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An Investigation into the Mode of Action and Selectivity of 3,6-dichloropicolinic acid.

by

LORNA MARY LOVE THOMPSON

A thesis submitted to the CNAA in partial fulfilment of the degree of Doctor of Philosophy.

Collaborating Establishment: The Dow Chemical Company
Letcombe, Oxfordshire.

DECLARATIONS

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

Signed..Lorna Thompson.....
(Candidate)

Signed..A. H. Cobb.....
(Director of Studies)

ABSTRACT

AN INVESTIGATION INTO THE MODE OF ACTION AND SELECTIVITY OF 3,6-DICHLOROPICOLINIC ACID

LORNA M.L. THOMPSON

The mode of action and selectivity of the herbicide 3,6-dichloropicolinic acid (clopyralid) was examined in three plant species under glasshouse and controlled environment conditions. At the growth stages used Matricaria inodora was susceptible, Galium aparine mildly susceptible and Beta vulgaris tolerant to the herbicide when applied at concentrations ranging from 6.25 to 400 g ai. ha^{-1}. Visual symptom development was characteristic of auxin-type compounds with rapid and sustained epinasty in susceptible species. This auxin-type activity was partially confirmed in a study of levels of key metabolites in treated plants. Herbicide was retained in significant amounts on the leaves of each species and differential sensitivity was not accounted for by differences in the extent of uptake or translocation, since the herbicide was absorbed into leaves of each species and rapidly translocated in the phloem to the primary growing points as shown by autoradiography. Differences were measured in uptake and translocation of radiolabelled clopyralid droplets between sprayed and unsprayed plants and according to the application method used. Differences in the extent and pattern of metabolism of clopyralid were recorded in the test species. Field rate clopyralid (100 g ai. ha^{-1}) increased the rate of photosynthesis in the third leaf of intact M. inodora plants up to twenty four hours after treatment. B. vulgaris showed no response. Preliminary experiments also recorded clopyralid effects on both abaxial and adaxial stomatal apertures in third leaf M. inodora and B. vulgaris tissue. Stomatal effects were greatest in the susceptible species where the range of stomatal apertures was restricted by clopyralid. Ethylene evolution was rapidly increased by a range of clopyralid concentrations in M. inodora third leaf explants. A clear dose response pattern emerged and it was shown by the use of the precursor ACC and ethylene biosynthesis inhibitors cobalt and AVG that the effect, which was absent in tolerant B. vulgaris, was a symptom of herbicide action and not the cause of the visually observed herbicide damage which appeared concurrently in the explants. These findings were discussed in relation to the literature and hypotheses presented to account for the observed selectivity of clopyralid in these species.

PUBLICATIONS

SANDERS, G.E., THOMPSON, L.M. and PALLETT, K.E. (1985): The influence of morphology of Galium aparine on the uptake and movement of clopyralid and fluroxypyr. Proc. 1985 British Crop Protection Conf. - Weeds. 419-425.

THOMPSON, L.M.L and COBB, A.H. (1986): Experimental studies into the selectivity of clopyralid in sugar beet. Aspects of Appl. Biol., 13, 17-24.

THOMPSON, L.M.L., SANDERS, G.E. and PALLETT, K.E. (1986): Experimental studies into the uptake and translocation of foliage applied herbicides. Aspects of Appl. Biol., 11, 45-53.

THOMPSON, L.M.L. and COBB, A.H. (1987): The selectivity of clopyralid in sugar beet; studies on ethylene evolution. Proc. 1987 British Crop Protection Conf. - Weeds. 1097-1104.

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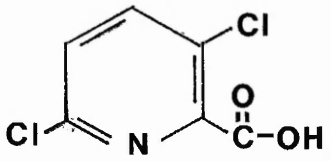
Chapter 1. INTRODUCTION

1.1 Clopyralid

The herbicide clopyralid (3,6-dichloropicolinic acid) is a synthetic pyridine compound introduced by The Dow Chemical Company in 1975 (Haagsma, 1975). Its structure and important chemical and physical properties are shown below:

structural formula:



empirical formula: $C_6H_3Cl_2NO_2$

molecular weight: 192

physical state: white, odourless, crystalline solid

melting point: 151-152 °C

vapour pressure: 1.2×10^{-5} mmHg at 25 °C

solubility: 1000ppm at 25 °C in H_2O

The compound is used as a selective, post-emergent herbicide in small grain cereals, including maize, sorghum and flax, and also in oil seed rape and sugar beet crops. Susceptible weed species include many species from the plant families Compositae, Polygonaceae, Umbelliferae and Leguminosae (Dow Technical Information).

Since the morphological effects of clopyralid and other pyridine compounds on growing plants appear similar to those caused by IAA and auxin-type herbicides, it has been assumed that their mode of action is similar to that of the auxin-type herbicides as represented by the phenoxyalkanoic acids. Structural similarities between compounds may also support this. However, over 40 years of research on auxin-type herbicides has provided no single or defined mechanism for herbicide action or

selectivity, and the observations that clopyralid is useful against many phenoxy-tolerant weeds, and that it can have a synergistic effect in mixtures with phenoxy compounds, may suggest that its mode of action is distinct from that of the phenoxyalkanoates.

The following sections briefly review our current understanding of auxin-type herbicides and highlight various areas of experimentation. This literature focuses on five areas of especial interest to this study, namely the effects of auxin-type herbicides on,

1. morphology and ultrastructure,
2. nucleic acid, protein and carbohydrate metabolism,
3. herbicide uptake, translocation and metabolism,
4. photosynthesis, and
5. ethylene metabolism.

For clarity, the literature pertaining to pyridine compounds and specifically to clopyralid is summarised in the relevant chapter introductions.

1.2 Physiological Effects of Auxin-type Herbicides

1.2.1 Morphological and Ultrastructural Effects

Martin and Fletcher (1972) studied the effects of sublethal doses of six growth regulator herbicides, including 2,4-D and 2,4,5-T on lettuce. Examination of the treated plants of this highly sensitive species revealed a wide range of morphological effects in response to these compounds. Symptoms included epinasty, reduced lamina growth, vein thickening, fusion of new leaves, stem splitting, adventitious root development and serration at leaf margins. Although the duration and severity of such symptoms depends on herbicide dose and plant age, as well as degree of susceptibility, they constitute the characteristic morphological effects of auxin herbicides on sensitive plants. Anderson and Thompson (1973) reviewed the effects of herbicides on the ultrastructure of plant cells. They described

how terminal changes in plant tissue associated with herbicide-induced plant death are similar, if not identical to, ultrastructural changes associated with death by other causes, and can seldom be distinguished from the final stages of senescence. However, many effects of auxin-type herbicides on plant cellular membranes have since been shown. Hallam and Sargent (1970) observed disruptions in the outer chloroplast envelope, plasma membrane and endoplasmic reticulum within 4 hours of application of 2,4-D to bean leaves. White and Hemphill (1972) indicated that the first visible effect of 2,4-D in leaves was a rupture and disintegration of the tonoplast.

Typical structural symptoms therefore include rapid internal membrane disorganisation and the manifestation of a range of growth abnormalities.

1.2.2 Effects of Auxin-type Herbicides on Nucleic Acid and Protein Synthesis

Examining a wide range of herbicides, Fedtke (1982) suggested that the inhibition of nucleic acid and protein synthesis was not a primary site of herbicide action in intact plant tissues. He concluded that the herbicidal inhibition of *in vivo* systems synthesising proteins or nucleic acids may usually be regarded as a secondary effect, induced as a result of some form of inhibition of the supply of metabolic energy to the process in the form of ATP. In a more detailed review, Fletcher and Kirkwood (1982) outlined such indirect effects of herbicides from many chemical groups. They also described findings of effects on nucleic acid and protein synthesis measured in tissue segments such as leaf discs, half seeds and roots, as well as in cells. In particular the inhibitory effects of the organophosphorous herbicide glyphosate were discussed.

Auxin and auxin-type herbicides, particularly the phenoxyalkanoic acids, have been found to increase plant nucleic acid and protein content. Skilberger and Skoog (1953) were the first to report auxin effects on RNA and DNA synthesis, this being in cultured tobacco tissue. Many researchers have since reported increases in RNA and protein following 2,4-D treatment (eg West,

Hanson and Key, 1960; Key and Shannon, 1964; Key, Lin, Gifford and Dengler, 1966; Chen, Switzer and Fletcher, 1972), and the mechanism producing this increase and its relation to auxin-type herbicide symptoms and the mode of action and selectivity of such compounds has been extensively studied.

Key (1963) found that in excised corn mesocotyl tissue the major effect of 2,4-D was on RNA metabolism, rather than on its synthesis. However, the literature on the subject supports the idea that auxins induce RNA synthesis. Of the two possible origins for increased synthesis of RNA and proteins, Cherry (1976) has suggested that regulation of transcription (ie DNA directed RNA synthesis) by auxin-type herbicides is more feasible than any effects on the translation of mRNA into proteins. He summarised several years findings stating that in vivo treatment of the soybean plant with 2,4-D increases the capacity of chromatin (and RNA polymerase solubilised from chromatin) to synthesise nucleic acids (O'Brien, Jarvis, Cherry and Hanson, 1968). Cherry (1967) demonstrated that there was no effect of 2,4-D on the capacity of isolated soybean nuclei to produce RNA, whilst nucleii isolated from 2,4-D treated plants did show a greater capacity to produce RNA than controls. This indicated that regulation of transcription was indirectly controlled by 2,4-D and experiments were carried out to ascertain how auxins mediated this effect. The involvement of a cytoplasmic factor as a 'signal' released from the plasma membrane in response to auxin-herbicide binding was examined. Hardin, Cherry , Morre and Lembi (1972) found that auxin caused the release of a "factor" from isolated soybean plasma membranes which was capable of stimulating RNA polymerase activity. The identity of the factor was not found although preliminary data indicated that it was inhibited by protease treatment and may be released when membranes were treated with lipase (Cherry, 1976).

A more detailed study of the increased rate of RNA synthesis has shown that RNA polymerase I is increased more than RNA polymerase II. In a study of isolated soybean nuclei (Chen, Lin, Chang, Guilfoyle and Key; 1975) it was found that those isolated from auxin treated tissues contained about 2.5 times as

much RNA polymerase I activity as control nuclei i.e. RNA polymerase II activity was increased approximately 25% by auxin over a series of experiments. In another study, Guilfoyle et al (1975) found that 2,4-D increased soluble RNA polymerase I activity by 80% , whilst increasing RNA polymerase II activity by only 30%. Teissere et al (1975) suggested that a factor which activates nucleolar RNA polymerase I(b) is doubled in response to 2,4-D. These findings are supported theoretically by Theologis, Huynh and Davis, (1985) who found that auxin increased the amount of specific mRNA's in pea epicotyl tissue and suggested that the speed of the response (15min lag) indicated a primary action. These authors found that there was no relationship between auxin-induced hydrogen ion excretion and mRNA induction and tentatively suggested that the early-induced mRNAs may code for cell wall polypeptides. This could influence cell wall extension and subsequent cell enlargement, with hydrogen ion excretion being a consequence of enhanced bio-synthetic activity induced by the hormone. Key (1964) had previously suggested that RNA and protein synthesis were essential processes for cell elongation and linked 2,4-D enhancement of the rate of cell elongation with formation of some specific RNA. Theologis and Ray (1982) found that the number of mRNA sequences responding to auxin increased with time of exposure, indicating a temporal programme of hormone-induced gene expression or possibly a cascade of responses, in which some early-induced mRNAs code for regulatory polypeptides which affect the transcription of other mRNAs. The mechanism by which transcription of specific mRNAs is induced by a hormone-mediated signal is not known. However, Cherry (1976) described two possibilities. Firstly, histones or basic proteins may be removed from DNA exposing specific sections for transcription, or secondly, the signal could bind to already exposed DNA sites and act by initiating RNA synthesis.

Although increased RNA content and ribosome number may result in increased protein synthesis, effects on protein metabolism may also occur. Fletcher and Kirkwood (1982) noted the findings of Krishchenko in 1973 who discovered that plants of spring wheat treated with 2,4-D had reduced protein synthesis but enhanced

accumulation of free amino acids. It has been suggested that excessive nucleic acid and protein synthesis precludes normal cell functions and results in the characteristic symptoms of auxin-type herbicide action, including tissue proliferation which leads to epinasty (Key et al, 1966). Malhotra and Hanson (1970) suggested that resistance of wheat and oats to picloram was due to their high levels of nucleases, with which they were able to minimise the phytotoxic effects of increased nucleic acid synthesis induced by the herbicide.

Thus there is much evidence in the literature to support the importance of auxin-type herbicide effects upon protein and nucleic acid synthesis.

1.2.3 Effects of Auxin-type Herbicides on the Carbohydrate Content of Plants

Carbohydrates play a significant role in the plant since they represent vital food reserves and constitute the structural framework of each cell (Kirkwood, 1976). The activity of 2,4-D on glycolysis and associated metabolic processes has been extensively studied and it has been found that the phenoxy-alkanoic acid herbicides can alter the carbohydrate content of plants. However, effects are complex and vary with species, tissue, dose and other conditions. Several effects were outlined by Wort (1964) in his review of herbicide effects of plant composition and metabolism. These included a 5% increase in the amount of sugar obtained from sugar cane as a result of 2,4-D treatment, and a reduction in reducing sugar concentration in soybeans treated with 2,4-D (this reduction was coupled to increased starch production). Rogerson, Bingham, Foy and Sterrett, (1972) analysed the effect of fenac (2,3,6-trichloro-phenyl acetic acid) on the carbohydrate levels in mugwort rhizomes and found effects within one day of treatment. Both glucose and fructose content were increased by the herbicide.

It may be the case that variations in reducing sugar content could indicate metabolic effects of auxin-type herbicides on a

number of related processes of intermediary metabolism rather than a discrete response.

1.2.4 Effects on Photosynthesis

Inhibition of photosynthesis forms the primary mode of action for a number of important herbicide groups (Van Oorschot, 1970) but unlike the direct effects of, for example the triazine and phenylurea compounds on the photosynthetic pathway, recorded effects for phenoxyalkanoic acid herbicides are generally indirect. Robertson and Kirkwood (1970) described indirect effects of 2,4,5-T on the Hill reaction, in which 1.2×10^{-4} M herbicide caused a 50% inhibition in electron flow in isolated pea chloroplasts. Other effects of the phenoxyalkanoic acid herbicides on assimilate production have been suggested to result from ultrastructural changes in the plant which cause alteration of the translocation profile thereby reducing or enhancing assimilate accumulation at various sites. Effects on photosynthesis have also been attributed to herbicide-induced disruptions of stomatal mechanism. Several synthetic herbicidal auxins are known to induce stomatal closure (Pemadasa and Jeyaseelan, 1976) and such effects may originate from herbicide-induced changes in guard cell permeability. Stimulatory effects of auxin (Turner and Bidwell, 1965) and auxin-type herbicides have also been noted. Cardenas, Slife, Hanson and Butler, (1967) found rapid stimulation of photosynthesis (0-15min) in cocklebur following spot application of 2,4-D and correlated this finding with an IAA effect in broad bean which causes the breaking of bud dormancy (Bidwell and Turner, 1966).

1.3 Uptake, Translocation and Metabolism of Auxin-type Herbicides

1.3.1 General Considerations

Investigations of absorption, translocation and metabolism of herbicides are vital to mode of action studies. The aims and objectives of such investigations, and the experimental methods commonly used are summarised below. Theoretical aspects of herbicide entry into plants and subsequent translocation and

patterns of metabolism are outlined with respect to foliage-applied auxin-type herbicides.

The rationale of conducting uptake and translocation studies can be divided into four areas:

1. They may indicate possible target tissues for herbicide action in susceptible species and thus highlight areas for subsequent experimentation.

2. Comparisons of uptake and translocation data from susceptible and resistant species may reveal, in part or whole, reasons for observed selectivity (Schafer and Chilcote, 1970; Hallmen, 1974; Sargent, 1976; Wyrill and Burnside, 1976; and Sanders and Pallett, 1987).

3. Detailed work with one compound, to assess uptake and translocation following various application regimes (formulation, spray volumes, droplet size etc.) under different environmental conditions and at various plant growth stages, allows a profile of herbicide behaviour to be constructed (Holly, 1976; Muzik, 1976; Radosevich and Bayer, 1977; and Coupland, 1983). This can be used to increase the efficiency and safety of herbicide usage in the field. Dose rates can be optimised and the timing of applications, with respect to plant growth stage and environmental conditions, may be selected to maximise herbicide effect and minimise crop damage.

4. Uptake and translocation studies with more than one compound may provide reasons for antagonism or synergism between herbicides in the field. For example, the rapid enhancement of the onset of glyphosate injury to Cirsium arvense shoots when low rates of picloram were incorporated was found to be due to the blocking, by picloram, of glyphosate movement from the shoots to the roots. This prevention of root kill also explained the antagonistic effect of picloram on long-term control of C.arvense by glyphosate (O'Sullivan and Kossatz, 1982).

Herbicide metabolism within plant tissues may result in inactivation. Hence, this process can greatly influence activity and selectivity, making it an important area of study in mode of action research. Additionally, metabolism studies are vital in conjunction with uptake and translocation experments which use radioassay techniques, in order to check that the isotope remains attached to the parent molecule (Wain and Smith, 1976; Kirkwood, 1983).

The most convenient and widely used method for following the uptake and translocation of foliage-applied herbicides is via the use of radiolabelled compounds. These are applied to test plants, usually in a manner relevant to field conditions, and their movement traced by qualitative and/or quantitative radioassay techniques. Where suitable, ^{14}C-labelled compounds are used since they are relatively cheap to produce and, because of their weak β-emission, pose negligible danger to the investigator. Such methods have been widely used for over 30 years (eg Crafts, 1956; Leonard, 1958; Yamaguchi and Crafts, 1958), although application and recovery methods vary greatly depending upon the aims of the experiment (Thompson and Cobb, 1986). In some studies, explant systems are used instead of whole plants (Lingle and Suttle, 1985). Amongst the literature there are several valid studies which do not use radiolabelled compounds (eg Davis, Bovey and Merkle, 1968; Scrifes, Baur and Bovey, 1973; Bovey, Ketchersid and Merkle, 1979), instead relying on chromatographic techniques to determine concentrations of extractable herbicide entering into and being distributed through plant tissues. For example, an accurate method has been reported for the detection, by gas-liquid chromatography, of clopyralid residues (Galoux, Van Damme and Bernes, 1982).

Metabolism studies are generally conducted in parallel with uptake and translocation studies and usually involve the chromatographic separation of radiolabelled compounds extracted from plant tissues. The form of recovered activity is recorded with reference to standards and the extent to which the parent molecule has been altered within the plant may be ascertained. In some studies, the chemical nature and herbicidal activity of metabolites is investigated (Feung, Hamilton and Mumma, 1977).

1.3.2 Herbicide uptake

The foliar absorption of herbicides may be divided into three processes, ie:

1. Movement through the plant cuticle.
2. Partitioning into the apoplast.
3. Uptake by underlying epidermal cells.

1. Movement through the plant cuticle: The cuticle is a lipoidal layer, the main function of which is to restrict plant water loss. It also serves as the primary barrier to penetration of materials into plants. The continuous structure of the cuticle is maintained by a cutin matrix in which cuticular waxes are embedded. This is covered with an outer wax layer (epicuticular waxes), the thickness and morphological characteristics of which vary depending upon both plant species and the environmental conditions under which waxes are deposited (Hull, 1970; Hull, Morton and Wharrie, 1975; Bukovac, 1976). Since the cuticle is largely of a hydrophobic nature it favours the entry of non-polar (lipophilic) compounds which are thought to be sorbed into and diffused through the lipoidal components of the cuticle. In this way the chemical nature of the herbicide molecule has a marked effect on its cuticular penetration, and it is generally accepted that the movement of organic materials across the cuticle is inversely correlated with the polarity (Richardson, 1977). Accordingly, Norris and Freed (1964) have shown that the ester form of 2,4-D is more rapidly absorbed than either the acid or the triethanolamine form in big leaf maple seedlings (Acer macrophyllum). Price and Anderson (1985) examined ten species and found that in six of them the iso-octyl ester of 2,4-D was absorbed more than the sodium salt over a 26 hour period. In three of the species, uptake of the two molecules was equal, and in Xanthium pennsylvanicum the sodium salt of 2,4-D was more greatly absorbed than the ester form. These and other studies (eg Sargent and Blackman, 1972) highlight the differences in uptake between plant species and illustrate that, although generalisations about the effect of the molecular structure of a herbicide on its uptake can be made, extrapolations between plant species cannot be assumed. The pathway by

which water-soluble or ionic herbicides penetrate the cuticle is poorly understood. The existence of an aqueous continuum between the plant and environment is suggested by measurements of cuticular transpiration, and the cuticle is frequently described as being sponge-like and hydrated, under conditions of high humidity, forming aqueous pores and channels. Schonherr (1979) proposed and tested a model in which polar compounds penetrate through these aqueous pores. He estimated a pore radius of 0.45nm in Citrus sinensis and found that polar molecules with a radius close to or greater than this value failed to penetrate . Although there is much controversy about their nature, areas have been found in the cuticle which are preferentially permeable to polar compounds and they serve as hydrophilic 'bridges' across the cuticle (Bukovac, 1976).

The presence of trichomes on leaf surfaces may enhance cuticular penetration by increasing the epidermal surface area exposed to the spray solution. Some authors suggest that the area around the base of trichomes is preferentially permeable to herbicides due to delayed cuticular development (Sifton, 1963).

Stomata may also play a two-fold role in foliar penetration. Under certain conditions entry of herbicides may occur through the stomatal pore, whilst the cuticle over the guard and accessory cells may be more permeable and therefore increased uptake may be noted in these areas (Bukovac, 1976).

2. Partitioning into the apoplast: From within the cuticle herbicides must move into the plant cell wall continuum prior to entering the protoplast. Whereas cuticular entry depends largely on the lipophilic characteristics of the herbicide molecule, this partitioning depends on the hydrophilic characteristics. This means that for successful penetration of the cuticle and subsequent partitioning into the aqueous phase, it is desirable for a herbicide to have a suitable lipophilic/hydrophilic balance in its formulation. This means that the relationship between the lipophilicity of a herbicide and its uptake is far more complex than that between lipophilicity and simple cuticular penetration. Ester formulations, which are

strongly lipophilic, are apparently hydrolysed (possibly by cuticular enzymes) and thereafter partition into the apoplast (Fletcher and Kirkwood, 1982).

3. Absorption by epidermal cells: There are several possible routes by which herbicide molecules may penetrate the plasma membrane of epidermal cells. Once again the lipophilic/hydrophi -lic balance of the molecule is important. Lipophilic herbicides probably have little difficulty in crossing the lipid bilayer of the cell membrane by a simple process of diffusion, the rate of which will depend on the size and shape of the molecule as well as the arrangement of the membrane phospholipids (Morod, 1976). Those compounds which are more polar may rely on active transport processes, usually reserved for sugars and amino acids, to transport them across the plasmalemma. This process will require an energy input and thus its rate will be influenced by factors which affect cell metabolism, eg temperature and light intensity. Such factors have been shown to influence the rates of absorption of 2,4-D and 2,4,5-T (Richardson, 1977). The entry of small organic molecules may be facilitated by small pores in the cell membrane. There has been a great deal of work published concerning the effect of such factors as molecular structure, plant species and growth stage and the environment (light, temperature and humidity) on the uptake of foliage applied herbicides as outlined in reviews by Robertson and Kirkwood (1969), Bukovac (1976) and Richardson (1977). There has, however, been little attempt (probably due to experimental restraints) to distinguish between effects upon cuticular absorption, partitioning of a compound into the apoplast, and its movement into the underlying cells. This means that, although we have a detailed understanding of factors likely to affect herbicide uptake, we may only postulate on the relative effect that each factor has on each stage of the absorption process.

It should be noted that some compounds move in the apoplast and then directly enter the phloem tissues, thus by-passing leaf cells. Others, which are normally root absorbed, do not penetrate the symplast and move only in the apoplast, and so remain within the treated leaf (Fletcher and Kirkwood, 1982).

1.3.3 Herbicide Translocation

The movement of foliage applied herbicides from their site of entry consists of three phases. Firstly, there is short distance movement of the compound from cell to cell. This is followed by phloem loading, after which the movement of herbicide from one part of the plant to another takes place in the sieve tubes.

As previously mentioned, short-distance transport can take place in the apoplast (with polar compounds moving in the non-living cell wall continuum) or in the symplast, where molecules which have penetrated the cell membrane can move from cell to cell via plasmodesmata (Robards, 1975). Although the mechanism of movement within the sieve tubes has yet to be fully elucidated , their loading via the cytoplasm of companion cells is an active process taking place against a concentration gradient (Hay, 1976).

Most accepted theories of translocation involve a stream of solutes moving from a 'source' tissue (photosynthetic stems or leaves) to a 'sink' (meristematic and developing tissues) and it has been assumed that herbicides and other exogenous compounds are carried along in this assimilate stream (Hay, 1976). Certain structural criteria for phloem transport of chemicals have been proposed by Crisp (1972). These include the presence of a free carbox ylic group or functional groups which can form conjugates with sugars or amino acids. The importance of sink activity in the movement of herbicides has been shown by several studies including Hunter and McIntyre (1974), and is discussed by Robertson and Kirkwood (1970). Compounds can also be carried in the transpiration stream if they 'leak' from sieve tubes into the apoplast.

1.3.4 Auxin-type Herbicide Uptake, Translocation and Metabolism

The uptake and translocation of the phenoxyalkanoic acid compounds has been investigated in a large number of plant species (eg Fites, Slife and Hanson, 1964; Neidermyer and Nalewaja, 1969; Coble, Slife and Butler, 1970; Chanikov, Mekeev, Pavlova and Dubovoi, 1971) and, although patterns of uptake and

movement do emerge, inter-species differences are large. In general, the uptake into treated leaves is rapid although its extent can vary (from less than 10% to over 90% of applied compound) depending upon peculiarities of application (chemical formulation, droplet size, pH, concentration, etc) as well as plant and environment factors (Robertson and Kirkwood, 1969; Richardson, 1977). The extent and rate of its subsequent translocation also depends on a great many factors, including the degree of metabolite immobilisation which takes place. A general pattern of auxin-herbicide translocation from treated leaves to the most active photosynthetic sinks is typical, and most papers cite accummulation in apical and developing tissues enforcing theoretical profiles of phloem movement. Hay, (1976) found little evidence of movement of these herbicides (in particular 2,4-D) to plant roots, and suggested that since these would be expected to act as photosynthetic sinks, the movement into roots may be prevented by their leakage into the apoplast and movement upwards in the transpiration stream. However, Fites, Slife and Hanson (1964), and more recently Turnbull and Stephenson (1985), have shown that in species with active root systems, 2,4-D does move into roots. Both groups also measured root exudation of 2,4-D. Most phenoxyaliphatic herbicides are ambimobile, i.e. they can move in the phloem and xylem, and so are absorbed by roots and foliage.

The metabolic degradation of herbicide molecules in higher plants may result from a wide range of chemical reactions, most of which are catalysed by specific enzyme systems (Hatzios and Penner, 1982). These include oxidation, decarboxylation, hydroxylation, hydrolysis and conjugation, all of which are mechanisms of detoxification of the phenoxyalkanoic herbicides (Fletcher and Kirkwood, 1982). These compounds can undergo rapid and extensive metabolism in certain species (Kirkwood, 1983).

1.4 Effects on ethylene Biosynthesis

1.4.1 Ethylene Biosynthesis

Ethylene is a gaseous plant growth regulator which initiates fruit ripening and regulates many aspects of plant growth, development and senescence. It is biologically active in trace amounts and is produced from almost all tissues of higher plants including leaves, stems, roots, flowers, fruits, tubers and seedlings (Lieberman, 1979). Current understanding of the biosynthetic pathway of the gas has been concisely reviewed by Yang and Hoffman (1984) and is outlined in summary form in figure 1.1.

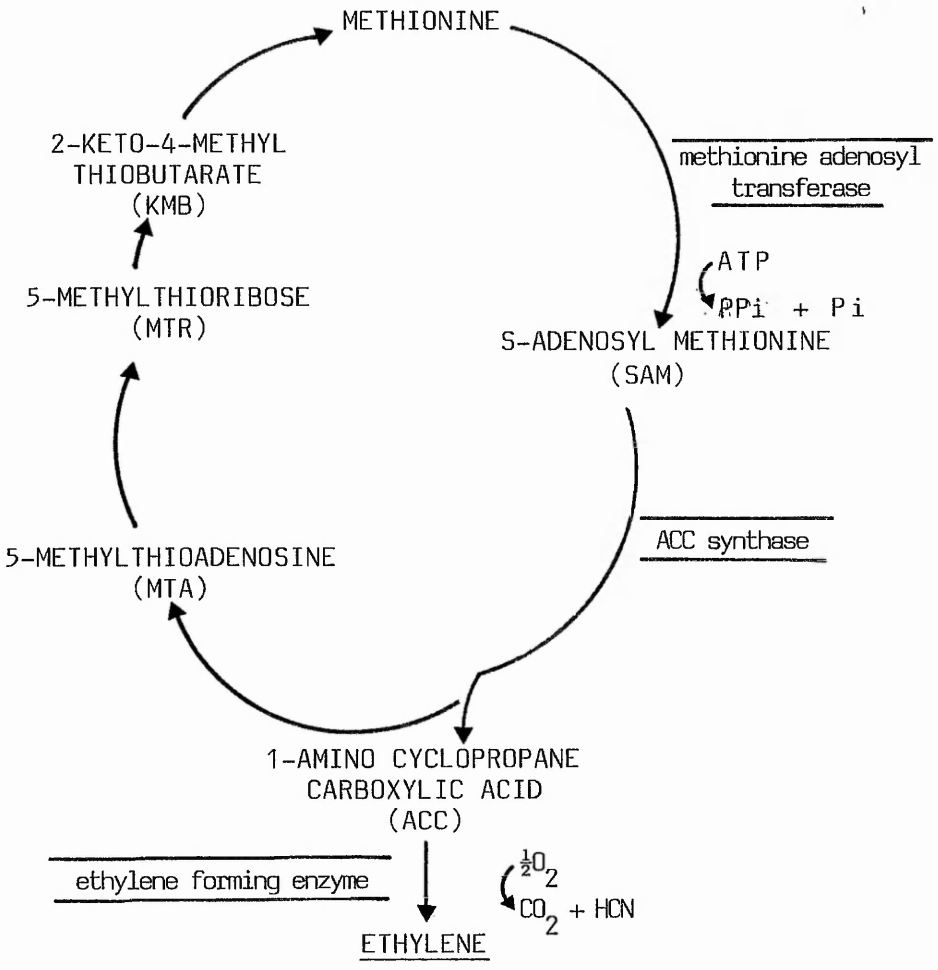


Figure 1.1. Ethylene biosynthetic pathway.

In the mid 1960's methionine was shown to be the precursor of ethylene in higher plants (Lieberman and Mapson, 1964; Lieberman Mapson, Kunishi and Wardale, 1965), and the mechanism of its conversion to ethylene and its recycling has been intensively studied. Ethylene is produced from methionine via the intermediates s-adenosylmethionine (SAM) and 1-aminocyclopropane carboxylic acid (ACC). The first step in the pathway involves the synthesis of SAM from methionine and adenosine triphosphate (ATP) via an adenosyl transferase reaction (Adams and Yang, 1977). In the absence of oxygen this conversion is followed by the metabolism of SAM to ACC and 5-methyl thioribose (MTR). In air, however ACC is rapidly converted to ethylene. The metabolism of SAM to ACC is catalysed by the pyridoxyl enzyme ACC synthase (Yu, Adams and Yang, 1979) and the importance of this as a key enzyme in the pathway is now established. The conversion of ACC to ethylene is oxygen-dependent and is catalysed by an enzyme system (ethylene forming enzyme, EFE) probably located in the tonoplast and plasma membrane (Yang, 1981; John, 1983; Yang and Hoffman, 1984), although ACC can be converted to ethylene non-enzymatically by oxidants (Boller, Herner and Kende, 1979). The recycling of methionine commences with the production of MTR (through rapid nucleosidase hydrolysis of the intermediate 5-methylthioadenosine, MTA) and *in vivo* studies have confirmed that MTR is converted into methionine via 2-keto-4-methylthiobutyrate (KMB) (Yang and Hoffman, 1984). It has been shown that KMB is efficiently converted into methionine (Baur, Yang, Pratt and Biale, 1971), thus completing the biosynthetic cycle.

1.4.2 Regulation of Ethylene Biosynthesis

Ethylene production is vital to a number of plant developmental processes and is regulated by a variety of plant and environmental factors. An essential role is played by ethylene in ripening fruits and senescing tissues, where changes occur in the ability of tissues both to produce ACC from SAM and to produce ethylene from ACC. The autocatalysis of ethylene, where ethylene presence stimulates its further production, is also a characteristic feature of these tissues (Yang, 1981). Auxin

(IAA) plays an important regulatory role in ethylene biosynthesis (Abeles and Rubenstein, 1964; Imaseki, 1981). IAA rapidly induces ethylene synthesis (Franklin and Morgan, 1978) by increasing the activity of ACC synthase (Jones and Kende, 1979; Yoshi, Watanabe and Imaseki, 1980; Yang and Hoffman, 1984). The increasing activity is dependant upon continuous protein and RNA synthesis (Yoshii and Imaseki, 1981), indicating that the stimulation is due to de novo synthesis of the enzyme, which has been found to be highly labile (Kang, Newcombe and Burg, 1971; Sakai and Imaseki, 1971). Ethylene production in plants may also be induced by various stress factors including mechanical injury (Imaseki, Uritani and Stahmann, 1968), environmental extremes and by chemicals and gaseous pollutants (Suttle, 1984; Pell and Puente, 1986). As with auxin-induced ethylene production, the enzyme ACC synthase plays a key role in the induction process and it appears that stress induces rapid accumulation of ACC synthase with resultant increase in ethylene production (Yang, 1981).

Regulation of ethylene biosynthesis is also thought to be influenced by light and carbon dioxide concentration. Bassi and Spencer (1982) found that a high CO_2 concentration (0.5%) increased the rate of ethylene production in intact sunflower plants (Helianthus annus L.). The response occurred only in the light. In the same species they examined the effects of light on ethylene production and concluded that, in an open experimental system, light has little effect upon ethylene production in intact plants (Bassi and Spencer, 1983). This is contrary to findings obtained when leaf tissues were used in closed systems and light was found to be inhibitory to ethylene production (Yang and Hoffman, 1984), and they suggest that differences may be attributed to techniques used for their measurement.

Pyridoxyl enzyme inhibitors inhibit the production of ACC from SAM. These fall into two groups; (1) vinylglycine analogues, the most potent of which is aminoethoxyvinylglycine (AVG), (Yu and Yang, 1979; Yoshii and Imaseki, 1982) and (2) hydroxylamine analogues, the most widely used of which is

aminooxyacetic acid (AOA, Amrhein and Wenker, 1979). The inorganic ions Co^{++} and Ni^{++} also inhibit ethylene production, having their effect via inhibition of the conversion of ACC to ethylene (Yu and Yang, 1979; Yang and Hoffman, 1984). Indirect inhibition of ethylene production may also be found, for example where physical conditions (eg high temperature or cold shock) or chemicals (eg 2,4-dinitrophenol (DNP) or carbonyl cyanide m-chlorophenyl hydrazone (CCCP)) act to disrupt plasma membrane structure, the integrity of which is thought to be essential for activity of the EFE.

1.4.3 Ethylene as a Mediator in Auxin-Type Herbicide Action

The application of ethylene gas to plant tissues may result in a great variety of measurable physiological responses depending upon plant/tissue sensitivity (determined by factors such as age, environment and current plant hormone regime), concentration of ethylene and duration of exposure. Crocker (1948) summarised the major responses, including epinasty of leaves, tissue proliferation, abscission of leaves, flowers and fruits, root and root hair initiation and chlorophyll breakdown. More recently measured responses include the inhibition of growth and cell division (Apelbaum and Burg, 1972A and B), and effects upon the regulation of stomatal behaviour (Madhavan, Chrominski and Smith, 1983; Tissera and Ayres, 1986). Many of these effects are similar to effects of auxin (Morgan, 1976) and it is now considered that many auxin effects are mediated by ethylene.

Ethylene production following herbicide application has been reported in a number of plant species (Maxie and Crane, 1967; Abeles, 1968; Holm and Abeles, 1968; Baur and Morgan, 1969; Stacewicz-Sapuncakis *et al*, 1973; Paradise, Ebert and Elstner, 1981; Hall *et al*, 1985; Baker and Hunt, 1986). The effects of herbicides on ethylene production have been reviewed by Morgan (1976) and production is thought to arise via one or both of the routes outlined in figure 1.2.

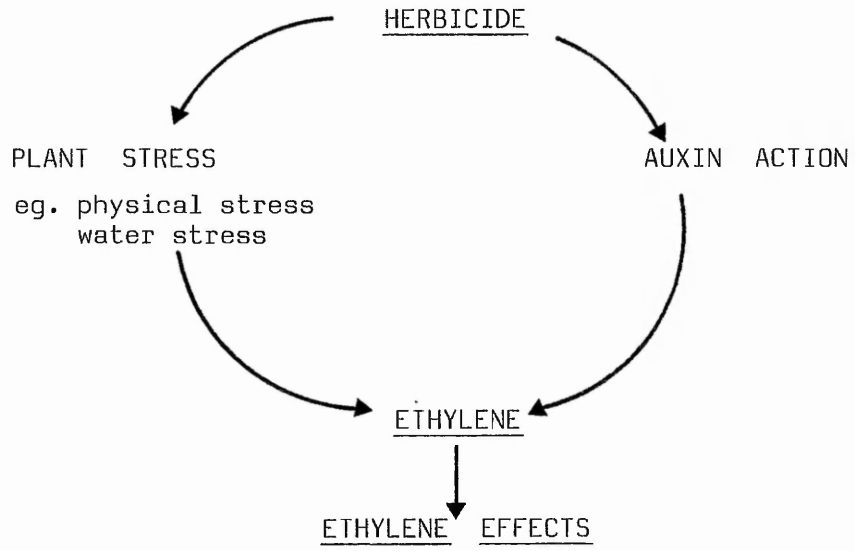


Figure 1.2. Possible routes for herbicide-induced ethylene production

A wide variety of herbicides put the target plant under some form of stress thus indirectly causing ethylene evolution. For example, the grass herbicide metolachlor induces ethylene production in sorghum seedlings which is a symptom of physical damage (Paradise, Ebert and Elstner, 1981). However, the auxin type herbicides such as 2,4-D and picloram have a more direct effect upon ethylene production and, in the same way that many auxin effects are mediated by ethylene, many symptoms of these herbicides are thought to be due to ethylene action. Although there is evidence that some auxin-type herbicide induced symptoms which precede plant death, such as leaf and stem epinasty, may be accounted for by ethylene, ethylene is not the agent by which these herbicides kill plants (Morgan, 1976). Several researchers have attempted to distinguish between and separate some of the effects of auxinic herbicides from those of ethylene (Abeles, 1968; Apelbaum and Burg, 1972A). Although this has proved difficult, it appears that a high concentration of auxin, especially a synthetic auxin like 2,4-D, can exert a herbicidal effect independant of or in addition to the action of auxin-induced ethylene (Apelbaum and Burg, 1972A). There are several instances where ethylene production is increased more greatly by auxin-type herbicides in susceptible compared to resistant or tolerant species. This was noted as early as 1962

when Morgan and Hall experimented with 2,4-D in susceptible cotton and resistant grain plants.

1.5 Aims of The Investigation

The aim of the present study is to investigate the biochemical and physiological mechanisms of action and selectivity of clopyralid using selected weed species. Two weed species were selected for investigation. Firstly, highly susceptible Scentless Mayweed (Matricaria maritima ssp, inodora (L.) Clapham, recently renamed Tripleurospermum maritimum ssp, inodorum (L.) Koch), referred to as M.inodora in this study. This is a member of the compositae and one of the designated target weeds for clopyralid (Dow technical information). The other weed used was the partially susceptible Cleavers (Galium aparine L.). This is an annual dicotyledonous weed of great economic importance which has proven difficult to control. The biology of the species is discussed by Froud-Williams (1985). Sugar beet (Beta vulgaris cv. Salohill) was used as a resistant target crop.

Chapter 2. PRELIMINARY INVESTIGATIONS

2.1 Introduction

2.1.1 Morphological and ultrastructural effects of pyridine herbicides

The effects of systemic pyridine herbicides on the morphology of sensitive plants are very similar to those of 2,4-D and other auxin herbicides as discussed in the general introduction. Fisher, Bayer and Weier (1968) studied the anatomical and morphological effects of picloram on red kidney bean (Phaseolus vulgaris). They recorded stem bending, growth inhibition and tumor-like growths near the plant apex. Early anatomical effects included the formation of adventitious root initials from the stem cambium. Sanders and Pallett (1987) examined the physiological and ultrastructural changes in Stellaria media following treatment with fluroxypyr (4-amino-3,5-dichloro-6-fluoro-2-pyridyloxyacetic acid), and found initial petiole curvature followed by stem elongation and thickening prior to leaf senescence, stem and petiole necrosis and plant death. In this study light and electron microscopy revealed leaf tissues containing cells with disrupted and swollen chloroplasts and a disrupted tonoplast. Within the stem tissue there was extensive meristematic differentiation and adventitious root development. These authors also measured increased levels of reducing sugars and amino acids in treated foliar tissue indicating reserve mobilisation during initial stages of symptom development. Both triclopyr and clopyralid showed morphological effects typical of auxin-type herbicides in peanut and these were comparable to 2,4,5-T injury in this species (Bovey and Meyer, 1981). Typical symptoms recorded were the upward rolling of the terminal leaves and callus growth on stems. Inhibition of overall foliar growth was accompanied by the appearance of swollen, shortened and twisted stems.

2.1.2 Pyridine herbicide effects on nucleic acid and protein synthesis

Effects of systemic pyridine compounds such as picloram and triclopyr on nucleic acid and protein synthesis are similar to those induced by the phenoxyalkanoic acids, such as 2,4-D and 2,4,5-T. Chen et al (1972) found that picloram increased RNA levels in cucumber roots by more than 200% over water controls, as well as increasing DNA and protein content. Malhotra and Hanson (1970) found that in sensitive cucumber and soybean, picloram caused increases in RNA content and protein (on g/fresh weight basis). However in resistant barley, wheat and corn there was no effect on RNA content and little change in DNA and protein values. These authors explained their results by the presence of higher levels of bound nucleases in the resistant species preventing the accumulation of nucleic acids. These results support the hypothesis that picloram functions as an auxin-type growth regulator with a herbicidal action similar to that of 2,4-D (Eisinger and Morre, 1971). The similarity of picloram action on nucleic acid synthesis to that of auxin allows its substitution for auxin in plant tissue culture (Goodin and Becher, 1967).

Chen and Lin (1977) showed that nuclei isolated from clopyralid treated plant tissues had around five times as much RNA polymerase I activity as nuclei isloated from untreated plants. This result is consistent with results obtained in experiments with nuclei from 2,4-D treated plants (Chen et al, 1975) as discussed in the general introduction.

2.1.3 Aims

The aim of the present study was firstly, to characterise the relative susceptibilities of the three test species to clopyralid herbicide, and to record morphological symptom development in each. Secondly effects on key metabolites were investigated and their contribution to herbicide activity and selectivity assessed. In these preliminary studies comparisons of clopyralid effects to established patterns for auxin-type herbicides were made.

2.2 Materials and Methods

2.2.1 Plant Material

All plants were glasshouse grown in J. Arthur Bowers seed and potting compost. Seeds were obtained from Herbiseed and sown in 90mm diameter plastic pots; Matricaria inodora on the surface, Galium aparine and Beta vulgaris var 'Salohill' at depths of 5mm and 10mm respectively. Plants were thinned to two per pot at the young seedling stage and transferred to an environmental chamber (Fisons model 600 G3, type TTL; 14h day photoperiod, 20°C and 100µmoles m^{-2} s^{-1} photosynthetic photon flux density (P.P.F.D.), 14°C at night). M.inodora and B.vulgaris plants were treated when three to four leaves were fully developed. G.aparine plants were treated when the 2nd leaf whorl was fully developed and the 3rd visible.

2.2.2 Herbicide Application

Clopyralid was applied as the monoethanolamine salt (Lontrel 100). Dose rates equivalent to 6.25, 25, 100 and 400 g ai.ha^{-1} (0.0625, 0.25, 1 and 4 times recommended field rate) were applied at the recommended volume rate of 200 l ha^{-1}. Spray applications were made using a laboratory pot sprayer fitted with a Tee jet H1/SS 8001 nozzle (Mardrive Engineering Company).

2.2.3 Assessment of susceptibility

Four replicate plants of each of the three test species were examined 3, 7, 14, 21 and 28 days after treatment (d.a.t.) with the 4 herbicide doses. Symptoms were visually assessed and recorded photographically. Aerial plant parts were then dissected and, following fresh weight determination, oven dried at 90°C for 48h. Dry weights were measured and results expressed as mean values of 4 replicates plus or minus standard error. Selected means were compared using Student's t-test. An examination of B.vulgaris plants 60 d.a.t. was also undertaken. In order that comparisons between species could be made dry weights were also expressed as % of untreated controls.

2.2.4 Symptom Analysis

M.inodora-petiole length: the petiole length of the 3rd leaf of 4 replicate M.inodora plants was measured 3, 7 and 14 d.a.t. with 4 clopyralid doses. Results were expressed as mean petiole length (mm) plus or minus the standard error.

G.aparine-internode length: the mean internode length of 4 replicate G.aparine plants 14 d.a.t. with 4 clopyralid doses was calculated by dividing total plant length by the number of internodes. Measurements were presented in mm plus or minus the standard error.

2.2.5 Assessment of levels of Key Metabolites

Four replicate plants of each of the 3 test species were biochemically analysed for levels of protein (insoluble, soluble and microsomal), amino acids and reducing sugar content at 1, 3, 7 and 14 d.a.t. with each of the clopyralid doses. The fresh weights of plants was measured, then aerial tissue extracted according to the scheme illustrated in figure 2.1 over. Volumes were adjusted to account for differing tissue mass.

Biochemical Tests

Proteins were assayed using the Coomassie Blue test (Sedmak and Grossberg, 1977). A 0.1ml aliqiot was mixed with 5ml of diluted protein reagent. (One hundred mg Coomassie Blue (G250) dissolved overnight with stirring in 50ml 95% aq(v/v) ethanol. To this was added 100ml 85% aq(v/v) phosphoric acid and the whole made up to $1dm^3$ with water. The reagent was diluted to 50% (v/v) with water immediately prior to use). The absorbance of the mixture at 595nm was measured after 15min at room temperature and compared to that of standard concentrations of bovine serum albumin (Sigma Fraction V, ranging from 100-1000μgml^{-1} by means of linear regression analysis.

Amino Acids were assayed using the ninhydrin assay (Yemm and Cocking, 1955)with hydrazine as reducing agent. A 1ml sample

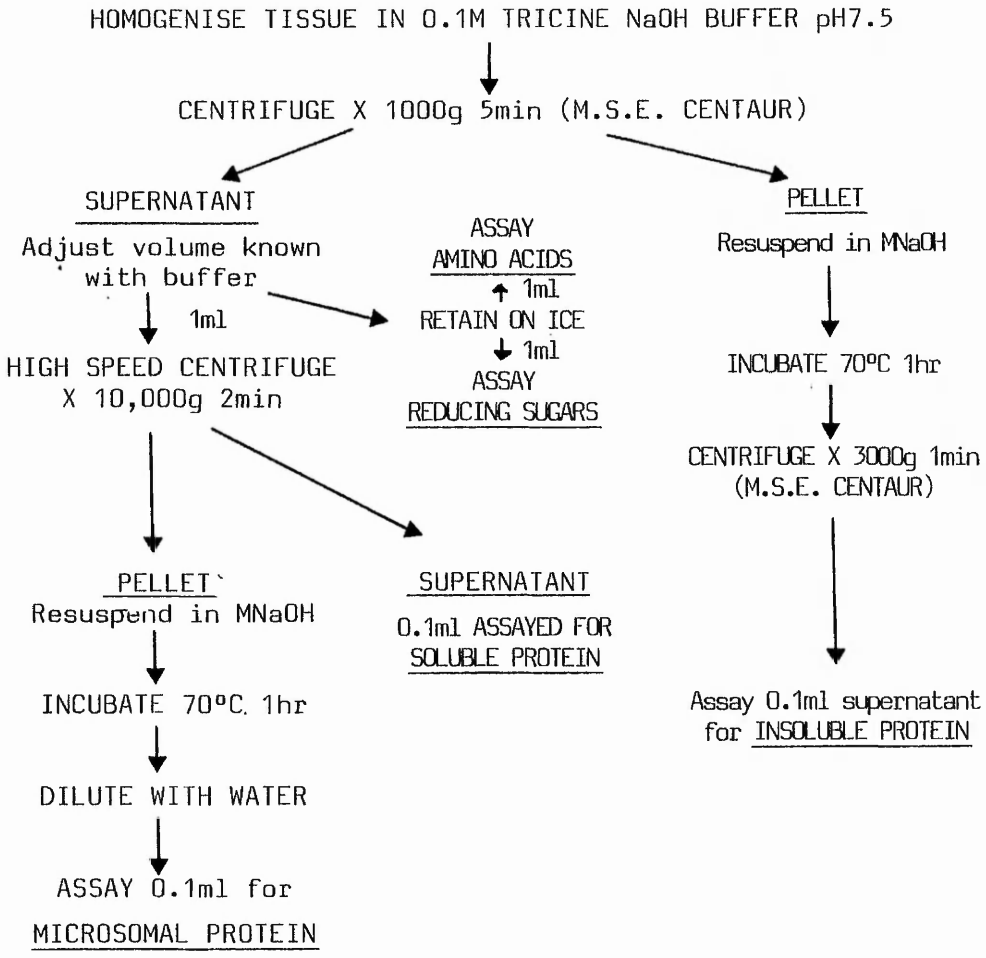


Figure 2.1. Tissue extraction procedure for assessment of key metabolites in whole plants. All preparations were held at 4°C whenever possible.

was mixed with 1.2ml of ninhydrin reagent (3.3g ninhydrin, 250ml methyl cellosolve, 50ml 4M acetate buffer (250g sodium acetate trihydrate dissolved in water to 400ml with heating. Fifty ml glacial acetic acid was added when cool and the volume made up to 500ml with water) 15ml glacial acetic acid - distilled water to 500mls), and 0.8ml hydrazine solution (0.13g hydrazine

sulphate in 500ml of water; add 1 drop concentrated sulphuric acid) added. The mixture was incubated at 100°C for 15mins and 3mls 50% aq. (v/v) ethanol added after cooling. The absorbance at 570nm was measured after 10mins at room temperature and compared to that of prepared glycine standards in the range 10-100µg ml^{-1}.

Reducing Sugars were assayed using Nelson's test (Nelson, 1944). To 1ml of sample 1ml Nelsons alkaline reagent was added. This was prepared by mixing 12.5ml of solution A (12.5g anhydrous sodium carbonate in 350ml distilled water, plus 12.5g potassium tartrate, 10g sodium bicarbonate and 100g anhydrous sodium sulphate, make up to 500mls with water) and 0.5ml of solution B (7.5g copper sulphate ($CuSO_4:5H_2O$) in 500mls water plus one drop concentrated sulphuric acid.) The solutions were mixed and incubated at 100°C for 20mins. After rapid cooling 1ml arsenomolybdate reagent (BDH Chemicals) was added. Seven ml distilled water was added after 5min and the absorbance of the solution measured at 540nm and compared to the absorbance of glucose standards in the range 10-200µgml^{-1}. All photometric measurements were made using a grating spectrophotometer (Cecil Instruments model CE 303). Results were calculated on µg/g fresh weight and µg/plant basis and recorded as mean values plus or minus standard errors. Selected means were compared using Student's t-test and comparisons between plant species were made using values expressed as a percent of untreated controls.

RNA Content of Leaf and Apical Tissues was assayed in 4 replicate plants of M.inodora and B.vulgaris 1, 3, 7 and 14 d.a.t. with field rate clopyralid (100gha^{-1}). The 3rd leaf of each plant and a 1cm apical portion were dissected and RNA extracted using 5% aq. (v/v) TCA (trichloroacetic acid; Parish, 1972) by the method illustrated in figure 2.2 over. RNA content was estimated using orcinol reagent to detect ribose moities on purine ribosides. To 1ml of sample 2ml orcinol reagent (100mg $FeCl_3.6H_2O$ dissolved in 100mls concentrated hydrochloric acid plus 3.5ml orcinol dissolved in ethanol (6% w/v)) was added and the solution incubated at 90°C for 30mins. After rapid cooling (under running water) absorbance was measured using a grating

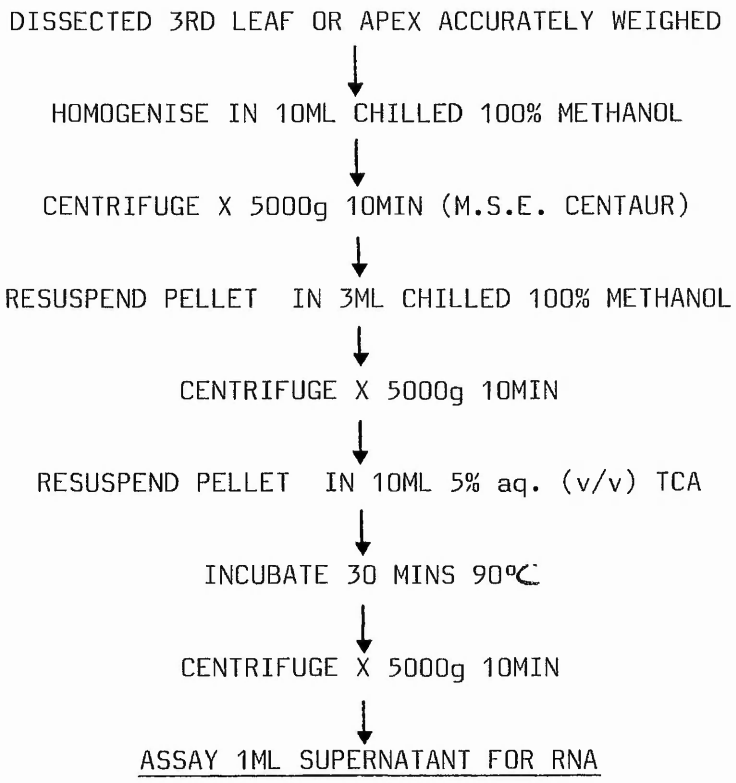


Figure 2.2 RNA extraction method

spectrophotometer (Cecil Instruments, Model CE 303). Adenosine monophosphate (AMP) was used as standard and results were calculated as purine riboside equivalents. Data is presented as mean values plus or minus standard error with data calculated on both a $\mu g g^{-1}$ fresh weight and μg per leaf or apex basis. For comparisons between species values were expressed as % untreated controls.

2.3 Results

2.3.1 Assessment of Susceptibility

M.inodora

M.inodora developed leaf epinasty within four to five hours following clopyralid application. Plate 2.1 shows clopyralid effects on the species 3 d.a.t. with 6.25, 100 and 400g $ai.ha^{-1}$. At the lowest dose clopyralid caused leaf epinasty particularly on the youngest and emerging tissues. This response was

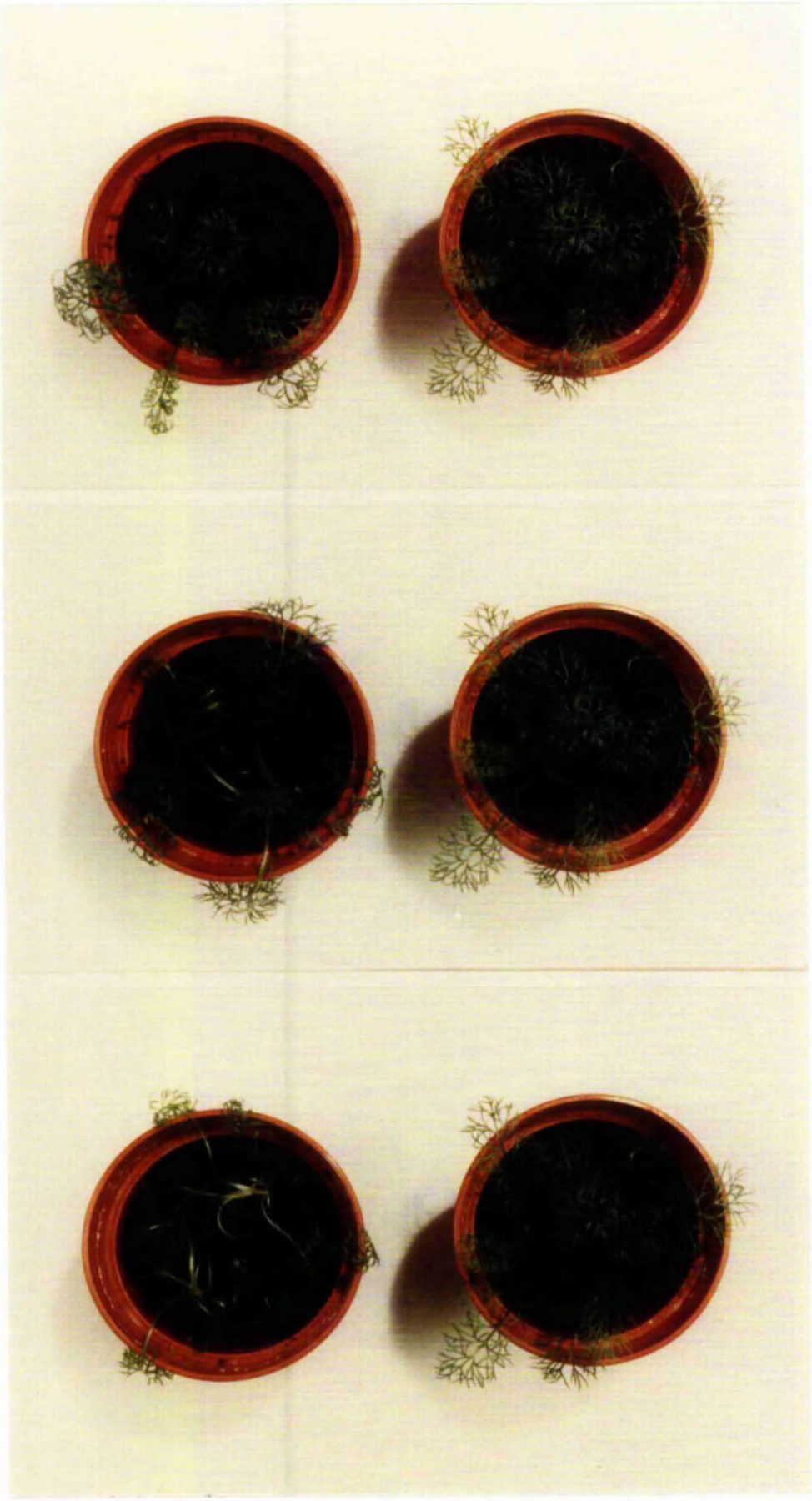
Plate 2.1 Effect of clopyralid on M.inodora 3 days after treatment with:

(A) 6.25g a.i. ha^{-1}

(B) 100g a.i. ha^{-1}, and

(C) 400g a.i. ha^{-1}

Control plants are on the right side of the plate.



accompanied by petiole curvature at 100g ai.ha^{-1} and at 400g ai.ha^{-1}, some thickening of the petioles was also noted. Plates 2.2 and 2.3 show the effects of 6.25 and 100g ai.ha^{-1} after 14 days. At the lower dose leaf curl persisted on older leaves and new growth was distorted. At 100g ai.ha^{-1} symptoms were severe; petiole curvature being most advanced and stem thickening pronounced. Development of new growth was arrested together with lamina expansion. In some plants the apex became elongated and swollen, and proliferations of tissue at the base of stems could be found. Adventitious roots were also observed just above the soil level and stem splitting usually preceeded necrosis. At 100 and 400g ai.ha^{-1} plants were killed 20-28 d.a.t. Applications of 25 and 6.25g ai.ha^{-1} did not lead to plant death within the four week experimental period.

Initial epinastic responses in M.inodora appeared to be accompanied by a stimulation in petiole elongation and so measurement of third leaf petiole length 3, 7 and 14 d.a.t. with 4 herbicide doses was conducted. The results are presented in table 2.1. Petiole lengths of plants treated with 6.25gai.ha^{-1} were greater than controls at each sample time, however the increases were not statistically significant. Reductions in 3rd leaf petiole length were measured after 3 and 14 days in plants treated with 400g ai.ha^{-1} clopyralid. This was statistically significant at 14 d.a.t. ($P \leq 0.01$).

Table 2.1 Effect of clopyralid on the length of the 3rd petiole of M.inodora (mm $\pm$ S.E.)

CLOPYRALID DOSE (g ai. ha^{-1})	DAYS AFTER TREATMENT		
	3	7	14
0	58.44 $\pm$ 6.09	52.94 $\pm$ 2.82	57.94 $\pm$ 3.39
6.25	62.50 $\pm$ 3.82	57.13 $\pm$ 1.51	64.94 $\pm$ 3.45
25	49.19 $\pm$ 1.86	46.50 $\pm$ 2.50	59.56 $\pm$ 10.38
100	63.82 $\pm$ 2.80	56.11 $\pm$ 3.87	56.06 $\pm$ 5.26
400	46.81 $\pm$ 2.32	50.10 $\pm$ 3.22	36.06 $\pm$ 3.99

Plate 2.2 Effect of clopyralid (6.25g ai.ha^{-1}) on M.inodora 14 days after treatment.

Plate 2.3 Effect of clopyralid (100g ai.ha^{-1}) on M.inodora 14 days after treatment.

B.vulgaris

Plate 2.4 shows the epinastic response of B.vulgaris plants 7 d.a.t. with 400g ai.ha^{-1} clopyralid, a large increase in axil angle (with petioles tending towards a horizontal position) being the major herbicide effect. Fourteen d.a.t. (plate 2.5) some recovery from this symptom was noted but leaf curl had developed and increased pigmentation of young tissues (due to enhanced anthocyanin production) was observed. Few herbicide-induced effects were recorded in plants treated with 6.25 and 25g ai.ha^{-1} and those treated with 100g ai.ha^{-1} showed similar but less severe symptoms to the ones discussed above. All B.vulgaris plants recovered and were indistinguishable from controls 40-60 d.a.t.

G.aparine

Plate 2.6 illustrates the response of G.aparine to 100g ai.ha^{-1} clopyralid 1 d.a.t., marked epinasty on youngest and emerging leaf whorls being evident. By 7 d.a.t. a clear dose response was noted in this species (plate 2.7). Treated plants appeared stunted compared to controls at all doses, the severity depending on rate. Epinasty progressed from the youngest tissues to the cotyledons in all but the lowest dose. Development of new growth was arrested and youngest tissues were severely distorted. Treated plants appeared to have more lateral growth than controls, with the suppression of apical dominance being a possible symptom of herbicide action. Table 2.2 indicates that mean internode lengths of G.aparine plants 14 d.a.t. were significantly reduced with respect to controls at each herbicde dose (with the exception of 25g ai.ha^{-1} where reduction was not statistically significant) and that the extent of this inhibition was clearly dose-dependent. Plate 2.8 shows G.aparine 14 d.a.t. with 100g ai.ha^{-1} clopyralid. Epinasty of stem tissue was apparent, new growth had severely reduced lamina area, and excess production of lateral shoots, particularly around the stem base could be clearly seen. Only the highest clopyralid dose (400g ai.ha^{-1}) killed G.aparine plants within the 28 days time course of this experiment. Necrosis, followed by plant death, appeared approximately 10 days later at 100g ai.ha^{-1}. At the lower 2 doses G.aparine plants survived clopyralid treatment although growth patterns were severely distorted.

Plate 2.4 Effect of clopyralid (400g ai.ha^{-1}) on B.vulgaris 7 days after treatment.

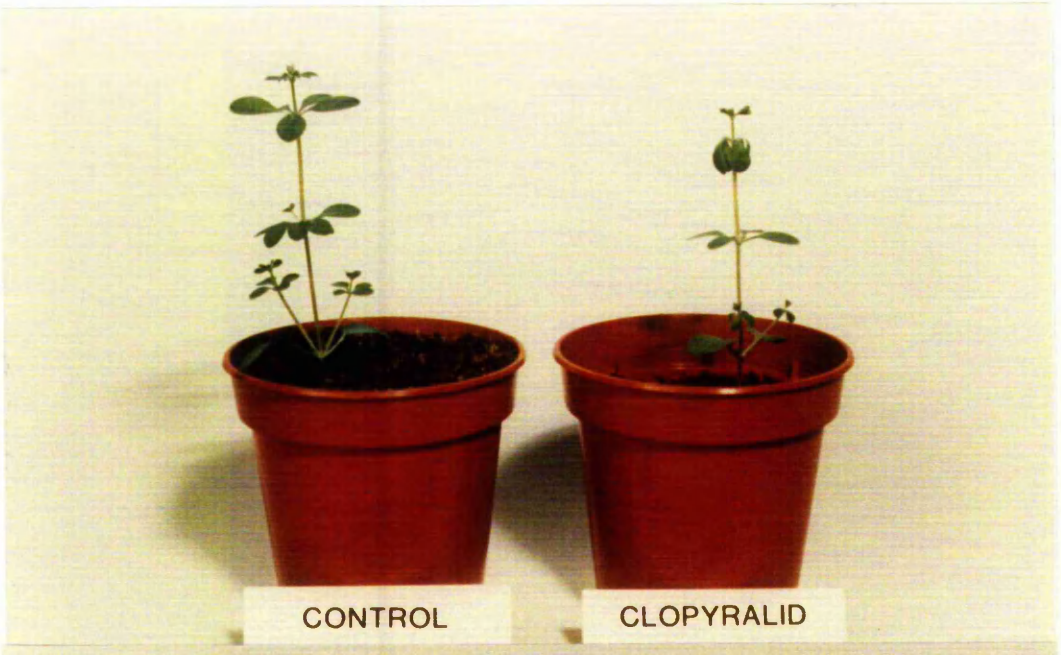
Plate 2.5 Effect of clopyralid (400g ai.ha^{-1}) on B.vulgaris 14 days after treatment.


4

Plate 2.6 Effect of clopyralid (100g ai.ha^{-1}) on G.aparine 1 day after treatment.

Plate 2.7 Effect of clopyralid on G.aparine 7 days after treatment - dose response, left to right; control, 6.25, 25, 100 and 400g ai.ha^{-1}.

Plate 2.8 Effect of clopyralid (100g ai.ha^{-1}) on G.aparine 14 days after treatment.


CONTROL
CLOPYRALID


0
1/4
1/2
1
4

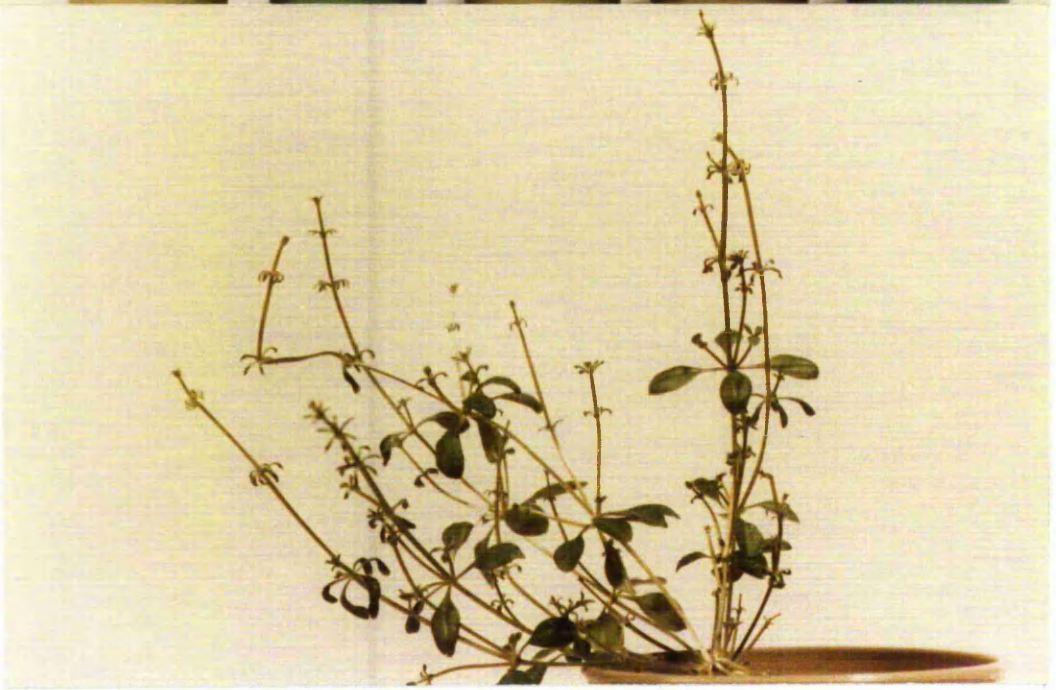


Table 2.2 Mean internode lengths of G.aparine plants 14 days after treatment with clopyralid (mm ± S.E.) n=4

CLOPYRALID DOSE ($g\ a.i.ha^{-1}$)	$\bar{X}$ INTERNODE LENGTH ± S.E.	PROBABILITY RATIO
0	93.28 ± 8.40	___
6.25	73.63 ± 0.43	$P<0.05$
25	78.29 ± 1.36	NS
100	57.48 ± 1.14	$P<0.01$
400	37.24 ± 0.82	$P<0.001$

2.3.2 Effect of clopyralid on plant dry weight

Figure 2.3 shows the effect of 6.25 and 400g $a.i.ha^{-1}$ clopyralid on the dry weight of M.inodora plants. The two doses produced significantly different responses after 14, 21 and 28 days ($P<0.05$, $P<0.05$, $P<0.05$), this being only due to the dry weight reductions induced by 400g $a.i.ha^{-1}$ after these periods. Dry weights were significantly reduced from controls by 400g $a.i.ha^{-1}$ clopyralid after 14 and 21 days ($P<0.01$, $P<0.02$). Treatment with 6.25g $a.i.ha^{-1}$ gave no significant deviation from control values.

Figure 2.4 illustrates that 6.25 and 400g $a.i.ha^{-1}$ gave similar dry weight responses in G.aparine, with significant reductions from controls at 6.25g $a.i.ha^{-1}$ after 21 and 28 days ($P<0.05$, $P<0.05$) and after the same intervals at 400g $a.i.ha^{-1}$ ($P<0.01$, $P<0.01$).

Sugar beet treatment with clopyralid at 6.25g $a.i.ha^{-1}$ and 400g $a.i.ha^{-1}$ gave no clear dose response with respect to dry weight (figure 2.5). Although there was a trend towards initial dry weight reductions, followed by recovery at 6.25g $a.i.ha^{-1}$, the only data points significantly different from controls were the high dose dry weight reductions after 7 ($P<0.01$) and 28 ($P<0.05$) days. By 60 d.a.t. dry weights of treated plants were not significantly different from controls (Table 2.3).

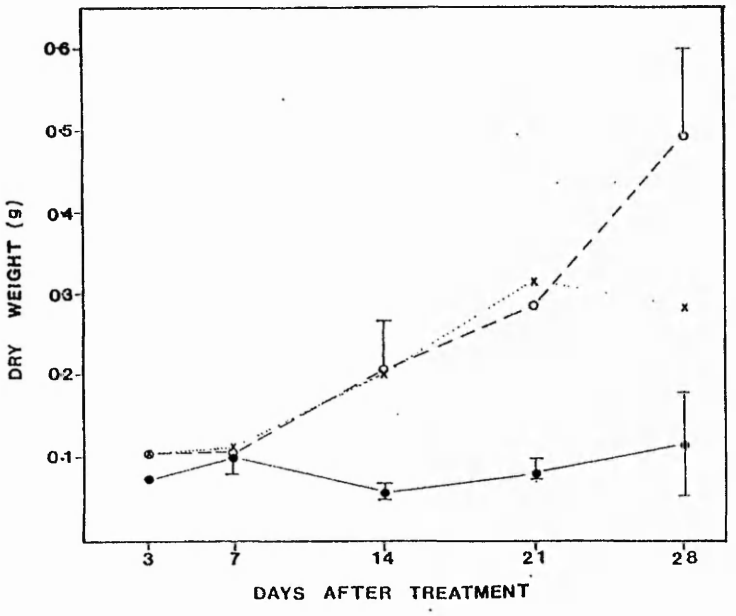


Figure 2.3 Effect of clopyralid at 6.25g a.i.ha^{-1} (--o--) and 400g a.i.ha^{-1} (—●—) on M.inodora dry weight compared to untreated controls (··x··). Results are the mean values of 4 replicates and bars represent standard errors.

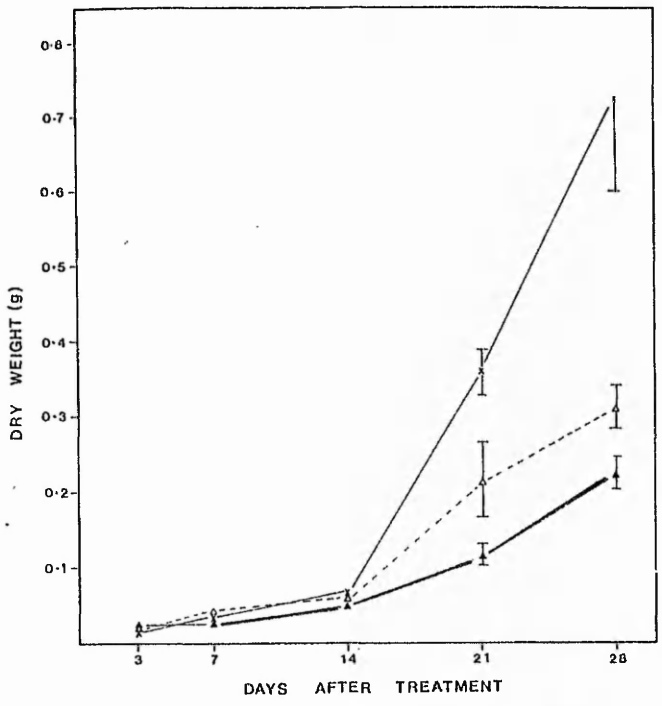


Figure 2.4 Effect of clopyralid at 6.25g a.i.ha^{-1} (--Δ--) and 400g a.i.ha^{-1} (—▲—) on G.aparine dry weight compared to untreated controls (—x—). Results are the mean values of 4 replicates and bars represent standard errors.

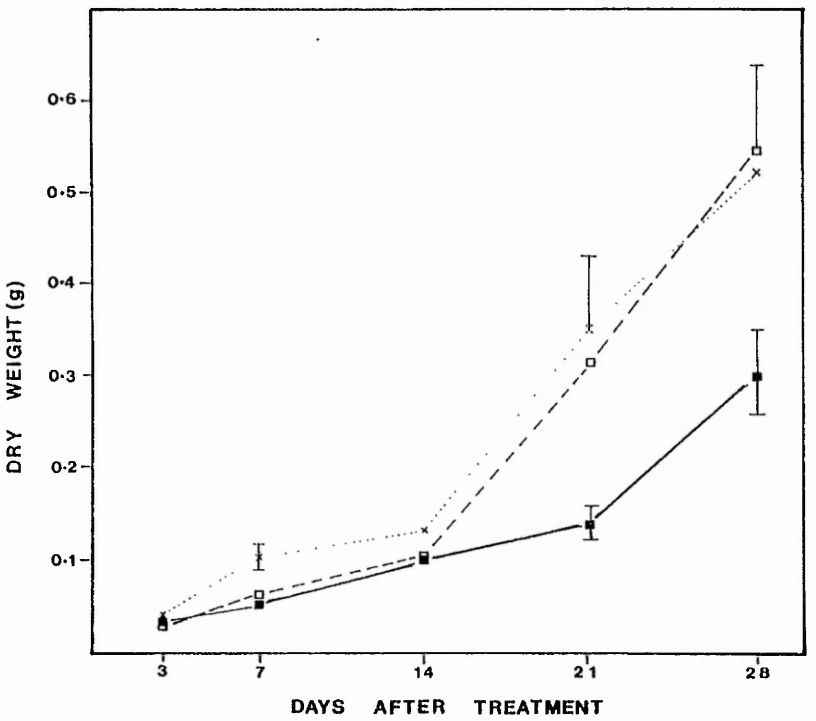


Figure 2.5 Effect of clopyralid at 6.25g a.i.ha^{-1} (--□--) and 400g a.i.ha^{-1} (——■——) on B.vulgaris dry weight compared to untreated controls (···×···). Results are mean values of 4 replicates and bars represent standard errors.

Table 2.3 Effect of clopyralid on B.vulgaris dry weight 60 days after treatment.

CLOPYRALID DOSE g a.i.ha^{-1}	$\bar{X}$ DRY WEIGHT (g) $\pm$ S.E.
0	0.8732 $\pm$ 0.1440
6.25	0.8870 $\pm$ 0.1690
25	0.8507 $\pm$ 0.1320
100	0.8924 $\pm$ 0.1750
400	0.9219 $\pm$ 0.3570

The effect of field rate clopyralid (100g a.i.ha^{-1}) on plant dry weight in each of the test species is compared in figure 2.6 where values are expressed on a % of control basis. M.inodora showed a progressive reduction in dry weight over the 28 day period with statistically significant reduction (as calculated from original data) after 21 days ($P< 0.02$). In B.vulgaris initial reductions in dry weight with respect to controls were noted 3 and 7 days after clopyralid application, this was statistically significant 3 d.a.t. ($P<0.05$), however values did not differ significantly from controls after 14, 21 or 28 days. In G.aparine dry weight was significantly reduced compared to controls after 21 and 28 days ($P<0.01$, $P<0.05$).

2.3.3 Fresh weight

Figure 2.7 shows dry and fresh weights of all 3 test species following treatment with 100g a.i.ha^{-1}clopyralid expressed on the basis of % of control. Fresh weight values generally paralleled the dry weight data. Although this was the case at all dose rates used, fresh weight values were generally more variable than dry weight measurements and large fluctuations were commonly observed.

2.3.4 Effect of clopyralid on metabolite content

Total Protein

M.inodora

Figure 2.8 shows the effect of 6.25 and 400g a.i.ha^{-1}clopyralid upon total protein content per plant, and plant fresh weight in M.inodora. The herbicide had no significant effects on total protein, with levels paralleling fresh weight changes throughout the time-course at both dose rates.

B.vulgaris

In B.vulgaris there was large variability in total protein measurements, however, stimulations in protein content with respect to controls were identified 1 d.a.t. These were highlighted when results were expressed as µg protein per g fresh weight prior to comparison with controls (figure 2.9).

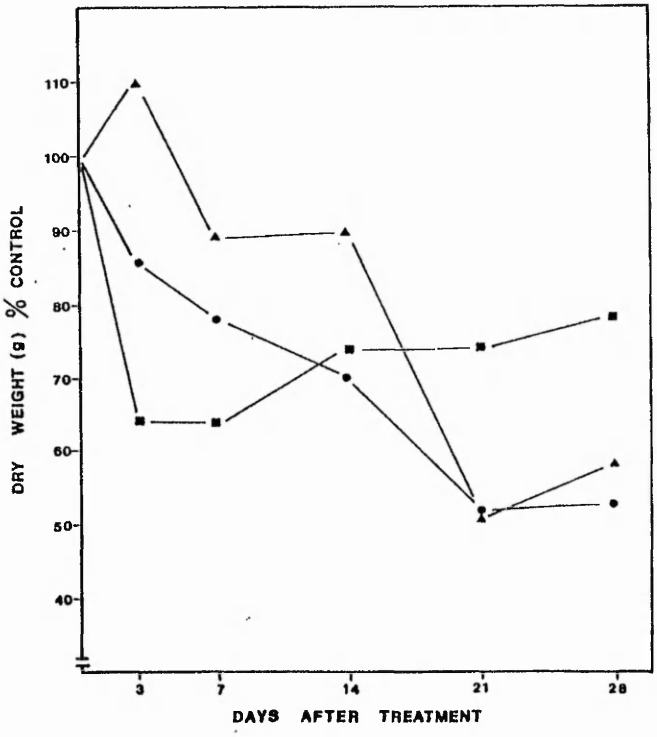


Figure 2.6 Effect of 100g a.i.ha^{-1} clopyralid on plant dry weight in M.inodora (—●—), B.vulgaris (—■—) and G.aparine (—▲—). Values are means of 4 replicates.

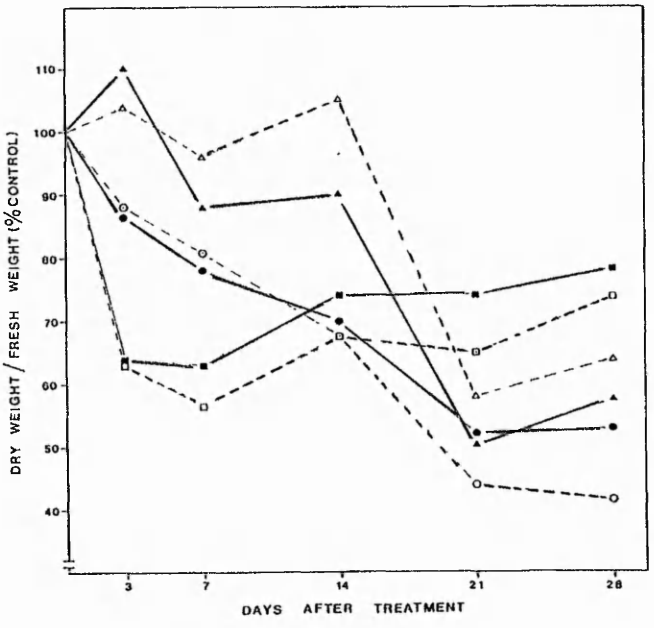


Figure 2.7 Effect of clopyralid (100g a.i.ha^{-1}) on fresh weight (broken lines) and dry weight (solid lines) in M.inodora (●/○), B.vulgaris (■/□) and G.aparine (▲/△). Values are the mean of 4 replicates.

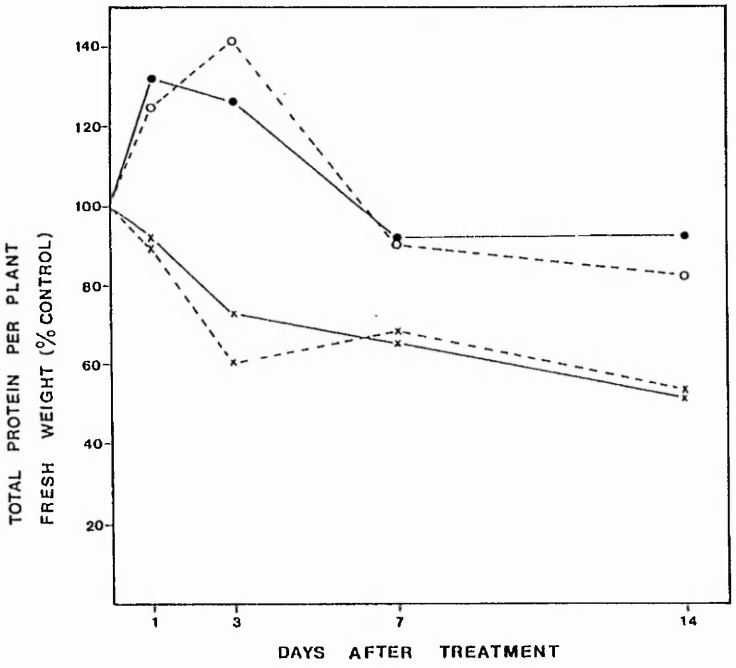


Figure 2.8 Effect of clopyralid on M.inodora total protein content (solid lines) and plant fresh weight (broken lines). Dose rates; 6.25g a.i.ha^{-1} (●/o) and 400g a.i.ha^{-1} (X).

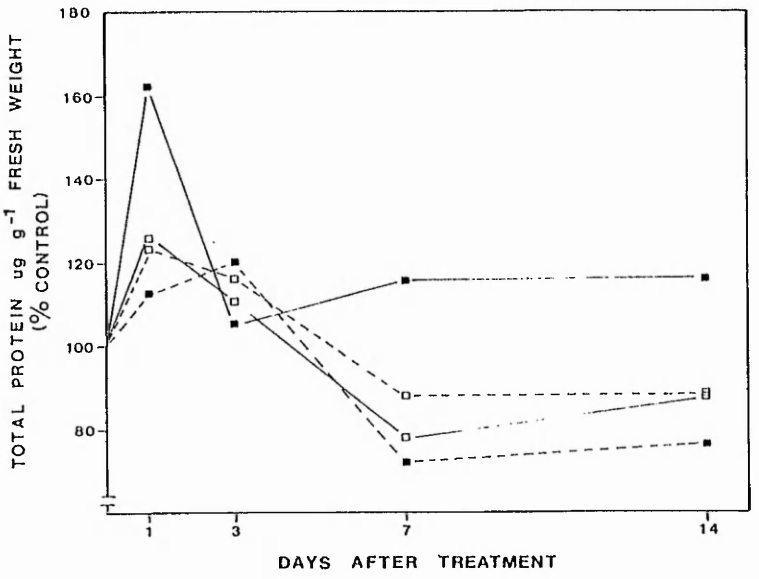


Figure 2.9 Effect of clopyralid on total protein in B.vulgaris. Dose rates; 400g a.i.ha^{-1}(—■—), 100g a.i.ha^{-1}(--■--), 25g a.i.ha^{-1} (—□—) and 6.25g a.i.ha^{-1} (--□--).

This transient increase appeared at all clopyralid doses and was statistically significant at 400g a.i.ha^{-1} ($P < 0.05$) where the concentration of protein was 26.25 $\pm$ 3.07 mg/g fresh weight compared to the control value of 16.20 $\pm$ 1.98 mg/g fresh weight. The response was not sustained and protein content was not significantly different from controls from 3 days onwards.

G.aparine

In G.aparine there were no significant changes in total protein content following clopyralid application. Protein content generally paralleled fresh weight changes over the experimental period as in figure 2.10 which illustrates results at 6.25 and 400g a.i.ha^{-1}, with results expressed as % of control.

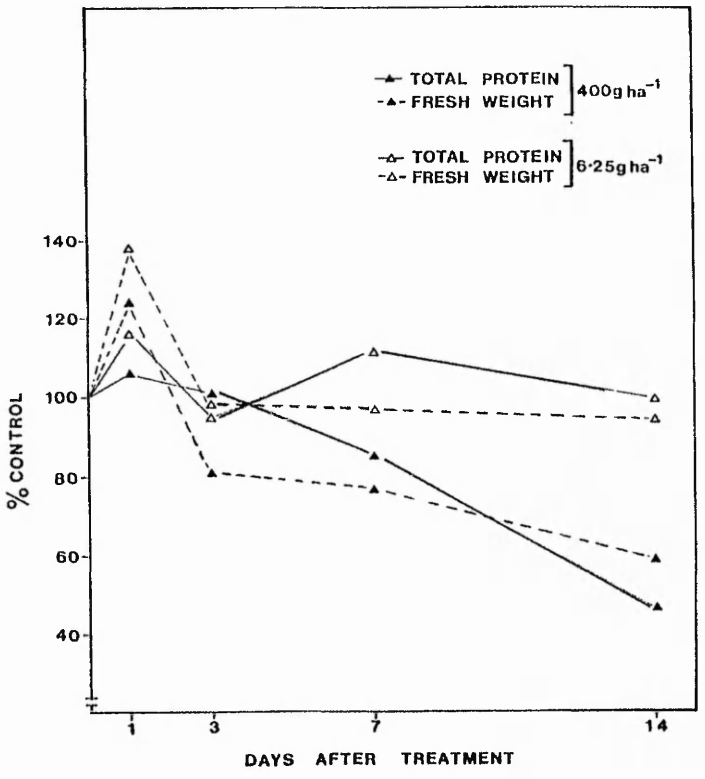


Figure 2.10 Effect of clopyralid; 400g a.i.ha^{-1} (▲) and 6.25g a.i.ha^{-1} (Δ) on G.aparine fresh weight (broken lines) and total protein (solid lines).

Protein fractions

The effects of clopyralid on protein content in each of the 3 test species 1 and 14 d.a.t. with 6.25 and 400g a.i.ha^{-1} are presented in tables 2.4A and 2.4B. The results are also plotted as % of control values in figures 2.11A, B and C.

In M.inodora, whilst there were no effects on total protein content after 1 day (fig 2.8), there were increases in the microsomal protein fraction, compared to controls at both rates shown. The increases at 400g a.i.ha^{-1} were statistically significant ($P<0.02$). At 6.25g a.i.ha^{-1} this increase was sustained up to 14 d.a.t. (not significant) and, accompanied at that time by non-significant increases in the other 2 fractions. At 400g a.i.ha^{-1} the slight reduction in total protein measured 14 d.a.t. was accounted for by reductions in soluble and microsomal protein fractions, with higher insoluble protein values with respect to controls.

The treatment of G.aparine with 6.25 and 400g a.i.ha^{-1} clopyralid resulted in a decrease in all protein fractions 1 d.a.t.. The reduction was found to be statistically significant for soluble proteins at 400g a.i.ha^{-1} ($P<0.01$). At the lower dose protein concentration returned to control values after 14 days. At 400g a.i.ha^{-1} however, the decrease was largely sustained although concentrations of insoluble protein were significantly greater than controls after 14 days ($P<0.05$).

The response of B.vulgaris to 6.25g a.i.ha^{-1} clopyralid involved a transient increase in the microsomal protein fraction, the concentration of which approached the control 14 d.a.t.. At 400g a.i.ha^{-1} the significant increase in total protein ($P<0.05$) 1 d.a.t. consisted of an increase in the microsomal protein fraction and a significant rise in the soluble protein concentration ($P< 0.02$); again concentrations approached control values after 14 days.

Table 2.4A Protein concentration (mg g^{-1} fresh weight) 1 d.a.t. with 6.25 and 400g a.i. ha^{-1} clopyralid.

SPECIES/DOSE	PROTEIN FRACTIONS			
	Total Protein	Microsomal	Soluble	Insoluble
M.inodora				
CONTROL	18.48 ± 4.72	2.63 ± 0.89	11.42 ± 3.60	4.41 ± 0.90
6.25g a.i. ha^{-1}	19.68 ± 2.07	3.69 ± 0.56	10.84 ± 1.16	5.12 ± 0.53
400g a.i. ha^{-1}	20.22 ± 4.32	3.77 ± 0.36	12.62 ± 2.44	4.15 ± 0.67
G.aparine				
Control	19.71 ± 0.84	5.55 ± 0.63	10.43 ± 0.95	3.71 ± 0.18
6.25g a.i. ha^{-1}	17.35 ± 0.86	4.65 ± 0.17	9.15 ± 1.18	3.64 ± 0.28
400g a.i. ha^{-1}	16.80 ± 1.88	5.02 ± 0.81	6.53 ± 0.51	3.60 ± 0.22
B.vulgaris				
CONTROL	16.20 ± 1.98	2.54 ± 0.68	11.43 ± 0.82	2.23 ± 0.48
6.25g a.i. ha^{-1}	20.26 ± 2.61	3.84 ± 0.42	14.06 ± 1.21	2.34 ± 0.96
400g a.i. ha^{-1}	26.25 ± 3.07	4.58 ± 0.91	19.62 ± 2.29	2.05 ± 0.32

Table 2.4B Protein concentration (mg g^{-1} fresh weight) 14 d.a.t with 6.25 and 400g a.i.ha^{-1} clopyralid.

	PROTEIN FRACTIONS			
SPECIES/DOSE	Total protein	Microsomal	Soluble	Insoluble
M.inodora				
CONTROL	22.18 ± 4.41	4.19 ± 1.39	12.57 ± 2.05	5.41 ± 1.02
6.25g a.i.ha^{-1}	24.86 ± 1.73	5.33 ± 1.00	13.04 ± 1.48	6.48 ± 0.66
400g a.i.ha^{-1}	21.36 ± 1.08	3.83 ± 6.06	10.23 ± 0.81	7.28 ± 0.31
G.aparine				
CONTROL	6.25 ± 1.10	1.18 ± 0.40	3.89 ± 0.55	1.25 ± 0.12
6.25g a.i.ha^{-1}	6.59 ± 1.13	1.20 ± 0.49	3.92 ± 0.55	1.47 ± 0.18
400g a.i.ha^{-1}	5.06 ± 0.51	1.10 ± 0.38	2.23 ± 0.61	1.72 ± 0.13
B.vulgaris				
CONTROL	6.62 ± 0.53	1.06 ± 0.18	4.34 ± 0.13	1.26 ± 0.08
6.25g a.i.ha^{-1}	5.83 ± 0.42	0.98 ± 0.10	3.38 ± 0.42	1.48 ± 0.18
400g a.i.ha^{-1}	7.68 ± 0.98	1.17 ± 0.08	4.96 ± 0.58	1.58 ± 0.18

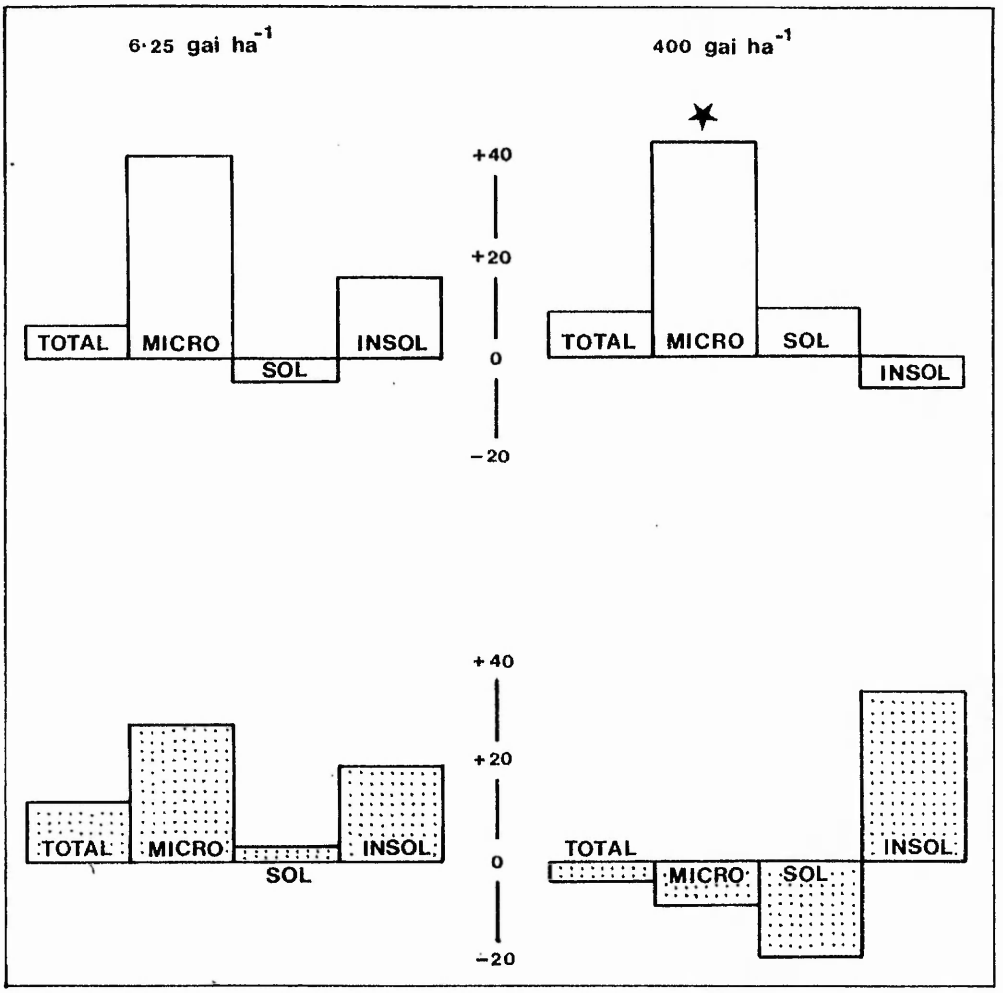


Figure 2.11A Percentage change (compared to controls) in protein content in M.inodora 1 (▭) and 14 (▭) days following clopyralid application. ★ denotes statistically significant difference.

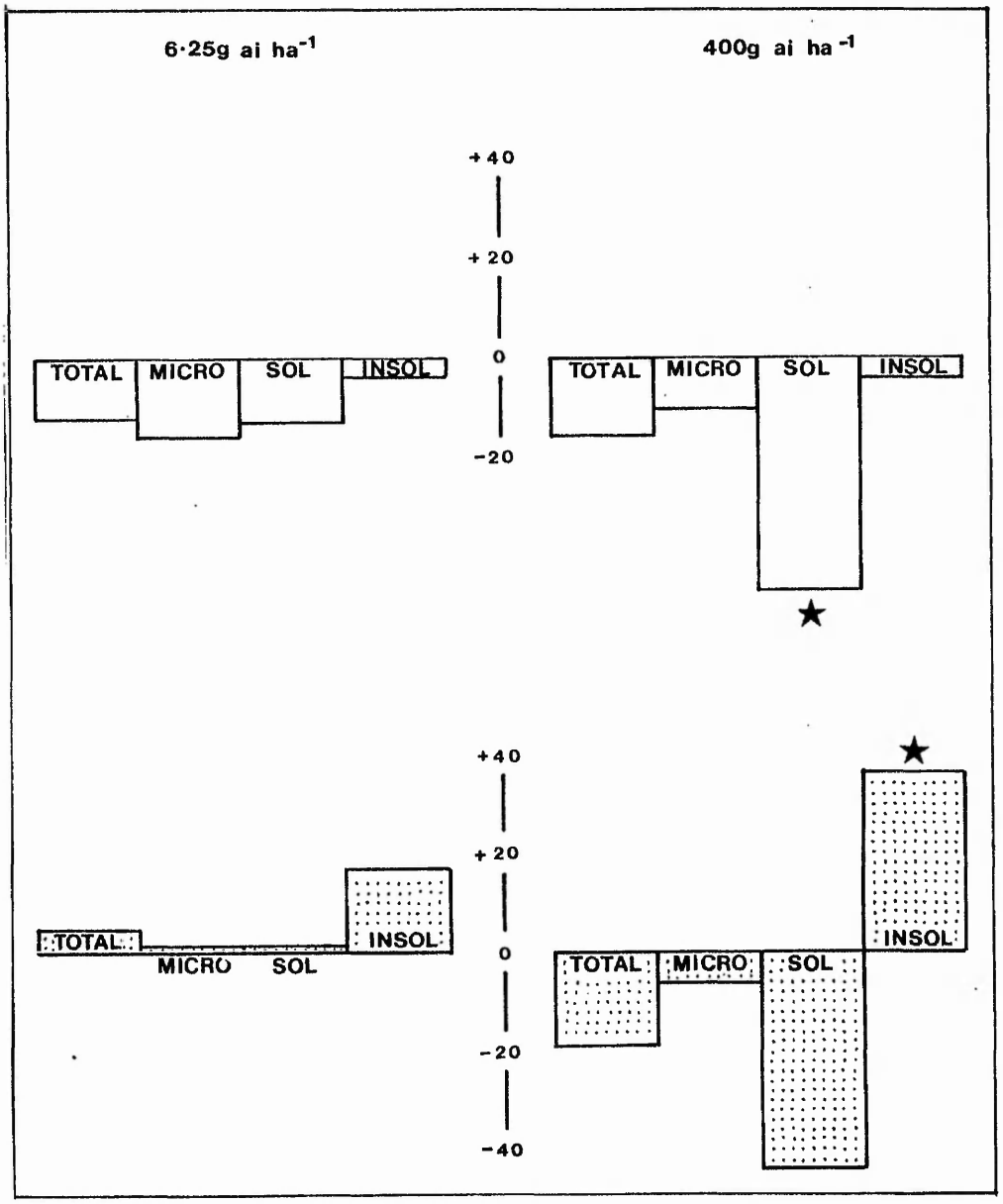


Figure 2.11B Percentage change (compared to controls) in protein content in G.aparine 1 (▭) and 14 (▦) days following clopyralid application. ★ denotes statistically significant difference.

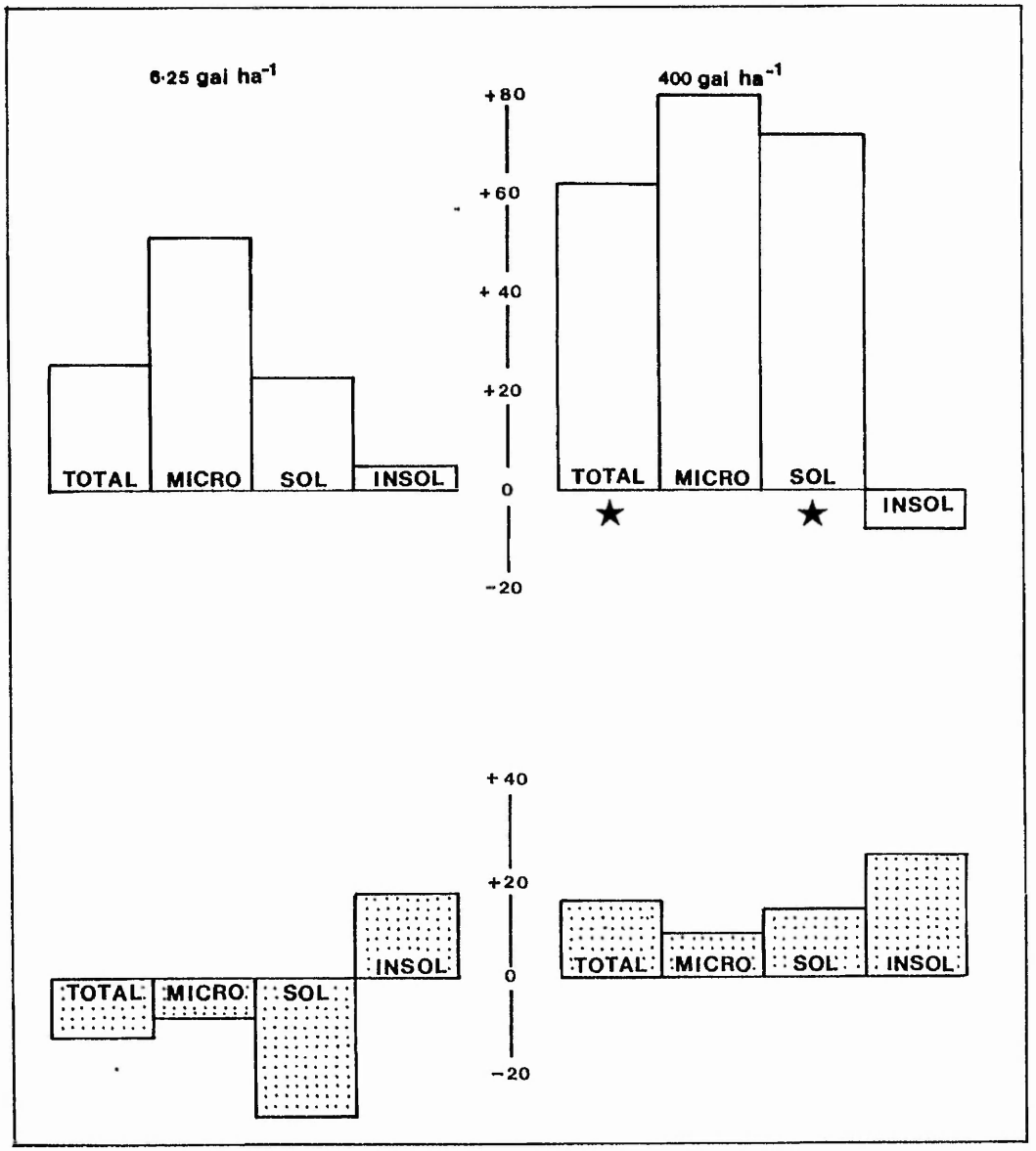


Figure 2.11C Percentage change (compared to controls) in protein content in B.vulgaris 1 (▭) and 14 (⁝⁝) days following clopyralid application. ★ denotes statistically significant difference.

Table 2.5 records the mean amounts of protein fractions extracted from control plants of the 3 test species 1 and 14 days into the experimental period. The relative proportions of total protein accounted for by each fraction is recorded. In each species, at both time points, soluble (cytoplasmic) proteins accounted for most of the total measured, with values ranging from 0.53 to 0.78 of the total amount. The remainder of the total was divided evenly between microsomal and insoluble fractions. Proportions were comparable between species.

Table 2.5 Relative proportions of protein fractions in control plants 1 and 14 days into the experimental period. Values in parenthesis represent relative proportions of total protein.

PROTEIN CONTENT (ug/plant)
(RELATIVE PROPORTIONS OF TOTAL PROTEIN)

DAYS	MICROSOMAL	SOLUBLE	INSOLUBLE	TOTAL
M.inodora				
1	354	1535	593	2482
	(0.14)	(0.62)	(0.24)	
14	1036	3110	1338	5484
	(0.18)	(0.58)	(0.24)	
B.vulgaris				
1	1220	5486	1072	7778
	(0.17)	(0.78)	(0.15)	
14	2687	11281	3274	17242
	(0.15)	(0.65)	(0.19)	
G.aparine				
1	1312	2467	879	3787
	(0.28)	(0.53)	(0.18)	
14	1929	6234	2040	10203
	(0.19)	(0.61)	(0.20)	

Amino Acids

The effects of clopyralid on amino acid content are compared with effects on microsomal proteins in M.inodora and B.vulgaris plants treated with 400g a.i.ha^{-1} in figures 2.12 and 2.14. In M.inodora, whilst microsomal proteins were significantly increased ($P<0.05$) 3 d.a.t., levels of amino acids deviated little from controls. As may be seen in figure 2.13, changes in amino acid content compared to control, closely parallel fresh weight changes following application of 400g a.i.ha^{-1} and 6.25g a.i.ha^{-1} clopyralid in a similar fashion to total proteins in this species (figure 2.8). In B.vulgaris amino acid levels followed changes in microsomal proteins throughout the period of the experiment. Apart from this finding, clopyralid generally had no significant effect on amino acid concentration regardless of plant species or herbicide dose.

Reducing Sugars

Large fluctuations in reducing sugar content over the 14 day experimental period were noted in all clopyralid-treated plants. No species specific or concentration dependent effects were demonstrated. Figures 2.15 and 2.16 illustrate these fluctuations in data obtained by analysis of plants treated with 100g a.i.ha^{-1} and 400g a.i.ha^{-1} clopyralid. Application of 400g a.i.ha^{-1} generally produced greater reducing sugar effects than 100g a.i.ha^{-1} in both tolerant and resistant species.

RNA Content

Tables 2.6, 2.7, 2.8 and 2.9 record the effects of 100g a.i.ha^{-1} clopyralid upon leaf and apical RNA content in M.inodora and B.vulgaris.

In M.inodora, apical RNA content was greatly reduced at each time point when expressed on a µgRNA per g fresh weight basis (table 2.6). The reductions were statistically significant at 1 ($P<0.001$) and 14 ($P<0.01$) days. This observation was a consequence of apical swelling, since values approached control levels when examined on the basis of RNA per apical section. After 14

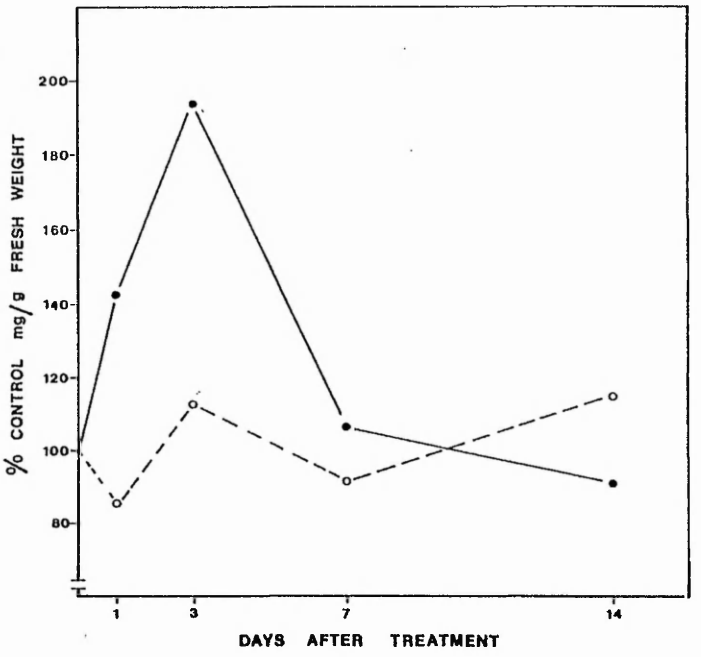


Figure 2.12 Effect of clopyralid (400g a.i.ha^{-1}) on amino acid content (-o-) and microsomal protein content (—●—) in M.inodora. Results expressed on a mg per g fresh weight basis.

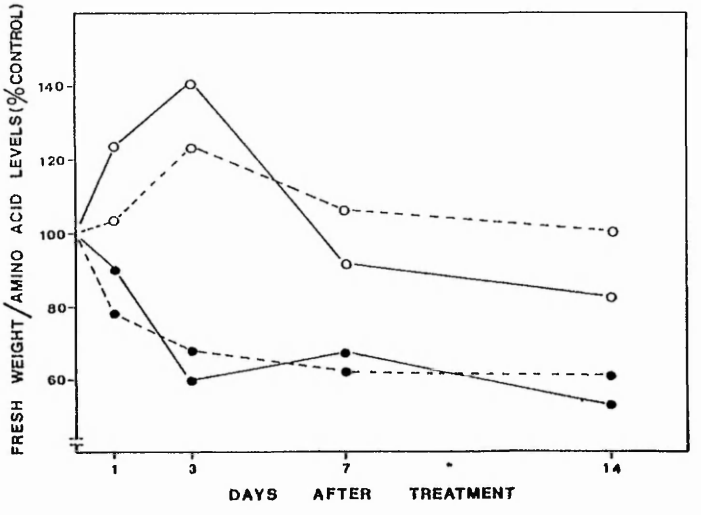


Figure 2.13 Effect of clopyralid at 400g a.i.ha^{-1} (●) and 6.25g a.i.ha^{-1} (o) on amino acid content (broken lines) and plant fresh weight (solid lines) in M.inodora.

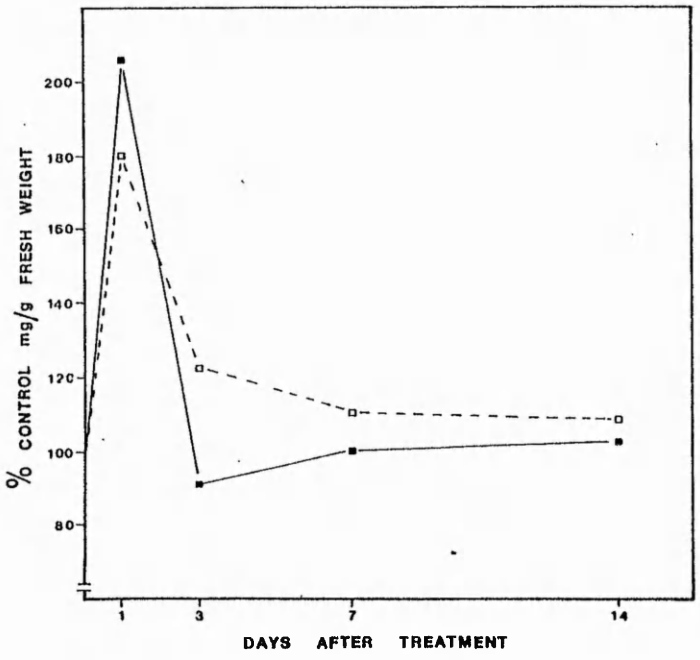


Figure 2.14 Effect of clopyralid (400g a.i.ha^{-1}) on amino acid (—■—) and microsomal protein (—□--) content in B.vulgaris.

days, clopyralid induced a significant increase ($P<0.05$) in RNA per apex in this species. No significant effects on 3rd leaf RNA were recorded (table 2.7).

In B.vulgaris, clopyralid significantly increased apical RNA 14d.a.t. ($P<0.05$), where RNA content calculated on a µg per g fresh weight basis, was 166% of control value. When converted to a value per apex there was no significant difference between control and treated plants, however expressing results on this basis revealed a significant reduction in RNA per apical section compared to control after 7 days ($P<0.05$). The only significant effect of clopyralid on B.vulgaris leaf RNA content was to reduce the concentration 1 d.a.t., this reduction being significant when results were expressed on a µg per g fresh weight basis.

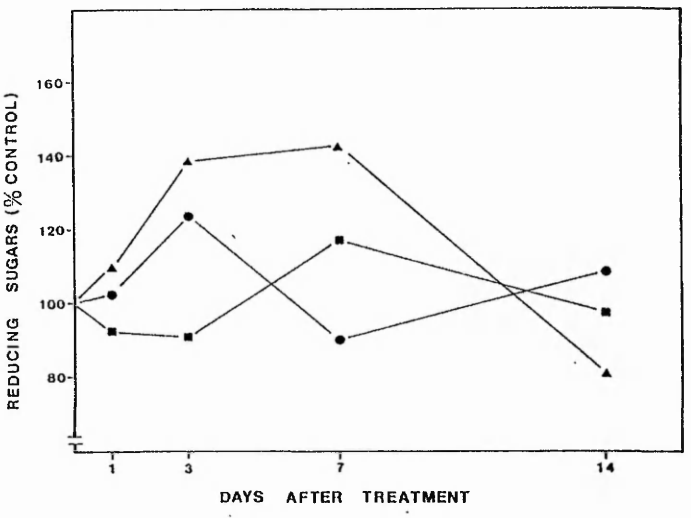


Figure 2.15 Effect of clopyralid (100g a.i.ha^{-1}) on reducing sugar content in M.inodora (—●—), B.vulgaris (—■—) and G.aparine (—▲—).

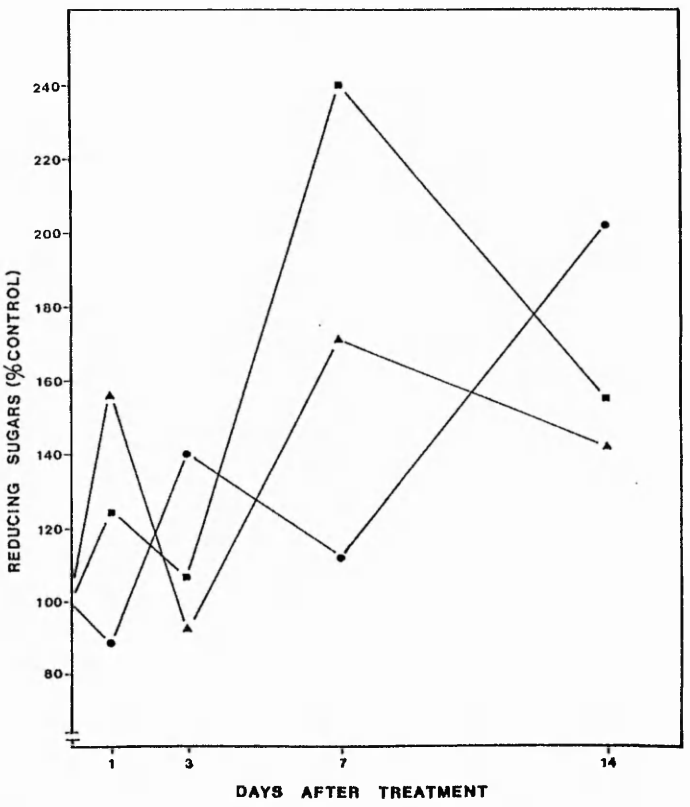


Figure 2.16 Effect of clopyralid (400g a.i.ha^{-1}) on reducing sugar content in M.inodora (—●—), B.vulgaris (—■—) and G.aparine (—▲—).

Table 2.6 Effect of clopyralid (100g a.i.ha^{-1}) on apical RNA content in M.inodora.

	µg g^{-1} fresh weight			µg/apex		
D.A.T	CONTROL	TREATED	% CONTROL	CONTROL	TREATED	% CONTROL
1	1645 ± 176	576 ± 19	35	252 ± 29	231 ± 40	91
3	3082 ± 1090	2393 ± 342	77	105 ± 31	107 ± 19	101
7	3625 ± 1134	1471 ± 690	40	218 ± 39	226 ± 35	103
14	14287 ± 1750	4291 ± 869	30	573 ± 12	717 ± 54	125

Table 2.7 Effect of clopyralid (100g a.i.ha^{-1}) on leaf RNA content in M.inodora .

	µg g^{-1} fresh weight			µg/leaf		
D.A.T	CONTROL	TREATED	% CONTROL	CONTROL	TREATED	% CONTROL
1	239 ± 19	256 ± 36	107	53 ± 13	28 ± 4	189
3	1354 ± 397	1458 ± 117	107	211 ± 48	241 ± 22	114
7	1525 ± 448	871 ± 143	57	205 ± 19	190 ± 18	92
14	3380 ± 448	3281 ± 362	97	586 ± 45	638 ± 80	108

Table 2.8 Effect of clopyralid (100g a.i.ha^{-1}) on apical RNA in B.vulgaris.

	$\mu g\ g^{-1}$ fresh weight			μg/apex		
D.A.T	CONTROL	TREATED	% CONTROL	CONTROL	TREATED	% CONTROL
1	556 ± 103	379 ± 47	149	40 ± 9	71 ± 12	178
3	2722 ± 445	2683 ± 381	98	281 ± 22	205 ± 18	73
7	1736 ± 282	1640 ± 146	94	64 ± 16	90 ± 2	140
14	2558 ± 317	4255 ± 608	166	525 ± 127	342 ± 20	65

Table 2.9 Effect of clopyralid (100g a.i.ha^{-1}) on leaf RNA in B.vulgaris.

	$\mu g\ g^{-1}$ fresh weight			μg/leaf		
D.A.T	CONTROL	TREATED	% CONTROL	CONTROL	TREATED	% CONTROL
1	351 ± 51	151 ± 26	48	33 ± 7	21 ± 2	64
3	810 ± 53	1034 ± 277	127	153 ± 3	176 ± 35	115
7	484 ± 96	314 ± 37	70	23 ± 5	20 ± 3	87
14	2241 ± 123	2650 ± 249	118	483 ± 25	543 ± 54	112

2.4 Discussion

Symptom development recorded in response to clopyralid in this study was quite typical of auxin-type herbicide action and was in accordance with that found in response to other pyridine compounds. Although symptoms appeared in all three test species, the degree of susceptibility of each could be appointed according to the severity and duration of symptoms and whether or not plants were killed within the experimental period. Thus M.inodora was highly susceptible to clopyralid, G.aparine mildly susceptible and B.vulgaris considered tolerant. These findings from glasshouse experiments correlate well with published field assessments (Brown and Uprichard, 1976; Mayer, Lush and Rose, 1976; Clay, 1983; Richardson and Parker, 1977; Sinclair and Cox, 1980; Lake, 1980)

The suppression of apical dominance by clopyralid found in G.aparine may have arisen indirectly via herbicide damage to the dominant apex. Alternatively, it could have been the result of increased 'auxin' concentration in tissues, this altering the complex balance of growth regulatory substances in such a way as to inhibit the suppressive effect of the dominant apex on the lateral outgrowths. Herbicide interference with apical dominance is very common but rarely observed without damage to the apex (Parker, 1976).

In M.inodora it was somewhat surprising that no significant stimulation of petiole elongation was measured since it commonly accompanies other auxinic responses which were recorded in this species. Sanders and Pallett (1987) found Stellaria media petiole length to be increased by fluroxypyr treatment 1 and 2 d.a.t. This would suggest that increases in petiole length may occur prior to 3 d.a.t when the present experiments commenced.

The examination of clopyralid effects on fresh weight and dry weight in the three test species provided quantification of relative susceptibility, with B.vulgaris showing recovery within the 28 days experimental period. In the 2 susceptible species dose responses were quantified to a certain extent by these measurements, however the small number of replicates in this

preliminary study coupled with the inherent variability of measurements prevented extensive meaningful comparison between effects of different application rates. Nevertheless, it appeared that G.aparine and M.inodora were being affected differently by clopyralid. In G.aparine, a simple dose response pattern emerged with the highest clopyralid rate (400g a.i.ha^{-1}) reducing dry weight more greatly than 6.25g a.i.ha^{-1}, but with no significant difference between the two effects (figure 2.4). However, in M.inodora there was a stimulation of plant dry weight by 6.25g a.i.ha^{-1} clopyralid, whilst the highest rate (400g a.i.ha^{-1}) produced consistant dry weight reductions over the experimental period. One interpretation of these findings could be that the herbicide is acting as a simple metabolic inhibitor in G.aparine with a more complex interaction in highly susceptible M.inodora. This would be supported by the findings that G.aparine responds to clopyralid application with a decrease in all protein fractions 1 d.a.t at 6.25 and 400g a.i.ha^{-1}. Epinastic responses and recorded stimulations in reducing sugars are however contrary to this highly simplistic interpretation since these indicate stimulatory activity.

Examination of clopyralid effects on RNA, amino acid and protein concentrations in M.inodora and B.vulgaris revealed important effects in both species, however, no distinct pattern emerged which could account for selectivity. The initial increases in apical RNA, microsomal and soluble proteins and amino acids in B.vulgaris could be related to the "classic" effects of auxin-type compounds on nucleic acid and protein synthesis outlined in chapter 1. However, since this pattern occurred in the tolerant plant species and was of a transient nature, with recovery from clopyralid effects taking place, its importance to herbicide activity is lessened. The fact that apical RNA was increased in B.vulgaris whilst leaf RNA was reduced with respect to control, demonstrates how herbicide effects can vary depending upon plant tissue. This presents a major problem of this type of study where, in assessing gross herbicide effects on key metabolites, whole plants were used, thus, in all probability masking effects on specific tissues. This concept may explain why few significant effects

on metabolite content were observed in highly susceptible M.inodora, whilst morphological symptom development was rapid and severe. The fact that significant increases were noted in microsomal protein (ribosome-associated) may indicate that consequent effects on other proteins and amino acids, in specific tissues are being masked in the process of whole plant extraction.

The large and variable increases in reducing sugar content found in all species indicated general metabolic stimulation and may have resulted from increased reserve mobilisation and/or increased photosynthetic activity.

Chapter 3. CLOPYRALID UPTAKE, TRANSLOCATION AND METABOLISM

3.1 Introduction

Published data concerning the uptake, movement and metabolism of the 4 major systemic pyridine herbicides; fluroxypyr, triclopyr, picloram and clopyralid highlights variations in the patterns of translocation and modification of these compounds. Uptake and translocation patterns of pyridine compounds are often comparable to the phenoxyalkanoic acid herbicides (Hay, 1976; Fletcher and Kirkwood, 1985; Hall, 1985) although species specific differences have been noted. For example, Hallmen and Eliasson (1972) found that picloram was absorbed faster than 2,4-D in wheat seedlings, whilst in rape and sunflower only small differences were noted in the rate of absorption of the two compounds (Hallmen, 1974).

Species differences in translocation patterns between the chemical groups are also notable. Turnbull and Stephenson (1985) found that, whilst the rate of absorption and translocation of ^{14}C was similar for both ^{14}C-2,4-D and ^{14}C-clopyralid, the distribution of the herbicides throughout C.arvense plants was very different. Radosevich and Bayer (1979) also found differences and reported that triclopyr and picloram were more ambimobile than 2,4,5-T in a range of plant species.

Large differences in the translocation behaviour of herbicides in the pyridine group may also be found. Radosevich and Bayer (1979) showed that triclopyr was more mobile than picloram in a range of species. In another study, Bovey (1979) found a greater accumulation of picloram than clopyralid in huisache leaf and stem tissue. More recently Sanders et al (1985) found greater uptake of fluroxypyr than clopyralid in G.aparine. This was a contributory factor in the greater activity of fluroxypyr in this species.

In general clopyralid has been found to be rapidly absorbed and extensively translocated, with the apical and young tissues forming the major sites of herbicide accumulation. (Devine and Vanden Born, 1985; Turnbull and Stephenson, 1985).

The translocation of herbicides is often associated with their metabolism. For example; translocation of picloram in rape was prevented by the formation of water-soluble complexes which remained in the treated leaf (Hallmen, 1974). The extent and pattern of metabolism of the pyridine herbicides varies with chemical and plant species. Picloram metabolism is slow in a number of species (Foy, 1976) but the formation of polar conjugates of the herbicides has been recorded (Hallmen, 1974; Hall, 1985), and these are thought to be sugar conjugates. Similar conjugates have been found with clopyralid (Hall, 1985), although their herbicidal activity is uncertain.

The aims of this investigation are to establish the patterns of clopyralid uptake, movement and metabolism and assess their contribution to the differential activity of clopyralid in the three test species.

3.2 Materials and Methods

3.2.1 Plant growth conditions

M.inodora, G.aparine and B.vulgaris plants were grown and maintained as in chapter 2, except that B.vulgaris seeds were sown sparsely in trays before being transplanted to 2 per 90mm pot at the young seedling stage.

3.2.2 Herbicide Application

Clopyralid was applied as the monoethanolamine salt. The recommended field dose of 100g a.i.ha^{-1} was used at a volume rate of 200lha^{-1}. Spray applications were made using a laboratory pot sprayer as in chapter 2.

3.2.3 Retention Studies

Tartrazine dye was used to determine the volume of spray drops retained by plant leaves. An aqueous solution of the dye (5gl^{-1}) was sprayed onto 6 replicate plants of each test species in the same way as herbicide (see above). The tissues of interest were then washed in 0.5% (v/v) blank herbicide formulant and the absorbance of the solutions measured at 434nm (Cecil instruments

spectrophotometer model CE 303). Measurements of retention were obtained using regression analysis to compare optical densities to those of standard dilutions of the dye prepared at concentrations ranging from 0.39mgl^{-1} to 50mgl^{-1}.

3.2.4 Uptake and Translocation in M.inodora and B.vulgaris

The extent of herbicide uptake from the 3rd leaf of 6 replicate M.inodora and B.vulgaris plants 8, 24, 48, 168 and 336 hours after application was determined using 2,6-^{14}C-pyridine ring labelled clopyralid (specific activity 24.7 mCi mmol^{-1}). These preliminary studies were conducted in order to assess any gross differences in uptake between tolerant and susceptible species. In another experiment, the uptake and translocation of ^{14}C clopyralid was examined in treated plants of both species 8, 24, 72, 168 and 336 hours after treatment. Figures 3.1 and 3.2 outline the experimental schemes adhered to. Droplets of 0.2µl volume were used to give the closest approximation to field conditions. These droplets were consistently obtained using a Burkard microdroplet applicator (Rickmansworth, UK.). However, in the case of B.vulgaris it was found that 0.2µl droplets could only be consistently applied over the area above leaf veins. The hydrophobic nature of the remainder of the leaf surface prevented transfer of droplets from the dispensing needle without damage to the cuticle. This problem led to the adoption of two application regimes with B.vulgaris. Random appication consisted of 4 x 0.5µl droplets placed across the leaf, whilst in vein application 10 x 0.2µl droplets were placed along the main vein.

Radiolabelled clopyralid was diluted with field rate herbicide to the required number of disintegrations per minute (d.p.m., concentration). Blank herbicide formulant was used at the same concentration present in field rate clopyralid (0.5%, v/v). Optisolve tissue solubiliser and Optiphase Safe scintillant were obtained from Fisons.

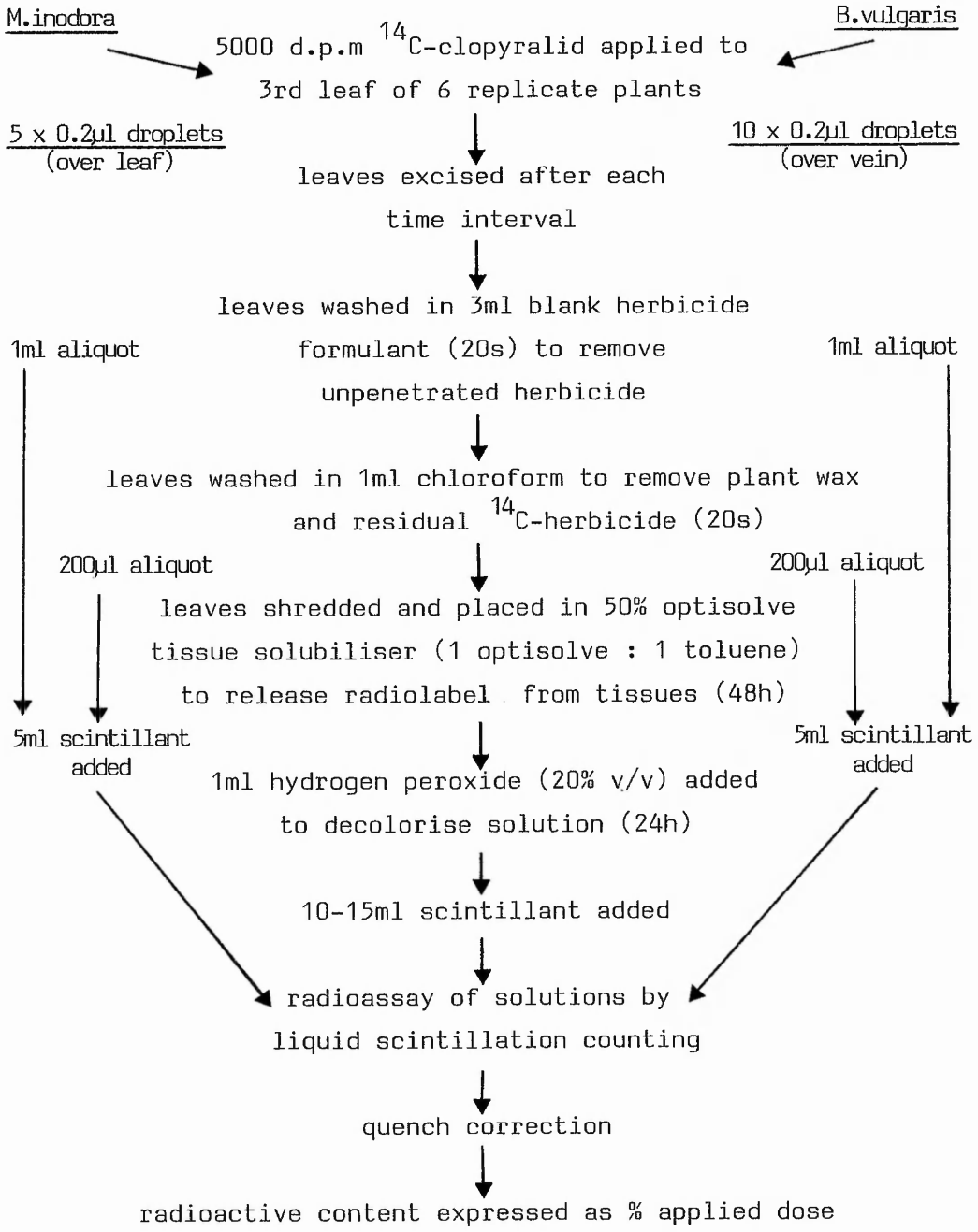


Figure 3.1 ^{14}C-clopyralid uptake studies - experimental procedure.

3.2.5 Uptake and Translocation in G.aparine

^{14}C-clopyralid uptake and translocation was measured in G.aparine plants treated with field rate clopyralid 8, 24, 72, 168 and 336 hours after application to 3 regions of 6 replicate plants; cotyledon (10 x 0.2µl droplets), first (eldest) leaf

whorl (1 x 0.2µl droplet per leaf), and the second leaf whorl (1 x 0.2µl droplet per leaf) of plants at the 2-3 leaf whorl stage (figure 3.3). Experimental procedures were as for M.inodora and B.vulgaris (figures 3.1 and 3.2). Approximately 20000d.p.m were applied to each plant.

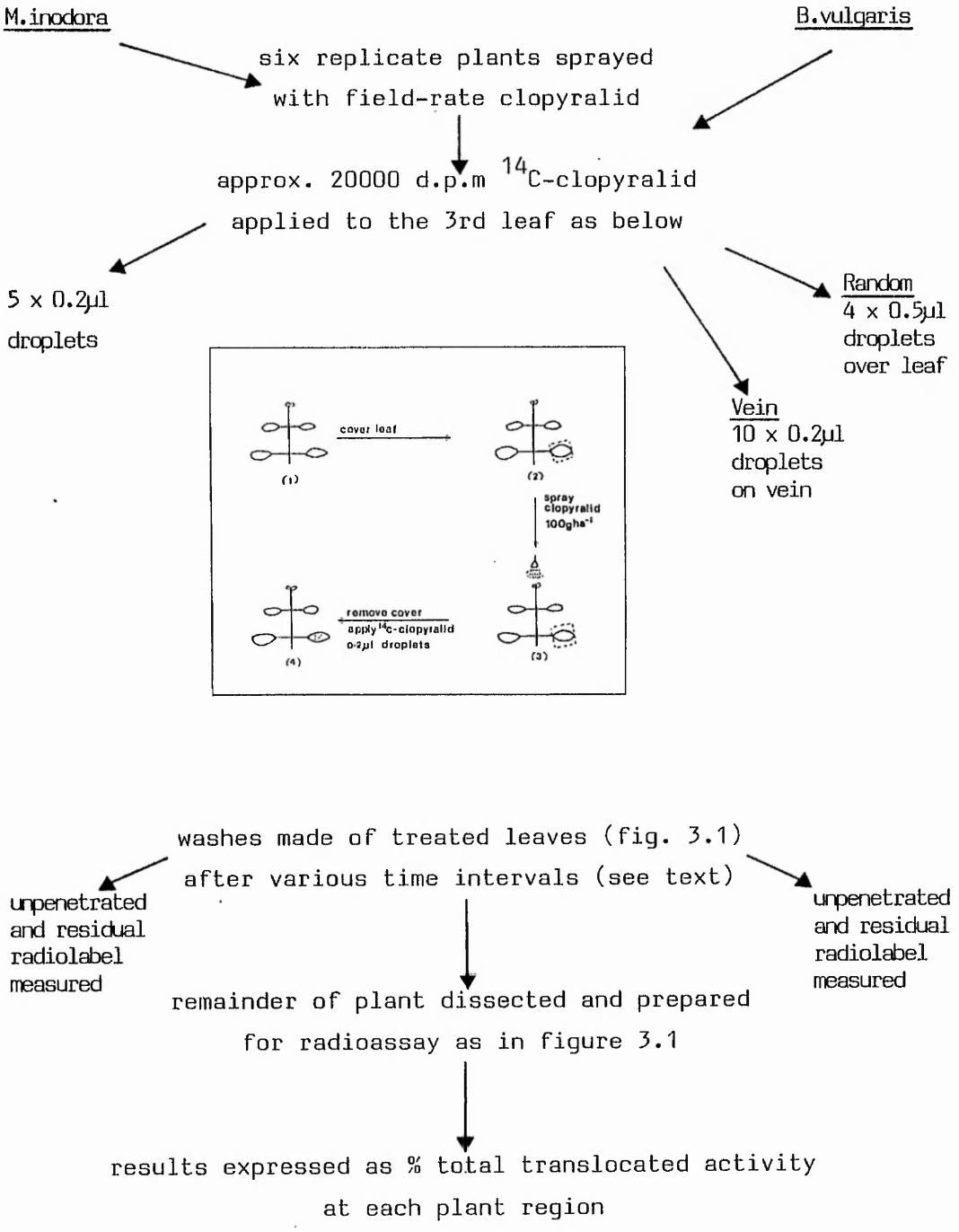
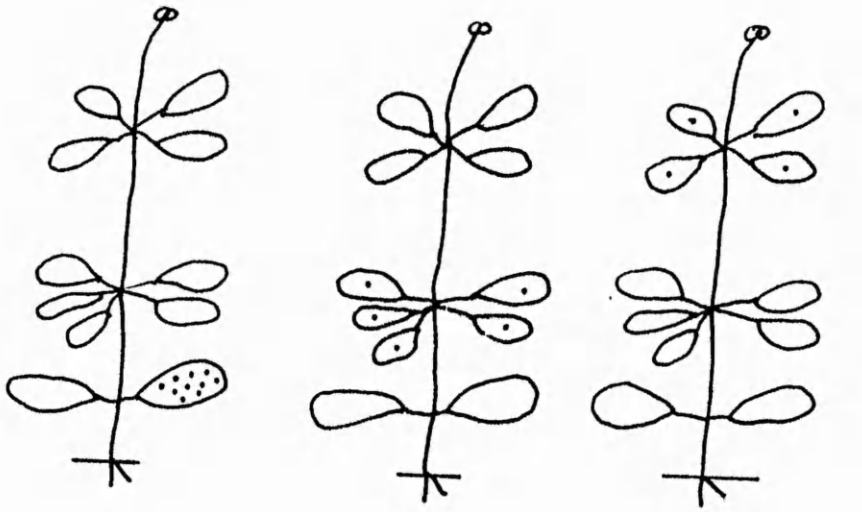


Figure 3.2 ^{14}C-clopyralid translocation studies - experimental procedure.



COTYLEDON	FIRST LEAF WHORL	SECOND LEAF WHORL
10 x 0.2µl	1 x 0.2µl/LEAF	1 x 0.2µl/LEAF

Figure 3.3 ^{14}C-clopyralid application regimes in G.aparine uptake and translocation experiments.

3.2.6 Autoradiography

2,6-^{14}C-clopyralid was applied to herbicide treated G.aparine plants according to the procedure described above. Since high levels of radioactivity were required radiolabelled clopyralid was diluted only slightly and approximately 500 000 d.p.m were applied to each plant. Two replicate treatments were made to 3 application sites; cotlyedon, first leaf whorl and second leaf whorl, with applications made as 15 x 0.2µl droplets.

After 7 days in an environmental chamber plants were removed from pots, mounted on absorbent paper, sandwiched between heavy glass sheets and allowed to dry in a warm room for 48 hours. The dried plant tissues were then mounted upon grey card (with the region of application of labelled herbicide clearly indicated) and photographed. Mounted tissue was then placed (under safe light) in contact with x-ray film (Kodak Saftey Film ARO) and left in total darkness for 28 days. At the end of this period,

films were developed and the fate of translocated ^{14}C-clopyralid qualitatively recorded by visual comparison of the negatives produced to the original photographs.

3.2.7 ^{14}C-clopyralid Volatilisation

Artificial leaf surfaces were prepared by extracting epicuticular waxes from each species into chloroform. The solvent was then allowed to evaporate and the waxes redissolved to a concentration of 2mg ml^{-1} . Twenty µl aliquots were placed onto glass coverslips and allowed to dry. After 24 hours 10 x 0.2µl droplets of ^{14}C-clopyralid were applied to the waxed areas (in the same manner as leaf application in the previous experiments) and the coverslips carefully placed in controlled environment chambers under the conditions outlined in chapter 2. After 8, 24, 96, 168 and 336 hours, 8 replicate coverslips were processed as outlined in figure 3.4 and the recovery of radiolabel recorded as % applied (d.p.m). Solvents and scintillant used were the same as for uptake and translocation work.

3.2.8 Contact Angles

The angle of contact of liquid droplets upon the abaxial and adaxial surfaces of leaves of the three test species was investigated. Leaf sections ($1cm^2$) were mounted on a plastic stage and 0.6µl droplets applied. Projection of the image of each droplet onto a screen using a slide projector allowed the angle of contact to be measured, as illustrated in figure 3.5. Contact angles of 10 replicate distilled water droplets were compared to those of 10 droplets of 0.5% v/v clopyralid formulant for leaves of each species (3rd leaf in *M.inodora* and *B.vulgaris*, cotyledon in *G.aparine*), and the percentage reduction in angle of contact due to the formulant noted.

3.2.9 Scanning electron microscopy

Sections of leaf tissue ($5mm^2$) were carefully dissected and mounted on metal studs using double sided tape. The sections were freeze-dried overnight (Edwards Minifast 600) and then coated with gold in a sputter coating unit (Nanotech SemPrep 2) before examination by scanning electron microscopy (Cambridge Stereoscan 600). Images were recorded photographically.

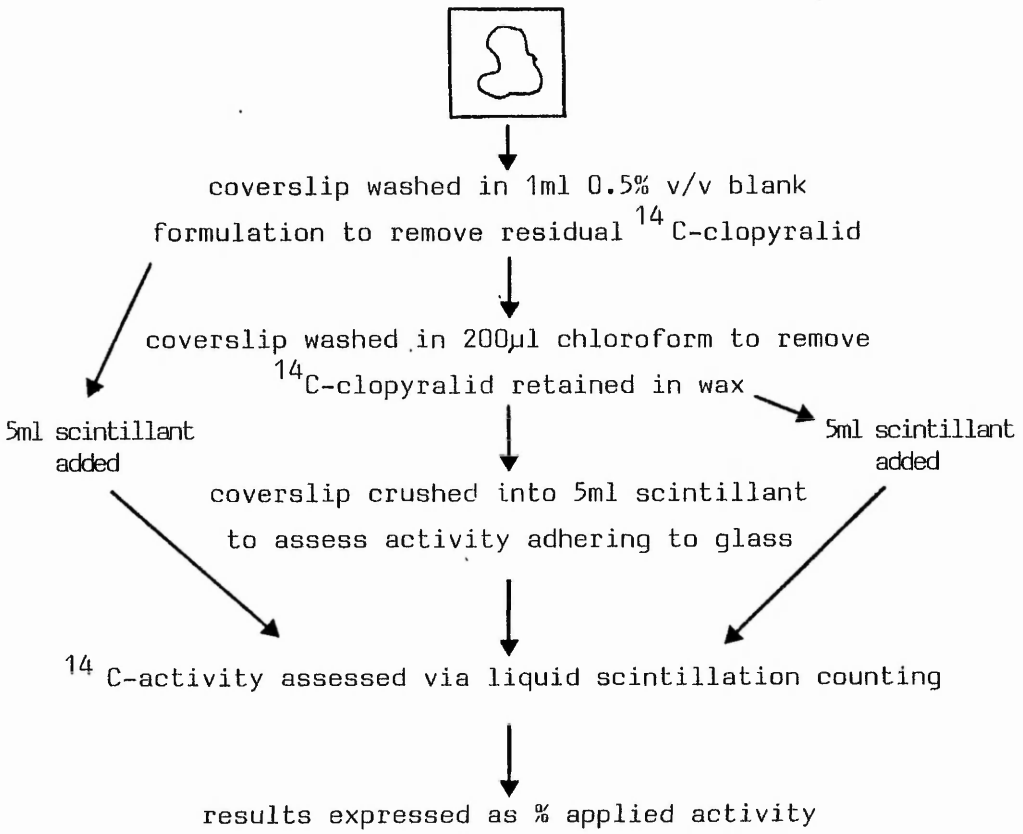


Figure 3.4 Experimental scheme for radiolabel recovery from artificial leaf surfaces.

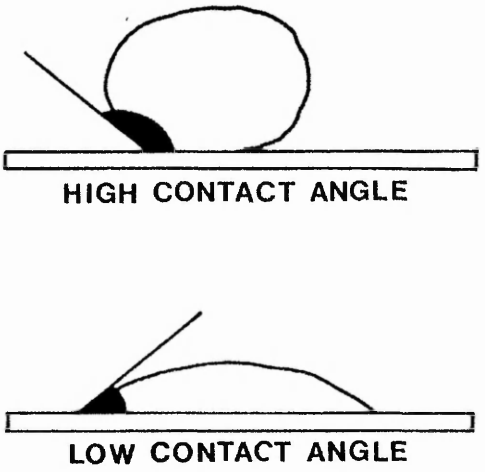


Figure 3.5 Measurement of contact angle.

3.2.10 Metabolism Studies

Fifteen to eighteen 0.2µl droplets of ^{14}C-clopyralid (approx. 200 000 d.p.m per plant) were applied to the cotyledons and first leaf whorl of G.aparine and the 3rd leaf of M.inodora and B.vulgaris (applied over veins in B.vulgaris). After 7 and 14 days the apex and young or emerging tissues of 4 replicate plants of each test species were homogenised in a minimal volume of acetone (500-1000µl) at 4°C. Each homogenate was centrifuged at maximum speed for 1 min in a microfuge (M.S.E Microcentaur) and the resulting supernatants concentrated in a nitrogen stream to a volume of 100µl. Pellets were solubilised in 450µl Optisolve tissue solubiliser and decolorised by the addition of 500µl hydrogen peroxide (20% v/v) prior to liquid scintillation counting to assess radioactive content.

Thirty to forty µl of each supernatant were spotted onto precoated plastic T.L.C plates (Merck, Kieselgel 60 F254) and developed in 80 chloroform : 20 methanol : 1 glacial acetic acid. After development 15mm wide strips within which the extracts had migrated, were excised from plates and sectioned into 5mm portions. Each portion was placed into 5ml Optiphase Safe scintillant (Fisons) and radioactive content assayed by liquid scintillation counting. In the solvent system used clopyralid (acid) had an Rf value of 0.2. The Rf values and distribution of any non-clopyralid forms of the applied ^{14}C-radiolabel were noted for each species after the 2 experimental periods. The experiments were then repeated in full giving a total of 8 replicate plants per treatment, and results expressed as mean percentage total recovered activity (>1%) found in each 5mm Rf band.

3.2.11 Qualitative Estimation of the Activity of Extracted Clopyralid Metabolites using a Sensitive Tissue Bioassay

Trays of M.inodora and B.vulgaris plants were treated with field rate clopyralid by wick application. A cotton wick impregnated with the herbicide was drawn over foliage allowing plant leaves (but not the apices) to come into contact with the

solution. After 7 days in an environmental chamber (conditions as previously described) the apical tissues of 10 plants were dissected and freeze-dried overnight (Edwards, Minifast 600). Dry apices were homogenised in 500µl 100% acetone at 4°C and, following centrifugation (M.S.E Microcentaur, high speed setting) and repeated washing, the volume of the solution was reduced under nitrogen before application to plastic T.L.C plates (Merck, Kieselgel 60 F254). Plates were run in 80 chloroform : 20 methanol : 1 glacial acetic acid. As soon as dry, plates were cut into regions corresponding to major bands of herbicide accumulation in the metabolism study. Adsorbant was scraped off these areas and washed repeatedly in 100% acetone to recover adsorbed compounds. Acetone was driven off the extracts under nitrogen and residues solubilised in 9ml 1/5 strength Nitches medium and solutions adjusted to pH 6 with sodium hydroxide. Residues were bioassayed according to a method devised by Dr Sanders in this laboratory (Sanders, Thompson and Pallett, 1985). Solutions were placed in a 5cm glass petri dish and covered with self sealing film into which 8 replicate portions of G.aparine shoot tip, with leaves removed, were inserted to a depth of 5mm. Each shoot tip was measured for extension growth following a 48 hours incubation period under controlled environment conditions as previously stated. Extension was compared to controls as well as to 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M solutions of clopyralid and IAA. The experiment was repeated 4 times.

3.3 Results

3.3.1 Retention Studies

Table 3.1 records the average tartrazine spray volumes retained by selected tissues of each test species. Although there was high variability, resulting from individual plant morphology and stature, differences between species were evident. Since the 3rd leaf of B.vulgaris retained most dye, the volumes of radiolabelled clopyralid applied in uptake studies were chosen to reflect this, in order that laboratory comparisons, particularly between B.vulgaris and M.inodora, should have relevance

and validity to the spray situation. Using the data it was decided that appropriate volumes of application in uptake and translocation work would be 1µl in the case of M.inodora (3rd leaf) and G.aparine (cotyledon) and 2µl for B.vulgaris (3rd leaf). In G.aparine it was found that leaf whorls retained 7-10µl of spray (Sanders et al, 1985). However for experimental purposes, the application of one 0.2µl droplet to each leaf of the whorl was found to be convenient.

Table 3.1 Tartrazine dye retention by plant tissues. Values are the mean of 6 replicate measurements plus or minus the standard error.

SPECIES	TISSUE	RETENTION (µl)
M.inodora	LEAF 3	0.72 ± 0.51
B.vulgaris	LEAF 3	2.72 ± 0.62
G.aparine	COTYLEDON	1.83 ± 0.41

3.3.2 Uptake and Translocation in M.inodora and B.vulgaris

Table 3.2 presents the results of preliminary ^{14}C-clopyralid uptake studies in M.inodora. The figures presented are percentages of applied ^{14}C-activity, the exact measurement of which was made by harvesting tissue at time zero. Total d.p.m's applied ranged from 4600 to 5500. The recovery of ^{14}C-activity from the leaf surface (ie that detected in aqueous and chloroform washes, fig 3.1) and the amounts in treated leaf tissues are recorded.

From the data it can be seen that movement of ^{14}C-clopyralid into the treated leaf was rapid, with 3.83% of applied activity being recovered from leaf tissue after 8 hours. It may also be noted that, although there is steady removal of activity from

the leaf surface over the 14 days experimental period, the levels of activity detected within the treated leaf did not rise. This indicates rapid translocation of absorbed ^{14}C-clopyralid.

Table 3.2 Uptake of ^{14}C-clopyralid from 3rd leaf M.inodora. Figures are the means plus or minus standard error from six replicate plants.

	(% applied ^{14}C-activity)	
TIME (h)	SURFACE RECOVERY	TREATED LEAF TISSUE
8	98.63 ± 2.82	3.83 ± 0.24
24	83.21 ± 0.70	5.88 ± 0.20
48	82.48 ± 0.74	3.19 ± 0.60
168	53.56 ± 2.82	4.16 ± 0.73
336	42.94 ± 1.58	4.51 ± 0.33

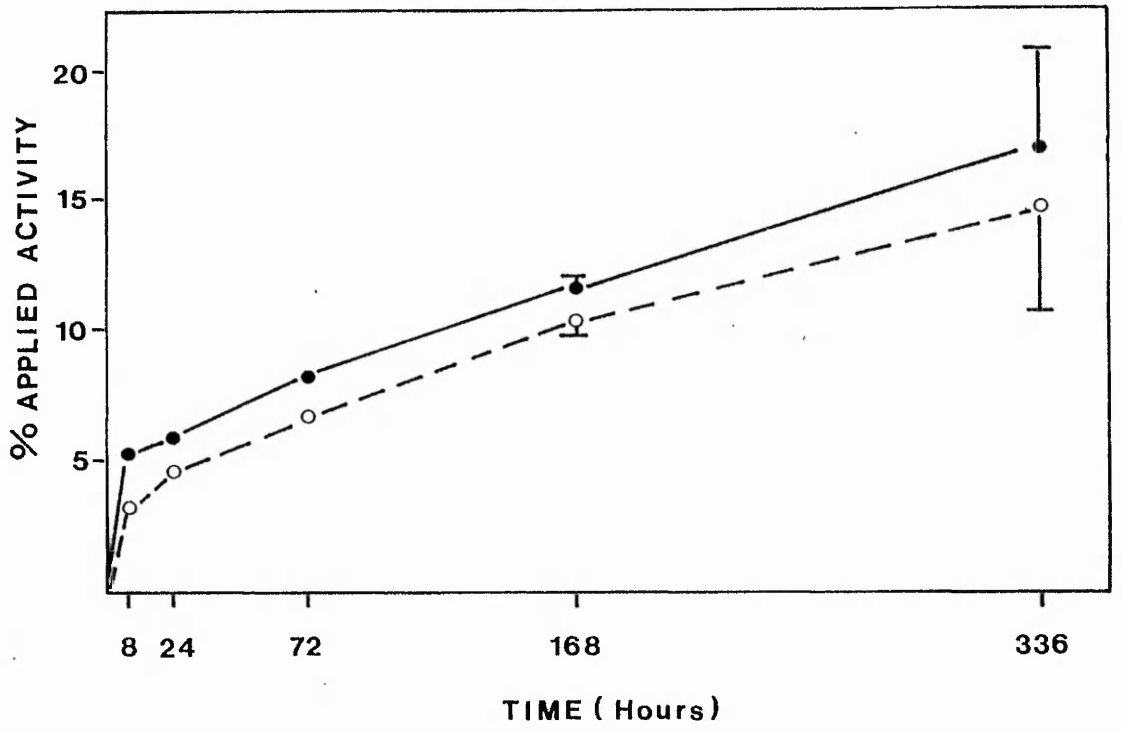


Figure 3.6 Uptake (—●—) and translocation (- -○- -) of ^{14}C-clopyralid in M.inodora. Data are presented as means of 6 replicates plus or minus standard error, and error bars are included where they exceed symbol size.

The results of the comprehensive study of uptake and translocation in M.inodora are expressed in figure 3.6 and table 3.3. Figure 3.6 plots uptake and translocation as percentage of applied activity against time. As found in the previous experiment, ^{14}C-clopyralid uptake into leaf 3 was rapid with 5% of applied ^{14}C-activity entering the leaf tissue within 8 hours. Its subsequent translocation was rapid and complete, illustrated by the proximity and parallel nature of the two plots. After 14 days 87% of absorbed activity was recovered remote from site of its application. Table 3.3 details the fate of absorbed clopyralid and maps its distribution, as percentage applied activity, over 6 tissue sites as well as itemising activities in treated leaf washes. Major points to emerge include the non-retention of ^{14}C-clopyralid in plant cuticular waxes, with less than 1% of applied activity being recovered in the chloroform wash at any time. There was no accumulation of ^{14}C-activity within the treated leaf. The most important sites of accumulation in treated plants were the young leaf and apical tissues where (336h after treatment, as highlighted in Table 3.3) the activity recovered accounted for 87% of all translocated activity. There was no accumulation of radioactivity within the roots of plants or in the cotyledons and primary leaves. Total recoveries in M.inodora (72, 168 and 336h after treatment) were 70, 73 and 64% of applied activity respectively. Possible reasons for this low recovery are outlined in the discussion.

Table 3.4 records the results of preliminary ^{14}C-clopyralid uptake studies in B.vulgaris. As with M.inodora, the movement of ^{14}C-clopyralid into the treated leaf was rapid, with 6.25% of applied activity being recovered from treated leaf tissue after only 8 hours. Also, in similarity with M.inodora (table 3.3), there was no accumulation of radiolabel in the treated leaf despite the progressive decrease in recoverable activity in surface washes over the experimental period. This was an indication of the herbicide's mobility within plant tissues. The surface recovery of ^{14}C-activity in B.vulgaris was generally lower than in M.inodora and this was considered to be a possible indication of there being greater uptake in B.vulgaris.

Table 3.3 Distribution of ^{14}C-activity at intervals following application to the 3rd leaf of M.inodora. Figures are mean % of applied activity plus or minus the standard error from 6 replicate plants.

REGION OF RECOVERY/TIME(h)	8	24	72	168	336
FORMULATION WASH	97.32 ± 3.79	91.15 ± 3.60	62.22 ± 1.01	61.02 ± 0.03	47.20 ± 1.26
CHLOROFORM WASH	0.54 ± 0.06	0.35 ± 0.14	0.28 ± 0.07	0.39 ± 0.00	0.53 ± 0.16
TREATED LEAF	2.03 ± 0.30	1.25 ± 0.16	1.49 ± 0.15	1.23 ± 0.12	2.27 ± 0.17
ELDEST LEAVES	0.48 ± 0.02	0.26 ± 0.04	0.58 ± 0.04	0.77 ± 0.87	2.44 ± 0.42
DEVELOPING LEAVES	0.88 ± 0.15	3.44 ± 0.15	4.59 ± 0.17	7.14 ± 0.87	8.78 ± 2.52
COTYLEDONS & PRIMARY LEAVES	0.39 ± 0.02	0.11 ± 0.01	0.17 ± 0.04	0.21 ± 0.03	0.22 ± 0.02
ROOTS	0.42 ± 0.06	0.16 ± 0.02	0.18 ± 0.01	0.33 ± 0.08	0.20 ± 0.03
APICAL TISSUES	1.07 ± 0.20	0.58 ± 0.06	1.15 ± 0.14	1.91 ± 0.31	3.16 ± 0.91
TOTAL RECOVERY	103.13 ± 4.60	97.30 ± 4.18	70.66 ± 1.63	73.00 ± 2.31	64.80 ± 5.49

Table 3.4 Uptake of ^{14}C-clopyralid from 3rd leaf of B.vulgaris. Figures are mean values plus or minus standard errors from 6 replicate plants.

	(% APPLIED ^{14}C-ACTIVITY)	
TIME (h)	SURFACE RECOVERY	TREATED LEAF TISSUE
8	68.85 ± 1.21	6.72 ± 1.37
24	65.29 ± 1.68	6.91 ± 0.61
48	63.39 ± 3.40	6.30 ± 0.59
168	34.38 ± 0.94	2.93 ± 0.17
336	27.90 ± 1.38	2.78 ± 0.20

Figures 3.7A and 3.7B and tables 3.5A and 3.5B record the results of uptake and translocation studies in B.vulgaris following both random and vein application of ^{14}C-clopyralid to the 3rd leaf. In each case, uptake of applied herbicide was rapid and subsequent translocation extensive. The application of 0.2µl droplets over the vascular tissue initially resulted in more rapid herbicide uptake with 11.29% of applied compound recovered from the 3rd leaf after only 8 hours, compared to 2.29% in the case of random application of 0.5µl droplets. However, the random application of 0.5µl droplets resulted in greater uptake after 168 and 336 hours (figure 3.6). Little difference was found between application regimes in the percentage of penetrated herbicide translocated out of the treated leaf. Translocation rates were high with 87% of vein-applied and 95% of randomly applied absorbed ^{14}C-activity recovered at tissue sites remote from the treated leaf.

Tables 3.5A and 3.5B outline the fate of absorbed ^{14}C-clopyralid and record its distribution according to the tissue dissection as listed. As in M.inodora, major sinks for translocated ^{14}C-activity were the youngest leaves and apical tissues, from which 65-70% of total translocated activity was recovered. However, after 336 hours a significant proportion of ^{14}C-activity was measured in older leaf and stem tissue.

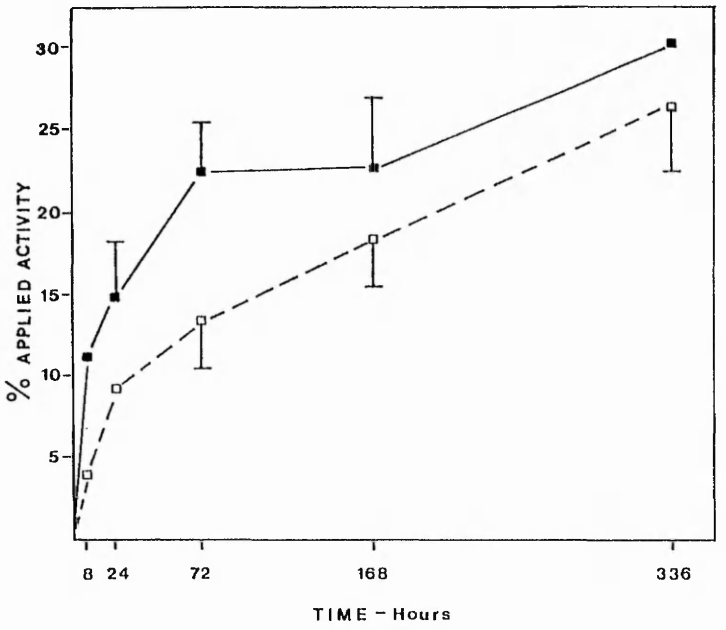


Figure 3.7A Uptake (—■—) and translocation (- -□- -) in 3rd leaf of B.vulgaris following vein application. Data are presented as mean values of 6 replicates plus or minus standard error.

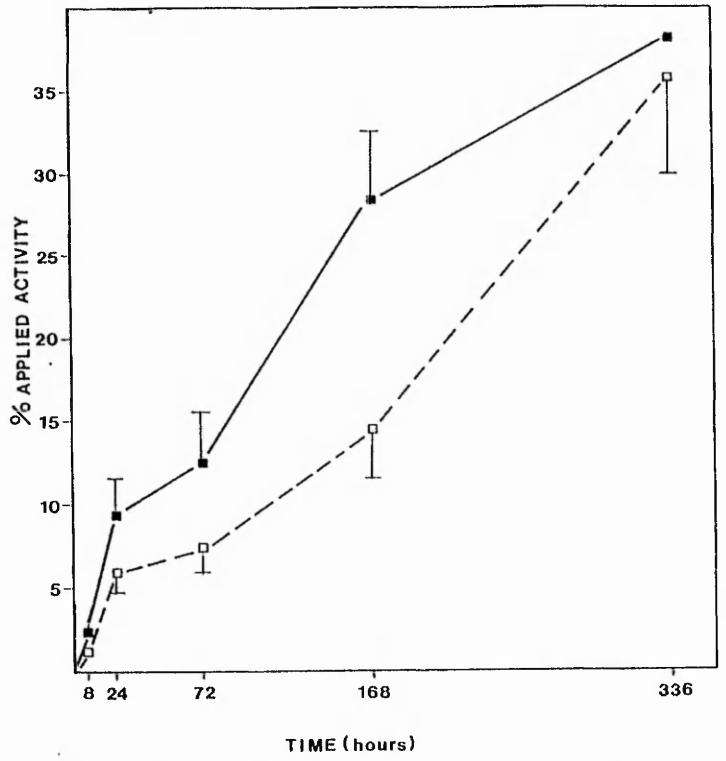


Figure 3.7B Uptake (—■—) and translocation (- -□- -) in 3rd leaf of B.vulgaris following random application. Data are presented as mean values of 6 replicates plus or minus standard error.

Table 3.5A Distribution of ^{14}C-activity at intervals following application of 10 X 0.2µl droplets of ^{14}C-clopyralid to vein tissue of 3rd leaf B.vulgaris. Figures are mean % of applied activity plus or minus the standard error from 6 replicate plants.

REGION OF RECOVERY/TIME(h)	8	24	72	168	336
FORMULATION WASH	74.49 ± 1.98	73.03 ± 9.81	67.57 ± 11.54	56.92 ± 3.58	39.50 ± 7.25
CHLOROFORM WASH	0.80 ± 0.09	0.53 ± 0.94	0.59 ± 0.05	1.15 ± 0.69	4.68 ± 1.58
TREATED LEAF	7.37 ± 1.76	5.75 ± 1.88	9.05 ± 0.37	14.35 ± 1.97	3.77 ± 0.37
COTYLEDONS	0.22 ± 0.10	0.42 ± 0.10	0.91 ± 0.10	0.34 ± 0.04	0.37 ± 0.09
YOUNGEST LEAVES	0.80 ± 0.02	1.43 ± 0.10	6.76 ± 0.74	5.50 ± 1.89	15.13 ± 0.96
ELDEST LEAVES	0.08 ± 0.01	0.73 ± 0.14	1.20 ± 0.73	0.78 ± 0.50	4.51 ± 0.92
APEX	2.11 ± 0.61	4.03 ± 0.32	2.62 ± 1.34	0.95 ± 0.10	2.27 ± 0.98
STEM	0.17 ± 0.02	1.41 ± 0.37	1.32 ± 0.36	0.25 ± 0.20	2.29 ± 0.49
ROOTS	0.62 ± 0.01	1.15 ± 0.26	0.64 ± 0.08	0.55 ± 0.05	1.88 ± 0.47
TOTAL RECOVERY	86.66 ± 4.58	88.48 ± 13.92	90.66 ± 15.31	80.69 ± 9.02	74.40 ±13.11

Table 3.5B Distribution of ^{14}C-activity at intervals following application of 4 X 0.5µl droplets of ^{14}C-clopyralid to the 3rd leaf of B.vulgaris. Figures are the mean % of applied activity plus or minus the standard error from 6 replicate plants.

REGION OF RECOVERY/TIME (h)	8	24	72	168	336
FORMULATION WASH	94.32 ± 2.79	88.17 ± 4.35	77.91 ± 3.52	47.47 ± 4.57	30.82 ± 5.34
CHLOROFORM WASH	1.63 ± 0.23	0.43 ± 0.20	0.81 ± 0.50	3.08 ± 1.03	0.74 ± 0.46
TREATED LEAF	1.13 ± 0.76	3.42 ± 1.25	5.22 + 1.63	14.14 ± 1.25	2.54 ± 0.13
COTYLEDONS	0.16 ± 0.02	0.28 ± 0.06	0.25 ± 0.01	0.61 ± 0.04	0.34 ± 0.08
YOUNGEST LEAVES	1.02 ± 0.17	2.01 ± 0.40	4.65 ± 1.47	10.44 ± 2.48	20.37 ± 2.96
ELDEST LEAVES	0.11 ± 0.10	0.47 ± 0.17	0.47 ± 0.05	0.40 ± 0.04	6.53 ± 2.46
APEX	0.51 ± 0.02	1.90 ± 0.43	0.76 ± 0.15	1.37 ± 0.23	4.47 ± 0.48
STEM	0.09 ± 0.10	0.64 ± 0.10	0.70 ± 0.16	0.90 ± 0.06	2.60 ± 0.31
ROOTS	0.12 ± 0.01	0.65 ± 0.11	0.49 ± 0.02	0.69 ± 0.09	1.69 ± 0.54
TOTAL RECOVERY	99.09 ± 4.20	97.97 ± 7.04	91.26 ± 7.51	79.10 ± 9.79	70.10 ± 12.76

3.3.3 Uptake and Translocation in G.aparine

Figure 3.8 illustrates the uptake and translocation of applied ^{14}C-activity from 3 regions of G.aparine plants, 1, 3 and 7 days after ^{14}C-clopyralid application. The pattern of rapid absorption and near complete translocation of applied radioactivity is similar to that found in M.inodora and B.vulgaris. The percentage of applied compound entering G.aparine is in the same range as that absorbed in M.inodora (fig 3.5). As may be seen in figure 3.8, uptake is greatest from the cotyledon with 13% of applied compound entering the plant 7 days after application. At the growth stage used, leaf whorls absorbed and translocated approximately equal amounts of activity.

Tables 6A, 6B and 6C describe the fate of absorbed ^{14}C-activity following ^{14}C-clopyralid application to the three designated tissues in G.aparine. Tissue dissection was modified according to the region of application in order that movement patterns could be discerned.

^{14}C-herbicide applied to the cotyledons (table 3.6A) was rapidly absorbed and transported to the youngest leaf whorls and apical tissues and the most proximate lateral outgrowths. There were only minimal amounts found in the opposite cotyledon. When applied to the 1st (eldest) leaf whorl (table 3.6B), ^{14}C-activity accumulated in youngest leaf and apical tissue as well as in the laterals both acropetal and basipetal to the site of application. Table 3.6C records the distribution of radiolabel applied to the 2nd (youngest) developed leaf whorl. Sites of accumulation included all developing lateral shoots and actively growing leaf and apical tissues.

As with B.vulgaris and M.inodora, a pattern of radiolabel movement from application site to primary areas of growth and development emerged in G.aparine, i.e. photosynthate sinks form the major regions of accumulation of ^{14}C-activity. No accumulation of ^{14}C-activity in epicuticular wax or roots was measured in G.aparine.

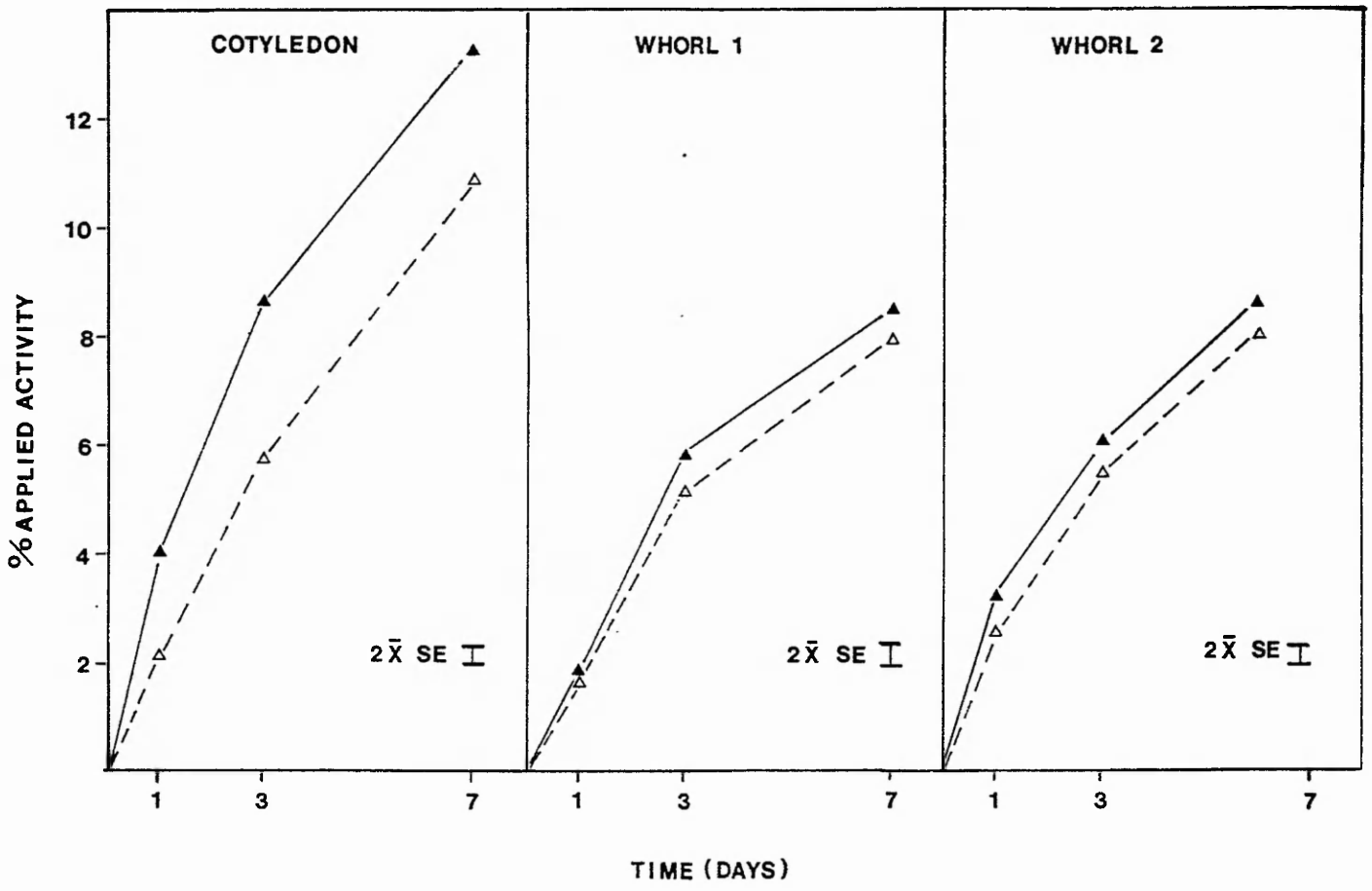


Figure 3.8 Uptake (—▲—) and translocation (-△-) of ^{14}C-clopyralid in G.aparine following application to 3 tissue sites. Figures are means of 6 replicates and twice mean standard error is indicated.

Table 3.6A Distribution of ^{14}C-activity at intervals following application of 10 X 0.2µl droplets of ^{14}C-clopyralid to one cotyledon of 6 replicate G.aparine plants. Figures are mean % of applied activity plus or minus the standard error.

REGION OF RECOVERY/TIME (h)	8	24	72	168	336
FORMULATION WASH	94.19 ± 0.96	93.10 ± 1.29	80.46 ± 3.30	68.60 ± 0.48	41.32 ± 2.01
CHLOROFORM WASH	1.49 ± 0.19	1.33 ± 0.10	2.18 ± 0.16	0.72 ± 0.15	0.92 ± 0.34
TREATED COTYLEDON	0.79 ± 0.07	1.88 ± 0.22	2.93 ± 0.49	2.38 ± 0.40	1.70 ± 0.10
OPPOSITE COTYLEDON	0.10 ± 0.01	0.20 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.24 ± 0.04
APEX & WHORL 3 OR TISSUE ABOVE	0.31 ± 0.02	0.52 ± 0.14	0.75 ± 0.15	1.78 ± 0.07	1.78 ± 0.26
LATERALS FROM WHORL 1	0.18 ± 0.02	0.78 ± 0.14	3.92 ± 0.35	8.07 ± 0.63	7.05 ± 1.11
LATERALS FROM WHORL 2	0.02 ± 0.01	0.18 ± 0.06	0.20 ± 0.02	0.40 ± 0.04	0.50 ± 0.12
WHORL 2 & STEM BELOW	0.04 ± 0.02	0.14 ± 0.02	0.24 ± 0.09	0.09 ± 0.01	0.13 ± 0.01
WHORL 1	0.05 ± 0.03	0.18 ± 0.04	0.16 ± 0.13	0.15 ± 0.01	0.26 ± 0.02
ROOTS	0.03 ± 0.02	0.17 ± 0.01	0.22 ± 0.02	0.18 ± 0.08	0.15 ± 0.02
TOTAL RECOVERY	97.20 + 1.35	98.48 ± 2.04	91.31 ± 4.73	82.63 ± 1.88	54.05 ± 4.03

Table 3.6B Distribution of ^{14}C-activity at intervals follpwing application of 1 X 0.2µl droplet of ^{14}C-clopyralid to each leaf of whorl 1 of 6 replicate G.aparine plants. Figures are the mean % of applied activity plus or minus the standard error.

REGION OF RECOVERY/TIME(h)	8	24	72	168	336
FORMULATION WASH	100.16 ± 5.60	102 03 ± 3.60	92.55 ± 3.44	82.76 ± 0.50	71.35 ± 4.55
CHLOROFORM WASH	0.72 ± 0.12	4.49 ± 1.68	0.76 ± 0.34	0.38 ± 0.21	0.03 ± 0.00
TREATED WHORL LEAVES	0.34 ± 0.01	0.45 ± 0.10	0.71 ± 0.07	0.60 ± 0.07	0.44 ± 0.01
COTYLEDONS & STEM TO SOIL	0.17 ± 0.02	0.18 ± 0.02	0.26 ± 0.04	0.32 ± 0.04	0.40 ± 0.08
WHORL 2 & STEM BELOW	0.34 ± 0.10	0.26 ± 0.04	0.17 ± 0.10	0.31 ± 0.07	1.20 ± 0.01
WHORL 3 & ABOVE & APEX	0.40 ± 0.03	0.60 ± 0.10	3.48 ± 0.33	3.63 ± 1.04	4.39 ± 1.01
LATERALS AT TREATMENT	——	——	——	0.31 ± 0.01	2.14 ± 0.24
LATERALS BELOW TREATMENT	0.15 ± 0.03	0.33 ± 0.07	1.15 ± 0.38	2.41 ± 0.42	3.76 ± 0.38
LATERALS ABOVE TREATMENT	——	——	——	——	0.28 ± 0.02
ROOTS	0.07 ± 0.01	0.08 ± 0.02	0.11 ± 0.02	0.90 ± 0.01	0.25 ± 0.01
TOTAL RECOVERY	102.35 ± 5.92	108.42 ± 5.63	99.19 ± 4.72	91.62 ± 2.37	84.24 ± 6.32

Table 3.6C Distribution of ^{14}C-activity at intervals following application of 1X 0.2µl droplet of ^{14}C-clopyralid to each leaf of whorl 2 of 6 replicate G.aparine plants. Figures are mean % of applied activity plus or minus the standard error.

REGION OF RECOVERY/TIME(h)	8	24	72	168	336
FORMULATION WASH	99.72 ± 5.56	95.64 ± 1.49	82.99 ± 2.13	86.67 ± 3.09	64.11 ± 1.41
CHLOROFORM WASH	0.56 ± 0.11	0.91 ± 0.09	1.07 ± 0.56	0.83 ± 0.40	0.02 ± 0.01
TREATED WHORL LEAVES	0.79 ± 0.30	0.70 ± 0.11	0.61 ± 0.12	0.59 ± 0.05	1.00 ± 0.15
COTYLEDONS & STEM BELOW	0.13 ± 0.02	0.14 ± 0.01	0.16 ± 0.00	0.17 ± 0.02	0.21 ± 0.03
WHORL 1	0.21 ± 0.07	0.17 ± 0.03	0.23 ± 0.04	0.15 ± 0.00	0.25 ± 0.34
WHORL 3 & ABOVE & APEX	1.14 ± 0.24	2.02 ± 0.23	4.04 ± 0.53	4.08 ± 0.97	5.38 ± 2.44
LATERALS AT COTYLEDONS	0.12 ± 0.01	0.14 ± 0.03	0.98 ± 0.14	2.73 ± 0.34	2.48 ± 0.16
LATERALS FROM WHORL 1	——	——	——	0.30 ± 0.06	0.96 ± 0.16
LATERALS AT TREATMENT	——	——	——	0.48 ± 0.04	0.42 ± 0.07
LATERALS ABOVE TREATMENT	——	——	——	——	1.06 ± 0.21
ROOTS	0.11 ± 0.01	0.12 ± 0.02	0.10 ± 0.03	0.11 ± 0.02	0.12 ± 0.06
TOTAL RECOVERY	102.78 ± 6.32	99.84 ± 2.01	90.18 ± 3.55	96.11 ± 4.99	76.01 ± 4.85

3.3.4 Autoradiography

Confirmation of the results of uptake and movement studies in G.aparine was gained when qualitative estimation of accumulation of ^{14}C-clopyralid was made using autoradiography. Plate 3.1 illustrates that the developing lateral shoots nearest the site of ^{14}C-clopyralid application and the young and apical tissues are the primary sites for ^{14}C-activity accumulation. Also revealed by the autoradiographs is the retention of ^{14}C-activity in stem tissue. This was not revealed in initial studies due to the dissection methods used.

3.3.5 ^{14}C-clopyralid Volatilisation

Table 3.7 records the recovery of applied ^{14}C-activity from artificial leaf surfaces of each test species. No significant volatilisation of herbicide was measured at any time during the experimental period.

3.3.6 Contact Angles

The angles of contact of distilled water droplets were similar on leaf tissues of each test species with values ranging from 61° to 64°. In all cases, reduced angles were measured where clopyralid formulation (0.5%, v/v) was applied in an identical manner (table 3.8). The extent of this reduction was greatest in M.inodora, with 21.56% and 31.94% reduction in contact angle on adaxial and abaxial surfaces respectively. The formulant solution was also effective at reducing contact angle in B.vulgaris. On G.aparine cotyledons, only a relatively small percentage reduction in contact angle due to the formulant was measured (table 3.8). The reason for the differential effectiveness of the formulant was sought by examination of leaf surfaces of each species using scanning electron microscopy.

3.3.7 Scanning Electron Microscopy

Plates 3.2, 3.3 and 3.4 are the SEM micrographs of adaxial surfaces of each of the 3 test species. Plate 3.2 clearly shows

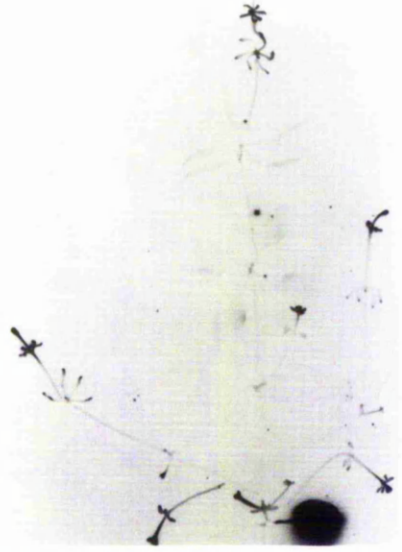
Plate 3.1 Autoradiographic examination of the fate of ^{14}C-clopyralid following application to:-

(A) COTYLEDON

(B) WHORL 1

(C) WHORL 2

COTYLEDON



WHORL 1

WHORL 2

that the adaxial surface of G.aparine has many trichomes. These are highly regular in shape and size and appear to be covered with wax platelets (plate 3.2C). On the other hand, M.inodora and B.vulgaris leaves appear to have a more uniform adaxial surface with epicuticular waxes coating epidermal cells in a wave-like formation.

Table 3.7 Recovery (% applied activity) of ^{14}C-clopyralid following application of ^{14}C-clopyralid to artificial leaf surfaces. Figures are mean values plus or minus standard error from 8 replicate coverslips.

M.inodora

Time (h)	FORMULATION WASH	CHLOROFORM WASH	GLASS	TOTAL
0	100	———	———	100
8	100.46 ± 1.26	3.14 ± 0.71	0.89 ± 0.14	104.49 ± 2.11
24	86.12 + 1.87	7.78 ± 0.83	1.41 ± 0.15	95.31 ± 2.85
72	97.10 ± 2.73	2.76 ± 1.45	0.86 ± 0.14	100.72 ± 4.32
168	74.95 ± 3.20	10.97 ± 1.04	3.44 ± 0.39	89.36 ± 4.63
336	64.67 ± 8.10	18.54 ± 5.41	9.93 ± 2.96	93.14 ± 16.47

B.vulgaris

Time (h)	FORMULATION WASH	CHLOROFORM WASH	GLASS	TOTAL
0	100	———	———	100
8	98.40 ± 1.58	2.00 + 0.24	2.92 ± 0.61	103.32 ± 2.43
24	97.01 ± 2.55	1.64 ± 0.67	2.29 ± 0.45	100.94 ± 3.67
72	93.55 ± 2.81	3.71 ± 0.16	3.05 ± 0.18	100.31 ± 3.15
168	81.67 ± 1.49	6.11 ± 0.99	10.11 ± 1.16	97.89 ± 3.64
336	67.66 ± 0.41	12.17 ± 1.16	27.66 ± 1.11	107.49 ± 2.68

G.aparine Time (h)	FORMULATION WASH	CHLOROFORM WASH	GLASS	TOTAL
0	100	———	———	100
8	95.76 ± 2.11	3.48 ± 0.69	0.82 ± 0.17	100.06 ± 2.97
24	94.41 ± 1.15	2.28 ± 0.41	1.82 ± 0.38	98.51 ± 1.94
72	82.88 ± 0.58	6.35 ± 0.50	1.95 ± 0.20	91.18 ± 1.28
168	79.70 ± 1.74	6.78 ± 0.25	4.44 ± 0.49	90.92 ± 2.48
336	59.86 ± 2.61	28.02 ± 3.88	18.82 ± 0.86	106.71 ± 7.35

Table 3.8 Effect of clopyralid formulation on the angle of contact of 0.6µl droplets. Figures are mean angles plus or minus standard error from 10 measurements. Average percentage reduction in angle due to formulant is indicated.

CONTACT ANGLES (°)

M.inodora	DISTILLED WATER	0.5% (v/v) FORMULANT	% REDUCTION DUE TO FORMULANT
ADAXIAL	61.44 ± 0.88	48.19 ± 1.36	21.56
ABAXIAL	63.27 ± 1.16	43.06 ± 1.20	31.94
B.vulgaris			
ADAXIAL	62.75 ± 0.48	52.12 ± 1.32	16.94
ABAXIAL	63.00 + 3.39	50.94 ± 1.42	19.14
G.aparine			
ADAXIAL	63.50 ± 0.28	60.13 ± 1.17	5.31
ABAXIAL	64.75 * 1.31	58.50 ± 1.11	9.65

3.3.8 Metabolism Studies

Extraction and separation of metabolites of ^{14}C-clopyralid from the apices of treated plants revealed that metabolism of clopyralid had occured, and that the pattern of this metabolism differed in each of the 3 test species (figure 3.9). Seven and 14 days after the application of ^{14}C-clopyralid to M.inodora and B.vulgaris, there was considerable formation of metabolites which were more polar than clopyralid acid, being recovered at Rf values 0-0.15. Rf values were a measure of the polarity of radiolabelled molecules separated in this solvent system, and 35-57% of ^{14}C-activity was measured in this region. At

Plate 3.2A S.E.M G.aparine adaxial cotyledon X 125

Plate 3.2B S.E.M G.aparine adaxial cotyledon X 300

Plate 3.2C S.E.M G.aparine adaxial cotyledon X 1250

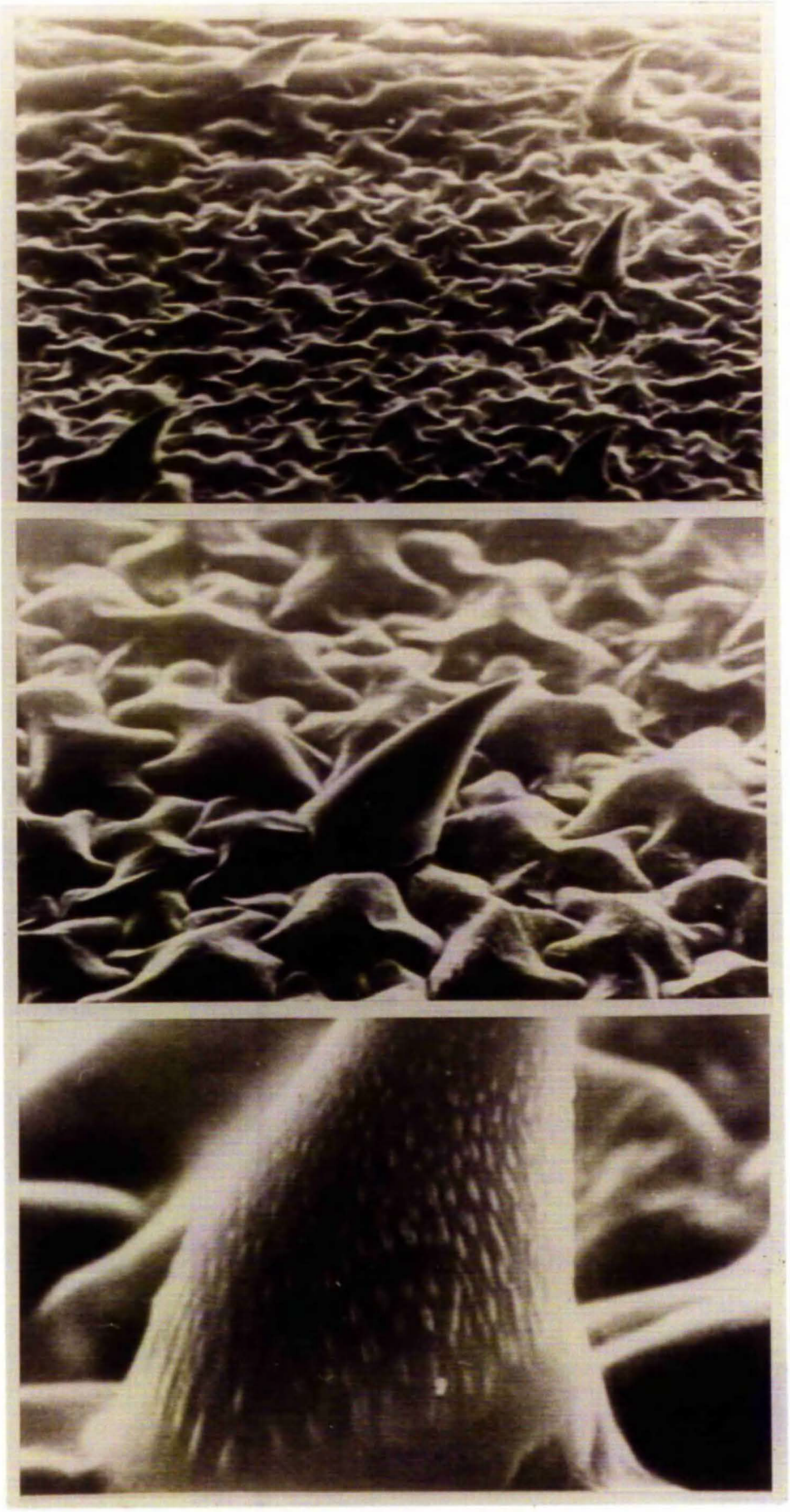
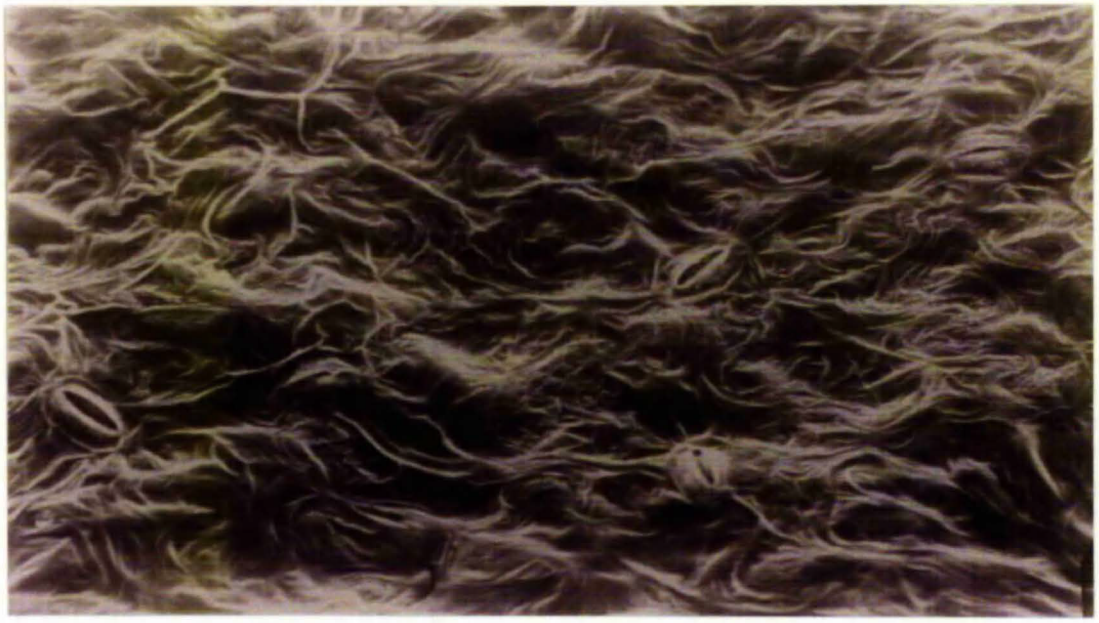
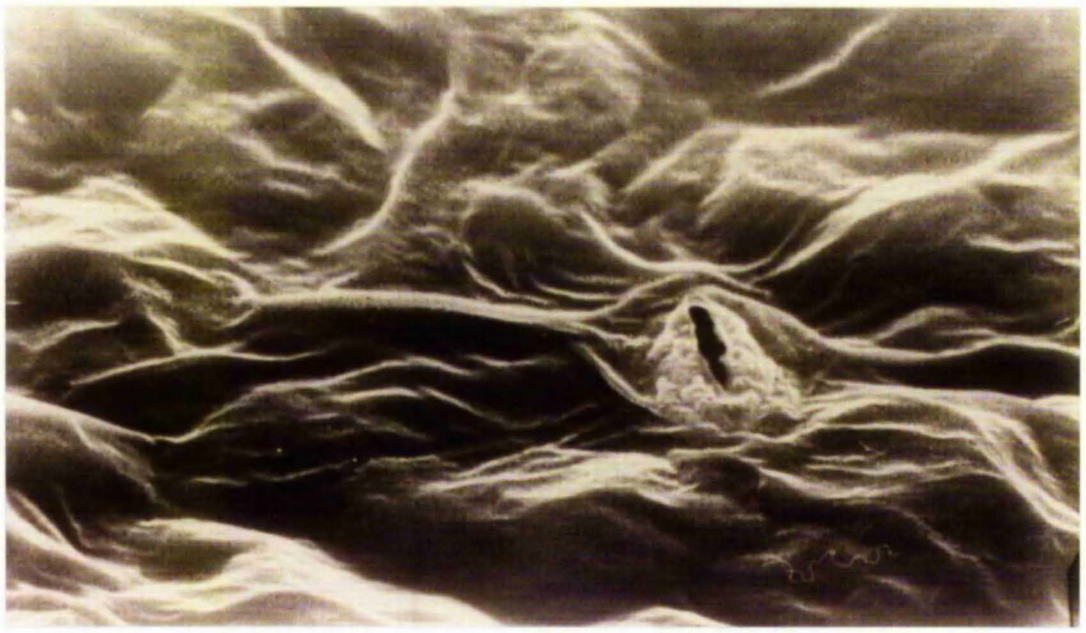


Plate 3.3 S.E.M B.vulgaris adaxial 3rd leaf X 300

Plate 3.4 S.E.M M.inodora adaxial 3rd leaf X 1250





both sample times there were comparable amounts of ^{14}C-clopyralid (free acid) recovered from these two species (Rf 0.15-0.25). This accounted for between 33 and 44% of total recovered activity. A metabolite, or group of metabolites, appeared in M.inodora at both 7 and 14 days after treatment which had an Rf value of 0.45-0.55. This was not detected in B.vulgaris.

TLC of metabolites of ^{14}C-clopyralid in G.aparine revealed that after 7 days approximately 60% of recovered activity was associated with free clopyralid acid. Approximately 50% of the remainder was recovered as more polar metabolites (Rf 0-0.15). After 14 days 50% of recovered radioactivity was in a form more polar than the parent molecule, with clopyralid acid accounting for 35% of recovered activity. The remaining ^{14}C-activity was recovered as compounds less polar than clopyralid-acid. Following extractions at both sample times, in each species, 10-15 % of applied ^{14}C-activity was measured in the tissue pellet.

3.3.9 Qualitative Estimation of the Activity of extracted clopyralid Metabolites using a sensitive tissue Bioassay

Figure 3.10 illustrates that clopyralid promoted elongation of G.aparine shoot explants at concentrations ranging from 10^{-6} M to 10^{-4} M with maximum elongation at 10^{-4} M. 10^{-3} M clopyralid produced no increased elongation with respect to controls. IAA and clopyralid acid have similar profiles of activity, although auxin was more active than clopyralid with maximum elongation being promoted by a concentration of 10^{-5} M. The elongation of tissue in response to IAA demonstrated the physiological relevance of this system in assaying hormonal activity.

Table 3.9 records the results of the qualitative estimation of clopyralid metabolite activity using the G.aparine shoot explant bioassay. Clopyralid acid recovered from both M.inodora and B.vulgaris was active, producing a significant increase ($p< 0.02$) in explant length compared to controls. In M.inodora metabolites, those both more and less polar than clopyralid showed significant activity ($p<0.001$) compared to control. This activity was not significantly different to that measured in

response to clopyralid acid. Metabolites in B.vulgaris also gave increased elongation ($p<0.02$) which again was not significantly different in extent to that induced by extracted clopyralid acid.

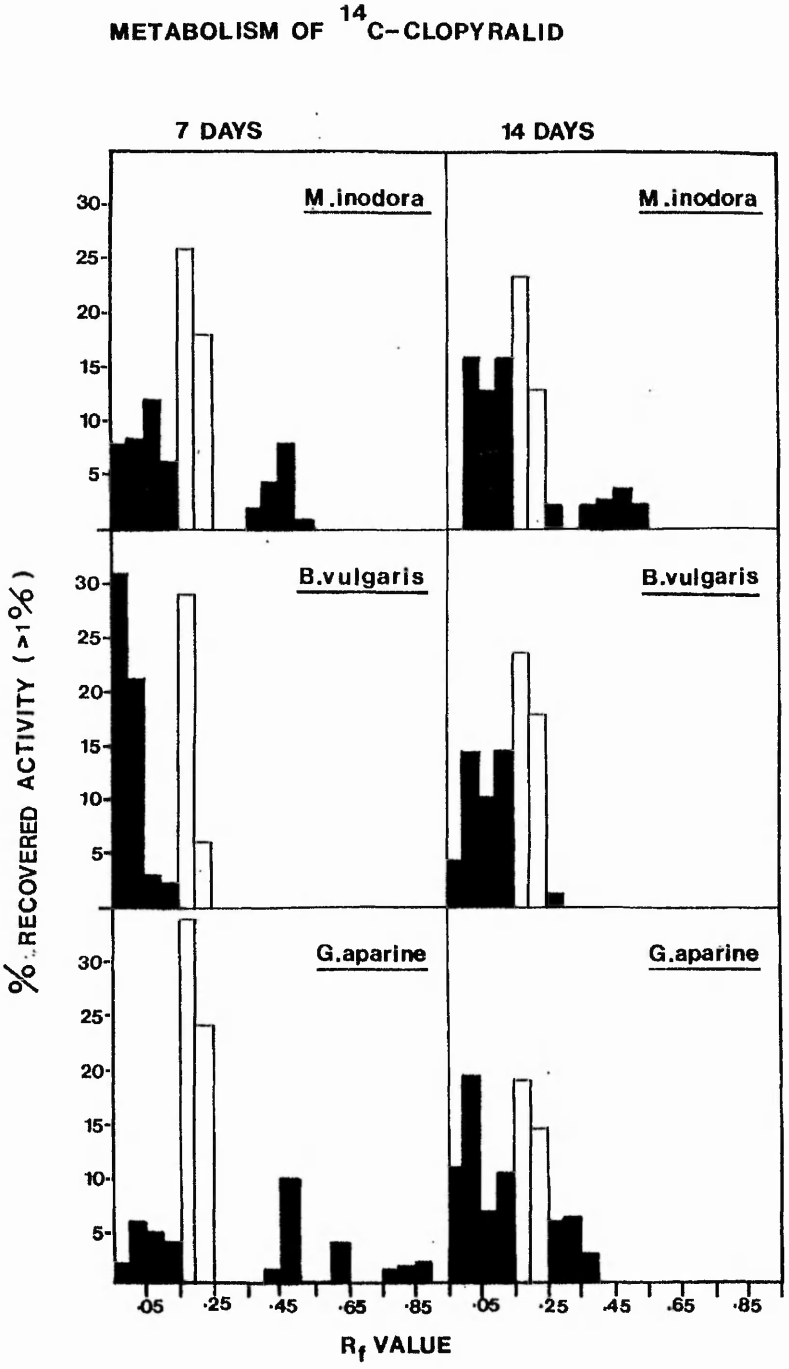


Figure 3.9 TLC of ^{14}C-clopyralid metabolites extracted from the apices of plants 7 and 14 days after herbicide treatment. (▭)- clopyralid acid, (■) - metabolite.

Table 3.9 Bioassay of clopyralid metabolites. Values are mean G.aparine shoot explant lengths plus or minus standard errors from 32 measurements on 4 occasions.

SECTION OF TLC PLATE (Rf)	M.inodora	B.vulgaris
CONTROL	23.03 ± 0.16	24.13 ± 0.23
0-0.15	25.84 ± 0.17	27.21 ± 0.32
0.15-0.25 (clopyralid acid)	26.03 ± 0.64	28.24 ± 0.81
0.45-0.55 (less polar metabolites)	24.14 ± 0.14	———

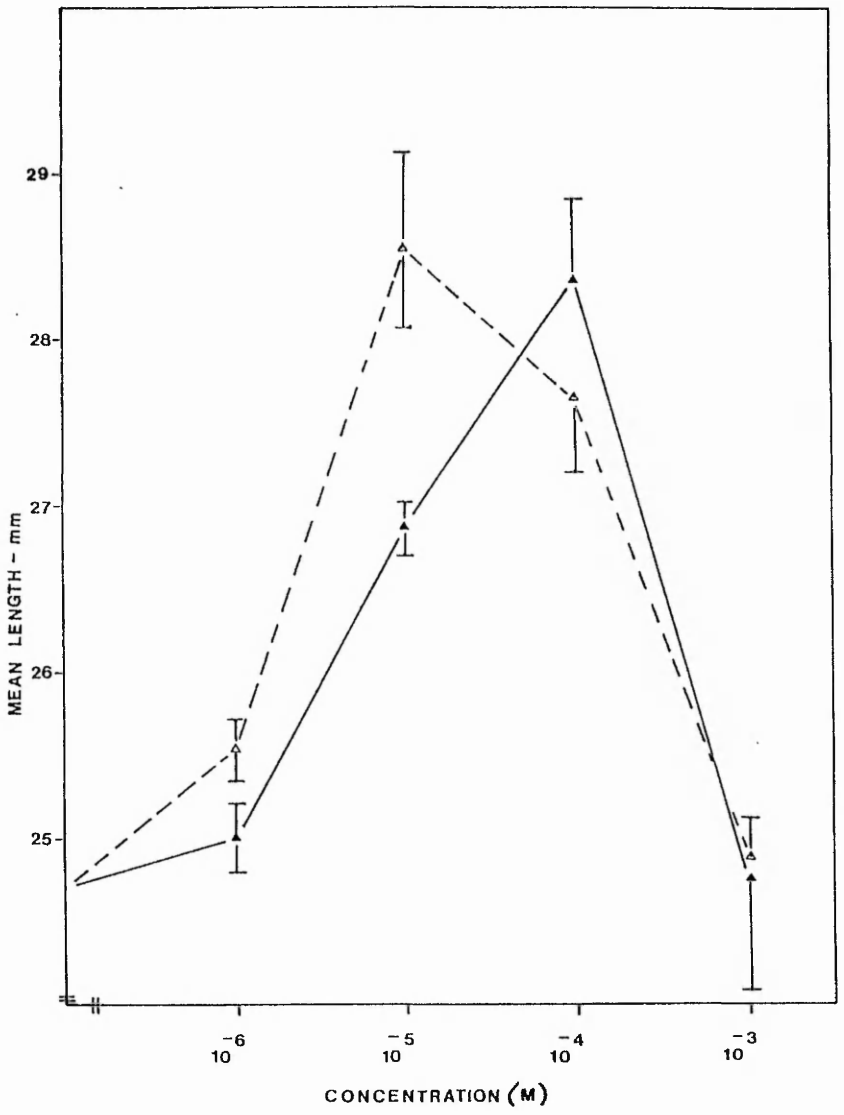


Figure 3.10 Extension of 20mm G.aparine shoot explants 48h after incubation with clopyralid acid (—▲—) and IAA (- Δ--) Values are mean lengths plus or minus the standard. error from 32 measurements on 4 occassions.

3.4 Discussion

Visual examination of the morphology of M.inodora and B.vulgaris plants at the growth stages studied suggested reasonable spray interception by leaves of the two species. Retention studies using tartrazine confirmed this and also showed that, on interception, there was spray retention in both species. Differences in volumes retained were attributed to differences in leaf shape, size and area rather than to gross differences in leaf surface properties since, using the spray parameters employed, distinct droplets were formed on the leaves of each species.

Preliminary uptake studies in M.inodora and B.vulgaris showed that clopyralid uptake occurred in both species. A pattern of constant removal of ^{14}C-activity from the leaf surface without its accumulation in treated leaf tissue indicated rapid translocation of ^{14}C-activity in both tolerant and susceptible plants (tables 3.2 and 3.4). These findings necessitated the in-depth experiment on uptake and movement of ^{14}C-clopyralid in sprayed plants, particularly since these initial experiments indicated greatest uptake in tolerant B.vulgaris. Results of the comprehensive study of ^{14}C-clopyralid uptake and translocation in M.inodora and B.vulgaris yielded a great deal of information about the behaviour of the compound within treated plants. The findings of the study are discussed in relation to previous work with clopyralid and picloram.

In both species, uptake of ^{14}C-clopyralid was rapid with 5% and 11% of applied activity entering M.inodora and B.vulgaris respectively after only 8 hours. These figures rose to 6% and 15% 24 hours after treatment. This rapid penetration of clopyralid has been reported in a number of plant species. Turnbull and Stephenson (1981) found 96% uptake after 24 hours in rapeseed and, over the same time period, O'Sullivan and Kossatz (1984) measured 25% uptake in Cirsium arvense. Both these values are greater than the findings of this study, however, apart from species differences the former study used 10% ethanol in their application solution which may have

hydrated or dissolved the plant cuticle maximising uptake. The latter work, although it used the monoethanolamine formulation of clopyralid, applied 5µl droplets held in position by lanolin, a procedure which can greatly alter uptake patterns (Schultz and Burnside, 1980). Both studies differed from this in using untreated plants. Even within the current study the effect of application regime and differences resulting from the use of sprayed versus unsprayed plants can be highlighted.

The application of 0.2µl droplets over the vein in B.vulgaris results initially in a more rapid uptake than the random placement of 0.5µl droplets (table 3.6). This was probably due to factors such as differences in cuticular properties over vascular tissue, the high surface area to volume ratio of small droplets, and the influence of increased speed of translocation (due to reduced long distance transport) on uptake. The fact that absorption is greatest following large droplet application 168 and 336 hours after treatment may result from an increased drying period rendering large droplets a greater potential for sustained absorption.

On comparison of preliminary uptake data using unsprayed plants to the comprehensive work on sprayed plants, differences were found in measured ^{14}C-clopyralid uptake. For example, after 168 and 336 hours, 34% and 28% respectively of applied ^{14}C-activity was recovered from the surface of unsprayed B.vulgaris plants where 0.2µl droplets were applied over the main vein. These figures were 56% and 40% in the sprayed plant experiment. This difference may have arisen as a consequence of decreased translocation in the sprayed plants. Differences in translocation in sprayed and unsprayed plants have been found with ^{14}C-fluroxypyr in Viola arvensis (Thompson, Sanders and Pallett, 1986). These examples highlight the dangers of close comparisons between experimental studies.

In this study the fact that a greater proportion of applied compound enters B.vulgaris than enters M.inodora eliminates differences in uptake being responsible for clopyralid selectivity.

Rapid and complete translocation of absorbed ^{14}C-activity was found in both species with up to 93% of absorbed activity being detected at sites remote from the treated leaf. Translocation rates were similar in the 2 species eliminating this factor as a possible selectivity determinant. Rapid translocation of clopyralid has been measured in many instances. Hall (1985) found that 87% of ^{14}C-clopyralid absorbed by Canada thistle had been translocated out of the treated leaf 6 days after treatment. In the same species, Devine and Vanden Born (1985) measured 78% translocation of applied clopyralid after 6 days. Using ^{14}C-picloram, Hallmen (1974) found 69% of recovered activity remote from the tissue of application 3 days after treatment of sunflower seedlings. Since clopyralid is readily transported out of the treated leaf, it's movement in the two species was ascertained in order that any differences in accumulation sites could be measured and their importance to herbicide selectivity assessed.

Accumulation of ^{14}C-activity in the apical and youngest leaf tissues of both species indicated a phloem mobile activity moving in the symplast in association with photosynthate on a route which could be correlated to photosynthate source to sink movement. This pattern of translocation was further demonstrated in the experiments with G.aparine. Uptake and translocation values were in the same range in this species as in M.inodora and B.vulgaris. ^{14}C-clopyralid application to 3 different tissues resulted in translocation of activity to the most proximate assimilate sink (plate 3.1), whether this be the main shoot apex or developing lateral tissues basipetal or acropetal to the site of application. There was little movement into developed tissues, eg cotyledon to cotyledon, and in all three test species there was little or no movement into root tissue. This pattern of clopyralid movement is consistent with many other studies. O'Sullivan and Kossatz (1984) found that the shoot apex accumulated most translocated ^{14}C-clopyralid in Canada thistle, and Hall (1985) measured 76% of recovered ^{14}C-clopyralid in the growing point of sunflower plants 6 days after application. Most work with ^{14}C-clopyralid supports the idea that movement of the herbicide in plants is from the site of application to the nearest major growing points. These may

be both acropetal and basipetal and are dependent on application site and plant growth stage. A great deal of work has been done on the perrenial weed Canada thistle, and it has been found that the translocation of a significant amount of herbicide to the highly active root system from foliar application is vital to its action. Devine and Vanden Born (1985) found 29% of applied ^{14}C-clopyralid in the roots of this species 6 days after foliar application, whilst Turnbull and Stephenson (1985) found 33% of applied ^{14}C-clopyralid in the roots after 9 days. The lack of accumulation in root tissue in this study most likely reflects the metabolic status of the roots in the test species at the growth stages used.

Ambi-mobility of clopyralid was shown in rapeseed by Turnbull and Stephenson (1981), and they suggested that herbicide tolerance in rapeseed may be due to the tendency of clopyralid to leak into the apoplast, thus preventing its reaching a symplastic site of action. From this study (particularly the absence of any accumulation of activity at leaf margins in G.aparine autoradiographs) no evidence was found of significant apoplastic movement of foliar applied clopyralid.

Losses of recoverable ^{14}C-activity were evident with each test species. Approximately 30% of applied radiolabel was lost after 3 days in M.inodora. In G.aparine and B.vulgaris losses were more acceptable at around 10% after this period. No volatilisation of ^{14}C-clopyralid was measured over the experimental period and root exudation was thought to be unlikely considering the lack of accumulation of activity in root tissues. However, root exudation of this compound has been found (Turnbull and Stephenson, 1985) in Canada thistle, and the possibility exists that leakage of activity into xylem vessels in the roots, coupled with exudation, could result in clopyralid being constantly being moved around the plant without root accumulation. The situation seems unlikely, since it would have resulted in very extensive distribution of activity around the plants and this was not found. Since the losses were most in the susceptible species it was felt unnecessary to undertake measurement of root exudation or possible losses of activity via metabolism to $^{14}CO_2$. The losses may be partially explained by the morphology of M.inodora. The delicate compound leaf meant

that following clopyralid damage, tissue came into contact with the compost making complete recovery difficult. This problem could have been avoided, in hindsight, by covering the pot surfaces with polythene. Nevertheless, the aims of the study were reached and it was conclusively shown that neither the extent of clopyralid uptake, nor the speed or direction of its subsequent translocation, were responsible for the differential activity of clopyralid in the test species.

Possible reasons for the higher ^{14}C-clopyralid uptake in B.vulgaris compared to the weed species were considered. The hydrophilic nature of the herbicide (in monoethanolamine salt formulation), and its consequent non-retention in the chloroform washes of cuticular waxes, suggests that a major role of the formulant solution is likely to be that of increasing the surface area of contact of clopyralid with the leaf, and so increase the potential for its entry into the epidermis via an aqueous route (Bukovac, 1976). Investigations of the effect of clopyralid formulant on the angle of leaf contact of water droplets revealed little difference between its effectiveness in B.vulgaris and M.inodora . That the contact angles measured were below 90° suggested that wax was not a prominent feature on these leaves (Holloway, 1968), and S.E.M photographs of the leaf surfaces confirmed this. Adaxial surfaces of the two species were visually similar with no structured waxes apparent. Possible explanations for the difference in herbicide uptake include B.vulgaris perhaps having a more greatly hydrated cuticle than M.inodora, offering more aqueous 'channels' . Another possibility is that B.vulgaris has a higher growth rate, at this development stage creating more powerful sinks for phloem movement and resultant uptake. In G.aparine, contact angles on cotyledon tissues were reduced little by formlulant solution, probably due to the abundant trichomes on the leaf surface. These trichomes may have offered sites of preferential herbicide entry due to delayed cuticular development at their base (Sifton, 1963). Wyrill and Burnside (1976) found good absorption of 2,4-D and glyphosate in common milkweed and attributed it, in part, to the presence of trichomes on the adaxial leaf surface.

It must be noted that syringe droplets are delivered under static rather than impact conditions and this should be borne in mind when discussing contact angle results and their relevance to the spray situation.

In this study, each species efficiently translocated ^{14}C-clopyralid out of the treated leaf and so it was decided that an investigation of the form of radiolabel recovered from young leaf and apical tissues (i.e. the major sites of herbicide accumulation) was appropriate in order to assess whether variations in the extent and pattern of clopyralid metabolism, measured after 7 and 14 days could contribute to clopyralid selectivity. A similar proportion of ^{14}C-activity (10-15%) was unextractable from tissues of each test species. This was thought to be due to irreversible binding of ^{14}C-clopyralid to proteins and structural carbohydrates, presumably associated with the cell wall. This is similar to the findings of Hall (1985) who measured an average of 9% of recovered activity bound in this way in Canada thistle over a 6 day period following treatment with ^{14}C-clopyralid.

Some researchers have found that clopyralid metabolism does not take place in certain plant species (Bauriedel et al, 1974; Turnbull and Stephenson, 1981) however in this study there was considerable formation of polar conjugates of ^{14}C-clopyralid in each test species after 7 and 14 days. These accounted for 18%-60% of recovered activity and may be correlated with the water-soluble metabolite(s) of clopyralid found by Hall (1985) to account for 16% of recovered activity in Canada thistle and 70% in rapeseed after 6 days. This author concluded that it was likely to be a disaccharide-ester with a glucose subunit farthest from the clopyralid molecule. The other sugar was thought to be variable. There have been many reports of the existence of water-soluble complexes formed in picloram treated plants. Hallmen (1974) suggested that the formation of these in rape leaves rendered the herbicide immobile and that this contributed to the tolerance of this species to the herbicide. Sharma and Vanden Born (1972) concluded that ^{14}C-picloram was conjugated with sugars in Canada thistle, and Hallmen and

Eliasson (1972) found water-soluble complexes of picloram in wheat. As well as polar complexes a ^{14}C-clopyralid metabolite was found which was less polar than the parent molecule in this solvent system, accounting for around 10% of recovered activity. There is a possibility that it could be correlated with a metabolite extracted by O'Sullivan and Kossatz (1985) from Canada thistle where a single unidentified clopyralid metabolite accounting for 10% of absorbed activity 48 hours after treatment was mentioned. Extrapolation of Hall's study on Rf values of the amide of clopyralid, allows the tentative suggestion that the less polar metabolite may be the amide of the herbicide. The compound was interesting particularly because it appeared only in the susceptible species. It was found in M.inodora at both time periods and in G.aparine after 7 days. Since relative amounts of parent herbicide were similar in each species after 7 and 14 days accounting for between 34 and 58% of recovered activity, it was considered that assessment of the biological activity of the metabolites was a more important direction for further work than the elucidation of their chemical identity.

In a "bioassay" using clopyralid-sensitive tissue it was shown that both groups of metabolites had activity, indicating that they are herbicidally active or rapidly converted to active clopyralid within plant tissues. The non-polar metabolites found in the susceptible species did not appear (on qualitative estimation) to be more active than the parent molecule. It is possible, however, that the true activity of the metabolites was not expressed due to possible breakdown during the incubation period. Despite experimental design to avoid this it may be that certain complexes are highly unstable and Hall (1985) has demonstrated this, particularly with respect to pH. Considering the free acid to be the herbicidally active form of clopyralid, it is concluded that differential metabolism is unlikely to contribute to clopyralid selectivity.

Chapter 4. THE EFFECT OF CLOPYRALID ON PHOTOSYNTHESIS AND STOMATAL APERTURE

4.1 Introduction

Literature concerning the photosynthetic effects of phenoxy-alkanoic acid herbicides is sparse. Furthermore, there have been no recent studies on the effects of the pyridine auxin-type compounds on this important process. This is surprising considering the rapid epinastic (accelerated growth) effects which occur following pyridine application, which perhaps indicate an increased demand for photosynthates, similarly the large but variable fluctuations in plant metabolites which can take place following application, particularly those changes in reducing sugar content (as shown for clopyralid in chapter 2), which may also imply changes in photosynthesis.

The aims of the following experiments were to investigate the effects of clopyralid on photosynthesis rate in susceptible and resistant species. Experiments were also designed to measure effects of clopyralid on stomatal aperture, particularly since the phenoxyalkanoic acid herbicides have been found to influence the stomatal mechanism (Pemadasa and Jeyaseelan, 1976). In both experiments the importance of any differential effects with respect to herbicide activity and selectivity were considered.

Infra-red gas analysis was used to monitor photosynthetic CO_2 uptake since the sensitivity of this technique is considered to provide the most accurate means of measuring photosynthetic rate. Several methods of measuring stomatal apertures were assessed including both direct and indirect measurements.

4.2 Materials and methods

4.2.1 Plant Material and Herbicide Application

M.inodora and B.vulgaris plants were raised in J.Arthur

Bowers seed and potting compost in a glasshouse with a 14h day (200-400μM photons m^{-2} s^{-1}, PPFD). At the young seedling stage plants were thinned to one per 90mm pot and transferred to an environmental cabinet (Fisons model 600G3, type TTL, 14h day 20°C and 100μM m^{-2} s^{-1}, PPFD, 14°C at night). Plants were treated with 100g ai. ha^{-1} clopyralid when 3-4 leaves were fully expanded. Herbicide was applied using a laboratory pot sprayer.

4.2.2 Measurement of photosynthetic carbon reduction by infra-red gas analysis

Figure 4.1 outlines the experimental system used for in vivo measurement of photosynthesis. Six identical plant chambers (25mm X 25mm X 10mm) were connected in series to an automatic gas sampler (The Analytical Development Company ADC:WA161) and an infra-red CO_2 analyser (GP Instruments Ltd) in differential mode. Chambers were uniformly illuminated by a Camrex Solarcolour LGH/PS/U sodium fluorescent tube, the intensity of which could be varied by adjusting the height of the lamp above the chambers. Constant temperature was maintained at 20°C $\pm$ 2°C by a circulating water jacket. Air was pumped in from outside the laboratory via an external air-line and its humidity controlled by bubbling first through water and then through 100% glycerol at 20°C. After cleaning by dust filters, air was passed through each leaf chamber at a flow rate of 0.013 dm^2 s^{-1}, then dried through silica gel before entering the IRGA. Air from each leaf chamber was sampled at 5 minute intervals and a measure of the difference in CO_2 concentration (Δ ppm) between the sample and external air directly entering the analyser was automatically and continuously recorded on a chart recorder (Bryans Instruments). In differential mode, the analyser measured CO_2 content in the range of plus or minus 35ppm.

4.3.2 Experimental Design

For each experiment, plants were removed from the cabinet at 9.00am, treated with clopyralid and returned to the cabinet for 30 minutes to allow spray deposits to dry. During this period,

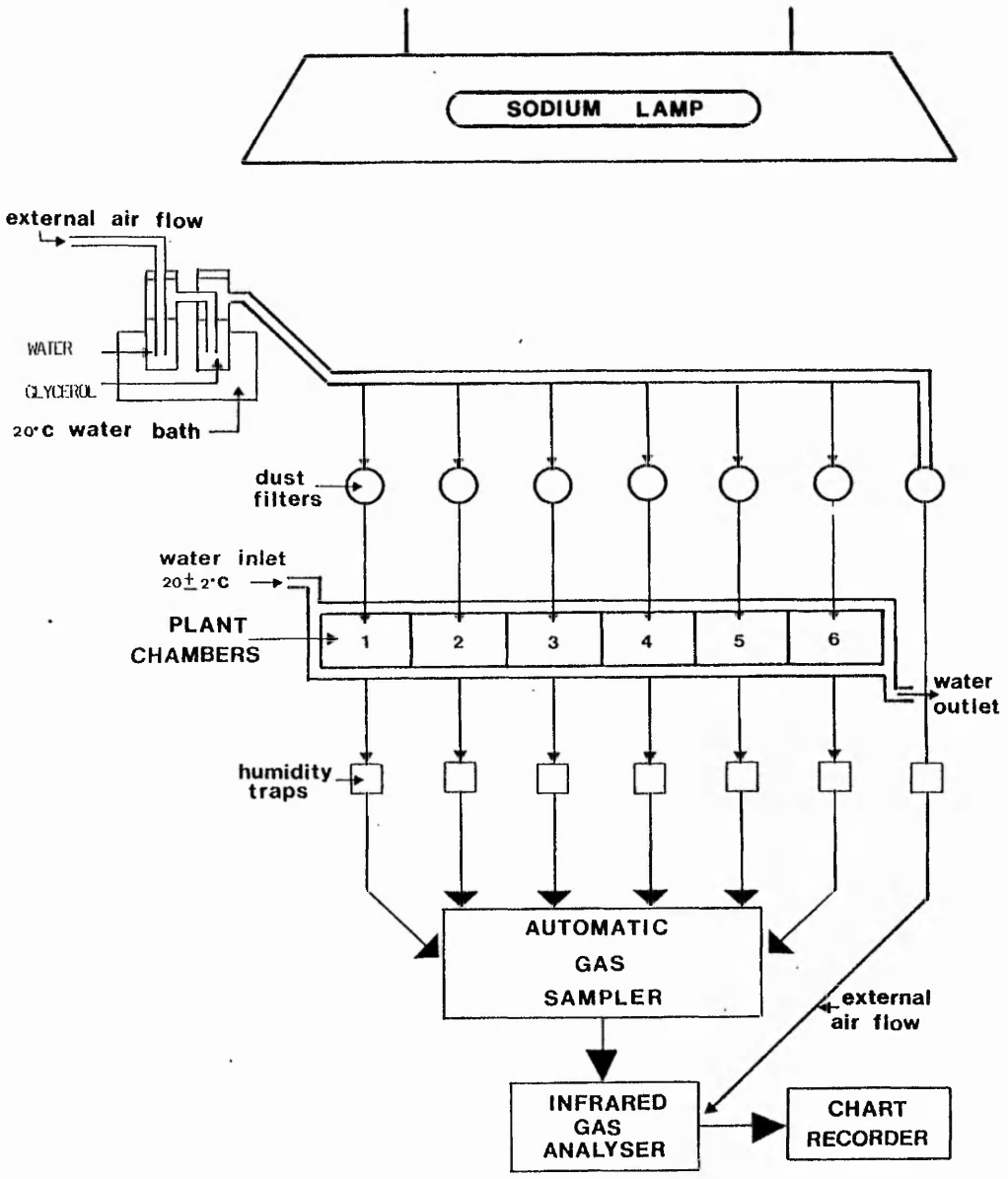


Figure 4.1 Experimental system for measurement of photosynthetic CO_2 evolution.

the gas analyser was calibrated using a standard gas containing 350ppm CO_2 (Air Products Ltd) and air made free of CO_2 by passage through soda lime. The third leaf of intact plants were then carefully secured in the experimental chambers using Kelseal Gel to provide an airtight seal. Control plant leaves were placed in two chambers, treated in two and two were sealed empty. After a 30 minute period of acclimatization, the chambers were connected to the anaylser and readings commenced at 15 minute intervals. Values were generally constant after 1 hour in the chambers and calculations commenced 4 hours after herbicide application. Rates of photosynthesis were calculated from the differences in CO_2 concentrations (Δ ppm) between chambers containing leaves and empty chambers, according to the method used by Sanders (1984), and were expressed as micromoles of carbon dioxide produced per square decimeter of leaf area per second ($\mu M\ CO_2 dm^{-2} s^{-1}$).

B.vulgaris leaf surface area (dm^2) was measured using a leaf area meter (LiCor, ΔT). With M.inodora the formula of Sanders (1984) was used, in which,

$$\text{Area } (dm^2) = \frac{[\ 2 \times (0.182 \times \text{length (cm)} \times \text{width (cm)}) + 0.285\ ]}{100}$$

Measurement of photosynthetic rates in M.inodora and B.vulgaris over the range 200-1000$\mu M\ m^{-2}\ s^{-1}$ (PPFD) was carried out to identify the optimal flux densities for photosynthesis in both species.

4.2.4 Effect of 100g a.i. ha^{-1} clopyralid on photosynthesis in M.inodora and B.vulgaris

The effect of 100g a.i. ha^{-1} clopyralid on third leaf photosynthesis in M.inodora and B.vulgaris was recorded at intervals over a 24 hours period. Six treated plants were compared to six control plants on 3 separate occasions for each species. Results were expressed as mean photosynthetic rate plus or minus standard error. For studies of photosynthesis over a

longer period, the third leaf of clopyralid-treated M.inodora plants were tagged with loose wire loops for identification purposes and all plants maintained in the environmental cabinet. After 1, 3, 7 and 14 days following herbicide treatment third leaf photosynthesis was measured as described above. For each time interval 6 treated plants were compared to 6 control plants and the whole procedure repeated three times. Results were expressed as photosynthetic rates plus or minus the standard error.

4.2.5 Effect of clopyralid on electron flow in B.vulgaris and M.inodora thylakoid fragments

Thylakoid fragments (type E, Hall, 1972) were prepared from M.inodora and B.vulgaris. Five g of leaf tissue was homogenised (Ultra-Turax) for 20 seconds in 20ml extraction medium (50mM Tricine-NaOH buffer, pH 7.5, 300mM NaCl, 2mM EDTA, 0.01% (w/v) BSA, 0.1% (w/v) PVP, 3mM $MgCl_2$) at 0°C and the homogenate filtered through 8 layers of muslin before centrifugation at 3000 X g for 1 minute (M.S.E Centaur). The pellet was resuspended in 6ml resuspension medium (5mM Tricine-NaOH buffer, pH 7.5, 100µM sucrose, 3mM $MgCl_2$, 2mM EDTA, 0.1% (w/v) BSA), and the suspension maintained in darkness at 4°C. A 0.5ml aliquot was assayed spectrophotometrically for chlorophyll content (Arnon, 1949), and the chlorophyll concentration of the preparation adjusted to 200µg ml^{-1}. Reaction mixtures (final volume 2ml) were prepared, in cuvettes, each containing 0.25ml thylakoid suspension (50µg chlorophyll), 0.2ml 20M Tricine-NaOH buffer pH8 and either 1.35ml H_2O (control) or 1.15ml H_2O plus 0.2ml clopyralid (treated) and 0.2ml 2,6-dichlorophenolindophenol (DCPIP) (0.18gl^{-1}) was added immediately before the cuvettes were exposed to saturating light (750µM photons m^{-2} s^{-1} PPFD, Griffin Halight 300 projector lamp). The absorbance of the solutions at 660nm was measured at one minute intervals using a grating spectrophotometer (Cecil Instruments CE303) and results calculated as µM DCPIP reduced mg Chl^{-1} min^{-1} by reference to a series of standard concentrations of the dye. The effects of clopyralid at final concentrations of 10^{-5} 10^{-4} and 10^{-3}M were determined.

4.2.6 Effect of Clopyralid on Stomatal Aperture in M.inodora and B.vulgaris

M.inodora and B.vulgaris seeds were sparsely sown in 5cm deep trays and maintained under glasshouse conditions as previously described. Plants at the 3-4 leaf stage were treated at 0730hrs with 100g a.i. ha^{-1} clopyralid using a Shandon chromatography sprayer (aerosol) to simulate a volume rate of 200lha^{-1}. Control plants were treated with distilled water at the same volume rate.

4.2.7 Measurement of Stomatal Aperture

Three 2.5mm^2 portions of third leaf tissue were dissected from 3 separate plants and immersed in 100% glycerol on a glass microscope slide. A coverslip was immediately (and carefully) placed over the sections and leaf surfaces examined using a light microscope (Vickers Instruments) at maximum light intensity. The stomatal apertures of 9-15 stomata on each section were measured at X400 magnification, with the use of an eyepiece graticule. Stomatal apertures were calculated as mean values plus or minus the standard error from both abaxial and adaxial leaf surfaces.

Two experiments were conducted using untreated plants to assess the suitability of the technique for measurements of herbicide effects on stomatal aperture. In the first, single stomata were observed and measured every 5 minutes for 20-25 minutes and any change in aperture noted. Secondly, stomatal apertures of untreated plants were measured from abaxial and adaxial surfaces of B.vulgaris and M.inodora every hour and the pattern of daytime fluctuations assessed. Mean values plus or minus the standard error were recorded. Stomatal ratio between leaf surfaces was also measured for both species. The effects of 100g a.i. ha^{-1} clopyralid on stomatal aperture in M.inodora and B.vulgaris were recorded hourly for 9-15 hours. Nine measurements were made from each of 3 sections at each time point and all experiments were repeated three times, making each value the mean of 81 individual stomatal measurements. Both surfaces were examined and results expressed as percentage of apertures from control plants.

4.3 Results

4.3.1 Measurement of Optimal Flux Density for Photosynthesis

Figures 4.2 and 4.3 demonstrate the effect of light intensity on the photosynthetic rates of M.inodora and B.vulgaris 3rd leaves. In M.inodora, rates ranged from 30.9μM CO_2 dm^{-2} s^{-1} at 240μM m^{-2} s^{-1}(PPFD) to 84.64μM CO_2 dm^{-2} s^{-1} at 720μM m^{-2} s^{-1}. In B.vulgaris rates were generally lower, ranging from 26.56 to a maximum 50μM CO_2 dm^{-2} s^{-1} at 750 μM photons m^{-2} s^{-1}. An optimum light intensity of 750μM photons m^{-2} $s^{-1}$was selected for further study with both test species.

4.3.2 Effects of 100g a.i. ha^{-1} Clopyralid on 3rd leaf photosynthesis rate in M.inodora and B.vulgaris

Figures 4.4 and 4.5 illustrate the effect of 100g a.i.ha^{-1} clopyralid on 3rd leaf photosynthesis rate in M.inodora and B.vulgaris up to 24 hours after treatment. In M.inodora, leaf photosynthetic rate was increased by the herbicide throughout the experimental period. Although treated plants showed high variability in rates, increases were statistically significant after 8, 12, 14, 18, 20 and 24 hours ($P<0.02$, $P<0.02$, $P<0.02$, $P<0.001$, $P<0.05$, $P<0.05$). In B.vulgaris 100g a.i. ha^{-1}clopyralid had no significant effect on the photosynthetic rate of the 3rd leaf up to 24 hours after treatment (figure 4.5).

The effects of clopyralid on 3rd leaf photosynthesis in M.inodora 1, 3, 7 and 10 days after treatment with 100g a.i. ha^{-1} are shown in table 4.1. The herbicide significantly increased photosynthetic rate to 318% of control values 1 day after treatment ($P<0.001$) and reduced it after 3 and 10 days ($P<0.01$, $P<0.001$). No result was recorded after 14 days since the condition of treated plants was highly variable making selection of representative samples impossible.

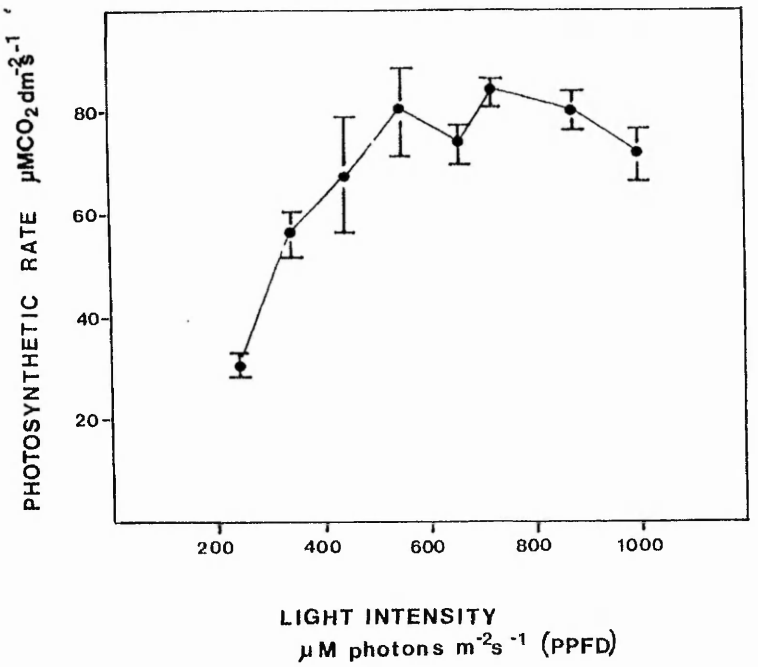


Figure 4.2 Effect of light intensity on photosynthetic rate in M.inodora 3rd leaf. Values are means plus or minus standard error from 4 replicate plants.

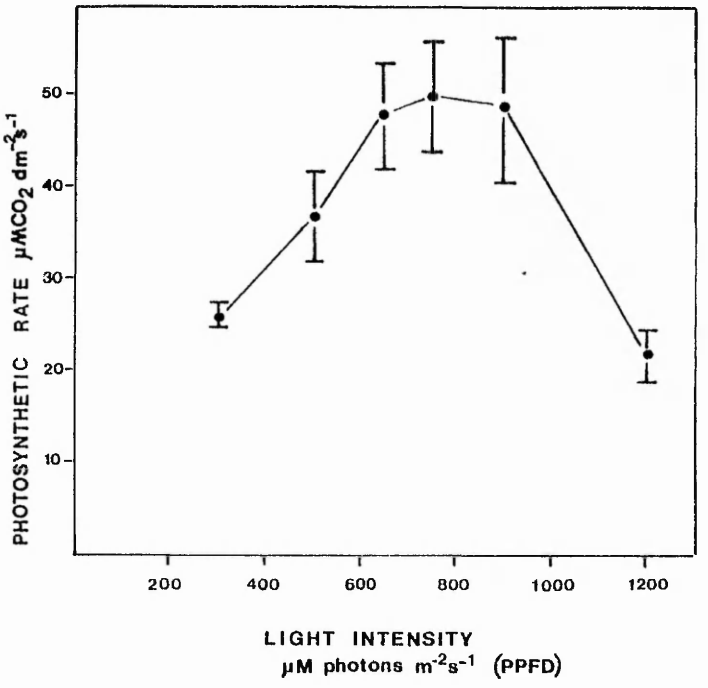


Figure 4.3 Effect of light intensity on photosynthetic rate in B.vulgaris 3rd leaf. Values are means plus or minus standard error from 4 replicate plants.

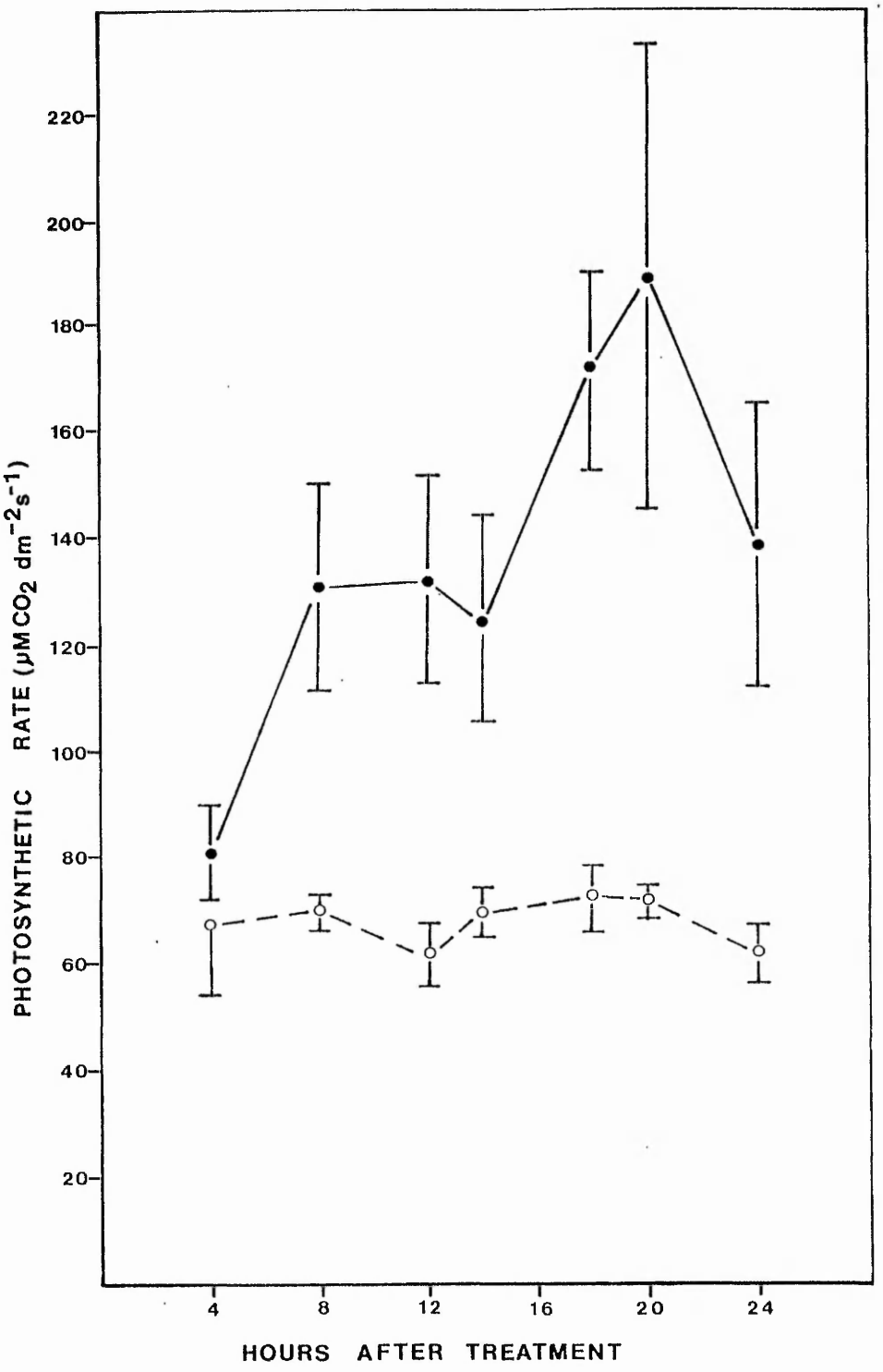


Figure 4.4 Effect of clopyralid (100g a.i. ha^{-1}) on rate of photosynthesis in M.inodora 3rd leaf. Values are means of 6 replicates and standard errors are indicated. Control (-o-), 100g a.i. ha^{-1} (—●—).

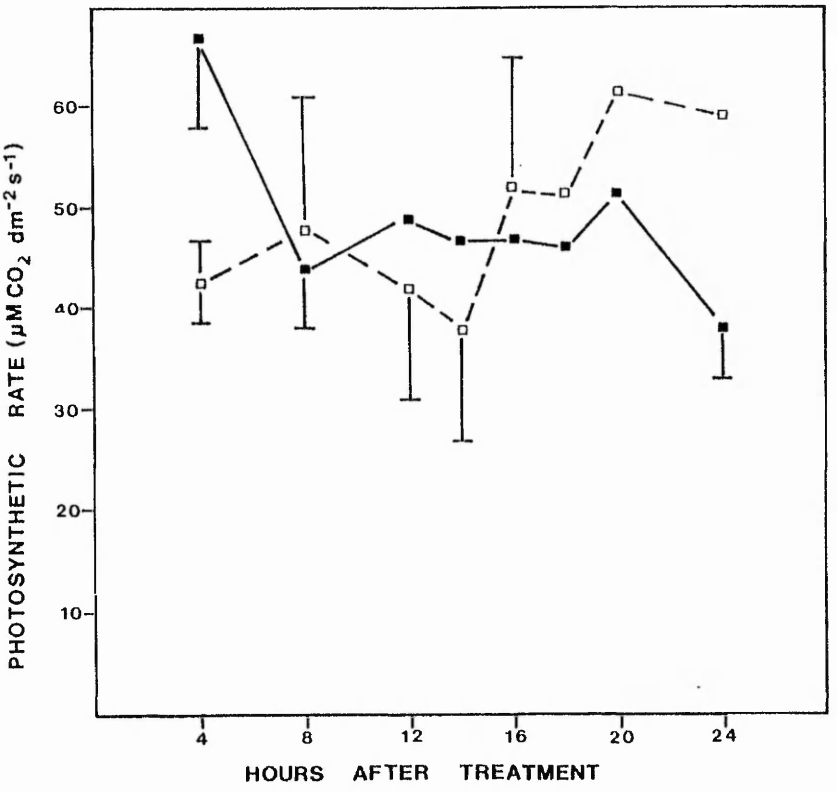


Figure 4.5 Effect of clopyralid (100g a.i. ha^{-1}) on rate of photosynthesis in B.vulgaris 3rd leaf. Values are means of 6 replicates and standard errors are indicated. Control (-□-), 100g a.i. ha^{-1} (—■—).

Table 4.1 Effect of clopyralid (100g a.i.ha^{-1}) on 3rd leaf photosynthesis in M.inodora over 14 days. Values are means of 6 replicates plus or minus standard error.

DAYS AFTER TREATMENT	PHOTOSYNTHETIC RATE (μM CO_2 dm^{-2} s^{-1})		
	CONTROL	100g a.i. ha^{-1} CLOPYRALID	% CONTROL
1	47.20± 8.52	149.99±17.39	318
3	96.49±12.69	31.16± 8.36	32
7	84.36±10.65	94.85± 8.00	112
10	95.15±10.56	22.53± 4.87	24
14	68.57± 9.12	—	—

4.3.3 Effect of clopyralid on electron flow in M.inodora and B.vulgaris thylakoid fragments

Figure 4.6 shows that clopyralid had no effect on DCPIP reduction in M.inodora or B.vulgaris thylakoid fragments at concentrations 10^{-5}M, 10^{-4}M or 10^{-3}M. Rates of DCPIP reduction in the presence of clopyralid did not differ significantly from those of controls.

4.3.4 Effects of clopyralid on Stomatal Aperture

Stomatal apertures of dissected leaf sections remained constant over a 20-25 min period of immersion in glycerol on a microscope stage. The clarity of images observed is indicated in plates 4.1 and 4.2 which record low and high power images of M.inodora and B.vulgaris abaxial leaf surfaces.

The patterns of daytime fluctuations in M.inodora and B.vulgaris adaxial and abaxial stomatal apertures are recorded in figures 4.7-4.10. A circadian rhythm in aperture was clearly apparent in these measurements, with stomatal closure commencing in the late afternoon. A midday-closure phenomenon was exhibited especially in M.inodora adaxial stomata.

Table 4.2 records the mean number of stomata (calculated from 45 fields of view) on the adaxial and abaxial third leaf surfaces of M.inodora and B.vulgaris. In both species there was a significant difference in stomatal density between the two surfaces. In B. vulgaris there were approximately twice as many stomata on the abaxial surface compared to the adaxial, whilst the opposite was true in M.inodora.

Table 4.2 Stomatal ratios in M.inodora and B.vulgaris

Species	Stomatal Density ($\bar{X}$ N° ± S.E.)		Stomatal Ratio
	ADAXIAL	ABAXIAL	adaxial/abaxial
M.inodora	4.95 ± 0.19	2.00 ± 0.15	2.48
B.vulgaris	6.69 ± 0.30	11.89 ± 0.30	0.56

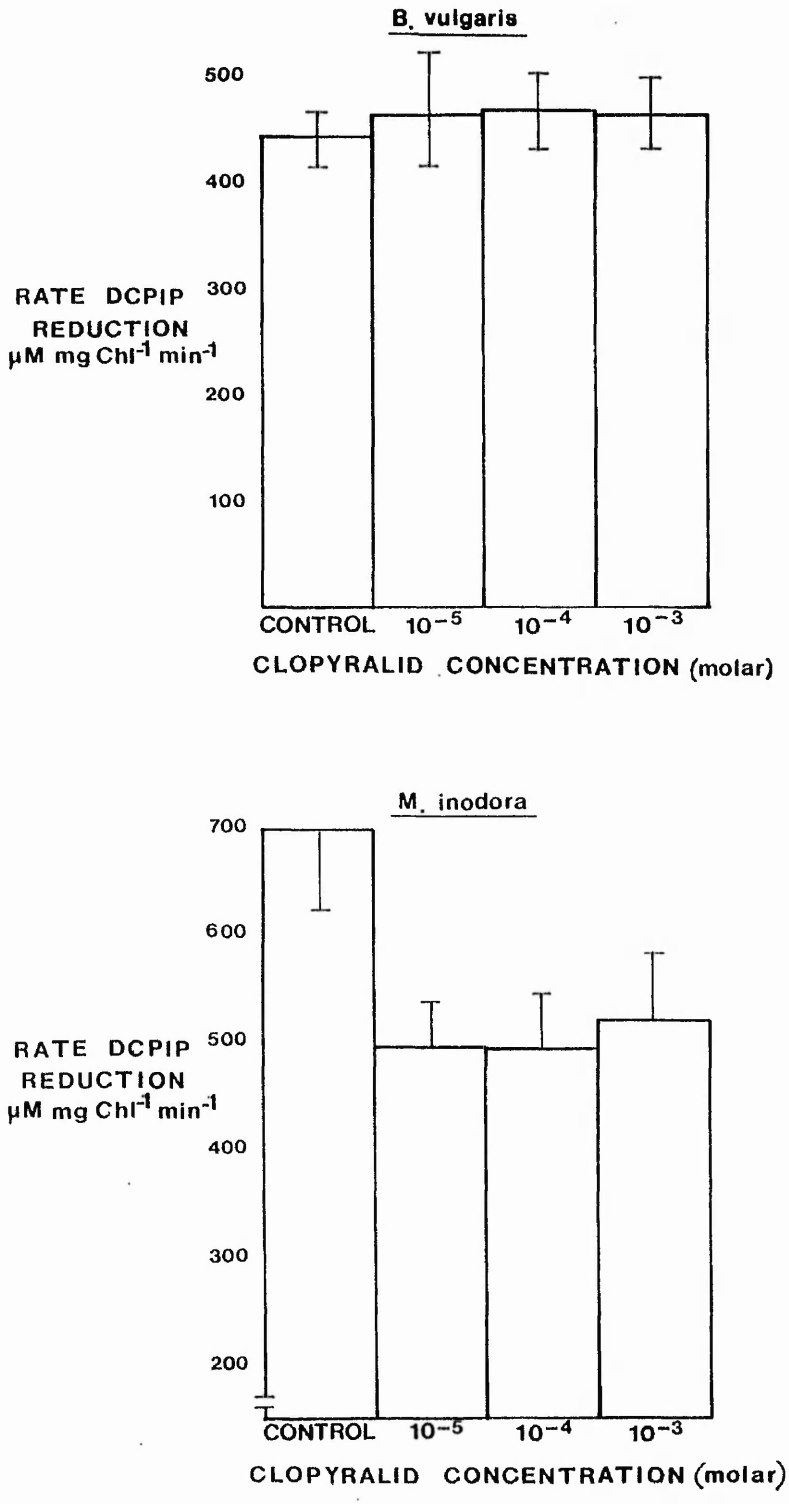


Figure 4.6 Effect of clopyralid on DCPIP reduction by electron flow in thylakoid fragments from M.inodora and B.vulgaris.

Plate 4.1A Abaxial leaf surface of M.inodora X 125.

Plate 4.1B Abaxial leaf surface of M.inodora X 500.

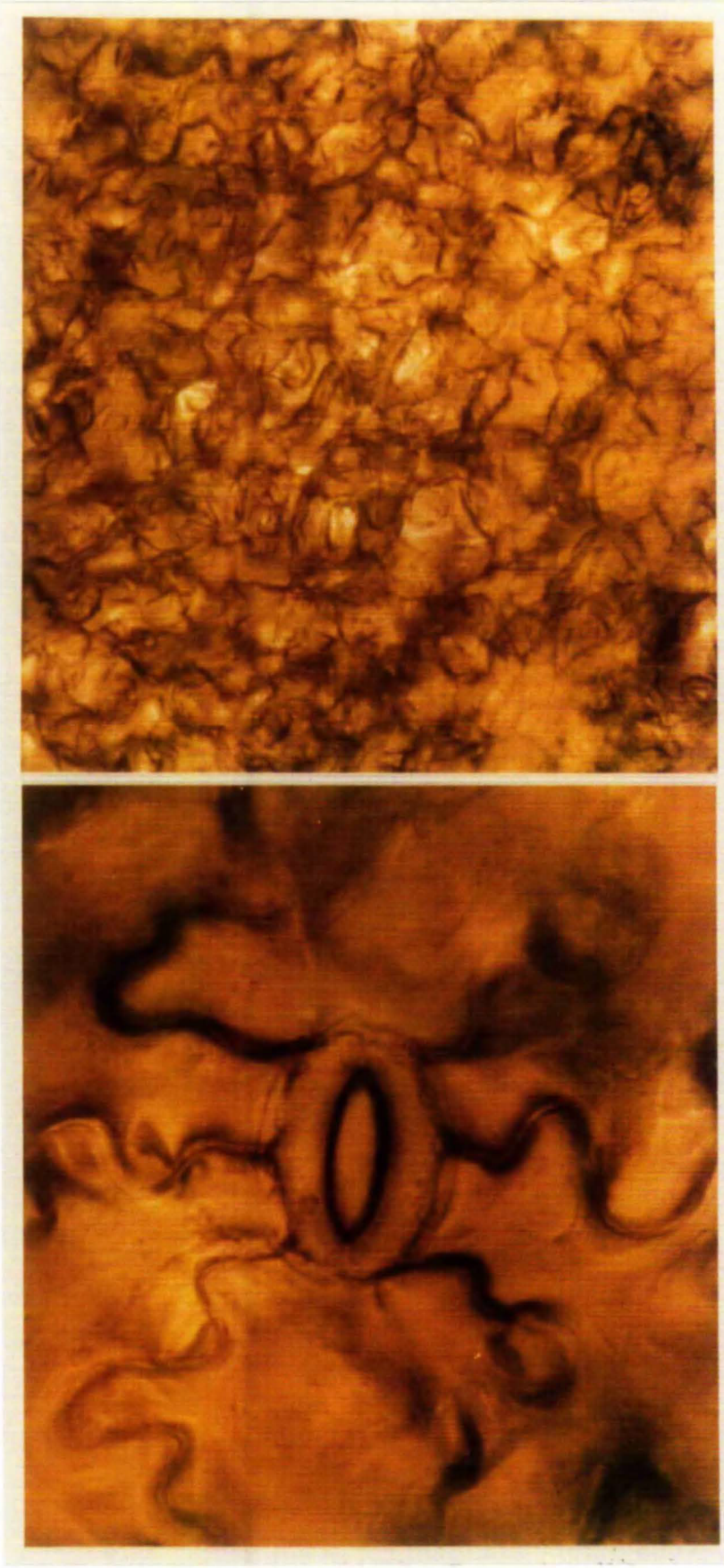
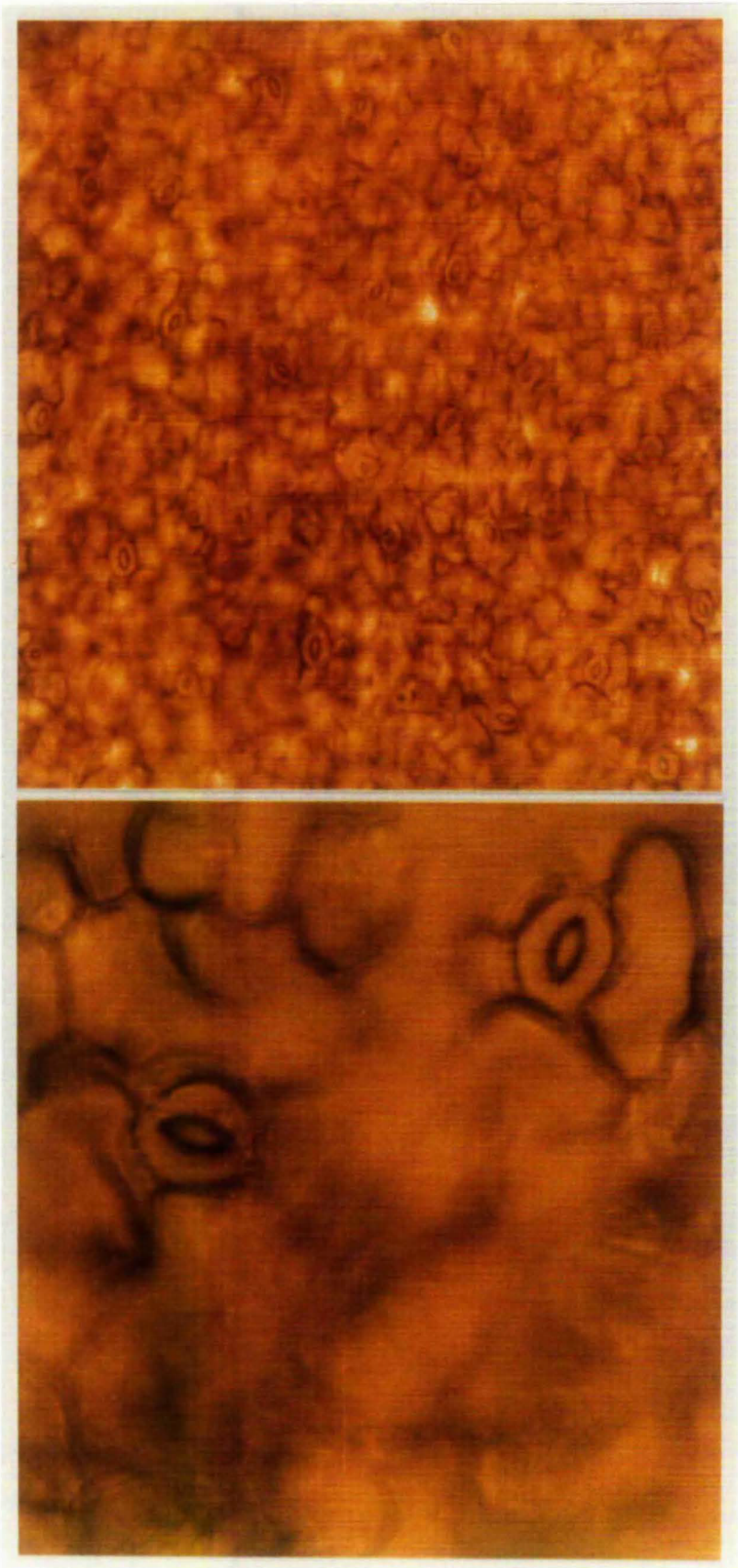


Plate 4.2A Abaxial leaf surface of B.vulgaris X 125.

Plate 4.2B Abaxial leaf surface of B.vulgaris X 500.



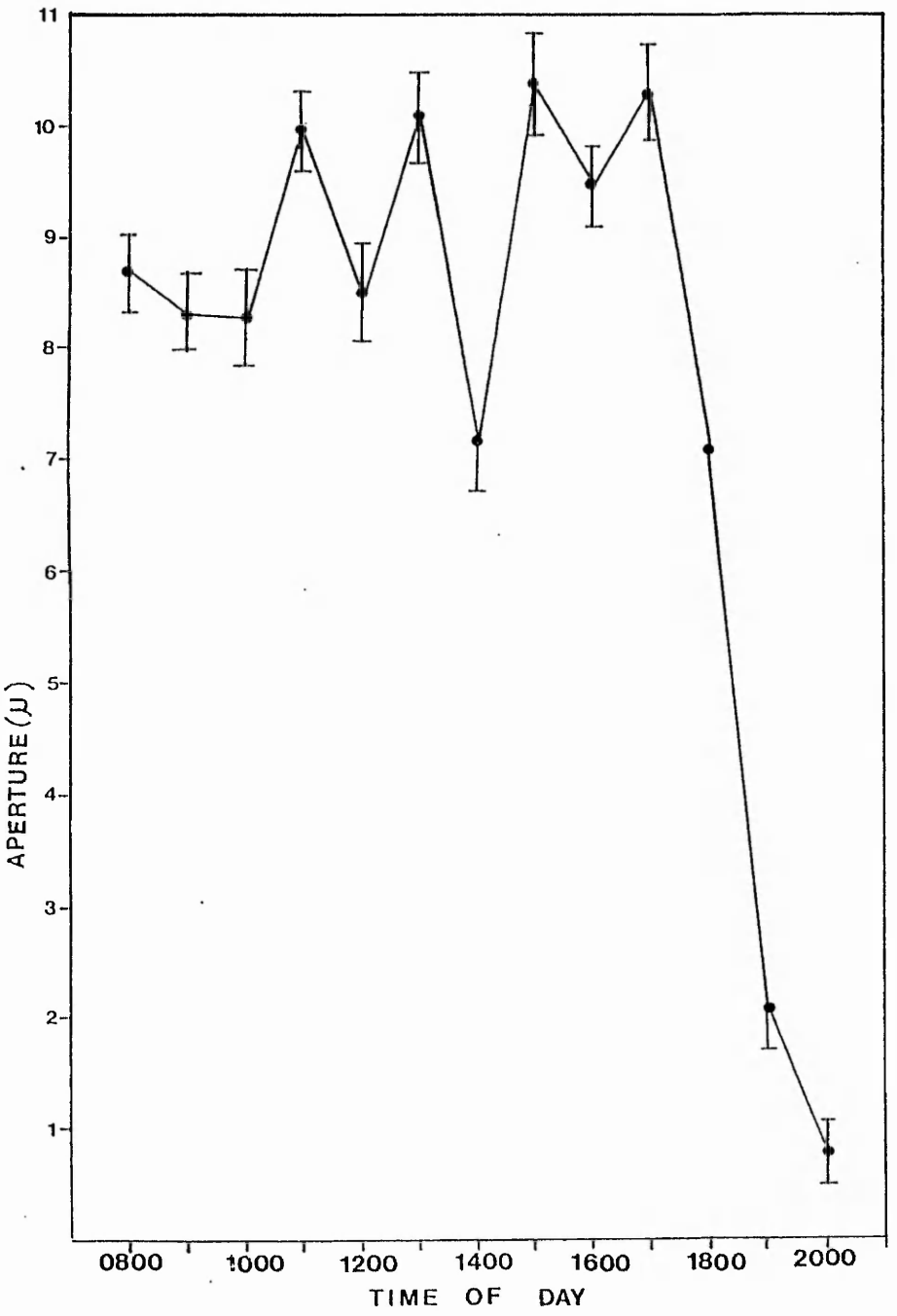


Figure 4.7 Daytime fluctuations in M.inodora abaxial stomatal aperture. Values are the mean of 45 replicates. Bars indicate standard error.

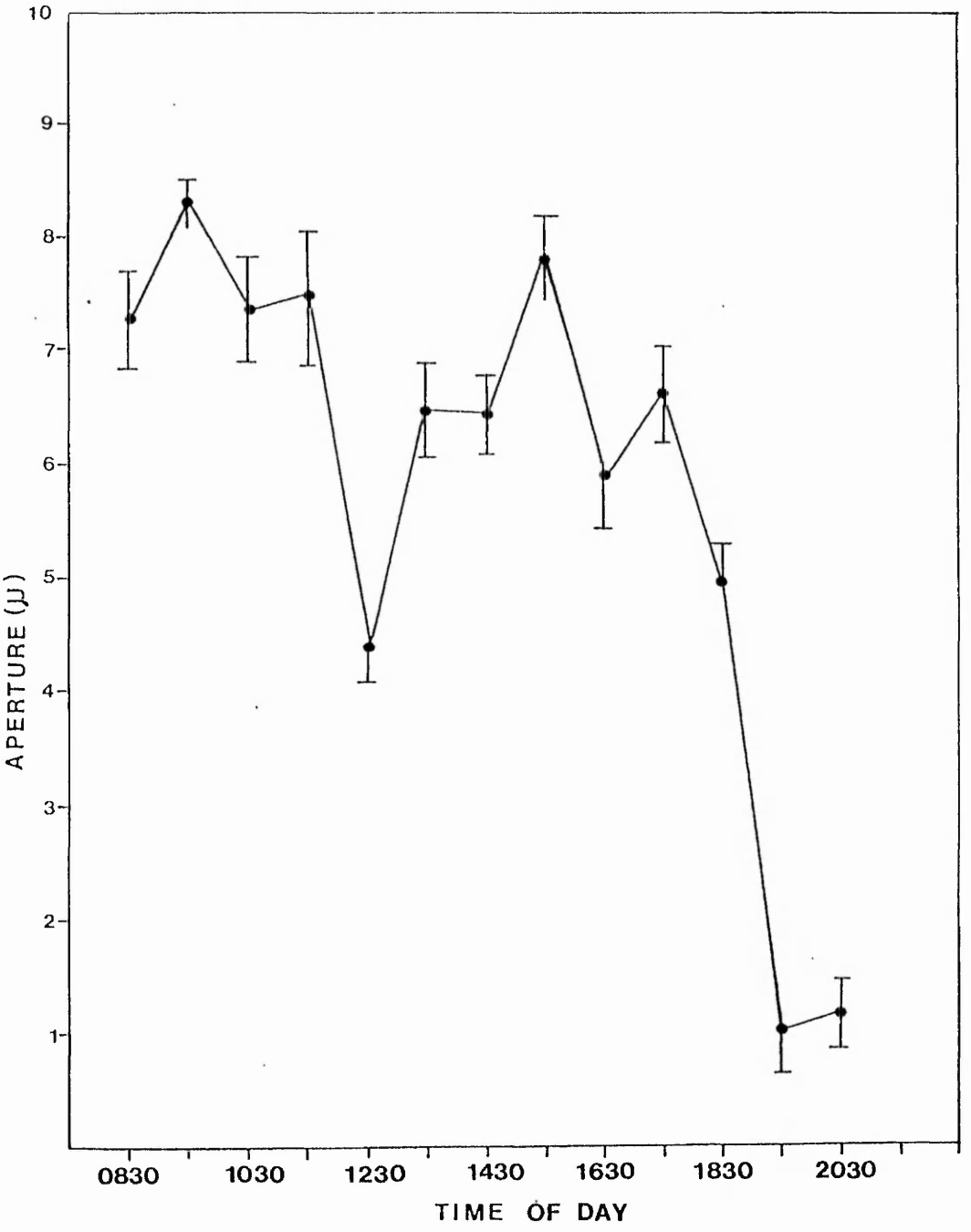


Figure 4.8 Daytime fluctuations in M.inodora adaxial stomatal aperture. Values are the mean of 45 replicates. Bars indicate standard error.

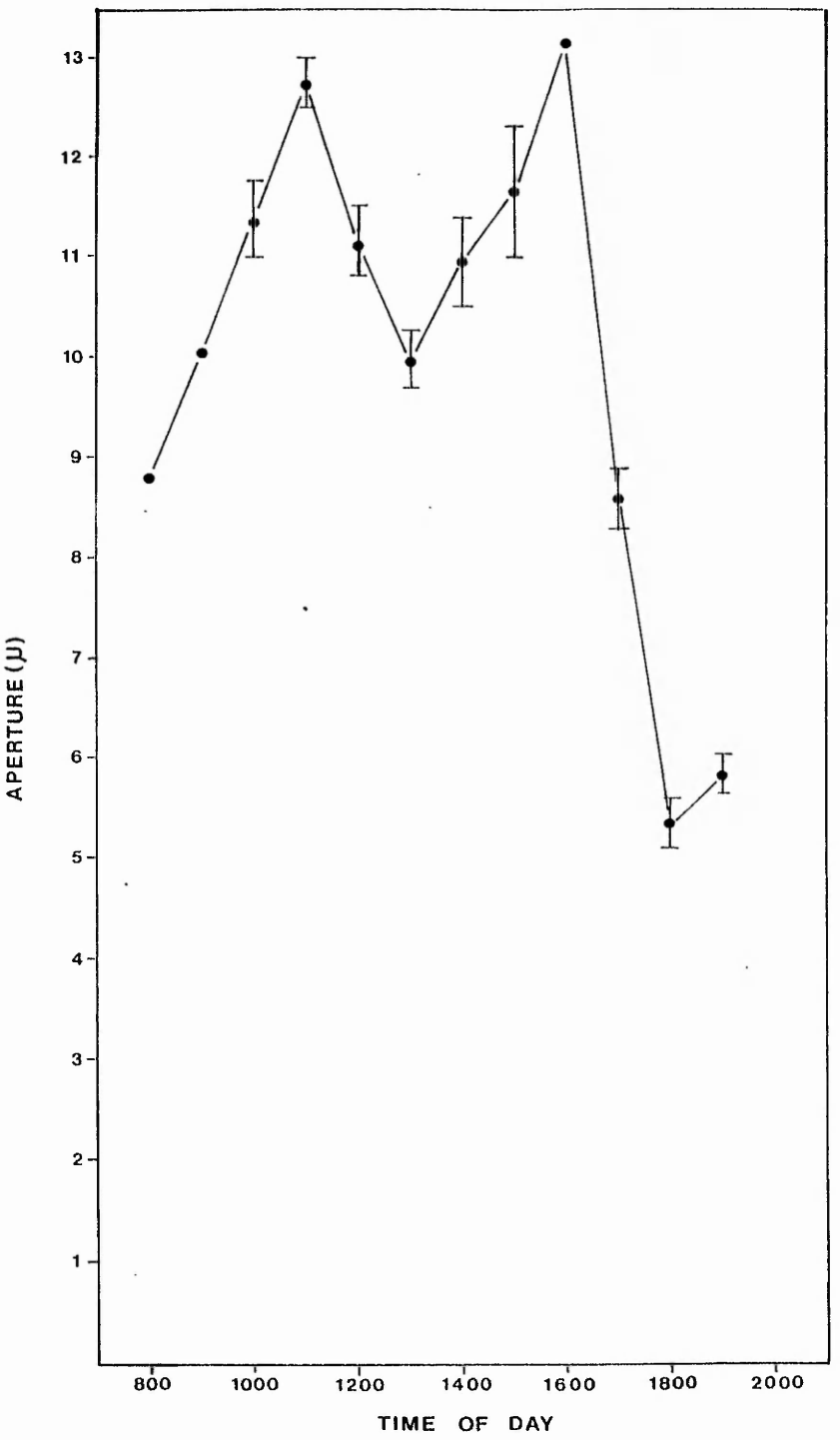


Figure 4.9 Daytime fluctuations in B.vulgaris abaxial stomatal aperture. Values are the mean of 45 replicates. Bars indicate standard error.

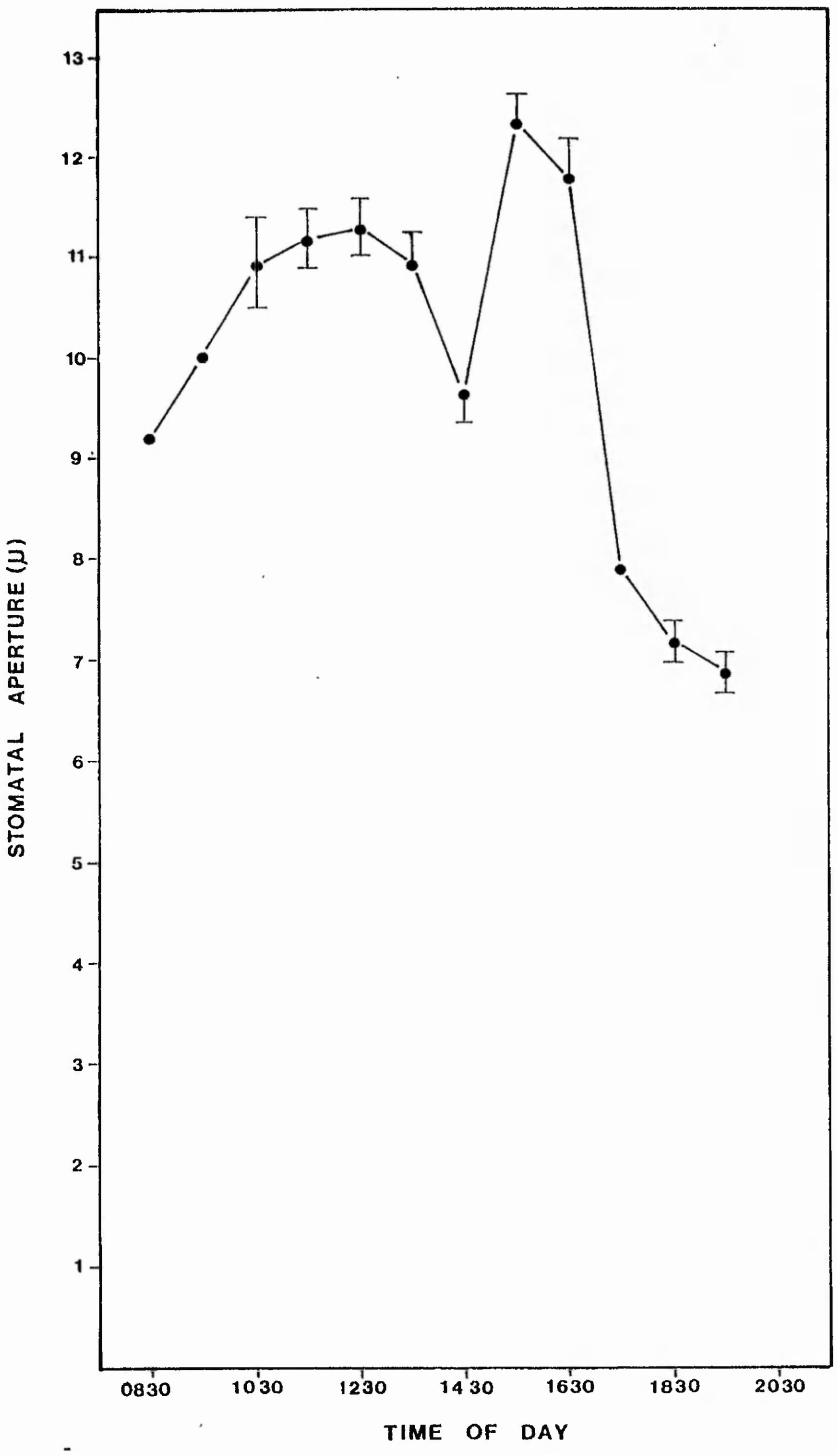
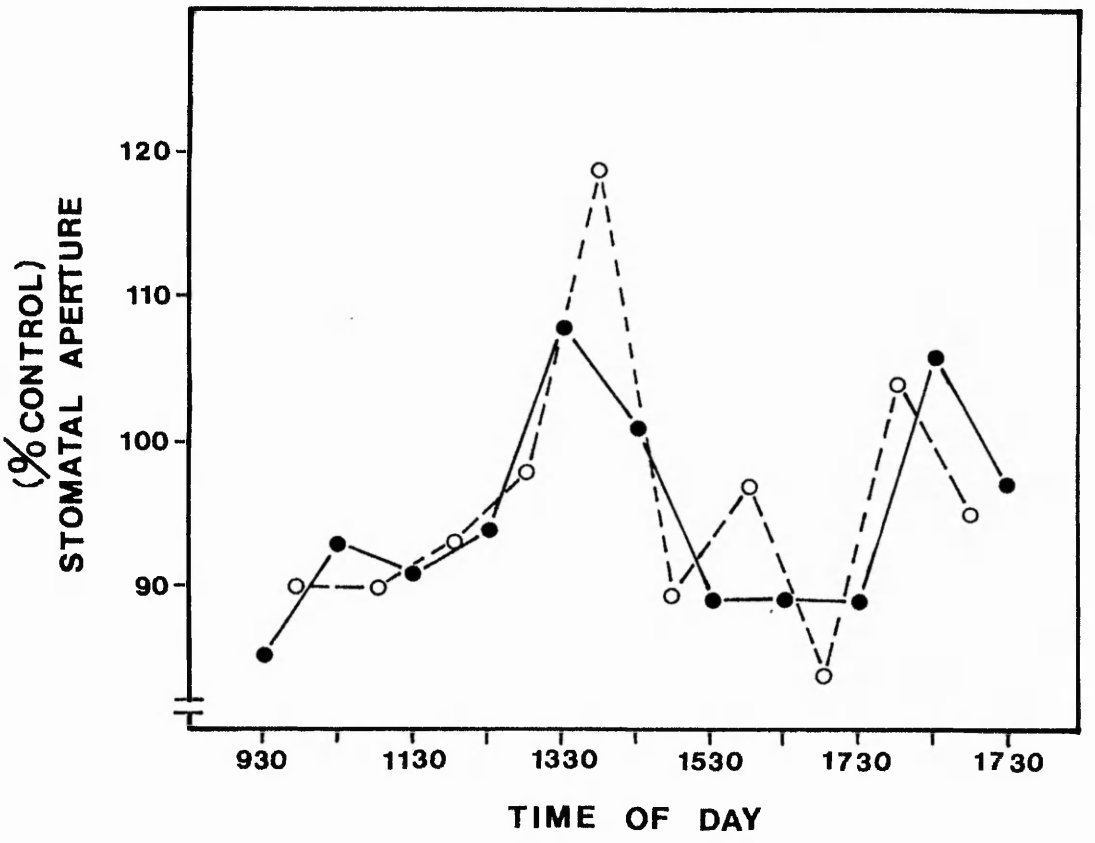


Figure 4.10 Daytime fluctuations in B.vulgaris adaxial stomatal aperture. Values are the mean of 45 replicates. Bars indicate standard error.

Clopyralid application to leaf surfaces during stomatal opening (0730hrs) resulted in altered apertures over the subsequent 18 hour period. In B.vulgaris only small effects were measured in response to the herbicide, the most noticable being increased stomatal apertures at 1400hrs (figure 4.11) which may correlate with reduced midday closure. Effects were very similar for both leaf surfaces. In M.inodora effects on adaxial and abaxial apertures are shown in figure 4.12. Initially, clopyralid reduced stomatal apertures with respect to controls (ie.prevented maximum opening), whilst after midday the general effect was that of increasing aperture with respect to controls (ie. delaying stomatal closure). Again both surfaces behaved similarly.



Figue 4.11 Effect of clopyralid (100g a.i. ha^{-1}) on abaxial (--O--) and adaxial (—●—) stomatal apertures in B.vulgaris. Values are the mean of 81 replicates over 3 occasions.

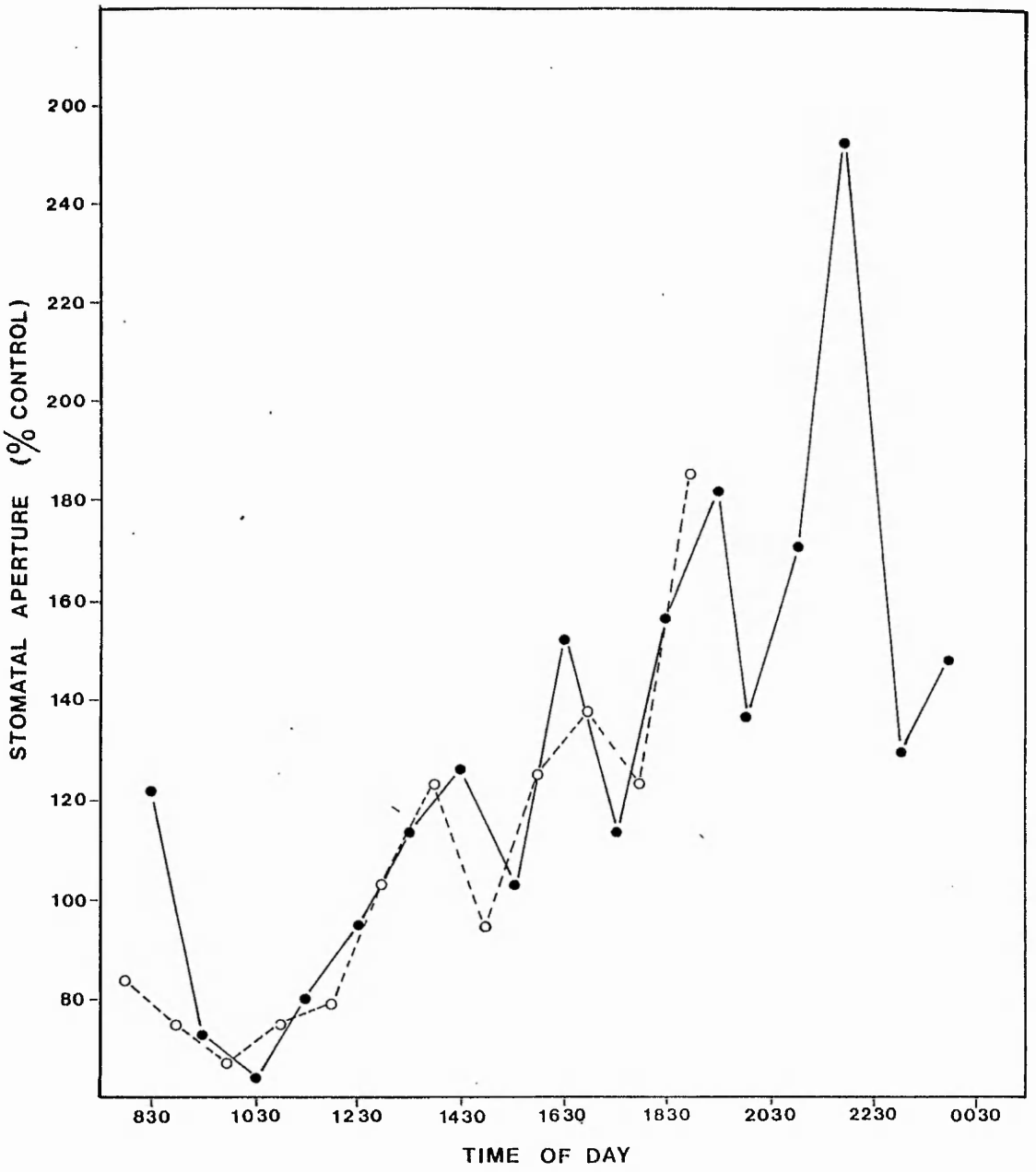


Figure 4.12 Effect of clopyralid (100g a.i. ha^{-1}) on abaxial (--O--) and adaxial (—●—) stomatal apertures in M.inodora. Values are the mean of 81 replicates over 3 occasions.

4.4 Discussion

The infra-red gas analysis system used was found to be troublesome and time consuming to construct and render leak-free but, once running, it yielded consistent and dependable measurements of leaf photosynthesis. One of the main pitfalls of the technique, that measured CO_2 may be reduced by dilution with water vapour from transpiration (Bruce and Ward, 1985), was avoided by passing air through absorbant silica gel before it entered the analyser.

The rapid, large increase in M.inodora photosynthesis up to 24 hours after clopyralid application was a clear and significant response which accompanied epinastic symptom development. The complete lack of response in B.vulgaris was equally clear. One criticism of the experiment was that no measurements of photo-respiration were made alongside those of photosynthesis. A more extensive study should undoubtedly consider photorespiration, however, since the increase in photosynthesis was so great, it was thought unlikely that inhibition of photorespiration could significantly contribute to the effect.

Although no compatible data exists with which to compare our results, Cardenas et al (1967) found initial stimulations of photosynthesis to be the most striking effect following spot treatment of cocklebur with 2,4-D. In this study $^{14}CO_2$ assimilation was increased to 155% of control 24 hours after treatment, and the authors showed that the rapid stimulation could also be measured immediately following 15 minute exposure of plants to $^{14}CO_2$. In an experiment with IAA, Turner and Bidwell (1965) showed that spraying bean leaves with this growth regulator increased the rate of photosynthesis, after 30 minutes, to a value almost double that prior to application. They observed that rates returned to normal within approximately 1 hour of the increase. They concluded that the stimulation was independent of stomatal effects, since the adaxial surface of the bean leaves (where herbicide was applied) bore no stomata. The possibility of systemic effects on abaxial stomata following rapid movement of herbicide across the leaf was not discussed.

The same authors (Bidwell and Turner, 1966) later demonstrated that 2,4-D did not mimic IAA in their experimental system, having no effect on bean leaf photosynthesis.

The long-term reduction in M.inodora photosynthesis was anticipated due to the observed cessation of growth of treated plants and eventual necrosis. Possible reasons for the rapid increase in M.inodora photosynthesis were sought. An initial experiment showed that there were no direct effects of clopyralid on photosynthetic electron transport in thylakoid fragments of either test species. Considering the literature, this was not surprising and research rapidly progressed onto investigation of stomatal effects of clopyralid which could perhaps contribute to the observed changes in photosynthesis. This area was one of particular interest since many published reports of the effects of auxin-type compounds on stomatal mechanism exist.

Firstly, several methods of measurement of stomatal aperture were assessed for suitability of use with the project test species. These included epidermal peeling, porometry, and the use of clear vinyl film (Redmann, 1984) and silicone rubber impressions (Weyers and Johansen, 1985) but, due largely to the fragile nature of M.inodora leaves, the direct observation method of Stalfelt (1962) was eventually selected. Glycerol was used as the immersion medium and aperture measurements were easily made by light microscopy with the aid of an eyepiece graticule. Preliminary experiments confirmed that this technique gave valid measurements, with apertures remaining constant during the examination period and showing predicted daytime fluctuations.

Interesting effects of clopyralid on stomatal aperture in M.inodora were discovered with restrictions in stomatal mechanism apparently preventing maximum aperture and delaying stomatal closure, with effects similar for both leaf surfaces. As mentioned, several effects of auxin-type compounds on stomatal apertures have been measured and the stimulatory effect of IAA on stomatal aperture established (Pemadasa, 1982). Pemadasa and Jeyaseelan (1976) examined the effects of 3 herbicidal auxins, 2,4-D, 2,4,5-T and MCPA, on stomatal opening and found that all 3

herbicides prevented wide opening of Stachytarpheta indica stomata, although the mechanism of the effect was different for 2,4-D than the other compounds. In a later experiment 1-napthylacetic acid (NAA) and 2-napthoxyacetic acid (NOXA) were found to restrict stomatal opening in the same species (Pemadasa, 1979). Mansfield (1967) studied effects of NOXA, NAA and 2,4-D on stomata of detached leaves. NOXA and NAA caused a marked closure of stomata of Xanthium pennsylvanicum which could not be reversed by flushing with CO_2 -free air. A closure effect of 2,4-D was also noted, however this was not reversible thus indicating a more direct effect on guard cells.

Proposals as to the origins of the stomatal effects discovered in the present study would be premature, considering the preliminary nature of the experiments and the complexity of factors involved in stomatal mechanism as summarised by Zeiger (1983). Two points however may be made. Firstly, the likelyhood of the stomatal effects found in this study for M.inodora accounting for the increase in photosynthesis is uncertain. Although CO_2 is the major limitation of whole plant photosynthesis and CO_2 entry is primarily controlled by stomata, some authors calculate the limitation of photosynthesis by stomata to be slight (Farquhar and Sharkey, 1982). Secondly, abaxial and adaxial responses to clopyralid were similar whilst the adaxial stomata were in contact with a greater amount of herbicide, indicating that the stomatal effects were indirect rather than contact initiated.

The fact that a clear difference in response to clopyralid appears between the resistant and susceptible species may be significant in terms of herbicide selectivity and further investigation is demanded. Throughout the present study two inherent differences in the test species have been revealed which must be acknowledged in future research. Firstly maximum photosynthesis rate in B.vulgaris is lower than that measured in M.inodora, when the rate is expressed on a leaf area basis. The second point is that adaxial to abaxial stomatal ratio is around five times higher in M.inodora as in B.vulgaris.

To conclude this discussion it is relevant to mention an interesting hypothesis put forward by Bidwell and Turner (1965) to explain why corn showed no increased photosynthesis in response to auxin whilst a range of other species did respond. These authors speculated that corn was already photosynthesising at its maximum rate and so IAA was not able to exert an effect. Could this be extrapolated to the present study with some form of genetic interaction of clopyralid causing stimulation of photosynthesis in the sensitive species which initiates subsequent phytotoxic effects, whilst the resistance of B.vulgaris is due to its functioning within its maximum photosynthetic range? Is there a case for the competitive nature of the annual weed M.inodora having photosynthetic capacity in reserve?

Chapter 5. INVESTIGATION OF CLOPYRALID EFFECTS ON ETHYLENE PRODUCTION

5.1 Introduction

In a recent paper Hall *et al* (1985) evaluated whole-plant ethylene production in response to clopyralid in rapeseed and sunflower plants. Their findings suggested that enhanced ethylene biosynthesis in response to herbicide application was a factor involved in the resulting morphological changes in the susceptible species. In view of this work, a series of experiments were designed to monitor ethylene production in response to clopyralid in tolerant *B.vulgaris* and susceptible *M.inodora*. Measurements were made of ethylene evolution from tissue - explants, in a closed vial system under controlled environment conditions. The maximum experimental period was restricted to 24 hours since the rapid response to clopyralid was considered to be the most important. Inhibitors of ethylene biosynthesis which have been greatly used in research towards an understanding of the biochemical pathway of ethylene production were employed in the present study in order to investigate to what extent epinastic symptom development was due to ethylene gas.

Hence, the aims of the study were to measure ethylene production in response to clopyralid in tolerant *B.vulgaris* and susceptible *M.inodora*, and to assess the relationship of these measurements to symptom development and selectivity. By expanding the study to include *G.aparine* and fluroxypyr the value of ethylene measurement as a tool in determining selectivity to auxin-type herbicides was also assessed.

5.2 Materials and Methods

5.2.1 Plant Material

Plants were propagated in J.Arthur Bowers seed and potting compost in 5cm deep trays. *B.vulgaris* and *G.aparine* seeds were sparsely sown at a depth of 5mm whilst *M.inodora* seeds were surface sown. Trays were maintained under glasshouse conditions

with a 14 hour day (200-400μM photons m^{-2} s^{-1}, (PPFD)) and 20-25°C. In all experiments B.vulgaris and M.inodora were used at the three to four leaf stage. G.aparine plants were used at the two to three leaf whorl stage.

5.2.2 Chemicals

All test chemicals were first dissolved in methanol (final concentration, <0.2% v/v), and then diluted with 10^{-3} M MES buffer adjusted to pH6 with NaOH. AVG, ACC, 2,4-D and IAA were obtained from the Sigma Chemical Company. Cobaltous chloride was obtained from BDH chemical. Technical grade clopyralid and fluroxypyr were gifts from the Dow Chemical Company.

5.2.3 Incubation System

Leaf 3 of M.inodora and B.vulgaris, and the youngest two leaf whorls of G.aparine were used for incubation. Following excision at the stems, leaves were exposed to chemicals in gas-tight vials as illustrated in figure 5.1. In those experiments where the effects of exogenously applied ethylene were examined, gases were introduced by injection through the seal. The vials were incubated in a controlled temperature water bath at 25°C and illuminated at 50μM photons m^{-2} s^{-1} (PPFD) using natural white fluorescent tubes (Phillips). One cm^3 gas samples were withdrawn at regular intervals using a gas-tight syringe.

5.2.4 Determination of Ethylene Concentration

The ethylene concentration in each vial was determined using a gas chromatograph (Sigma 3B Perkin Elmer) equipped with a Poropak Q 80-100 mesh column (Perkin Elmer) and a flame ionisation detector. The oven temperature was maintained at 100°C and a laboratory computing integrator (Perkin Elmer LC1-100) was used to calculate the amount of ethylene in each sample with respect to appropriate standards. Standard gases were obtained from Air Products Ltd. After each withdrawal, gas volumes in each vial were amended with ambient air and results adjusted to account for dilution during sampling. Each experiment was fully replicated

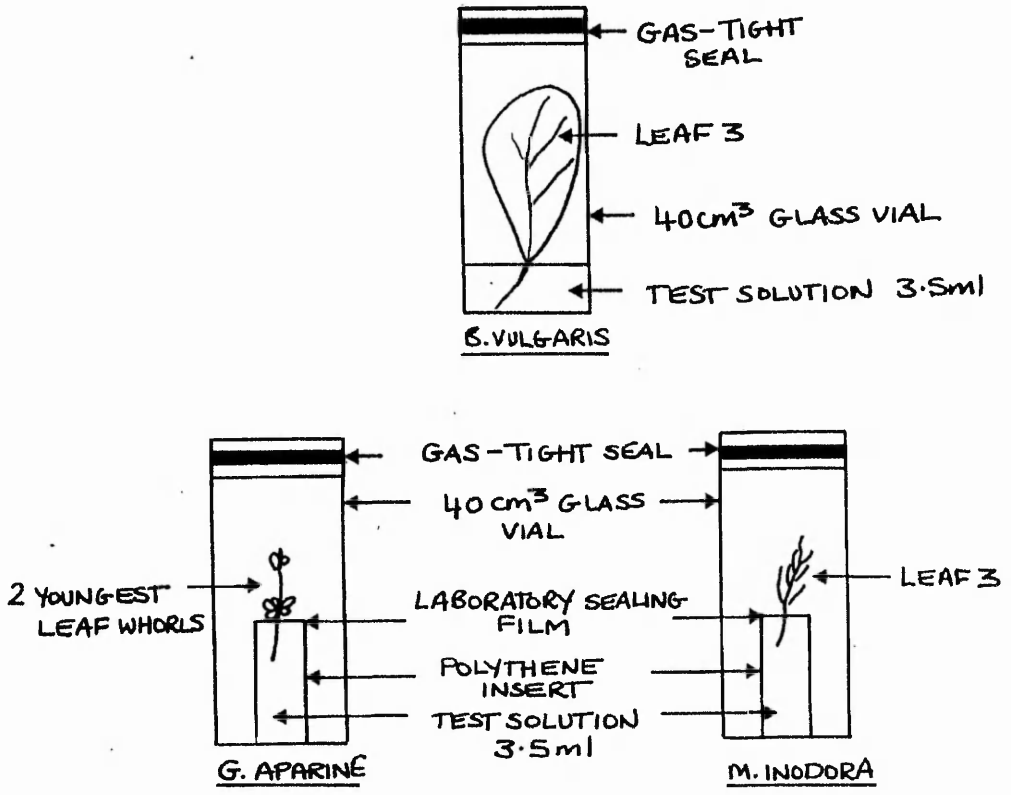


Figure 5.1 Experimental system for measurement of ethylene production by excised plant tissues.

on 3 or 4 occasions and mean values plus or minus standard errors are presented. Comparisons between selected means were made using Student's t-test. Data are expressed on a fresh weight basis and are corrected, where necessary, for any ethylene present in blank tubes. Epinastic symptom development was both visually assessed and recorded photographically.

5.3 Results

Clopyralid induced rapid, concentration-dependent increases in ethylene production in M.inodora over 6 hours (figure 5.2). Increases were statistically significant ($P < 0.01$) after only 2 hours at the highest clopyralid concentrations, and after 3 hours at 10^{-4} M. Clopyralid concentrations of 10^{-5} M and 10^{-6} M consistently increased ethylene production, although values were not statistically

different from the controls. Incubation of excised leaves with 10^{-3} M clopyralid gave the greatest ethylene production with 2.6 times this amount yielding no further increases. Visible symptom development commenced after approximately 4 hours at the highest clopyralid doses, and by 6 hours leaf epinasty could be noted at all clopyralid concentrations. After 24 hours, symptom development was well defined and a clear dose-response pattern emerged in the degree of epinastic symptoms (plates 5.1 and 5.2).

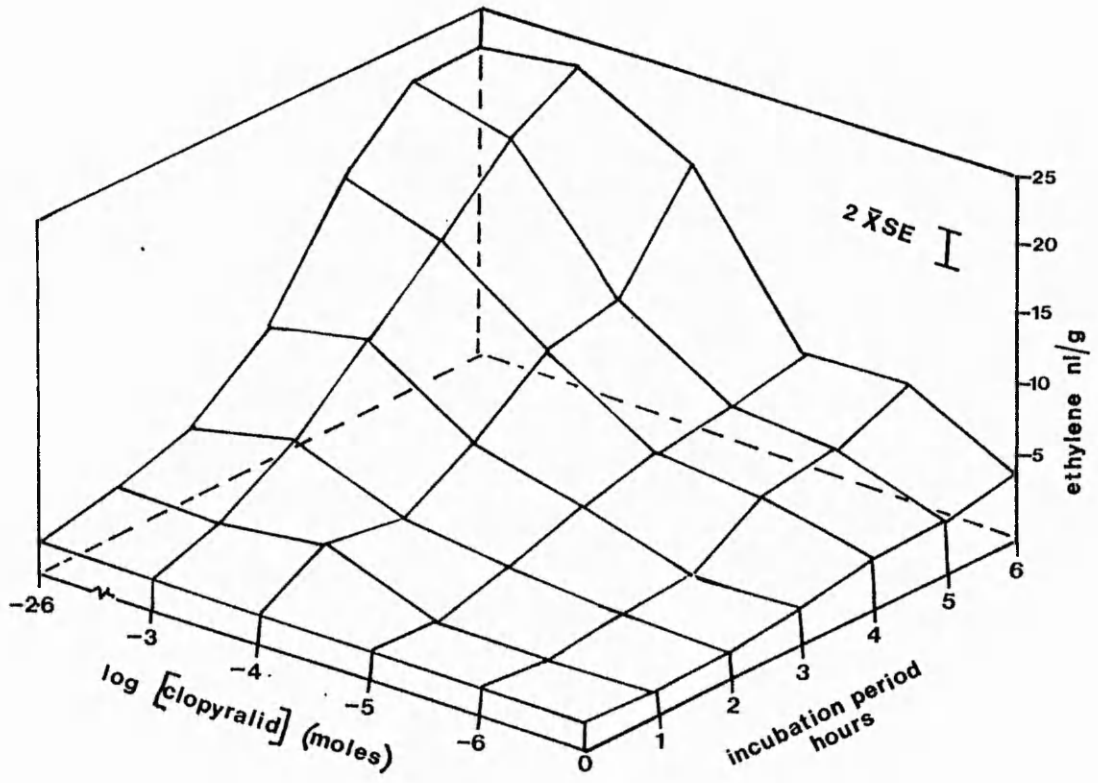


Figure 5.2 Effect of clopyralid on ethylene evolution in M.inodora, dose response and time-course over 6h.

Comparisons of ethylene production in response to clopyralid in susceptible M.inodora and tolerant B.vulgaris over 24 hours revealed a differential response (figure 5.3). B.vulgaris showed no increased ethylene production with respect to the controls at any of the clopyralid concentrations studied and leaves appeared unaffected by the herbicide after the 24 hour period (plate 5.3). In M.inodora, clopyralid induced a concentration dependent increase in ethylene production of up to 10 fold. As in the short term study (figure 5.2), 10^{-3} M clopyralid was the optimal

concentration for the response. However, on comparison with figure 5.2, the values measured were not consistent with linear ethylene production over the 24 hour period, indicating that ethylene production levelled off between 6 and 24 hours.

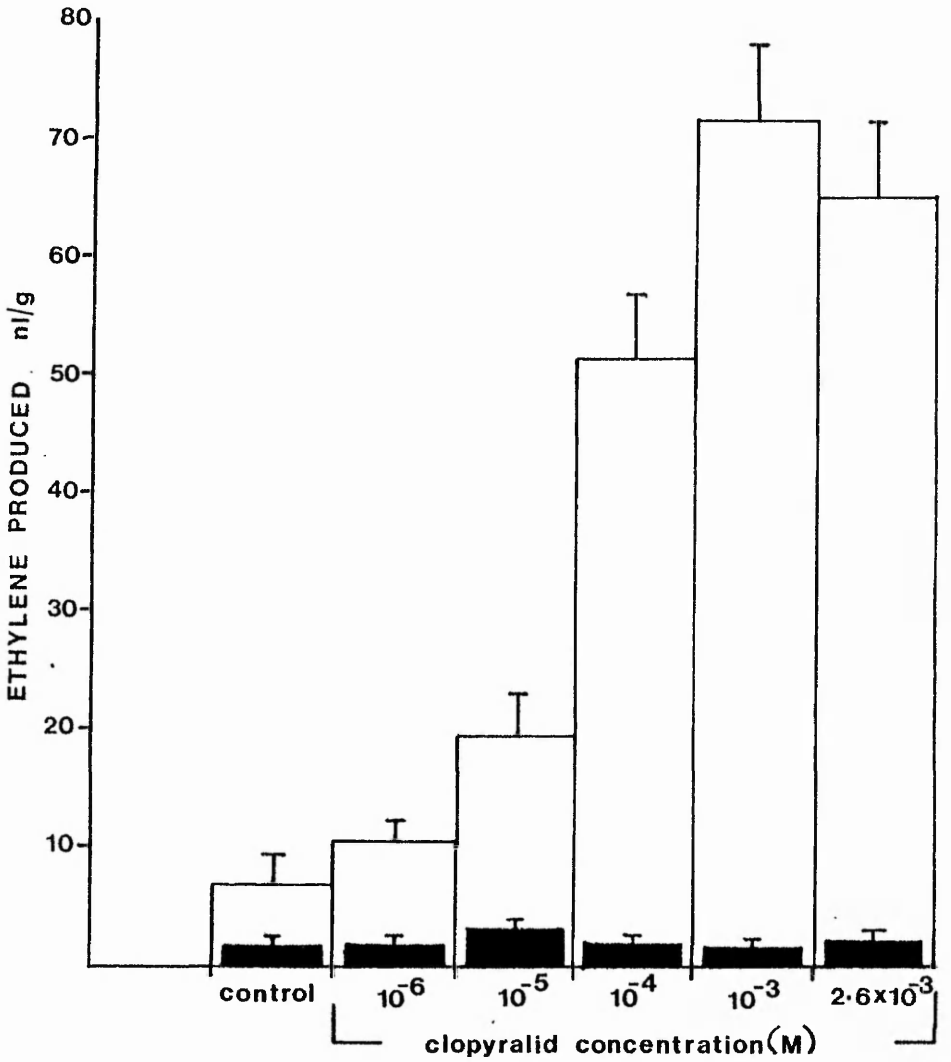


Figure 5.3 The effect of clopyralid on ethylene evolution in M.inodora (☐) and B.vulgaris (■) following incubation of excised leaves for 24 hours. Each point is the mean value from 8 plants on at least 3 occasions. Bars represent standard errors.

Plate 5.1 Effect of 10^{-3}M clopyralid on M.inodora leaf explant following 24h incubation.

Plate 5.2 Dose response of M.inodora leaf explants to clopyralid following 24h incubation.

Plate 5.3 Effect of 10^{-3}M clopyralid on B.vulgaris leaf explant following 24h incubation.

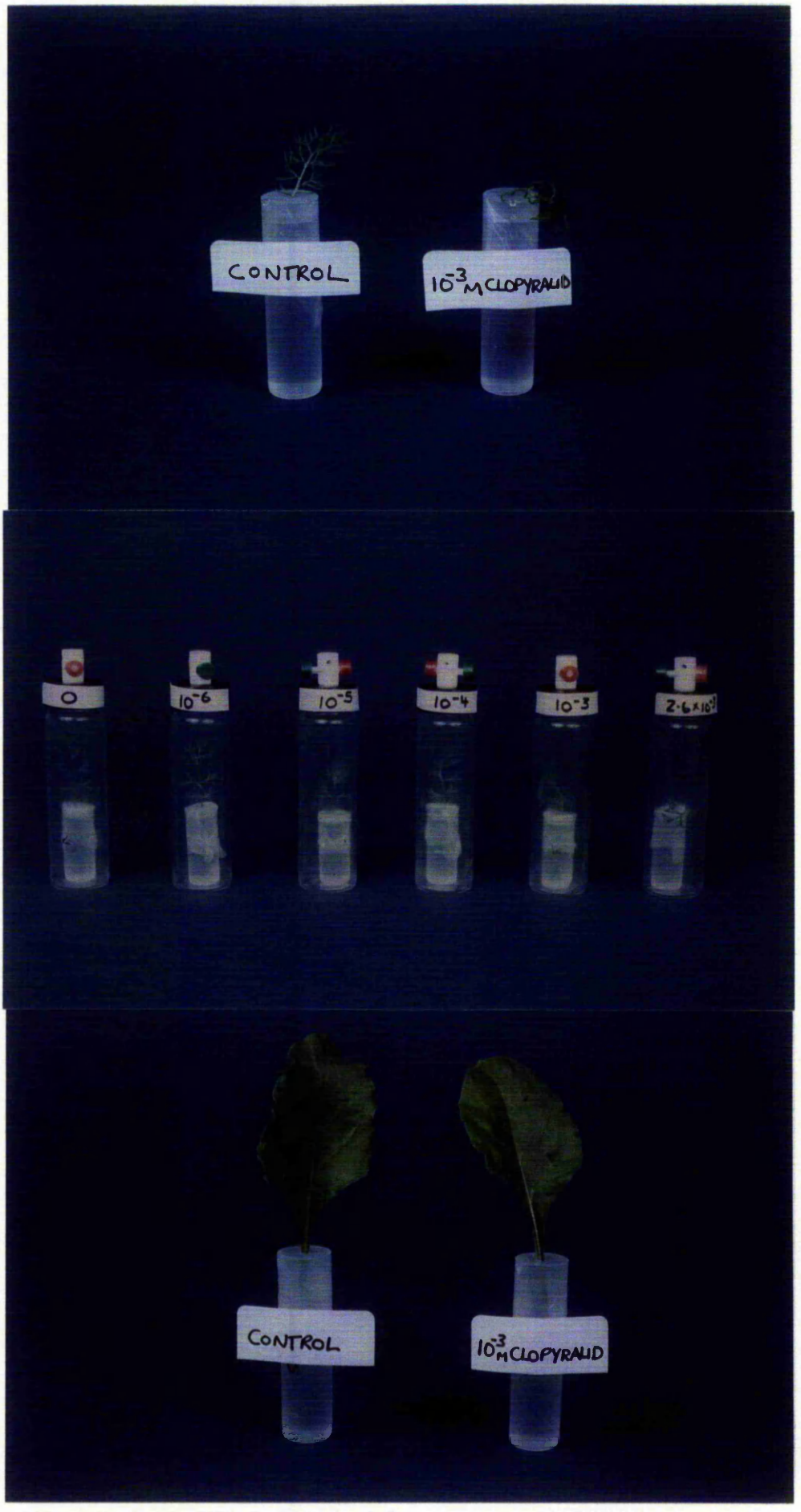

CONTROL
10^{-3} M CLOPYRALID
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
2.6×10^{-3}
CONTROL
10^{-3} M CLOPYRALID

Plate 5.4 Effect of 10^{-3}M 2,4-D and 10^{-3}M IAA on M.inodora leaf explants following 24h incubation.

Plate 5.5 Effect of 10^{-3}M 2,4-D and 10^{-3}M IAA on B.vulgaris leaf explants following 24h incubation.

Plate 5.6 Effect of AVG (10^{-4}M) and Co^{++} (10^{-3}M) on 10^{-3}M clopyralid effects on M.inodora leaf explants following 24h incubation.


CONTROL
10^{-3}_{M} IAA
10^{-3}_{M} 2,4-D
CONTROL
10^{-3}_{M} IAA
10^{-3}_{M} 2,4-D
AVG + COBALT
AVG + COBALT + 10^{-3}_{M} CLOPYRALID
10^{-3}_{M} CLOPYRALID

Ethylene evolved by M.inodora in response to 10^{-3} M clopyralid was equivalent to that induced by 10^{-3} M IAA over both 6 and 24 hours as illustrated in figure 5.4. Symptom development was also similar in response to the 2 chemicals (plates 5.1 and 5.4). Whilst 2,4-D induced similar symptom development as IAA and clopyralid after 24 hours (plate 5.4), ethylene produced was around five times greater (figure 5.5). Although B.vulgaris did not respond to clopyralid, ethylene production in this species was induced by IAA and 2,4-D (figure 5.6), and this species was efficient at converting supplied ACC to ethylene (Table 5.1). Epinastic symptoms were also induced by IAA and 2,4-D in B.vulgaris after 24 hours (plate 5.5).

Table 5.1 Production of ethylene from 10^{-3}M ACC in M.inodora and B.vulgaris. Data are the means of six replicates plus or minus standard errors.

	ETHYLENE PRODUCTION nl/g fresh weight	
INCUBATION PERIOD (h)	M.inodora	B.vulgaris
2	20.03 ± 3.92	11.69 ± 1.17
4	38.99 ± 4.52	21.12 ± 1.19
24	163.62 ± 10.90	245.91 ± 32.41

The ethylene biosynthesis inhibitors AVG and Co^{++} reduced M.inodora ethylene production in response to 10^{-3} M clopyralid by approximately 50% over 6 and 24 hours incubation (Figure 5.6). However, although ethylene production was reduced in the presence of these inhibitors, symptom development was not diminished (plate 5.6).

Exogenous ethylene gas applied at a concentration range from 1 to 500nl/40cm^3 vial failed to induce epinastic symptoms in either M.inodora or B.vulgaris (plates 5.7 and 5.8). Furthermore, despite the high levels of ethylene produced by both M.inodora and B.vulgaris in response to supplied ACC (table 5.1), no visible symptom development was recorded (plates 5.9 and 5.10).

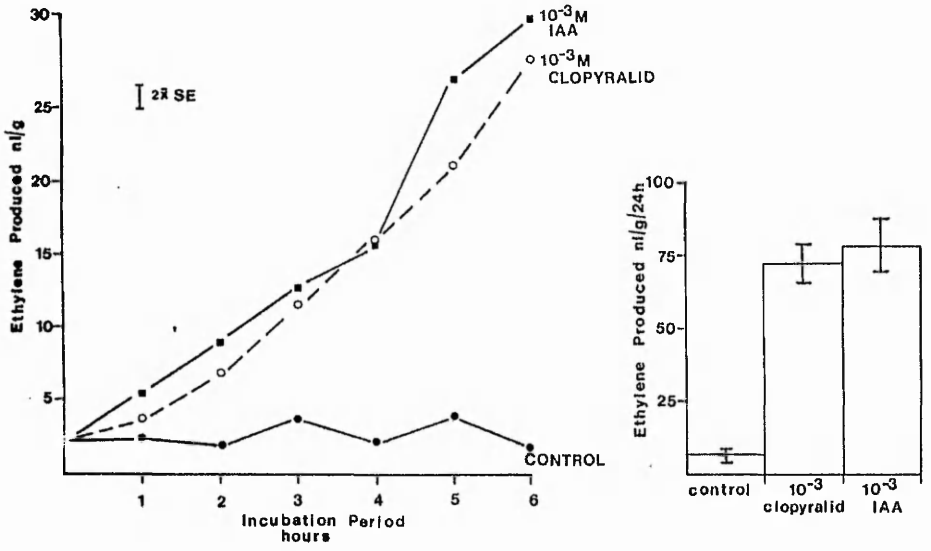


Figure 5.4 Effect of 10^{-3}M clopyralid and 10^{-3}M IAA on ethylene evolution in M.inodora over 6 and 24 h. Each point is the mean from 8 replicates on at least 3 occasions. Bars represent standard errors.

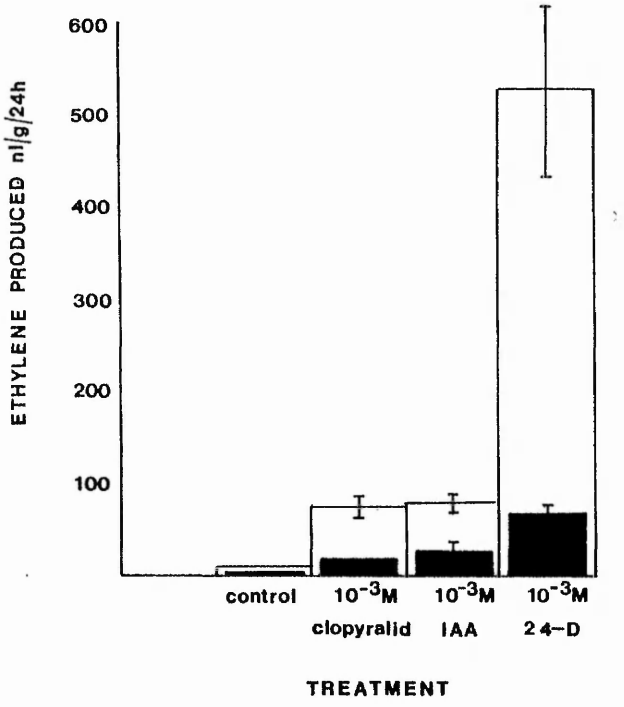


Figure 5.5 Effect of clopyralid, IAA and 2,4-D on ethylene evolution in M.inodora (□) and B.vulgaris (■) following 24h incubation. Values are the means from 8 replicates and bars represent standard errors.

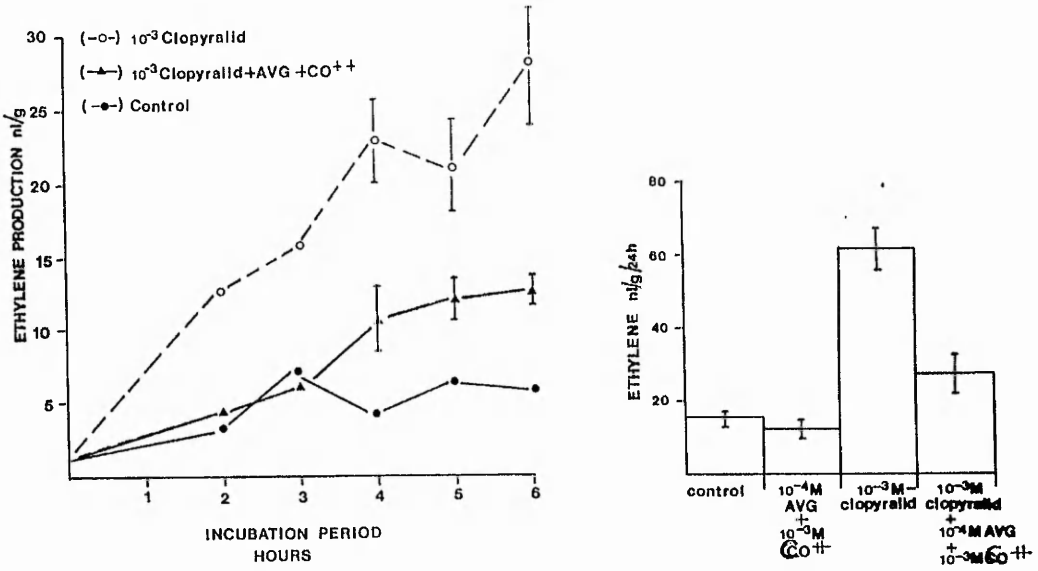
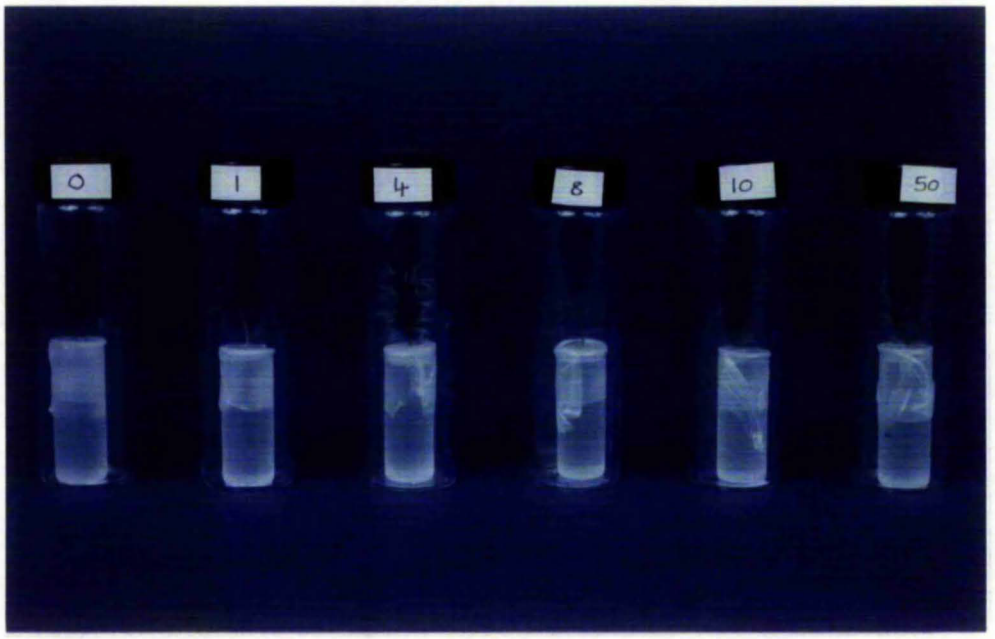


Figure 5.6 Effect of AVG (10^{-4}M) plus Co^{++} (10^{-3}M) on clopyralid induced ethylene production in M.inodora over 6 and 24 hours. Data are means of 6-8 replicates. Bars represent standard errors.

Although difficult to quantify, it often appeared, on visual assessment, that symptoms induced by 2.6 X 10^{-3}M clopyralid were more severe after 24 hours than those due to 10^{-3} M clopyralid. Ethylene production in response to 10^{-3} M clopyralid compared to controls in M.inodora, B.vulgaris and G.aparine reflected herbicide selectivity in these species (figure 5.7 and plate 5.11). G.aparine is mildly susceptible to clopyralid and fell between tolerant B.vulgaris and highly susceptible M.inodora in terms of ethylene production. When another pyridine compound, fluroxypyr (highly active on G.aparine, Sanders et al, 1985) was applied to G.aparine in this system, its activity was also reflected by ethylene production (figure 5.8 and plate 5.12). Fluroxypyr induced up to five times more ethylene than clopyralid after 2 and 24 hours in G.aparine.

Plate 5.7A Effect of 0-50nl/vial ethylene gas on M.inodora leaf explants following 24 hours incubation.

Plate 5.7B Effect of 0-500nl/vial ethylene gas on M.inodora leaf explants following 24 hours incubation.


0
1
4
8
10
50

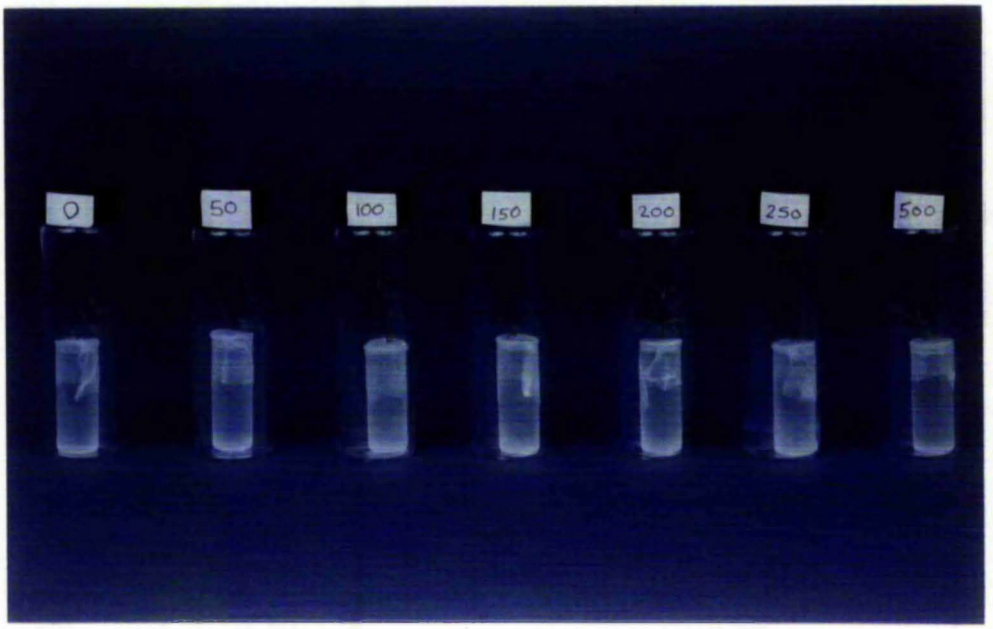
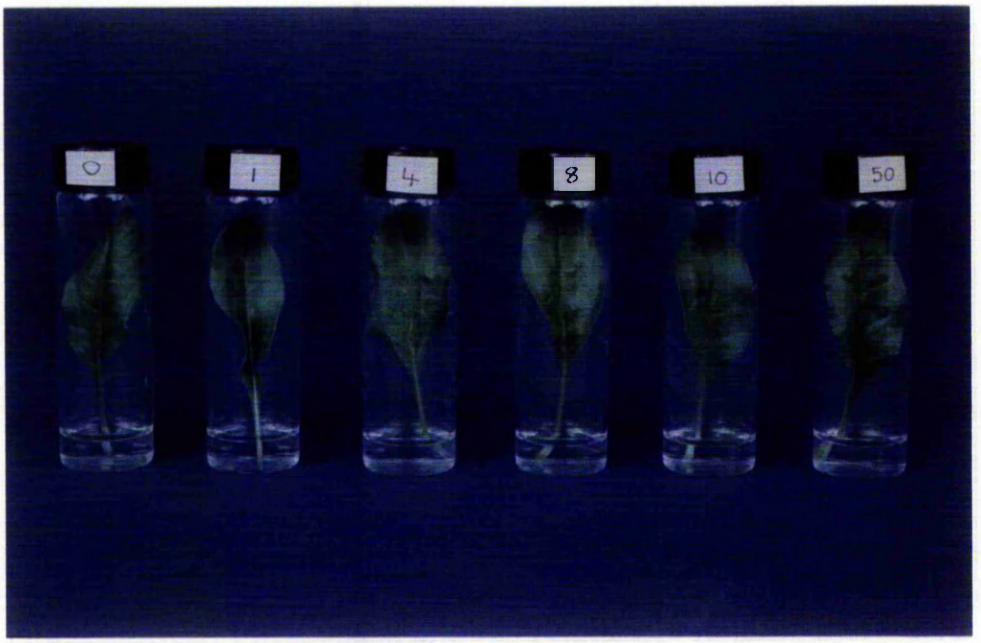

0
50
100
150
200
250
500

Plate 5.8A Effect of 0-50nl/vial ethylene gas on B.vulgaris leaf explants following 24 hours incubation.

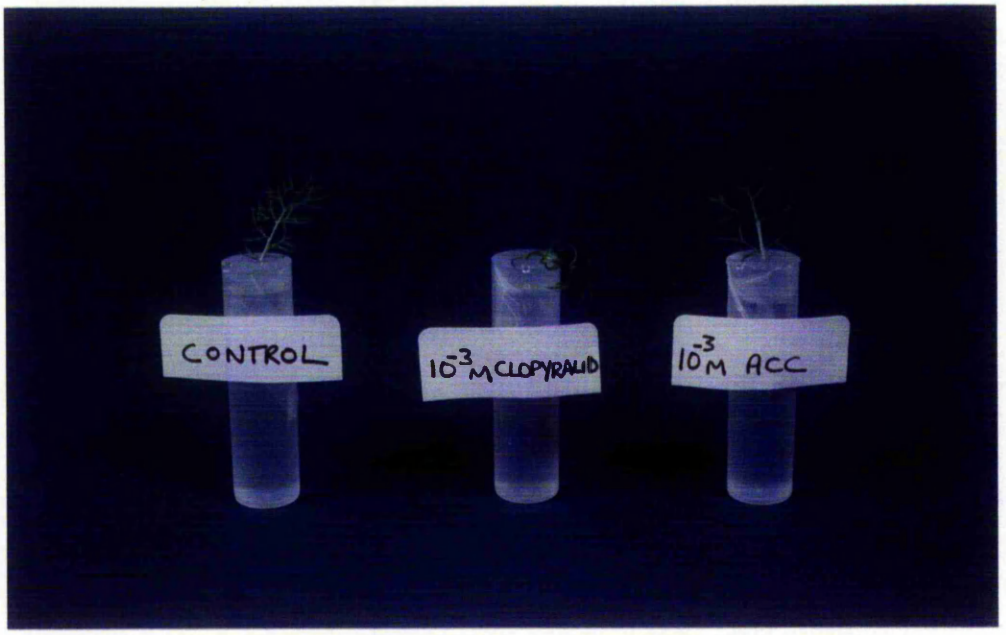
Plate 5.8B Effect of 0-500nl/vial ethylene gas on B.vulgaris leaf explants following 24 hours incubation.



2

Plate 5.9 Effect of 10^{-3}M clopyralid and 10^{-3}M ACC on M.inodora leaf explants following 24 hours incubation.

Plate 5.10 Effect of 10^{-3}M clopyralid and 10^{-3}M ACC on B.vulgaris leaf explants following 24 hours incubation.


CONTROL
10^{-3}M CLOPYRALID
10^{-3}M ACC


CONTROL
10^{-3}M CLOPYRALID
10^{-3}M A.C.C.

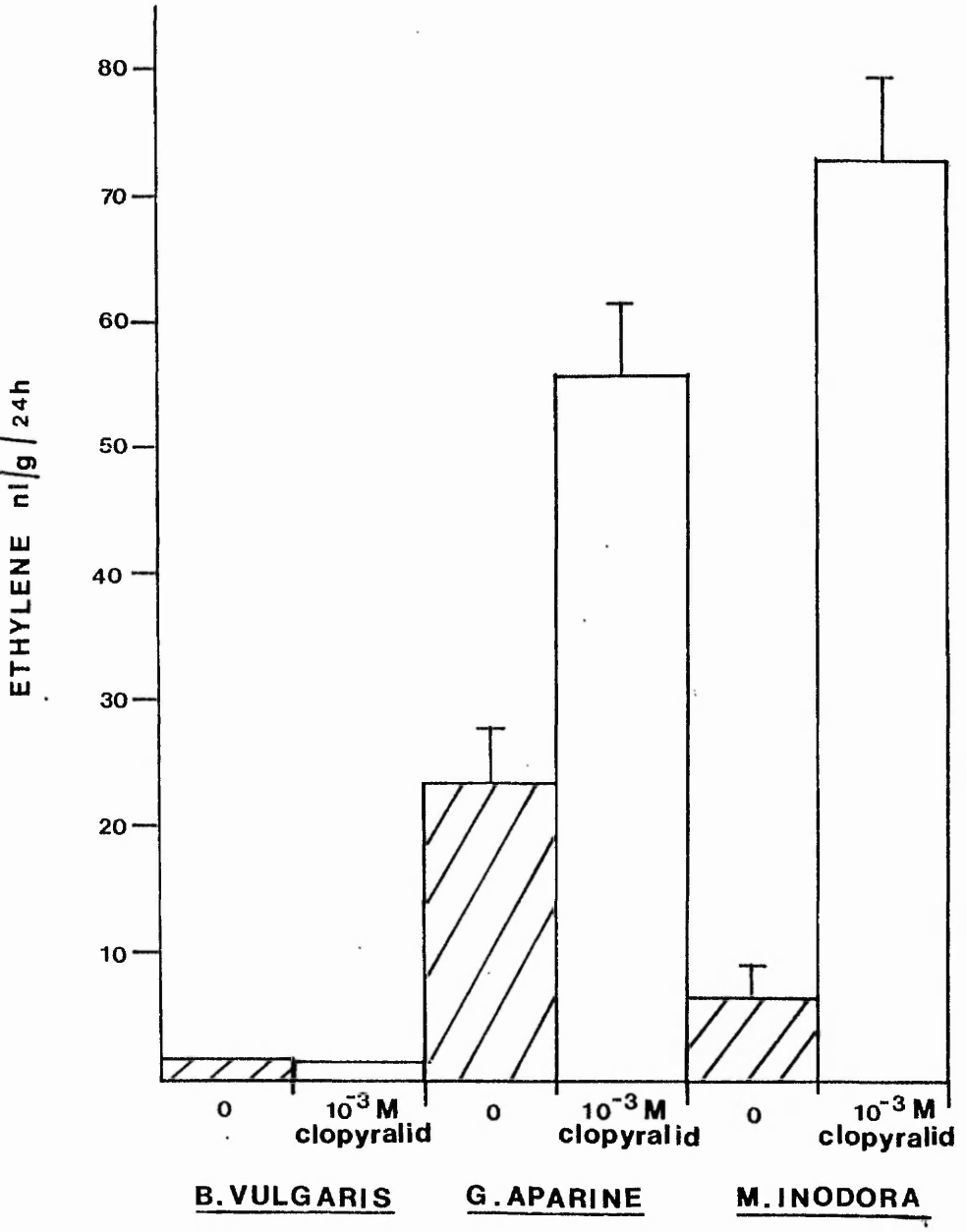


Figure 5.7 Effect of 10^{-3}M clopyralid on ethylene evolution in 3 test species following 24 hours incubation. Each value is the mean of 8 replicates. Bars represent standard errors.

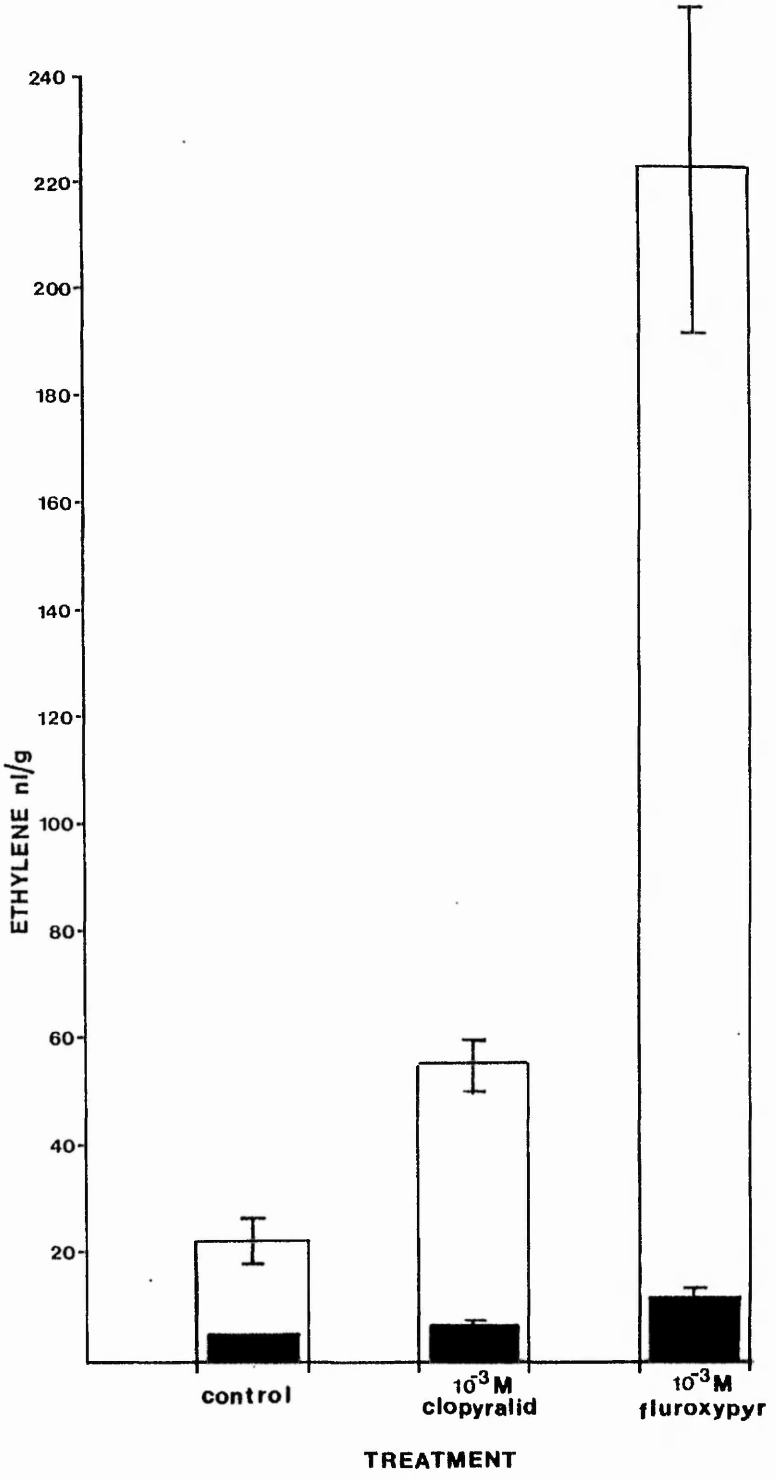
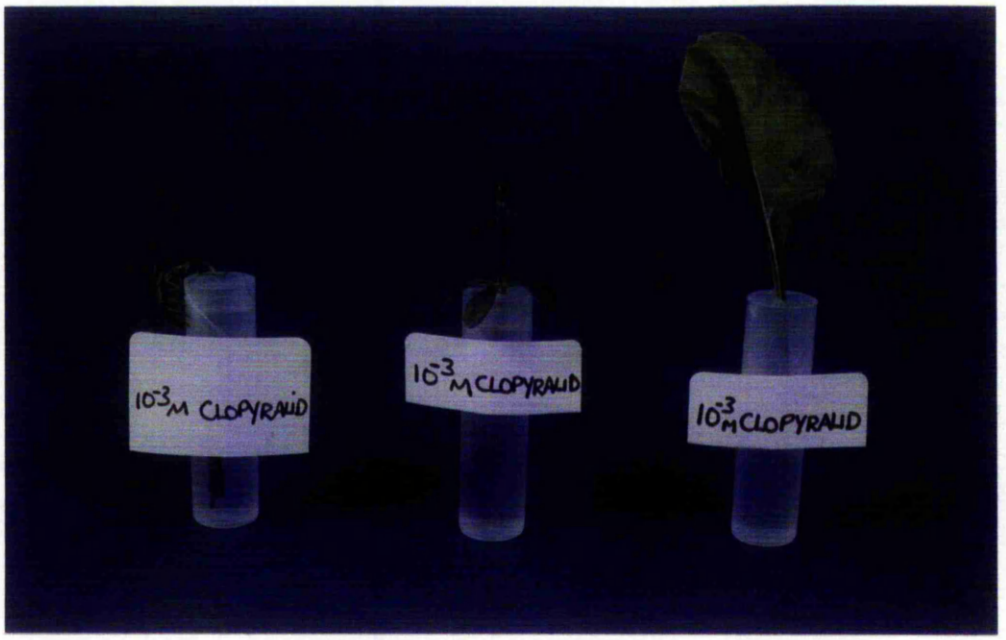


Figure 5.8 Effect of clopyralid (10^{-3}M) and fluroxypyr (10^{-3}M) on ethylene evolution in G.aparine after 2 (■) and 24 (□) hours. Values are the mean of 8 replicates, bars represent standard errors.

Plate 5.11 Effect of 10^{-3}M clopyralid on explants of M.inodora, G.aparine and B.vulgaris following 24 hours incubation.

Plate 5.12 Effect of 10^{-3}M clopyralid and 10^{-3}M fluroxypyr on G.aparine explants following 24 hours incubation.


10^{-3}M CLOPYRALID
10^{-3}M CLOPYRALID
10^{-3}M CLOPYRALID


CONTROL
10^{-3}M CLOPYRALID
10^{-3}M FLUROXYPYR

5.4 Discussion

A recent paper by Hall *et al* (1985) strongly criticized the use of closed experimental systems, as used in this study, for the measurement of ethylene production, rightly stating that changes in the gaseous environment around plant tissues may become interfering factors in such experiments. The justification for using a closed vial system in this study is two fold:

1. All experiments were conducted over a short time period, with the longest incubation time being 24 hours. Over such a time, the dilution of evolved ethylene in an air stream would make measurements of very small amounts unreliable.

2. The use of a simple system such as that described above allows adequate replication, thereby ensuring reliability of resulting data.

The main reason for using leaf explants was to examine clopyralid action on ethylene production following direct vascular uptake of the active molecule (i.e. clopyralid acid), thus eliminating factors such as formulation, translocation and metabolism. The use of this model system revealed an apparent inconsistency with previously reported whole plant data (Thompson and Cobb, 1986), that whilst *B.vulgaris* explants showed no visual symptoms in response to clopyalid, spray application of the herbicide to whole plants resulted in transient epinastic symptoms. These symptoms appeared on leaf tissues after 1 day and progressed to petioles and stems between 1 and 3 days after treatment. By 14 days after herbicide application recovery from these symptoms was shown by *B.vulgaris*. That *B.vulgaris* plants, but not leaf explants, showed epinastic symptoms could have resulted from a variety of possible factors including;

1. sensitivity to formulant components,
2. some form of apical interaction where clopyralid application resulted in stimulation of auxin production leading to epinasty as an indirect herbicide effect, and
3. the possibility that epinasty was a symptom of clopyralid -induced plant stress (eg. water stress).

This inconsistency clearly highlights the danger of direct comparison or extrapolation between studies using whole plant and explant systems.

Gas-chromatography is the standard measurement and identification technique for ethylene (Ward et al, 1978), and whilst photoionisation detectors (PID) have been found to be more efficient at ethylene detection (Bassi and Spencer, 1985), this study has found that, using a closed system, flame ionisation detection (FID) was sufficiently sensitive to detect the levels of gas encountered in these experiments. The use of a PID would only have been necessary if a hydrocarbon-free system was in use where any appearance of ethylene would be significant. The use of closed systems also meant that the need to concentrate ethylene physically, on pre-columns, or chemically (DeGreef, DeProft and DeWinter, 1976), was avoided. The experimental system was thus kept uncomplicated, accurate and reliable.

Initial findings suggested that observed leaf epinasty is directly caused by ethylene production in M.inodora. However, this apparent link is disproven by the following further observations.

(a) Although ethylene evolution was induced by incubation with the precursor ACC alone, leaf epinasty was not observed (plate 5.9).

(b) Exposure to a wide range of concentrations of ethylene gas failed to produce epinastic symptoms (plates 5.7A and 5.7B).

(c) AVG and Co^{++} inhibited clopyralid-induced ethylene production by 50% whilst symptom development appeared unchanged (plate 5.6).

(d) Incubation with 2.6×10^{-3} M clopyralid resulted in the production of more severe symptoms in M.inodora after 24 hours than was noted following incubation with 10^{-3} M clopyralid. This was despite the similar or lower levels of ethylene gas induced by the higher herbicide dose (plate 5.2).

In agreement with Hall et al, 1985, these results demonstrated a differential ethylene response in susceptible and tolerant plant species. B.vulgaris did not produce ethylene or visible symptoms in response to clopyralid in this system. That B.vulgaris is capable of producing ethylene was demonstrated in its response to ACC, 2,4-D and IAA each of which induced ethylene production. As with M.inodora, no visible symptoms were noted following incubation with ACC or in response to exogenously applied ethylene in this tolerant species (plates, 5.8A, 5.8B and 5.10). However, the species demonstrated it's physiological capacity to produce leaf epinasty in response to herbicides (in this system) via its response to IAA and 2,4-D (plate 5.8).

From this data it is suggested that ethylene production is a symptom of herbicide activity in susceptible species and it is considered to be one of a cascade of events initiated by binding at an auxin receptor site. There are many published reports of responses to auxin which occur on a time scale from a few minutes to several hours (Evans, 1974), and figure 5.9 describes some of these effects in relation to the findings of the present study. Herbicide effects are initiated by binding of molecules to auxin receptor sites located in the plasmalemma. The binding event stimulates the production of secondary messenger(s) (Brummel and Hall, 1987). One well documented and immediate auxin effect is the change in cell membrane permeability (Evans, 1985) where H^+ ion efflux from the cytoplasm into the cell wall is brought about by the activation of a plasmalemma-bound H^+-pumping ATPase (Hager et al, 1971). The consequent acidification of the cell wall results in loosening which leads to cell elongation. Fitzsimmons et al, (1987) have reported H^+ion efflux from oat coleoptiles in response to 100 and 1000μM clopyralid. The diversity of auxin effects indicate activity via altered gene expression and so the involvement of a secondary messenger in the form of a regulator of gene expression. Regulation at the genome could result in the observed increase in ethylene via stimulation of ACC synthase production, as well as increasing RNA and protein synthesis to a level necessary to support continued cell elongation and growth stimulation resulting in epinasty. In this experimental system the production of ethylene and epinastic symptoms appear to be

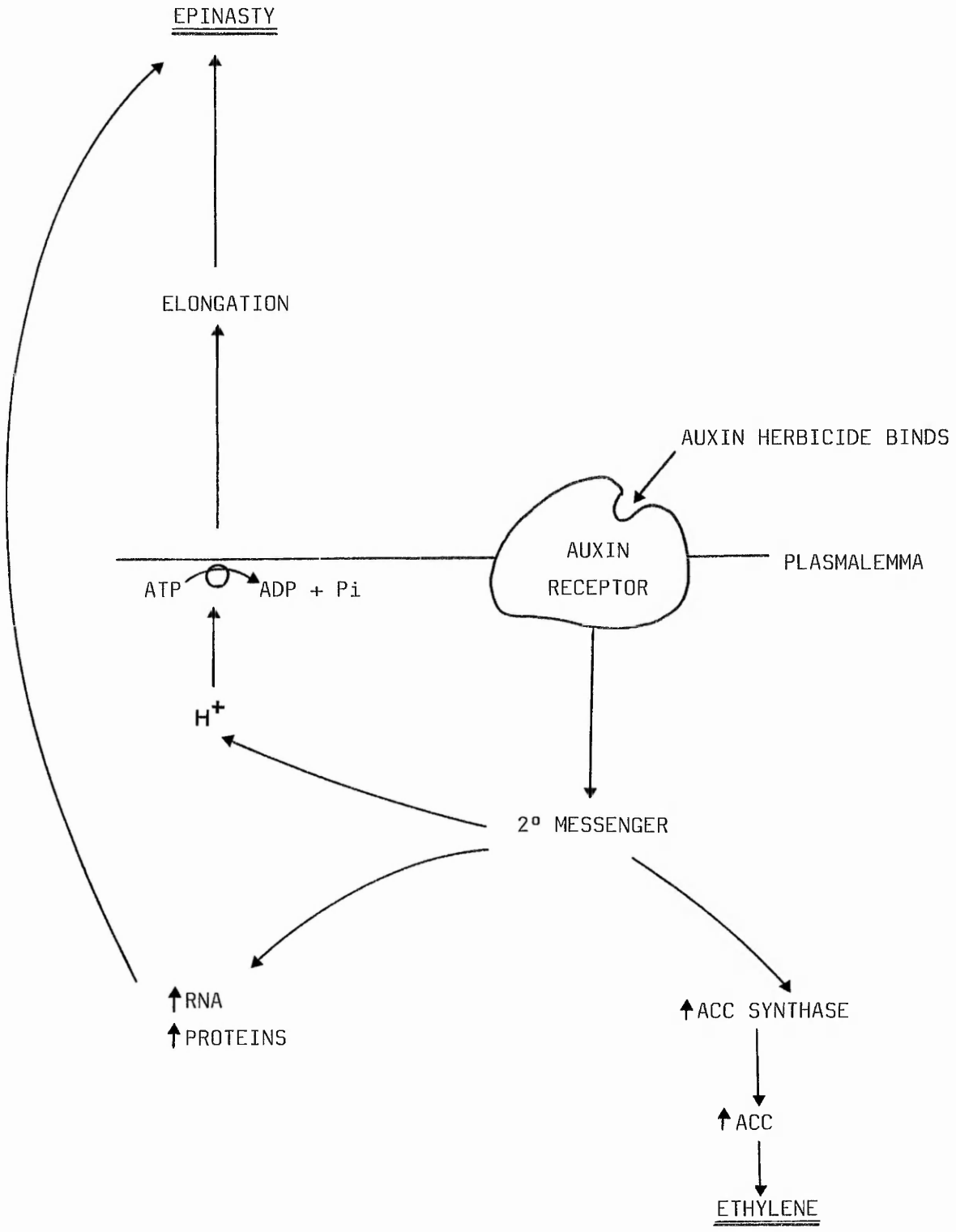


Figure 5.9 Production of ethylene and epinasty following herbicide binding at an auxin receptor.

separate and measurable responses to the common stimulus of herbicide binding, the two effects possibly mediated by the same secondary messenger.

Assuming, from our knowledge of clopyralid movement patterns, that the herbicide is freely entering B.vulgaris leaves, it is speculated that clopyralid may not induce epinasty in this species due to a relatively poor affinity for the auxin binding site. Since ethylene alone is not the cause of epinastic symptoms in response to clopyralid in M.inodora, it is concluded that differential ethylene production is unlikely to form the basis of clopyralid selectivity in M.inodora and B.vulgaris. However, measurement of ethylene production may be a valuable tool in determining sensitivity to auxin type herbicides since in both figures 5.8 and 5.9 the known sensitivity of plant species is clearly reflected in ethylene production values, with respect to controls. Ethylene production is a potential indicator of lethal herbicide binding and may be very useful in assessing selectivity and structure/activity relationships of novel auxin-type herbicides.

Chapter 6. DISCUSSION

The aims of this chapter are to summarise the findings of this study in relation to published literature, and to appraise the experimental techniques employed. The considered direction of future research will be indicated.

In major research programmes it is easy to lose sight of the original aim. It is therefore useful to ask the question why studies of herbicide action are undertaken, and note that the purpose of research into herbicide mode of action and selectivity arises from the ideal of making weed control a safer, more efficient and thus cheaper undertaking. From an understanding of herbicide effects on plant systems we may forsee the design of 'custom-built' compounds, and, via genetic manipulation, the ever-more realistic goal of breeding herbicide-resistant crop plants, possibly resulting in cheaper and safer foodstuffs. This philosophy has a substantial effect on experimental studies in that academically interesting findings may not demand futher investigation within terms of the original brief, eg. herbicde uptake patterns in a particular species may generate questions relating to the plant's systemic apparatus but, if uptake is eliminated as a factor in herbicide selectivity, further investigation would be irrelevant. This situation appears in the present study in that each area of experimentation yielded results which could have formed the basis of further intensive study and although this was advantageous in providing broad research experience, it presented problems in terms of the reference literature available. An extremely wide range of published material had some relevance to the project eg. herbicide studies, material on auxin and other plant growth regulators and so on, but, due to the molecule-specific and species-specific nature of each study, there was little particularly relevant material available.

The preliminary experiments in this study as described in Chapter 2 were carried out to fulfil the need for species-specific information on clopyralid action under our experimental conditions. This essential groundwork clearly defined the

sensitivity of the test species, with M.inodora proving susceptible to clopyralid, G.aparine mildly susceptible and B.vulgaris tolerant. This was in agreement with other selectivity studies eg. Brown and Uprichard, (1976) and Lake, (1980). The work also vindicated the study with respect to the field situation in that application of clopyralid by pot sprayer resulted in similar symptom development as that recorded in field studies (Richardson and Parker, 1977). Forming a unique study, the results nonetheless found several areas of correlation with the fragmented literature on clopyralid effects on other plant species and with the effects of similar compounds. Generally the visible symptoms of clopyralid action were typical of auxin-type compounds.

A criticism of initial experiments is that too few replicates were used in fresh and dry weight analyses and in the analysis of biochemical effects, since the variability of measurements prevented accumulation of meaningful data. In order that the initial experiments not be excessively lengthy, greater reliability could be achieved by reducing the number of plant species used, the duration of experiments, or the number of herbicide doses used. Reducing the number of plant species would probably have meant abandoning the mildly susceptible G.aparine which proved to be an extremely useful species in both uptake and translocation studies (Sanders, Thompson and Pallett, 1985), and ethylene experiments due to its growth mode. This would have been a substantial loss to the study. Reducing the duration of the experiments may have been advisable but recovery of B.vulgaris would not have been represented leaving its susceptibility characterisation incomplete. It is however considered that any future studies should concentrate more upon early effects (0-3 days), since the primary responses are most likely to be correlated with the mode of action. Reducing the number of herbicide doses would, on reflection, be the most appropriate route to follow should similar studies be carried out. Small scale experiments with several doses would establish one rate upon which to concentrate further work, alternatively recommended field rate could be used as in later stages of the present study.

In future biochemical analyses, the use of isloated cells could solve the problem of possible 'masking' of effects and would allow any cell-type specificity to be identified. The microscopic examination of herbicide effects on cell organelles as reviewed by Anderson and Thomson (1973) could also be valuable and ultrastructural effects may be correlated with biochemical responses. However the development of cell isolation methods and cell-study techniques could be a lengthy procedure and the relevance of cell effects to the whole plant situation uncertain. In summary, the preliminary data formed an essential basis for further experimentation.

Results of the uptake and translocation studies generally agreed with published material for clopyralid, eg. O'Sullivan and Kossatz, (1984); Devine and VandenBorn, (1985); Turnbull and Stephenson, (1985); Hall and VandenBorn, (1988); and Bovey, Hein and Meyer, (1988)A. Uptake and the rapid phloem translocation of clopyralid to photosynthetic 'sinks' occurred in all 3 test species with neither factor apparently being responsible for differential activity (Thompson and Cobb, 1986). Well established experimental methods were followed (Thompson et al, 1986) and radioassay procedures consulted (Coupland, 1986), thus reliability of resulting data was assured and the figures interpreted with the assumption that radiolabelled herbicide molecules behave in the same way as non-labelled compound. This assumption is acceptable since similar patterns of clopyralid uptake have been measured using non-radioassay methods (Bovey, Hein and Meyer, 1988B).

Two main criticisms of the experiments arose, firstly, dissection of plant tissue did not reveal the retention of clopyralid in stem tissue of G.aparine which appeared on autoradiographs. In future experiments greater attention should be paid to the sectioning of plants depending on the aims of the study in order that important patterns of translocation are not overlooked. Secondly, the expense and availability of 14-C-clopyralid only allowed the production of 2 replicates of each of the autoradiographs, ideally 4 or 6 would have been produced. Any further research in this area should concentrate on identifying and characterising and form of compartmentation of herbicide molecules following entry into plant cells. This

could result in sequestration of active molecules, or accumulation of molecules at an active site, and so affect selectivity. The use of isolated cells, as previously mentioned would be a possible route to follow, however, since behavior of these cells would be difficult to correlate to the whole-plant situation, it could be of more value to be able to 'fix' treated plants and extract organelles from these tissues to determine sites of accumulation. During extraction, however, herbicide may "leak" or be excreted from organelles making localisation of compound extremely difficult to measure (Hallam and Sargent, 1970).

The conclusion reached from metabolism studies in Chapter 3 was that differential metabolism does not contribute to clopyralid selectivity in the test species. Although this agrees with reports for other plant species eg Hall and VandenBorn (1988), further experimentation is demanded for 2 reasons.

(1) The application of large quantities of herbicide to plants in the metabolism study was necessary to ensure non-contact with the tissue of extraction and to provide a detectable amount of extractable material. This may have altered the metabolism of clopyralid compared to 'normal' amounts. The use of HPLC as a more accurate and sensitive technique than the relatively crude TLC would allow the use of low-volume droplet application by reducing the concentration of material needed for measurement of metabolites. Also, via this method metabolites could be chemically identified and then possibly synthesised in suitable quantities to allow the investigation of any physiological activity which they may have

(2) The bioassay used in this study provided only an indication of the activity of extracted compounds. Any further experiments should characterise the sensitivity of the assay by exposure to a wide range of auxin-type and non auxin-type compounds. Research must concentrate on this area if assumptions that clopyralid free-acid is the only active form of the herbicide are to be validated.

In his book on herbicide mode of action, Fedtke (1982) stated that one symptom in the phytotoxicity of auxin-type herbicides was the stimulation of photosynthesis. He gave no reference to the source of this statement and no modern reports have been uncovered to confirm it. This study clearly recorded an initial increase in photosynthesis in M.inodora in response to clopyralid which was undetected in tolerant B.vulgaris. The origin of the increase and its importance to herbicide selectivity strongly demands further investigation. Its relationship to the observed stomatal effects also needs to be clarified. A major flaw of this study was that in the short term IRGA experiment (Chapter 4), plants were held in the illuminated chamber for a continuous period of 24 hours. Such stressing of plants was considered justifiable since the procedure of sealing leaves into the chambers was often time-consuming and so accurate time intervals could not have been observed. Also, removing plants from the apparatus caused tissue damage and so different plants would have been needed for each time point and, with only one analyser available, the experiment could not have been completed within the research period. The use of a more modern hand-held IRGA, which clamps over the leaf surface without causing tissue damage, would have been a solution to the problem. However, in trials with a similarly designed porometer it was found that the leaf morphology of M.inodora was totally unsuitable for such experimentation since no substantial area of leaf surface area was available for use. Despite experimental problems, the validity of the results was maintained by reference to appropriate controls. A major consideration for any future research into herbicide effects on photosynthesis and stomatal mechanism would be to use plant species which have a large leaf area and a regular leaf shape, thus increasing the range of potential experimental techniques.

During measurement of the complex stomatal effects of clopyralid, a report was published indicating that ethylene gas generation was increased in response to clopyralid in susceptible but not tolerant s pecies (Hall et al, 1985). Noting that ethylene can effect the reguation of stomatal behaviour as well as being a possible mediator of other auxin effects, the investigation of any herbicide-induced ethylene production in the test species was undertaken. In agreement

with Hall et al (1985) a differential response to clopyralid was measured in tolerant and susceptible species. Since the explant system was extremely simple, the use of inhibitors of ethylene biosynthesis and precursors of ethylene biosynthesis as well as the close correlation to symptom development, allowed the important conclusion to be made that ethylene gas is not the cause of visual herbicide damage but is produced as a symptom of herbicide action in susceptible M.inodora (Thompson and Cobb, 1987). The measurement of this symptom as an effective and convenient means of testing new compounds for auxin-herbicide activity was shown to have some potential. The examination of a wide range of compounds and plant species of defined sensitivity would allow the method to be fully evaluated.

This study did not discover the mode of action nor the mechanism of selectivity of clopyralid in the test species. It did clearly define the species response to the herbicide and characterised clopyralid effects on stomatal aperture, photosynthesis and ethylene production. Differential uptake, translocation and metabolism were firmly excluded from a role in clopyralid selectivity.

In conclusion, clopyralid selectivity must be accounted for by one, or a combination of both of two phenomena. Firstly, compartmentation of compound within cells as discussed earlier and secondly, and perhaps more importantly, differential binding activity of herbicide molecules at one or more receptor site(s) within the plant cell. Assuming, as for auxin, that herbicide symptoms are initiated following binding at a receptor probably located in the plasmalemma (Brummell and Hall, 1987) (figure 5.9), the strength of that receptor affinity will influence the response produced. Further investigation should use the results of the present study as a basis from which to investigate binding activity. A recent paper by Fitzsimons, Barnwell and Cobb, (1988), describes an experiment on auxin binding which could indicate possible methods for future work. These authors used hydrogen ion efflux from Avena coleoptiles as a direct response to auxin binding at the plasmalemma, and measured the

rate of that efflux in response to IAA and auxinic compounds including clopyralid. In this system clopyralid was measured to be 6000 times less active than IAA. This report provides a valuable starting point for experiments with relevant tissues, possibly isolated cells, which may generate answers to questions of activity and selectivity. Investigation of binding activity may also explain observed cases of antagonism or synergism of herbicides such as the antagonism by clopyralid of picloram activity in rapeseed recently reported by Hall and Soni, (1989). Such work may eventually lead into an understanding of why and how binding initiates such a wide range of phytotoxic symptoms.

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Crop protection of sugar beet and crop protection and quality of potatoes: part I

Experimental studies into the selectivity of clopyralid in sugar beet

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SUMMARY

Under glasshouse and growth cabinet conditions sugar beet was found to be tolerant to 100 gai/ha clopyralid, whilst Tripleurospermum maritimum ssp inodorum (L.) Koch (Scentless Mayweed, Matricaria perforata Merat.) was susceptible. Experimental studies using ^{14}C-clopyralid, revealed that this selectivity did not arise from differences in the uptake of the compound from foliage or in the extent of movement from the treated leaf. Translocated material followed a similar distribution in both test species and metabolism did not appear to provide a detoxification route in sugar beet. Thus uptake, movement and metabolism do not appear to form the basis of clopyralid selectivity in these species.

INTRODUCTION

Annual broad-leaved weeds, particularly of the family Compositae, form the major weed problem in sugar beet (Fryer & Makepeace, 1977). Clopyralid, a discovery of The Dow Chemical Company, is a post-emergent broad-leaved herbicide for control of Compositae weeds selective in a variety of crops including sugar beet. In glasshouse trials 50 g a.i./ha clopyralid gave a 71% reduction in vigour of Tripleurospermum maritimum ssp inodorum (L.) Koch (Scentless Mayweed, Matricaria perforata Merat.) with only a transient 7% reduction in beet crop vigour twenty days after application (Richardson & Parker, 1977). The use of clopyralid alone and its addition to other sugar-beet herbicides, increases flexibility of weed control, especially during early crop growth when competitive ability is poor (Dow Agriculture, Technical Information).

Herbicide selectivity may result from differences in uptake into crop and weed species or differences in the rate and extent of subsequent movement of the compound within the plant (Thompson, Sanders and Pallett, 1986). Differential metabolism of a herbicide molecule by plant enzyme systems may also result in selective action (Schafer & Chilcote, 1970; Hallmen, 1974; Kirkwood, 1983). If an understanding of the reasons for herbicide tolerance and susceptibility can be reached, this may lead to increased efficiency and safety of usage via informed choice of optimum application rates, environmental conditions and growth stages. The design of more effective molecules and formulations may also be aided.

This paper describes a laboratory and glasshouse-based study which set out to assess the selectivity of clopyralid towards Tripleurospermum maritimum ssp inodorum (L.) Koch (Scentless Mayweed, Matricaria perforata Merat.) in sugar beet. The extent towards which selectivity depended upon differences in uptake, translocation and metabolism of the compound was investigated using techniques established in this laboratory (Sanders, Thompson & Pallett, 1985; Carr, Davies, Cobb & Pallett, 1986; Thompson et al, 1986).

MATERIALS AND METHODS

Plant growth conditions

Plants were raised in a glasshouse and J. Arthur Bowers potting compost was used throughout propagation. Sugar-beet seeds (cv. Salohill) were sparsely sown in trays to a depth of 1 cm and transplanted at the young seedling stage to two per 90 mm pot. T. maritimum seeds were surface sown in 90 mm pots and thinned to two per pot at the young seedling stage. When the first true leaf had emerged the pots were transferred to an environmental chamber (Fisons model 600G3, type TTL), 14 h day photoperiod, 20°C and 100 µmoles m^{-2} sec^{-1} (photosynthetically active radiation), 14°C at night. Plants were treated when three to four leaves were fully developed.

Herbicide application

Clopyralid was applied as the monoethanolamine salt. The recommended field dose of 100 g a.i./ha was used at a volume rate of 200 litres/ha. Spray applications were made using a laboratory pot sprayer (Mardrive Engineering Company) fitted with an 80° Tee Jet nozzle.

Assessment of susceptibility

Four replicate plants of each test species were examined 1, 3, 7, 14, 21 and 28 days after treatment. Symptoms were visually assessed and recorded photographically. Aerial plant parts were then dissected and oven-dried for 48 h at 90°C. Dry weights were measured and calculated as a % of control.

Uptake and translocation

^{14}C-pyridine-ring labelled clopyralid (supplied by The Dow Chemical Company) was applied to the third leaf of treated plants. The general methodology employed was as previously described (Sanders et al, 1985; Carr et al, 1986) and discussed (Thompson et al, 1986). Only the area over sugar-beet leaf veins was receptive to the application of 0.2 µl droplets. The minimum droplet size which could be consistently applied to the remainder of the leaf surface was 0.5 µl. This led to the adoption of two application regimes for the treatment of sugar-beet plants. Vein application consisted of ten 0.2 µl droplets placed above veins. Random application involved random placement of four 0.5 µl droplets on the leaf. In T. maritimum five 0.2 µl droplets were applied. Each application contained a total of 20,000 disintegrations per minute.

Metabolism studies

Methodology was as previously reported for clopyralid (Sanders et al, 1986). Essentially, apical regions from sugar beet and T. maritimum were

extracted into acetone 7 and 14 days after treatment and metabolites separated by thin layer chromatography (TLC) using chloroform, methanol and glacial acetic acid as solvents (80:20:1). Droplet application was made only over the veins of sugar beet.

RESULTS

Susceptibility assessment

Initial visual symptom development following foliar application of clopyralid was similar in sugar beet and T. maritimum. After one day leaf curling was observed in both species and this epinastic response progressed to petiole and stem tissue between one and three days after treatment. In T. maritimum, petiole elongation was stimulated up to seven days, after which cessation of growth was noted and necrosis developed preceding plant death, which occurred 21 to 28 days after treatment. Sugar beet showed recovery from initial symptoms by 14 days and, although new growth was distorted and plants appeared stunted compared to controls, recovery was nearing completion after 28 days. These observations were reflected in the shoot dry weight values in which an initial reduction in sugar-beet dry weight was followed by recovery towards control values within the test period. Conversely, T. maritimum showed a progressive reduction in shoot dry weight over the 28 day period (Fig. 1).

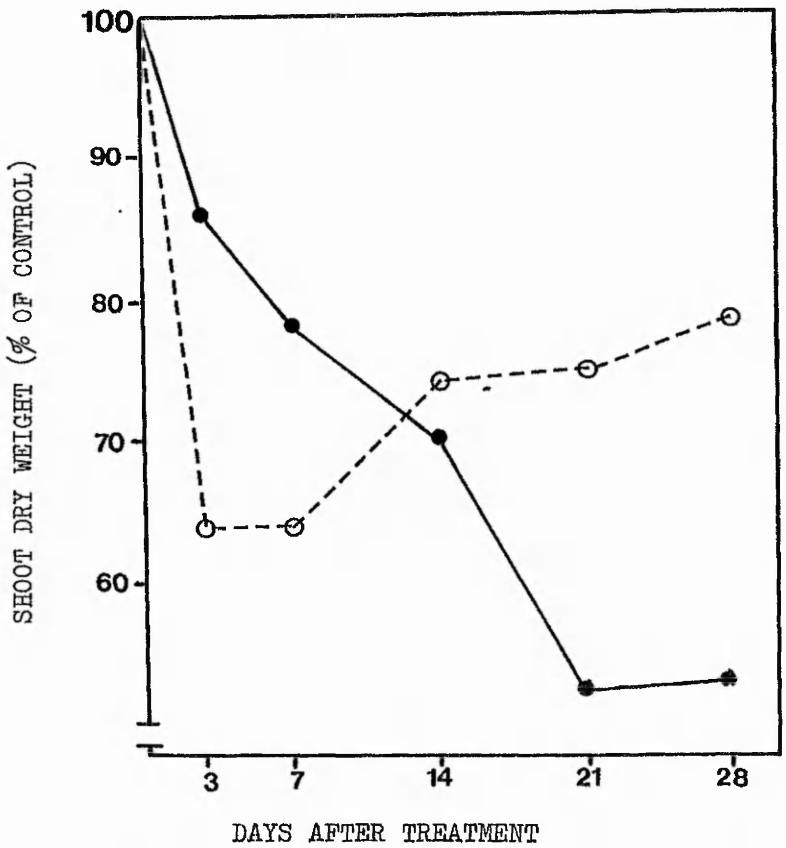

Fig. 1 The effect of clopyralid (100 g a.i./ha) upon shoot dry weight in T. maritimum (—●—) and sugar beet (--○--) Values plotted are means of four plants

Uptake

The uptake of ^{14}C-clopyralid, defined as the % applied ^{14}C-activity recovered from within plant tissues, was found to be rapid in both test species, with up to 11% of applied activity entering the plants after only eight hours. After 3, 7 and 14 days uptake into sugar beet was greater than the uptake measured in T. maritimum. In sugar beet ^{14}C-clopyralid applied to the vein surface as 0.2 µl droplets penetrated the leaf more rapidly than 0.5 µl droplets applied randomly, however the latter application gave more extensive uptake after 7 and 14 days (Table 1).

Table 1. Uptake of ^{14}C-clopyralid in sugar beet and T. maritimum. Values are the mean of six replicates and S.E.s are included

		Uptake of ^{14}C-clopyralid (% applied)			
Species	Application	8 hours	3 days	7 days	14 days
Sugar beet	Vein	11.29 ± 2.79	22.50 ± 3.45	22.62 ± 4.75	30.22 ± 4.22
	Random	2.29 ± 0.99	12.54 ± 3.49	28.55 ± 4.19	38.36 ± 6.69
T. maritimum	Random	5.27 ± 0.09	8.16 ± 0.08	11.59 ± 0.27	17.07 ± 4.07

Translocation

Translocation of ^{14}C-clopyralid out of the treated leaf was rapid in both sugar beet and T. maritimum. After eight hours 50% of ^{14}C-activity taken up from random application in sugar beet had been translocated, and the corresponding figure for T. maritimum was 61%. Translocation patterns were similar in the two species, with around 90% of the absorbed herbicide translocated after 14 days. Thorough analysis of treated plant revealed that the major sites of accumulation in both species were apical and developing leaf tissues. Vein and random applications to sugar beet resulted in similar translocation profiles after 14 days (Table 2).

Table 2. Major sites of ^{14}C-clopyralid translocation in sugar beet and T. maritimum 14 days after treatment. Values represent the mean of six replicates and S.E.s are given

	% Of total translocated ^{14}C-activity		
	Sugar beet		T. maritimum
	Vein	Random	Random
Young leaves	57.20 ± 3.63	56.88 ± 8.26	59.32 ± 9.53
Apical tissue	8.58 ± 3.70	12.48 ± 1.34	21.35 ± 6.76
Total	65.78 ± 7.33	69.36 ± 9.60	80.67 ± 16.29

Metabolism

Extraction of metabolites of ^{14}C-clopyralid from the apices of treated plants revealed after TLC, that the two species had different patterns of clopyralid metabolism (Fig. 2), Rf values being a measure of the polarity of radiolabelled molecules separated in this solvent system. Although there was considerable formation of polar conjugates in both species after 7 and 14 days, the amount of free acid (i.e. clopyralid) recovered from each species was comparable. However in T. maritimum a metabolite appeared with a Rf value of 0.5, which was not detected in sugar-beet extracts (Table 3).

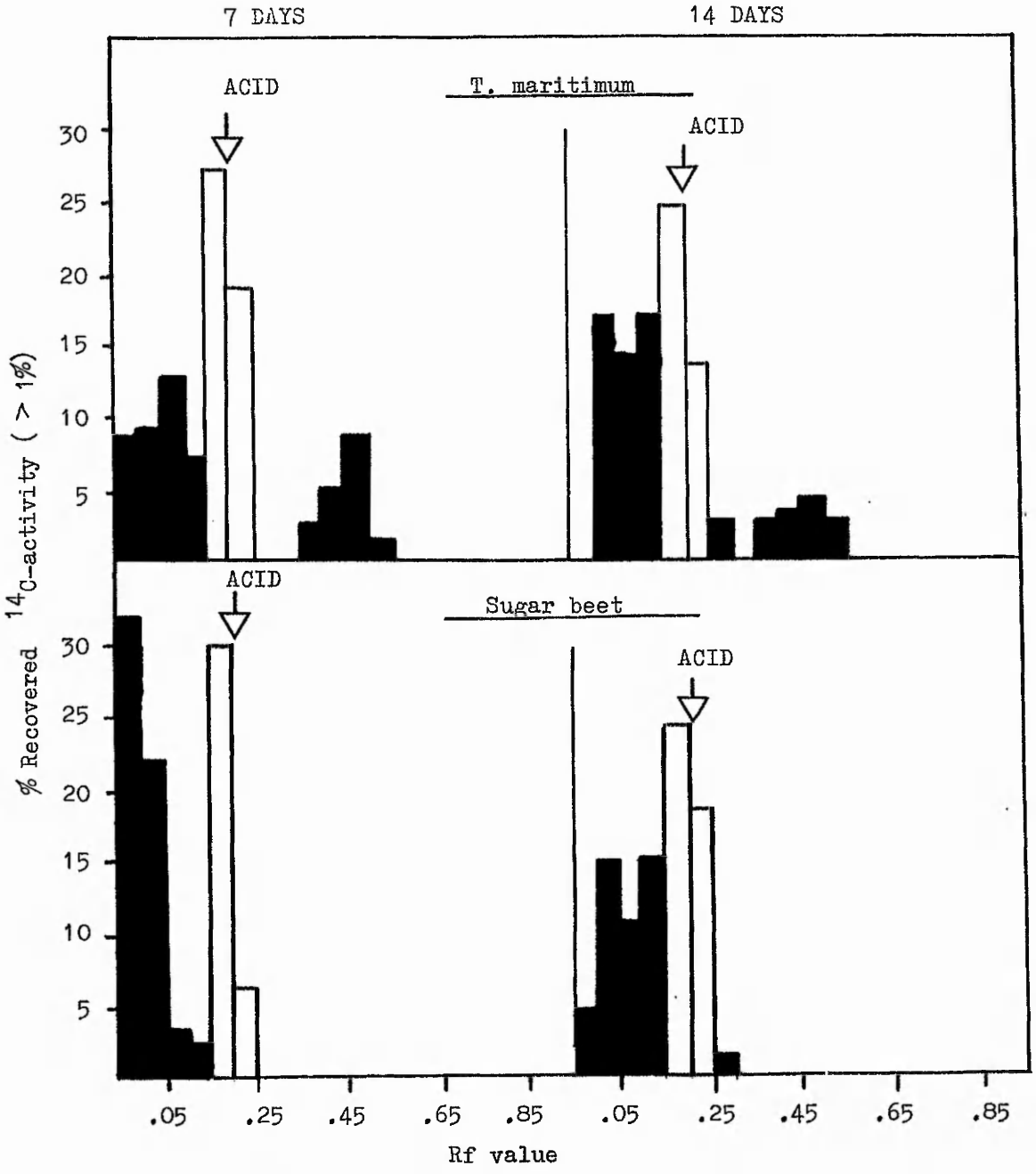

Fig. 2 TLC of ^{14}C-clopyralid metabolites extracted from the apices of T. maritimum and sugar beet 7 and 14 days after treatment

Table 3. Percentage of recovered ^{14}C-activity 7 and 14 days after treatment in three regions of TLC plates. Means are of 8 values and S.E.s are given

	Sugar beet		T. maritimum	
Rf value	7 DAYS	14 DAYS	7 DAYS	14 DAYS
0-0.15	57.70 ± 5.62	45.60 ± 11.51	35.17 ± 3.79	46.49 ± 3.64
0.15-0.25	33.94 ± 5.41	40.54 ± 4.60	44.15 ± 2.67	37.24 ± 4.58
0.45-0.55	-	-	12.91 ± 1.20	7.51 ± 2.21

DISCUSSION

Susceptibility and tolerance to a herbicide are relative terms and are influenced by several factors including herbicide dose, plant age and the degree of competition, as detailed for the phenoxyalkanoic herbicides by Pilmoor and Gaunt (1981). Under the experimental conditions used in this study, sugar beet could be regarded as tolerant to 100 g ai/ha clopyralid, whilst T. maritimum was highly susceptible. Sugar beet is not, however, resistant to the herbicide since sub-lethal symptom development was expressed in an epinastic response typical of auxin-type herbicides. Symptom development in sugar beet however was transient and plants showed a complete recovery. Dry weight data reflected visual symptoms and, although variation was very large, a response trend was obtained by this simple quantification. An extension of the test period to 60 days demonstrated complete recovery of sugar-beet plants (data not presented).

The rapid penetration of clopyralid into both test species is characteristic of this herbicide as shown in Cirsium arvense (L.) Scop. (Creeping Thistle) (O'Sullivan & Kossatz, 1984; Turnbull & Stephenson, 1985), Prosopis juliflora (Honey mesquite) (Bovey & Mayeux, 1980) and Brassica napus cv. 'Tower' (rapeseed) (Turnbull & Stephenson, 1981). The fact that a greater proportion of applied compound enters sugar beet than enters T. maritimum eliminates differences in uptake being responsible for clopyralid selectivity. Although the activity of foliage applied herbicides can depend, to a certain extent, upon the proportion entering the leaf, the amount reaching the biochemical site of action is more important (Price & Anderson, 1985).

The sensitive tissue for auxin-type herbicides is still uncertain with apical meristems, stems and roots being obvious candidates (Pilmoor & Gaunt, 1980). The extensive and complete translocation of clopyralid has been previously reported in Galium aparine L. (Cleavers) (Sanders et al, 1985), and translocation studies aimed to compare patterns of clopyralid movement in the test species. Accumulation in developing leaf and apical tissue indicates a phloem-mobile compound. The slight difference observed in the translocation rate is unlikely to account for observed selectivity and, although percentage accumulation at the apices is less in sugar beet than T. maritimum, the actual amount of herbicide reaching the apex this represents is likely to be comparable in each species when the uptake data is taken into account (Tables 1 and 2).

Metabolism studies were conducted to assess whether variations in the pattern and extent of clopyralid metabolism in the two species could contribute to selectivity. A similar proportion of ^{14}C-activity (10-15%) was

inextractable in each of the species due to irreversible binding to cell components (data not presented). Metabolites with ^{14}C-activity that were more polar than clopyralid in this system were thought to be conjugates of this acid molecule, presumably to amino acids, proteins and sugars. Conjugates may represent immobilisation and so inactivation of the acid, or may represent a potential reservoir of the herbicide. This was not further investigated since similar proportions of clopyralid were observed in each species after 7 and 14 days (Fig. 2). The less polar ^{14}C-clopyralid metabolite which appeared in the susceptible species at Rf value 0.45-0.55 may be a molecule with activity greater than clopyralid which could account for T. maritimum susceptibility. It may correspond to the finding of an unidentified metabolite in C. arvense by O'Sullivan and Kossatz (1984). In a bioassay of clopyralid-induced elongation of G. aparine explants, the unidentified metabolite showed activity but was not more active than the parent herbicide molecule (Thompson, unpublished data). The likelihood is that it is rapidly converted to the free acid which all published evidence cites as the active molecule. The compound may, however, have other activity which will be revealed only by further study which its discovery surely demands.

From our basic metabolism studies we find it unlikely that differential metabolism accounts for the selectivity of clopyralid in sugar beet.

From the investigations carried out in this study no clear indication of the process responsible for selectivity has yet emerged. Identification of the site or major sites of action of auxin-type herbicides may lead to the discovery of variations in sensitivity at that level. It does, however, seem unlikely that a single factor is responsible for the selective toxicity of clopyralid and a combination of factors restricting the effective herbicidal concentration in sensitive tissues of an apparently tolerant species is perhaps more probable.

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Aspects of Applied Biology 11, 1986
Biochemical and physiological techniques in herbicide research

Experimental studies into the uptake and translocation of foliage applied herbicides

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Summary: A summary of experimental methods for the investigation of uptake and translocation of foliage applied herbicides is presented. Consideration is given to factors including mode of herbicide application, site of application, the use of sprayed or unsprayed plants and the harvesting of treated material. Data is presented for several herbicides to demonstrate the type of information obtained from uptake and movement studies. To aid interpretation of such data the advantages and disadvantages of the major methods are highlighted.

INTRODUCTION

Studies of herbicide uptake can reveal information on its absorption into the plant cuticle and the rate of penetration into the underlying tissues. Translocation studies are carried out to assess the rate and direction of any herbicide movement from the site of application, and the major sites of herbicide accumulation within a treated plant.

When conducting herbicide mode of action research uptake and translocation studies can be vital to establish possible target tissues in susceptible species. Comparisons of uptake and translocation data from susceptible and resistant species may reveal, in part or whole, the reasons for observed selectivity (Schafer and Chilcote 1970).

Detailed work with one compound, to assess uptake and translocation at various application rates, environmental conditions and plant growth stages, allows a profile of herbicide behaviour to be constructed. This can be used to increase the efficiency and safety of herbicide usage in the field. Dose rate can be optimised and the timing of application with respect to plant growth stage and environmental conditions may be selected to maximise herbicide effect and minimise crop damage.

Uptake and translocation studies with more than one compound can often provide reasons for observed synergism. The rapid enhancement of the onset of glyphosate injury to Cirsium arvense shoots when low rates of picloram were incorporated, was found to due to the blocking, by picloram, of glyphosate movement from the shoots to the roots. This prevention of root kill also explained the antagonistic effect of picloram on long-term control of C.arvense by glyphosate (O'Sullivan and Kossatz 1982).

This paper aims to introduce the major experimental methods in use for investigation of uptake and translocation of foliage applied herbicides. Reference to specific data will highlight practical considerations involved in the design of such investigations.

METHODS OF STUDYING UPTAKE AND TRANSLOCATION

Uptake and translocation of foliage-applied herbicides is most easily followed using radiolabelled herbicides. This involves the application of radiolabelled compound to the test plant and the tracing of its subsequent fate. ^{14}C-labelled compounds are normally used. They are relatively cheap to produce and, because of their weak β-emission pose negligible danger to the investigator.

The two methods most commonly used differ in the technique employed to monitor distribution of the ^{14}C-activity. The first method, which is largely qualitative uses the property of ^{14}C to expose an X-ray emulsion. ^{14}C-labelled herbicide is applied to the test plant and, at various times after treatment, the plant is killed, dried and mounted securely on a flat surface. This is photographed and then placed in contact with an X-ray film, in darkness, for a suitable length of time. Following exposure, the film is developed, to produce an autoradiograph. By comparison of this to the original photograph, the distribution of ^{14}C-activity throughout the plant can be deduced. The intensity of exposure can give an indication of quantity of translocation. This method is useful since a simple, visual representation of herbicide distribution is produced. The problems involved in autoradiography include mounting of the material in order that all parts of a sample are in equal contact with the X-ray film. The morphology of some species makes this difficult. The method also necessitates application of relatively high amounts of activity and, even using highly sensitive X-ray film, the exposure time may be considered too great. The method of killing and drying plants for autoradiography is critical since re-distribution of herbicide within the plant between the time of harvesting and the presentation of material to X-ray film must be avoided. Freeze drying has been found to be the best method, (Crafts 1956) although the dissecting of material immediately into small sections before oven drying provides a suitable alternative means of limiting label re-distribution.

The second major method of measuring uptake and translocation of foliage applied herbicides concerns tracing the fate of an applied ^{14}C-labelled herbicide using quantitative liquid scintillation counting (LSC). At harvest the residual ^{14}C-labelled herbicide on the leaf surface is usually washed into blank formulation. ^{14}C-labelled herbicide in the cuticular wax is extracted by a chloroform wash and measured by LSC. ^{14}C-activity within plant tissues may be released either by solubilization using tissue solubilizers or, as $^{14}CO_2$, by sample oxidation. Where a known quantity of ^{14}C-activity is applied to a plant, quantitative radioassay can provide an accurate profile of ^{14}C-herbicide distribution. Uptake and translocation are normally stated as a percentage of the applied dose. Herbicide uptake is the amount of ^{14}C-herbicide washed from the leaf surface and the cuticular waxes, subtracted from the total applied. The amount of herbicide accumulating at sites remote from the site of application is regarded as the translocated herbicide.

MODE OF APPLICATION OF RADIOLABELLED HERBICIDE

The major consideration in uptake and translocation studies is the means of applying radiolabelled herbicide. Ideally, to mimic field conditions, the compound should be sprayed onto plants. This is impractical for three reasons: firstly because of the danger to the experimenter, secondly, because a known amount of activity can not be accurately applied and thirdly because of the expense involved.

Although micro-sprayers have been developed for the application of small volumes of labelled pesticides over known areas (Coggins and Baker 1983), common practice is to place droplets onto the plant surface using a hand

syringe or a mechanical microdroplet applicator. The size of droplets used is limited by the accuracy with which they may be consistently produced. This laboratory has found that 0.2µl droplets can be dispensed consistently using a Burkard microdroplet applicator (Rickmansworth UK). This gives the closest practical approximation to field conditions since field spraying equipment produces droplets in the volume range 0.005-0.25ul (Calculated from Fryer and Makepeace 1977).

Although 0.2µl droplets can often be used, limitations on their application may be posed by variations in plant surface characteristics. During a study of uptake and translocation of 3,6-dichloropicolinic acid in Beta vulgaris, (Thompson 1985 unpublished data) it was discovered that 0.2µl droplets could only be applied over the veinal area of the leaf. The hydrophobic nature of the remainder of the leaf surface prevented transfer of droplets from the dispensing needle without damage to the cuticle. To allow the randomised positioning of droplets over the leaf surface the droplet volume had to be increased to 0.5µl. This was considered too large for results to be directly relevant to field conditions. The difficulty in application resulted in a comparison being made between uptake and translocation of 10 x 0.2µl droplets from the surface of veins, and following random application of 4 x 0.5µl droplets: Uptake results (Figure 1) show that although herbicide placed on

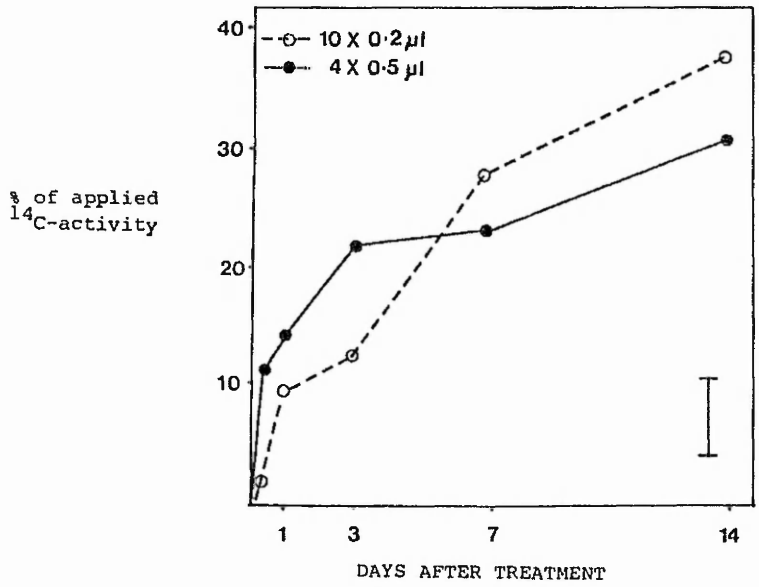

FIGURE 1. Uptake of ^{14}C-3,6-dichloropicolinic acid by 3rd leaf of Beta vulgaris. Plants were treated at the 4 leaf stage and maintained in a constant environment chamber throughout the experiment. (14 hour day 20°C, 10 hour night 14°C).
Data is mean of 6 replicates and bar represents mean standard error.

the vein surface more rapidly penetrated the treated leaf, the random application of 0.5µl droplets resulted in more extensive uptake after 7 and 14 days. Little difference was found in the percentage of penetrated herbicide which was translocated out of the treated leaf. (Table 1).

TABLE 1. Translocation of ^{14}C-3,6 dichloropicolinic acid in B.vulgaris

Days After Treatment	% total applied activity 1	3	7	14
Veins (10 x 0.2µl)	61.5 ± 2.55	60.0 ± 3.26	81.2 ± 3.77	87.8 ± 4.03
Random (4 x 0.5µl)	63.5 ± 1.90	58.4 ± 2.68	50.4 ± 3.52	93.4 ± 6.90

Data is a mean of six replicates and standard errors are included. Plant growth stage and treatment conditions as figure 1.

Several papers have been published where ^{14}C-labelled herbicide has been applied in droplets of 5µl (O'Sullivan and Kossatz 1984) or 10µl volumes (Hunter and McIntyre 1974). The relevance of this work, using such large droplets must be questioned. The use of lanolin rings in such studies, to contain the herbicide and prevent runoff from the leaf surface has also been criticised. It has been shown that such procedures significantly alter absorption and translocation of both glyphosate and 2,4-D in the leaves of Apocynum cannabinum and that their use is best avoided if field conditions are to be simulated. (Schultz and Burnside 1980).

The total volume of labelled compound applied in uptake and translocation studies should be comparable to the amount retained by that region of the plant during a field treatment. This may be determined by spraying plants with a coloured, water soluble dye eg. tartrazine. A known concentration of dye washed into a known volume immediately following spraying may be measured colorimetrically and retention on any part of the plant can be calculated. (Pallett and Caseley 1980).

A known amount of ^{14}C-activity must be contained within the volume to be applied. Generally, the greater the amount of activity applied then the greater is the accuracy achieved. Limits in this respect are set by the availability of labelled compound and its specific activity. It must be noted that ^{14}C-labelled herbicide should be prepared in its most commonly used formulation and any dilution should be with unlabelled herbicide at field rate.

SITE OF APPLICATION

An important factor in uptake and movement studies is the site of herbicide application. It has recently been shown in Galium aparine that consideration of the site of spray interception is important with ^{14}C-fluroxypyr uptake, (Sanders, Thompson and Pallett 1985). There was greatest spray interception by the second whorl from which greatest uptake of ^{14}C-fluroxypyr occurred (Table 2).

Enhanced uptake by younger leaves has been reported for other herbicides (Norris 1974). The site of spray interception was also found to influence the direction of translocation. ^{14}C-fluroxypyr penetrating the cotyledons was primarily translocated to adjacent developing laterals whereas that absorbed by the second whorl moved mainly to the shoot apex, (Sanders, Thompson and Pallett 1985).

TABLE 2. Retention of tartrazine dye and uptake of ^{14}C-fluroxypyr by G.aparine after 7 days

Region of plant	cotyledon	whorl 1	whorl 2
Dye retention	13.3 ± 0.98	26.7 ± 3.73	38.7 ± 1.85
^{14}C-fluroxypyr uptake	39.9 ± 5.45	47.7 ± 2.41	75.6 ± 5.70

Data represents % of total applied. It is a mean of 6 replicates and standard errors are included. Plants were treated at 2-3 whorl stage. Environmental conditions as figure 1.

Several studies of the effect of placement of herbicide upon uptake and translocation have been conducted (eg. O'Sullivan and Kossatz 1984) and it is considered to be an important factor governing the extent of uptake and the direction of any herbicide translocation. For this reason any valid and relevant study of uptake and translocation must include consideration of the site of application.

SPRAYED VS UNSPRAYED PLANTS

A great deal of uptake and translocation work has been conducted on unsprayed plants. Since differences have been found in uptake and movement patterns between sprayed and unsprayed plants, this laboratory considers it more relevant to record the movement of herbicides in sprayed plants. This is carried out by covering the area to which radiolabelled herbicide is to be applied with a polythene cover (Figure 2).

The plant is then sprayed with unlabelled herbicide using a laboratory pot sprayer. The cover is then removed and radiolabelled compound applied to the unsprayed region.

In a comparison of uptake and movement of ^{14}C-fluroxypyr in sprayed and unsprayed Viola arvensis there was no difference in the uptake but unsprayed plants showed over three times more translocation than sprayed plants (Table 3).

TABLE 3. The uptake and translocation of ^{14}C-fluroxypyr by the 4th leaf of Viola arvensis. A comparison of movement in fluroxypyr-sprayed plants

	% applied ^{14}C-activity		
	uptake	translocation	recovery
Unsprayed	56.1 ± 5.1'	29.5 ± 2.29	85.1 ± 1.82
Sprayed	51.1 ± 8.0ᵕ	9.7 ± 2.02	85.6 ± 2.52

Plants were treated at 4-5 leaf stage. Data is the means of 6 replicates and standard errors are included. Growth conditions as figure 1.

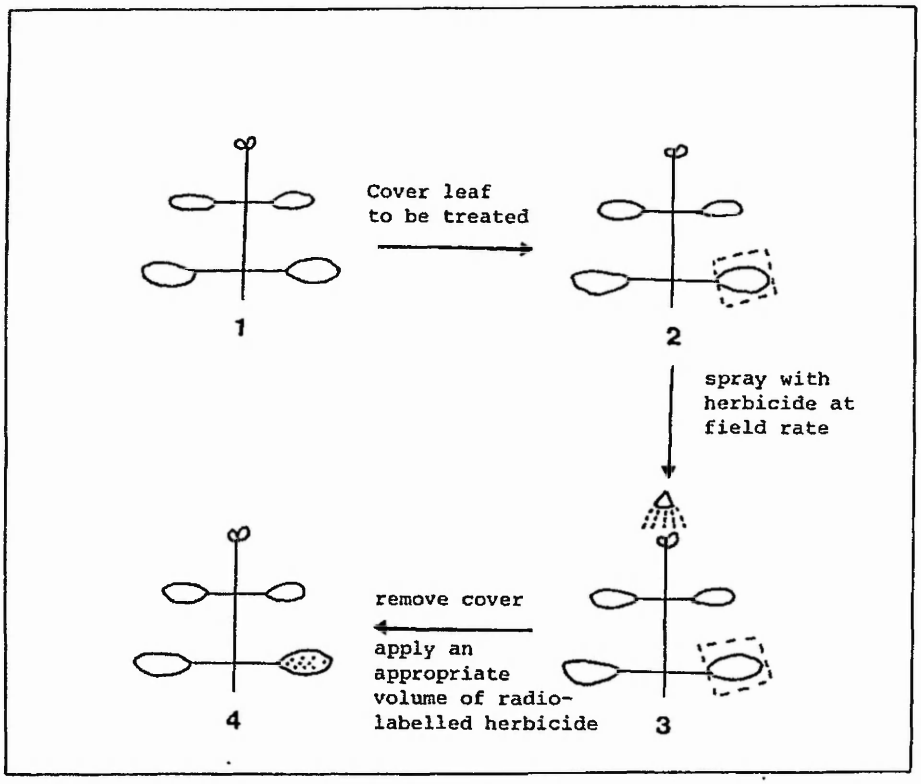

FIGURE 2. A procedure for uptake and translocation determination in sprayed plants.

The distribution pattern of translocated herbicide was also different (Table 4). In particular, the % translocation to the roots was much greater in unsprayed plants than sprayed plants.

TABLE 4. Distribution of translocated ^{14}C-fluroxypyr in V.arvensis 7 days after treatment

	% of translocated ^{14}C-activity		
	Older leaves	Young leaves+apex	Roots
Unsprayed	11.84 ± 0.80	44.74 ± 1.63	43.41 ± 5.36
Sprayed	29.47 ± 5.38	66.67 ± 11.07	8.09 ± 1.34

Data is mean of 6 replicates and standard errors are included. Growth and treatment conditions as Table 3.

Studies of uptake and movement in sprayed plants are more relevant to field conditions than those carried out in unsprayed plants.

HARVESTING OF EXPERIMENTS

Following application of ^{14}C-labelled herbicide, plants are harvested at specific time intervals. The intervals selected will depend upon the purpose of the experiment and the herbicide in question. Three major considerations arise:- The first is the necessity of conducting a harvest at time zero where washing of applied ^{14}C-labelled herbicide into blank formulation is carried out immediately following application.. This gives an accurate figure for the amount of recoverable activity applied.

Secondly it must be ensured, throughout the time course, that distribution of the ^{14}C-activity may be regarded as indicative of the distribution of the herbicide molecule (Kirkwood 1983). In short term experiments or in the case of a very stable compound, which remains relatively unmetabolised, distribution of the ^{14}C-label is generally indicative of the herbicide but this will not be the case if the herbicide is rapidly metabolised. Metabolism studies concurrent to uptake and translocation work are often necessary. Many herbicides, particularly ester forms are metabolised in leaves prior to translocation, (Richardson 1977; Sanders, Thompson and Pallett 1985; Carr, Davies, Cobb and Pallett 1986).

Important properties of herbicides may be masked by the harvest intervals chosen. An example is shown in the uptake of ^{14}C-fluroxypyr in V.arvensis (Sanders unpublished data). Harvesting after 4 hours, it was found that almost 50% of the applied label was not recoverable into blank formulation but had penetrated into the cuticular waxes and was recoverable by washing the treated area in chloroform. This explains the rainfastness property of fluroxypyr which may contribute to its effectiveness as a herbicide. The cuticular waxes were found to act as a resevoir for continued herbicide uptake (Figure 3).

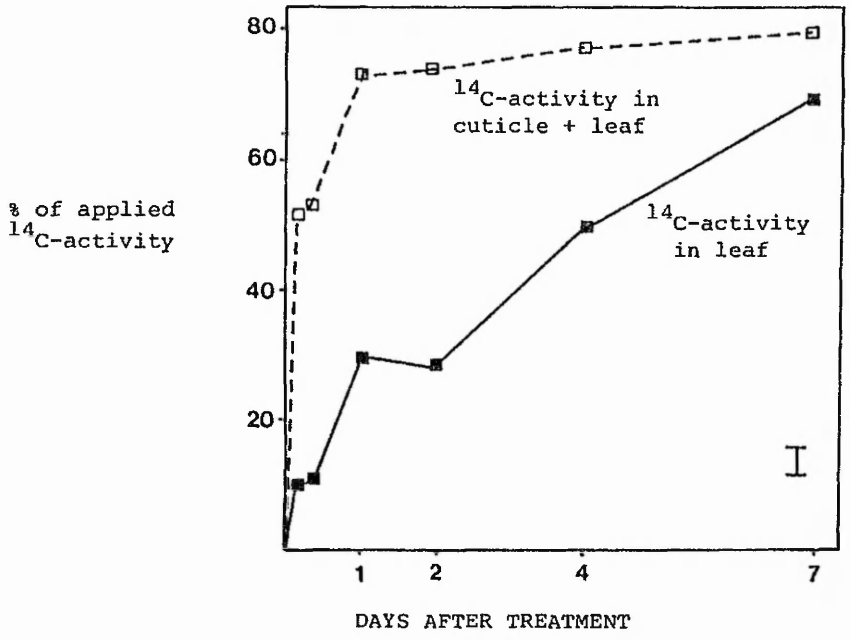

FIGURE 3. Uptake of ^{14}C-fluroxypyr by V.arvensis.
Growth conditions as Figure 1, plant treatment as.table 3. Data is mean of 6 replicates. Bar represents mean standard error.

Sectioning of plant material prior to quantitative radioassay can be crucial to the results gained. Tissues must be dissected in such a way that a distribution profile may be constructed. The choice of plant division is generally made in response to the questions set by a particular study. Autoradiographs can be useful as a guide to dividing tissue. When a treated plant is sectioned, tissue must be small enough to solubilise or oxidise. Details of tissue processing for LSC are given by Coupland (this volume).

In sprayed Stellaria media ^{14}C-fluroxypyr is translocated to and retained in the stem to a significant degree (Table 5). This discovery has led onto further research on the mode of action of fluroxypyr in the stem. In a great many studies the stem has not been regarded as a separate tissue but has been

included with other sections of the plant. Caution is therefore needed in the interpretation of data from such studies.

TABLE 5. Uptake and translocation of ^{14}C-fluroxypyr in S.media after 7 days

Region of plant	% of applied ^{14}C-activity
Aqueous leaf wash	2.7 ± 0.16
Chloroform leaf wash	14.0 ± 1.00
Treated leaves	20.0 ± 2.68
Leaves below	0.4 ± 0.09
Stem below	9.5 ± 2.26
Leaves above	3.9 ± 0.69
Stem above + apex	26.6 ± 2.19
Lateral shoots	1.3 ± 0.57
Roots	0.6 ± 0.05
Total	79.1 ± 8.05

Data represents mean of 6 replicates. Standard errors are included. Treatment was to the 3rd leaf pair of plants at the 3-4 leaf pair growth stage.

LOSSES OF RADIOLABELLED HERBICIDE

An ideal study of herbicide uptake and translocation would include a balance whereby 100% of applied activity could be accounted for. This is rarely the case and, in many publications, total recoveries are not stated.

When examining losses in recovery, 3 immediate routes exist. The first is loss by volatilisation from the leaf surface which is obviously a factor in the field as well as in the laboratory. To assess the extent of volatilisation of a herbicide under laboratory conditions plant waxes can be removed from leaves and placed on glass coverslips. This forms artificial leaf surfaces from which loss of ^{14}C-labelled herbicide may be monitored independant of any uptake factors. Some compounds show measurable volatility eg. unformulated loxynil and Bromoxynil salts show approximately 20% volatilisation 7 days after application to Viola arvensis and Matricaria inodora isolated waxes (Table 6).

TABLE 6. Evaporation of ^{14}C-loxynil and Bromoxynil salts from V.arvensis and M.inodora isolated cuticles after 7 days at 20°C

	% applied ^{14}C-activity	
	V.arvensis	M.inodora
Ioxynil-Na	20.3 ± 2.67	17.3 ± 1.53
Bromoxynil-K	22.7 ± 1.99	20.3 ± 2.0

Data is mean of 8 replicates. Standard errors are included.

A limitation of this technique is that extracted waxes are unstructured therefore the applicability of data to intact leaf surfaces must be questioned (Holloway 1969).

Root exudation may contribute to losses and this can be studied by growing test plants hydroponically in liquid media and recording exudation of foliar applied ^{14}C-labelled herbicide into the solution. In a study of herbicide translocation in C.arvense, (Turnbull and Stephenson 1984), 48% of applied ^{14}C-2,4-D was found to be exuded from the roots after 9 days. This fact, coupled with the information that the distribution of ^{14}C-2,4-D within plants grown hydroponically was very similar to the distribution within soil grown plants allowed root exudation to be considered a viable source of loss and not merely an effect of hydroponic cultivation. Since, however, in some species hydroponic cultivation can alter the translocation pattern of certain herbicides it is recommended to carry out both hydroponic and soil experiments.

Metabolism of herbicide where ^{14}C-activity is lost to the atmosphere as $^{14}CO_2$ is another possiblity for loss. This may be minimised by using ring labelled compounds where possible, (Kirkwood 1983). Ring structures show greater metabolic stability than chain structures, but even where no loss is found, it is necessary to identify the nature and biological activity of the ^{14}C-label recovered, to ensure that the herbicide has not been conjugated or metabolised to an inactive form.

Although the source of losses of radiolabelled herbicide must be examined, losses of 15-20% over a 14 day period are commonly considered acceptable in published work.

CONCLUSION

Although uptake and translocation studies are considered vital in herbicide research, interpretation of papers is often difficult because of the many different methods used. Major points may be overlooked and the relevance of resulting data to field conditions is often limited by practical considerations in the laboratory.

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THE SELECTIVITY OF CLOPYRALID IN SUGAR BEET; STUDIES ON ETHYLENE EVOLUTION.

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ABSTRACT

Clopyralid induces rapid, concentration-dependent evolution of ethylene from excised scentless mayweed leaves, which precedes the onset of visible epinastic symptoms, and is comparable to that induced by exogenous auxin. However, incubations using inhibitors (AVG and Co^{++}) and a precursor of ethylene biosynthesis (ACC) have shown symptom development to be independent of ethylene production in this experimental system. Incubation of excised sugar beet leaves with clopyralid does not result in increased ethylene production or the development of visual epinastic symptoms after 24 h. However, an intact ethylene biosynthetic pathway does exist in sugar beet since sensitivity to IAA and 2,4-D was demonstrated. These observations indicate that the selectivity of clopyralid is not solely related to differential ethylene evolution, but is more likely to result from differential binding affinity to an "auxin site".

INTRODUCTION

The herbicide clopyralid, a discovery of The Dow Chemical Company, is recommended for selective weed control in Brassica crops and sugar beet. It is particularly active against weeds of the families Compositae and Leguminosae (Dow Technical Information). Recent studies in this laboratory have shown scentless mayweed (Matricaria perforata Merat.) to be highly susceptible to clopyralid whilst sugar beet (Beta vulgaris cv. Salohill) is tolerant. The basis of this selectivity remains obscure, being independent of differential herbicide uptake, translocation or metabolism (Thompson & Cobb, 1986).

Clopyralid is a synthetic pyridine compound which, like certain benzoic acid and phenoxyalkanoic acid derivatives, is described as an "auxin-type" herbicide. This classification arises from the fact that many effects of such herbicides on plant growth and anatomy are similar to those caused by exogenously applied auxin (Haagsma, 1975; Hall et al, 1985). One common symptom is the rapid induction of young stem and leaf epinasty which has been linked to the production of ethylene gas by treated tissues (Baur & Morgan, 1969; Hall et al, 1985). This association is supported by the findings that auxin increases the rate of ethylene production in vegetative tissue (Imaseki, 1981), and that exposure of whole plants to ethylene at very low concentrations can lead to epinastic symptom development (Crocker, 1948). It is now established that auxin-type herbicides stimulate ethylene production in a number of species, and a recent report suggests that enhanced ethylene production following the application of clopyralid to sunflower (Helianthus annus) is a factor involved in resulting symptom development (Hall et al, 1985). These authors also cite differential ethylene production in clopyralid-tolerant and susceptible species, although no inference is made of the significance of this difference in terms of a possible contribution to clopyralid selectivity.

This paper describes the measurement of ethylene production in response to clopyralid application in excised leaves from tolerant sugar beet and

susceptible mayweed. Its relationship to symptom development and selectivity has also been assessed with reference to our current understanding of ethylene biosynthesis. Ethylene is produced from methionine via intermediates S-adenosylmethionine (SAM) and 1-aminocyclopropane carboxylic acid (ACC) (Yang & Hoffman, 1984). The conversion of SAM to ACC is catalysed by the enzyme ACC synthase and forms the rate-limiting step in the pathway. The production of ACC synthase is auxin sensitive and the enzyme is specifically inhibited by aminoethoxyvinylglycine (AVG) (Imaseki, 1981). The conversion of ACC to ethylene is catalysed by an enzyme complex referred to as the ethylene forming enzyme (EFE) and is not rate-limiting. This final reaction step may be inhibited by cobalt ions (Yang, 1981).

MATERIALS AND METHODS

Plant Material

Plants were propagated in J. Arthur Bowers seed and potting compost in 5 cm deep trays. Sugar beet seeds were sparsely sown at a depth of 5 mm, whilst mayweed seeds were surface sown. Trays were maintained under glasshouse conditions with a 14h day (200-400 µmole photons/m^2/s, photosynthetic photon flux density, PPFD) and 20-25^{o}C. In all experiments plants were used at the three to four leaf stage.

Chemicals

All test solutions were first dissolved in methanol (final concentration (<0.2%), and diluted with 10^{-3}M MES (2-[N-Morpholino]-ethane sulfonic acid) buffer adjusted to pH6 with NaOH. AVG, ACC, 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) were obtained from the Sigma Chemical Company. Cobaltous chloride was obtained from BDH chemicals. Technical grade clopyralid was a gift from The Dow Chemical company.

Incubation system

For consistency with previous work (Thompson & Cobb, 1986), leaf 3 of both species was used. Following excision at the stem, leaves were exposed to test chemicals in gas tight vials as illustrated in figure 1.

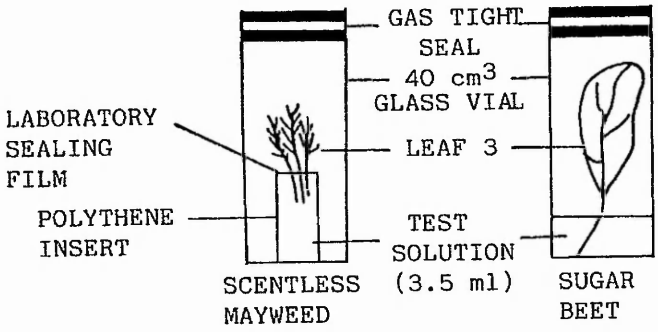

Fig. 1. Experimental system for measurement of ethylene production by excised leaves.

The vials were incubated in a controlled temperature water bath at 25^{o}C and illuminated at 50 µmoles photons/m^2/s (PPFD) using natural white fluorescent tubes (Phillips). One cm^3 gas samples were withdrawn at regular intervals

using a gas-tight syringe. Ethylene concentration was then determined in a gas chromatograph (Sigma 3B-Perkin Elmer) equipped with a Poropack Q. 80-100 mesh column (Perkin Elmer) and a flame ionisation detector. Oven temperature was maintained at 100°C. A laboratory computing integrator (Perkin Elmer LC1-100) was used to calculate the amount of ethylene in each sample with reference to appropriate standards. After each withdrawal, gas volumes in each vial were amended with ambient air and results adjusted to account for dilution during sampling. Data are expressed on a fresh weight basis and are corrected, where necessary, for any ethylene present in blank tubes. Epinastic symptom development was both visually assessed and recorded photographically. Each experiment was fully replicated on 3 or 4 separate occasions and mean values plus or minus standard errors are presented.

RESULTS

Initial studies clearly demonstrated a differential ethylene response in clopyralid - susceptible and tolerant species (Figure 2).

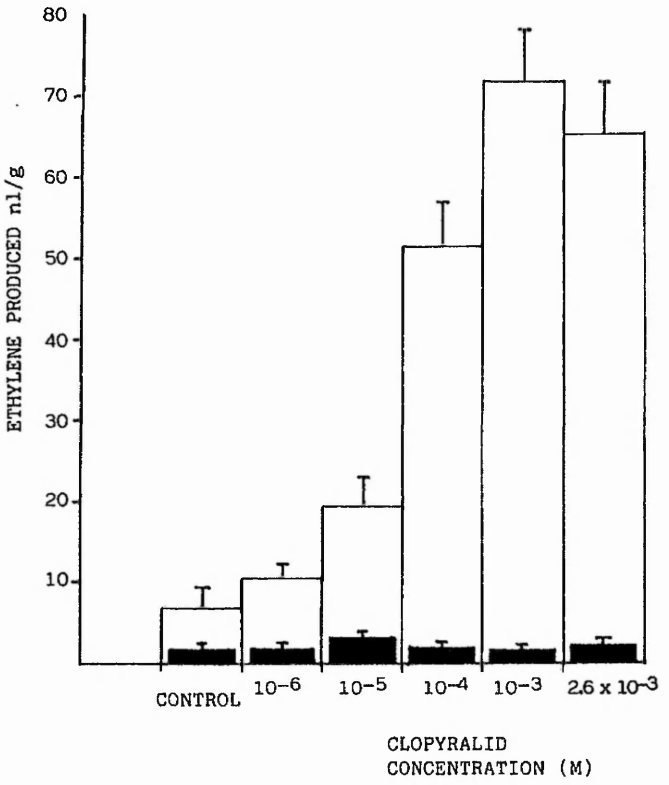

Fig. 2. The effect of clopyralid upon ethylene evolution in mayweed (□) and sugar beet (■) following incubation of excised leaves for 24h. Each point is the mean from 8 separate plants. Bars represent S.E.'s.

After 24h incubation of excised leaves with a range of clopyralid concentrations, tolerant sugar beet showed no increased ethylene production with respect to controls, whilst the identical treatment of scentless mayweed resulted in a concentration-dependent increase in ethylene production of up to 10 fold. This increase was highly significant ($P<0.001$) at 2.6×10^{-3}M and 1×10^{-3}M clopyralid and significant ($P<0.01$) at 10^{-4}M. Sugar beet leaves appeared unaffected by the herbicide after the 24h period, whereas mayweed exhibited classic epinastic symptoms which increased in severity with increasing herbicide dose. Short-term studies revealed that the clopyralid-induced increase in ethylene production in mayweed was an extremely rapid and concentration-dependent response (Figure 3), being measurable after only 1h incubation and sustained for the following 5h.

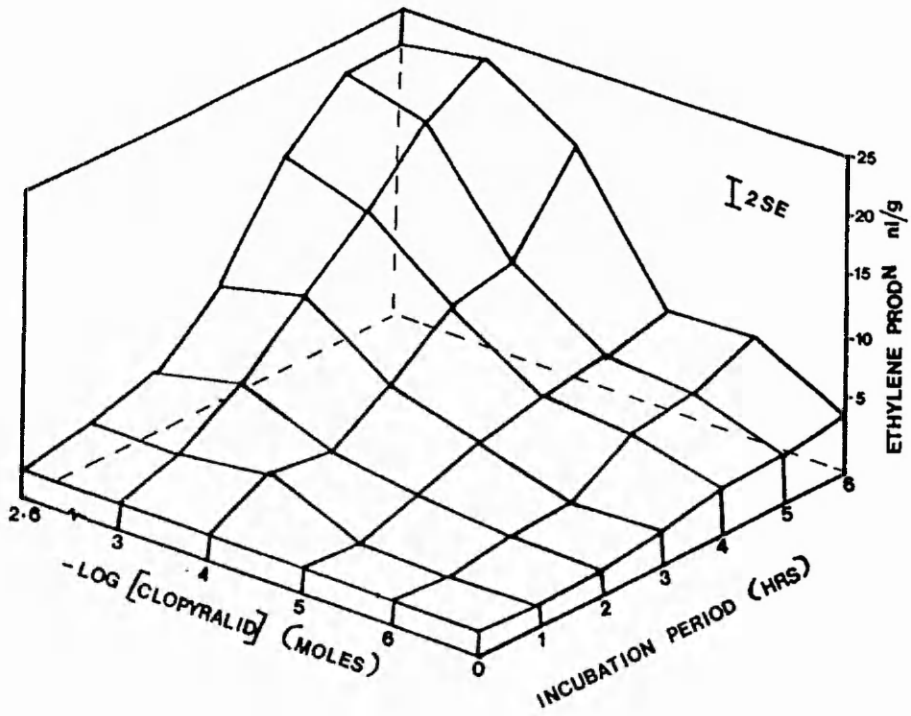

Fig. 3. Effect of clopyralid upon ethylene evolution in scentless mayweed, dose-response and time-course over 6h. Each point is the mean from 8-12 separate plants. Average standard error is indicated.

Increases were statistically significant ($P<0.01$) after only 2h at the two highest clopyralid concentrations and after 3h at 10^{-4}M. Clopyralid concentrations of 10^{-5}M and 10^{-6}M also gave consistently increased ethylene production although values were not statistically different from controls. As found in the 24h experiment, incubation of excised mayweed leaves with 10^{-3}M clopyralid gave the greatest ethylene production with 2.6×10^{-3}M yielding no further increase. Symptom development commenced after 4h at the highest clopyralid doses, and by 6h leaf epinasty was noted at all concentrations. The response of mayweed to 10^{-3}M clopyralid was equivalent to that induced by 10^{-3}M IAA over both 6 and 24h, as illustrated in figure 4.

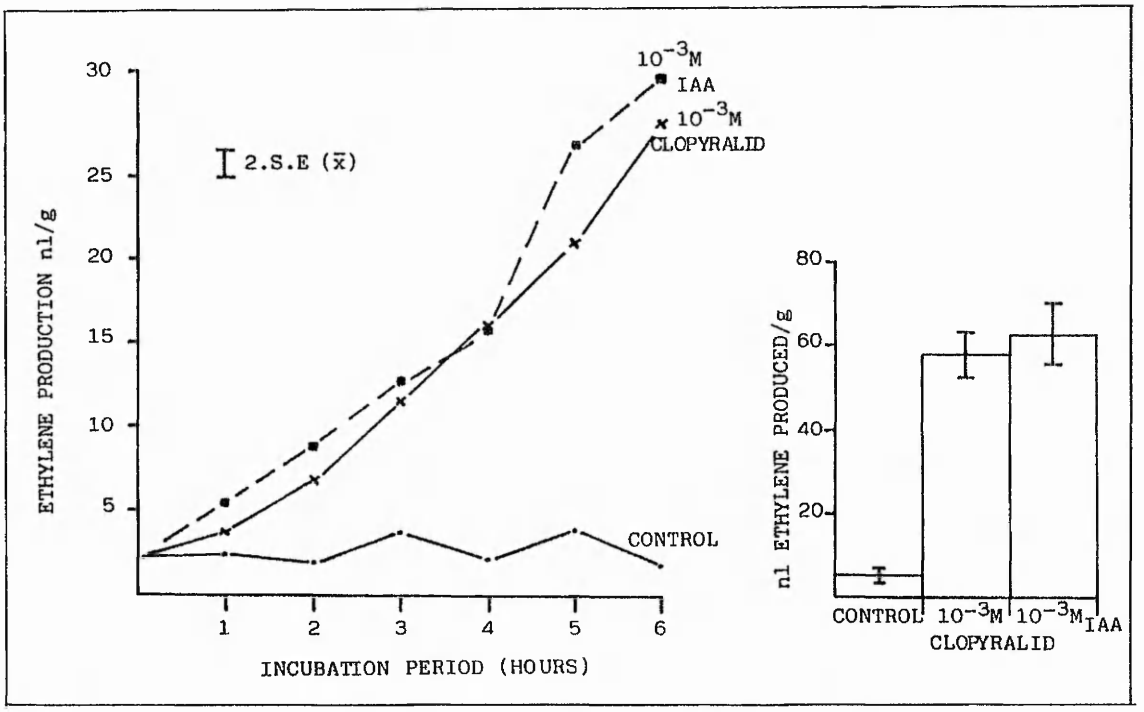

Fig. 4. Effect of clopyralid and IAA on ethylene evolution in scentless mayweed over 6 and 24h. Each point is the mean from 8 separate plants. Bars represent S.E.'s.

The onset and development of visual epinastic symptoms was similar with both clopyralid and IAA. The ethylene biosynthesis inhibitors AVG and Co^{++} reduced mayweed ethylene production in response to 10^{-3}M clopyralid by approximately 50% over 6 and 24h incubations (Figure 5). However, although ethylene production was reduced in the presence of these inhibitors, symptom development was not diminished.

Although sugar beet showed no symptom development or increased ethylene production in response to 10^{-3}M clopyralid, the same concentration of IAA or 2,4-D increased ethylene production by 5 and 12 fold respectively (Figure 6).

These marked increases were accompanied by typical symptom development. However, although sugar beet was insensitive to clopyralid, both species were capable of converting exogenous ACC to ethylene over a 24h period. (Table 1). No symptom development was noted with either species after 24 hours.

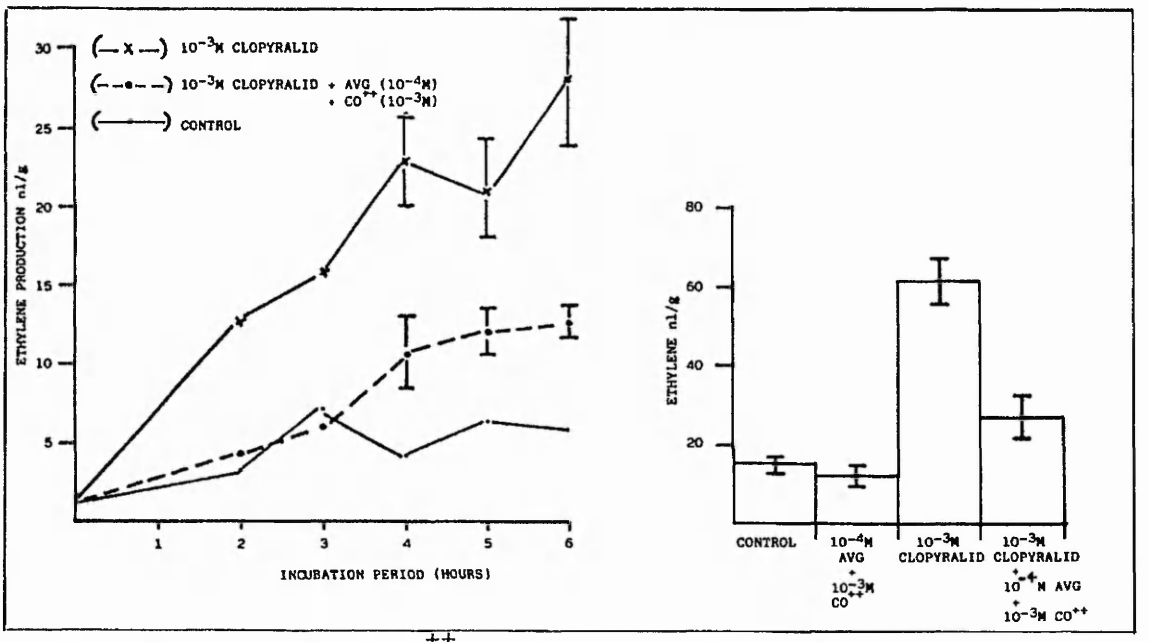

Fig. 5. Effect of AVG and Co^{++} on clopyralid-induced ethylene production in mayweed over 6 and 24h. Data are means of 6-8 replicates. Bars represent S.E.'s.

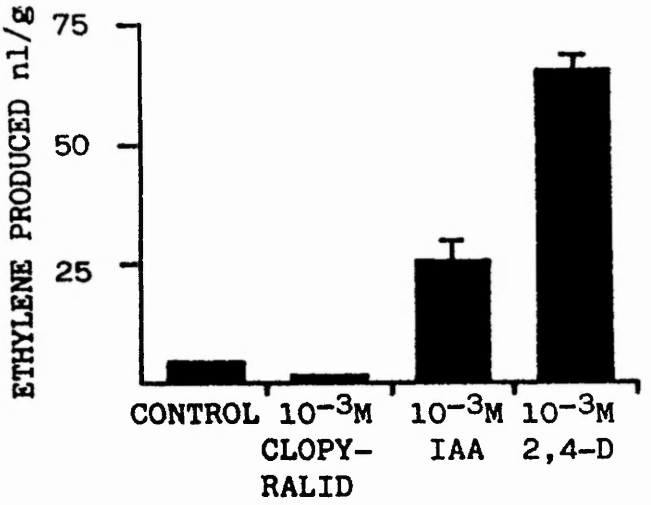

Fig. 6. Effect of clopyralid, IAA and 2,4-D on ethylene evolution in sugar beet after 24h incubation. Each point is the mean from 8-12 separate plants. Bars represent S.E.'s.

Table 1. Production of ethylene from 10^{-3}M ACC in mayweed and sugar beet. Data are means of six replicates plus or minus standard errors.

INCUBATION PERIOD (H)	ETHYLENE PRODUCTION, nl/g Mayweed	Sugar beet
2	20.03 ± 3.92	11.69 ± 1.17
4	38.99 ± 4.52	21.12 ± 1.19
24	163.62 ± 10.90	245.91 ± 32.41

DISCUSSION

Using a relatively simple assay system of incubating excised leaves in herbicide solutions, this study has demonstrated that clopyralid induces a rapid and concentration-dependent production of ethylene from a susceptible species, scentless mayweed, that precedes the onset of epinastic symptom development. Furthermore, no epinastic symptom development was observed in sugar beet as a result of clopyralid application in this system. This observation reveals an apparent inconsistency between our previously reported whole plant data (Thompson & Cobb, 1986) and the current study, in that symptoms were observed in the previous but not the present study. However, it should be borne in mind that the former study used whole plants with foliar application of formulated clopyralid, whilst the current experiments employed the direct, vascular uptake of unformulated clopyralid-acid into leaf explants.

These findings suggested that the observed leaf epinasty is directly caused by ethylene production in mayweed. However, the apparent link is disproven by the further observations that (1) although ethylene evolution was induced by incubation with the precursor ACC alone, leaf epinasty was not observed, (2) exposure to ethylene gas (using prepared standard gases, data not shown) also failed to produce epinastic symptoms in our system, and (3) whilst AVG and Co^{++} inhibited clopyralid-induced ethylene evolution by 50%, symptom development was not affected. Hence, we conclude that ethylene evolution alone is not the sole cause of epinastic symptoms in response to clopyralid incubation in our experimental system.

Neither ethylene production nor epinastic symptom development were observed in excised sugar beet leaves in response to clopyralid incubation. This species is, however, capable of producing ethylene when supplied with the precursor ACC or with IAA and 2,4-D, but symptom development was only observed following incubation with IAA and 2,4-D. Thus, this species has the physiological capacity to produce leaf epinasty in response to herbicides in this experimental system, but is insensitive to clopyralid over 24h. From our present results we conclude that differential ethylene production is not the sole basis of clopyralid selectivity in sugar beet and scentless mayweed.

It is our opinion that ethylene production is a symptom of auxin-type herbicide activity in susceptible species, and we consider it to be one of a cascade of events initiated by binding at an auxin receptor site. There are many published reports of responses to auxin which occur on a time scale from a few minutes to several hours (Evans, 1974). These effects range from almost immediate changes in cell membrane permeability (Fitzsimons et al,

1987), to stimulations in protein synthesis and carbohydrate deposition after 1 to 2 hours, which later appear as measurable growth responses (Evans, 1974). The production of ethylene and visible symptoms therefore appear to be separate measurable responses to the common stimulus of herbicide binding. In this sense, we speculate that clopyralid does not induce epinasty in sugar beet, in this system, due to a relatively poor binding affinity for the auxin binding site in this species.

Finally, the measurement of ethylene production may be a valuable tool in determining potential sensitivity to auxin-type herbicides, and assessing selectivity and structure/activity relationships.

ACKNOWLEDGEMENTS

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ERRATA

1. In the photosynthetic studies described in Chapter 4 , the symbol μM was erroneously used to describe carbon dioxide reduction. This should read μmoles throughout.

2. The following abbreviations have been used in the text but have not been defined.

ABA . abscisic acid
IAA , indol-3yl-acetic acid
BSA , bovine serum albumin
MCPA , 2-methyl,4-chlorophenoxyacetic acid
PVP , polyvinylpyrollidone