that the 32-34 kD protein is located on the surface of the thylakoid membrane, and is more accessible, with the 41 kD protein hidden underneath, and consequently less accessible (Oettmeier et al., 1982).

The biphasic nature of the inhibition with SiMo as acceptor (Figure 2) may be explained by the two binding sites of the herbicides and two electron acceptor sites of SiMo (Figure 3). Site 2 will have a higher affinity for the herbicide than Site 1 because of its location on the membrane surface. At lower herbicide concentrations

electron acceptance of SiMo from plastoquinone will only be inhibited, however at higher concentrations acceptance from both Q and plastoquinone will be inhibited (Fig. 3). Inhibition of DCPIP reduction will occur irrespective of which binding site is occupied.

The I_{50} values would be expected to be the same for the two electron acceptors or possibly higher values would be necessary for SiMo. However, the values for SiMo were 2-3 times lower than DCPIP for ioxynil (Table 1). A possible explanation for this may be that the close proximity of the two binding sites with the electron acceptor sites may lead to an interaction of ioxynil with SiMo rendering its reduction more sensitive to this herbicide.

In conclusion ioxynil is the more effective inhibitor of electron transport. This may contribute to the greater symptoms induced by this compound with S. media, however other factors such as penetration, translocation and metabolism must be responsible for the different responses of M. inodora and V. arvensis. These factors are currently under investigation.

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APPENDIX I

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ERRATUM

PAGE	LINE	AMENDMENT
6	28	Fat hen for fathen
7	35	Fat hen for fathen
11	8	Fleeker for Fletcher
12	34	Schmidt for Smidt
14	29	Insert Fietz after Doell
25	13	Demeter for Demeks
32	32	OH· for OH.
35	6	Insert Shear after Foy
65	19	Chluroxuron for chlortoluron
103	16	1983, 1984 for 1983 a + b
106	29	than for and
130	18,32	Anderson + Thomson 1973 for Anderson + Shaelling 1973
165	2	Insert Gross after Coombs
191	28	Greunhagen for Greuhagen
198	12	Doell for Doll
202	7	COWETT for COWLETT
202	28	BLACKMAN for BLACKMANN
202	32	BLANKENDAAL for BLAKENDAAL
206	1	hydrolysis for hydrolsis
206	4	FEIERABEND for FEIRABEND
206	16	Insert Plant after and
218	29	susceptible for susceptical
222	4	ZAPPI, E. for ZAPP, I