UPTAKE AND EFFECTS OF HERBICIDES ON

THE LAWN MOSS *RHYTIDIADELPHUS SQUARROSUS*

by

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ABSTRACT

Herbicide absorption and its effects on the moss *Rhytidiadelphus squarrosus* were investigated in the field, glasshouse and laboratory.

Application of selected herbicides to *R. squarrosus*-infested turf caused chlorosis of the moss shoots, or blackening in the case of iron salts. Regrowth from herbicide-damaged moss was observed following all treatments, with only dichlorophen, chloroxuron with iron salt, thiram, oxadiazon, and ammonium sulphate reducing *R. squarrosus* cover in the long term (>17 wk).

Glasshouse-maintained monocultures of *R. squarrosus, Dicranum scoparium* and *Polytrichum juniperinum* treated (sprayed) with paraquat, 2,4-D, dichlorophen, and ferrous sulphate showed similar toxic effects to those observed in the field.

In laboratory studies, growth of *R. squarrosus* was inhibited by dichlorophen and 2,4-D and was sensitive to incubation time; this was attributed to a slow continuous permeation process. Paraquat inhibited *R. squarrosus* growth but was not sensitive to incubation time due to rapid absorption. Ferrous sulphate, in contrast, stimulated growth at low concentrations (200-1000 μM Fe) and short incubation times (5 min). ^{14}C-paraquat and iron were rapidly exchanged to the cell walls with poor intracellular uptake. During prolonged incubations (96 h), redistribution of extracellularly-bound ^{14}C-paraquat to intracellular sites was noted. Moss shoots immersed in dilute ^{14}C-paraquat solutions showed preferential accumulation in the leaves compared to the stems.

Comparisons of ^{14}C-paraquat uptake and distribution between *R. squarrosus* and *Lolium perenne* tissues, using efflux analysis, were made. Intracellular paraquat uptake did not differ but a smaller vacuolar volume in *R. squarrosus* tissues may reflect preferential accumulation of paraquat in the vacuole. The quantity of cell wall-bound paraquat was ten times greater for *R. squarrosus* tissues than for *L. perenne*.

Uptake of ^{14}C-2,4-D by *R. squarrosus* was not observed. In contrast, rapid uptake of dichlorophen was found. Paraquat, dichlorophen, 2,4-D, and iron salts were found to cause some loss of membrane integrity.

Photosynthesis and respiration in *R. squarrosus* were rapidly reduced by dichlorophen treatment (spray), as was chlorophyll *a* content. Photosynthesis was also reduced several weeks (8 wk) with paraquat and 2,4-D after spraying, but no effect was observed following ferrous sulphate treatment. Analysis of *R. squarrosus* PI curves, after treatment with dichlorophen or paraquat indicate that both chemicals inhibit electron transport.

R. squarrosus was sensitive to all herbicides tested although application method was an important contributing factor in their performance.

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To my husband Rob, my Mother and in memory of my Father

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ABBREVIATIONS

A	absorbance
α	photosynthetic efficiency
ai	active ingredient
ADP	adenosine 5′-diphosphate
A_{max}	maximum uptake at equilibrium
ATP	adenosine 5′-triphosphate
chl	chlorophyll
CO_2	carbon dioxide
df	degrees of freedom
dpm	disintegrations per minute
2,4-D	2,4-dichlorophenoxyacetic acid
E	Nernst potential
EDTA	ethylenediaminetetra-acetic acid
F	Faraday's constant
$FeCl_3$	ferric chloride
$Fe(NO_3)_3$	ferric nitrate
$FeSO_4$	ferrous sulphate
ha	hectare
HCl	hydrochloric acid
HNO_3	nitric acid
H_2SO_4	sulphuric acid
I_k	light saturation point
IRGA	infra-red gas analyser
k	rate constant
kDa	kilo-Dalton
ln	natural logarithm
µM	micromole
mA	milliamp
mM	millimole
mV	millivolt
n	number of replicates
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
P	net photosynthesis
PI	photosynthetic irradiance
P_{max}	maximum rate of photosynthesis
PSI	photosystem I
PSII	photosystem II
PSU	photosynthetic unit
R^2	coefficient of variation
S	single exponential
SD	standard deviation
SO	single exponential origin through zero
SED	standard error of the difference
SE	standard error
spp	species
t	time
T	absolute temperature
$t_{\frac{1}{2}}$	time to half maximum uptake or efflux
v/v	volume to volume ratio
wk	week
wt	weight
z	valency

CHAPTER 1

GENERAL INTRODUCTION

1.1 MOSS WEEDS IN TURF

Mosses have been described, collectively, as the commonest weeds of neglected turf (Dawson, 1977; Blandy,1954). Moss weeds can be categorised into two broad groups, pleurocarpous and acrocarpous, whose growth forms are, respectively, a loose prostrate habit and dense erect tufts. The important moss weeds of turf are pleurocarpous, producing long branching horizontal shoots capable of covering the soil surface quickly (Blandy, 1954; Brauen, Goss and Nus, 1986). Acrocarpous mosses, in contrast, are more commonly found on bare soil.

Blandy (1954) emphasised the need to know and understand the moss weed species present, their methods of propagation and the conditions under which they thrive, prior to studying their control in turf. However, literature referring to moss control in turf does not always specify the moss species present but treats them collectively (Brauen, Goss and Nus, 1986; Ondrej, 1985; Kuttruff and Kleve-Kellen, 1980; Svensson, 1976).

The following moss species (nomenclature follows Corley and Hill, 1981) have been categorised by Dawson (1977) and Blandy (1954) as weeds in turf:

Barbula convoluta

Barbula fallax

Brachythecium rutabulum

Bryum argentum

Bryum capillare

Campylopus paradoxus

Ceratodon purpureus

Dicranum scoparium

Eurhynchium praelongum

Hypnum cupressiforme

Polytrichum juniperinum

Pottia truncata

In addition the species *Rhytidiadelphus squarrosus* and *Calliergon cuspidatum* have been described as weeds of turf (Anon, 1982).

1.2 CONTROL OF MOSS WEEDS IN TURF

Mosses can be controlled in turf by cultural or chemical methods.

1.2.1 Cultural methods of control

Cultural techniques for controlling moss generally involve improving health and vigour of the turf, thereby rendering the conditions less suitable for growth and colonisation by mosses. Methods of cultural control are therefore specific to individual turfs and their associated deficiencies. The conditions encouraging moss growth, and techniques for their remedy, based on methods recommended by Pycraft (1984), Anon. (1982, 1985), Blandy (1954) and Dawson (1977) are presented in Table 1.1.1.

Table 1.1.1 illustrates the ability of mosses to thrive in environments unfavourable to grasses, commonly due to poor lawn management practices.

1.2.2 Chemical control

Early methods of chemical control of mosses in turf, described by Blandy (1954) and Dawson (1977), involved the use of either potassium permanganate, ferrous sulphate or mercurous chloride (calomel).

Currently recommended formulations for chemical control of mosses in lawns (Pycraft, 1984; Anon., 1982, 1985) include dichlorophen di(5-chloro-2-hydroxydiphenyl)methane, lawn sand (ammonium sulphate +

Table 1.1.1 Causes of moss infestation of lawns and their cultural remedies

Causes	Remedies
Soil pH. Moss weeds in turf are often considered problems solely of acid soils (Anon 1982, 1985; Pycraft, 1984) indicating a need to raise pH. However, Barbula fallax is an example of a moss weed in turf on calcareous soils (Blandy, 1954).	Addition of lime or chalk to acid soils.
Nutrient-deficient soil. Low fertility will weaken growth of grasses increasing the likelihood of infection by diseases and competition from mosses.	Top dressing and fertilisers applications.
Compaction of soil. Grasses require loose soil structure for nutrient uptake and growth of roots. This is in contrast to mosses which are rootless, sometimes possessing rhizoids predominantly for the function of anchorage.	Addition of sand or top soil and spiking.
Poor drainage. Mosses require damp conditions to thrive unlike grasses which may be inhibited by bad aeration.	Spiking, raking, addition of charcoal or an irrigation system.
Excessive shade. Many moss species require less light than grass species and grow readily under trees and bushes.	Pruning of overhanging vegetation and planting of shade tolerant grass species.
Thatch build up. Mosses thrive in lawns with excessive thatch build up (layers of dead grass) at the soil surface.	Raking during the summer months when moss growth is slow and the use of a grass collection box when mowing.
Presence of broad leaved weeds. These weaken the grasses by competition enabling mosses to establish themselves.	Hand weeding or the use of ~~the use of~~ selective broad leaved weed herbicides.
Height of grass sward. Mowing too short or long encourages acrocarpous and pleurocarpous mosses, respectively.	Adjust cutting height.

ferrous sulphate + fine sand), ferrous sulphate, chloroxuron (3-[4-(4-chlorophenoxy)phenyl]-1,1-dimethylurea) with iron, and tar oil. Nabam (1,2-ethanediylbis (carbamodithioate)) and endothal (3,6-epoxycyclohexane-1,2-dicarboxylic acid) have also been recommended for moss control in turf by Pearce (1971) and Brauen, Goss and Nus (1986), respectively, but are not marketed for this use.

Mercurous chloride, a previously recommended lawn moss killer, has been withdrawn from the pesticide market due to concern about possible build-up of mercury levels in the environment (Anon., 1985).

Data relating to action of the above chemicals are sparse. The traditional ferrous sulphate treatment has been reviewed by Kuttruff and Kleve-Kellen (1980), Blandy (1954) and Anon. (1982, 1985) and its more common application as a mixture with ammonium sulphate (lawn sand) is discussed by Dawson (1977), Jackson (1961), Svensson (1976) and Ondrej (1985). Dichlorophen initially superseded mercurous chloride for herbicidal control of moss in lawns (Jackson, 1961; Woolhouse, 1973; Brown, Ougham and Beckett, 1986, Anon., 1982, 1985) and was later joined by chloroxuron, the latter often formulated with $FeSO_4$ (Kuttruff and Kleve-Kellen, 1980; Svensson, 1976 and Anon., 1985).

Literature on the effects of other herbicides for moss control, whether in lawns, orchards, or nursery stock containers, is extremely limited. Often they concern different moss species, habitats and application methods for each herbicide making judgements of relative efficacy and general moss sensitivity difficult. Table 1.2.2 summarises published literature on the sensitivities of a range of moss species from different habitats to various herbicides.

Table 1.2.2 A survey of published literature on the effects of herbicide on mosses (species where known)

Herbicide	Habitat (species)	Score of toxicity[1]	Author
Potassium permanganate	Turfs	3	Blandy, 1954
Ferrous sulphate	Turfs	3	
Calomel	Container grown plants	5	
Calomel	Turf	4	Jackson, 1961
Dichlorophen		4	
2,4-D	Sphagnum	1	Dana, 1966
2,4,5-T	Bogs	1	
Fenuron		1	
Diquat		1	
Bromoxynil		1	
Silvex		1	
Dicamba		1	
Pichloram		1	
Nabam	Turf	*	Pearce, 1971
Calomel	Turf	5	Woolhouse, 1973
Dichlorophen		5	
Glyphosate	Tea plantations	5	Ronoprawiro, 1975
Paraquat and Diquat		3	
2,4-D + MSMA + Diuron		3	
Captafol		3	
Chloroxuron	Turf	5	Svensson, 1976
Ferrous sulphate		3	
Chloroxuron	Turf	5	Kuttruff and Kleve-Kellen, 1980
Iron sulphate		3	
Ammonium sulphate		2	
Bordeaux mixture	Trees	*	Rosa, 1980
Tar oil		*	
Aminotriazole	Orchards (soil)	5	Atkinson, Abernathy and Crisp, 1980
Bromacil		3	
Chlorthiamid		5	
Dichlobenil		4	
Diuron		5	
Glyphosate		3	
Propyzamide		4	
Simazine		2	
Glyphosate	Tea plants	2	Ronoprawiro, 1981
Fentin acetate		4	
DNOC		4	

Table 1.2.2 (cont'd)

Herbicide	Habitat (species)	Score of toxicity[1]	Author
2,4-D	Woodlands	1	Stjernquist, 1981
MCPA	(Dicranum sp)	1	
Triclopyr	(Pleurozium sp)	1	
Glyphosate		2	
Chloroxuron	Container	5	Van Himme and Stryckers,
Thiram	grown	4	1981
Binapacryl	plants	1	
Chinonamid		4	
EPTC	Laboratory experiments (P. commune)	3	Bowers, 1982
Atrazine	Embankments	4	Skender, 1983
Oxadiazon	Container	5	Ryan, 1983
Dodine acetate	grown plants	4	
Thiram		4	
Ziram		1	
Captan		1	
Tribasic copper		1	
Bordeaux mixture	seed beds	5	Walker and Reynolds, 1984
Oxadiazon	Container	2	Wilson and Hughes, 1985
Diphenamid + mogeton	grown plants (Funaria hygrometrica)	4	
Oryzalin		4	
" + mogeton		4	
Mogeton		4	
Ammonium sulphate	Turfs	3	Ondrej, 1985
Iron sulphate			
Bensulide		2	Brauen et al. 1986
Endothal		5	
Dichlorophen	Laboratory experiments (R. squarrosus)		Brown, Ougham and Beckett, 1986
Paraquat	Ornamental	(2)	Balcerkiewicz and
Glyphosate	plants	(2)	Rusinka, 1987
Atrazine		(2)	
Simazine		(2)	(not quantitative)

[1] = score of toxicity (1 = no effect; 2 = slight visible damage; 3 = moderate damage; 4 = severe damage; 5 = death of moss)

* = no toxicity data available

1.3 GENERAL CHARACTERISTICS OF BRYOPHYTES

The Bryophyta contain three classes: Bryopsida (mosses), Hepaticopsida (liverworts) and Anthoceratopsida (hornworts). Typically, bryophytes possess two distinct stages in their life cycle *viz* :- green gametophytic stage characterised by the possession of multicellular sex organs called antheridia and archegonia, and a partially-dependent sporophytic stage, not green at maturity, concerned with shedding spores.

Gametophytes range in size from barely visible to 1 m in height, their size being limited by the absence of lignin. Moss gametophytes generally show a definite axis or stem (caulid) on which small unstalked leaves (phyllidia), sometimes possessing a midrib, are borne. The stem is more weakly constructed (non-lignified) than in vascular plants, without their elaborate internal tissue differentiation although functional vascular tissues are present in some groups, notably the Polytrichales. Bryophytes are often attached to their substrate by rhizoids, similar to root hairs in vascular plants (Schofield, 1985), but rhizoids are completely absent or sparse in pleurocarpous species including the lawn weed *Rhytidiadelphus squarrosus.*

Many moss and liverwort gametophytes can reproduce asexually by means of gemmae, small clusters of cells which break away to form a new plant, or else by propagula, brittle specialised branches that are easily fragmented and dispersed to new sites (Schofield, 1985). The simple process of branching followed by decay of attachments is an important method of vegetative propagation in many bryophytes.

Sexual reproduction involves the formation of antheridia and archegonia on the vegetative gametophyte. The antheridia are multicellular sacs containing spermatazoids (male gametes) and are intermixed with paraphyses (filamentous sterile structures).

Archegonia contain a single egg which, following fertilisation by a male gamete, will give rise to the sporophyte.

In general, the sporophytic stage of bryophytes is dependent on the gametophyte. Photosynthate must pass from the gametophore through the transfer cells of the foot into the sporophyte. When mature, capsules shed their spores as a fine dust which will subsequently germinate to form prothalli and then develop into the new gametophytes. Many bryophytes including some lawn weeds never produce sporophytes and reproduce by vegetative methods.

1.4 RHYTIDIADELPHUS SQUARROSUS

The bryophyte selected for the experimental work in this thesis was *Rhytidiadelphus squarrosus*. This species is one of the major moss weeds of lawns on both calcareous and non-calcareous soils in Britain and North Europe and is plentiful in the lawns of Silwood Park, around which much of the work was based. Details of the biology of *R. squarrosus* are given below.

Rhytidiadelphus squarrosus (Hedw.) Warnst.(Hypnobryales:Hypnaceae) is a common pleurocarpous moss, indifferent to pH of the soil, found in moist grassy places growing in dense patches or as single shoots. The stems (caulidia) are often red (Schofield, 1985) and between 5 and 12 cm long, the shoot length being partially dependent on the presence of other vegetation. The leaves (phyllidia) spread in a squarrose manner from a wide sheathing base and give the shoot apex a star-like appearance (Plate 1.4.1). The leaves are unistratose, 2-3 mm long and taper to an acute point. They are faintly plicate with bluntly toothed margins and and have short forked nerves. Rhizoids are absent or sparse in *R. squarrosus* under field situations, although visible when grown in laboratory conditions. Reproduction of *R. squarrosus* is generally by vegetative means following the detachment of tissue from



Plate 1.4.1. Scanning electron micrograph of a leafy gametophore apex of Rhytidiadelphus squarrosus showing star-like spreading of leaves at the apex (Magnification x144)

the gametophyte and its subsequent regeneration to form a new gametophyte plant. Successful sexual reproduction is rarely observed. It is probable that the spread of the species is dependent on the detachment of viable fragments by mowing machinery and grazing animals.

1.5 CYTOLOGY OF R. SQUARROSUS

An appreciation of cell structure of bryophytes is central to an understanding of the action of herbicides. The following account is based partly on published work on bryophytes in general and partly on my own observations of stem and leaf sections of *R. squarrosus* using transmission electron microscopy.

The cellular organisation of bryophytes is closely similar to that of vascular plants (Duckett, 1986). The cellular organisation of *R. squarrosus* with regard to lead uptake and its location within cells has been studied by Skaar, Ophus and Gullvag (1973), Gullvag, Skaar and Ophus (1974) and Halvorsen, Gullvag and Cunningham (1978).

The cell walls of bryophytes are structurally simple and are composed of cellulose microfibrils embedded in non-cellulosic carbohydrate (Duckett, 1986). Histochemical studies on stem tissue of the Polytrichaceous moss *Dendroligotrichum dendroides* indicate that cellulose microfibrils are embedded in pectin, aldehydes, and small amounts of polyuronides with free carboxyl groups, and are not lignified (Scheirer, 1973). Plate 1.5.2 reveals the polylamellated structure of the leaf cell wall of *R. squarrosus* with lamellae possessing microfibrils aligned at various angles. A similar polylamellated cell wall can be seen in the cells comprising the stem tissue (Plate 1.5.3). Here it is easy to differentiate the inner, middle and outer cell wall. This layering of the stem cell walls resembles that of *D. dendroides* (Scheirer, 1977). The lamellated structure in both species is only visible in the middle and outer cell

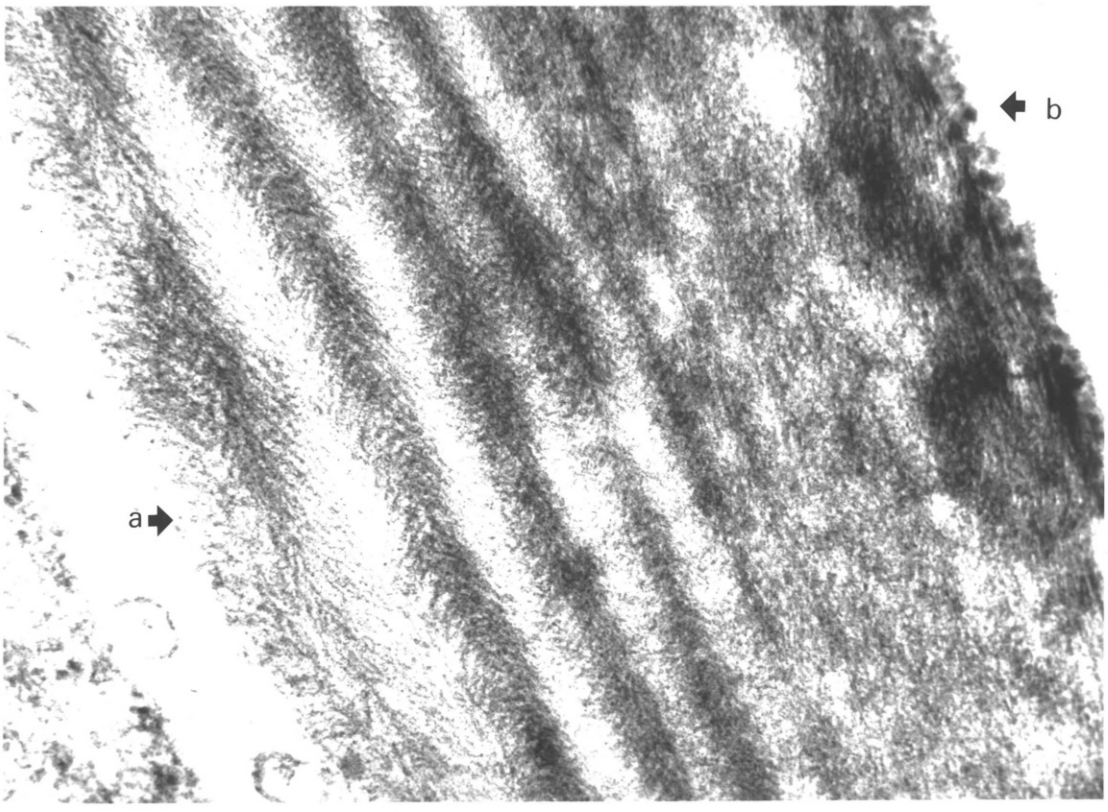


Plate 1.5.2. Transmission electron micrograph of the polylamellated leaf cell wall of Rhytidiadelphus squarrosus (Magnification x138260) (a) inner cell wall and (b) outer cell wall.

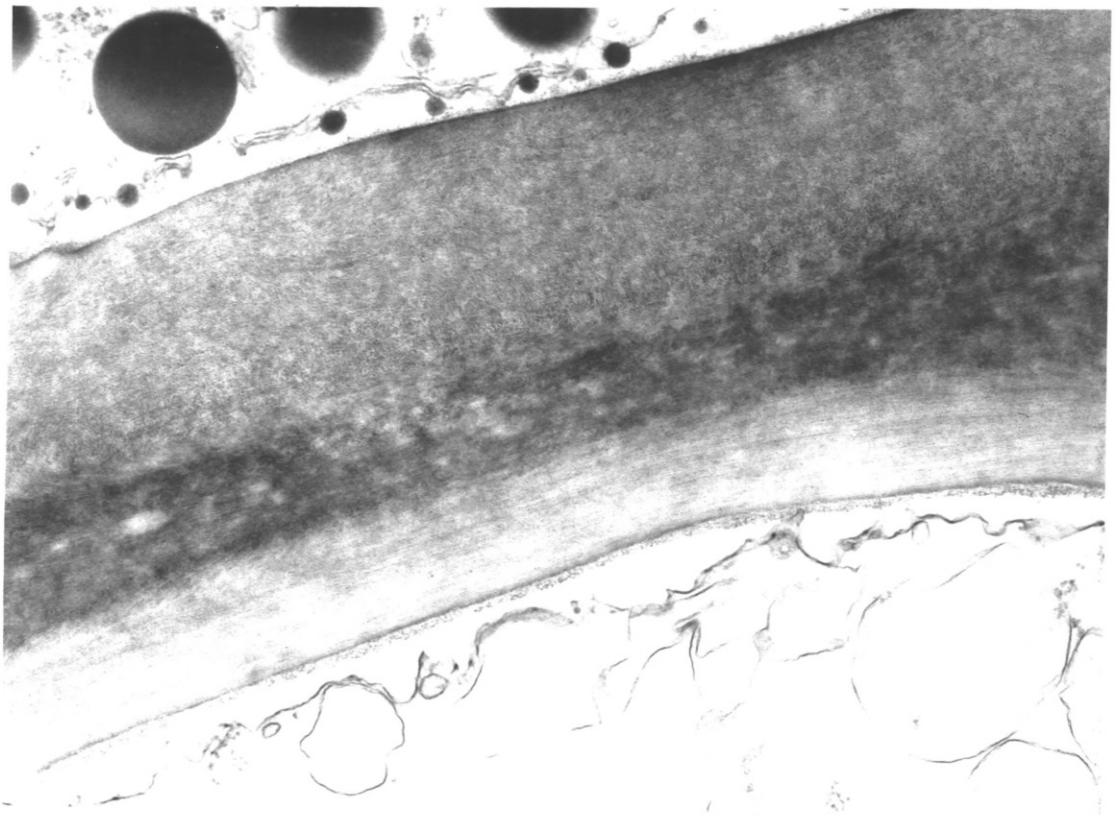


Plate 1.5.3. Transmission electron micrograph of the polylamellated stem cell walls of Rhytidiadelphus squarrosus (Magnification x 33509)

wall layers. The lamellation and orientation of microfibrils in these layers is similar to that described for Polytrichaceous mosses which in turn have been likened to nacreous conductive tissue (thickened lateral cell walls) of vascular plants (Stevenson, 1974; Warmbrodt and Evert, 1974). Transmission electron micrographs of the leaf cell of *R. squarrosus* also indicate the presence of two polylamellated cell wall layers (Plate 1.5.2).

Cells similar to the leptoids (sieve elements) of *Dendroligotrichum dendroides* which possess oblique end walls (Scheirer, 1977) are visible in *R. squarrosus* (Plate 1.5.4). Pores or plasmodesmata between the sieve element-like cells may exist but were not clearly visible in the sections observed. The plasmalemma is clearly visible against the cell wall (Plate 1.5.3) but plasmolysis has occurred in some stem cells.

Plasmodesmata in leaf and stem cells of *R. squarrosus* were observed by Gullvag *et al.* (1974) and Halvorsen *et al.* (1978) with smooth endoplasmic reticulum or tubules passing through the pores. The presence of plasmodesmata in stem cells of *R. squarrosus* was described by Halvorsen *et al.* (1978) and related to leptoid structures. Plate 1.5.5 shows plasmodesmata in *R. squarrosus* stem cells with extensive associated endoplasmic reticulum around the area and desmotubules penetrating the plasma membrane-lined pores. The stem cells of *R. squarrosus* observed to contain plasmodesmata resemble those described as leptoids in the Polytrichales (Warmbrodt and Evert, 1974; Scheirer, 1973, 1977; Stevenson, 1974; Eschrich and Steiner, 1968 a, b). Clarkson (1974) described desmotubules as not possessing a unit membrane structure; consequently they are finer than endoplasmic reticulum and plasma membrane. The plasmodesmata observed in *R. squarrosus* (Plate 1.5.5) share the characteristics of those found in vascular plants; narrow ends, such that there is a close association of

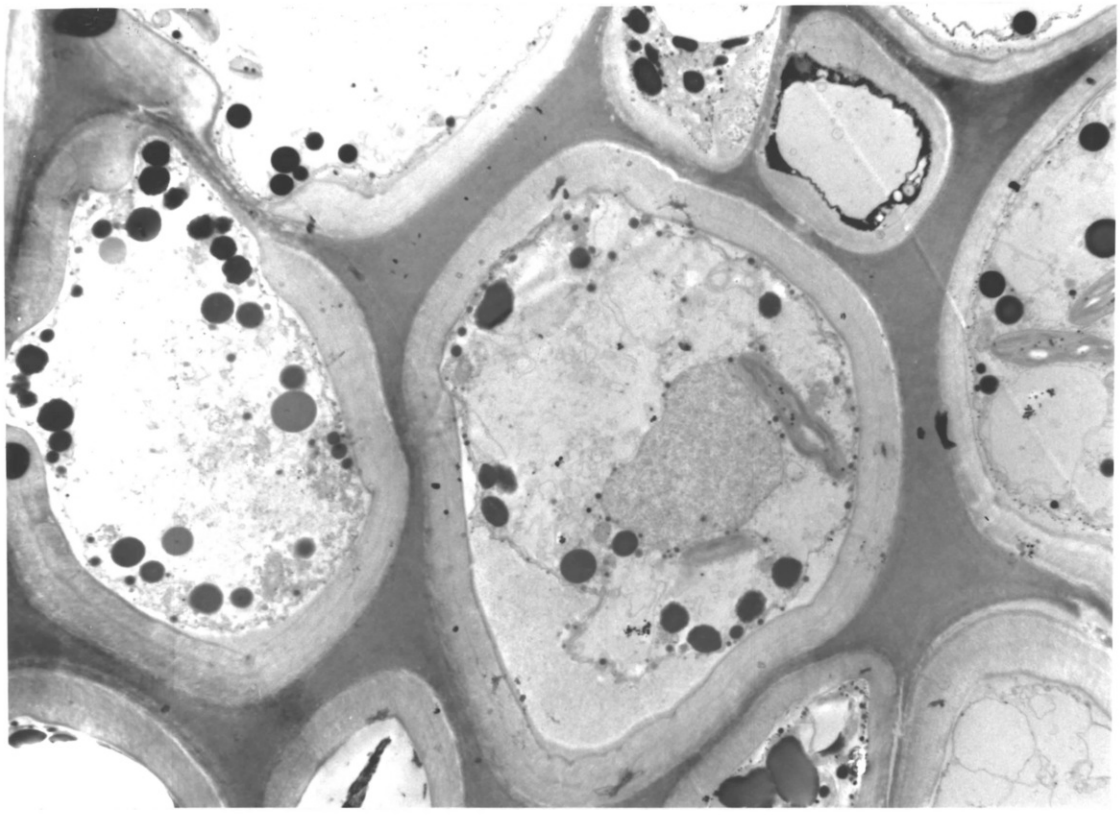


Plate 1.5.4. Transmission electron micrograph of Rhytidiadelphus squarrosus stem cells (T.S) showing leptoid-like oblique cell walls resembling those described by Schofield and Hébant (1984) for Polytrichales (Magnification x20998)

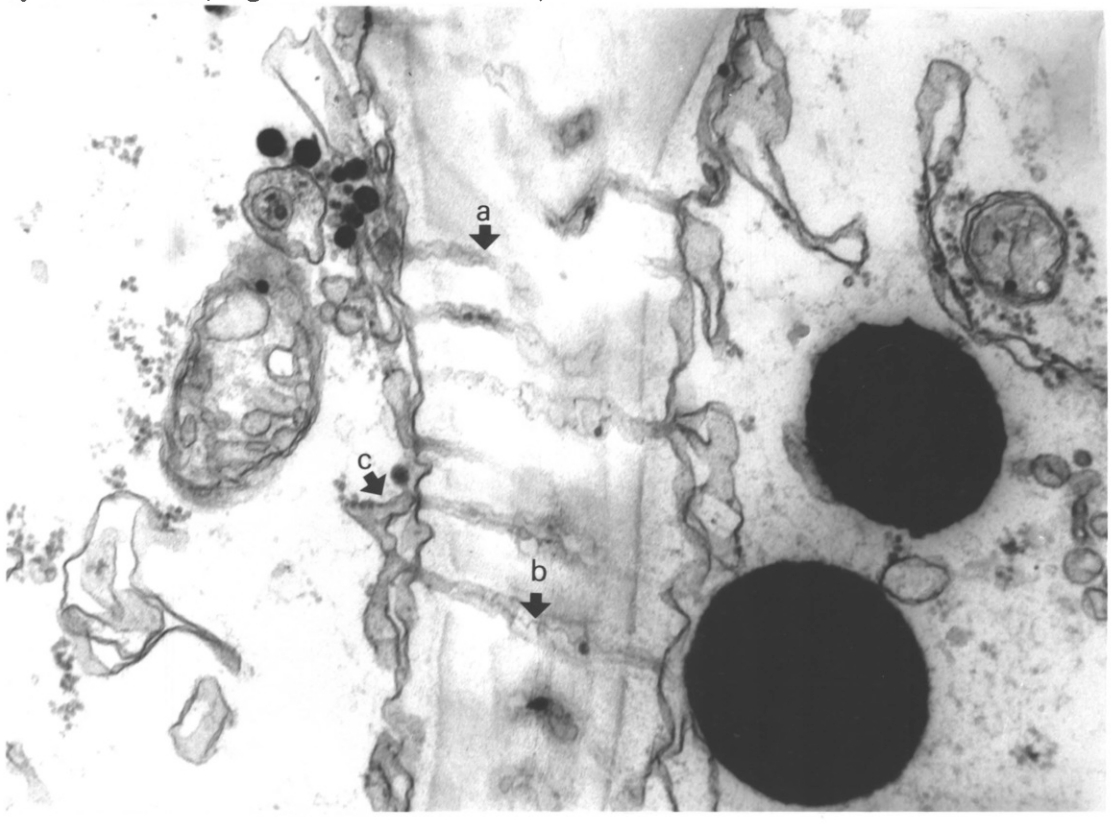


Plate 1.5.5. Transmission electron micrograph of stem cells of Rhytidiadelphus squarrosus showing (a) plasmodesmata; (b) desmotubules and (c) associated endoplasmic reticulum (Magnification x32718)

plasma membrane and desmotubule, and a wider central zone. The close association of the plasmalemma and desmotubule ensures continuity between the cells but not via open pores.

The plasmodesmata of *R. squarrosus* stems (Plate 1.5.6) are congregated in specific areas of the cell wall, characterised by large indentations of the inner surface. Such areas are described as pits in vascular plants and are characteristic of young sieve elements. In vascular plants, as these elements mature, callose tissue is laid down on the surface of sieve areas and down through the pores (Fahn, 1982; Clarkson, 1974). Therefore, in older sieve areas, depressions of the inner cell walls are not apparent. Callose-containing cells have been observed in mosses (Duckett, 1986; Hébant, 1977) but were not apparent in or around the plasmodesmata of *R. squarrosus*. Uncallosed pores have also been observed in the club-moss *Lycopodium lucidulum* (Warmbrodt and Evert, 1974) which was described as possessing numerous primary pit fields with plasmodesmata on lateral and tapering walls between differing cell types in the phloem. Translocation studies in *R. squarrosus* were not undertaken in the present investigation but the parietal system of endoplasmic reticulum indicates that the perforated areas may function as a transport system for assimilates or water.

The plasmodesmata observed in the cell wall pits of *R. squarrosus* stem cells (Plate 1.5.5) are numerous and large and were also found by Halvorsen *et al.* (1978). It is possible that the apparent large plasmodesmata sizes are artefacts of microscopy due to interference from other plasmodesmata visible below the surface of the stem section. Alternatively, the perforations may be simple pits in the cell wall rather than true plasmodesmata of the type described by Duckett (1986) at the junctions between leptoids.

A comparison of the morphology of *R. squarrosus* stem cells showing conductive tissue properties with those of leptoid cells from Polytrichales indicates some similarities. Leptoid cells of

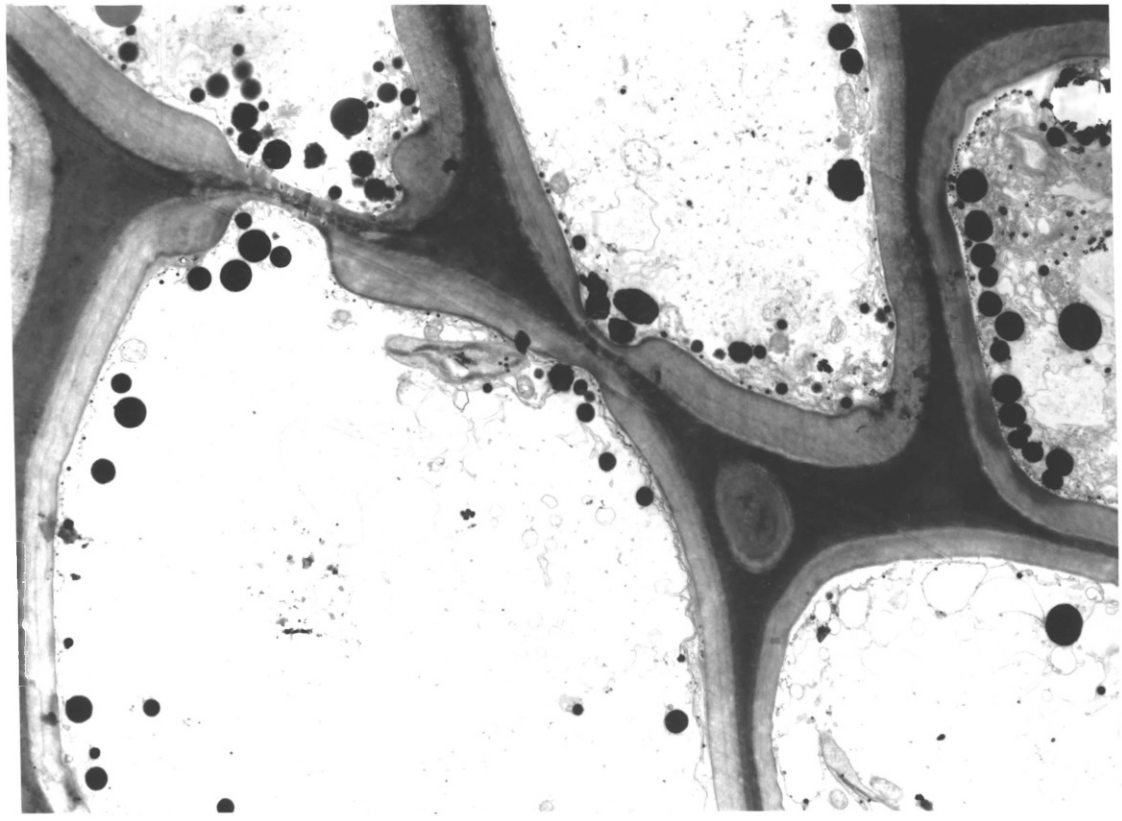


Plate 1.5.6. Transmission electron micrograph of stem cells of Rhytidiadelphus squarrosus showing the congregation of plasmodesmata in a specific area (pit) of the cell wall (Magnification x9008)

Polytrichales (Hébant, 1977; Schofield and Hébant, 1984) are characterised by: 1) elongated shape with terminal end walls tending to be oblique and possessing many plasmodesmata; 2) lateral cell walls possessing plasmodesmata but these are fewer in number than in the terminal end walls; 3) mitochondria (plastids) and extensive stacked endoplasmic reticulum (sieve element reticulum) are identifiable; 4) frequent, but developmentally late, degeneration of the nucleus. Schofield and Hébant (1984) also described the existence of many intermediate types of cellular organisation in less differentiated species. Halvorsen *et al.* (1978) observed many leptoid-like features in *R. squarrosus* stem cells. Also X-ray energy dispersive analysis indicates that nutrient translocation may occur via the plasmodesmata and associated endoplasmic reticulum. In the present study, no electron micrographs showing end wall perforations or stacked endoplasmic reticulum were recorded, although lateral wall perforations were observed (Plate 1.5.6). Therefore the apparent conductive stem tissue of *R. squarrosus* is best described, using the terminology of Hébant (1979), as conductive parenchyma.

Mitochondria, chloroplasts, smooth and rough endoplasmic reticulum polysomes, and nucleus are present in stem cells of *R. squarrosus* (Plate 1.5.7). Chloroplasts resembling those found in vascular plants are also present with lamellated grana, starch grains and plastoglobuli. The chloroplasts have poorly developed lamellae systems possibly reflecting the high light environment of *R. squarrosus* growing in turf.

Lipid bodies were also observed in large numbers in the stem cells but not in the leaf cells of *R. squarrosus* (Plate 1.5.8). Lipid bodies are major food reserves for bryophyte tissues (Duckett, 1986). Swanson, Anderson, Gellerman and Schlenk (1976) described similar lipid bodies in *Ceratodon purpureus* containing mainly triglycerides.

Plate 1.5.7. Transmission electron micrograph of cellular plastids of a Rhytidiadelphus squarrosus stem cell (Magnification x31040)

(a) chloroplast

(b) plastoglobuli

(c) starch grains

(d) lipid bodies

(e) mitochondria

(f) nucleus

(g) rough endoplasmic reticulum

(h) smooth endoplasmic reticulum

(i) polysomes

(j) vacuolated area





Plate 1.5.8. Transmission electron micrograph of a leaf cell of Rhytidiadelphus squarrosus showing the polylamellated cell wall, cytoplasm, mitochondria and a chloroplast (Magnification x14750)

Electron micrographs of *R. squarrosus* stem tissue show a preponderance of lipid bodies around the outer edges of the cells (Plates 1.5.4-1.5.7 and 1.5.9). This may be indicative of lipid excretion to the plasmalemma or through the plasmalemma for deposition on the inner cell wall. Moore, Luff and Hallen (1982) describe lipid bodies in desiccation-tolerant mosses as possible sources of short chain carbon compounds for membrane repair. The close association of endoplasmic reticulum and lipid bodies at the cell wall (Plate 1.5.9) may indicate that lipid bodies can be incorporated into the membranes and exocytosed. However, the Golgi apparatus is generally considered to function in cell wall deposition (Fahn, 1982) and was not observed in the present study. Similar membrane-bound inclusions termed 'refractive spherules' were described by Schofield and Hébant (1984) in *Polytrichum commune*.

Halvorsen *et al.* (1978) did not observe large single vacuoles within stem cells of *R. squarrosus*, as observed in Plate 1.5.7, but numerous small ones. Leaf sections of *R. squarrosus* presented by Skaar, Ophus and Gullvag (1973) also do not show large vacuolated areas. Leaf tissue electron micrographs were not sufficient to determine vacuole form in the present study as no whole cell micrographs were obtained. The large plastid free area visible in Plate 1.5.8 could therefore be attributed to ruptured tissue during preparation or sectioning rather than a single vacuole. The cell organisation of *R. squarrosus* found in the present study thus resembles that of the leafy hepatic *Jungermannia vulcanicola* described by Satake and Miyasaka (1984).

1.6 WATER AND SOLUTE RELATIONS IN BRYOPHYTES

Bryophytes differ fundamentally from vascular plants in the manner in which they obtain moisture and mineral nutrients and in their abilities to withstand low tissue water potentials.

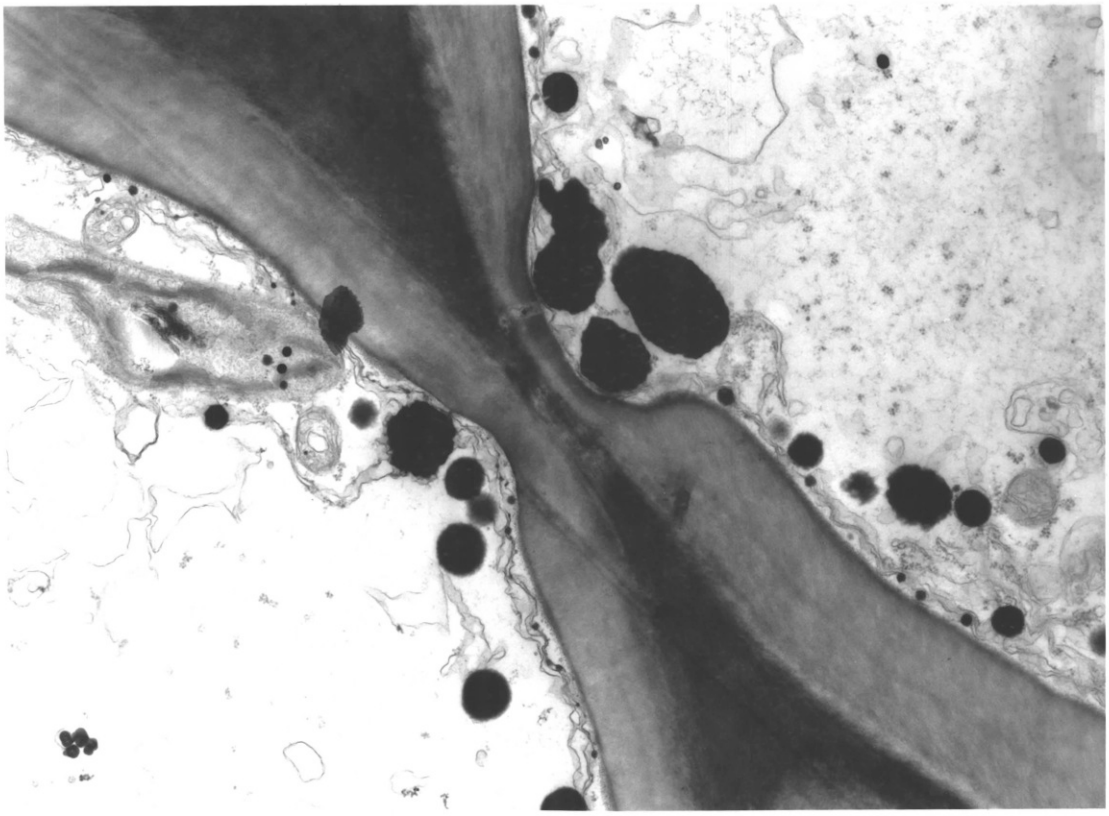


Plate 1.5.9. Transmission electron micrograph of stem cells of Rhytidiadelphus squarrosus showing the preponderance of lipid bodies around the edges of cells and association with endoplasmic reticulum (Magnification x20998)

The majority of mosses obtain their water and nutrients from atmospheric moisture (Schofield, 1985). Although mosses probably do not acquire water and nutrients from their substratum, they may depend on the substratum and its water holding capacity for their survival, an example being that of weed mosses in turf and their preference for a moist micro-environment within the grass swards, especially in the presence of leaf litter.

Vascular plants have evolved a conducting system that will bring water from the soil to the photosynthesising shoots. The Polytrichales contain the best examples of endohydric mosses. These species possess simple vascular tissues, the hydroids and leptoids, for conduction of water and assimilates respectively. These structures are analogous but not homologous to the xylem and phloem of vascular plants. In the Polytrichales water is passed to the stems by a network of rhizomes. In contrast, the ectohydric mosses (such as *R. squarrosus*) lack a conductive system and rely totally on the uptake of surface water. To complement the two different water uptake and conduction systems, the endohydric and ectohydric mosses possess different strategies of adaptation to their water conduction. Endohydric mosses usually possess a water repellent cuticle and often a layer or deposits of a substance that resembles the epicuticular waxes of vascular plants (Proctor, 1979a). Ectohydric mosses possess a thin cuticle, if any, and generally no wax-like deposits. Therefore, the endohydric mosses repel water from their surfaces, possibly to aid CO_2 exchange, and ectohydric mosses depend on the accumulation of water across their surfaces. The scanning electron micrograph (Plate 1.6.1) shows the absence of waxy granular deposits on the adaxial surface of *R. squarrosus* leaves.

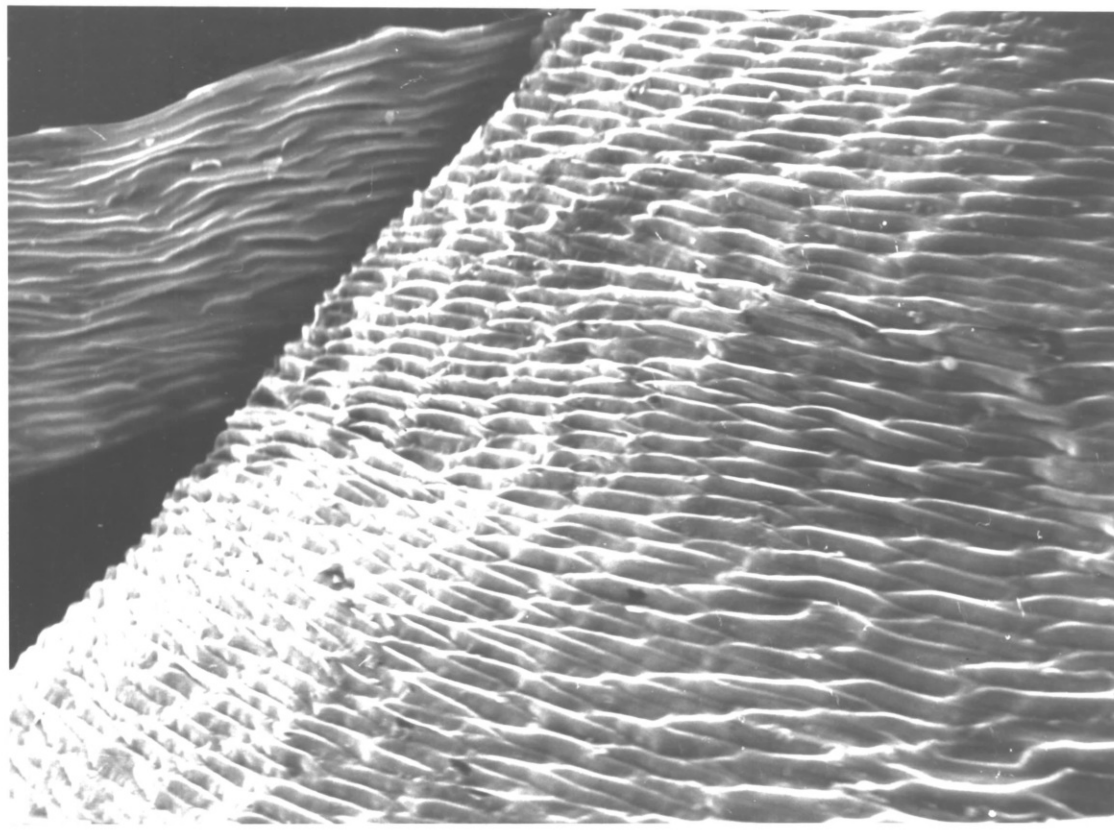


Plate 1.6.1 Scanning electron micrograph of the adaxial surface of a Rhytidiadelphus squarrosus leaf showing the leaf cell shape and absence of waxy surface deposits (Magnification x2571)

Ectohydric mosses often conduct water over their surface by capillary action (Proctor, 1979a, b; Dilks and Proctor, 1979; Bowen, 1933). Plate 1.6.2 shows the existence of longitudinal surface plictations in the leaves of *R. squarrosus* which may serve this function. Proctor (1979a) described similar plictations in *Neckera crispa* as structural elements within the cell wall. Schofield (1985) described increased rates of water movement, by capillary action, over cells with a thick cell wall size to protoplast size and suggested that increased cell wall thickness reduced the loss of water to the cells. Overall movement of water along the moss shoot is probably dependent on capillary networks between overlapping leaves, leaf bases and rhizoids.

R. squarrosus may be best classified as mixohydric as it has been shown by Halvorsen *et al.* (1978) to possess leptoid like cells in the shoot which, although normally associated with assimilate transport in *Polytrichum* spp., may also aid in water transport. Transmission electron micrographs (Plates 1.5.4 and 1.5.6) confirm the findings of Halvorsen *et al.* (1978). Schofield (1985) described stereids in a number of the subclass Bryidae although these are generally considered to be of supporting function.

The water associated with the external surfaces of bryophytes and within the matrix of the cell walls is an essential factor in the acquisition of nutrients by bryophytes (Schofield and Hébant, 1984). Ectohydric moss species, such as *R. squarrosus*, have the potential to absorb nutrients over their entire surface. The intracellular uptake of dissolved substances from the external solution is controlled by a selectively permeable lipoprotein membrane (Brown, 1984). The high cation exchange capacity of the cell wall may also enable accumulation of substantial quantities of dissolved substances from the external solution, as well as from particulate minerals trapped in the overlapping tissues. The role of cation exchange on bryophyte cell walls and its relationship with intracellular uptake are unclear but

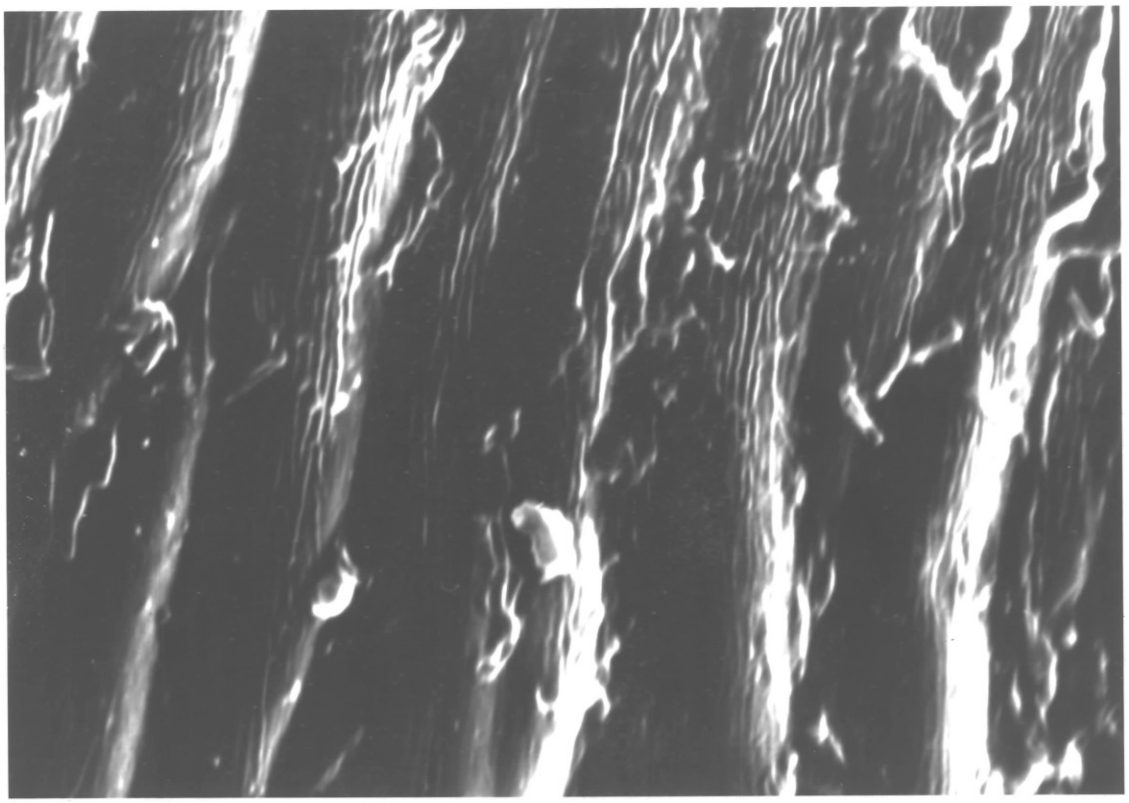


Plate 1.6.2. Scanning electron micrograph of the longitudinal plictations on the adaxial leaf surface of Rhytidiadelphus squarrosus (Magnification x19200)

are presumed to have only a small immediate effect (Brown, 1984). However, the high cation exchange capacities of bryophytes have been suggested to be buffers against excessive concentrations of ions reaching the plasma membranes or reservoirs for intracellular uptake (Schofield, 1985). Wells and Brown (1987) showed in *R. squarrosus* that redistribution of cadmium from extracellular exchange sites to an intracellular location can occur.

The ease with which ectohydric mosses acquire water over their surfaces also exposes them to circumstances where water loss occurs leading to desiccation of the tissue. Many mosses are poikilohydric in nature in that their tissues, including the protoplast, can have a variable water content, closely resembling that of their surrounding atmosphere. Some moss species have also been found to be desiccation sensitive (Proctor, 1984). Desiccation tolerant species can withstand drying to water contents equivalent to 10% of their dry weights (Dilks and Proctor, 1974)

Many mosses are morphologically adapted to prevent water loss. Those species possessing a cuticle may initially experience impeded water loss, and mosses with leaf apices that extend into points can reduce evaporation by up to 20% (Proctor, 1984). Tufted growth forms also help to conserve water due to the development of a boundary layer which resists evaporation.

Desiccation tolerant species resume respiration immediately upon remoistening, rising to levels greater than normal (Dilks and Proctor, 1976a). Photosynthesis recovers more slowly. Between 12-24 h after remoistening, both respiration and photosynthesis return to normal (Proctor, 1984). Recovery times, following desiccation, have been observed to depend on desiccation period (Moore, Luff, and Hallam, 1982), seasons (Dilks and Proctor, 1976b), temperature (Schofield,

1985) rate of desiccation (Dilks and Proctor, 1974; Krochko, Bewley and Pacey, 1978) and degree of chloroplast degradation (Streusand, Weber and Ikuma, 1986).

The cells of desiccation tolerant mosses are generally small or long and narrow, as for *R. squarrosus*, with dense contents and small vacuoles (Proctor, 1984). On drying, the protoplasts of these cells have been observed to shrink to half their normal volume. Large numbers of lipid bodies have also been found in desiccation tolerant mosses; these are thought to be involved in supplying short carbon chain compounds for membrane repair mechanisms (Moore, Luff and Hallam, 1982).

Brown and Buck (1979) described a sudden loss of ions from desiccated moss cells upon rewetting of the tissue, believed to be due to a loss of plasma membrane integrity. Some of the released ions are reabsorbed on restoration of membrane integrity. Adsorption of cations to the cell walls of the moss may serve in the reabsorption of the ions (Brown and Buck, 1979).

Desiccation tolerance is attributed to the stability, during desiccation and subsequent rehydration, of the moss proteins and cytoplasmic ribosomal RNA (Dhindsa and Bewley, 1977; Bewley, 1979). In addition, the ability to resume protein synthesis, RNA synthesis, respiration and photosynthesis immediately upon rewetting is essential in desiccation tolerant species (Bewley, 1979). On resumption of these activities, reconstitution of the damaged plasmalemma can occur, followed by the readsorption of leaked ions.

1.7 EFFECTS OF HERBICIDES ON PLANTS

Studies on the uptake and mode of action of herbicides are primarily related to their activity on vascular plants.

1.7.1 Uptake of herbicides

To exert an effect, a herbicide applied to the surface of a plant must enter into the living tissues. The processes involved in

penetration are well documented in vascular plants (Baker, 1980; Bukovac, 1976; Price, 1982, 1983; Chamel, 1986). Here her bicides must initially traverse a layer of epicuticular wax, which may be in the form of a smooth film, microcrystalline deposits, or in some cases absent (Baker, 1980; Chamel, 1986) and a cuticle before reaching the cell wall of the epidermal cells. The epicuticular waxes are composed in general of long-chain (C26-34) aliphatic hydrocarbons, ketones, aldehydes, alcohols and alkyl esters (C35-60) (Baker, 1980; Chamel, 1986). Cutin and cuticular waxes comprise the bulk of the cuticle and are composed of unesterified hydroxycarboxylic acids derived from C16-18 acids and long chain fatty acids respectively (Baker, 1980). The chemical compositon of the cuticle confers both hydrophilic and lipophilic properties and at physiological pH a negative charge exists (Chamel, 1986). The epicuticular wax and cuticle therefore represent a barrier for the penetration of foliar applied chemicals. The efficiency of a chemical in overcoming this barrier may determine its activity and selectivity.

The next layer, the cell wall, is mainly composed of cellulose and polyuronic acids in vascular plants, the composition and synthesis of which have been reviewed by Robinson (1977). Price (1982) described chemicals which are capable of diffusing across the cuticle and entering the aqueous continuum of the cell wall free space. The two factors preventing movement of herbicide molecules on penetrating the cell wall are interactions with wall structures leading to partition or binding. The cell walls possess anionic exchange sites formed by uronic acids associated with cellulose and pectins, plus some attributable to proteins and polyphenols (Clarkson, 1974). Cations exchanged to anions of the cell walls are said to occupy the 'Donnan Free Space'. This follows Donnan's description of the equilibria established when inorganic ions are separated by a membrane permeable to such ions but impermeable to large organic anions present in one compartment of the

system (Clarkson,1974; Street and Opik, 1976). The influence of cation exchange on the uptake of herbicides following spray application has been described by Price (1978). He related evidence for the reversibility of the Donnan exchange to some organic cations possessing relatively high mobilities in wheat. However, in some situations adsorption may be irreversible, as is the case of 1-methyl- pyridinium chloride in apple leaves, possibly due to binding to lignins or tannins. The quantitative and qualitative composition of the cuticle and cell wall and the chemical properties of the herbicide will therefore influence the rate of herbicide uptake into vascular plants (Rivera and Penner, 1979; Chamel, 1986; Bukovac, 1976).

Finally, the herbicide must penetrate the plasmalemma. Plant membranes are mainly composed of lipids and proteins plus other constituents such as sterols, metal ions and water, and they possess both structural and functional properties (Rivera and Penner, 1979). The main functions of the membrane include regulation of cell permeability, and ion and electron transport. The ability of a herbicide to penetrate the plasmalemma is, therefore, a function of cell permeability, environmental conditions, plant age, chemical properties of the herbicide and its lipid solubility, and the lipid composition of the membrane (Rivera and Penner, 1979).

1.7.2 Mode of action of herbicides

Herbicide activity is generally directed towards metabolic pathways which are specific to plants. These include amino acid biosynthesis, photosynthesis, plant hormone action and the regulation of cell division (Fedtke and Trebst, 1987). However, many of the mechanisms and modes of action of herbicides have still to be elucidated.

Herbicidal action can be divided into primary and secondary mechanisms (Moreland, 1980). The primary mechanisms refer to those that can be measured within a few seconds. These are relatively few and include free radical formation, photo-oxidation and inhibition of electron transport.

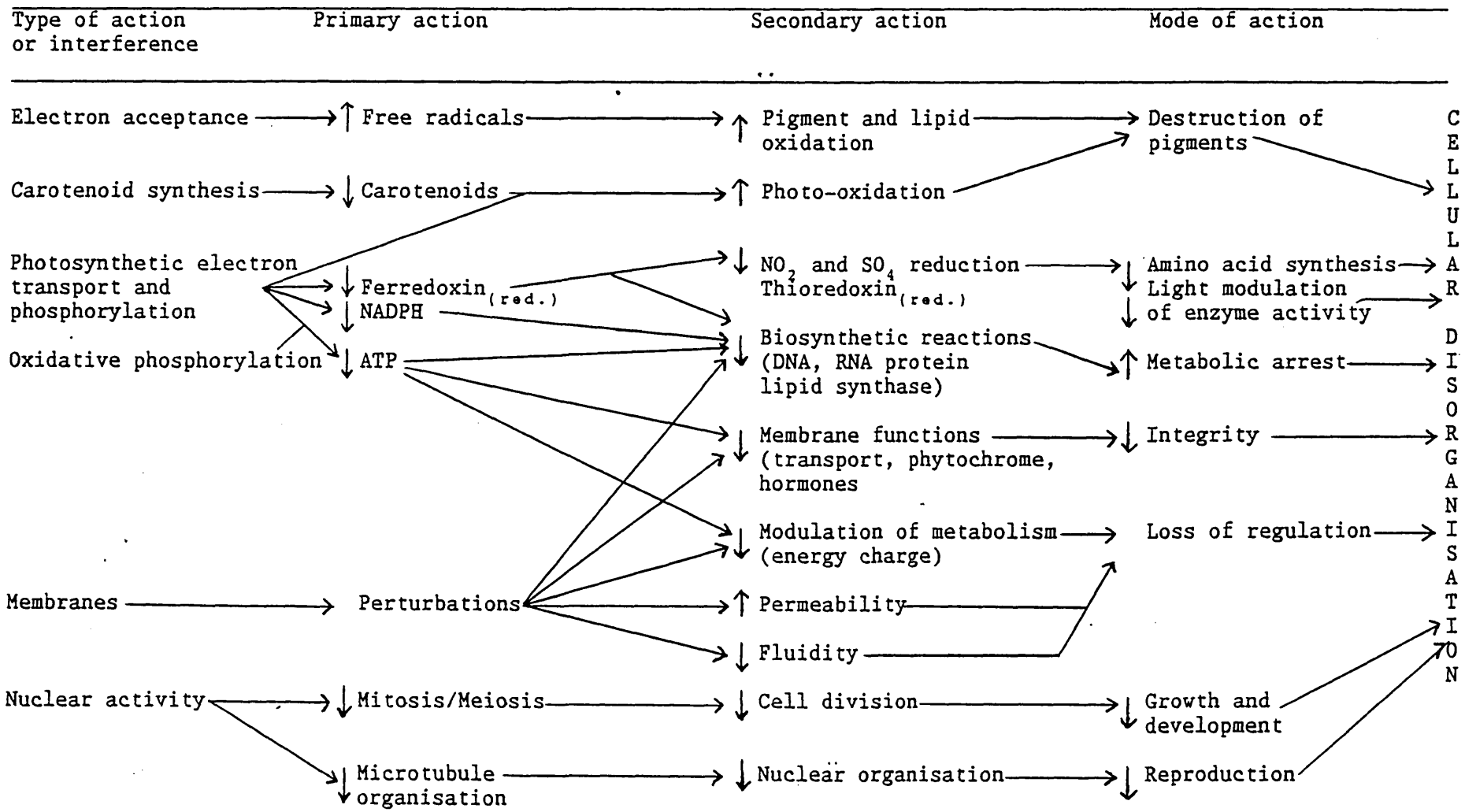
Secondary actions are measured in minutes, after suppression of the availability of ferredoxin, $NADPH_2$ and ATP. These include biosynthesis reactions, membrane functions, modulation of metabolism and accumulation of nitrite. The mechanisms and modes of action of herbicides are summarised in Table 1.7.1 (Moreland, 1980).

In general the modes of action of herbicides can be categorised into those affecting respiration, photosynthesis, protein synthesis or lipid synthesis (Fryer and Makepeace, 1977). Herbicide classification into a particular category is not rigid and overlaps exist. The general activity within each group is described below:

1) Inhibitors of respiration may interfere with a) glycolysis, the oxidation of glucose to pyruvate, b) the Krebs cycle and the oxidation of various organic acids, c) electron transport, and d) oxidative phosphorylation, the mechanism by which oxidation is coupled with phosphorylation of ADP to ATP. Herbicides having these effects are generally termed uncouplers (dinoseb, ioxynil), inhibitors of oxidative phosphorylation (carbamates, phenoxy acids) or electron transport inhibitors (possibly dinitroanilines) (Corbett, Wright and Baillie, 1984). Many herbicides classified as uncouplers have been found to do so at low concentrations but to also inhibit oxidative phosphorylation at high concentrations.

2) Inhibition of photosynthesis is the most common form of herbicidal action. Many photosynthetic inhibitors affecting the light reaction also inhibit ATP formation in isolated mitochondria. The main inhibitory action is that of electron transport and the sites of action

Table 1.7.1 Mechanisms and Mode of Action of Herbicides (cited Moreland, 1980)



↑ increase; ↓ decrease

are generally in photosystem II at a) the O_2 evolution pathway (triazines, ureas), or b) a site adjacent to plastoquinone (ioxynil, bromoxynil). Bipyridylium herbicides also act on photosynthesis; they inhibit electron transport by accepting electrons in photosystem I.

3) Protein synthesis and nucleic acid metabolism are the sites of action for several groups of herbicides. Many of the herbicides which inhibit nucleic acid metabolism and protein synthesis also affect ATP synthesis by oxidation or photophosphorylation. This overlap in actions makes the identification of indirect and direct effects of herbicides on protein synthesis difficult (benzoic acids, carbamates, dinitroanilines, phenoxy acids).

4) Interference of lipid synthesis is generally of slow occurrence in terms of visible effects on the plant. Action is on both the organelle and cell membranes (dichlobenil, endothal).

The work presented in this thesis investigates the effects of four herbicides, paraquat, 2,4-D, dichlorophen and $FeSO_4$, on the moss *R. squarrosus*. A summary of their action in vascular plants follows.

Paraquat

Paraquat is a non-selective foliage-applied herbicide which when present within the plant chloroplast, undergoes rapid reductions and reoxidations with the continual production of hydrogen peroxide. Hydrogen peroxide is believed to be the phytotoxic entity (Calderbank and Slade, 1975). The process of reduction and reoxidation interferes with photosynthesis by diverting photosynthetic energy away from carbon dioxide fixation. For this process to occur, both light and oxygen are required. Rapid cell destruction due to membrane damage is caused by the release of hydrolytic enzymes from the vacuole plus the formation of toxic radicals and peroxides (Corbett, Wright and Baillie, 1984).

2,4-Dichlorophenoxyacetic acid (2,4-D)

2,4-D is a selective, foliage-applied herbicide which exerts growth regulatory/hormonal type activity on most annual and perennial broad leaved species (Fryer and Makepeace, 1977). Susceptible species show symptoms of chlorosis, altered stomatal function, stem tissue proliferation, root initiation from stem tissue, disintegration of root tissue and abnormal apical growth (Corbett *et al.*, 1984). High concentrations of 2,4-D may adversely effect photosynthesis; low concentrations may stimulate it (Audus, 1964).

Grasses are generally very tolerant to 2,4-D but some damage may occur if the herbicide is applied at a very early growth stage (Fryer and Makepeace, 1977).

Dichlorophen

Dichlorophen is marketed in various formulations for the control of fungi, bacteria, algae and mosses. Vascular plants are not affected by the foliar application of dichlorophen. This has been attributed to the low aqueous solubility of dichlorophen leading to poor foliar absorption (Purkiss, 1983).

Dichlorophen has been classified as an uncoupler of oxidative phosphorylation in fungi, bacteria and rat mitochondria (Corbett *et al.*, 1984; Nakaue, Caldwell and Buhler, 1972). However, in Moreland's (1980) classification system for inhibitors of plant photosynthesis and respiration, dichlorophen is grouped as an inhibitory uncoupler of oxidative phosphorylation. Figure 1.7.1 is a schematic representation of electron transport and phosphorylation in plant mitochondria showing postulated sites of activity of inhibitory uncoupler herbicides. Inhibitory uncouplers at low molar concentrations, were described by Moreland (1980) to, satisfy the criteria of uncouplers by :-

Fig. 1.7.1 An outline of electron transport and phosphorylation in plant mitochondria (Moreland, 1980).

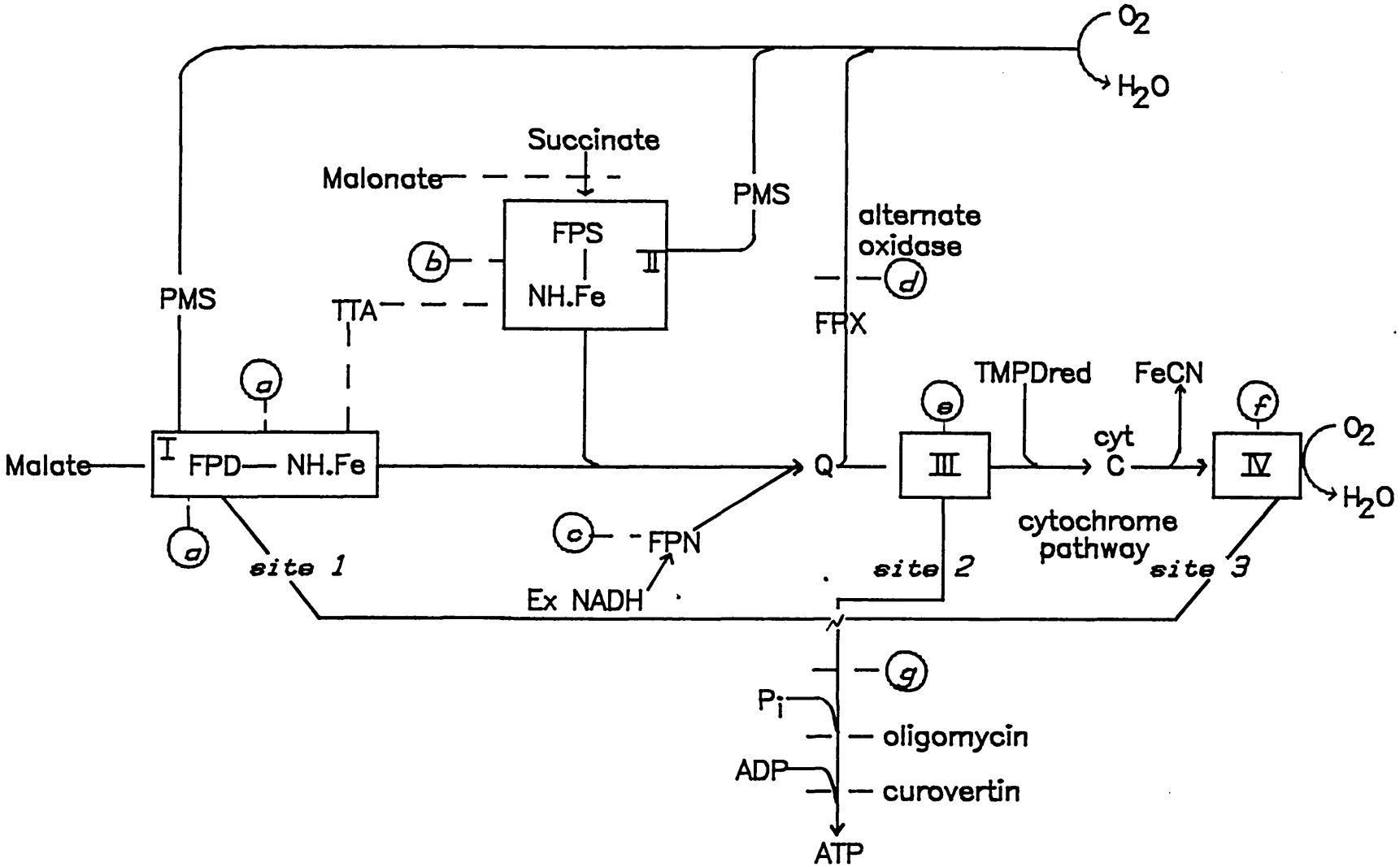
Abbreviations:

Italics letters in circles show the postulated sites of action of inhibitors.

a - *f* = are electron transport inhibitors, *g* = uncoupler

ExNADH, exogenous NADH; FPD, flavoprotein associated with complex I; FPS, flavoprotein associated with complex II; FPX, flavoprotein associated with alternate pathway; FPN, flavoprotein associated with oxidation of exogenous NADH; NH-Fe, non-heme proteins; Q, ubiquinone; FeCN, ferricyanide; I, II, III, IV, sites of phosphorylation.

Fig. 1.7.1


O2
H2O
Succinate
Malonate
PMS
FPS
NH.Fe
II
b
alternate
oxidase
d
PMS
TTA
FRX
a
TMPDred
FeCN
e
f
Malate
I
FPD
NH.Fe
Q
III
cyt
C
IV
O2
H2O
a
site 1
c
FPN
Ex NADH
site 2
cytochrome
pathway
site 3
g
Pi
oligomycin
ADP
curovertin
ATP

(a)stimulating state four respiration (Fig. 1.7.1),

(b)inducing ATPase activity,

(c)circumventing oligomycin imposed inhibition of state three respiration (Fig. 1.7.1),

(d)inhibiting various exchange reactions catalysed by mitochondria in the absence of substrates that involve ADP, ATP and water.

At high molar concentrations, inhibitory uncouplers act as electron transport inhibitors and will affect chloroplast reactions.

Ferrous sulphate ($FeSO_4$)

Iron is an essential micro-nutrient for plants. $FeSO_4$ is not used as a vascular plant herbicide but it is marketed both independently and in mixtures with other chemicals for the control of mosses in lawns. In vascular plants iron deficiency is inhibitory to photosynthesis (Spiller and Terry, 1980). Ferrous iron enters solution in toxic quantities in anerobic soils (Wheeler, Al Farraj and Cook, 1985) and is a principal cause of the absence of many plant species from waterlogged soils. In aerobic conditions, ferric iron is the predominant form. Ferric iron compounds have limited solubility and generally do not reach toxic concentrations in natural environments.

1.8 ANATOMICAL AND PHYSIOLOGICAL PROPERTIES OF BRYOPHYTES IN RELATION TO HERBICIDE UPTAKE

Bryophytes differ from vascular plants in that they generally do not possess a well developed cuticle (Section 1.6; Duckett, 1986). Proctor (1979a) found that 23 out of the 43 moss species he observed lacked a microcrystalline surface wax. The remaining twenty species were described as possessing well developed superficial wax comparable with the epicuticular wax of vascular plants, or traces of surface wax

of similar character. Correlation was also found between the presence of surface wax and endohydric water relations (Proctor, 1979a) indicating the importance of surface wax in water conservation. However, all bryophytes retain a need for external water conduction. Like vascular plants, the cell walls of mosses are composed of a framework of cellulose microfibrils embedded in pectins. Modifications to the cell wall of bryophytes have been observed in a few species, mainly jungermannialean hepatics, in the form of more than one cell wall layer, coloration due to tannins, and ornamentation (sometimes cutinized) (Schofield, 1985). However, despite the similarities with vascular plants, differences between the two groups appear to exist causing moss cell walls to resist resin impregnation for TEM studies (Apostolakos and Galatis, 1985). Mosses also possess higher cation exchange capacities than vascular plants. Clymo (1963) attributed the extraordinarily high cation exchange properties in the genus *Sphagnum* to high concentrations of unesterified polyuronic acids.

The high cation exchange capacity, absence of a well developed cuticle, and the ability to absorb nutrients and water via their external surfaces have made ectohydric mosses favoured organisms for air pollution (Nash and Nash, 1974; Goossens, 1980; Ruhling and Tyler, 1970; Ferguson and Lee, 1980; Thomas and Schunke, 1984; Hermann and Hubner, 1984; Gilbert, 1968) and aquatic pollution studies (Burton, 1979; Satake and Miyasaka, 1984). These studies are based on the fact that accumulation of minerals will, mainly occur from the atmosphere or water but not the substratum. In addition, the ability of mosses to accumulate and tolerate high levels of "heavy" metals has been the subject of many studies on uptake, cellular location and metal specificity (Brown and Beckett, 1985; Brown and Bates, 1972; Brown and Buck, 1985; Burton, 1979; Ruhling and Tyler, 1970; Brown and Whitehead, 1986; Wells and Brown, 1987). The ability of mosses to

withstand environments with high metal content has been principally attributed to their cell wall cationic binding capacities preventing intracellular uptake and, therefore, cell damage.

In contrast to the extensive studies of pollutant and metal uptake, there is no information on the uptake of herbicides by mosses. Absence of the physical barriers associated with the initial uptake of herbicides into vascular plants (epicuticular waxes and a cuticle) would perhaps suggest a more direct passage into moss cells compared with that of vascular plants. However, some vascular plant herbicides have limited effect on bryophyte tissues. These include simazine, glyphosate, bromacil (Atkinson, Abernathy and Crisp, 1980), paraquat with diquat, 2,4-D, captafol (Ronoprawiro, 1975), 2,4,5-T, bromoxynil, dicamba, pichloram (Dana, 1966) and atrazine (Balcerkiewicz and Rusinska, 1987) (Table 1.2.2). This suggests that intracellular uptake does not occur or that the mosses are biochemically different to vascular plants. However, sensitivity has been shown in bryophytes to dichlorophen (Jackson, 1961; Woolhouse, 1973; Ougham, 1983; Brown, Ougham and Beckett, 1986), chloroxuron (Svensson, 1976; Van Himme and Stryckers, 1981), aminotriazole (Atkinson *et al.*, 1980), oxadiazon (Ryan, 1983) and endothal (Brauen *et al.*, 1981).

1.9 OBJECTIVES

1) Investigation of the efficiency of commercially available herbicide preparations for the control of mosses in turf and also on the effects of a range of vascular plant herbicides on the control of mosses in turf.

2) Study of growth and recovery of *R. squarrosus*, under controlled conditions, following herbicide treatments.

3) Examination of cell absorption and efflux of selected herbicides by *R. squarrosus*.

4) Investigation of the effects of herbicides on cell physiology of *R. squarrosus*.

CHAPTER 2

EFFECTS OF FIELD APPLICATION OF HERBICIDES

ON LAWN MOSSES

2.1 INTRODUCTION

The experiments described in this chapter were undertaken to observe 1) the effects of commercially available lawn moss killers and a range of other herbicides on mosses in turf, and 2) to determine whether similar effects could be reproduced in glasshouse studies and on a wider range of moss species.

Field trials were performed to observe the problems encountered when moss control applications are made to turf. The presence of grasses and broad leaved weeds may restrict the quantity of the sprayed herbicide reaching the mosses. In addition, herbicide damage to the grass and broad leaved weed populations of the turf would cause changes in the immediate environment of the mosses, possibly influencing their response. *Rhytidiadelphus squarrosus* was the predominant moss species present in experimental plots.

Two field trials were performed in consecutive years (1984, 1985). The objective of the first year's trial (1984) was to test the efficiency of the commercially available moss killers dichlorophen, chloroxuron with iron salt, ferrous sulphate and ammonium sulphate over a range of doses. Additionally, the non-selective contact-acting vascular-plant herbicide paraquat, described in the literature as having little effect on mosses (Balcerkiewicz and Rusinska, 1987) and the fungicide treatment thiram, observed by Webster (1975) to be an effective bryophyte killer, were included.

The objective of the second field trial (1985) was to assess the effects of 9 different pesticides, including the most effective moss killers of the first years trial, on the control of *R. squarrosus* in a lawn situation. Chemicals used were acifluorfen, oxadiazon, oxyfluorfen, 2,4-D, Gloquat C and paraquat, dichlorophen and chloroxuron with iton salt. The chemicals were selected on the basis of their toxic effects on mosses, as observed by other workers.

Acifluorfen and oxyfluorfen were observed to cause damage to moss species growing at the soil surface, by Turner, D.H (1984, personal communication), during herbicide studies on Rhododendrons. Oxadiazon was described, by Ryan (1983), as an effective herbicide for the control of both mosses and liverworts at the soil surface of container-grown plants. Severe toxic effects in mosses on using 2,4-D and paraquat have not been observed (Balcerkiewicz and Rusinska, 1987; Stjernquist, 1981 ; Dana, 1966), although both are used extensively for the, respective, control of broad leaved weeds and vascular plants. Gloquat C is a recommended moss killer for garden paths but not for lawns due to its toxic effects on grasses. However, despite this Gloquat C was included in this trial to assess its toxicity on *R. squarrosus* along with the proprietary lawn moss killers dichlorophen and chloroxuron with iron salt. The recommended lawn moss killers, dichlorophen and chloroxuron with $FeSO_4$ were used again in the second field trial to assess their performance under different experimental conditions (Section 2.2.4). In addition, the toxic effects of dichlorophen and chloroxuron with iron salt on *R. squarrosus* serve as standards of toxicity to which other herbicide treatments can be compared.

A glasshouse trial was performed to determine the reproducibility of the field trial results on the effects of *R. squarrosus* growing under controlled environmental conditions and also in the absence of potentially interacting grasses and broad leaved weeds. Two other mosses were included in the trial, *Polytrichum juniperinum* and *Dicranum scoparium*, to observe whether their biological reponses to herbicide treatments were consistent with those of *R. squarrosus*. *P. juniperinum* was chosen for this study as it is typically found in areas where herbicides are applied (e.g. under fruit bushes and trees). *P. juniperinum* is, also, endohydric with cutinised leaves and, therefore,

shows a closer resemblance to a vascular plant than *R. squarrosus*. *D. scoparium* was selected on the basis that although it is an ectohydric moss like *R. squarrosus* it differs in being acrocarpous with rhizoidal attachments to the soil. The herbicides paraquat, dichlorophen, 2,4-D and ferrous sulphate were selected due to their different effects on *R. squarrosus* in the previous field trials.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

1) Field Experiments

R. squarrosus-infested lawns at Silwood Park, Ascot, Berkshire (SU 948684) and in a garden in Eversley, Hampshire (SU 769622) were used for moss control studies in 1984 and 1985, respectively. The soil types at the Ascot, Berkshire and Eversley, Hampshire sites were sandy:sandy loam and loam, respectively. The most abundant grass species present in the lawns were *Agrostis tenius*, *Festuca rubra* and *Holcus mollis* at Ascot, Berks. site and *Poa pratensis* and *Holcus mollis* at the Eversley, Hants site. Both sites were similar in their broad leaved weed and moss species composition, although the broad leaved weed population was greater at the Hampshire site whilst thatch accumulation was more evident at the Berkshire site. The predominant moss species at both sites was *R. squarrosus*. Other mosses present, but not abundantly, were *Pseudoscleropodium purum* and *Pleurozium schreberi*. The broad leaved weed species present, at both sites, were *Ranunculus* spp., *Trifolium* spp., and *Bellis perennis*.

2) Glasshouse cultivation

Clumps of the acrocarpous mosses *Dicranum scoparium* and *Polytrichum juniperinum* were collected from the grounds of Silwood Park, Ascot, Berkshire. Whole clumps of the mosses were transferred to

seed trays (20 X 15 cm) containing acid washed sand. Transplanting moss colonies by this method minimised damage to individual shoots and to the overall growth habit which is of importance in moisture conservation.

Gametophyte shoots of the pleurocarpous moss *R. squarrosus* were collected from lawns at Silwood Park. The shoots were teased from the turf and transferred to seed trays (20 x 15 cm) containing acid washed sand.

Trays of moss were kept in an unheated glasshouse (*ca.* 15°C) receiving no supplementary lighting. A two week conditioning period was given prior to applying herbicides to ensure that the mosses had survived transplanting. Mosses were watered, from below, twice a day by adding distilled water to the standing trays.

2.2.2 Experimental layout of field plots

One metre square plots, with 0.5 m guard rows between adjacent plots, were marked in two corners with bamboo canes. Where this was not feasible for aesthetic or lawn maintenance reasons brightly coloured golf tees were pushed into the turf as markers. The layout of the plots, for both experiments, was irregular and the treatments were assigned randomly. Each treatment was replicated three times.

2.2.3 Herbicide application

Herbicide treatments were applied using a hand held Buchier Sprayermatic 5S hydraulic sprayer fitted with a HSS8002 Teejet nozzle. Chemicals were applied at their commercially recommended (or multiples of) rates, at 3 bar pressure, diluted with water and without the addition of extra adjuvants.

2.2.4 Application conditions for field experiments

1984 Field Trial. Weather conditions were still and sunny with an air temperature of 6°C and no rainfall for 72 h after spraying; heavy dew was noted during this period. Grass length was approximately 5 cm but accurate assessment was hindered by litter accumulation at the soil surface.

1985 Field Trial. Weather conditions were moderately sunny and still with an air temperature of 16°C and no rainfall for 48h after treatment. Grass length was *ca.* 4 cm.

2.2.5 Herbicide treatments

In the first field trial (1984) the objective was to assess the effects of commercially available lawn moss killers. Herbicidal treatments applied were dichlorophen, chloroxuron, thiram, paraquat, ferrous sulphate and ammonium sulphate. Sensitivity of *R. squarrosus* to these treatments was tested by applying the herbicides at 3.00, 1.00 and 0.33 times the manufacturers recommended rates.

In the second field trial (1985) a range of vascular plant herbicides were applied at the manufacturers recommended rates for their normal use. The herbicides used were acifluorfen, oxyfluorfen, oxadiazon, paraquat, 2,4-dichlorophenoxy acetic acid (2,4-D), Gloquat C, dichlorophen and chloroxuron with iron salt.

The effects of four herbicides, showing both efficient and inefficient control of *R. squarrosus* in the field trials, were tested on *P. juniperinum*, *D. scoparium* and *R. squarrosus* in the glasshouse experiment. Herbicide treatments used, at recommended rates, were paraquat, dichlorophen, 2,4-D and $FeSO_4$.

Herbicide details:

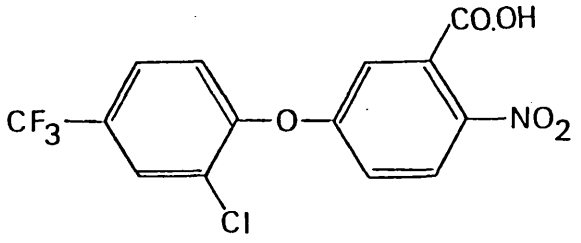
1. Acifluorfen

Supply: Blazer (Rohm and Haas)

Recommended rate: 280g in 200l/ha (Pesticide Manual,1983)

Chemical name: 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid

Structure:



Use: Post-emergence herbicide against broad leaved weeds in wheat, groundnuts, rice and soyabeans

2. Ammonium sulphate

Supply: Gem fertilisers.

Recommended rate: 25kg in 1350l/ ha.

Chemical name: hydrated $(NH_4)_2SO_4$.

Use: fertiliser

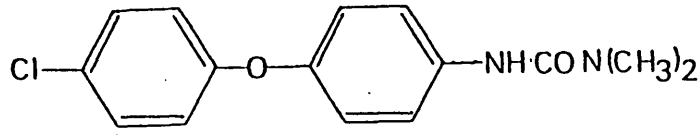
3. Chloroxuron

Supply: Tumblemoss (Murphy agrochemicals) formulated with ferrous sulphate and urea

Recommended rate: 100kg in 1000l/ha (Recommended rate refers to application with a watering can to small areas of lawn. For application with a hydraulic sprayer the volume was reduced by 10%).

Chemical name: 3-[4-(4-chlorophenoxy)phenyl]-1,1-dimethylurea

Structure:



Use: Soil and foliage acting herbicide for use in strawberries, onions, leeks,ornamentals and moss control in lawns.

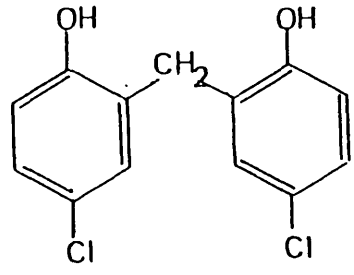
4. Dichlorophen

Source: Supermosstox (Rhône Poulenc)

Recommended rate: 50l in 1000 l /ha.

Chemical name: di-(5-chloro-2-hydroxyphenyl)methane

Structure:



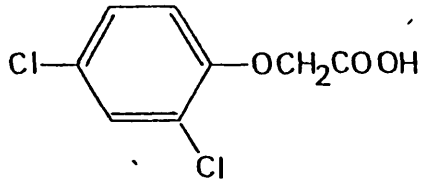
Use: Fungicide, bactericide, algal control, anthelmintic, and selective moss killer for lawns.

5. 2,4-Dichlorophenoxy acetic acid (sodium salt)

Supply: Technical (ICI)

Recommended rate: 1.4kg in 200l/ha (Pesticide manual,1977)

Structure:



Use: Systemic post emergence herbicide for control of broad leaved weeds in cereals.

6. Ferrous sulphate

Supply: Gem fertilisers.

Recommended rate: 56.6kg in 8333 l/ ha.

Chemical formula: hydrated $FeSO_4$

Use: micronutrient, control of moss in lawns.

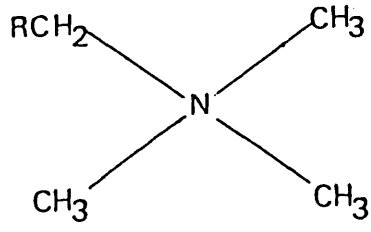
7. Gloquat C

Supply: (quarternary ammonium biocide) ABM chemicals

Recommended rate: 300 l in 4500 l/ha (ABM technical bulletin)

Chemical name: alkyl aryl trimethyl ammonium chloride

Structure:



Use: Moss, liverwort and algal control of hard surfaces and prevention of 'rooting through' in container grown plants.

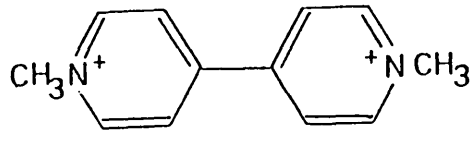
8. Paraquat dichloride

Supply: Gramoxone (ICI).

Recommended rate: 3 l in 500 l/ha (Pesticide Manual,1977)

Chemical name: 1,1'dimethyl-4,4'bipyridinium dichloride

Structure:



Use: Non-selective contact herbicide, stubble cleaning, inter-row weeding.

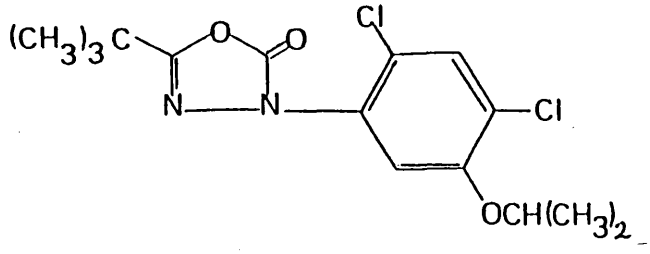
9. Oxadiazon

Supply: Ronstar (Rhône Poulenc).

Recommended rate: 4.4 kg in 200 l/ha (Technical literature, 1978)

Chemical name: 2-tert butyl-4-(2,4-dichloro-5-isopropyl-oxyphenyl)-1,3,4-oxadiazolin-5-one

Structure:



Use: Pre and post emergence herbicide for control of grasses and broad leaved weeds in cotton, rice, transplanted plants and turf.

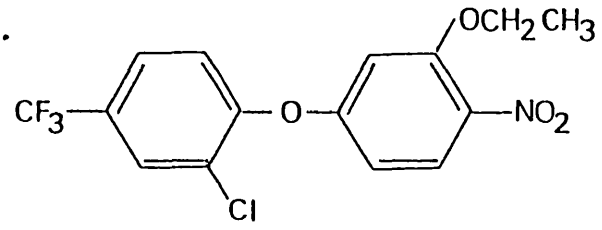
10. Oxyfluorfen

Supply: Goal (Rohm and Haas)

Recommended rate: 250g in 200 l/ha (Meakin and Orpin,1984)

Chemical name: 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene

Structure:



Use: Pre residual and post contact emergence herbicide for control of weeds in banana, citrus, coffee, top fruit.

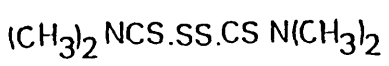
11. Thiram

Supply: Tripomol 80 (Bos)

Recommended rate: 97.5 g in 1300 l/ha (Webster, 1975)

Chemical name: tetramethylthioperoxydicarbonic diamide

Structure:



Use: Fungicide for foliage and seed treatments.

2.2.6 Field and glasshouse assessments of herbicide effects

Quantitative assessments of plant abundance before and after herbicide treatments were made objectively in the field experiments. Visual estimates of damage were made at intervals after herbicide application in both the field and glasshouse experiments. The visual scores for damage were assessed using the European Weed Research Councils scoring system - 'Simple percentage rating of phytotoxicity effects' (cited, Guidelines for Field Evaluation of Herbicides, 1979) (Table 2.2.1). The scoring system was based on the percentage of the whole plant visibly damaged, distorted or scorched compared to that of the control plots. 100 scores were recorded from each plot using a 50 cm quadrat sectioned into 100 $(5\ cm)^2$ units.

Quantitative estimates of percentage cover of the three plant categories, mosses, grasses and broad leaved weeds, were made using a point quadrat method. A point frame with 10 fine (<2 mm diameter) points spaced linearly at 5 cm intervals was used to take 100 readings in a random 50 x 50 cm area.

Final point quadrat measurements were taken 5 or 9 months after treatment for the 1984 and 1985 trials, respectively. The final plant cover values for each plot were expressed as percentages of the initial values giving a percentage change in cover value attributable to the effects of the herbicide treatments.

Table 2.2.1 European Weed Research Council's Simple Percentage Rating of phytotoxic effects (cited Auatralian Weeds Committee - Guidelines for field evaulation of herbicides, 1979)

% Rating	Effects
0	None evident
10	Negligible: discoloration, distortion and/or stunting barely seen
20	Slight: discoloration, distortions and/or stunting clearly seen
30	Moderate damage: moderate discoloration, marked distortions and/or stunting. Recovery expected
40	Substantial damage: much discoloration, distortions and/or stunting; some damage probably irreversibly
50	Majority of plants damaged, many irreversibly; some necrosis; discoloration and distortions severe
60	Nearly all plants damaged, most irreverisibly; some plants killed (<40%); substantial necrosis and distortion
70	Severe: substantial number of plants killed (40-60%); much necrosis and distortion
80	Very severe: majority plants killed (60-80%); remainder show much necrosis and wilting
90	Remaining live plants (<20%) mostly discolored and distorted permanently or desiccated
100	Complete loss of plant and (or) crop yield

All visual scores were subjected to an arc sine transformation before statistical analysis. Visual scores and cover data were statistically analysed by analysis of variance.

2.3 RESULTS AND DISCUSSION

The first year's field trial (1984) was designed to test the efficiency of *R. squarrosus* control in turf using the commercial lawn moss killers, dichlorophen, chloroxuron with iron salt, ferrous sulphate and ammonium sulphate. The efficacies of the vascular plant herbicide paraquat, described by Balcerkiewicz and Rusinska (1987) as having limited effects on mosses, and of the fungicide thiram, observed by Webster (1975) to have toxic effects on bryophytes, were also tested.

2.3.1 1984 Field trial

Dichlorophen, chloroxuron with iron salt, ferrous sulphate, ammonium sulphate, thiram and paraquat were applied to *R. squarrosus* infested turf at Silwood Park, Ascot at x0.33, x1.00 and x3.00 the recommended application rates. Applications were made on the 29 January, 1984.

2.3.1.1 Visible damage assessment and changes in the percentage cover of R. squarrosus after herbicide treatment

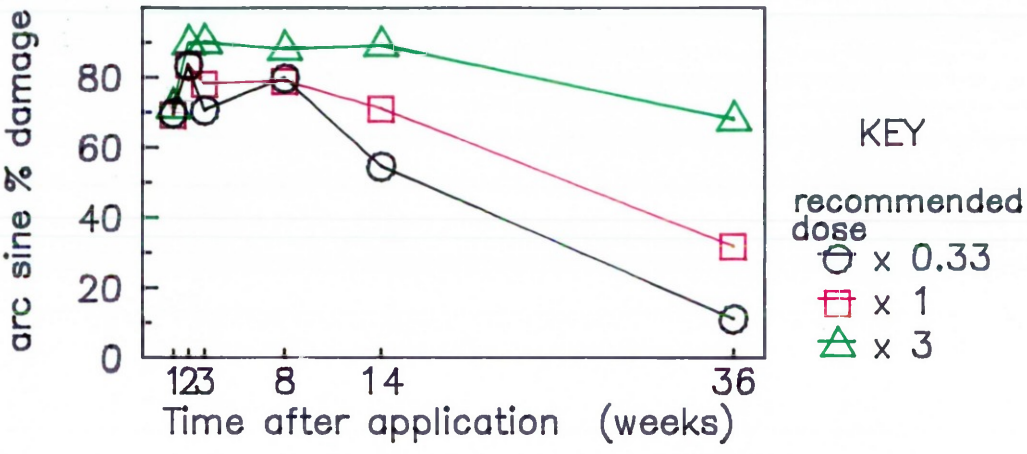
Herbicide damage scores and changes in percentage cover for *R. squarrosus* in turf following the different treatments are presented in Figs 2.3.1 - 2.3.3.1.

Dichlorophen

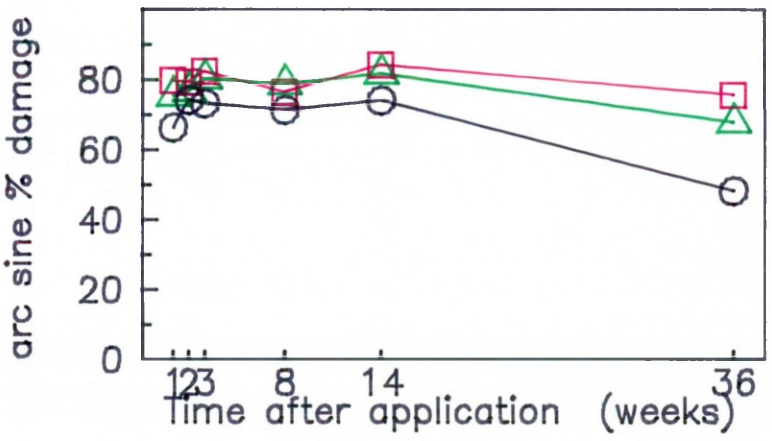
The highest damage score recorded was 90% obtained 3 weeks after spraying with the x3.00 rate of dichlorophen (Fig. 2.3.1). This, however, was not significantly different from high rates (x3.00) of chloroxuron with iron salt, thiram and $FeSO_4$ (Figs 2.3.1 and 2.3.2).

Fig. 2.3.1
Percentage visible damage, following herbicide treatments, to *Rhytidiadelphus squarrosus* in turf. (site: Ascot, Berks.)

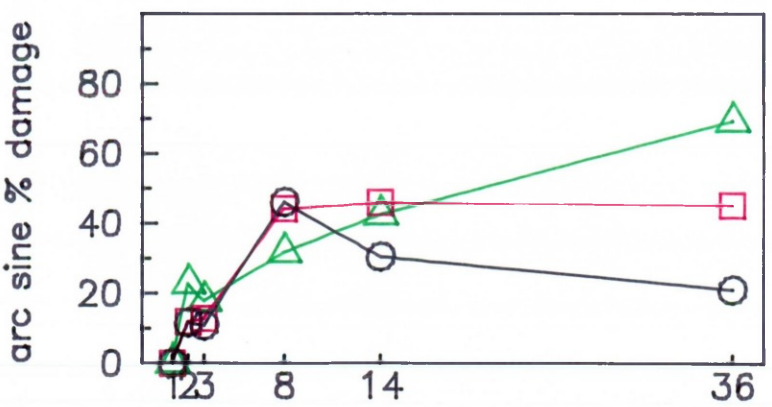
Dichlorophen



Chloroxuron with iron



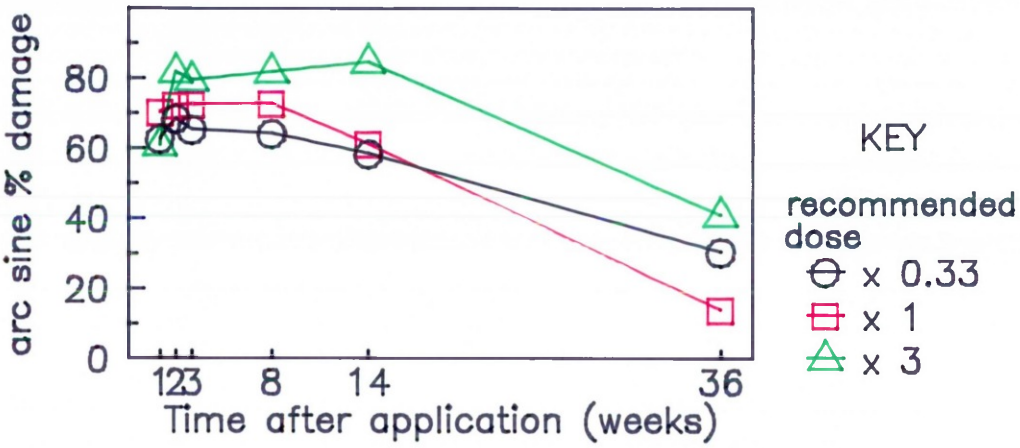
Paraquat



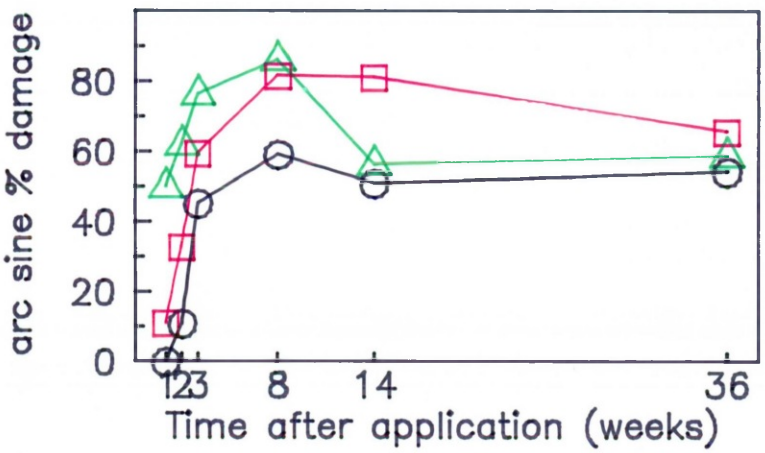
Time after application (weeks)

Fig. 2.3.2
Percentage visible damage, following herbicide treatments, to *Rhytidiadelphus squarrosus* in turf. (site: Ascot, Berks.)

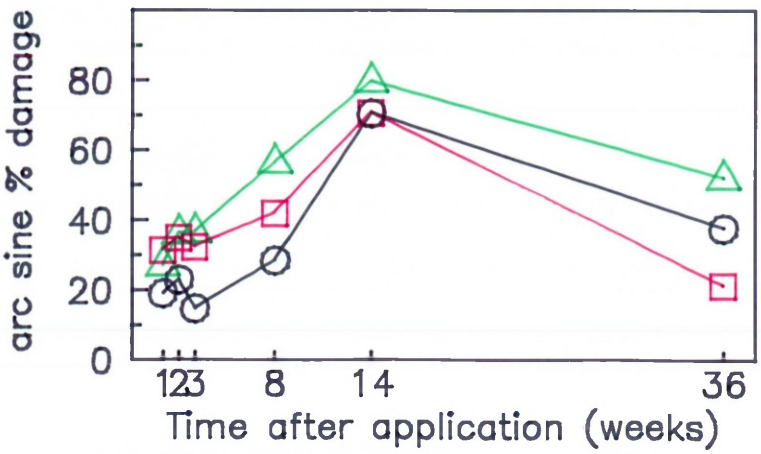
Ferrous sulphate



Thiram



Ammonium sulphate



SED 10.09
d.f = 306
n = 3

Dichlorophen, yielded similar damage scores irrespective of concentration over the first 3 weeks, as did chloroxuron. However, 36 weeks after treatment, damage scores were not significantly different from the control in the plots treated with x1.00 and x0.33 rates of dichlorophen. Regrowth from the stem or occasionally from the apex of the moss shoot accounted for the reduced damage scores.

The visible symptoms of dichlorophen toxicity in *R. squarrosus* were the loss of leaf and apex tissue colour. At high dichlorophen application rates, some of the *R. squarrosus* stems were observed to have lost their natural red coloration and stem turgidity.

A significant reduction (-89%) in cover of *R. squarrosus* compared to the control plots was obtained for the highest dichlorophen rate (x3.00) after 36 weeks (Fig. 2.3.3.1). The recommended dichlorophen rate (x1.00) reduced *R. squarrosus* cover after 36 wk, but not significantly (*ca.* -47%).

The visible damage scores and the percentage reduction in cover measurements, 36 wk after dichlorophen treatment, of *R. squarrosus* increased with application rate. The absence of dichlorophen damage to grasses suggests that it is initially an effective herbicide for the control of *R. squarrosus* in turf. However, long term control of *R. squarrosus* was poor at recommended application rates (x1.00), although increasing the rate three fold did significantly reduce moss cover.

Chloroxuron with iron salt and $FeSO_4$

Chloroxuron, formulated as 'Tumblemoss', also includes $FeSO_4$. The effects of this formulation are here compared with those of $FeSO_4$ applied alone. The chloroxuron formulation blackened the moss tissue and gave similar initial damage scores to $FeSO_4$ (Plate 2.3.1). The visible damage scores for both the combined chloroxuron and iron salt formulation and the separate $FeSO_4$ treatment were based on the

Fig. 2.3.3.1 Net changes in the percentage cover of *Rhytidiadelphus squarrosus*, 36 weeks after herbicide treatments. (site: Ascot, Berks.)

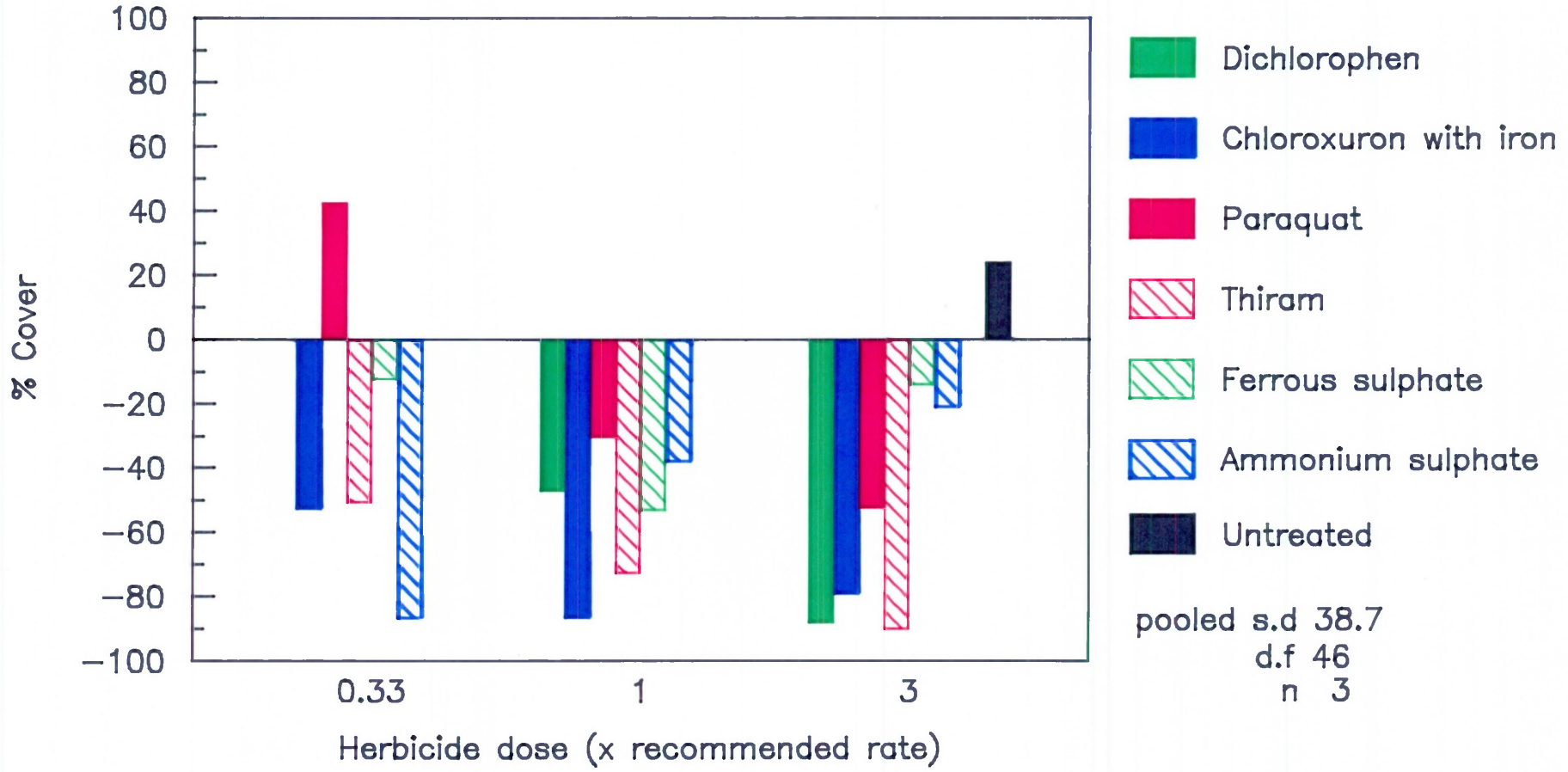




Plate 2.3.1. Blackening effect of ferrous sulphate, 1 wk after application to moss-infested turf.

intensity and overall distribution of the blackened *R. squarrosus* shoots. The blackening effect of iron salts has been described as scorch (Anon., 1982). This may, however, be a misleading description as this effect has also been ascribed to the formation of pigmented iron complexes with tannins at the moss cell wall (Field, 1969). Blackening of the moss tissues has been scored, in this section, as evidence of damage but later work (Sections 3.3.7 and 6.3.2) does not corroborate it. The visible damage scores immediately after application for the chloroxuron with iron salt formulation and $FeSO_4$ applied separately were similar (Figs 2.3.1 and 2.3.2). *R. squarrosus* treated with the chloroxuron with iron salt formulation, following all three rates of application, showed significantly less recovery (*ca.* 80% damage) after 36 weeks than equivalent rate of $FeSO_4$ (*ca.* 25% damage). Kuttruff and Kleve-Kellen (1980) and Svensson (1976) observed similar effects of chloroxuron and $FeSO_4$ on regrowth of mosses. In the present study observations were made using light microscopy (mag. x100) on small samples of leaf tissue of *R. squarrosus* blackened by treatment with $FeSO_4$ in the laboratory. No differences in the colour or cellular constituents of the blackened tissue, compared to untreated leaves, were apparent confirming that the blackening was located on the walls of the moss (Field, 1969).

Chloroxuron with iron salt reduced *R. squarrosus* cover significantly at the x1.00 (-86%) and x3.00(-79%) application rates 36 wk after treatment (Fig.2.3.3.1). $FeSO_4$ did not significantly reduce the percentage cover of *R. squarrosus* in turf (Fig. 2.3.3.1).

Chloroxuron, formulated as Tumblemoss, would appear to be more effective in the long term control of *R. squarrosus* in turf than dichlorophen, at their recommended rates. Regrowth from blackened chloroxuron with iron salt or $FeSO_4$ treated mosses was easily observed as new tissue was green in colour. Unlike that of $FeSO_4$ treated *R. squarrosus*, where regrowth generally occurred at the shoot apex,

chloroxuron with iron salt treated *R. squarrosus* showed regrowth a few centimetres below the shoot apex. The addition of $FeSO_4$ to a moss killer preparation such as chloroxuron may aid only in the production of a visible blackening effect assumed by the user to be damage. Chloroxuron, however, was apparently responsible for inhibiting regrowth from the stem apices of *R. squarrosus* and for reducing its cover in turf.

Iron salts, independently or in mixtures with other herbicides, may act as a grass fertiliser, improving their vigour and competitive ability towards other plants including mosses.

Thiram

Thiram caused a characteristic whitening of moss tissue after spray application (Webster, 1975) due to a residual film of the thiram formulation (wettable powder) left on drying. A gradual bleaching at the apex was observed with time after thiram treatment. Webster (1975) observed similar symptoms in the liverworts *Marchantia* spp. and *Lunularia* spp. growing in plant containers. The highest damage score obtained following treatment with thiram was 86% for the x3.00 rate, after 8 wk. Thiram treatments were slower in achieving a visible effect than those of dichlorophen, chloroxuron with iron salt or $FeSO_4$. However, recovery from thiram treatments was slight with a significant reduction in *R. squarrosus* cover following x1.00 (-73%) and x3.00 (-90%) application rates (Fig. 2.3.3.1). Effective moss control, in plant containers, was achieved by Van Himme and Stryckers (1981) and Ryan (1983) using thiram. Here, significantly less recovery was observed in plots receiving thiram treatments (54%, 56% damage) than in those receiving dichlorophen (11%, 32% damage) for the x0.33 and x1.00 rates, respectively, 36 wk after treatment.

From these results, thiram would appear to be as effective as the proprietary lawn moss herbicides dichlorophen and chloroxuron with iron salt. The field trial results described in this chapter would also indicate that thiram has greater potential for the long term control of mosses than dichlorophen.

Paraquat

Visible effects of paraquat on *R. squarrosus* during the first three weeks after treatment were slight (Fig. 2.3.1). However, 8 wk after treatment, approximately 40% of the tissue had become straw coloured. The severity of paraquat effects observed on *R. squarrosus* were related to concentration. A significant recovery (*ca.* 20% damage) of *R. squarrosus* following the x0.33 application rate and a further increase in damage to *ca.* 70% from the x3.00 application rate was observed 36 weeks after treatment. Regrowth of *R. squarrosus*, following the x0.33 paraquat application, was from the stem tissues *ca.* 2 cm below the shoot apex.

The paraquat treatments did not significantly reduce *R. squarrosus* cover over the 36 wk experimental period. Surprisingly, *R. squarrosus* cover increased (42%) from the starting value, following the lowest (x0.33) rate of paraquat application (Fig. 2.3.3.1). Balcerkiewicz and Rusinska (1987) observed a similar expansion of mosses following paraquat treatment of grassy embankments. Increased moss cover, in both cases, can probably be attributed to reduced competition from vascular plants due to the severe scorching of their foliage by paraquat.

Ammonium sulphate

Visible damage following ammonium sulphate treatment of *R. squarrosus* resembled those of paraquat with bleaching of the shoot apices. No significant differences in damage scores were recorded

between the different application rates of ammonium sulphate. The greatest damage (*ca.* 70%) was observed 14 wk after treatment, for all doses, but by 36 wk regrowth from the stems had occurred, reducing the damage score (to *ca.* 20-50%) (Fig. 2.3.2). Ondrej (1985) and Kuttruff and Kleve-Kellen (1980) also noted the limited duration of effects of ammonium sulphate treatments on lawn mosses.

A reduction in *R. squarrosus* cover (-87%) was observed following treatment with ammonium sulphate at x0.33 rate (Fig. 2.3.3.1). No significant changes in moss cover were determined for the higher (x1.00 and x3.00) rates of ammonium sulphate (Fig. 2.3.3.1). The variations in the effects of different concentrations of ammonium sulphate on cover are not easily explained. Possibly, the low rate of ammonium sulphate acted as a grass fertilizer and reduced moss cover through a competitive effect. This would then suggest that increasing the application rates of ammonium sulphate to x1.00 and x3.00 may have induced toxic effects on the grasses.

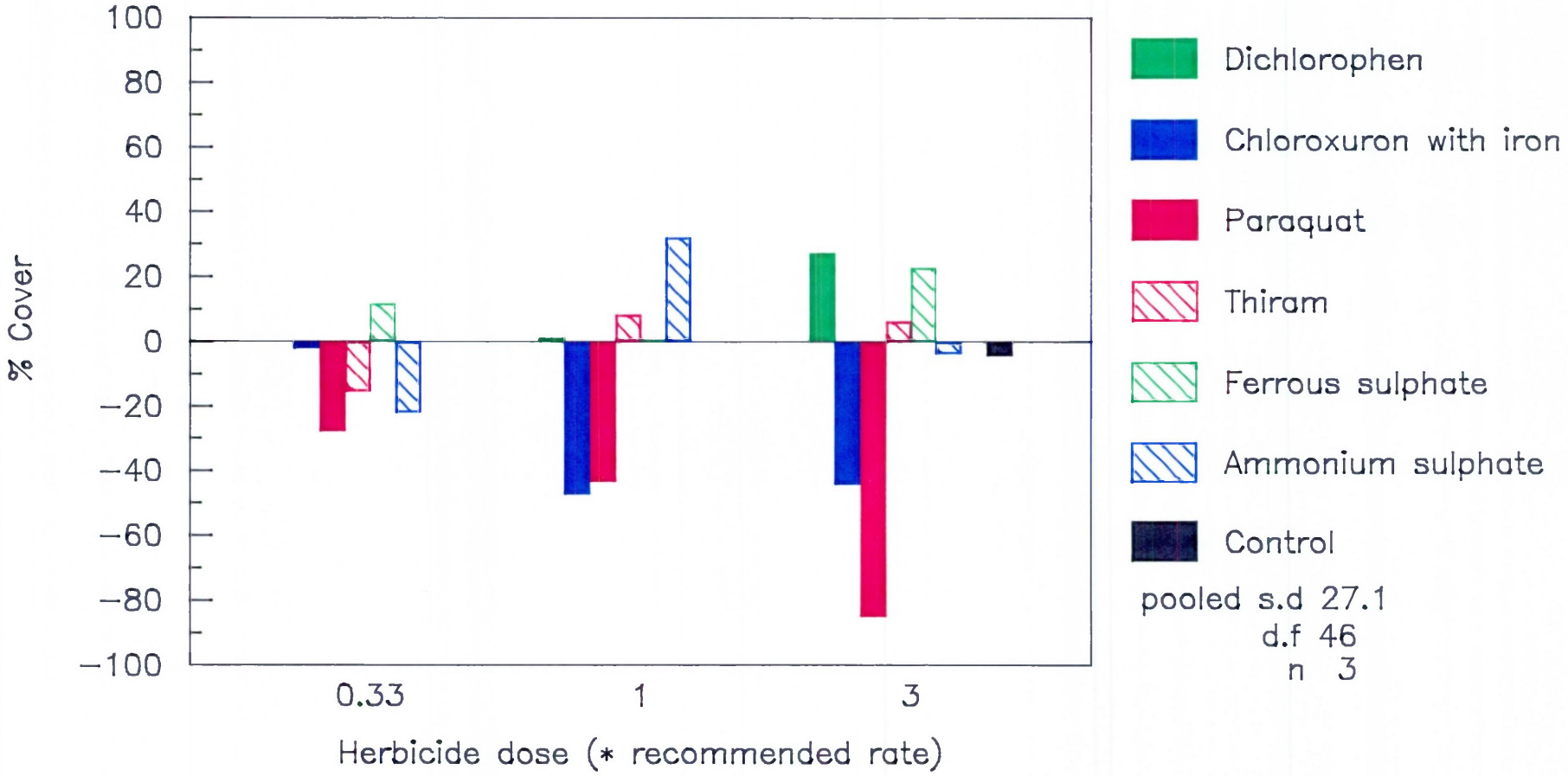
The damage scores recorded after 36wk were, in general, good indicators of the final percentage cover measurements for all herbicide treatments.

2.3.1.2 Visible damage assessment of turf grasses after herbicide application

The most abundant grass species present at the Ascot, Berks. site were *Agrostis capillaris*, *Festuca rubra* and *Holcus mollis.*

Visible damage to grasses by dichlorophen, chloroxuron with iron salt, thiram, $FeSO_4$ and ammonium sulphate was slight and damage scores were not systematically recorded. However, paraquat initially caused scorching and desiccation of the foliage. The resultant reduction in grass foliage following paraquat application also resulted in the loss of the underlying thatch which had previously been anchored by it. The x3.00 paraquat rate was the only treatment to show a significant

Fig. 2.3.3.2 Net changes in the percentage cover of grasses 36 weeks after herbicide treatments. (site: Ascot, Berks.)



reduction in grass cover 36 wk after application (Fig. 2.3.3.2). Regrowth from the root system of grasses, treated with low paraquat doses, accounts for the absence of a significant reduction in grass cover in these treatments (Fig. 2.3.3.2). The loss of grass vegetation may have enhanced paraquat damage of *R. squarrosus* after 3 wk by exposing it to direct sunlight and thus creating higher temperatures and causing desiccation. Such conditions could enhance the effects of paraquat on *R. squarrosus* by reducing growth rate. They also rendered the tissues susceptible to dislodging by wind or removal during mowing.

Significant differences were also found between the effects of dichlorophen and chloroxuron with iron salt on grass cover although neither treatment differed significantly from the control. The x1.00 and x3.00 chloroxuron with iron salt rates reduced grass cover by 47% and 44% respectively, compared to an increase of *ca.* 27% for the x3.00 dichlorophen rates. In addition, no major toxic effects on grass foliage were observed following treatment with dichlorophen or chloroxuron with iron salt. This suggests that chloroxuron with iron salt may have inhibited the growth of the grasses without causing visible damage to the foliage. In addition, chloroxuron uptake by the root system of the grasses may have aided in the long term effects on their growth and the subsequent reduction in grass cover. This is supported by the marketing of chloroxuron for use as a non-selective, soil-applied herbicide for the control of weeds in strawberries, ornamental plants and onions (Pesticide Manual, 1977).

2.3.2 1985 Field trial

The sensitivity of mosses to vascular plant herbicides has not been extensively studied. In this experiment the susceptibility of *R. squarrosus* to a range of vascular plant herbicides was tested. Comparisons of the early and long term (17 wk) biological effects of

each herbicide treatment on *R. squarrosus*, and on the grasses and broad leaved weeds were made. Herbicide treatments used in this experiment were chosen on the basis of effects on mosses observed by other authors, as described in Section 2.1. The treatments applied were acifluorfen, oxyfluorfen, 2,4-D, oxadiazon, chloroxuron with iron salt, dichlorophen, Gloquat C and paraquat, each at their recommended rate.

The major broad leaved weed species present in the turf were *Trifolium* spp., *Ranunculus* spp., *Bellis perennis* and *Taraxacum officinalis*. The broad leaved weed population of the turf was greater in this experiment than the last although the distribution, percentage cover and species composition varied over the experimental site. For those reasons broad leaved weeds in the experimental plots were assessed collectively. The predominant grass species present in the turf were *Poa pratensis* and *Holcus mollis*. Herbicide damage was again collectively assessed for all grass species.

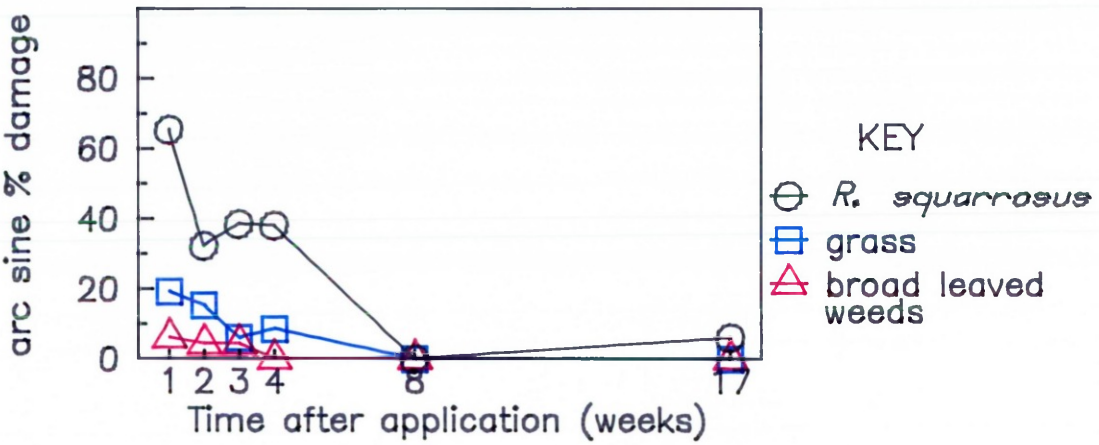
Visible damage scores and percentage cover results for all treatments and plant groups are presented in Figs 2.3.4 to 2.3.8

Chloroxuron with iron salt

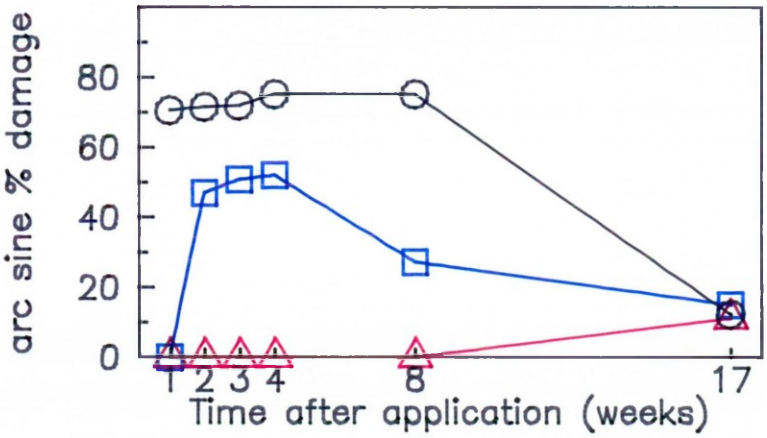
The greatest damage score observed for *R. squarrosus* was 75%, 4 to 8 wk after treatment with chloroxuron with iron salt (Fig. 2.3.4). However, unlike in the 1984 field trial (Fig. 2.3.3.1), the percentage cover reduction (-17%) 17wk after treatment was not significantly different from the control (Fig. 2.3.5). In contrast to the 1984 field trial, damage (*ca.* 50%) of young grass leaves was observed although the final grass cover was little affected (-5%) (Figs. 2.3.4 and 2.3.6). The toxic damage of young grasses in this trial may be due to the selective activity of chloroxuron towards annual and seedling grasses (Fryer and Makepeace, 1977). Chloroxuron with iron salt significantly reduced the percentage cover of the broad leaved weeds present in the turf although visible damage was slight (Figs 2.3.4 and 2.3.6).

Fig. 2.3.4
Percentage visible damage following herbicide treatments to *Rhytidiadelphus squarrosus*, grasses, and broad leaved weeds, in turf.
(site: Eversley, Hants.)

Dichlorophen



Chloroxuron with iron salt



Acifluorfen

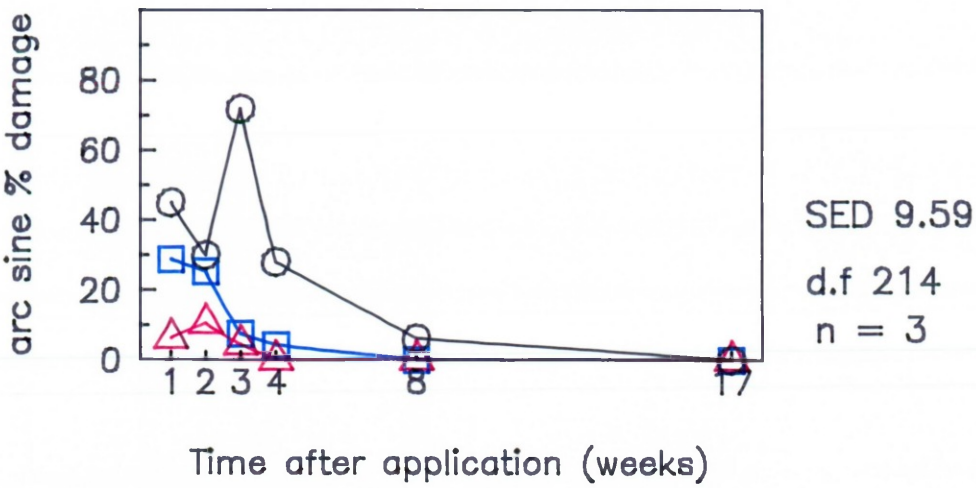
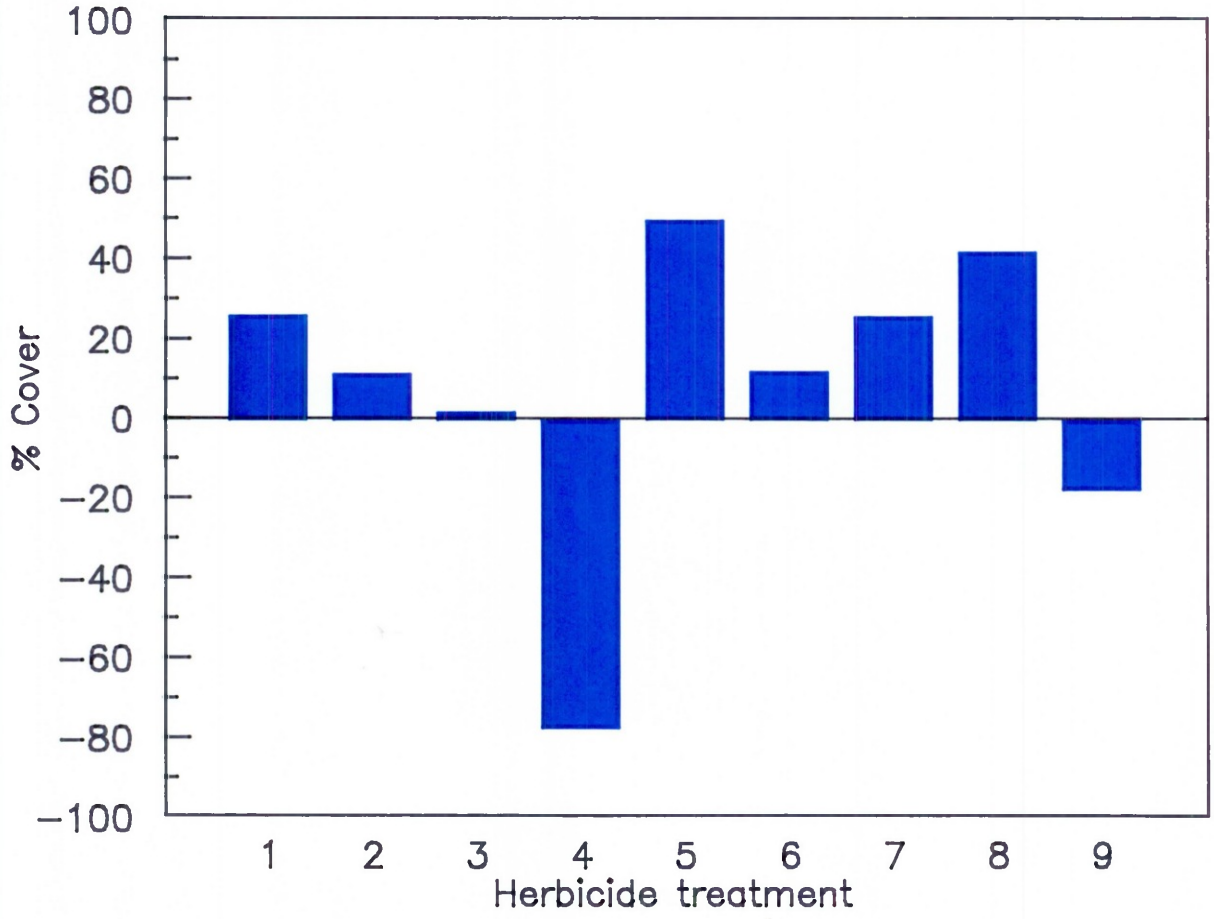


Fig. 2.3.5 Net changes in the percentage cover of *Rhytidiadelphus squarrosus* 17 weeks after herbicide treatments. (site: Eversley, Hants.)



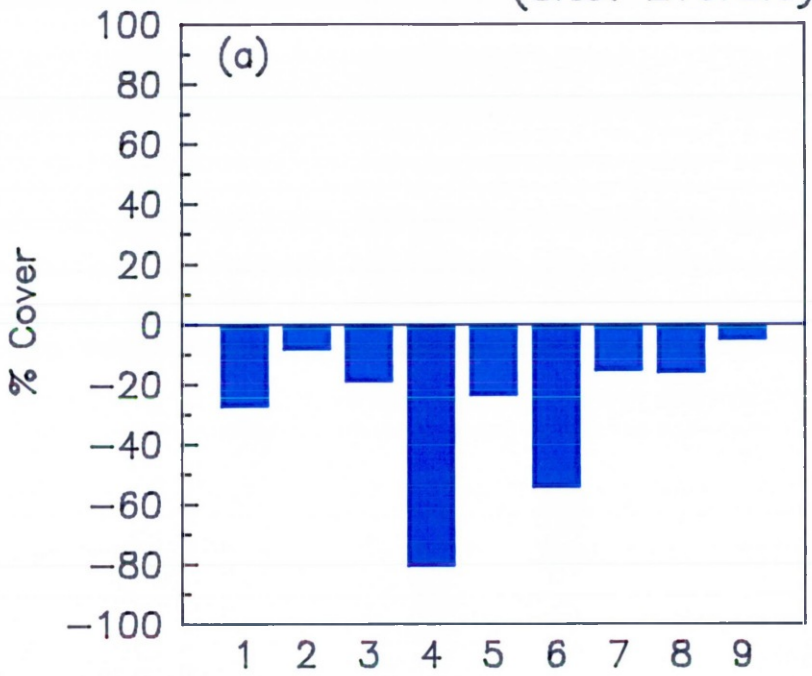
KEY

1 Untreated
2 Acifluorfen
3 Oxyfluorfen
4 Oxadiazon
5 2,4—D
6 Paraquat
7 Gloquat C
8 Dichlorophen
9 Chloroxuron

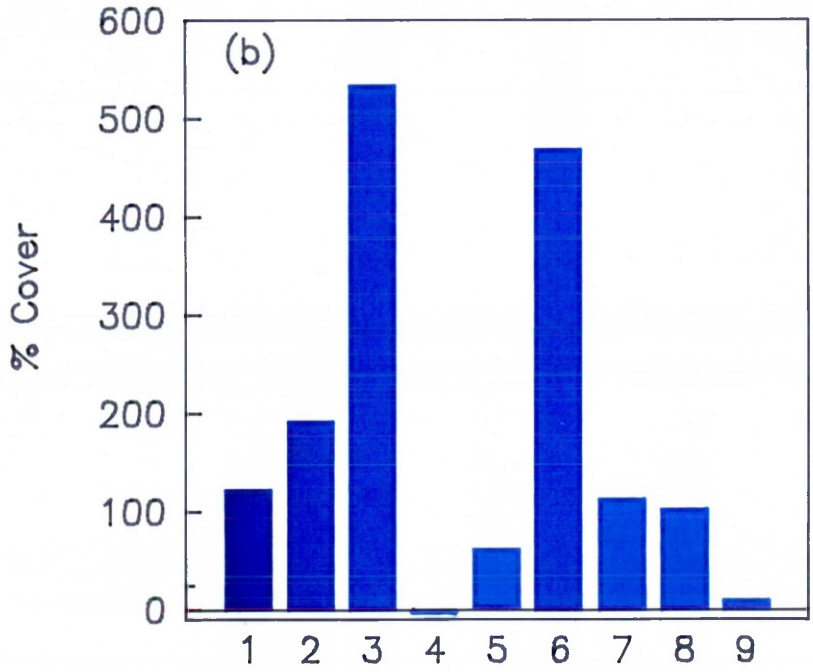
pooled s.d 30.2
d.f 29
n 3

Fig. 2.3.6
Net changes in percentage cover of a) grasses, and b) broad leaved weeds, in turf, 17 weeks after herbicide treatments.

(site: Eversley, Hants.)



pooled s.d 19.8
d.f 26
n 3



pooled s.d 302
d.f 26
n 3

KEY

1	Untreated	4	Oxadiazon	7	Gloquat C
2	Acifluorfen	5	2,4−D	8	Dichlorophen
3	Oxyfluorfen	6	Paraquat	9	Chloroxuron

The reduction in cover of the broad leaved weeds following the chloroxuron with iron treatment may have allowed the growth and expansion of *R. squarrosus*. The 1984 field trial, following paraquat treatment, produced results suggesting that a loss in vascular plant cover could increase the cover of *R. squarrosus* (Section 2.3.1.1). Chloroxuron effects on the broad leaved weeds may have been enhanced by uptake via the roots as well as the foliage (Section 2.3.1.2) as the damage scores following foliage application were low. In addition, chloroxuron's persistence in the soil extends its period of biological activity to several months after application (Pesticide Manual, 1977). However, in turf situations contact of growing *R. squarrosus* shoots with the soil is poor. The control of broad leaved weeds in turf by the chloroxuron with iron salt treatment could encourage the spread of mosses, by removing competition, thus reducing its efficiency in moss control.

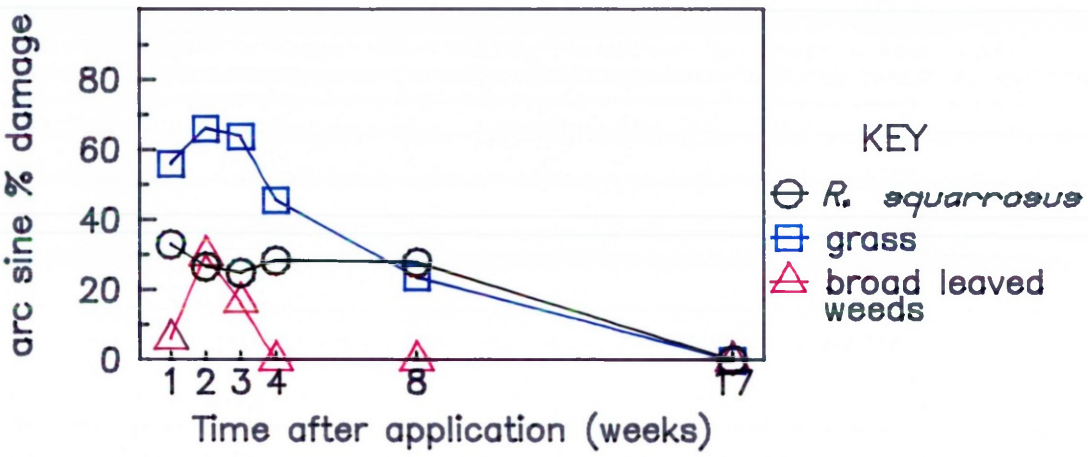
Dichlorophen

Dichlorophen caused bleaching (65% damage) of *R. squarrosus* shoots 1 wk after treatment (Fig. 2.3.4). Regrowth from the damaged stems occurred within 4 wk with no visible damage after 8 wk. *R. squarrosus* cover following dichlorophen treatment was not affected (Fig. 2.3.5). A 20% damage score was recorded for grasses 1 wk after dichlorophen treatment although no significant reduction in cover was noted (Figs. 2.3.4 and 2.3.6). Moss control trials, conducted by Woolhouse (1973), also indicated a toxic effect of dichlorophen on grasses. Broad leaved weeds treated with dichlorophen showed no significant visible damage or long term (17 wk) effects on cover (Figs. 2.3.4 and 2.3.6)

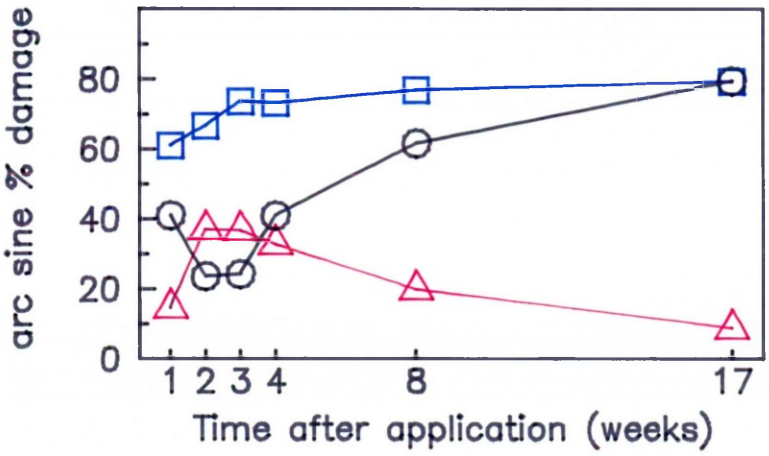
Chloroxuron with iron salt and dichlorophen both caused scorch of grass tissue in the 1985 field trial but this was absent in the 1984 field trial (Section 2.3.1.2). This disparity may arise from the different application dates (January 1984 and May 1985). On the

Fig. 2.3.7
Percentage visible damage following herbicide treatments to *Rhytidiadelphus squarrosus*, grasses, and broad leaved weeds, in turf
(site: Eversley, Hants.)

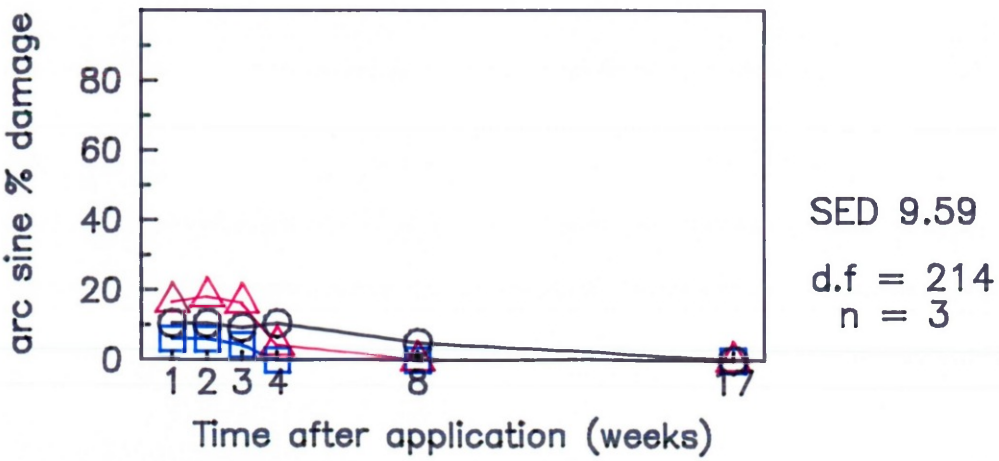
Oxyfluorfen



Oxadiazon



2,4—D



latter occasion much fresh growth was present in the turf and this is generally more sensitive to herbicides than mature tissues (Fryer and Makepeace, 1977).

Oxyfluorfen and Acifluorfen

Oxyfluorfen and acifluorfen produced similar biological effects on *R. squarrosus* (25-40%), on grasses (30-60%) and on broad leaved weeds (10-30%) (Figs 2.3.4 and 2.3.7). Regrowth of *R. squarrosus* from beneath the stem apex (*ca.* 2 cm), reduced the visible damage scores after 4 wk. The treatments caused no significant reduction of percentage cover of *R. squarrosus*, grasses or broad leaved weeds (Figs. 2.3.5 and 2.3.6).

R. squarrosus leaves thus appear to be sensitive to both oxyfluorfen and acifluorfen applications although eventual regrowth from the stems occurred. Oxyfluorfen and acifluorfen are known to inhibit photosynthetic electron transport and cause peroxidation of unsaturated fatty acids in vascular plants. This leads to the general destruction of chloroplasts and membrane damage. Resistance to these herbicides has been tentatively attributed to reduced levels of unsaturated fatty acids in vascular plants. However, Suire and Asakawa (1979) described mosses as containing arachidonic and linoleic acids, both unsaturated fatty acids, although their percentage composition varies between moss species and their ecological environments.

The limited long term effects of acifluorfen and oxyfluorfen on the stems of *R. squarrosus* may reflect poor penetration of the herbicides due to protection by the leaves. Alternatively, the stems may only play a small part in photosynthesis and, therefore, be less sensitive to photosynthetic inhibitor herbicides.

Oxadiazon

Oxadiazon produced chlorosis in the leaves of *R. squarrosus* over the first 4 wk (Fig. 2.3.7), similar to the toxic effects of acifluorfen and oxyfluorfen (Fig. 2.3.4 and 2.3.7). This increased in severity to 80% after 17 wk and the percentage cover was reduced by 77%. Effective control of the moss *Leptobryum pyriforme*, in potted plants, by oxadiazon has been observed by Ryan (1983). However, similar work by Wilson and Hughes (1985) showed poor oxadiazon control of *Funaria* spp. in container-grown crops.

As a lawn moss killer, oxadiazon is not desirable as this was the only herbicide used which reduced grass cover (-80%) significantly over the 17 wk period (Fig. 2.3.6). The broad leaved weeds were not severely affected by oxadiazon reaching a damage score of 37% 4wk after treatment.

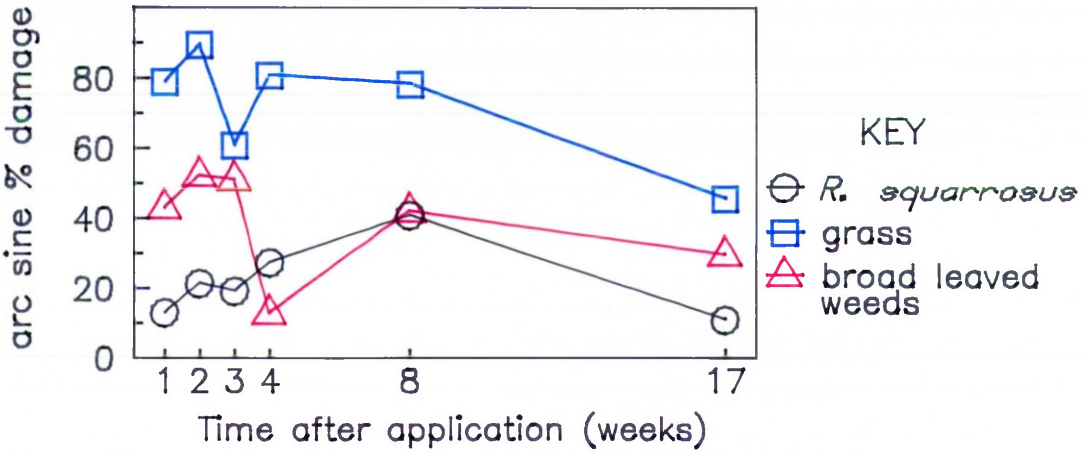
The severe toxic effects of oxadiazon on the turf grasses may have influenced the effects exhibited by *R. squarrosus*. Reduction in grass cover would expose the mosses to direct sunlight and desiccation.

Paraquat

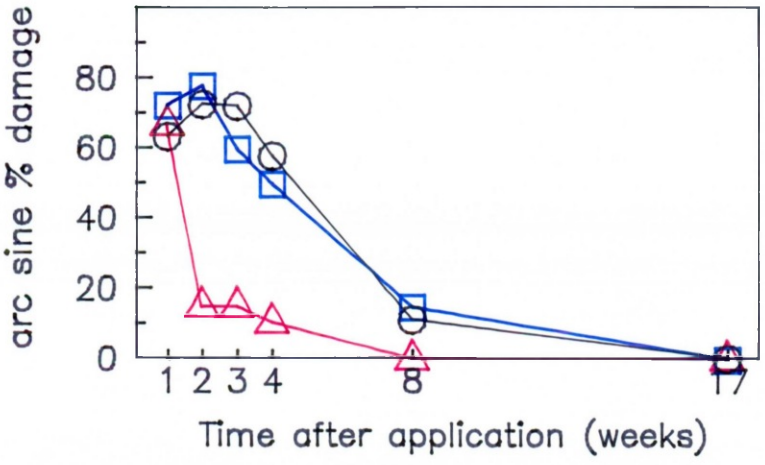
Effects of paraquat on *R. squarrosus* were slow to appear (Fig. 2.3.8). The highest damage score (41%) was reached 8 wk after treatment. The percentage cover of *R. squarrosus*, following paraquat treatment, did not significantly alter over the 17 wk period (Fig. 2.3.5). The effects on both grasses and broad leaved weeds were initially severe giving a damage score of 90% and 52%, respectively, 2 wk after treatment (Fig. 2.3.8). However, both the grasses and broad leaved weeds recovered from the paraquat treatment. In the case of broad leaved weeds, cover was increased (470%) although this was not significantly different from the control. Grass cover was reduced by 54% although this did not differ significantly from the control (Fig.

Fig. 2.3.8
Percentage visible damage following herbicide treatments to *Rhytidiadelphus squarrosus*, grasses, and broad leaved weeds, in turf.
(site: Eversley, Hants.)

Paraquat



Gloquat C



SED 9.59

d.f 214

2.3.6). Paraquat treatment, as observed in the previous field trial (Section 2.3.1.1), caused a slow but progressive visible damage of *R. squarrosus*. *R. squarrosus* would, therefore, appear to be sensitive to paraquat but requires time for the biological effects to become visible. This may possibly reflect a slow uptake of paraquat by *R. squarrosus*, possibly due to binding of the paraquat cation to the extracellular anionic binding sites of the cell wall (see Section 4.3.1.6).

2,4-D

2,4-D caused bleaching of the leaves of *R. squarrosus* at the shoot apices (*ca.* 1 cm) (Fig. 2.3.7). The highest visible damage score recorded was 10% 1-2 wk after treatment. 2,4-D treatment increased the cover of *R. squarrosus* by 50% 17 wk after treatment, although this increase was not statistically significant (Fig. 2.3.5). The reduction in broad leaved weeds following 2,4-D treatment may have allowed spread of *R. squarrosus*. 2,4-D caused *ca.* 20% damage of broad leaved weeds 2 wk after treatment and reduced cover slightly. Effects of 2,4- D on grasses were slight.

Phenoxyalkanoic herbicides, including 2,4-D, are frequently used in lawn herbicide preparations for broad leaved weed control. Thus, increased *R. squarrosus* cover following 2,4- D treatment, indicates that a moss killing preparation is required following 2,4-D treatment to prevent their spread in lawns.

The limited effect of 2,4-D on *R. squarrosus* suggests tolerance to its growth regulatory activity as is also found in grasses.

Gloquat C

Gloquat C is a quarternary ammonium compound recommended for moss control on paths. Toxic effects reached 70% after 3 wk but

none were visible 17 wk after treatment due to regrowth from the damaged stem tissues (Fig. 2.3.8). Consequently, no significant effects were observed for *R. squarrosus* cover following Gloquat C treatment (Fig. 2.3.5).

Severe toxic effects of Gloquat C were observed on grasses (78% damage) and broad leaved weeds (68% damage) after 1-2 wk (Fig. 2.3.8). However, due to regrowth no significant change in cover was observed after 17 wk (Fig. 2.3.6).

In summary, *R. squarrosus* was sensitive, to some degree, to all the herbicide treatments applied. In all cases, except the chloroxuron treatment where the addition of $FeSO_4$ caused blackening of the moss, bleaching of the leaves occurred. Recovery of *R. squarrosus* leaves following herbicide treatments did not occur. Regrowth from the stem tissue *ca.* 2 cm from the shoot apex was the predominant response to herbicide treatment 2-4 wk after treatment. The stem tissue of *R. squarrosus* was, therefore, not severely affected by the herbicide treatments. This may be due to protection from herbicide penetration of the stem by the surrounding leaves, or due to the high density, compaction of cells or thick cell walls of *R. squarrosus* stem tissue (Section 1.5). The different herbicide uptake properties of leaves and stems of *R. squarrosus* are pursued further in Chapter 4.

2.3.3 Study of herbicide damage to mosses in a glasshouse trial

A glasshouse trial was designed to study visual damage to mosses caused by selected herbicides under controlled environmental conditions. The herbicides tested were paraquat, 2,4-D, dichlorophen and ferrous sulphate. Comparisons were made of the effects of these herbicides on *R. squarrosus*, *Polytrichum juniperinum* and *Dicranum scoparium* to determine whether the activity of any herbicide was species specific. Competitive interactions with grasses and broad

leaved weeds were excluded by cultivating pure tufts of the selected species in plastic trays in the glasshouse.

Damage scores were made over a period of 12 weeks and are presented in Figs 2.3.9 and 2.3.10. Plates 2.3.2 - 2.3.6 show mosses of *D. scoparium* and *P. juniperinum* one month after application of the herbicides.

Dichlorophen

Dichlorophen treatments caused significantly greater visible damage (*ca.* 70%) than the other herbicides employed in all three moss species (Fig. 2.3.9). The main symptoms, following dichlorophen application, were chlorosis or browning of the tissue a few days after application (Plate. 2.3.2). Regrowth was apparent 12 wk after treatment from the damaged stem tissue of *R. squarrosus*, generally 2-3 cm beneath the shoot apex. Dichlorophen-treated *P. juniperinum* and *D. scoparium* showed slight recovery from the least severely damaged shoots and occasional new growth from the tuft bases.

2,4-D

2,4-D caused a gradual loss of moss tissue colour. Peak damage scores of 50%, 22% and 33% were obtained for *R. squarrosus*, *D. scoparium* and *P. juniperinum*, respectively, 8 wk after treatment (Fig. 2.3.9). *P. juniperinum* differed from the other two species in showing growth distortions (Plate 2.3.4) in response to 2,4-D. The young green apices of the *P. juniperinum* were observed to bend approximately at right angles to the stem. Distortion of dicotyledonous plants is a classic symptom of 2,4-D growth regulatory type activity (Fryer and Makepeace,1977). Growth of spores and protonemata of *P. commune* have previously been shown to be inhibited by the herbicide EPTC (S-ethyl dipropylthiocarbamate) which also causes growth regulatory effects in



Plate 2.3.2 Untreated Dicranum scoparium (left) and Polytrichum juniperinum (right) plants

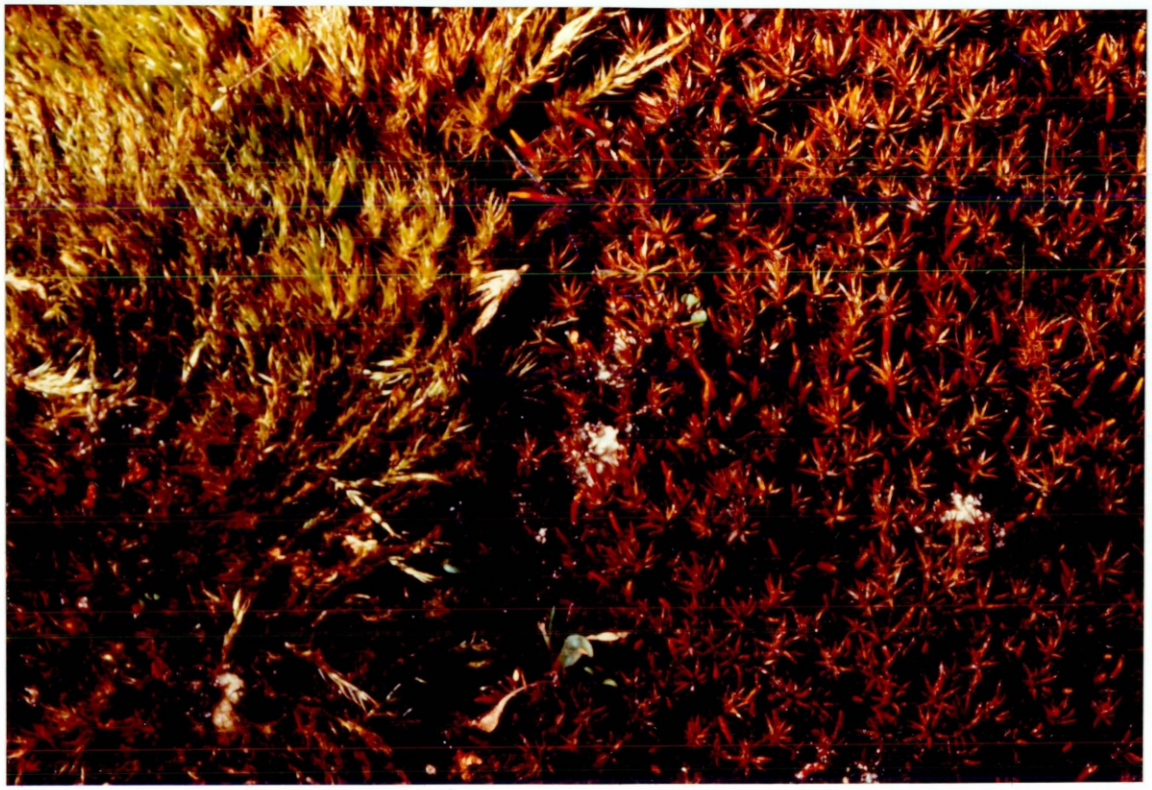


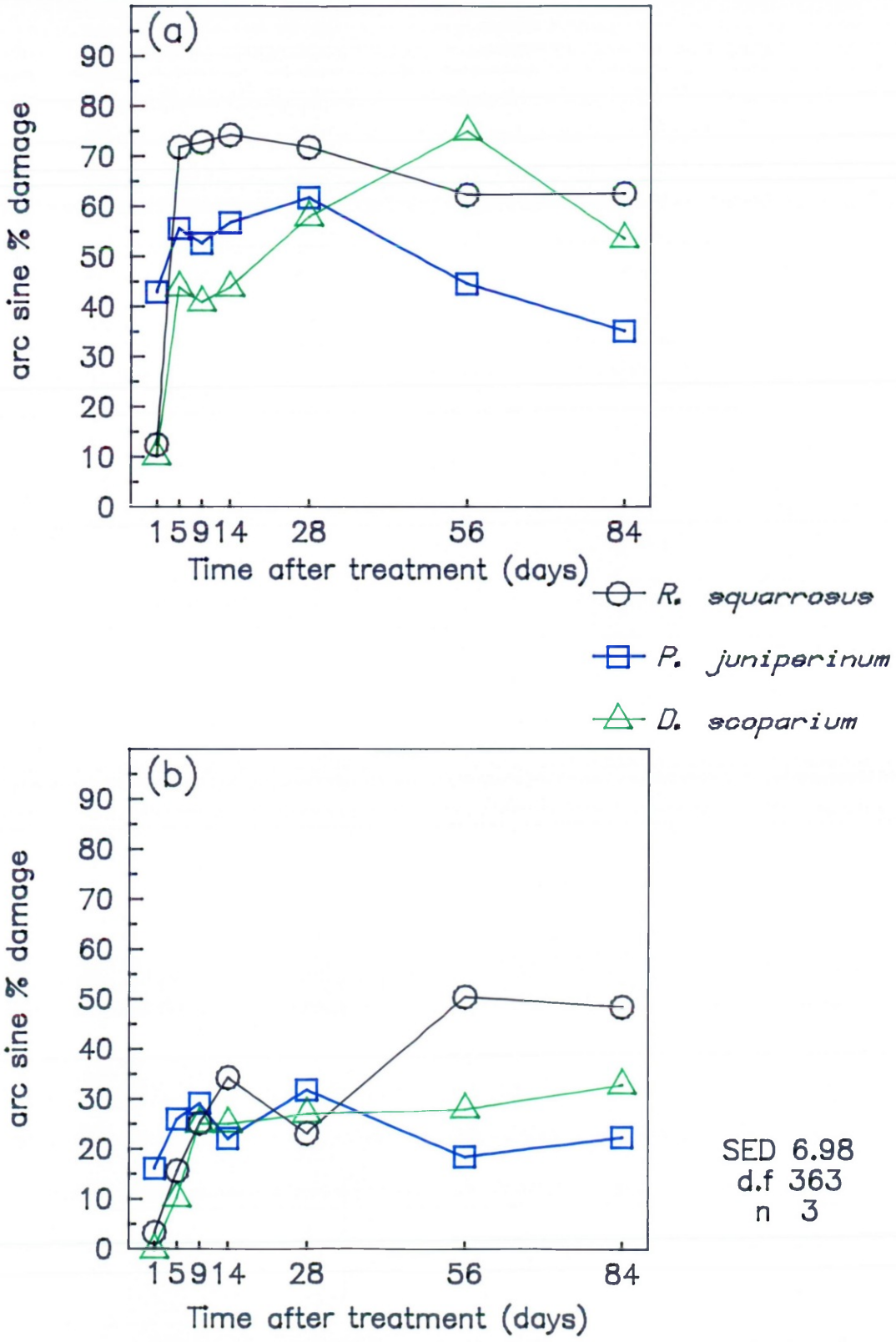
Plate 2.3.3 Effect of dichlorophen on Dicranum scoparium (left) and Polytrichum juniperinum (right) plants one month after treatment



Plate 2.3.4 Effect of 2,4-D on Dicranum scoparium (left) and Polytrichum juniperinum (right) plants one month after treatment

Fig. 2.3.9

Percentage visible damage following treatments of (a) Dichlorophen, and (b) 2,4–D to 3 species of glasshouse cultivated moss.



dicotyledonous plants (Bowers, Rule, Froiland, Richards and Wilkinson, 1982). Growth regulatory activity observed within *P. juniperinum* probably reflects a similarity in its hormonal regulation to that of dicotyledonous plants together with its well developed vascular system. No effects were noted in the ectohydric *R. squarrosus* and *D. scoparium*

Paraquat

The effects of paraquat were similar to those observed in the field experiments (Sections 2.3.1.1 and 2.3.2) with peak damage (*ca.* 47%) in *R. squarrosus* visible 12 wk after treatment (Fig. 2.3.10). Paraquat caused chlorosis in the shoot apex of *R. squarrosus*. *P. juniperinum* and *D. scoparium* showed little visible damage following paraquat application until week 12 when slight chlorosis of the tissues was noted (Plate 2.3.4). The gradual effect of paraquat in the glasshouse trial corroborates the field results (Section 2.3.1.1 and 2.3.2) and suggests that they were not solely due to changes in habitat following the death of the grass foliage.

$FeSO_4$

Rapid blackening of moss tissue following $FeSO_4$ treatments was observed for all three moss species. However, the intensity of blackening was less for *P. juniperinum* and *D. scoparium* (Plate 2.3.6) and only 20% damage(=blackening) was observed for *P. juniperinum* and *D. scoparium* compared to 56% for *R. squarrosus* following $FeSO_4$ application (Fig. 2.3.10). The difference in intensity of the blackening, following $FeSO_4$ treatment, between the three moss species may reflect differences in their cell wall compositions altering the formation of pigmented iron complexes. The waxy coat of the apical cells of the leaf lamellae of *P. juniperinum* (Proctor, 1979a) may severely restrict entry of the iron solution. Regrowth of *P. juniperinum* produced a



Plate 2.3.5 Effect of paraquat on Dicranum scoparium (left) and Polytrichum juniperinum (right) plants one month after treatment

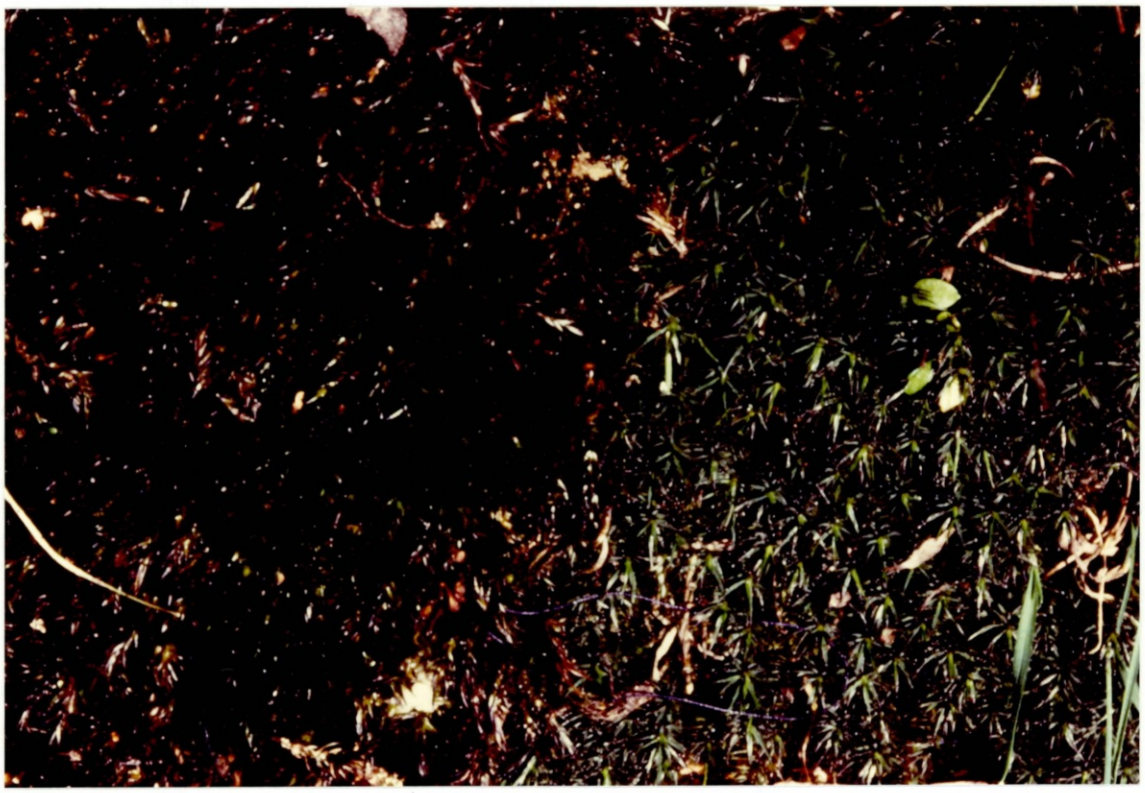
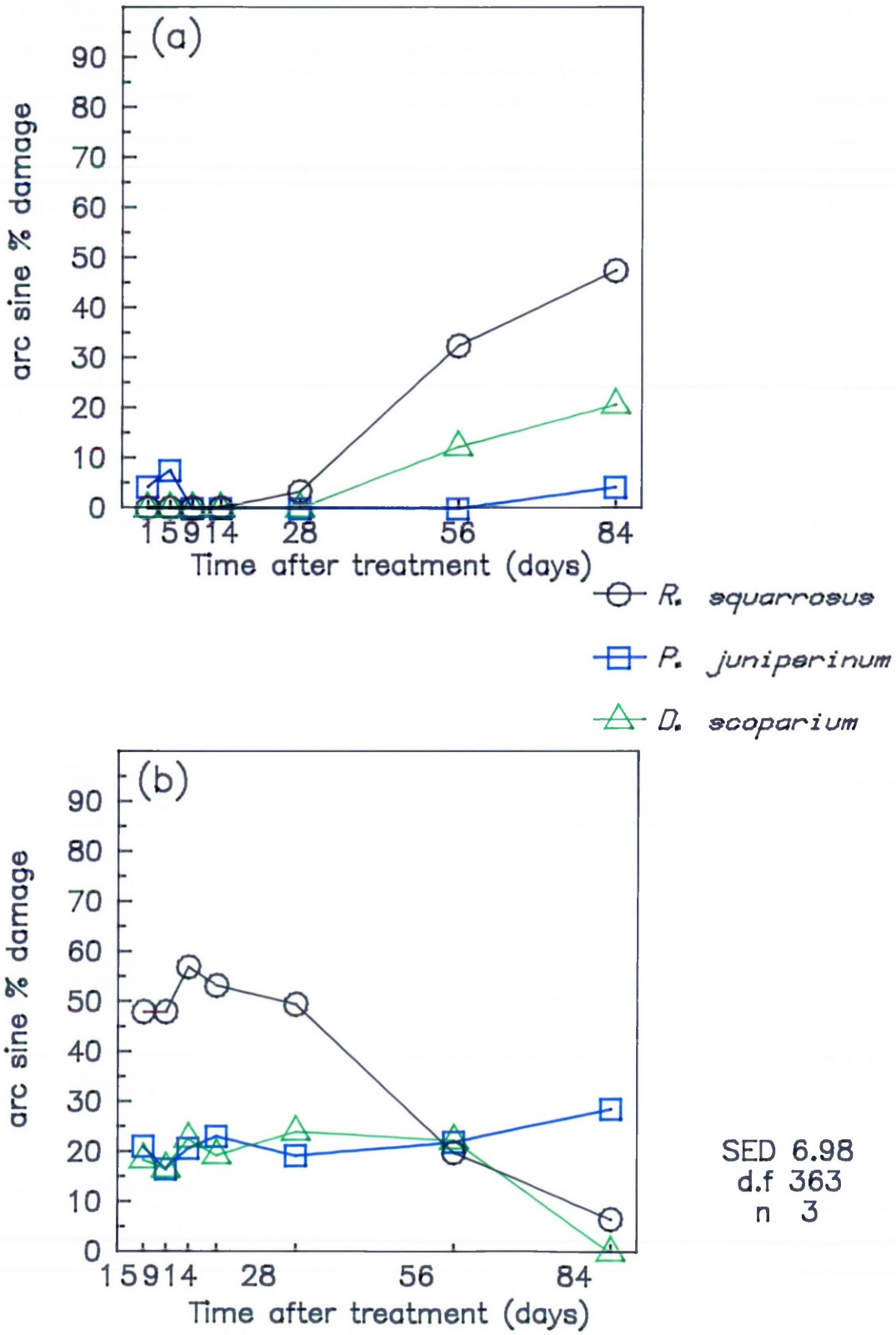


Plate 2.3.6 Effect of ferrous sulphate on Dicranum scoparium (left) and Polytrichum juniperinum (right) plants one month after treatment

Fig. 2.3.10

Percentage visible damage following treatments of (a) Paraquat, and (b) Ferrous sulphate to 3 species of glasshouse cultivated moss.



bright green star of new leaves from the apex within the outer blackened leaves. *R. squarrosus* resumed growth by producing side shoots from the stems and in some cases from the apex. *D. scoparium* showed only slight regrowth producing lighter green tissue at the leaf bases and apex.

Each of the herbicides tested had a strikingly different effect but the three mosses behaved similarly. The exception was *P. juniperinum* which showed growth distortions following the 2,4-D treatment not seen in *D. scoparium* or *R. squarrosus*. Dichlorophen was the most toxic of the four herbicides applied although regrowth eventually occurred. Regrowth, following all treatments, from the stems of *R. squarrosus* was the most rapid of the three mosses. This suggests, as in the field trials (Section 2.3.1.1), that the herbicide has not been able to penetrate and damage the stem tissue. Regrowth from *P. juniperinum* and *D. scoparium* was not easy to study because it occurred from the shoot apices and tuft bases and also by extension of the leaves.

The closely packed growth habit of the moss shoots, especially that of acrocarpous mosses, may prevent adequate herbicide penetration. The ability of mosses to grow from small fragments of tissue indicates that complete coverage is required for effective control. Thus, a possible explanation for the variable or limited effects of herbicides on mosses cited in the literature (e.g. Balcerkiewicz & Rusinska, 1987) is given by differences in the efficiency of herbicide application due to the wide range of growth forms.

2.4 CONCLUSIONS

Visible damage to *R. squarrosus* was observed following field applications of dichlorophen, chloroxuron with iron salt, ferrous sulphate, paraquat, thiram, oxadiazon, acifluorfen, oxyfluorfen, 2,4-D, and Gloquat C. However, in most cases regrowth occurred within 2-3 wk so that *R. squarrosus* cover in turf was not signficantly reduced in the long term.

Chloroxuron with iron salt, dichlorophen and thiram all caused a long term (36 wk) reduction in *R. squarrosus* cover when applied at rates greater than recommended. A low application rate (x0.33 recommended) of ammonium sulphate also reduced cover of *R. squarrosus* in turf, at Ascot, in 1984. In the 1985 field trial carried out at Eversley, only oxadiazon was successful in reducing *R. squarrosus* cover. The limited effects of dichlorophen and chloroxuron with iron salt on *R. squarrosus* cover were attributed to regrowth and possibly to a residual soil activity inhibiting vascular plant growth, respectively.

Paraquat and 2,4-D both reduced the vascular plant population in the field experiments, and in so doing tended to increase *R. squarrosus* cover.

The glasshouse trial simulated herbicide effects obtained in the field experiments. Similar herbicide effects were observed in a glasshouse trial which excluded competitive interactions with vascular plants and included *P. juniperinum* and *D. scoparium* in addition to *R. squarrosus.* Growth regulatory effects were observed in *P. juniperinum* following 2,4-D treatments but these were not seen in *D. scoparium* and *R. squarrosus.*

Several herbicides, including dichlorophen and chloroxuron with iron salt, caused visible damage to grasses but only oxadiazon significantly reduced grass cover. This excludes oxadiazon from use in moss control of lawns.

CHAPTER 3

EFFECTS OF PARAQUAT, DICHLOROPHEN, IRON SALTS AND 2,4-D ON THE GROWTH OF *RHYTIDIADELPHUS SQUARROSUS*

3.1 INTRODUCTION

The initial trials (Ch. 2) demonstrate the range of phytotoxic effects of proprietory moss killers and other herbicides on *R. squarrosus*, predominantly under field conditions. However, field experiments incorporate many variables which may influence the net biological effect and its interpretation. These include 1) the method of herbicide application which will determine contact, retention and the total dose applied to the moss, 2) the turf density which will influence penetration of the spray into the moss canopy and cause secondary shading and competition effects, and 3) environmental factors such as temperature, water regime and light quality and quantity which may influence the rate of uptake and action of the herbicide. The above factors may all interact to prevent adequate interpretation of the biological response of *R. squarrosus* to herbicides applied in field trials.

Experiments were therefore undertaken to obtain a better understanding of the effects of herbicides on the growth of *R. squarrosus*. The compounds selected for this work were dichlorophen, paraquat, 2,4-D and $FeSO_4$, based on their varied performances in the field and glasshouse experiments (Ch. 2) and their availability as radio-labelled herbicides. In addition, the iron salt $FeCl_3$ was included to determine whether the effect of $FeSO_4$ on *R. squarrosus* is specific or a general feature of iron salt solutions. In contrast to the previous experiments (Ch. 2), treatments were made by complete immersion of moss tissue in the herbicide solutions, thereby ensuring thorough wetting. Environmental conditions after treatment of the tissue were then standardised in a controlled environment chamber.

The study of moss growth has often been used as a method for elucidating the effects of environmental factors (e.g. Busby, Bliss and Hamilton, 1978; Oliver, 1971; Furness and Grime, 1982a, b). The

measurement of moss growth as a technique for assessing biological efficacy of herbicides has been used by Stjernquist (1981) using coniferous forest mosses *in situ* and Brown, Ougham and Beckett (1986) in laboratory experiments. Brown *et al.* (1986) used gemmae of *Marchantia polymorpha* and segments of the moss *R. squarrosus* to assess growth response to a range of dichlorophen concentrations. A similar system was used in the present experiments. The degree of sensitivity of *R. squarrosus* to herbicides can be gauged from its ability to survive or recover following a range of different herbicide treatments and concentrations.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Rhytidiadelphus squarrosus samples were collected from the grounds of Silwood Park, Ascot, Berks. Shoots were teased from the turf and placed in seed trays (250 x 150 mm) containing a 2 cm layer of acid-washed silver sand. Trays of *R. squarrosus* shoots were transferred to a glasshouse where the temperatures were maintained (*ca.*15°C) by thermostatically controlled fan ventilators. Plants were misted twice daily (each for 1 h) with distilled water from a Defensor humidifier. Humidity was kept high (>85%) by enclosing the area in a polythene cage. A quartz halogen lamp provided supplementary lighting, to give a 14 h photoperiod.

3.2.2 Preparation and cultivation of moss shoots

Apical segments of *R. squarrosus* (3 cm) were excised from moss plants kept in a glasshouse for two weeks prior to use (see Section 3.2.1). Moss segments were washed three times in distilled water to remove surface debris and to ensure complete hydration of the tissue. Ten segments were randomly chosen and placed on paper towels to remove

excess water. Segments were then transferred to 20 ml glass vials containing 5 ml of herbicide solution. The vials, containing the fully immersed moss segments, were incubated for either 5 min or 24 h in a gently shaking water bath (20°C, tungsten filament lamp ca. 40 µmol m^{-2} s^{-1}). Control moss segments were incubated in 5 ml of distilled water. Each treatment was replicated three times. On completion of incubation, the treatment solution was withdrawn and the moss segments washed twice for 5 min in 5 ml of distilled water. The length of the 10 moss segments in each vial were then accurately measured before transferring to plastic Petri dishes (*ca.* 9 cm diameter) lined with moistened Whatman No. 1 filter paper. The closed Petri dishes were transferred to a growth cabinet where they were regularly moistened with distilled water to prevent desiccation. Conditions within the growth cabinet were kept constant throughout the experimental period (18°C ,14 h daylength, fluorescent lighting *ca.* 275 µmol m^{-2} s^{-1}).

Growth of the moss segments was recorded by measuring their length at regular intervals during a period of *ca.* 10 wk, with rerandomisation of the dishes within the growth cabinets at each time interval.

3.2.3 Herbicide treatments

The selected (Section 3.1) compounds were those used in previous experiments (Ch. 2): paraquat, dichlorophen, 2,4-D and $FeSO_4$. A range of herbicide dilutions was tested to assess sublethal as well as the more damaging effects of high concentrations. All test chemicals were prepared as solutions in distilled water.

3.2.4 Data presentation and statistical analysis

Segment length was used as a measurement of growth in preference to biomass increment because it is non-destructive and therefore enables successive measurements of the same samples to be taken.

Biomass measurements are also disadvantageous in experiments involving herbicide treatment of moss due to a tendency for new growth to be fine and fleshy, thereby constituting only a small proportion of the dry weight of the moss shoot. Furness and Grime (1982b) noted a reduced sensitivity of dry weight measurements, due to variations between populations, compared with other methods such as elongation growth. Elongation growth of moss tissues have been measured by Brown, Ougham and Beckett (1986), Bates (1988), Busby, Bliss and Hamilton (1978), Furness and Grime (1982a, b) and Green and Clayton-Green (1981).

Samples of ten segments were replicated three times for each treatment. Elongation growth data for different herbicides, concentrations and incubation times are presented graphically as 1) cumulative growth (segment length - initial segment length) at regular time intervals after treatment, depicting the effects of the herbicides on the reduction and recovery of elongation growth over a ten week post treatment period, and 2) the overall mean growth rate *viz* : (final segment length - initial segment length)/period of growth. This provides an overall measurement of the effect of the herbicide treatment on *R. squarrosus.* The significance of treatment effects was investigated by analysis of variance.

3.3 RESULTS AND DISCUSSION

Shoots of *R. squarrosus* were incubated in herbicide solutions for 5 min or 24 h and their subsequent elongation growth over 10 wk recorded (Figs 3.3.1 - 3.3.10). Measurements after two different incubation times (5 min and 24 h) were made to provide information on the importance of herbicide contact time.

3.3.1 Effects of 2,4-D on the growth of R. squarrosus

Effects on cumulative elongation growth

A 5 min incubation in 2,4-D solutions produced no significant

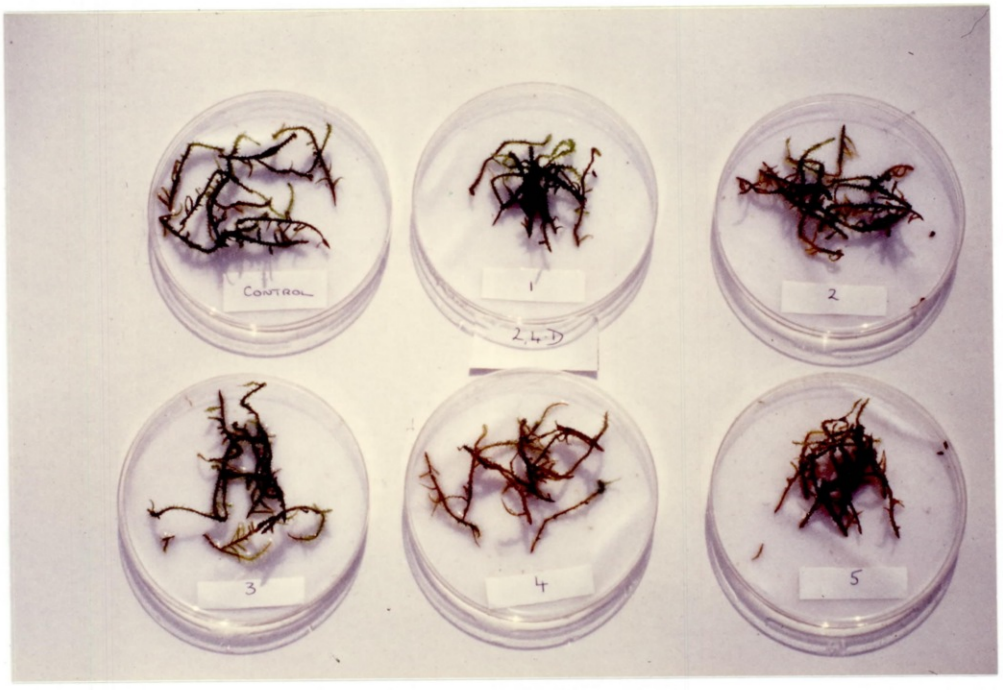


Plate 3.3.1 The effect of a 5 min incubation in 2,4-D solution on the growth of Rhytidiadelphus squarrosus 8 wk after treatment.

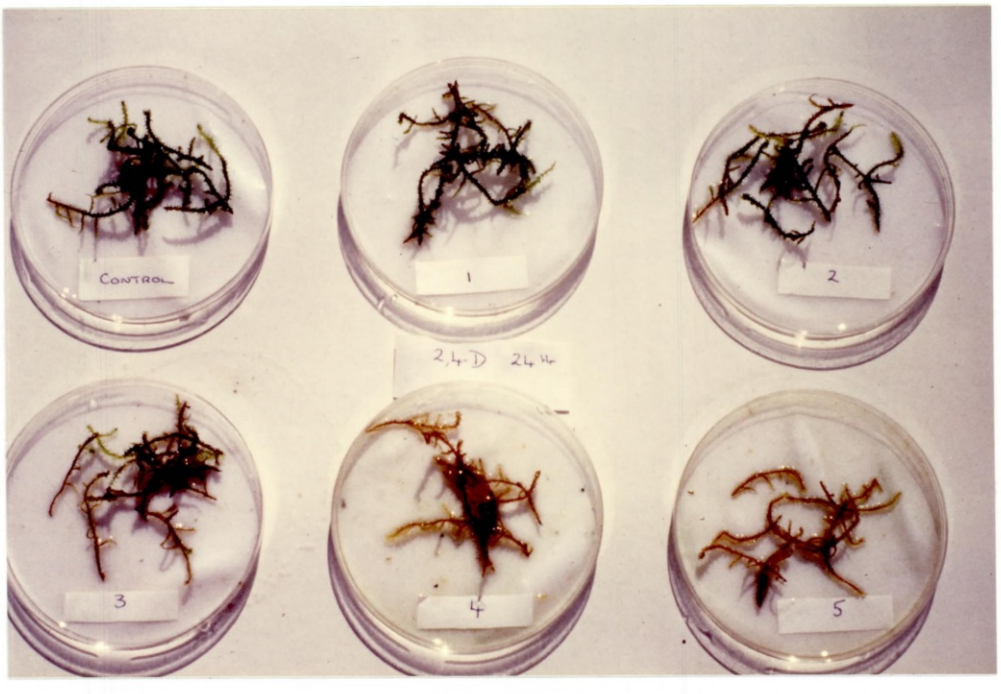


Plate 3.3.2 The effect of a 24 h incubation in 2,4-D solution on the growth of Rhytidiadelphus squarrosus 8 wk after treatment.

Treatments are 1 = untreated; 2 = 75 µM; 750 µM; 4 = 7.5 mM; 5 = 75 mM
6 = 150 mM

reduction or stimulation of the cumulative elongation growth, nor differences between treatments in the segments throughout the first 5 wk after treatment (Fig. 3.3.1) (Plate 3.3.1). However, at the final harvest, the cumulative elongation growth following 5 min treatments with the two highest 2,4-D concentrations was significantly lower than the control values.

At the lowest concentrations of 2,4-D (75 and 750 µM), growth after the 24h incubation treatment did not differ significantly from the controls. However, the two highest concentrations (75 and 150 mM) caused chlorosis of the shoots and a reduction in cumulative elongation growth due to atrophy of the damaged apical tissues (Fig. 3.3.1). Significant growth reductions were detected in these treatments within the first week after treatment (Fig. 3.3.1). Four weeks after treatment all 24 h 2,4-D treatments had reduced growth compared to the controls (Fig. 3.3.1).

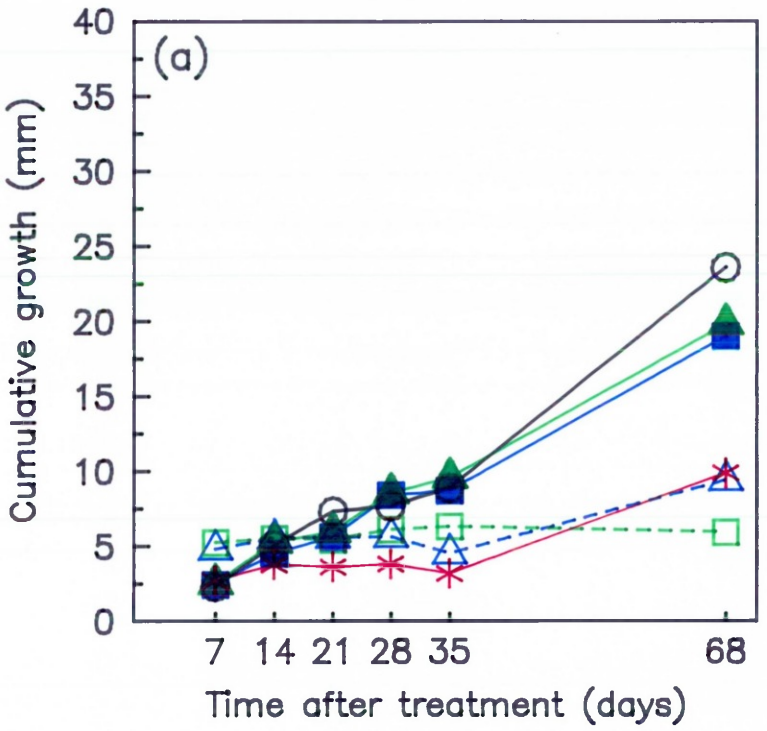
Presentation of the data as cumulative elongation growth draws attention to a greater initial and final growth phase following a 5 min incubation for the low compared with the high concentrations of 2,4-D (Fig. 3.3.1). This may be due to the slow development of inhibitory effects of 2,4-D on *R. squarrosus* followed by its recovery.

Clearly, 2,4-D significantly inhibits the elongation growth of *R. squarrosus* and the reduction is concentration-dependent. Increasing the length of time the moss was in contact with 2,4-D also reduced its growth. Figure 3.3.1 shows that the 24 h incubation is roughly equivalent to a 100 fold increase in concentration following a 5 min incubation.

Effects on the mean elongation growth rate

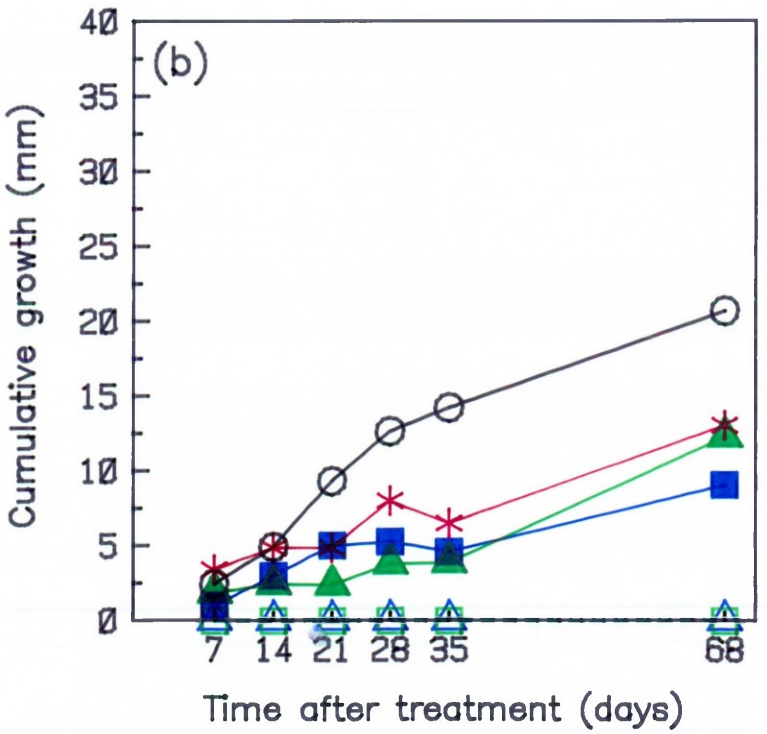
The histograms of mean elongation growth rates of *R. squarrosus* segments for the different 2,4-D concentrations show similar trends for

Fig. 3.3.1
Elongation growth of *Rhytidiadelphus squarrosus* shoots after treatment with 2,4–D, following (a) 5 min, (b) 24 h incubations.



Day	SED (a)	(b)
7	2.19	0.87
14	1.93	0.99
21	2.38	2.02
28	2.86	1.85
35	3.42	2.27
68	5.05	3.04
d.f	17	17

n = 3



both the 5 min and 24 h incubations (Fig. 3.3.2). After 24 h incubation, *R. squarrosus* showed a rapid and statistically significant decline in the growth rate irrespective of concentration. However, after a 5 min incubation, the growth rates declined significantly only at the two highest concentrations (75 and 150 mM) (Fig. 3.3.2).

The 2,4-D results indicate that moss segments require an uptake period greater than 5 min to reduce growth at the lower concentrations (Fig. 3.3.1). Thus, for 2,4-D to be useful as a herbicide against *R. squarrosus*, there is a requirement for extended contact times and at concentrations greater than those usually recommended (280-2300 g a.i ha^{-1} in a 200 l volume = *ca.* 6-50 mM) for the control of dicotyledonous weeds in pastures. 2,4-D is not used for the control of mosses in lawns but from the present results, it could act as a moss retardant when applied to turf. However, this needs to be balanced against any subsequent increase in moss growth following 2,4-D application due to a reduction in competition from broad leaved weeds (Section 2.3.2). Plates 3.3.1 and 3.3.2 show that 2,4-D caused limited visible damage to *R. squarrosus* when applied at low concentrations or for short incubation times. It is probable that the use of 2,4-D for the control of broad leaved weeds in lawns also reduces the growth of *R. squarrosus*, but that this passes undetected due to the lack of visible injury.

3.3.2 Effects of Dichlorophen on the growth of R. squarrosus

Effects on cumulative elongation growth

Following 5 min incubations in dichlorophen solutions, a significant reduction in cumulative elongation was observed 3 wk after treatment at the two highest concentrations (600 µM and 6 mM) (Fig. 3.3.3). The 24 h incubation in dichlorophen caused a more pronounced reduction in cumulative elongation growth and at all dichlorophen

Fig. 3.3.2
The mean growth rate, over ten weeks, of *Rhytidiadelphus squarrosus* shoots after treatment with 2,4–D, following (a) 5 min, and (b) 24 h incubations.

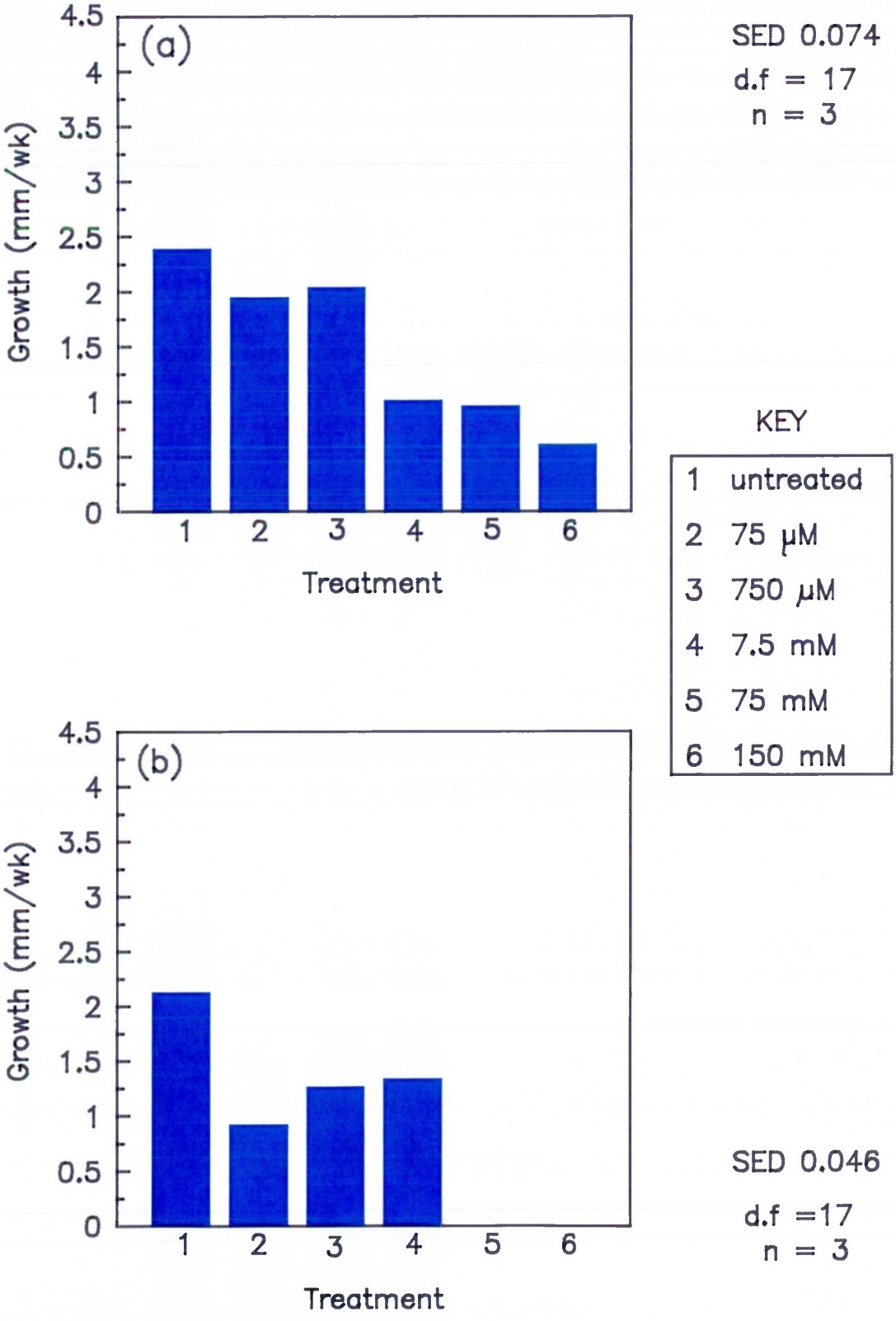
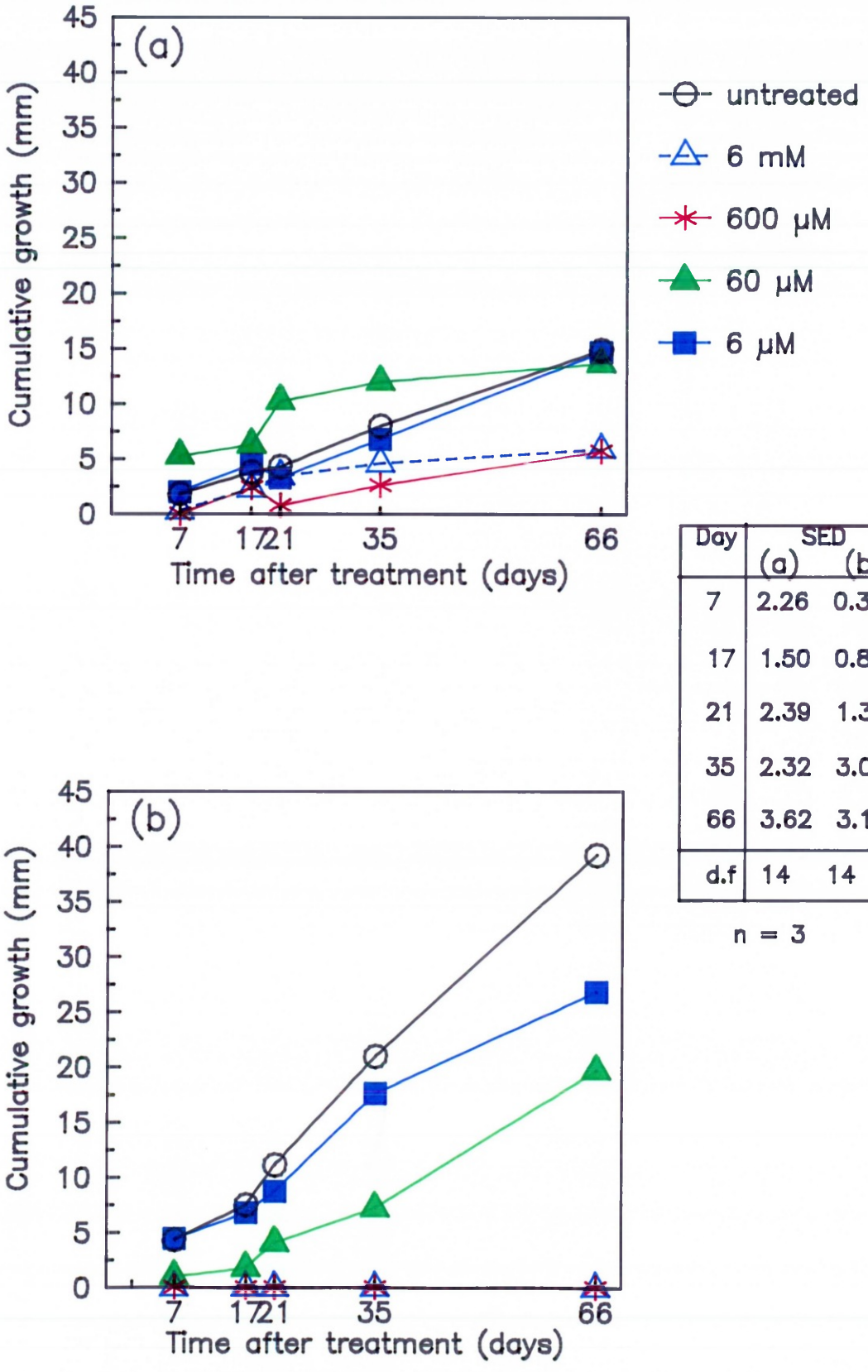


Fig. 3.3.3

Elongation growth of *Rhytidiadelphus squarrosus* shoots after treatment with dichlorophen, following (a) 5 min, and (b) 24h incubations.



Day	SED (a)	(b)
7	2.26	0.31
17	1.50	0.87
21	2.39	1.33
35	2.32	3.00
66	3.62	3.14
d.f	14	14

n = 3

concentrations (Fig. 3.3.3) compared with the control. The 24 h incubation treatments with 600 µM and 6 mM dichlorophen killed the moss (Plate 3.3.3).

Effects on the mean elongation growth rate

Mean elongation growth rates declined significantly following 5 min treatments with 600 µM and 6 mM dichlorophen (Fig. 3.3.4). No effect was noted at the two lower concentrations (6 and 60 µM) following a 5 min incubation (Fig. 3.3.4). The 24 h incubations, at all concentrations, significantly reduced the mean elongation growth rate (Fig. 3.3.4).

These results resemble those obtained by Brown, Ougham and Beckett (1986) for *R. squarrosus* segment growth over a range of dichlorophen concentrations. These authors observed reductions in dry weight, chlorophyll and potassium content following dichlorophen treatment. They attributed the effects of dichlorophen to substantial plasma membrane damage.

For both the dichlorophen and 2,4-D treatments, in the present study, death of moss segments occurred only after (or during) a 24 h incubation in the most concentrated solutions. The two highest dichlorophen concentrations (600 µM and 6 mM) used are equivalent to a hundredth and tenth dilution of the recommended rate for moss control, respectively. It would appear that on a molar basis, under laboratory conditions, dichlorophen is ten times more potent than 2,4-D.

3.3.3 Effects of Paraquat on the growth of R. squarrosus

Effects on cumulative elongation growth

The effects of paraquat on growth of *R. squarrosus* following a 5 min incubation were different from those of 2,4-D and dichlorophen. The herbicide did not kill the moss segments at low concentrations

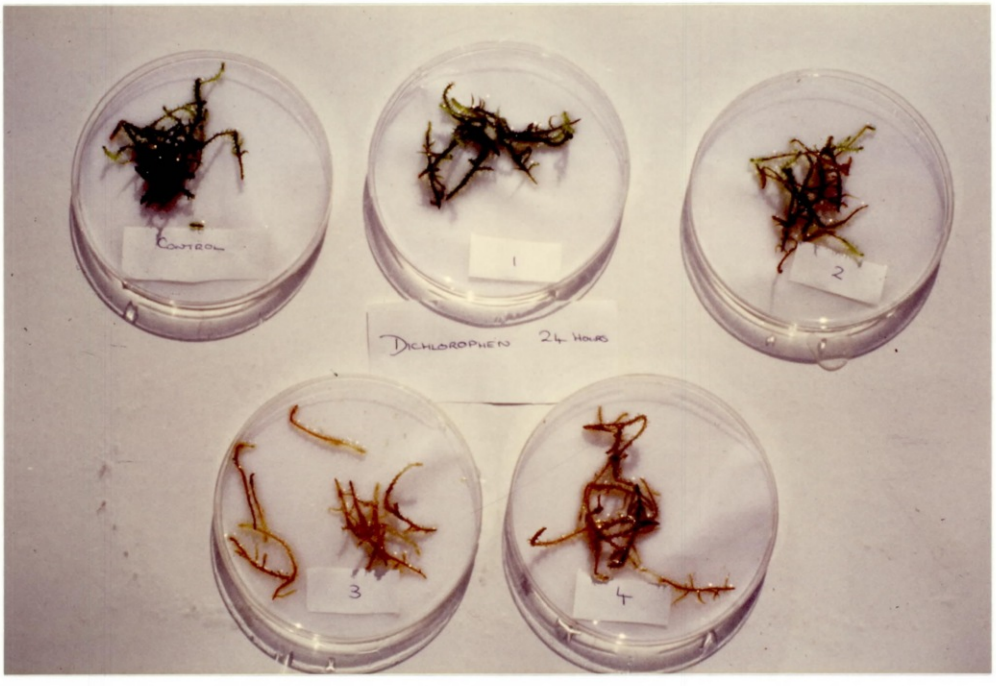
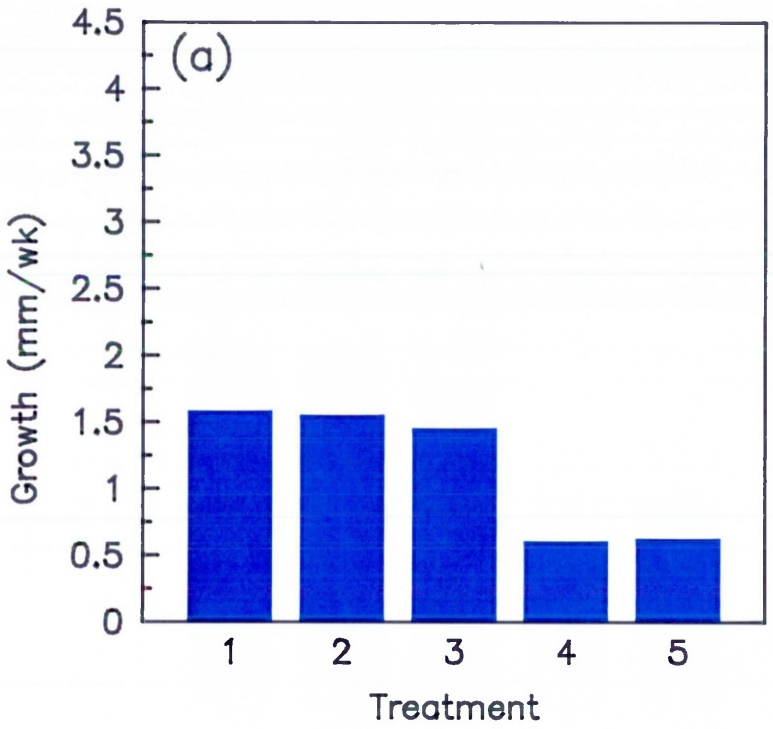


Plate 3.3.3 The effect of a 24 h incubation in dichlorophen solution on the growth of Rhytidiadelphus squarrosus 8 wk after treatment

Treatments are: 1 = untreated; 2 = 6 µM; 3 = 60 µM; 4 = 600 µM; 5 = 6 mM

Fig. 3.3.4
The mean growth rate, over ten weeks, of *Rhytidiadelphus squarrosus* shoots after treatment with dichlorophen, following (a) 5 min, and (b) 24 h incubations.

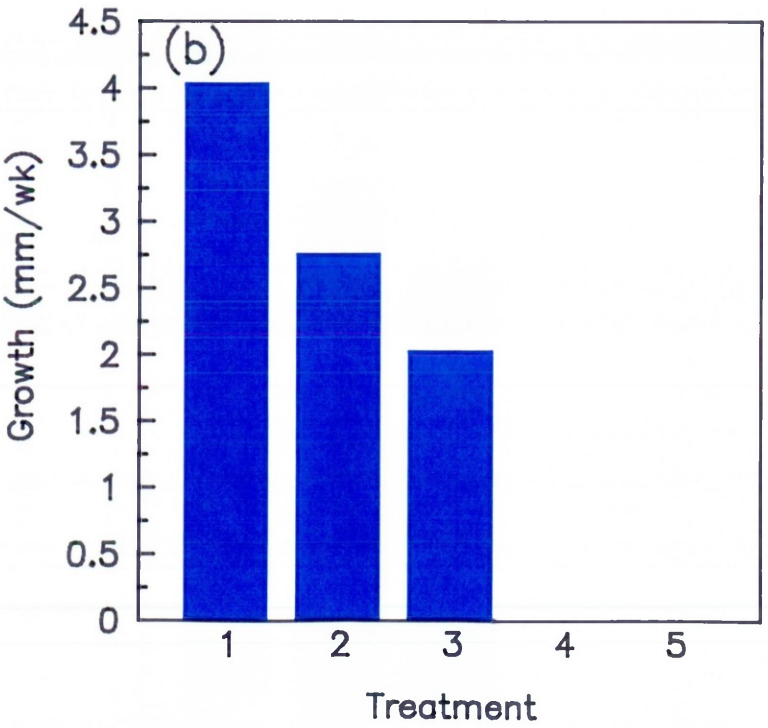


SED 0.389

d.f = 14
n = 3

KEY

1	untreated
2	6 µM
3	60 µM
4	600 µM
5	6 mM



SED 0.333

d.f = 14
n = 3

(7.25, 72.5 and 725 µM) but a reduction in the cumulative elongation growth was observed three weeks after treatment (Fig. 3.3.5). Higher concentrations of paraquat (7.25 and 72.5 mM) completely inhibited growth. Only the 7.25 and 72.5 mM treatments caused chlorosis of *R. squarrosus* shoots.

After 24 h incubation in paraquat, identical effects were noted (Fig. 3.3.5), *viz.* growth continued at low concentrations (7.25 and 72.5 µM) for the first three weeks after treatment, and then ceased. Therefore, at low concentrations, the effect of paraquat is delayed but at high concentrations it has immediate effect. This delay in biological activity, observed following the low paraquat treatments, resembles the delayed appearance of visible injury in the field experiments (Sections 2.3.1.1 and 2.3.2) and later photosynthesis experiments (Section 6.3.1).

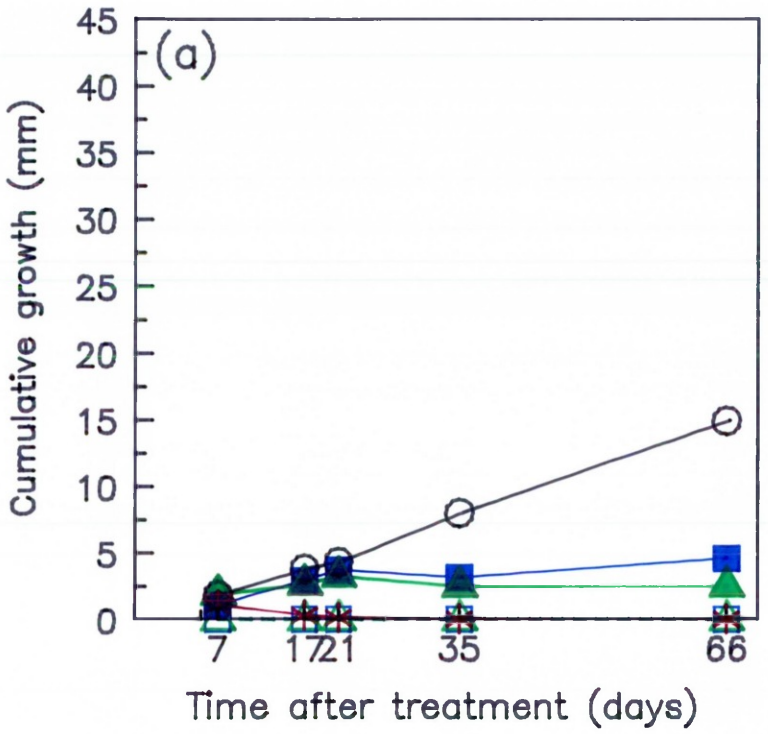
Effect on the mean elongation growth rate

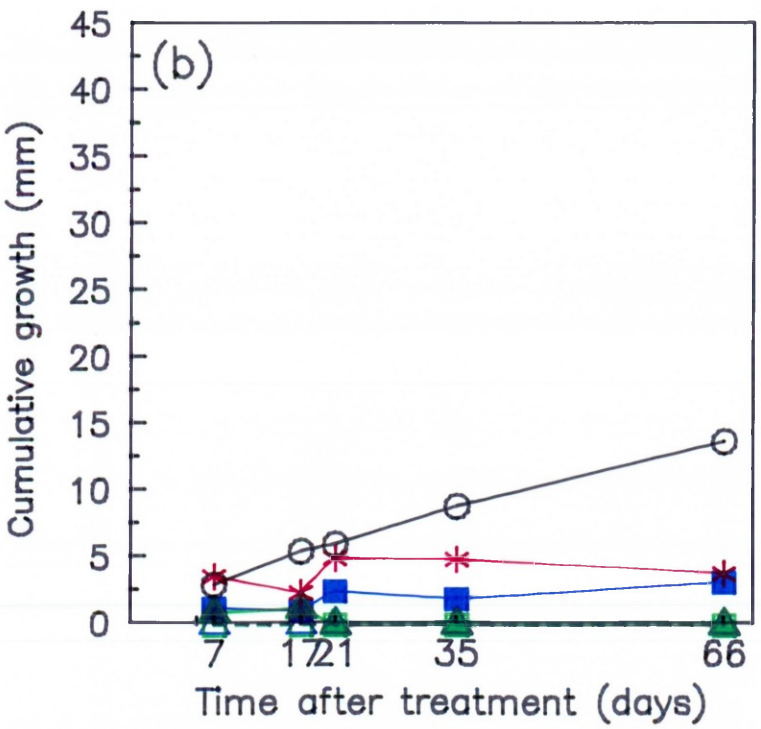
The mean elongation growth rates declined significantly with increase in paraquat concentration. No significant differences were determined between growth rates for the 5 min and 24 h incubation times (Fig. 3.3.6).

The generally recommended application rate of paraquat in field situations is 600g ai ha^{-1} (in 200 l volume = *ca.* 20 mM) which is slightly less than the maximum concentration used in the growth experiments (72.5 mM). However, it is apparent from the present study that *R. squarrosus* is sensitive to paraquat applied by immersion of the moss segments at concentrations lower than those normally applied in the field. Paraquat killed the moss segments at high concentrations, whilst the lower concentrations inhibited growth after 3 wk without producing visible injury.

The effectiveness of short incubation periods indicate that paraquat uptake is rapid. However, there are indications that freely

Fig. 3.3.5
Elongation growth of *Rhytidiadelphus squarrosus* shoots after treatment with paraquat, following (a) 5 min, and (b) 24 h incubations.

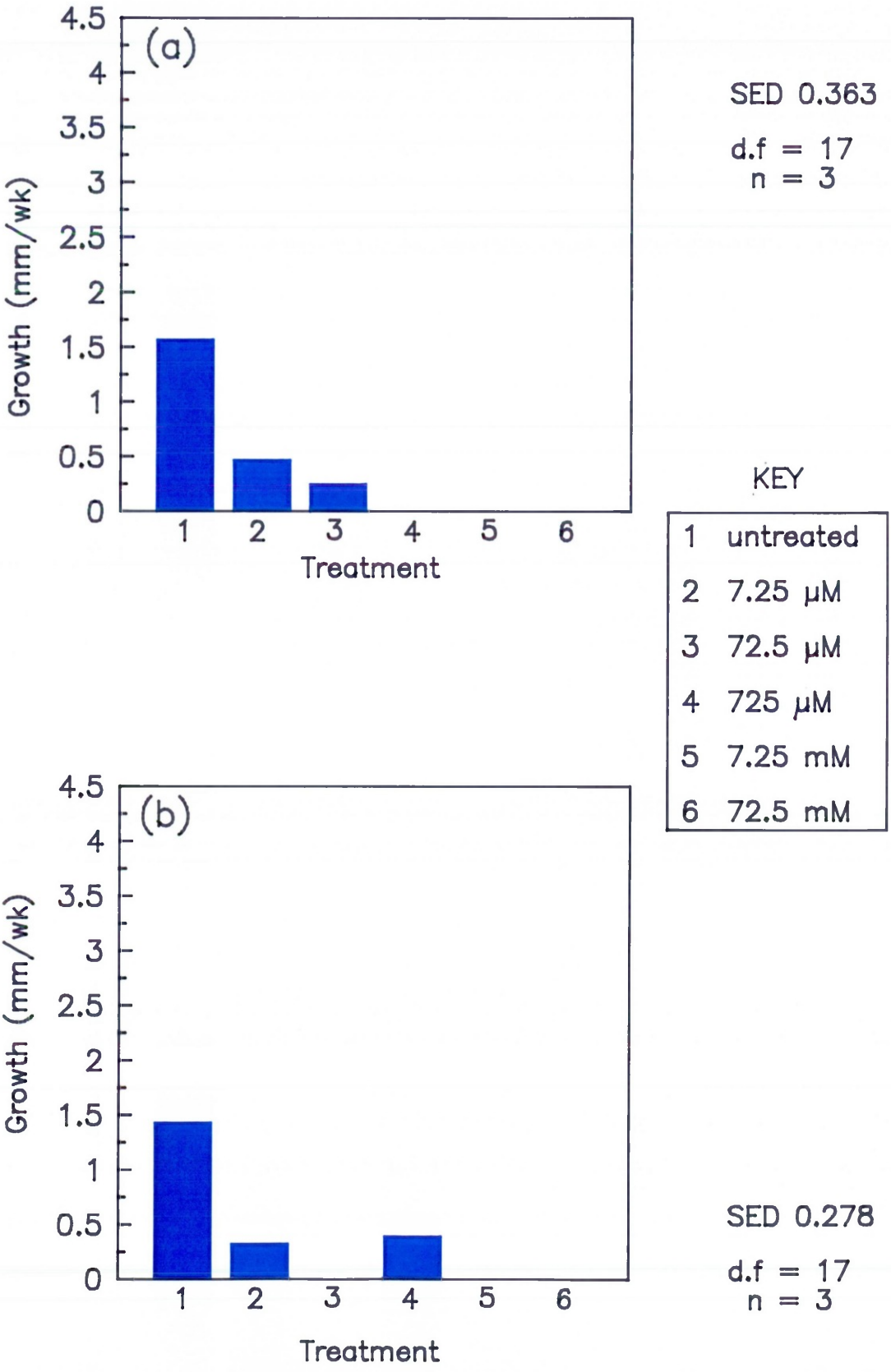




Day	SED (a)	(b)
7	1.05	1.58
17	1.51	2.20
21	1.47	1.68
35	2.71	2.03
66	3.43	2.62
d.f	17	17

n = 3

Fig. 3.3.6
The mean growth rate, over ten weeks, of *Rhytidiadelphus squarrosus* shoots after treatment with paraquat, following (a) 5 min and (b) 24 h incubations



available paraquat was exhausted in the solutions after quite short incubation periods. The delayed effect of low concentrations of paraquat could be due to paraquat initially binding to the cell wall exchange sites followed by slow uptake into the protoplasts (Section 4.3.1.6).

3.3.4 Effects of iron salts ($FeSO_4$, $FeCl_3$) on the growth of R. squarrosus

Effect on cumulative elongation growth

The moss segments turned black during incubation with each iron salt. The intensity of the black colour was related to the concentration of Fe used but was not affected by incubation time (Plates 3.3.4 - 3.3.6).

The growth of *R. squarrosus* was greater following 5 min treatments with 200 µM or 1000 µM Fe, irrespective of the salt used, than in the controls (Figs. 3.3.7 and 3.3.8). The 2000 µM Fe treatments produced a slight but significant reduction of growth. However, 24 h incubations in the iron solutions produced a reduction in growth following the 1000 µM and 2000 µM Fe treatments (Figs. 3.3.7 and 3.3.8) whereas the lower concentrations neither stimulated nor inhibited growth compared with the controls.

Effects on mean elongation growth rate

The mean elongation growth rates of *R. squarrosus* were similar at each incubation time (5 min or 24 h) following $FeSO_4$ and $FeCl_3$ treatments (Figs. 3.3.9 and 3.3.10). The 5 min incubations in $FeCl_3$ solutions of 200 µM and 1000 µM Fe led to a significant increase in growth rate (Fig 3.3.10). However, the 20 µM and 2000 µM Fe ($FeCl_3$) treatments were without effect. The same phenomenon was observed following the 5 min incubation in the corresponding $FeSO_4$ solutions (Fig. 3.3.9) but here the stimulation at low concentrations was

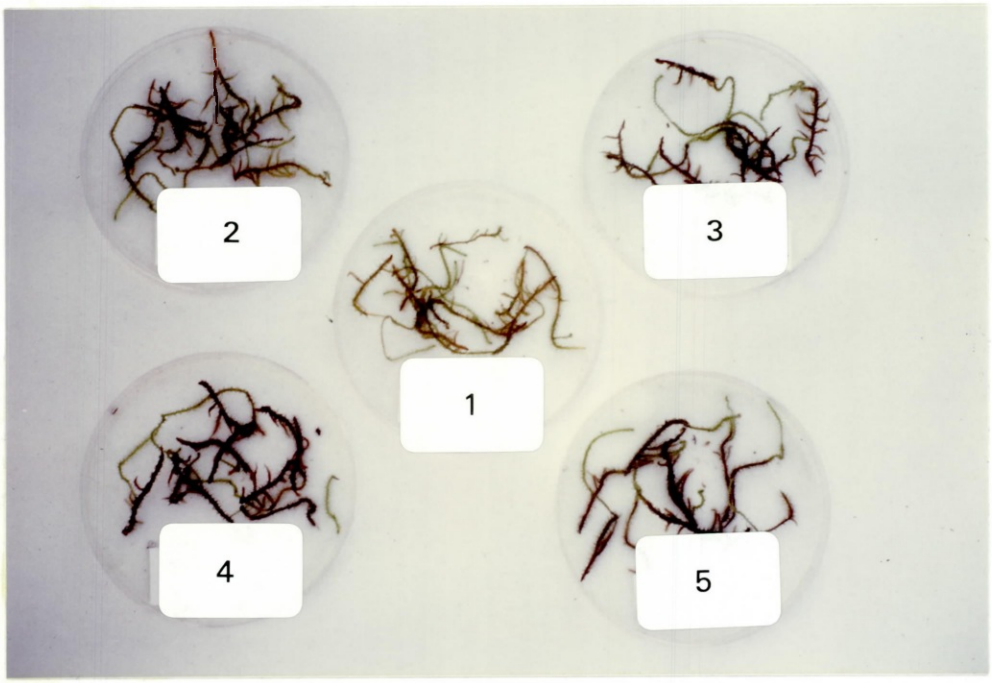


Plate 3.3.4 The effect of a 5 min incubation in $FeSO_4$ solutions on the growth of Rhytidiadelphus squarrosus 8 wk after treatment
Treatments as Fe are :
1 = untreated; 2 = 20 µM; 3 = 200 µM; 4 = 1000 µM; 5 = 2000 µM



Plate 3.3.5 The effect of a 5 min incubation in $FeCl_3$ solutions on the growth of Rhytidiadelphus squarrosus 8 wk after treatment

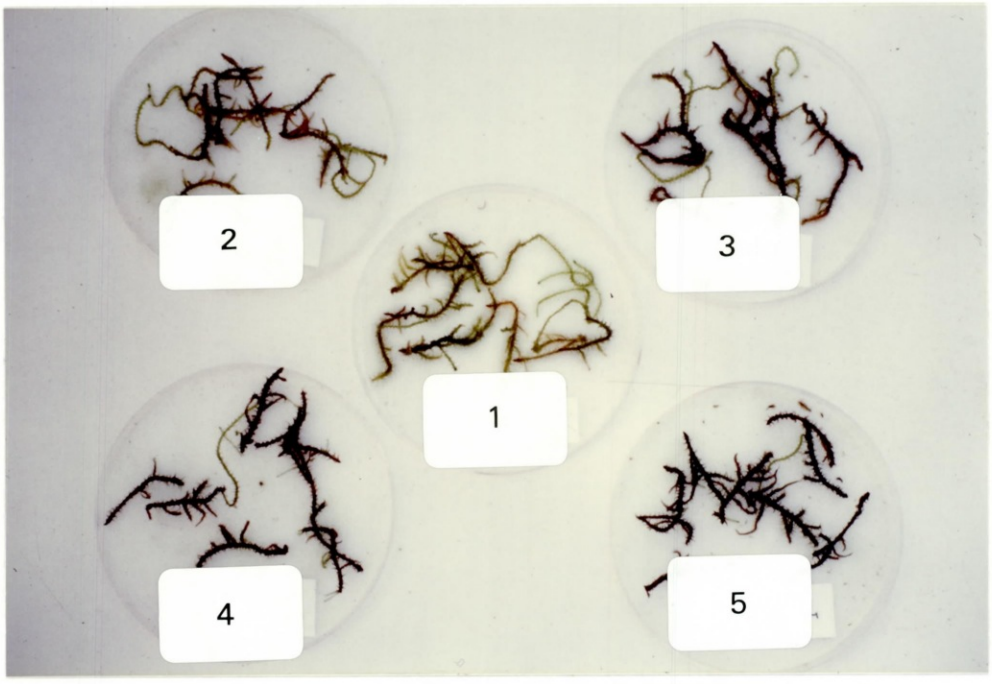
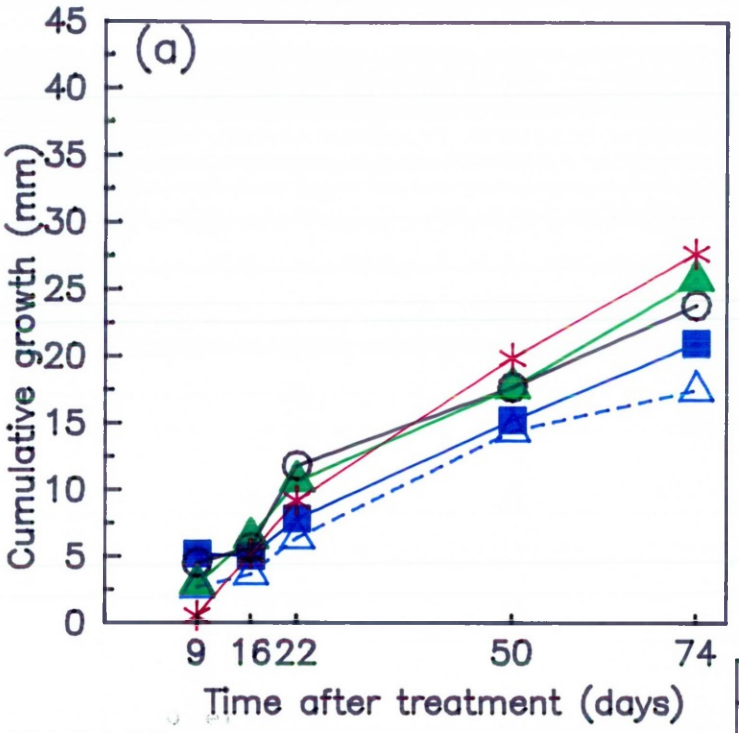


Plate 3.3.6 The effect of a 24 h incubation in $FeCl_3$ solutions on the growth of Rhytidiadelphus squarrosus 8 wk after treatment
Treatments as Fe are :
1 = untreated; 2 = 20 µM; 3 = 200 µM; 4 = 1000 µM; 5 = 2000 µM

Fig. 3.3.7
Elongation growth of *Rhytidiadelphus squarrosus* shoots after treatment with $FeSO_4$, following (a) 5 min, (b) 24 h incubations.



5 min		24 h	
day	SED	day	SED
9	2.64	11	1.29
16	1.49	17	0.87
22	2.04	24	1.35
50	2.75	45	2.60
74	3.06	69	3.62

df = 14
n = 3

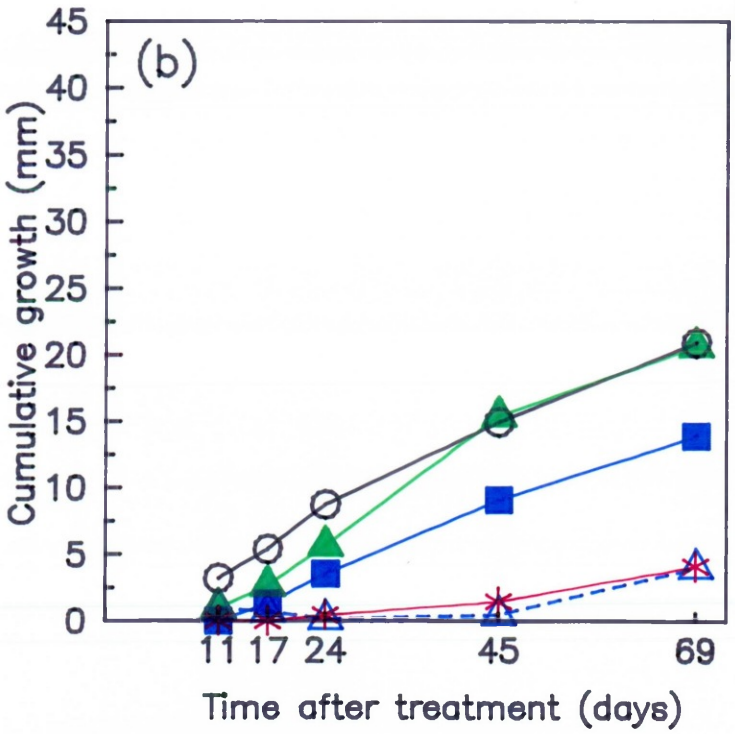
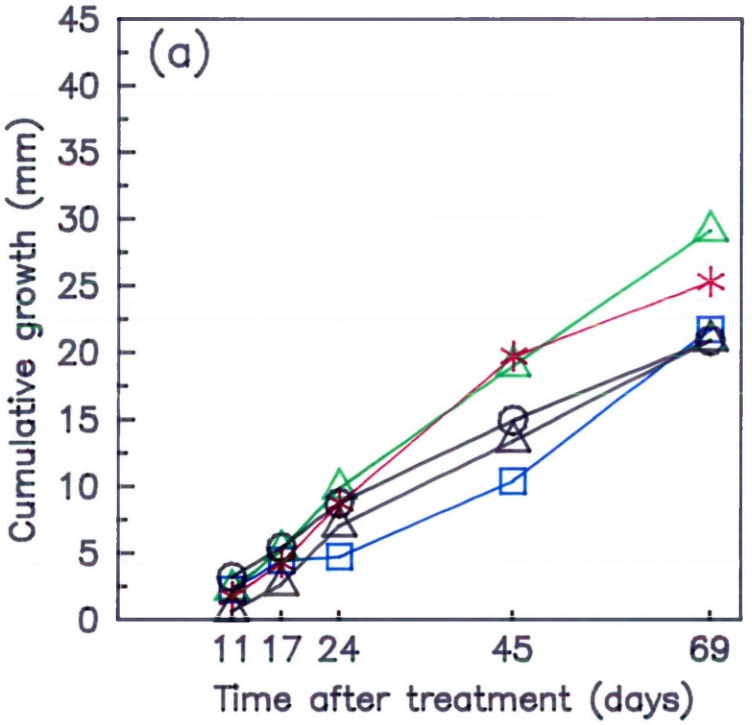


Fig. 3.3.8
Elongation growth of *Rhytidiadelphus squarrosus* shoots after treatment with $FeCl_3$, following (a) 5 min, (b) 24 h incubations.



SED

day	5 min	24 h
11	0.99	1.32
17	1.73	1.82
24	1.60	1.69
45	2.52	2.94
69	3.05	3.61

df = 14
n = 3

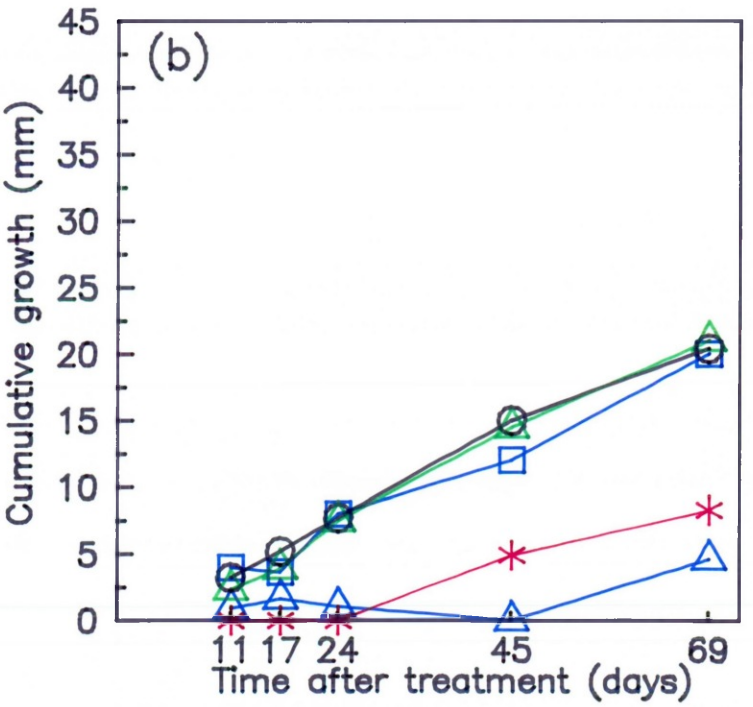


Fig.3.3.9
The mean growth rate, over ten weeks, of *Rhytidiadelphus squarrosus* shoots after treatment with $FeSO_4$, following (a) 5 min and (b) 24 h incubations.

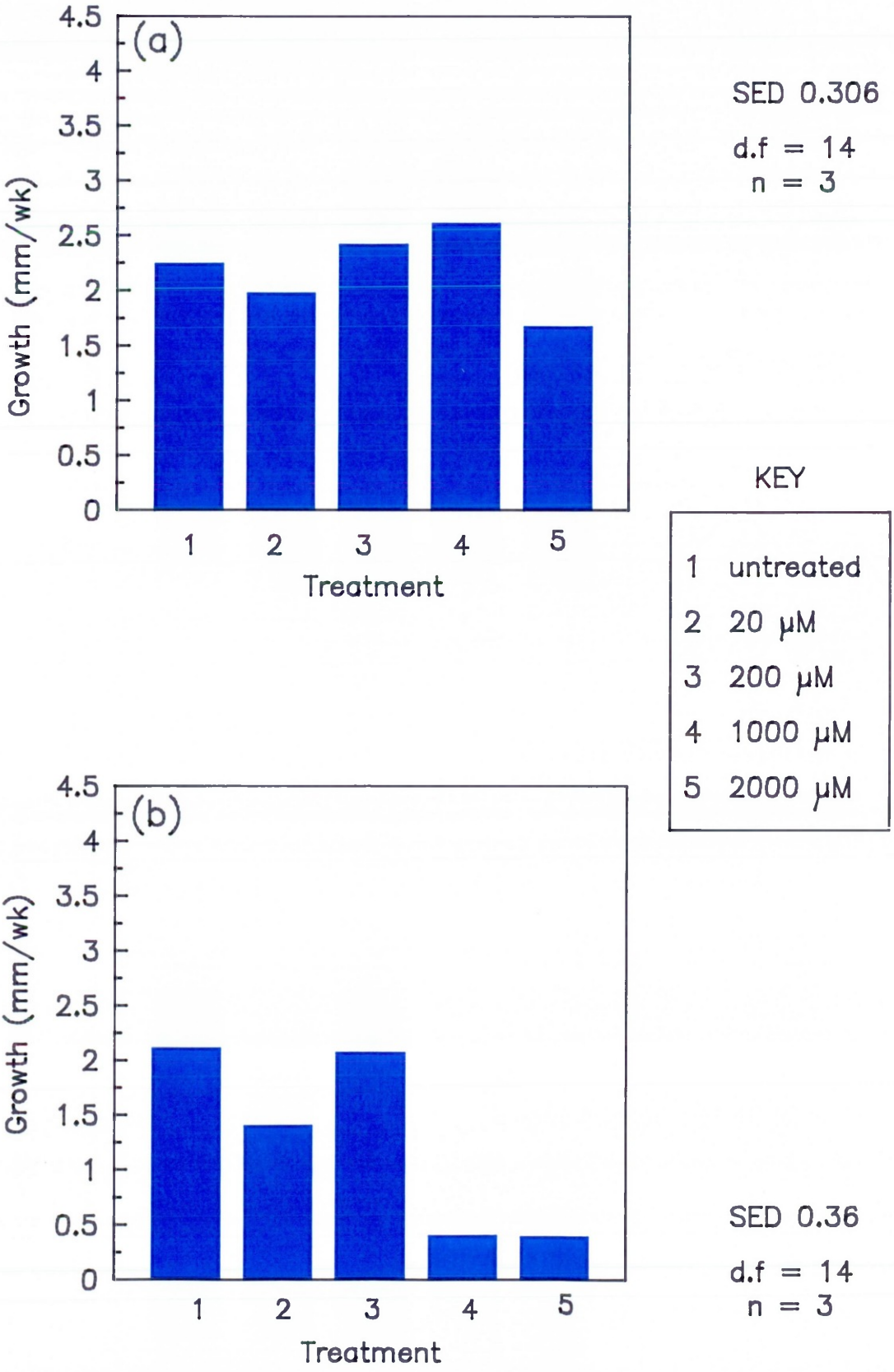
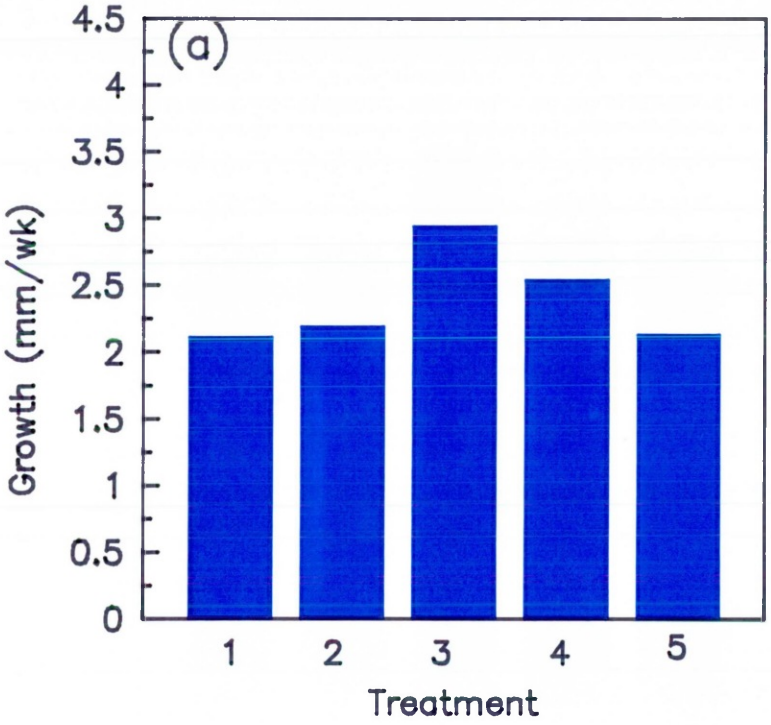


Fig. 3.3.10
The mean growth rate, over ten weeks, of *Rhytidiadelphus squarrosus* shoots after treatment with $FeCl_3$ following (a) 5 min, and (b) 24 h incubations.

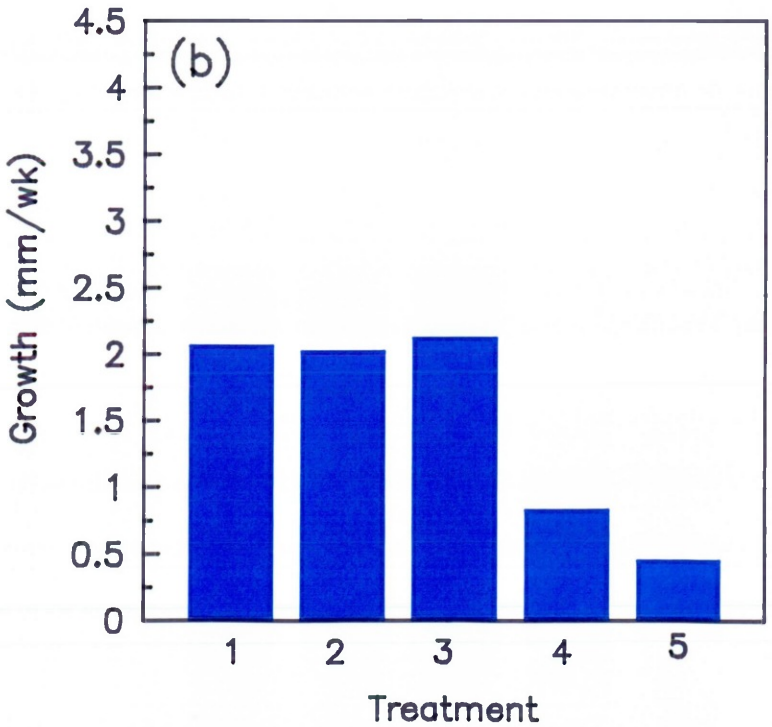


SED 0.309

d.f = 14
n = 3

KEY

1	untreated
2	20 µM
3	200 µM
4	1000 µM
5	2000 µM



SED 0.36

d.f = 14
n = 3

statistically insignificant. The growth rate for the 2000 µM Fe ($FeSO_4$) treatment was reduced significantly compared to the 200 µM and 1000 µM treatments, but not with respect to the control.

The mean growth rates following the 24 h incubations showed a significant reduction with the 1000 µM and 2000 µM concentrations of Fe from both iron salts (Figs. 3.3.9 and 3.3.10). No significant effects were found following incubation in the most dilute solutions.

There was little difference in activity between the two iron salts tested indicating that ferrous and ferric iron have equivalent herbicidal activity or that the supplied ferrous iron is quickly oxidised in the incubation medium. The results also suggest that the observed blackening of the moss tissue following iron treatment is not detrimental to growth (Plates 3.3.4 - 3.3.6). In fact, at low concentrations, where substantial blackening was observed, growth appeared to be stimulated (Figs 3.3.7 and 3.3.8). Conceivably, this may be due to etiolation in reponse to reduced light penetration. However, the photosynthesis experiments (Ch. 6) do not indicate reduced activity due to impeded light penetration in the iron salt treated shoots. Increased growth might indicate initial Fe deficiency within the moss shoots. Iron salt solutions with concentrations of 2000 µM Fe did impede *R. squarrosus* growth, either due to direct poisoning effect or shading. The limited effects of low concentrations of Fe in solution on moss growth might be due to binding in the cell walls as proposed for the cationic herbicide paraquat (Section 3.3.3).

3.4 CONCLUSIONS

The growth of *R. squarrosus*, in a controlled environment, was inhibited by 2,4-D, paraquat, dichlorophen, $FeSO_4$ and $FeCl_3$ treatments. The sensitivity of the moss to each treatment was a function of both concentration and the incubation time. Treatments with 2,4-D and dichlorophen caused reduction of moss growth at the highest concentrations (75 mM and 150 mM; 600 µM and 6 mM respectively), following a 5 min incubation. Following 24 h incubations, all concentrations of these herbicides inhibited moss growth. The effects of 2,4-D on moss growth were surprisingly pronounced considering that it is not commercially recommended as a moss killer. In the present experiments, dichlorophen appears to be 100 times more potent than 2,4-D. Moreover, a 24 h incubation period was required by both 2,4-D and dichlorophen for maximum biological effect, indicating that uptake is a relatively slow process and likely to be a limiting factor in field situations.

Paraquat differed from 2,4-D and dichlorophen in causing a reduction in moss growth which is related to concentration but which appears to be independent of the incubation time. The insensitivity to incubation time indicates that either a rapid uptake occurs (within 5 min), or, that continuing uptake into the moss protoplast is excluded due to rapid binding of the paraquat cations to the cell wall. In the latter case paraquat released from the cell wall may become available for intracellular uptake by the moss.

The effect of iron on moss growth (as $FeSO_4$ and $FeCl_3$) was apparently independent of the valency state of the iron and of its accompanying anion. Iron concentrations lower than those recommended for application to lawns (200 µM and 1000 µM) were found to inhibit growth of *R. squarrosus* following a 24 h incubation. However, in contrast to the other chemicals tested, 5 min iron treatments of 200 µM

and 1000 µM increased the rate of growth of the moss. Stimulation of growth might be due to 1) etiolation of the blackened moss shoots in reponse to reduced light penetration, or 2) the correction of iron deficiency present in the moss shoots at the onset of the experiment. The use of iron salts as a method of moss control in lawns must, therefore, be undertaken with care if the stimulation of growth is to be avoided.

CHAPTER 4

THE UPTAKE OF PARAQUAT, 2,4-D, DICHLOROPHEN AND IRON

BY RHYTIDIADELPHUS SQUARROSUS

4.1 INTRODUCTION

The present chapter describes experiments on the uptake and cellular location of the herbicides paraquat, Fe, 2,4-D, and dichlorophen in whole shoots and separated leaves and stems of *R. squarrosus*.

Paraquat is a cationic, broad spectrum vascular plant herbicide whereas 2,4-D is an anionic, dicotyledonous plant herbicide. Both caused slight or gradual growth retardation in *R. squarrosus* (Ch. 3). In contrast the anionic herbicide dichlorophen (a fungicide, algicide, bactericide and commercial moss killer) and the cation iron (moss killer) caused rapid bleaching or blackening of the moss shoots, respectively. Binding of the cationic chemicals to the cell walls of *R. squarrosus* might prevent intracellular uptake and therefore reduce biological activity. Uptake of the four herbicides by *R. squarrosus* was therefore studied to determine whether activity was linked to efficiency of penetration.

The following experiments were based on the differential displacement procedure of Brown and Buck (1979). Moss tissues were incubated in the herbicide treatment solutions and then the absorbed chemicals were extracted to quantitatively determine cellular location. Using this procedure the redistribution of paraquat between cellular compartments of the moss over long incubation times was studied. Also, the importance of the valency state and accompanying anion of iron ($FeCl_3$, $Fe(NO_3)_3$ and EDTA (Fe^{3+})) was investigated.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material

R. squarrosus was collected and grown as described in Section 3.2.1.

4.2.2 Herbicides used

Methyl-^{14}C-paraquat dichloride (Amersham) in an aqueous solution containing 2% ethanol, and 2,4-dichlorophenoxy[2-^{14}C]acetic acid (Amersham) in a toluene solution, were stored at 4°C and -20°C, respectively, prior to use.

Stock solutions of ^{14}C-paraquat were prepared by transferring 20 µl aliquots of ^{14}C-herbicide to 100 ml polythene storage bottles and allowing to evaporate to dryness. The radioactive herbicide was then redissolved in 100 ml of unlabelled paraquat solution of known concentration.

Incubation solutions were prepared by diluting the stock solutions with an equivalent concentration of unlabelled herbicide to obtain an activity of between 10000-15000 disintegrations per minute (dpm) per 5 ml aliquot.

The unlabelled herbicides used were those described in Section 2.2.5 and dilutions were made using distilled water.

The pH values of a range of unbuffered paraquat, 2,4-D, dichlorophen and Fe salts solutions are presented in Table 4.2.1.

4.2.3 Preparation of plant material

2.25 cm apical segments of *R. squarrosus* were cut and immediately placed in a 500 ml beaker containing distilled water for approximately 20 min to prevent desiccation. Shoots were then shaken with approximately 5 changes of distilled water to remove debris and freely soluble ions associated with the moss surface. The segments were then placed on paper towelling to remove excess water. The apical region (*ca.* 0.25 cm) was removed and the remaining 2 cm segments transferred quickly to glass scintillation vials containing 5 ml of distilled water. The apical 0.25 cm of the *R. squarrosus* segment was removed to standardise the segment age, thereby reducing differences in the cation and anion content (Bates, 1979; Brown and Buck, 1978) which might

Table 4.2.1 pH of herbicide dilutions used for uptake experiments

Herbicide	Concentration (µM)	pH
Paraquat	7.25	4.1
	72.5	4.1
	725	3.5
	7250	3.5
	72500	2.6
2,4-D (Na salt)	75	5.9
	750	6.3
	7500	6.1
	75000	7.1
	150000	7.5
Dichlorophen	6	6.9
	60	8.1
	600	10.2
	6000	11.0
$FeSO_4$	20	4.6
	200	4.5
	2000	4.2
$FeCl_3$	20	3.6
	200	3.1
	2000	2.5
$Fe(NO_3)_3$	20	4.2
	200	3.3
	1000	2.9
	2000	2.6
EDTA (Fe^{3+})	20	5.3
	200	5.1
	1000	5.1
	2000	5.2

influence uptake. Each vial contained ten 2 cm segments of *R. squarrosus*.

Preparations of separated leaves and stems followed the same procedure with the exception that, prior to the removal of the apex, the leaves were separated from the stems and placed in separate vials. To remove leaves from the stems, two pairs of fine pointed forceps were used, the first pair to maintain a firm hold on the apical area to be disposed of and the second to gently push off the leaves. The leaves were severed at their bases by the pointed tips of the forceps. No major visible injury to the stems or leaves was observed when viewed under the microscope. The stripped stems appeared intact with the occasional leaf remaining. The remaining leaves were picked off with forceps and discarded. Before transferring the stems to the vials, the apices were excised as described above. Leaves and stems were left for 30 min in 5 ml of distilled water to remove solutes from the fractured tissue edges. Each vial contained the leaves or stems of 10 moss segments. Tests were made for cellular damage following tissue preparation by analysing the potassium content in different cellular compartments (Section 8.3.1). No major damage to the leaves and stems was found on the basis of this method.

4.2.4 Incubation conditions

Herbicide applications were made by completely immersing 10 segments or, independently, the leaves or stems of 10 segments, in 5 ml of the treatment solution. Samples were incubated in a gently shaking water bath at 20°C receiving diffuse lighting (40 µmol m^{-2} h^{-1}) from a tungsten bulb.

The solutions were extracted from each vial using a vacuum extraction method. This technique provided rapid simultaneous extraction of incubation solutions from all replicates and treatments,

a factor critical for short incubation times. The extraction equipment consisted of the incubation vial (20 ml) and an accompanying collection vial (20 ml). Both vials were firmly stoppered and interconnected by narrow bore plastic tubing. The tubing reached the base of the incubation vial but only protruded a short way into the collection vial. Attaching a vacuum to a port in the collecting vial stopper drew the treatment solution through from the incubation vial. Multiple pairs of vials were used by connecting a vacuum pump to a glass junction with multiple ports. A vent in the stopper of the incubation vial was used to inject liquid and balance the pressure. To prevent the moss tissue leaving the vial with the liquid, glass beads were initially used to separate the opening of the extraction tube from the tissue (Plate 4.2.1). However, the glass beads also adsorbed cations from the incubation solutions and were superseded by maintaining the extraction tube orifice flush with the vial bottom.

4.2.5 Herbicide extraction technique.

Uptake results described in this chapter are based on the quantity of herbicide detected in the moss tissue and values are expressed as the quantity of herbicide per gram of oven dry (80°C) tissue.

Where applicable and where analytical methods were sensitive enough to detect low concentrations of herbicide, the absorbed herbicide has been resolved into separate components. These are: the quantity bound to the cell wall of the moss tissue (EXCHANGEABLE FRACTION), that soluble within the moss cells (INTRACELLULAR FRACTION), and that which is neither exchangeable from the cell walls nor free in solution within the cell (RESIDUAL FRACTION).

The analytical methods used differed for each herbicide (Section 4.2.6), which in turn influenced the extraction procedure required. The extraction method employed for each cellular compartmental analysis

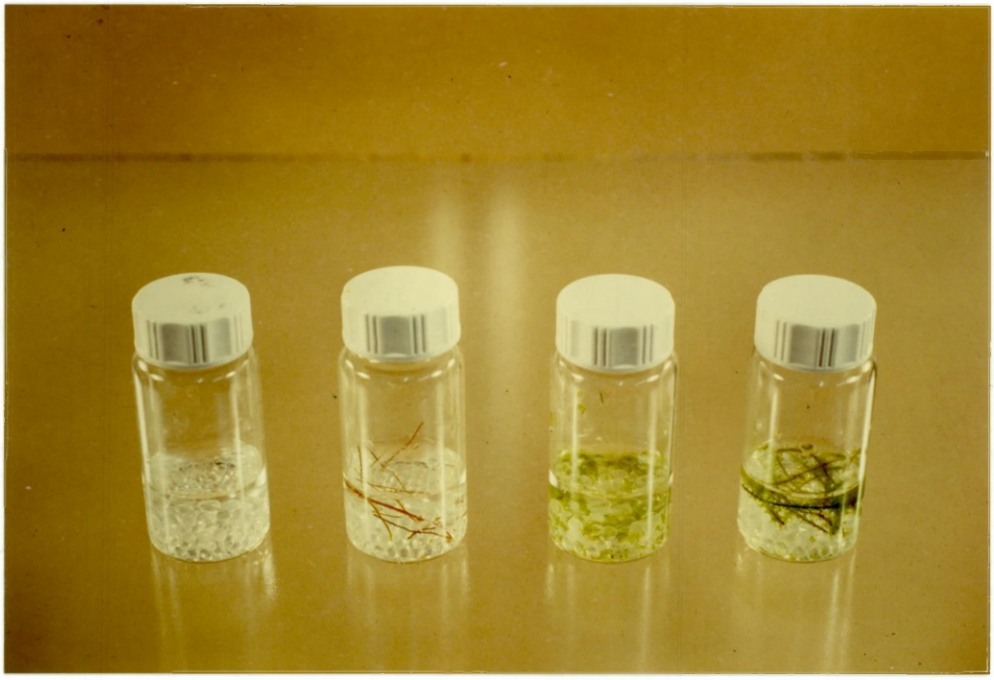


Plate 4.2.1 Typical tissue preparation of Rhytidiadelphus squarrosus for uptake experiments

(From left to right) blank preparation, stems, leaves and whole shoots

was based on the technique of Brown and Buck (1979). All herbicide application and extraction stages employed 10 moss segments in 5 ml of eluant (Fig. 4.2.1), gentle agitation of the vials in a water bath at 20°C and 40 μmol m^{-2} h^{-1} irradiance from a tungsten lamp.

Tissue solubilizations, performed as the final process in the extraction procedure to determine the residually bound herbicide, varied with the herbicide employed as described below.

4.2.5.1 Radioactive herbicides

For extraction of ^{14}C herbicides, the moss tissue was dissolved in 'NCS' tissue solubilizer (Amersham) and bleached with benzoyl peroxide using the procedure of Burrell and Brunt (1981). Samples, previously oven dried (80°C), were weighed and placed in glass scintillation vials and rehydrated with 0.5 ml of distilled water to which 1ml of a 50% solution of 'NCS' in toluene (0.33N) was added. The samples were capped and thoroughly shaken before placing in a 50°C incubator where they remained for 2-3 days until the tissue became translucent. If the tissue had not cleared within this time, a further 0.5 ml 'NCS' solution was added and the samples reincubated. Benzoyl peroxide (70 mg/0.5 ml toluene) was added to the 'NCS' solubilized tissue and left overnight to bleach the sample. The benzoyl peroxide solution was prepared in warmed toluene, the solution being filtered (Whatman No. 1) when cool. The bleached moss tissue was neutralised with 0.3 ml of glacial acetic acid before the addition of 10 ml of scintillation cocktail (Section 4.2.6).

4.2.5.2 Iron

Dry ashing of moss tissues followed by dissolution in acid was used to solubilize residual iron. Oven dried, weighed samples of moss were transferred to Pyrex test-tubes and heated in a muffle furnace

1. 10 x 2 cm shoots detached and washed in distilled water

↓

2. Incubated in 5 ml herbicide solution (20°C, 40 µmol m^{-2} s^{-1})

↓

3. Extrusion, by suction of the herbicide solution followed by washing (twice, 5 min) in distilled water

↓

4. Moss shoots washed three times in unlabelled herbicide (15, 15, 30 min) and washed twice (5 min) in distilled water ------→ EXCHANGEABLE FRACTION

↓

5. Extracted in boiling water for 1 h and washed twice with distilled water (5 min) ---------→ INTRACELLULAR FRACTION

↓

6. Shoots dried at 80°C and weighed

↓

7. Shoots solubilized using methods specific to the herbicide (see below) --------→ RESIDUAL

Solubilization techniques for specific herbicides
1) ^{14}C-paraquat and ^{14}C 2,4-D - 'NCS' tissue solubilizer (Section 4.2.5.1)
2) Iron - dry ashing and solubilization in 1:1 H_2SO_4:HNO_3 (Section 4.2.5.2)
3) Dichlorophen-boiling in 5M HCl

Stages 4 and 5 are absent in experiments measuring total herbicide uptake or dichlorophen uptake by R. squarrosus tissue

Fig. 4.2.1 Flow diagram showing the herbicide incubation and extraction stages employed with R. squarrosus

(Gallenkamp) for 12 h at 500°C. On cooling, the ashed tissues were dissolved in 0.3 ml of a 1:1 (v/v) mixture of conc. H_2SO_4 and HNO_3; where necessary samples were reheated to aid solution or an additional 0.2 ml of acid was added. Finally, the solutions were diluted with 5 ml of distilled water.

4.2.6 Herbicide analysis

1) Determination of ^{14}C-labelled herbicides

^{14}C-paraquat or 2,4-D in the incubation and tissue extracts were determined using an Intertechnique SL30 liquid scintillation counter. All results were corrected for quenching and background values.

The scintillation cocktail 'Scintran Cocktail T' (BDH), containing toluene, triton X-100, 2,5-diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) was used in all samples. A quench curve for 'Scintran Cocktail T' was determined using a ^{14}C-hexadecane standard. A series of vials containing a known quantity of ^{14}C-hexadecane in 10 ml of scintillation cocktail were quenched by the incorporation of small aliquots of chloroform. The counting efficiency of the samples was determined over a range of quenching values to obtain a calibration curve of % counting efficiency.

Incubation solutions and tissue extracts were transferred directly into clean scintillation vials containing 10 ml of scintillation cocktail. The ^{14}C-extraction from tissues by boiling in water or tissue solubilization was also performed in scintillation vials requiring only the addition of scintillant on completion.

All samples were counted for 10 min, or 20 min for low count samples.

2) Determination of iron

Iron in solution was determined by flame absorption

spectrophotometry using an Instrumentation Laboratory I.L. Model 151 instrument. All measurements were recorded at 248.3 nm, the principal line in the iron spectrum. A hollow cathode lamp (10 mA), a fuel lean air-acetylene flame, and a slit width of 80 µm were selected for iron analysis (Instrumentation Laboratory Inc., 1975). The spectrophotometer was calibrated, after allowing adequate time for the lamp to stabilize, by zeroing first with distilled water and then with the calibration blank of 6% conc HNO_3 in water. All experimental samples and iron solution standards were acidified (300 µl 6% HNO_3) to prevent precipitation of the iron salts. A calibration curve was determined using a standard working concentration range of 0-20mg Fe l^{-1}. Fresh standards were prepared before each analysis by diluting, with 3% HNO_3, a 'Spectrosol-Ferric chloride standard solution' (BDH) containing 20 mmol Fe in 1 mol HCl. Standards were stored in polythene, rather than glass, bottles to prevent cation adsorption to the glass surfaces.

Samples were aspirated alternately with distilled water and with a calibration standard check after every tenth sample.

3) Determination of dichlorophen

Dichlorophen analysis was based on a UV photometric procedure determined by Clements and Newburger (1954) for soap and cosmetic preparations. The detection wavelengths for dichlorophen were 260, 288 and 316 nm. The percentage of dichlorophen in the moss sample extracts was calculated as follows:

$$\frac{C}{100} \times \frac{\underline{A}\ \text{sam 288 nm} - \frac{\underline{A}\ \text{sam 260 nm} + \underline{A}\ \text{sam 316 nm}}{2}}{\underline{A}\ \text{std 288 nm} - \frac{\underline{A}\ \text{std 260 nm} + \underline{A}\ \text{std 316 nm}}{2}} \times \frac{1}{\text{sample weight}}$$

$\underline{A}$ sam = absorbance of sample

$\underline{A}$ std = absorbance of standard

C = concentration of standard in mg l^{-1}

4.2.7 Presentation and analysis of results

Results are expressed as µg or mg of herbicide g^{-1} dry weight of moss tissue.

Analysis of variance was used for statistical analysis of the experiments. Plots of the residuals versus means indicated that a logarithmic transformation was needed to stabilise the variance; all data were logarithmically transformed then re-analysed. All tables and graphs present means of the transformed data (or back transformed (antilog)), with standard errors of difference presented for the transformed means.

Critical single and double exponential curves were fitted to the uptake data using the Maximum Likelihood Program (Ross, 1987). The curves fitted were:

1. a single exponential (one rate-dependent component involved)

$$Y = A + B\ e^{-kt}$$

(The parameters A and $t_{\frac{1}{2}}$ (ln2/k) equate to the kinetic constants V_{max} and K_m under conditions of saturation kinetics)

2. a double exponential (two components with different rates)

$$Y = A + Be^{-kt} + C\ e^{-lt}$$

where

A = asymptote (maximum uptake at equilibrium)

k and l = rate constants

(ln2/k rate constant = $t_{\frac{1}{2}}$ to maximum uptake)

B and C = constants

t = time

Y = uptake at time t

The simplest curve which adequately describes the data has been presented in each case. A test was carried out to see whether the 2-component curve gave a significantly better fit than the 1-component curve. The test statistic used was similar to that used by Cousens, Firbank, Mortimer and Smith (1988):

$$F = \frac{\{RMSq(1)-RMSq(2)\}/\ (df(1)-df(2))}{RMSq(2)/df(2)}$$

RMSq = residual mean square

df = degrees of freedom

This is approximately F-distributed on ((df(1)-df(2))/df(2)).

The proportion of explained variance (R2) for each fitted curve is presented and represents the fraction of variation in Y, accounted for by the fitted model.

4.3 RESULTS AND DISCUSSION

4.3.1 The uptake of paraquat by R. squarrosus

This section describes the results of kinetic studies of paraquat uptake and distribution in the different cellular compartments of *R. squarrosus*. The effect of pretreatment with $FeCl_3$ on the exchangeable and intracellular uptake of paraquat was also studied. Prior to these studies initial experiments were undertaken to determine 1) the efficiency of ^{14}C paraquat displacement from the cell wall anionic binding sites, 2) possible inhibition of paraquat penetration into the stems of *R. squarrosus* shoots by the surrounding leaves, 3) the effects of paraquat concentration, 4) the efficiency of paraquat uptake from dilute incubation solutions, 5) the total paraquat exchange capacity. Using the information gained from the initial studies, investigations of the kinetics of paraquat uptake were undertaken (Section 4.3.1.6).

4.3.1.1 Exchange of extracellularly bound paraquat

Determination of the quantity of ^{14}C-paraquat (a divalent cation) exchanged on external anionic binding sites of *R. squarrosus* was achieved by substitution with unlabelled paraquat. Preliminary experiments were therefore necessary to determine the concentration of unlabelled paraquat required to efficiently displace externally bound ^{14}C-paraquat. Unlabelled paraquat solutions ranging in concentration from 7.25 µM to 72.5 mM were used as displacing solutions for leaves, stems and whole moss shoots treated for 24 h in solutions of 725 µM ^{14}C-paraquat.

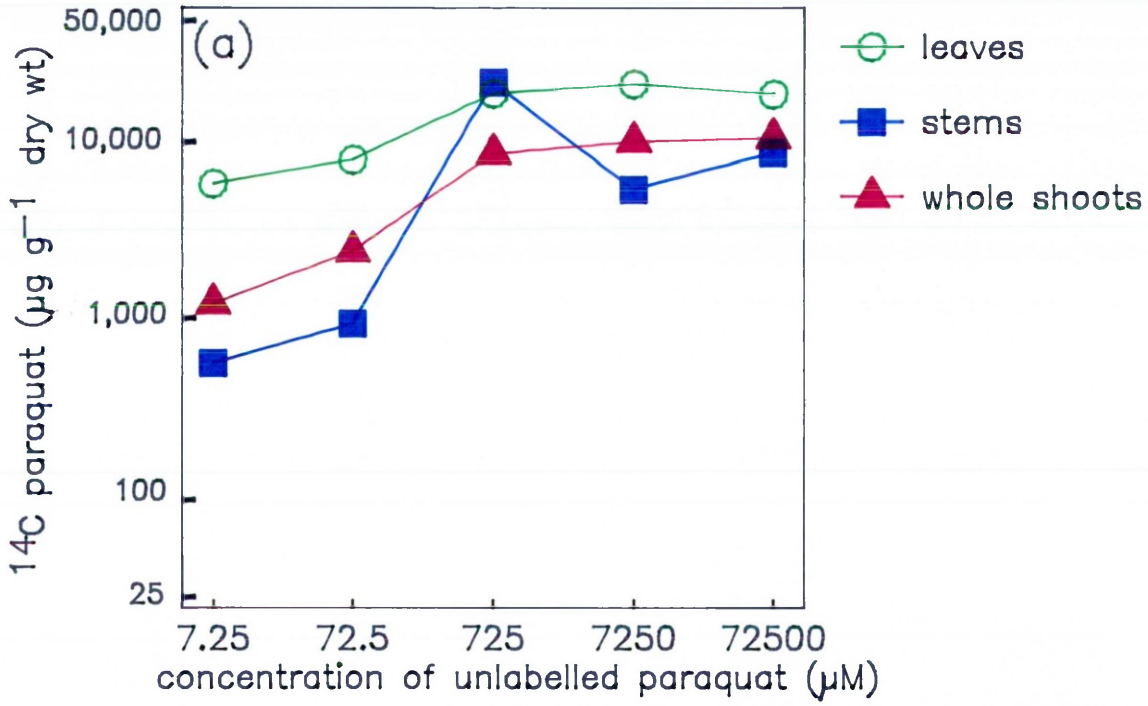
Total ^{14}C-paraquat uptake by leaves was greater (18.4 mg g^{-1} dry weight, SE ± 1.36) than stems (12.65 mg g^{-1} dry weight, SE ± 1.03) and whole moss tissue (11.70 mg g^{-1} dry weight, SE ± 0.59). This may be due to a greater number of anionic binding sites, per g dry weight, in the leaf cell walls than in the stem cell walls of *R. squarrosus*.

Significantly more ^{14}C-paraquat was displaced from leaves than from stems or whole moss shoots following all unlabelled paraquat treatments (Fig. 4.3.1). This may reflect a weaker binding of ^{14}C-paraquat to leaf cell walls than to stem cell walls. Another explanation could be that as the leaves of *R. squarrosus* are unistratose, compared with the multicellular stem tissues (Section 1.5), penetration of them by the unlabelled paraquat is more efficient.

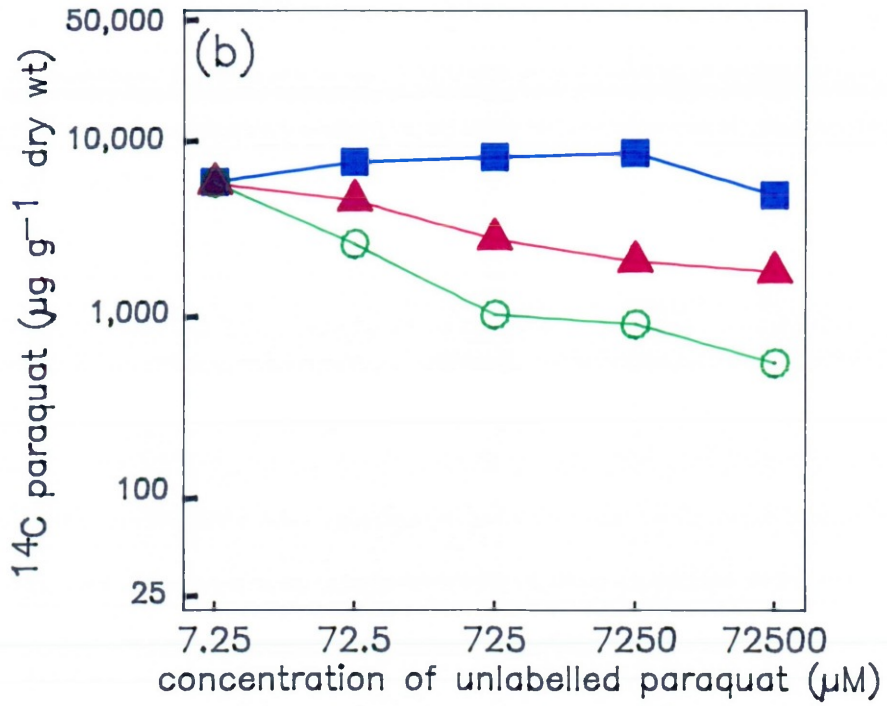
The quantity of ^{14}C-paraquat in the residual plus intracellular fractions did not differ significantly between the leaves and whole moss shoots following displacement with unlabelled paraquat. The residual ^{14}C-paraquat concentration of the stems (8.3 mg g^{-1} dry weight) was similar to that of the leaves (8.2 mg g^{-1} dry weight) and whole moss shoots (8.04 mg g^{-1} dry weight) following displacement with 7.25 µM unlabelled paraquat. However, the concentration of the residual plus intracellular fraction was higher in the stems (4.97 mg g^{-1} dry weight (35% of total uptake)) following displacement with 72.5 mM unlabelled paraquat than in the leaves (1.01 mg g^{-1} dry weight (5%)) or whole moss shoots (1.76 mg g^{-1} dry weight (10%)). These results suggest that ^{14}C-paraquat displacement from the stems was not as efficient as from the leaves and whole moss shoots. The absence of a large residual plus intracellular fraction in the whole moss shoots, compared to that of the stems, may reflect inadequate penetration of the initial ^{14}C-paraquat treatment.

Thus, inefficient displacement of paraquat from stems may be due to 1) poor penetration of the solution because of their multicellularity and lack of intercellular spaces (Duckett, 1986); 2) a greater proportion of the ^{14}C-paraquat being intracellularly located in the stems than it is in the leaves; 3) binding sites which have a greater affinity for paraquat than those found in the leaves. Price (1978) described immobilisation of substituted pyridinium salts, such

Fig. 4.3.1
^{14}C paraquat a) exchanged from, and b) residual in *R. squarrosus* after unlabelled paraquat washes following pretreatment (24h) with 725 µM ^{14}C paraquat.



ln SED 0.335
n = 2



Vertical axis is marked in a logarithmic scale

as paraquat, due to adsorption and partitioning into lipids of isolated cabbage leaf cuticles.

The minimum concentration of unlabelled paraquat for effective displacement of ^{14}C-paraquat from extracellular binding sites of *R. squarrosus* was 725 µM (Fig 4.3.1). In later work, a 7250 µM unlabelled paraquat solution was used to achieve effective displacement of extracellularly bound ^{14}C-paraquat. Further increases in the concentration of the unlabelled paraquat displacement solution could cause damage to the moss cell membranes. In addition, increasing the concentration of unlabelled paraquat in solution would also pose problems of colour quenching, thereby reducing the efficiency of liquid scintillation counting.

4.3.1.2 The influence of leaves on the uptake of paraquat by the stems

The previous results (Section 4.3.1.1) strongly suggest that the leaves impede entry of paraquat into the stems in whole moss shoots. Also, regrowth from herbicide treated segments of *R. squarrosus* (Chs 2 and 3) was from the stems which may have been protected by the leaves. An experiment to determine whether the presence of leaves impedes entry of paraquat into the stems was conducted. Total uptake of ^{14}C-paraquat (725 µM, 1 h) into separated leaves, stems and whole shoots was determined by tissue solubilization. In addition, leaves and stems were separated after incubation of the whole moss shoots in the ^{14}C-paraquat and the two components were analysed separately.

The uptake of ^{14}C-paraquat by stems separated before and after incubation was not significantly different although greater uptake was found in the post- than in the pre-separated leaves (Table 4.3.1). ^{14}C-paraquat uptake by the leaves was significantly greater than the stems when separated post-incubation. These results suggest that the

Table 4.3.1 Total ^{14}C-paraquat uptake (725 µM) by leaves and stems of Rhytidiadelphus squarrosus

Moss Tissue	^{14}C-paraquat uptake (µg g^{-1} dry wt) following a 24 h incubation period	
	tissues separated from whole shoots before incubation	tissues separated from whole shoots after incubation
Leaves	12201	19493
Stems	7198	10175

Uptake by whole moss shoots is 11409 µg g^{-1} dry wt; SED = 2663.9; n = 4

leaves might impede ^{14}C-paraquat uptake by the stems of *R. squarrosus* shoots during immersion in dilute ^{14}C-paraquat solutions.

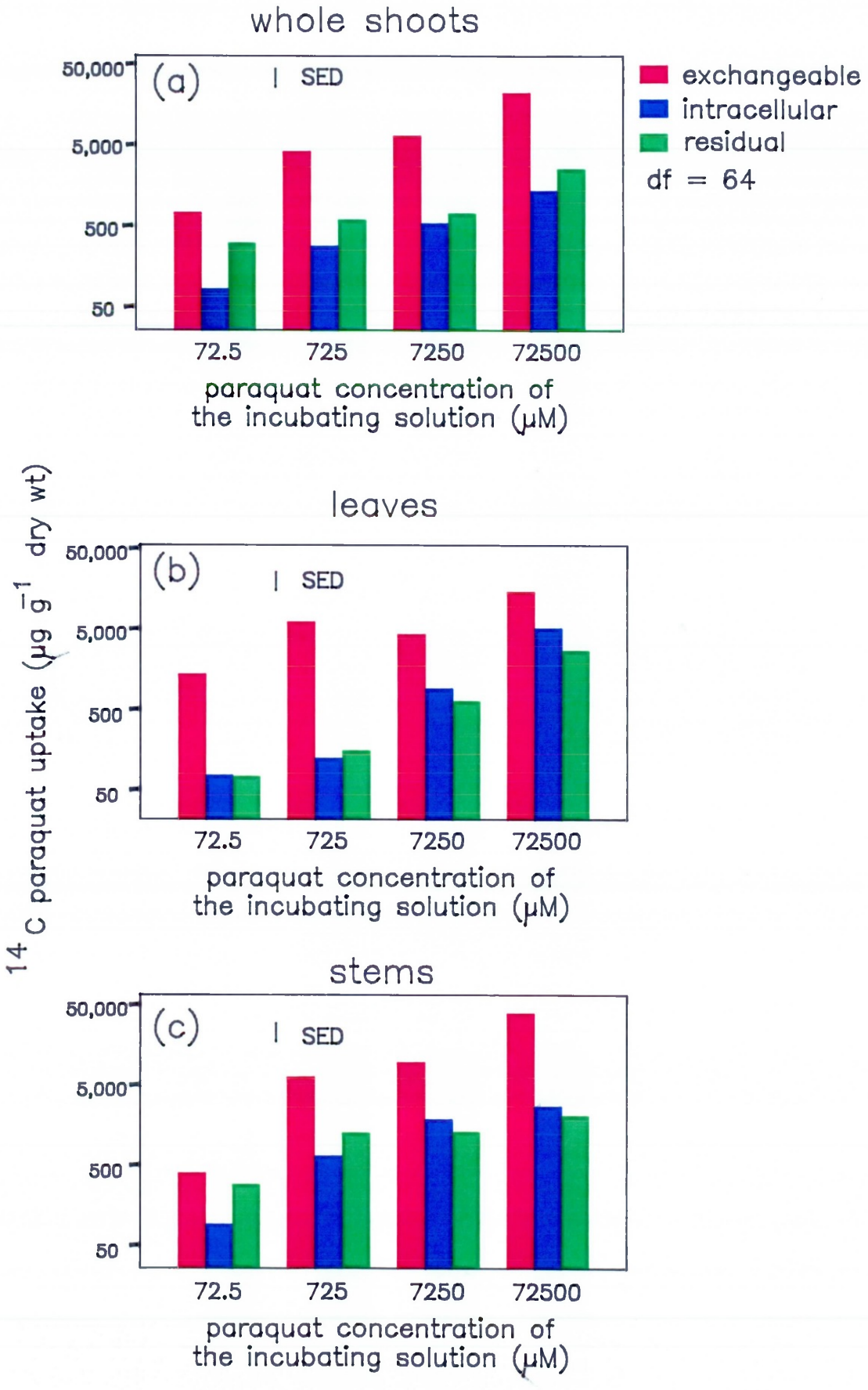
Under field conditions, using spray application, there is an even greater probability that the leaves may hinder penetration and uptake to the underlying stems than is shown in total immersion under laboratory conditions. Therefore a large volume application may be required for adequate penetration to the stems to ensure biological activity.

4.3.1.3 The effect of paraquat concentration on uptake by R. squarrosus

Increasing the ^{14}C-paraquat concentration (72.5 µM to 72.5 mM) significantly increased total uptake of the herbicide by leaves, stems and whole moss shoots over a 24 h incubation period (Fig. 4.3.2). The concentrations of paraquat in the exchangeable (externally bound), intracellular (free in solution in the protoplast) and residual fractions (released by tissue solubilization) all increased with increasing external concentration. Statistical analysis (ANOVA) revealed a significant difference between tissue types, cellular locations and incubation concentrations for the uptake of paraquat ($p<0.001$).

Fig. 4.3.2 The effect of ^{14}C-paraquat concentration on uptake (24 h) into (a) whole shoots, (b) leaves, (c) stems of Rhytidiadelphus squarrosus

Fig. 4.3.2



Vertical axis is marked in a logarithmic scale

Whole shoots and stems retained proportionately more of their total paraquat content in the exchangeable fraction than did leaves at the 72.5 mM external paraquat concentration.

Approximately 5% of the total paraquat uptake of the moss tissues entered the intracellular fraction following a 72.5 μM ^{14}C-paraquat treatment. The percentage of the total ^{14}C-paraquat uptake in the intracellular fraction of the moss tissues increased with ^{14}C-paraquat concentration of the incubating solution. This suggests that at low external paraquat concentrations the intracellular uptake, and therefore the toxic effects, of paraquat are limited due to paraquat binding to the cell wall.

Leaf and stem cells both appeared to contain more paraquat in their intracellular fractions than those of the complete moss tissue (Fig. 4.3.2). This may be a factor of both greater tissue density and dry weight of the whole moss shoots than those of the separated leaves and stems.

The residual paraquat extracted from the moss tissues increased significantly with concentration of the incubation solution ($p<0.001$) but did not differ significantly between the leaves, stems and whole moss shoots.

The proportion of total paraquat uptake found in the intracellular fractions of leaves, stems and whole moss shoots increased, with increasing external paraquat concentrations (Fig. 4.3.2). At high paraquat concentrations, the anionic binding sites of the leaves may have become saturated, enabling free paraquat to penetrate to the membranes. Saturation of the anionic binding sites of *Ipomoea* spp. leaves with paraquat increased the quantity of paraquat freely available for translocation (Price, 1978). An alternative explanation is derived from the existence of two uptake processes, system 1 and system 2, in plant cells (Clarkson, 1974). The first system maintains

uptake at low external concentrations and the second participates at high external concentrations possibly by increased permeability of the plasmalemma. It is unknown whether this model applies to bryophytes but Lüttge and Bauer (1968) stated that incubation of *Mnium cuspidatum* in solutions of 1 mM potassium chloride interfered with the functioning and permeability of the plasmalemma. By analogy, high paraquat concentrations in the external solution may cause an increase in the permeability of the plasmalemma and stimulate its entry into the intracellular fraction of the leaves. Saturation of the exchange sites at high paraquat concentrations is also supported by Fig. 4.3.2.

A slow release of paraquat from extracellular sites could allow net uptake equivalent to that of a high concentration of free paraquat presented for a short period. Indeed, Wells and Brown (1987) observed intracellular uptake of exchangeably bound cadmium in *R. squarrosus*. The delayed effect of paraquat on photosynthesis (Ch. 6) and the slow development of visible damage in the field and growth experiments (Chs 2 and 3) are compelling evidence that such a process is more important than direct uptake from the external solution into the cells.

4.3.1.4 The efficiency of paraquat uptake by whole moss shoots

The efficiency of ^{14}C-paraquat uptake by moss shoots of *R. squarrosus*, during 1 and 24 h incubations, was determined using solutions of low concentration (72.5 µM and 725 µM); quantities of radiolabel recovered from solution and bound to shoots is shown in Table 4.3.2.

The results indicate an almost complete removal of paraquat from the incubation medium (less than 2% of label being recovered from the treatment solutions after 1 and 24 h incubations at either concentration). Total uptake did not differ significantly between the 1 h and 24 h incubation times at both paraquat concentrations

Table 4.3.2 Equilibration of paraquat bound to R. squarrosus shoots with free paraquat in the incubation solution (μg g^{-1} dry wt)

Concentration (μM)	Incubation Time 1 hour In solution	1 hour Bound	24 hours In solution	24 hours Bound
725	190.1	10992.8	296.6	12493.9
72.5	18.7	3133.8	20.5	2901.5

ln SED = 0.2979; df = 23; n = 3

($p<0.001$). Increasing the concentration of paraquat from 72.5 μM to 725 μM significantly increased total uptake by moss tissue from *ca.* 3.0 to 12.0 mg g^{-1} dry weight and the concentration of the remaining paraquat in the incubating solution from *ca.* 20 μg g^{-1} dry weight (0.5% of the total applied) to 300 μg g^{-1} (2% of the total applied). This indicates that even at the low paraquat concentration (72.5 μM) a small proportion (0.5%) of the paraquat remains in solution and that this does not differ between the 1 h and 24 h incubation times. The small but significant increase in the proportion of paraquat remaining (2%) in solution after the 725 μM paraquat incubation, and the limited effects of incubation time suggest the existence of an equilibrium between the concentration of adsorbed paraquat and that remaining in solution.

Previous experiments (Section 4.3.1.3) have shown the majority of absorbed paraquat in *R. squarrosus* to be bound to the cell walls (exchangeable fraction). Mosses sprayed in the field could be expected to rapidly adsorb the paraquat to their cell walls. Possibly, as the concentration in the surface water declines, some of the adsorbed paraquat may be released into solution due to a reversal of the previous binding equilibrium. It can be expected that the extent of release would be a function of the total quantity of paraquat

exchanged. Such release could provide a supply of paraquat for continuing intracellular uptake.

4.3.1.5 Estimation of paraquat binding capacity of moss tissue

Mosses have the ability to bind cations passively to their cell walls (Ruhling and Tyler, 1970; Brown and Bates, 1972; Brown and Beckett, 1985; Wells and Brown, 1987). However, anionic binding sites within the protoplast, due to proteins or membrane constituents, could contribute to the total cation binding capacity of the mosses (Schofield, 1985). ^{14}C-paraquat uptake by living and boiled (cell damaged) *R. squarrosus* leaves, stems and whole shoots was assessed to determine if cation binding differed following disruption of the plasmalemma. Moss tissues were incubated (15 min or 24 h) in 725 µM ^{14}C-paraquat and total uptake measured by tissue solubilization after treatment.

Significantly ($p<0.001$) greater total quantities of paraquat were absorbed by leaves (*ca.* 15 mg g^{-1}) than either stems (*ca.* 9.5 mg g^{-1}) or complete moss shoots (*ca.* 9.7 mg g^{-1}). However, no significant differences were found between living tissues and moss tissue killed by boiling (Table 4.3.3). This implies that the majority of the paraquat

Table 4.3.3 Uptake of ^{14}C-paraquat (725 µM) by intact and dead (boiled) leaves, stems and whole moss shoots of R. squarrosus (µg g^{-1} dry wt)

Tissue form	Incubation Time					
	15 min			24 h		
	Leaves	Stems	Whole Shoots	Leaves	Stems	Whole Shoots
Intact	10208	5619	8383	14733	14414	13849
Dead	12810	9358	8450	23576	8946	8358

ln SED = 0.325; df = 28; n = 3

exchange is due to passive binding to the cell walls of the moss. This confirms similar findings of Brehm (1968) for the limited difference in cation exchange capacity of living and dead *Sphagnum* spp. However, Nieboer *et al.* (1976b) detected increases in the uptake of nickel by dead thalli of the lichen *Umbilicaria muhlenbergii.* In the present study, it is conceivable that intracellular cations released during boiling may have occupied anionic binding sites of the moss tissues reducing the measured paraquat binding capacity.

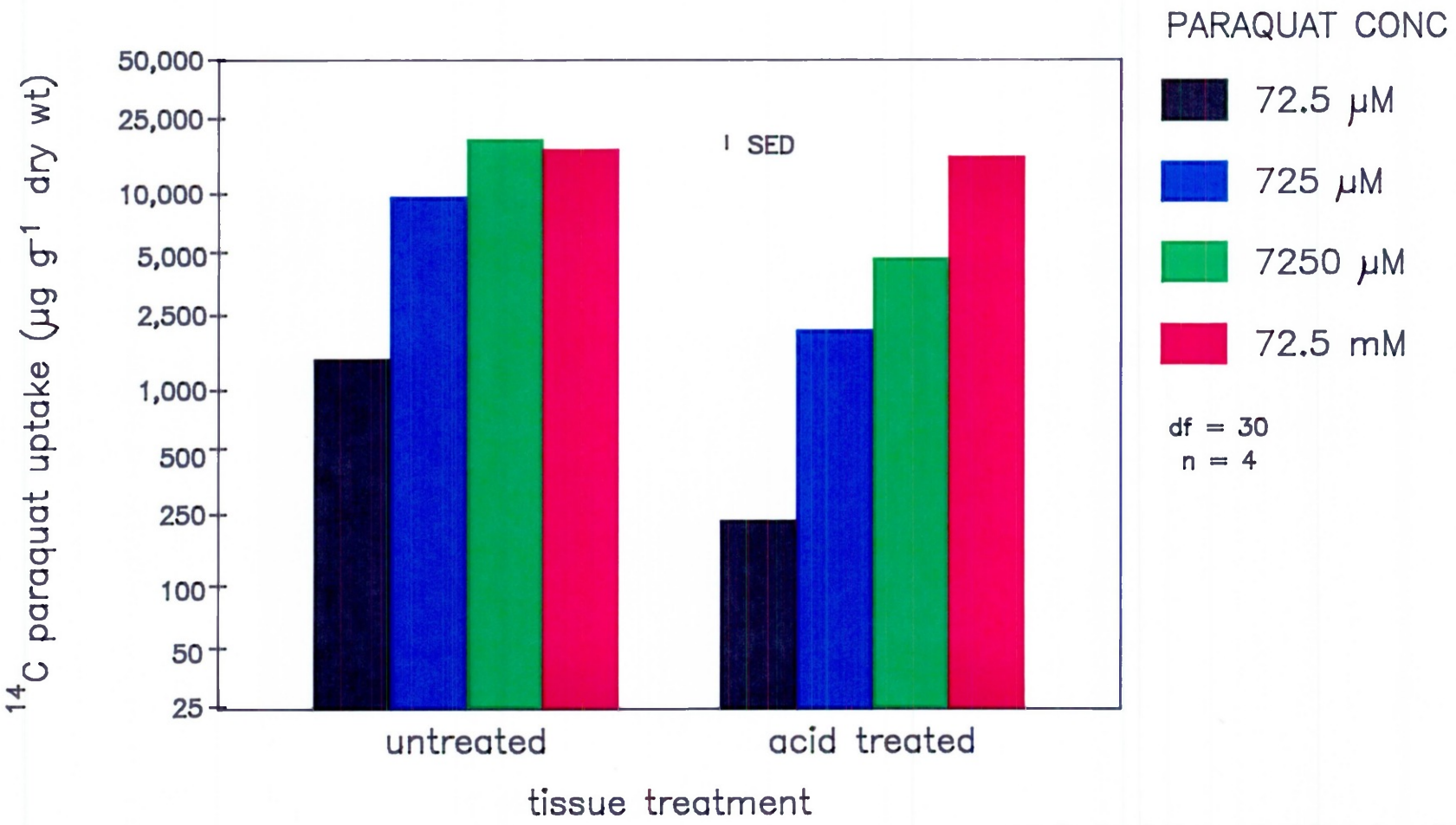
Slightly higher paraquat absorption values were obtained in both the living and dead tissue following long incubation times. In a similar study Pickering and Puia (1969) were unable to account for increased zinc binding to freshly killed *Fontinalis antipyretica* tissue over short, compared with long, incubation periods.

In a second experiment on paraquat binding capacity, boiled moss tissue was treated with acid prior to ^{14}C-paraquat incubation to displace bound cations which might interfere with the capacity measurement. Whole moss shoots were killed by boiling water treatment, immersed in 5 ml 2.5M hydrochloric acid (30 min) and then washed twice in 5 ml distilled water (each 5 min) before incubating in a range of ^{14}C-paraquat solutions for 24 h.

Total paraquat uptake was reduced significantly from 1500 µg g^{-1} dry weight to 204 µg g^{-1} dry weight as a result of the acid treatment when the shoots were supplied with 72.5 µM paraquat (Fig. 4.3.3). At the highest paraquat concentration (72.5 mM) uptake was similar for both tissue treatments (*ca.* 19.0 mg g^{-1} dry weight). The reduced uptake by acid-washed boiled tissue from the low paraquat concentrations (Fig. 4.3.3) was probably due to suppressed ionisation of the binding sites. Carry over of protons into the poorly buffered paraquat treatment solutions would ensure that this situation was not appreciably reversed. Wainwright and Beckett (1975) demonstrated in

Fig. 4.3.3

Uptake of ^{14}C Paraquat by killed (boiled) shoots of *R. squarrosus* following treatment with 2.5M HCl.



Vertical axis is marked in a logarithmic scale

studies with the lichen *Usnea florida* that uptake of zinc is pH-dependent due to proton competition for the binding sites. The results presented here provide little evidence that intracellular cations released by boiling inhibit the exchangeable binding of paraquat.

4.3.1.6 Kinetic studies of paraquat uptake and redistribution by R. squarrosus

For paraquat to exhibit its herbicidal action on *R. squarrosus*, it must enter the protoplast of the moss cells. Therefore, kinetic studies of ^{14}C-paraquat uptake in *R. squarrosus* were performed to investigate the cellular location of paraquat.

^{14}C-paraquat uptake by *R. squarrosus* tissues was studied over a 96 h incubation period. Initial paraquat uptake rates were used to calculate times for half maximum uptake ($t_{\frac{1}{2}}$) and maxima (A_{max}) for the exchangeable, intracellular, and residual fractions of the whole moss shoots, leaves, and stems. Additionally, measurement of paraquat uptake over a 96 h incubation time involves a period when exhaustion of the incubation medium has occurred (Section 4.3.1.4). The 96 h incubation would provide an opportunity to investigate the hypothesis of paraquat transfer from the cell wall to the protoplast.

Whole moss shoots, leaves, and stems were incubated in paraquat solutions for time intervals up to 96 h. Two paraquat concentrations were used in these experiments, 72.5 µM and 725 µM. In earlier experiments (Section 4.3.1.4) these solutions became exhausted due to uptake by *R. squarrosus* during a 1 h incubation period in a 5 ml sample.

The analysis presented here uses 1) the uptake data for the initial 4 h uptake periods, and 2) data for the whole 96 h incubation period. These time periods were chosen to overcome difficulties in

quantitatively assessing and comparing uptake curves for the different cellular fractions which were observed to fluctuate over the 96 h incubation time (Figs 4.3.4 to 4.3.9).

1. Uptake during the first 4 h

A single exponential curve adequately described the initial (4 h) uptake of ^{14}C-paraquat into the different cellular fractions of *R. squarrosus.* Ideally, kinetic studies are performed on initial uptake processes, utilising short incubation periods. In this study, the uptake period was extended to 4 h. This, however, did not interfere with the paraquat uptake profile and the data are suitable for kinetic analysis using methodology also employed for describing enzyme reaction kinetics (Morris, 1976; Wells and Brown, 1987). The parameters which describe maximum uptake (A_{max}) and the time required for half maximum uptake ($t_{½}$ = ln 2/-ln R) were obtained from the fitted curves and are used for quantitative comparisons between tissue types and herbicide treatments. In cases of rapid uptake, as for the exchangeable fraction of the leaves and for the total uptake, a single exponential function with an origin not through zero best described the data (Figs 4.3.4 to 4.3.7). Absence of a zero intercept is a consequence of the very fast initial rates and resulting lack of data values in the initial stages. This in turn, influences the accuracy of the curve fitting close to the origin. In addition, inefficiency of the paraquat extraction procedure could also produce a residual component equivalent to a non-zero y axis intercept (Wells and Brown, 1987). The values of A_{max} and $t_{½}$ for ^{14}C-paraquat uptake into each cell fraction in stems, leaves, and whole moss shoots are presented in Tables 4.3.4 and 4.3.5. The fitted curves and original data are displayed in Figs 4.3.4 to 4.3.7.

Table 4.3.4 Kinetic parameters for ^{14}C-paraquat uptake (± SE) from a 72.5 µM solution by Rhytidiadelphus squarrosus

Moss Tissue	Fraction	Curve x axis intercept	A_{max} (Maximum uptake at equilibrium) (µg g^{-1})	$t_{½}$ (min)	R^2	df
Leaves	Exchangeable	S	15223 (299)	28.8 (7.7)[3.1]	0.97	11
	Intracellular	SO	444 (55)	9.5 (6.2)	0.17	11
	Residual	SO	445 (50.6)	*	*	11
	Σ Exchangeable + residual	S	15826 (324)	29.4 (8.1)	0.9	11
	Total	S	16199 (397)	86.5 (9.6)	0.94	11
Stems	Exchangeable	SO	3671 (297)	13.1 (12.2)	0.70	11
	Intracellular	SO	-101 (64)	170.6 (17.3)	0.95	11
	Residual	SO	6724 (1529)	104.8 (11.3)	0.87	11
	Σ Exchangeable + residual	S	11476 (3076)	107.4 (27.4)	0.89	11
	Total	S	11876 (3351)	112.2 (27.9)	0.89	11
Whole Shoots	Exchangeable	S	6594 (2027)	133.4 (60.8)[2.2]	0.71	11
	Intracellular	SO	187 (29)	19.9 (20.9)	0.88	11
	Residual	SO	649 (112)	5.5 (35.3)	0.24	11
	Σ Exchangeable + residual	S	6803 (819)	79.2 (34.9)	0.74	11
	Total	S	7367 (1188)	109.4 (25.2)	0.79	11

R^2 = coefficient of variation; * = not determinable; S = Single exponential; SO = single exponential, origin through zero; figures in [ ] are estimates of $t_{½}$ if origin at zero.

Table 4.3.5 Kinetic parameters for ^{14}C-paraquat uptake (± SE) from a 725 µM solution by Rhytidiadelphus squarrosus

Moss Tissue	Fraction	Curve x axis intercept	A_{max} (Maximum uptake at equilibrium) (µg g^{-1})	$t_{½}$ (min)	R^2	df
Leaves	Exchangeable	S	34868 (*)	*[1.3]	0.38	5
	Intracellular	S0	694 (95)	4.15 (2.7)	0.36	5
	Residual	S0	5263 (*)	*	*	5
	Σ Exchangeable + residual	S	39883 (374)	32.2 (71.6)	0.18	5
	Total	S	40439 (1646)	30.4 (432.7)	0.12	5
Stems	Exchangeable	S0	18717 (605)	33.8 (3.6)	0.69	5
	Intracellular	S0	1541 (2241)	120.3 (33.7)	0.94	5
	Residual	S0	10622 (623)	30.8 (6.6)	0.57	5
	Σ Exchangeable + residual	S0	29480 (1011)	34.8 (3.8)	0.8	5
	Total	S0	34101 (1175)	42.9 (3.6)	0.82	5
Whole Shoots	Exchangeable	S0	16001 (150)	4.1 (2.1)	0.48	5
	Intracellular	S0	2511 (359)	47.2 (14.6)	0.97	5
	Residual	S0	2843 (167)	7.19 (10.9)	0.36	5
	Σ Exchangeable + residual	S0	18992 (243)	4.97 (0.22)	0.81	5
	Total	S0	19525 (204)	4.97 (0.22)	0.68	5

R^2 = coefficient of variation; * = not determinable; S = Single exponential; S0 = single exponential, origin through zero; figures in [ ] are estimates of $t_{½}$ if origin at zero.

Fig. 4.3.4 The relationship between ^{14}C-paraquat (72.5 µM) uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and incubation time over a 4 h period

The fitted curves are single exponentials

Fig. 4.3.4

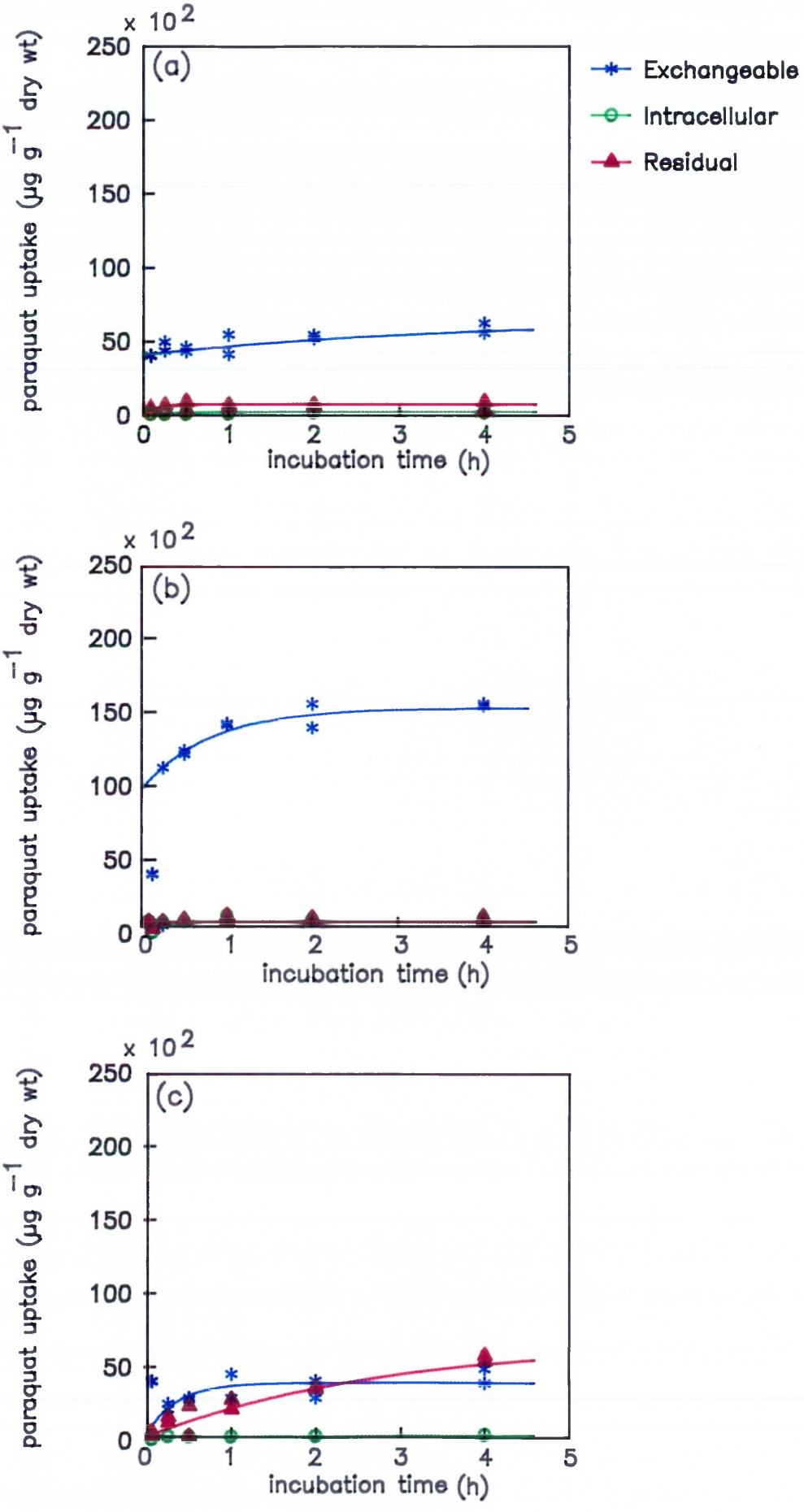
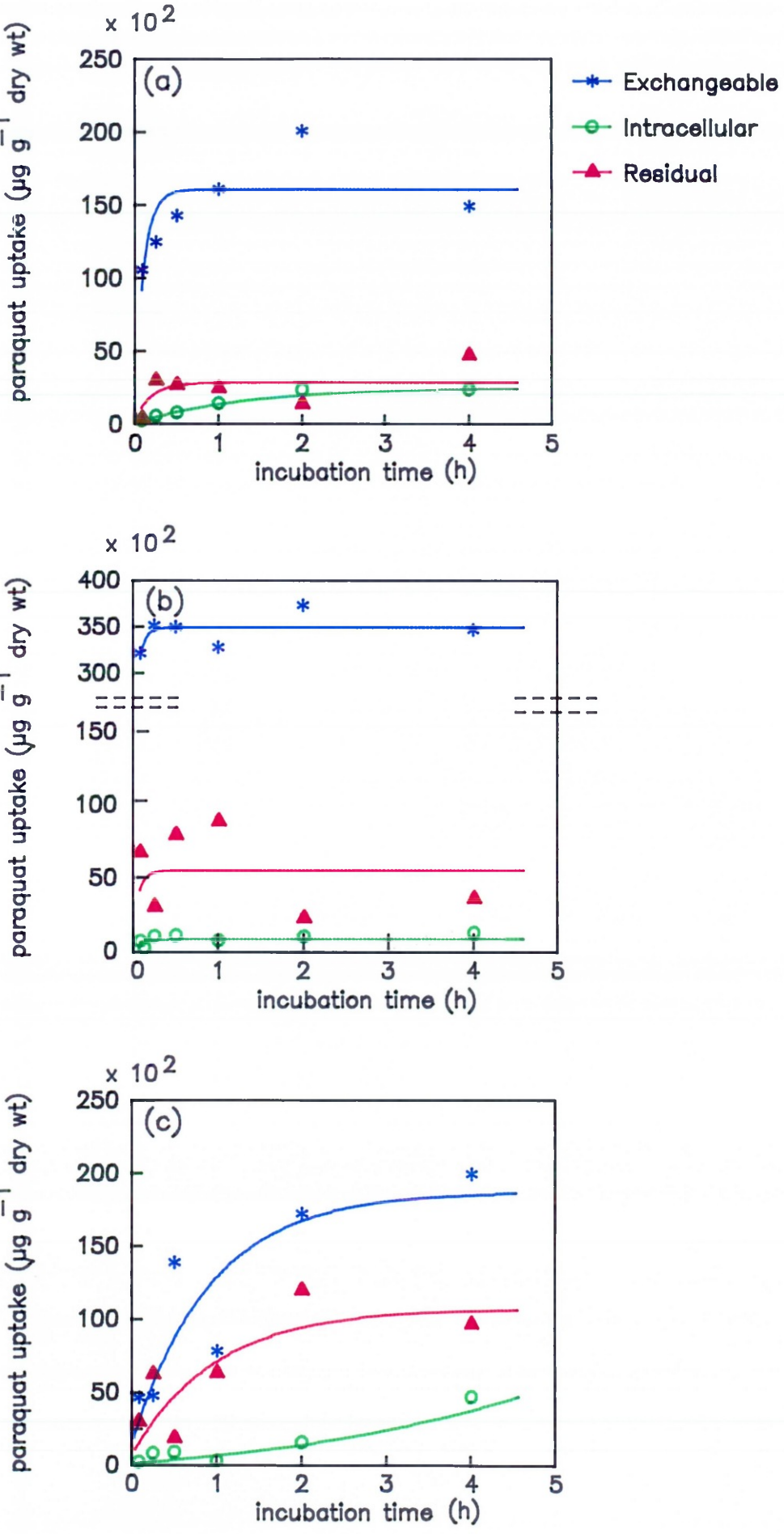

x 10 2
250
200
150
100
50
0
(a)
(b)
(c)
paraquat uptake (µg g -1 dry wt)
0
1
2
3
4
5
incubation time (h)
Exchangeable
Intracellular
Residual

Fig. 4.3.5 The relationship between ^{14}C-paraquat (725 µM) uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and incubation time over a 4 h period

The fitted curves are single exponentials

Fig. 4.3.5


x 10 2
(a)
paraquat uptake (µg g −1 dry wt)
250
200
150
100
50
0
0
1
2
3
4
5
incubation time (h)
Exchangeable
Intracellular
Residual
x 10 2
(b)
paraquat uptake (µg g −1 dry wt)
400
350
300
150
100
50
0
0
1
2
3
4
5
incubation time (h)
x 10 2
(c)
paraquat uptake (µg g −1 dry wt)
250
200
150
100
50
0
0
1
2
3
4
5
incubation time (h)

Exchangeable fraction

The $t_{\frac{1}{2}}$ values for exchangeable paraquat uptake in leaves and whole moss shoots of *R. squarrosus* following the 72.5 µM treatment were 28.8 min and 133.4 min, respectively. However, this is an overestimate due to the absence of zero intercepts (Figs 4.3.4 and 4.3.5). Use of a single exponential curve constrained through the origin would seriously underestimate A_{max}. The $t_{\frac{1}{2}}$ values for exchangeable uptake, described using a single exponential through the origin, in leaves (*ca.* 3.1 min) and whole moss shoots (*ca.* 2.2 mins) did not differ significantly but were smaller than that obtained for stems (13.1 min) following incubation in 72.5 µM paraquat. A similar pattern emerged following incubation in 725 µM paraquat (Table 4.3.5).

The largest A_{max} values were obtained for paraquat exchange by detached leaves for both the 72.5 µM (A_{max} = 15.2 mg g^{-1} dry weight) and 725 µM (A_{max} = 34.8 mg g^{-1} dry weight) paraquat treatments.

Intracellular fraction

The $t_{\frac{1}{2}}$ values for paraquat uptake into the intracellular fraction of the stems (170.6 min and 33.8 min) were significantly greater ($p<0.05$) than those for leaves (9.5 min and 1.3 min) and whole moss shoots (19.93 and 4.11 min) for both the 72.5 µM and 725 µM treatments. No significant differences in the A_{max} values were detected between the three tissue types for the 725 µM paraquat treatment (Fig. 4.3.5). At the lower paraquat concentration A_{max} values were highest in the leaf tissue. The stems, therefore, accumulated less paraquat intracellularly from the 72.5 µM paraquat than the leaves.

Residual fraction

The $t_{\frac{1}{2}}$ and A_{max} values for paraquat accumulation into the residual fraction were significantly greater for stems than those for whole moss

shoots ($p<0.001$) at both paraquat concentrations (Figs 4.3.4 and 4.3.5). Leaves yielded smaller $t_{\frac{1}{2}}$ values for both paraquat concentrations than stems, but not significantly so.

It is probable that paraquat in the residual fraction represents either binding to internal anionic sites or incomplete displacement of the externally bound paraquat. The residual and exchangeable components of each tissue type were therefore combined and the data re-analysed (Table 4.3.4 and 4.3.5). An improved fit would imply that the two fractions were of one origin.

The combined data when analysed produced a significant increase in total paraquat in the leaves but not in the stems and whole moss shoots for the 725 µM paraquat treatment (Table 4.3.5). This is probably due both to a greater paraquat availability to leaves and possibly greater numbers of anionic binding sites in the leaves than the stems. The coefficient of variation (R^2) values (Tables 4.3.4 and 4.3.5) show improved fits for the combined data compared with the analysis of the individual fractions (Tables 4.3.4 and 4.3.5). The final parameters for the combined data resemble that for the total ^{14}C-paraquat uptake by the moss tissues (Tables 4.3.4 and 4.3.5; Figs 4.3.6 and 4.3.7). Paraquat in the residual and exchangeable fractions, therefore, probably represents one true cellular location.

2. Uptake over the full 96 h incubation period

^{14}C-paraquat uptake by whole moss shoots, leaves and stems into the three cellular locations (extracellular, intracellular and residual) was analysed by fitting single and double exponential curves to the data. In all cases, the single exponential whose origin was not through zero gave the best description of the data. Wells and Brown (1987) also observed a significant non-zero y-axis intercept for graphs describing cadmium uptake by *R. squarrosus*. They attributed the

Fig. 4.3.6 The relationship between total ^{14}C-paraquat (72.5 µM) uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and incubation time over a 4 h period

The fitted curves are single exponentials

Fig. 4.3.6

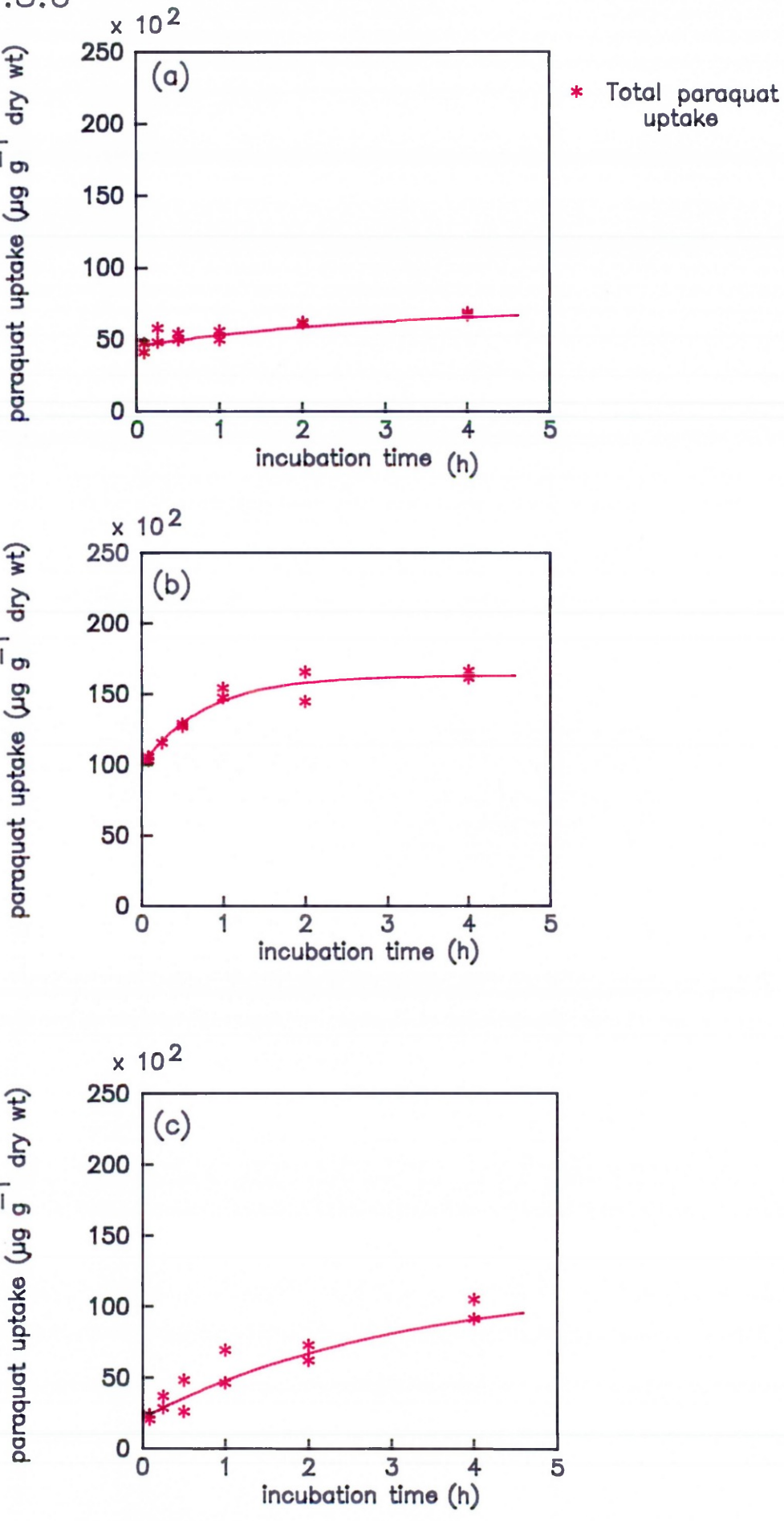
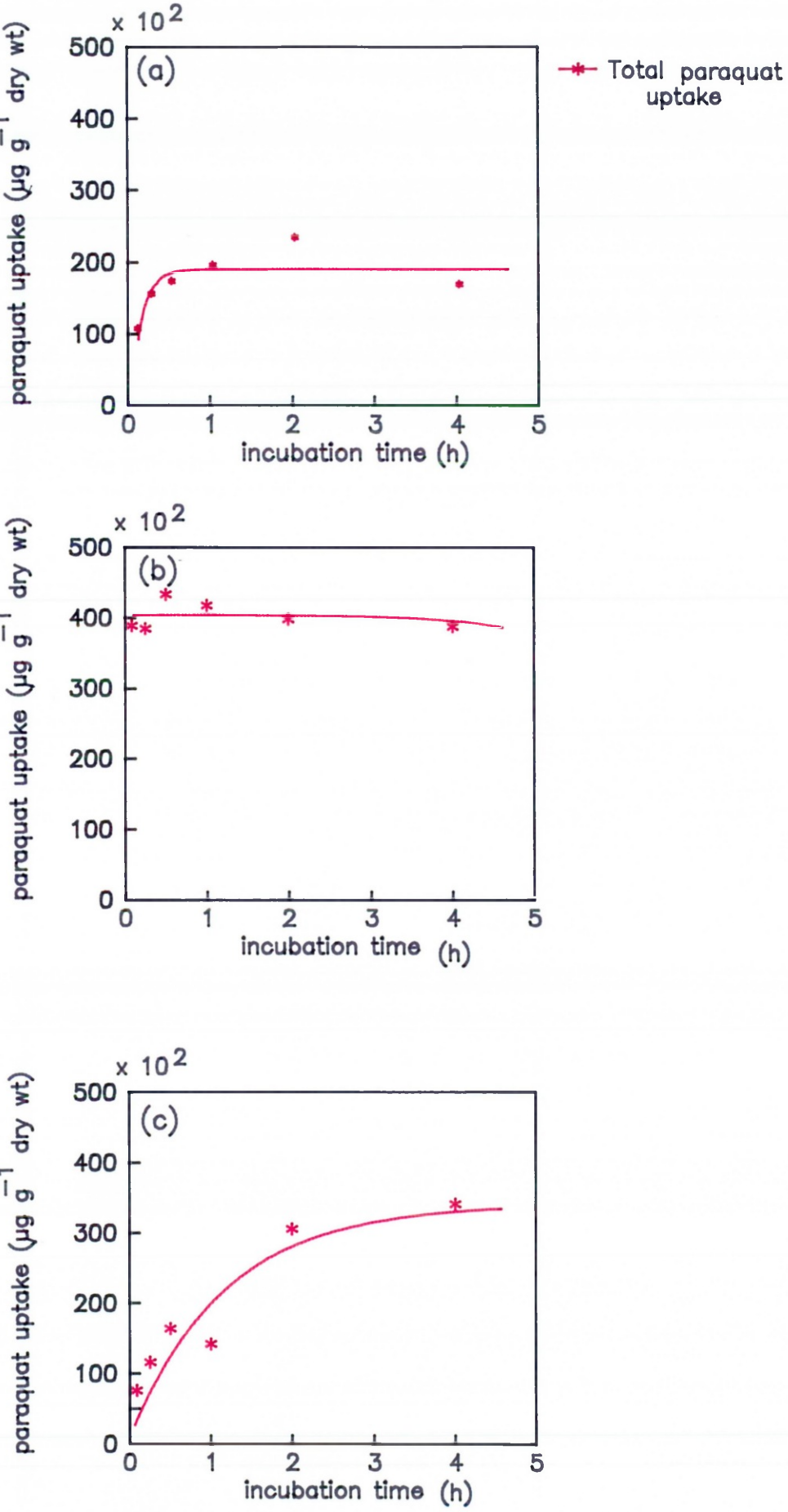

x 10 2
250
200
150
100
50
0
(a)
paraquat uptake (µg g −1 dry wt)
0 1 2 3 4 5
incubation time (h)
* Total paraquat uptake
x 10 2
250
200
150
100
50
0
(b)
paraquat uptake (µg g −1 dry wt)
0 1 2 3 4 5
incubation time (h)
x 10 2
250
200
150
100
50
0
(c)
paraquat uptake (µg g −1 dry wt)
0 1 2 3 4 5
incubation time (h)

Fig. 4.3.7 The relationship between total ^{14}C-paraquat (725 µM) uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and incubation time over a 4 h period

The fitted curves are single exponentials

Fig. 4.3.7


x 10 2
500
400
300
200
100
0
(a)
paraquat uptake (µg g −1 dry wt)
0
1
2
3
4
5
incubation time (h)
Total paraquat uptake
x 10 2
500
400
300
200
100
0
(b)
paraquat uptake (µg g −1 dry wt)
0
1
2
3
4
5
incubation time (h)
x 10 2
500
400
300
200
100
0
(c)
paraquat uptake (µg g −1 dry wt)
0
1
2
3
4
5
incubation time (h)

non-zero intercept to a residual Cd fraction arising from inefficiency of the displacement technique. Extrapolating uptake curves through zero would seriously under-estimate the uptake and maxima in this study.

Paraquat uptake curves for the exchangeable, intracellular and residual fractions of the whole moss segments (Figs 4.3.8 and 4.3.9) were, in general, intermediate between those of the separated leaves (Figs 4.3.8 and 4.3.9) and stems (Figs 4.3.8 and 4.3.9). Similarities in the uptake curves of the leaves and stems with those of whole moss shoots confirm that little damage to the tissues had occurred during their separation.

The total exchangeable paraquat concentration of the leaves after a 96 h incubation in 72.5 μM paraquat was at least ten times greater than that of the stems and five times that of whole moss shoots (Fig. 4.3.8). In contrast, the residual paraquat content of the stem tissue was ten times greater than that of the leaves (Fig. 4.3.8). Again, the whole moss tissue was intermediate in its residual paraquat content (Fig. 4.3.8). Increasing the paraquat concentration of the incubation solution increased the exchangeable and residual fractions of both leaf and stem tissue (Fig. 4.3.9).

Total paraquat content of the extracellular and residual fraction in all three tissue types declined with time following the initial rapid uptake phase (Figs 4.3.8 and 4.3.9). Leakage of intracellular cations following damage to cell membranes may have occurred, causing displacement of ^{14}C-paraquat. However, the intracellular fraction continued to increase in its paraquat content compared to the decreasing extracellular and residual fractions, suggesting that most cells were still intact and that redistribution of the paraquat had occurred.

Table 4.3.6 Summary of cation uptake parameters (± SE) for *R. squarrosus*

Parameter	Up to a 4 h incubation in 72.5 µM paraquat	Up to a 4 h incubation in 725 µM paraquat	30 min incubation in 100 µM $CdSO_4$ (cited Brown and Beckett, 1985)
Time required for half maximum total uptake (min)	109 (35.2)	4.9 (0.002)	
Maximum total uptake (µmol g^{-1})	40.9 (6.6)	108.5 (1.13)	
Time required for half maximum exchangeable uptake (min)		4.97 (2.71)	4.5 (2.5)
Maximum exchangeable uptake (µmol g^{-1})	37.8 (4.5)	105.5 (1.35)	107 (12)
Time required for half maximum intercellular uptake (min)	19.93 (20.9)	47.2 (14.6)	
Maximum intercellular uptake (µmol g^{-1})	1.04 (0.1)	13.95 (2.0)	14.0 (3.0)

Figures expressed as dry weights

Fig. 4.3.8 The relationship between ^{14}C-paraquat (72.5 µM) uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and incubation time over a 96 h period

The curves fitted are single exponentials

Fig. 4.3.8

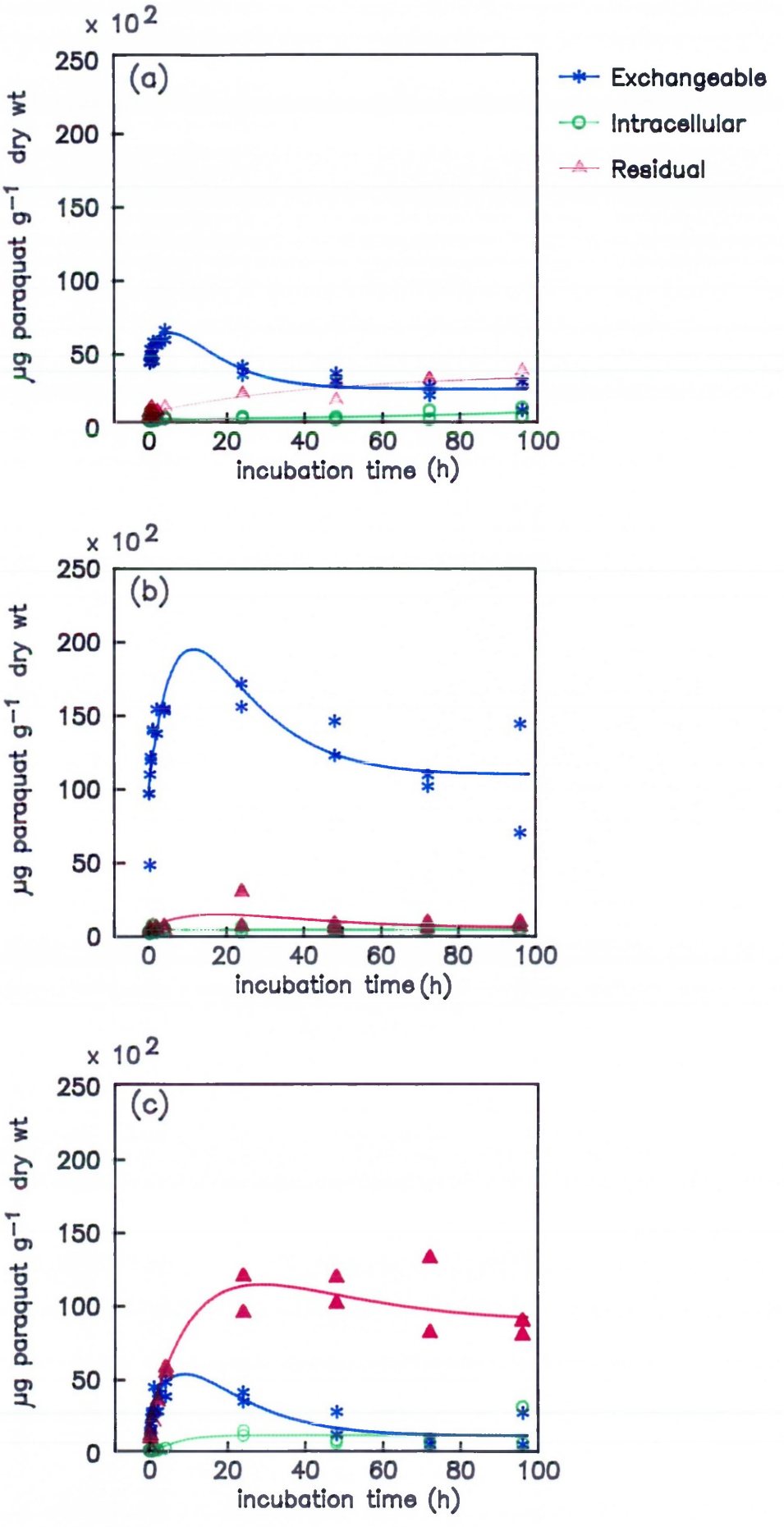
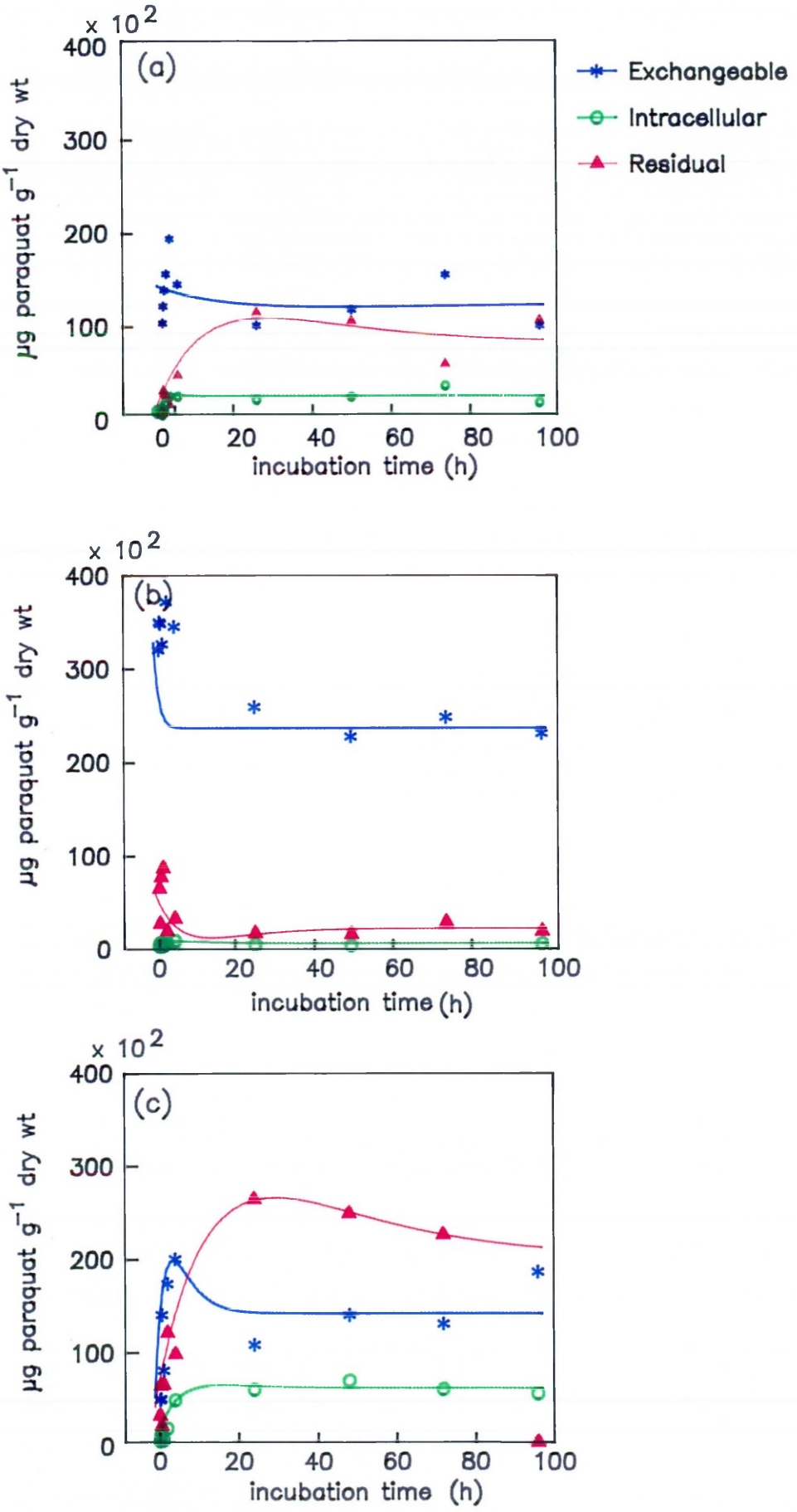

x 10 2
250
200
150
100
50
0
(a)
µg paraquat g−1 dry wt
0
20
40
60
80
100
incubation time (h)
Exchangeable
Intracellular
Residual
x 10 2
250
200
150
100
50
0
(b)
µg paraquat g−1 dry wt
0
20
40
60
80
100
incubation time (h)
x 10 2
250
200
150
100
50
0
(c)
µg paraquat g−1 dry wt
0
20
40
60
80
100
incubation time (h)

Fig. 4.3.9 The relationship between ^{14}C-paraquat (725 µM) uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and incubation time over a 96 h period

The curves fitted are single exponentials

Fig. 4.3.9


x 10 2
400
300
200
100
0
(a)
µg paraquat g−1 dry wt
0
20
40
60
80
100
incubation time (h)
Exchangeable
Intracellular
Residual
x 10 2
400
300
200
100
0
(b)
µg paraquat g−1 dry wt
0
20
40
60
80
100
incubation time (h)
x 10 2
400
300
200
100
0
(c)
µg paraquat g−1 dry wt
0
20
40
60
80
100
incubation time (h)

Good evidence for transfer of exchangeably held paraquat to the intercellular fraction has been obtained. Because paraquat in the incubation solution had been exhausted after 1 h incubation (Section 4.3.1.4), no further increases in concentration within the different cellular fractions could occur without redistribution. For all tissue types, paraquat concentration in the intracellular and residual fractions increased beyond 1 h suggesting an origin in the extracellular fraction. Figure 4.3.9 shows that the increase in intracellular paraquat is accompanied by a progressive decline in the exchangeable concentration.

The 4 h kinetic analysis of the data revealed greater intracellular $t_{½}$ values for the leaves than those for the stems for both paraquat incubation concentrations, although A_{max} was similar for both leaves and stems for the 725 µM concentration. The smaller $t_{½}$ values for extracellular paraquat accumulation by leaves and the greater total uptake by leaves suggests that their binding sites are more freely accessible than those of the stems on a gravimetric basis. Differences observed in the exchangeable paraquat content of leaves and stems may be attributed to:

1. The thickness of *R. squarrosus* stems and the absence of intercellular spaces (section 1.5) (Duckett, 1986), in contrast to the unistratose leaves. This may reduce the efficiency of the exchange washes.
2. Electrochemical affinity of the displacing chemical. Substitution of a cation by another of equal affinity may not be as rigorous as displacement by a cation of greater affinity for the binding sites.
3. Variation in strength of paraquat due to differences in the binding sites of the different tissues. High electrostatic charge densities in the binding sites may confer different paraquat binding

properties on the stem tissue. Clarkson (1974) and Clymo (1963) both describe high electrostatic charge densities as a characteristic of some cell walls. In addition, substituted pyridinium salts, such as paraquat, have been found to increase the cation binding capacities recorded in plant tissues due to their partitioning into lipids or binding to tannins and lignins (Price, 1978).

Wells and Brown (1987) observed inefficiencies in the exchange of metal cations from moss cell walls and suggested improvement of the sequential extraction technique such as increasing the concentration, volume or electrochemical affinity of the displacing chemical. Increasing the concentration or volume of unlabelled paraquat might cause cellular damage or interfere with the accuracy of ^{14}C-paraquat detection. However, an increase in electrochemical affinity of the displacing agent may have improved efficiency of paraquat exchange from the stem tissue.

Total paraquat uptake by leaves and stems was greater than that of the whole moss shoots. This was presumably due to exhaustion of, or equilibration (ratio of free:bound paraquat, (see Section 4.3.1.4)) with, the incubation solution by the whole moss shoots.

In the whole moss shoots, depletion of paraquat in the incubation medium may have restricted binding to the freely accessible leaf sites. It is possible that inadequate agitation of the incubation medium accounts for low uptake by the stems and whole moss shoots and low extraction efficiency. Poille and Jenny (1971), cited by Clarkson (1974) described a reduction in the initial uptake of rubidium by barley roots due to inadequate agitation and the formation of a boundary layer. This prevented complete homogeneity of the incubation medium with the tissue surface. The presence, if any, of a boundary layer may not impede uptake into the unistratose layer of moss leaf

tissue to the same degree as the more dense stem or whole moss tissue.

Results obtained from the 96 h and 4 h uptake analyses in the above experiments indicate that paraquat does not penetrate the stem tissue efficiently under the experimental conditions used. In earlier experiments, regrowth of *R. squarrosus* occurred from the stems but not the apex, following severe herbicidal effects. This observation supports the view that there is poor penetration of the herbicide into the stem tissue.

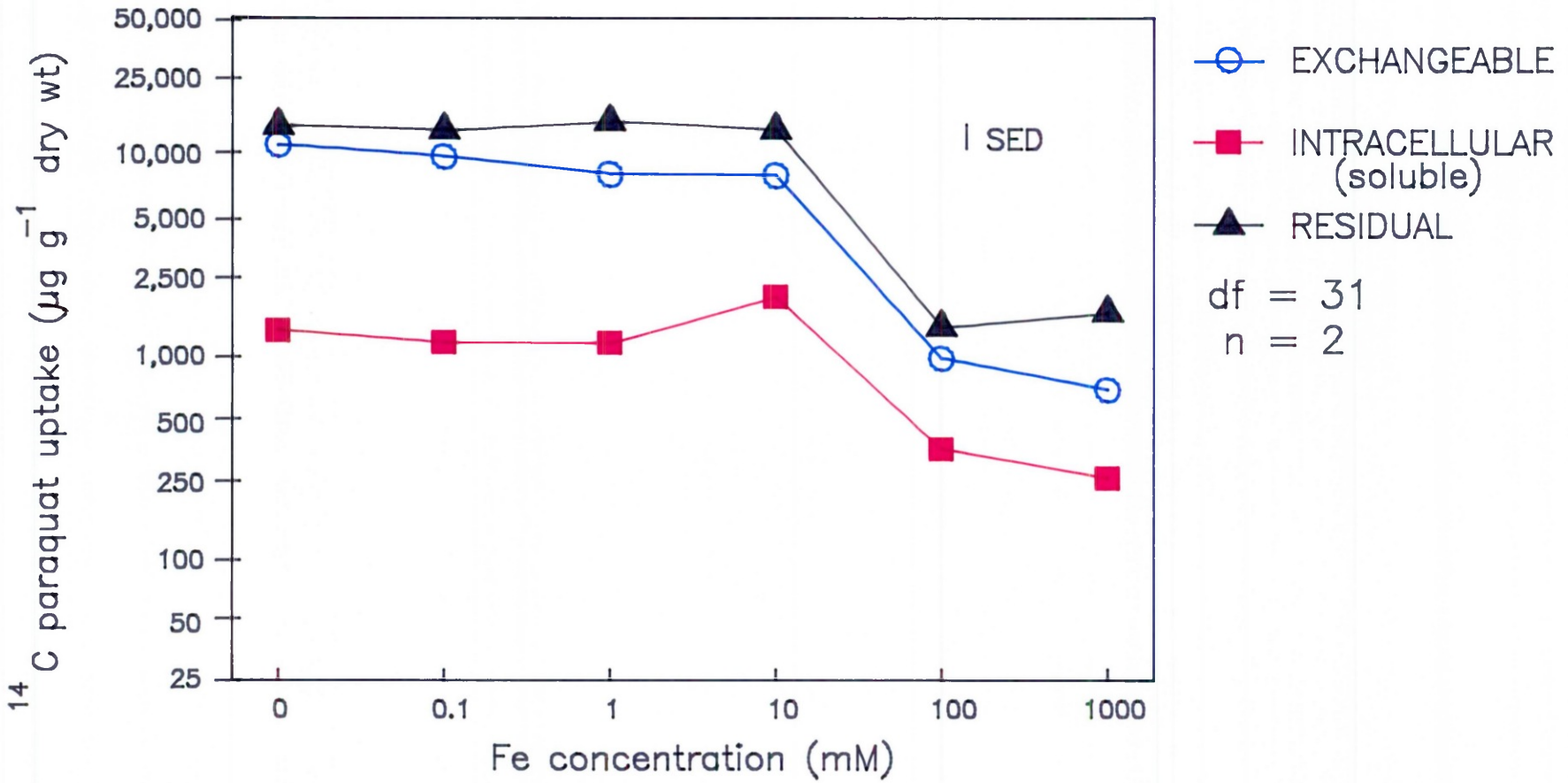
Finally, comparison of the estimated kinetic parameters for paraquat uptake with those determined by Brown and Beckett (1985) for cadmium uptake (100 µM) shows close similarities (Table 4.3.6).

4.3.1.7 Interaction between iron and paraquat uptake

A large proportion of the total iron (see Section 4.3.2) and paraquat absorbed by *R. squarrosus* becomes exchangeably bound to the cell walls. Previous experiments (Section 4.3.1.6) have shown that progressive redistribution of paraquat can occur from the exchangeable cell wall binding sites to the intracellular fraction of *R. squarrosus*. However, in the presence of other cations exchanged on the cell walls of *R. squarrosus*, less paraquat binding would be expected thereby reducing intracellular paraquat uptake. The following experiment was designed to determine whether paraquat and iron occupy the same exchangeable sites on the cell wall of *R. squarrosus* and whether pretreatment with $FeCl_3$ affects intracellular paraquat uptake.

R. squarrosus shoot segments were pretreated in different concentrations of ferric chloride for 1 h. The moss shoots were then incubated in 725 µM ^{14}C-paraquat for 24 h. Data for exchangeable, intracellular and residual ^{14}C-paraquat uptakes, following pretreatment in different $FeCl_3$ concentrations, are shown in Fig. 4.3.10.

Fig. 4.3.10 Inhibition of ^{14}C paraquat uptake by whole shoot segments of *R. squarrosus* following pre-incubation in ferric chloride solutions



Axes are marked in a logarithmic scale

More exchangeable paraquat was absorbed in untreated moss than in shoots pretreated with $FeCl_3$ (Fig. 4.3.10). Inhibition of paraquat exchange by $FeCl_3$ was progressive with increasing $FeCl_3$ concentration. Paraquat uptake at the highest $FeCl_3$ concentration (1.0M) was only 5% of that in the control. A similar response was found for paraquat uptake into the intracellular fraction. These results strongly suggest that intracellular paraquat uptake depends on initial uptake onto the cell wall. Alternatively, paraquat uptake may have been inhibited by iron saturation of intracellular uptake pathways or, perhaps, by a change in internal electrochemical potential.

Paraquat uptake into the residual fraction was also inhibited by $FeCl_3$ pretreatments (Fig. 4.3.10). This fraction is also believed to represent part of the extracellular pool (Section 4.3.1.6).

Exchangeably bound Fe was not readily displaced by the later incubation with the divalent cation paraquat. This is expected as polyvalent cations have a greater affinity for the anionic sites of moss cell walls than those of a lesser valency (Clymo, 1963). Therefore, paraquat can be described as possessing a weaker affinity for the anionic binding sites of the cell wall of *R. squarrosus* than does ferric iron. In general, cations of high affinity, and already present in the moss tissue, could hinder the uptake of cationic herbicides, such as paraquat, and reduce their effectiveness. Therefore, cationic herbicides applied to mosses in formulations with, or following, iron salts or fertilisers, could have reduced efficiency compared to a simple preparation. In certain situations, high levels of heavy metal pollutants might also limit the effects of cationic herbicides.

4.3.2 Iron uptake

Iron uptake by *R. squarrosus* was studied because ferrous sulphate is a recommended moss killer for lawns (Fryer and Makepeace, 1977). Unlike the cationic vascular plant herbicide paraquat, ferrous sulphate does not possess herbicidal properties towards vascular plants (Section 1.7.2). The following experiments were designed to observe the effects of concentration, incubation time and type of iron salt on total iron uptake by leaves, stems and whole moss shoots. Ferrous and ferric salts were both studied to determine if they were absorbed differently, but this is unlikely as ferrous ions are rapidly oxidised. Earlier growth experiments on *R. squarrosus* segments confirm the lack of difference between the ferrous and ferric salts (Ch. 3). Intracellular and exchangeable iron uptake was only measured following treatment with $FeCl_3$.

4.3.2.1 Intracellular and extracellular uptake of iron by R. squarrosus

R. squarrosus tissue was incubated in 200, 2000 and 6000 µM $FeCl_3$ over a 4 h period and levels of Fe in the extracellular (exchangeable) and intracellular (soluble) fractions were determined by the sequential elution technique (Table 4.3.7). Intracellular uptake of Fe did not vary with incubation time but significantly increased with increasing $FeCl_3$ concentration ($p<0.001$). Extracellular Fe uptake significantly increased with increasing incubation time ($p<0.001$) and $FeCl_3$ concentration ($p<0.001$). Free intracellular Fe was not detectable in untreated moss tissue but a natural Fe content of 154.6 µg g^{-1} dry wt (SE ± 22.9, n = 16) was determined following acid extraction and is presumably held primarily in an exchangeable form on the cell wall.

Table 4.3.7 The (a) intracellular and (b) residual (not soluble) uptake of iron from ferric chloride solutions by R. squarrosus ($\mu g\ g^{-1}$ fresh wt)

(a)

Concentration Fe(μM)	Incubation Time 5 min	30 min	1 h	2 h	4 h
200	509	670	427	389	494
2000	501	671	434	540	515
6000	1290	773	800	764	666

(b)

Concentration Fe (μM)	Incubation Time 5 min	30 min	1 h	2 h	4 h
200	951	1863	2184	2380	3770
2000	2246	2654	3415	3601	4573
6000	3920	4846	6142	5642	3837

ln SED = 0.2532; d.f. = 238; n = 8

The results for intracellular Fe uptake (Table 4.3.7) apparently indicate a rapid initial uptake phase with no appreciable uptake afterwards, possibly due to exhaustion of the incubation solutions. However, the increase in intracellular iron content with increased concentrations of iron in the treatment solution suggests the existence of an equilibrium between the intracellular and external iron solutions. Cadmium uptake in *R. squarrosus* is probably via a nonspecific transport system and displays saturation kinetics (Wells and Brown, 1987). Intracellular cadmium uptake was also found to continue over a 30 min period. Intracellular iron uptake values obtained for the 5 and 30 min incubations were not significantly different.

Wells and Brown (1987) observed a non-zero y-axis intercept for the plots of intracellular cadmium uptake which they suggested were due to a poor extraction procedure. The results obtained for Fe in the present study may also be attributed to a poor extraction procedure. Intracellular Fe may bind to the cell walls of the moss during release of intracellular contents by boiling. Additionally, release of a small component of the exchangeable fraction may occur during the boiling procedure. However, this was not observed by Brown and Bates (1972) in studies of lead uptake by *Grimmia donniana*. The disparity between the intracellular uptake profiles observed for Fe and cadmium (Wells and Brown, 1987) may be attributable to the different fractions measured. In the present study only soluble intracellular Fe was measured whereas Wells and Brown (1987) measured total intracellular cadmium uptake. The latter is equivalent to the combined soluble and insoluble intracellular fractions of the present investigation. Assuming no fundamental differences in uptake of the two metals, the lack of further intracellular Fe uptake after 30 mins may be due to intracellular soluble Fe binding or complexing with protoplasmic components, thereby reducing the concentration of soluble Fe detected.

4.3.2.2 Total uptake of iron supplied as $FeSO_4$

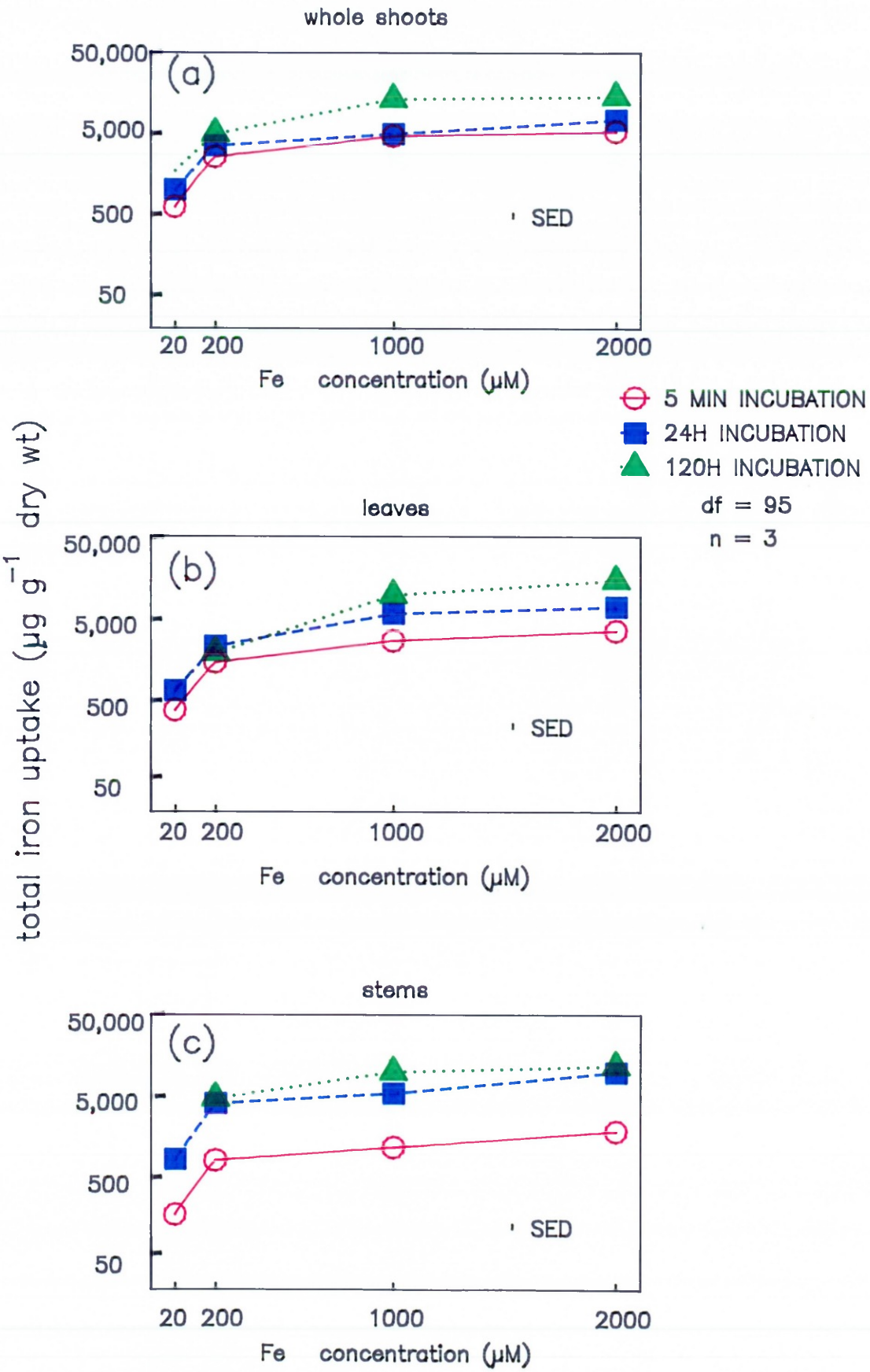
Fe uptake from ferrous sulphate solutions was studied to determine the effects of incubation time and Fe concentration on uptake by leaves, stems and whole moss shoots of *R. squarrosus*. Total uptake measurements only were recorded due to the small $FeSO_4$ concentrations used and the limited differences in intracellular Fe concentrations observed above (Section 4.3.2.1) over a similar concentration range. High Fe concentrations were avoided in this experiment because of their tendency to cause rapid exchange at the cell wall. This could possibly obscure differences which may exist between the tissue types and their affinity for Fe. The curves (Fig. 4.3.11) showing uptake in relation to concentration begin to plateau at an external concentration of 1000 µM Fe, regardless of the incubation time. However, increasing the incubation period led to enhanced total Fe uptake for all tissue types. Uptake beyond 24 h is probably due to equilibration of the incubation solution with less accessible cell wall binding sites and to continuing intracellular uptake.

Significantly greater uptake was observed in leaves than in the other tissues at all Fe concentrations, during the 5 min incubation. Intact segments showed greater uptake than stems over this period. However, over the 24 h incubation, no major differences between tissue types were apparent. Complete uptake of all the supplied iron in the 200 µM Fe treatment had occurred by 120 h for all tissues.

The results support the findings of the paraquat experiments (Section 4.3.1.6) where rate of uptake by leaves was faster than that of stems. Fe uptake by whole moss shoots, as in the paraquat experiments, was intermediate between that of the component tissues for the 1000 µM and 2000 µM Fe treatments. The rapid accumulation of Fe by the leaves, as observed for paraquat uptake (Section 4.3.1.2), may impede Fe uptake to the underlying stems.

Fig. 4.3.11 The effect of incubation time and $FeSO_4$ concentration on the total uptake of Fe by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus

Fig. 4.3.11



Vertical axis is marked on logarithmic scale

4.3.2.3 Total Fe uptake capacity

The parameters $t_{½}$ and A_{max} for total Fe uptake were determined for the leaves, stems and whole moss shoots.

To determine the Fe uptake capacities of the stems, leaves and whole moss shoots of *R. squarrosus*, tissues were incubated for 24 h in a wide range of $FeSO_4$ concentrations (20 µM to 110 mM). To ensure saturation, the incubation time employed (24 h) was greater than that used to study initial uptake rates (Section 4.3.1.6).

Uptake data for Fe by leaves, stems and whole moss shoots (Fig. 4.3.12) resemble those obtained for paraquat (Section 4.3.1.6).

A single exponential curve with a non-zero origin best described the data (Fig. 4.3.12). The kinetic constants (A_{max} and $½_{max}$) derived from the fitted curves might be expected to represent the sum of intracellular and extracellular components (Table 4.3.8). Intracellular and extracellular cadmium uptakes were found to differ in their kinetic parameters in *R. squarrosus* (Brown and Beckett, 1985; Wells and Brown, 1987). However, intracellular uptake is likely to represent a negligible proportion of the total uptake of Fe (see Section 4.3.2.1) and it can be assumed that the kinetic parameters will be mainly attributable to the exchangeable component.

The parameters A_{max} and $½_{max}$ represent the maximum total Fe uptake for the tissues and the concentration of Fe in the incubation medium which will give half of A_{max}, respectively, under the experimental conditions used. A_{max} and $½_{max}$ for stems (10.2 mg g^{-1} and 34.0 µg) are significantly smaller ($P<0.001$) than those of leaves (17.4 mg g^{-1} and 386 µg) and whole shoots (15.57 mg g^{-1} and 663.4 µg). The estimated A_{max} for whole moss shoots is intermediate between those for leaves and stems. The rate of extracellular cation exchange was observed by Pickering and Puia (1969) and Brown and Beckett (1985) to be concentration dependent. Therefore, depletion of the Fe concentration

Fig. 4.3.12 The relationship between total iron uptake by (a) whole shoots, (b) leaves, (c) stems of R. squarrosus and Fe ($FeSO_4$) concentration

The curves fitted are single exponentials

Fig. 4.3.12

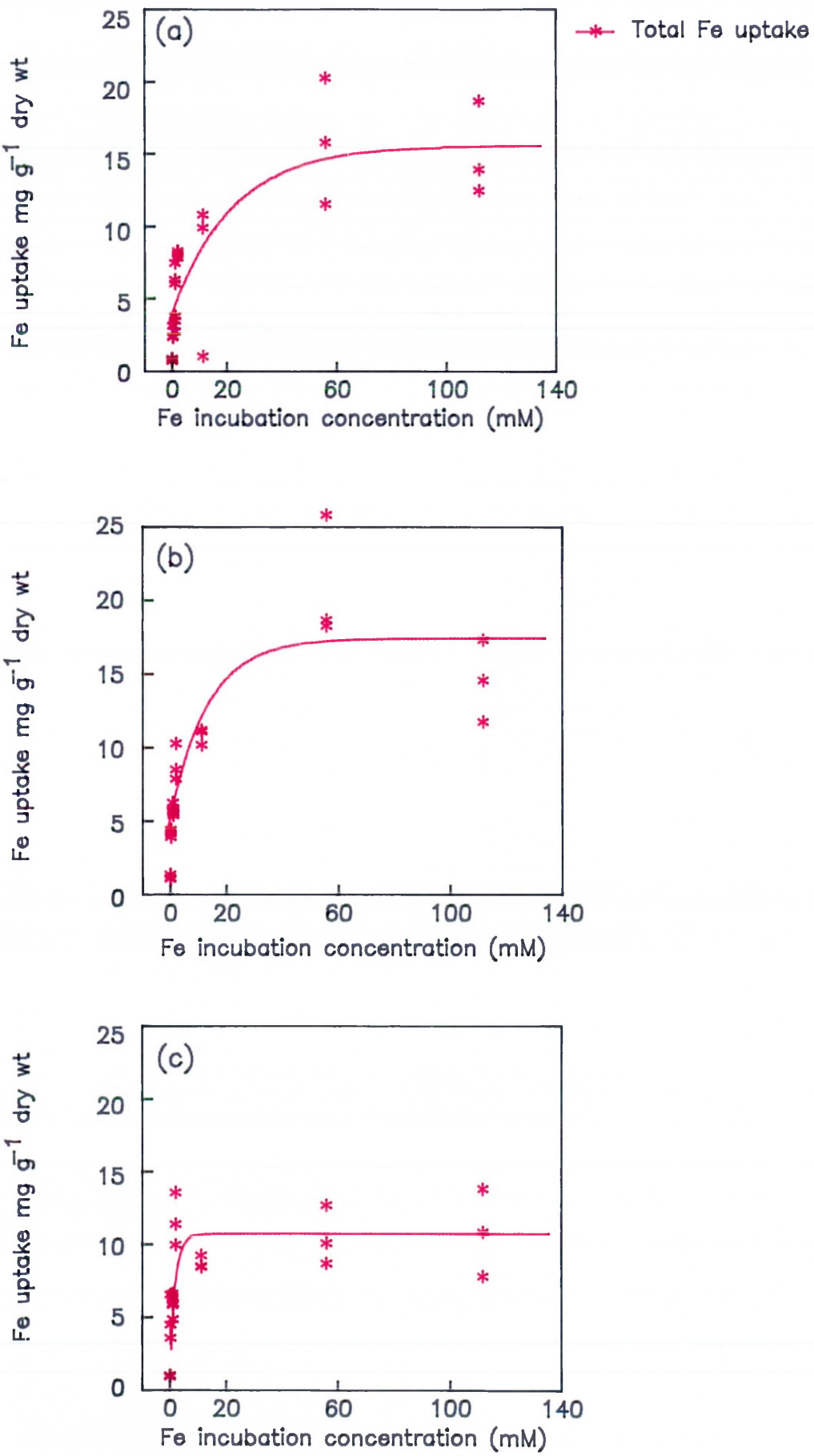

(a)
(b)
(c)
Total Fe uptake
Fe uptake mg g^{-1} dry wt
Fe incubation concentration (mM)
0
5
10
15
20
25
0
20
60
100
140

Table 4.3.8 Kinetic parameters for total iron uptake from $FeSO_4$ solutions (20 µM to 110 mM Fe) by leaves, stem and whole shoots of **R. squarrosus**

Tissue	Curve	Maximum iron uptake at equilibrium (µg g^{-1} dry wt)	½ max (µg Fe l^{-1})	R^2	n
Leaves	S	17466.23 (786.6)	386.8 (95.08)	0.80	24
Stems	S	10224.45 (654.61)	34.95 (9.827)	0.674	24
Whole shoots	S	15575.4 (1369.5)	663.46 (313.9)	0.805	24

S single exponential origin not through zero
Figures in parentheses are standard errors of the means

in the incubation medium should depress further uptake. The effect of depleted concentration on Fe uptake rates may be greater for leaves and whole moss shoots which possess higher A_{max} values than stems.

A_{max} for Fe uptake by whole moss shoots (Table 4.3.8) was greater than reported by Brown and Beckett (1985) and Wells and Brown (1987) for cadmium uptake by *R. squarrosus* (Table 4.3.6). These differences could be attributable to electrochemical charge following oxidation of the ferrous ions. However, binding of cations by the lichen *Usnea florida* was shown by Wainwright and Beckett (1975) to be independent of electrochemical charge, implying that only one charge was utilised in binding. In contrast Nieboer, Lavoie, Sasseville, Puckett and Richardson (1976a) noted that cation exchange in the lichen *Umbilicaria muhlenbergii* was consistent with an ion exchange process where the displaced charges are balanced. Different affinities for the binding sites of *Hylocomium splendens* have also been shown to exist between

cations, (Cu, Pb > Ni > Co > Zn, Mn) by Ruhling and Tyler (1970). Clymo (1963) showed selectivity to cation species for different exchange sites in *Sphagnum* spp which hindered accurate measurement of exchange capacity employing only a single ion. This selectivity effect may be due to cellular uptake as suggested by Nieboer *et al.* (1976a). Differences in total cation exchange capacity of mosses can also be attributed to population source, environment (Clymo, 1963) and storage conditions prior to experimentation (Wells and Brown, 1987). However, the greater total Fe uptake by *R. squarrosus* determined in this experiment, compared with those for cadmium obtained by Brown and Beckett (1985) and Wells and Brown (1987), can be related to the long incubation period (24 h). Pickering and Puia (1969) showed that 50% of zinc uptake by the aquatic moss *Fontinalis antipyretica* occurred during the first half hour, from a 0.5 mM $ZnSO_4$ solution, but that maximum uptake was not achieved until *ca.* 5 days. Also, there may be exchange of Fe to sites in the moss tissue not available to other metal ions, as described by Büscher (1986).

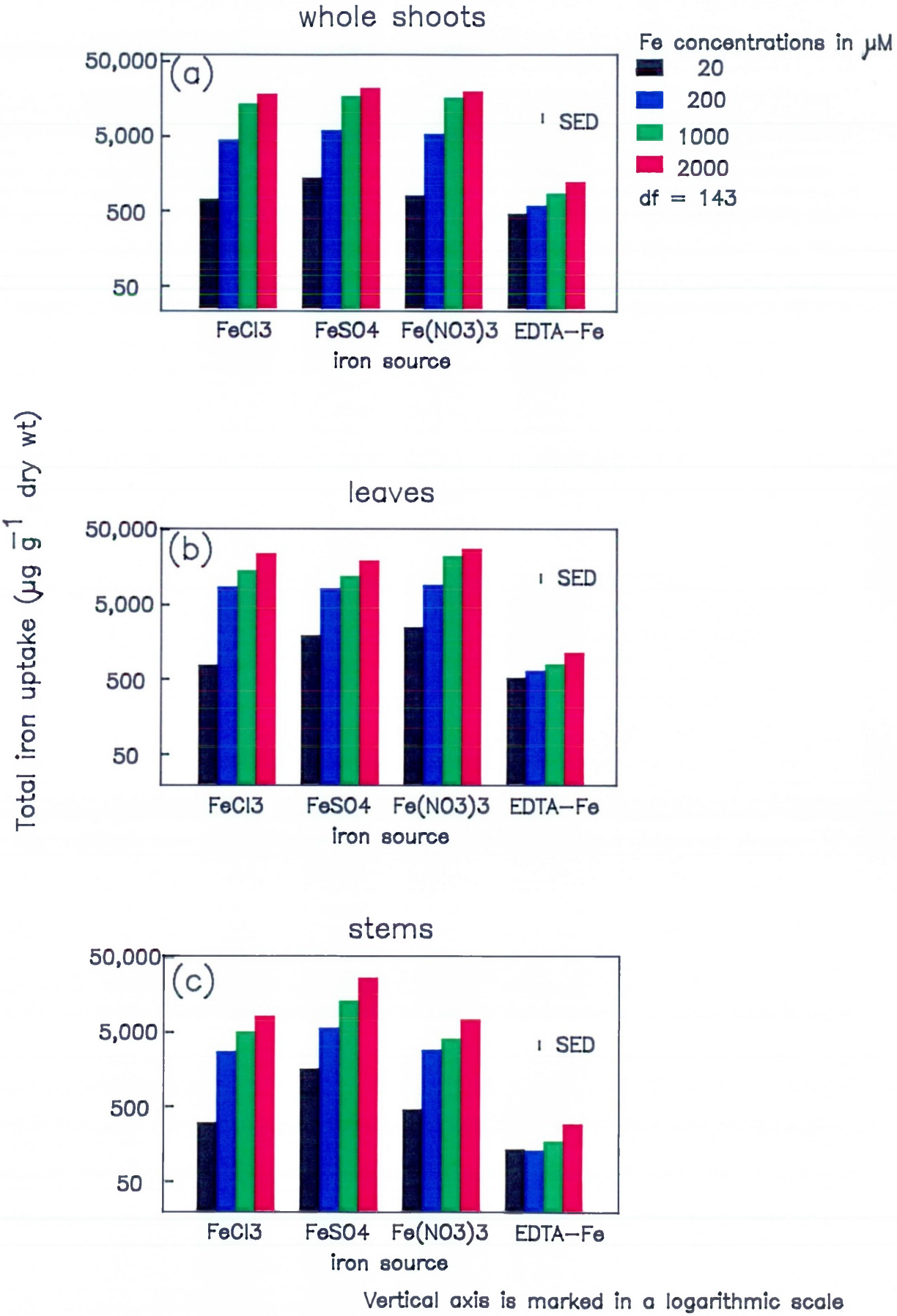
4.3.2.4 The influence of the form of iron supplied on total uptake

Ferrous suphate is the iron salt commercially used for the control of moss in lawns. The ability of *R. squarrosus* to absorb Fe from solutions of this salt was examined in the previous Section 4.3.2.3. A range of soluble iron salts including ferrous sulphate, ferric chloride, ferric nitrate and chelated iron in the form of the ferric monosodium salt of ethylenediaminetetra-acetic acid (EDTA) was chosen to study the effects of the anionic component.

The pH of the iron solutions differed considerably (Table 4.2.1), therefore affecting the dissociation of the iron salts. The anionic binding sites of the moss cell wall might also be affected by the pH of

Fig. 4.3.13 Total iron uptake by (a) whole, (b) leaves, (c) stems of R. squarrosus from different iron sources

Fig. 4.3.13



Vertical axis is marked in a logarithmic scale

the incubation solution, affecting their affinity for Fe (Section 4.2.1).

Analysis of variance of the data (Fig. 4.3.13) indicates significant differences in iron uptake between tissue types and iron salts ($p<0.001$).

Uptake by stems was significantly greater for $FeSO_4$ than for $FeCl_3$, $Fe(NO_3)_3$ or EDTA although this difference was not observed for the leaves or whole moss shoots (Fig. 4.3.13).

Low uptake from solutions of EDTA is probably related to the high stability of the chelated iron complex (stability constant = 14.3) and resultant low availablity of Fe.

It is unlikely that the greater Fe uptake by leaves compared to stems and whole moss shoots (Fig. 4.3.13) is due solely to exhaustion of Fe from the incubation solutions because this pattern was also observed in the EDTA treatments where Fe was certainly not exhausted. The greater uptake of Fe from $FeSO_4$ by stems may result from the higher pH of the $FeSO_4$ solutions compared to $Fe(NO_3)_3$ and $FeCl_3$, thereby increasing ionisation of the binding sites (Bates, 1982; Büscher, 1986). Wainwright and Beckett (1975) also observed increased zinc uptake from the lichen *Usnea florida* with increasing pH of the medium. A similar effect on cadmium uptake from the acetate salt, of slightly higher pH than other salts used, was observed by Wells and Brown (1985). Uptake of Fe by *R. squarrosus* tissue, therefore, showed little difference between iron salts except that poor uptake occurred from EDTA solutions. No major difference was found between ferrous and ferric salts, suggesting that the ferrous ion is rapidly oxidised to the ferric form.

4.3.3 2,4-D uptake by R. squarrosus

2,4-D uptake by *R. squarrosus* was studied to determine whether the limited herbicidal activity observed under field conditions, (Ch. 2) was due to poor uptake. The following experiments were designed 1) to determine if 2,4-D became exchangeably bound in *R. squarrosus* and 2) to measure intracellular 2,4-D uptake.

4.3.3.1 2,4-D exchange capacity of R. squarrosus tissue

Previous investigations (Sections 4.3.1.5 and 4.3.2.2) of uptake of paraquat and Fe by *R. squarrosus* determined the quantities exchanged to the cell walls. 2,4-D is an anionic compound and the anionic binding capacity of mosses is known to be small (Clymo, 1963). Initial experiments using the ^{14}C-labelled anion 2,4-D were designed to quantify the anionic exchange capacity of *R. squarrosus* shoots using a similar procedure to that adapted for ^{14}C-paraquat.

Passive binding of 2,4-D was studied by examining uptake in tissues killed by boiling. These were incubated in ^{14}C-2,4-D solutions. The pH values of the incubating solutions are presented in Table 4.2.1. The pK_a of 2,4-D is 2.8, therefore in the pH range used (5.9 - 7.5) 2,4-D will be largely dissociated.

Table 4.3.9 Percentage uptake of 2,4-D by dead (boiled) R. squarrosus tissue

2,4-D incubation concentration	% uptake (% total supplied)	S.D.
75 µM	0.50	0.103
750 µM	0.58	0.157
7.5 mM	0.78	0.124
75 mM	0.81	0.196
150 mM	0.56	0.202

After incubation for 24h in 2,4-D (75 µM - 150 mM) followed by two distilled water washes, the shoots were solubilized and ^{14}C-2,4-D was determined using liquid scintillation counting (Section 4.2.6). The values for uptake are estimates of the total anion exchange capacity for the tissues and were found to be very small, equivalent to less than 1% uptake of the total ^{14}C-2,4-D supplied (Table 4.3.9). Accurate determination of the anionic exchange capacity of *R. squarrosus* was not possible due to the very low percentages of exchanged ^{14}C-2,4-D measured. Clymo (1963) also found low values of anion exchange in *Sphagnum* spp, 460-fold lower than cation exchange capacity.

4.3.3.2 Intracellular uptake and anionic exchange of 2,4-D

Intracellular and exchangeable uptakes of ^{14}C-2,4-D by living leaves, stems and whole moss shoots of *R. squarrosus*, after incubation in 7.5 mM 2,4-D, were very low (Table 4.3.10). Results are presented as dpm per sample due to their very low activity and the large inaccuracies associated with correction of such values into specific 2,4-D weights. The uptakes of ^{14}C-2,4-D into the residual and intracellular fractions, expressed as percentages of the total supplied in solution, were between 0.02 - 0.25%. Results for the exchangeable 2,4-D fraction were beyond the detection limits. A significantly ($p < 0.001$) greater quantity of 2,4-D entered the intracellular fraction than was detected in the residual fraction. Uptake into both fractions increased with incubation period in 2,4-D (Table 4.3.10).

The absence of a detectable 2,4-D exchange fraction confirms the results of the previous experiments. The values presented here for *R. squarrosus* intracellular uptake, although small, exceed the external exchange. This is in complete contrast to the paraquat results (Section 4.3.1.6) where intracellular uptake was small compared to the exchangeable binding.

Table 4.3.10 Intracellular and residual intracellular uptake (dpm) of 2,4-D (7.5 mM) by leaves, stems and whole shoots of R. squarrosus

Tissue	Incubation Time 10 mins	2 h	24 h	48 h	72 h	96 h
Leaves						
Intracellular (soluble)	16.9	27.7	28.9	44.5	36.0	36.1
Intracellular (residual)	3.9	14.2	5.6	11.8	14.8	8.8
Stems						
Intracellular (soluble)	18.1	39.1	40.4	52.1	34.7	30.5
Intracellular (residual)	4.5	11.5	14.2	15.8	42.5	8.6
Whole shoots						
Intracellular (soluble)	19.7	34.9	27.3	43.6	38.4	47.5
Intracellular (residual)	7.5	17.4	20.5	22.3	45.4	31.1

ln SED 0.3277; df = 141; n = 4

(1) A cell wall exchangeable fraction for 2,4-D was not detected.
(2) Total dpm supplied ca. 12000.

Penetration of herbicides through the cuticle into the cells of vascular plants can occur via a lipoidal or aqueous route. 2,4-D is believed to penetrate vascular plant shoots and leaves through the lipoidal route, principally in an undissociated, non polar form. However, it has also been proposed that 2,4-D penetrates via an aqueous route because uptake increases with increased humidity (Bovey and Young, 1980). The minimal 2,4-D uptake results for *R. squarrosus* show that this aqueous route is unlikely to exist.

2,4-D uptake by *Ricinus communis* roots and barley protoplasts has been studied by Briggs, Rigitano and Bromilow (1987) and Rigitano, Bromilow, Briggs and Chamberlain (1987), respectively. They showed an increase in 2,4-D concentration in the protoplasm of barley and *Ricinus communis* roots with decreased pH of the incubating solution.

Penetration of plant membranes by undissociated 2,4-D is believed to be primarily by diffusion. On penetration of the membrane, dissociation of the molecule occurs due to the lower pH of the protoplasm compared with the bathing solution. This is believed to prevent the ready movement of the anions out of the cells (Rigitano *et al.*, 1987). In the present study, the pH of the 7.5 mM 2,4-D solutions (Na salt, pK_a 2.8) was 6.05, at which point 2,4-D would be largely present in the dissociated form. Limited uptake of 2,4-D would therefore be expected by *R. squarrosus* if it resembles vascular plants in predominantly absorbing the undissociated molecule (Briggs, Rigitano and Bromilow, 1987).

The uptake of anions by the moss *Hylocomium splendens* was studied by Wells and Richardson (1985); they obtained enhanced uptake of arsenate as the monocharged anion compared with the undissociated molecule. In this case they discounted the possibility of uptake by diffusion due to the hydrophobic core of the plasmalemma acting as a barrier to the passage of charged species. Although uptake was greatest as the monocharged ion, some uptake occurred at lower pH where the molecule was largely dissociated and therefore poly-charged. This was attributed to diffusion. In contrast, Wells and Richardson (1985) also found that the uptake of selenite was only affected by pH when the undissociated molecule was present in solution. Uptake of selenate could not be detected at all. The conclusion from anion competition experiments was that arsenate and selenite in *H. splendens* were accumulated via two different transport systems located at the plasmalemma (Wells and Richardson, 1985). Assuming similarities in the physiology of *R. squarrosus* and *H. splendens*, the 2,4-D anions do not appear to be accumulated via the anion uptake systems hypothesised by Wells and Richardson (1985) for the uptake of arsenate and selenite.

The low level of 2,4-D detected in the intracellular fraction of *R. squarrosus*, therefore, probably results from uptake of the very low levels of the undissociated molecule present in the incubation solution at pH 6.05.

An increase in 2,4-D recovered in the residual fraction of *R. squarrosus* was noted with time (Table 4.3.10). This was also apparent in experiments by Jenner, Saunders and Blackman (1968) on uptake of 2,4-D by segments of *Avena* sp. mesocotyl. In *Avena* sp. continuing uptake into the residual fraction was attributed to the formation of metabolic products. Furthermore, the accumulation of these metabolic products, and therefore their absence in the freely mobile intracellular fraction, was believed to promote further intracellular uptake (Jenner *et al.*, 1968).

The application of 2,4-D to mosses in the field experiments may, therefore, benefit from regulation of the pH of the formulation. Although pH regulation has proved an important aspect of laboratory and glasshouse studies of 2,4-D uptake, little benefit has been obtained in field experiments with vascular plants (Bovey and Young, 1980).

4.3.4 Dichlorophen uptake by R. squarrosus

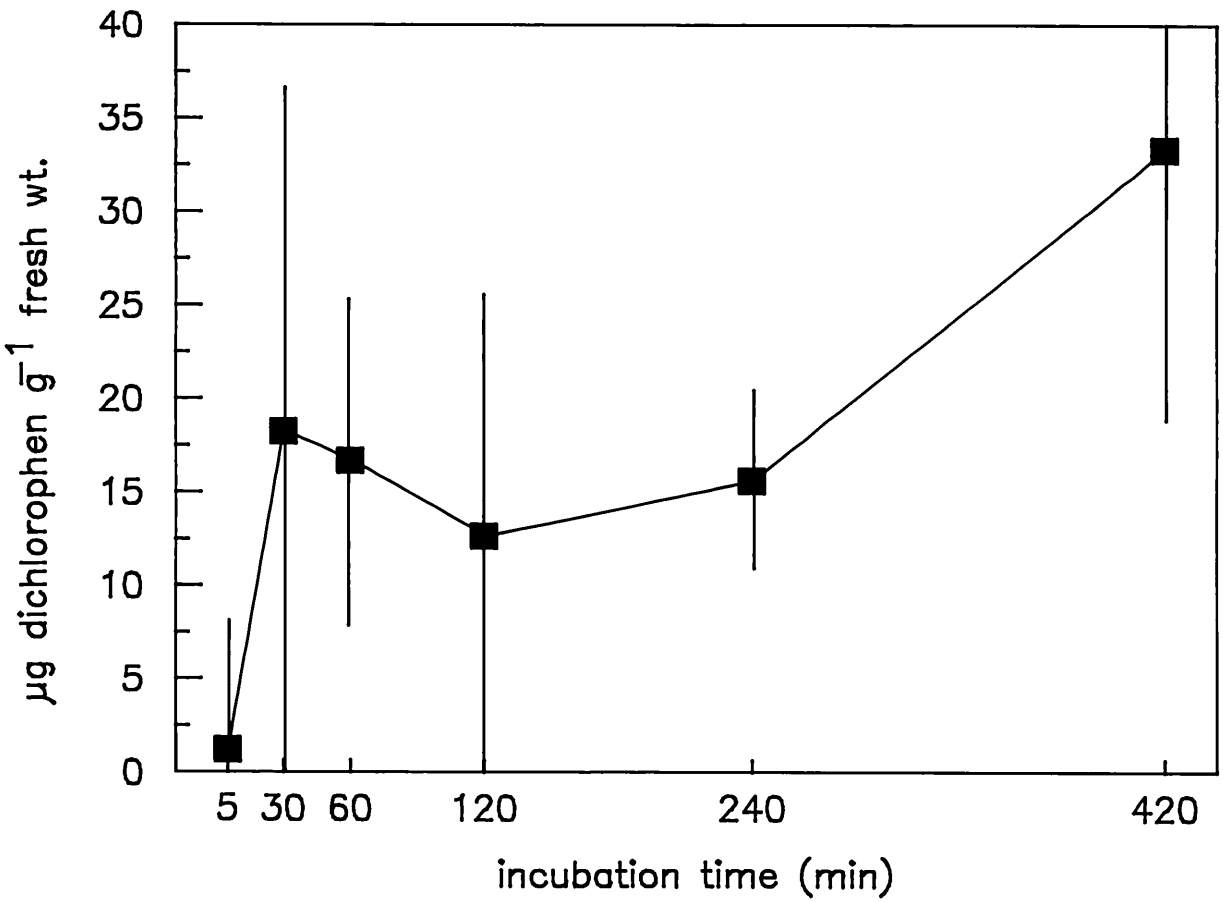
Dichlorophen caused rapid bleaching of *R. squarrosus* during field trials (Sections 2.3.1 and 2.3.3) suggesting rapid uptake of the herbicide. The following experiment was designed to measure dichlorophen uptake by *R. squarrosus* whole moss shoots. Analysis of dichlorophen was by the spectrophotometric technique of Clements and Newburger (1954).

R. squarrosus tissues were incubated in 60 µM dichlorophen solutions (pH 8.1). A 60 µM concentration was chosen as it could be determined accurately using UV spectrophotometry. Unfortunately, leakage of cell contents into the incubation solution (Brown, Ougham

and Beckett, 1986) interfered with the detection of dichlorophen. Attempts to reduce the interference were made by either adding known quantities of dichlorophen to the solutions or by subtracting the interference caused using a spectrophotometer blank containing moss extract. However, both techniques were insufficiently sensitive for accurate dichlorophen measurements. In spite of these problems, it was apparent that uptake of dichlorophen increased significantly ($p<0.05$) with incubation period (Fig. 4.3.14).

Dichlorophen is capable of entering the cells of *R. squarrosus* and its mode of uptake may be similar to that proposed for the entry of 2,4-D into vascular plants as the undissociated molecule (Section 4.3.3.2). Dichlorophen is formulated as the mono- and di-sodium salt for use in moss killers. The pK_a values for the two salts are 7.6 and 11.5, respectively. The unbuffered pH of the dichlorophen solution used was 8.1. The monosodium salt will, therefore, exist as the undissociated molecule to a large extent but the disodium salt will be highly dissociated. Thus from a theoretical viewpoint, dichlorophen is likely to give better uptake through cell membranes than 2,4-D. However, the intracellular pH is not likely to be high enough to cause significant dissociation of dichlorophen as was suggested for 2,4-D (Rigitano *et al.*, 1987). This situation might be expected to favour passive outward diffusion of dichlorophen when external levels fall. The loss of adsorbed organochlorines from *Pleurozium schreberi* exposed to low external concentration has been demonstrated by Larsen, Lokke and Rasmussen (1985). They described the accumulation of organochlorines as a dynamic equilibrium in contrast with the passive equilibrium process of metal accumulation.

Fig. 4.3.14
Uptake (+/− sd) of dichlorophen (conc. 60µM) by *Rhytidiadelphus squarrosus*.



4.4 CONCLUSIONS

Uptake of paraquat, iron, 2,4-D and dichlorophen by *R. squarrosus* tissues was studied in the laboratory using a range of solution concentrations and incubation periods.

The cations paraquat and iron were rapidly bound to extracellular binding sites and only small quantities were accumulated intracellularly in the moss tissue. In general, combining the paraquat uptake results for the separated leaves and stems of *R. squarrosus* gave an overall uptake profile resembling that for complete moss segments, and a similar pattern was observed with iron. The cation exchange capacity of the moss tissue was not increased following disruption of the cells, indicating that the majority of binding sites are extracellularly located.

Paraquat uptake into the stem tissue of the moss segments was impaired by the surrounding leaf tissue. Therefore, with an inadequate supply of paraquat in the incubation medium, binding of the herbicide was limited to the more accessible exchange sites of the leaves. Protection afforded by the leaves may enable regrowth to occur from undamaged regions of the stems following paraquat application.

Intracellular uptake and extracellular exchange of paraquat by leaves, stems and shoots of *R. squarrosus* can be adequately described by a single exponential curve. Kinetic studies showed that leaves and whole shoots possess greater paraquat exchange capacities than stems but total uptake (all fractions combined) of paraquat was greater in stems than in leaves. Intracellular uptake was similar in the different tissue types. During a 96 h incubation, redistribution of paraquat from the extracellular to intracellular sites occurred and this may be responsible for the slow herbicidal effect of this chemical.

Iron and paraquat possibly utilize the same extracellular and intracellular uptake mechanisms, as pre-incubation of moss tissue in iron solutions inhibited the extracellular and intracellular uptake of paraquat. Varying the iron salt did not significantly affect total iron uptake unless the iron was presented in chelated form, where uptake was lower, or the pH of the solution changed. The absence of an uptake difference between the ferrous and ferric salt solutions was probably due to the rapid oxidation of the former.

The uptakes of 2,4-D and dichlorophen were small in comparison with those of paraquat and iron. Dichlorophen, however, was capable of producing extensive cell damage indicated by the loss of intracellular solutes which interfered with its spectrophotometric detection. Uptake of 2,4-D by *R. squarrosus* might be improved by the use of formulations of low pH to increase the proportion of the undissociated molecule in solution, 2,4-D uptake having been shown in vascular plants to be greater in the undissociated form, thereby accumulating within the protoplasm via a lipoidal route (Rigitano *et al.*, 1987).

Under field conditions cationic herbicides can be expected to bind predominantly in an extracellular manner, particularly to leaves, and this may limit their impact. Extracellularly bound paraquat, however, is progressively transferred to the protoplasts. The anionic herbicides 2,4-D and dichlorophen, by comparison, do not bind in large quantities to moss cell walls and their action appears to be dependent on immediate uptake. Dichlorophen may be directly damaging to the plasmalemma so that cellular uptake is not a prerequisite for herbicidal action.

CHAPTER 5

COMPARTMENTAL ANALYSIS OF PARAQUAT UPTAKE IN

RHYTIDIADELPHUS SQUARROSUS

5.1 INTRODUCTION

Herbicide selectivity or resistance can be attributable, in some cases, to an inability of the compound to penetrate plant cells (Orwick, Schreiber and Hodges, 1976). To improve our understanding of herbicide effects and biological performance, a knowledge of the subcellular distribution, concentrations and permeabilities of herbicides in the plant tissue is required.

The majority of uptake experiments in moss tissue use the empirical method of Brown and Buck (1979). Intracellular uptake can be defined as the quantity of chemical remaining after subtraction of the extracellular components (Brown and Beckett, 1985; Wells and Brown, 1987) or as the quantity of the chemical released by boiling of the tissue to fracture the cells (Brown and Bates, 1972; Bates and Brown, 1974; Bates, 1987). An alternative approach for quantifying uptake and subcellular distribution of chemicals is that of compartmentation by influx and efflux studies. The technique is based on the equations of MacRobbie and Dainty (1958) for the movement of inorganic ions in the cells of the alga *Nitellopsis obtusa,* and involves the determination of rate constants between the cells and bathing solution. Three phases were identified in *N. obtusa*, the free space and two components of the non-free space, the cytoplasm and vacuole. Models with four compartments have been derived in work on giant algal cells where the fourth compartment represents plastids (Walker and Pitman, 1976). The concentration in each phase can be determined by the analysis of an isotope diffusing into a series of non-radioactive solutions from tissue previously treated in a radioactive solution. Measurements of herbicide fluxes and their distribution across membranes of vascular plant tissues have been made by Pillmoor, Roberts and Gaunt, (1982); Orwick, Schreiber and Hodges, (1976); Balke and Price, (1988). Related techniques have been used on moss tissue by Pickering and Puia (1969)

and Clymo (1963) to determine the mechanism of zinc uptake by *Fontinalis antipyretica* and sodium and calcium distribution in *Sphagnum papillosum*, respectively. In addition, Lüttge and Bauer (1968) studied ion uptake isotherms and individual ion fluxes of KCl solutions containing ^{86}Rb-label in *Mnium cuspidatum*.

The technique of efflux analysis has been used for the analysis of cellular locations of many different substances in plant tissues, for example, the subcellular location of inorganic ions (MacRobbie and Dainty, 1958; MacRobbie, 1969), flamprop metabolite distribution (Pillmoor, Roberts and Gaunt, 1982), herbicides in resistance/ selectivity studies (Orwick, Schreiber and Hodges 1976; Bland and Brian, 1975), gibberellins (Drake, 1979; Drake and Carr, 1981), model systemic compounds (Price, 1973) and host specific pathotoxins (Keck and Hodges, 1973).

Membrane permeability of flax leaves was shown by Dodge and Harris (1970) to increase following paraquat treatment. The experiments described in this chapter were designed to investigate the effects of paraquat on membrane permeability of *R. squarrosus* tissue and its quantitative intracellular distribution. Paraquat was chosen for this study as previous experiments (Section 4.3.1) showed efficient uptake by *R. squarrosus*. In addition, a sensitive analytical method was available for paraquat detection. To provide comparative data, efflux analysis was also performed on leaf slices of *Lolium perenne*. Calculations of the Nernst potential for the paraquat-treated tissues were also made for comparison with the known electrochemical potentials of other plant species and for determinations of hypothetical transport mechanisms.

5.1.1 Principles of efflux analysis

Estimates of the ion concentration in subcellular compartments are made by observing the kinetics of the absorption or elution of radioactive tracers by homogeneous samples of tissue in which inward and outward fluxes are approaching a steady state. Healthy and mature cells, which are no longer growing, come to a state of flux equilibrium if left in a solution where concentration is kept constant. Under these conditions it is assumed that the net flux of a given tissue is zero.

At equilibrium, influx and efflux can be measured. Influx is a measure of the initial rates of increase in cellular ionic activity following placement of the tissue in a tracer solution of known specific activity. Efflux is measured by loading the cells for a number of hours with tracer in a labelled solution, washing quickly with water and replacing in a solution of similar ionic concentration but lacking radioactive tracer. The external solution is regularly changed so that the external concentration of isotope remains at zero. Therefore, the radioactivity within the cells gradually decreases. The internal concentration of the ion remains constant during efflux studies. The loss of radioactivity across the cells is normally exponential indicating that a constant proportion of the remaining isotope is lost in each unit of time. In a one-compartment system, the plot of the natural logarithm of the loss of tracer against time, will therefore, result in a straight line whose slope represents the rate of efflux.

A major assumption in these experiments is that no membrane selectivity occurs for the unlabelled or labelled eluted materials. The high cation exchange capacity of plant material also complicates the interpretation of the data. For cations this will lead to an initial rapid efflux following the introduction of the unlabelled

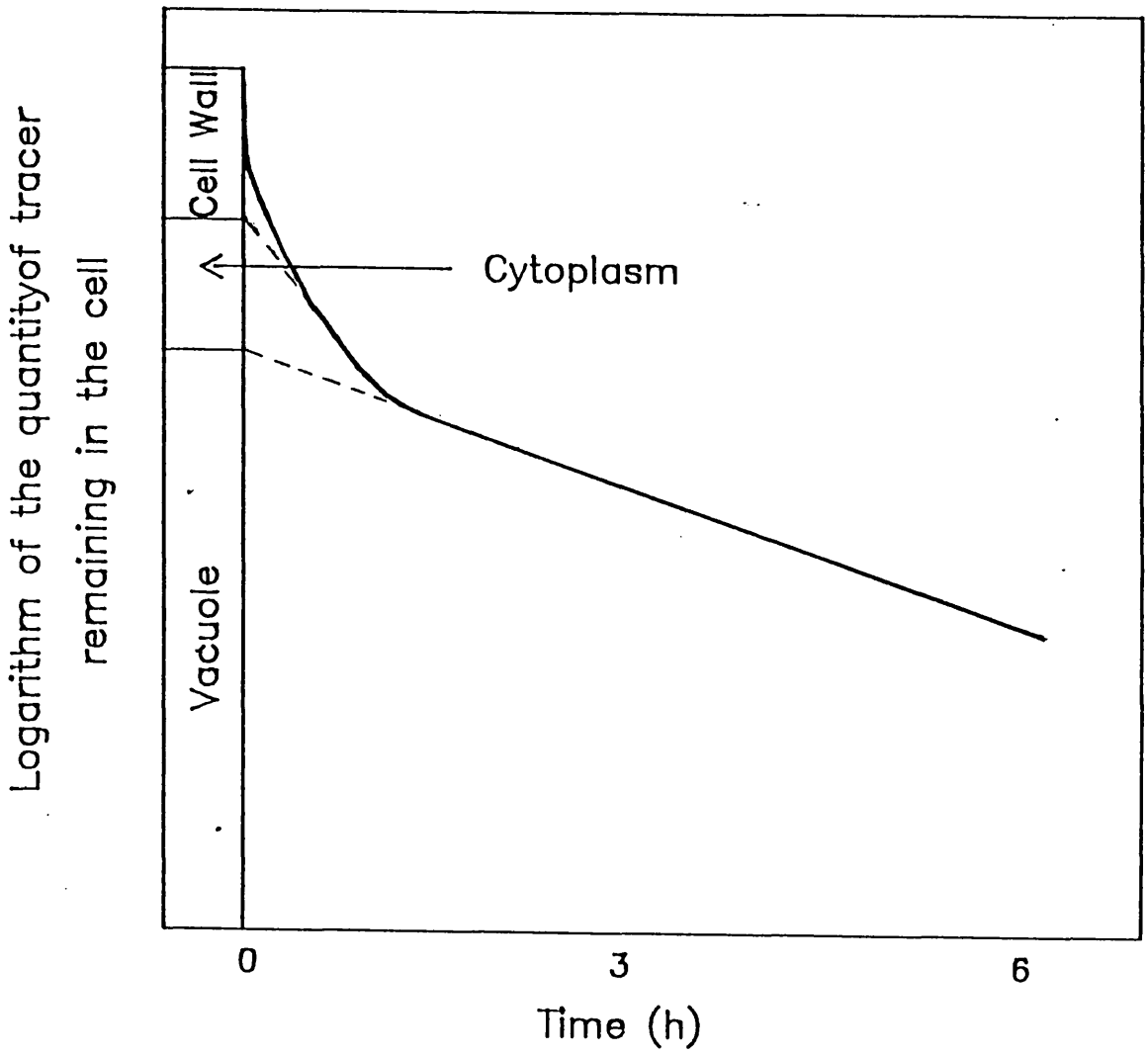


Fig. 5.1.1 Illustration of various phases in the elution of a tracer from a labelled cell into a tracer-free medium (Clarkson, 1974)

medium. The plot of the total isotope remaining in the cells, following the exchange process, is usually curvilinear (Fig. 5.1.1). Extrapolation of the linear portion to the ordinate gives a measure of the total quantity of isotope accumulated at the exchange sites of the cell wall.

If the flux of isotope across the plasmalemma and tonoplast differs, then the efflux curve resolves into two components. Extrapolation of these two linear components of the curve gives estimates of the concentrations in the vacuole and cytoplasm and rates of efflux across the respective membranes.

The sum of the radioactivities eluted at each stage and of the residual labelled fraction equals the activity in the cells at efflux time zero. Subtracting the activity in each elution sample from the total activity in the tissue at the start of the elution gives the quantity of isotope remaining in the tissue at each time interval.

5.1.2 Interpretation of results obtained from efflux analysis

The efflux of tracer from a previously labelled, multicompartment tissue is the sum of several exponential processes. A hypothetical plot of the natural logarithm of the quantity of radioactivity in the tissue against time is presented in Fig. 5.1.1. When the plot of the quantity reaching the bathing medium, and therefore leaving the tissue, is non-linear then it is reasonable to assume that there is more than one compartment. In the example (Fig 5.1.1), the curve can be resolved into three components which have logarithmic rates of elution.

Normally, towards the end of the experiment (Fig. 5.1.1), the curve becomes linear and is assumed to be derived from one compartment. If a constant rate of efflux from this compartment is assumed then the linear portion of the curve can be extrapolated back to the y axis. The y axis intercept, in ln dpm, is transformed back to dpm and

subtracted from the remaining data points (dpm), not included in the linear section of the curve. A second curve can be plotted of the logarithmically transformed data. Extrapolation of the linear component of the first curve relates to the vacuole (Fig. 5.1.1). Extrapolation, as above, of the remaining radioactivity in the tissues is normally attributable to efflux from the cytoplasm (Fig. 5.1.1). The final logarithmic plot defines the exchangeable and free space components (Fig. 5.1.1). The y-axis intercepts for each straight line component plot represent the total radioactivity present in the individual compartments at the start of the analysis. The half times for the rates of efflux from each compartment are based on the slopes of the linear components.

The efflux rates are influenced by temperature (except for that of the exchangeable component), tissue permeability, intracellular and extracellular concentration, permeability of cell membranes, the electrochemical potential of the cellular compartments and the chemical properties of the eluting molecule.

Efflux experiments may also be based on the principle of serial compartments (Fig. 5.1.2) as opposed to parallel compartments (Fig. 5.1.2). Serial compartments describe the flux of molecules along a fixed pathway of compartments. Parallel compartments describe the movement of molecules between any compartment such as between the tonoplast and external medium without traversing the cytoplasm. An example of parallel compartments was described by MacRobbie (1971) as vesicular transport of ions to, or from, the tonoplast. This mechanism of ion transfer is, however, considered to be relatively unimportant compared to the serial component.

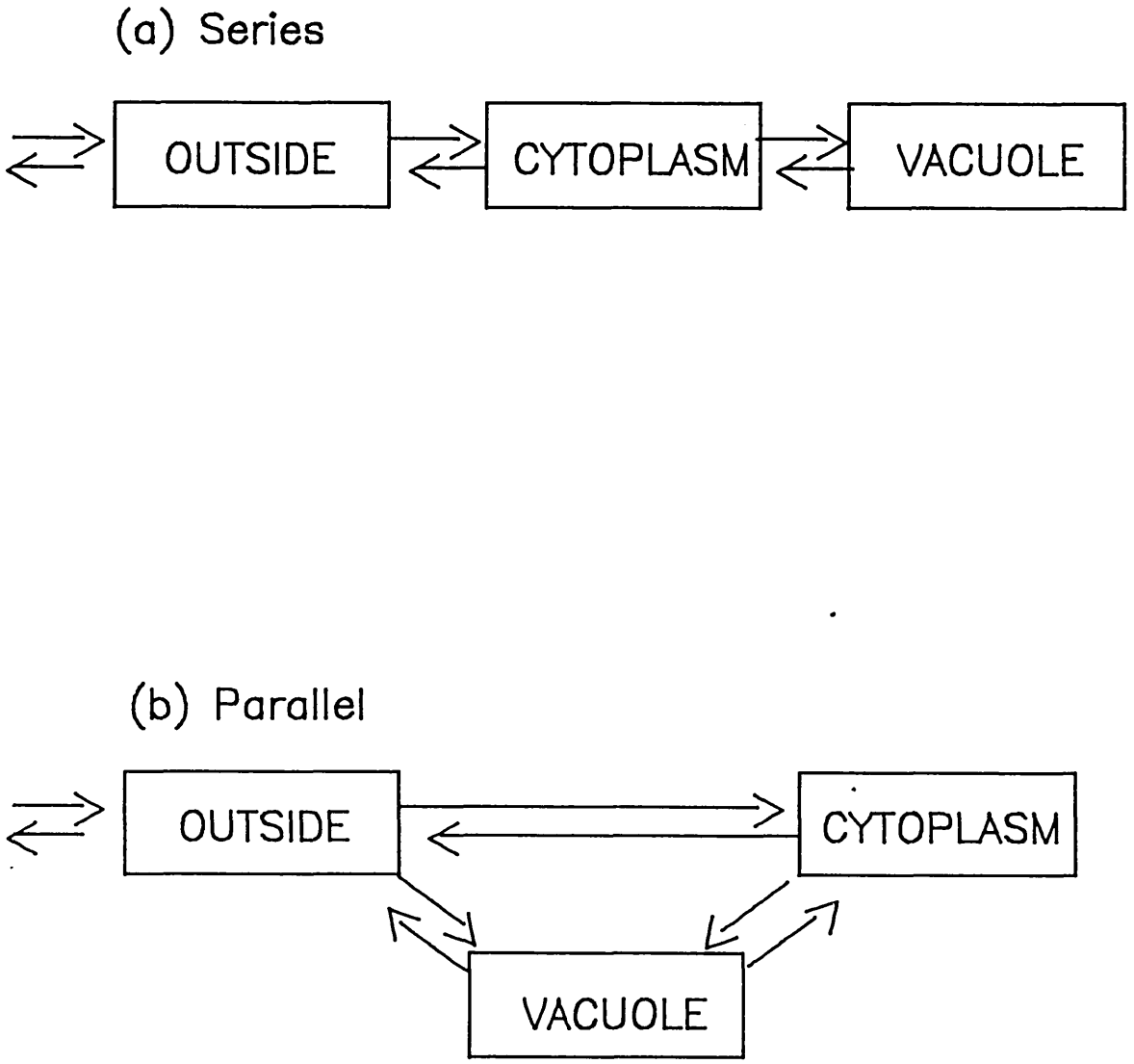


Fig. 5.1.2 Diagrammatic representation of (a) series and (b) parallel associations in a three compartment system (solution, cytoplasm and vacuole). The arrows indicate the direction of fluxes

5.2 MATERIALS AND METHODS

5.2.1 Plant material

R. squarrosus tissue was collected and stored as described in Section 3.2.1.

Lolium perenne was grown from seed in 6 inch pots containing John Innes No. 2 compost raised under glasshouse conditions. Natural lighting was supplemented with quartz halogen lamps to give a 14 h photoperiod and the temperature was maintained at *ca.* 15°C.

The youngest fully mature leaves from 5 wk old plants of *L. perenne* were sectioned into 400 µm leaf slices using a gel slicer (Joyce Loebl and Co. Ltd). To prevent excessive damage to the leaf tissue during sectioning, and to enable multiple sectioning, ten 2 cm segments of leaf tissue were supported in soft polystyrene and sectioned simultaneously. Leaf slices were transferred immediately to vials containing distilled water. Each experimental vial contained 100 leaf slices.

Whole shoot segments of *R. squarrosus* and leaf slices of *L. perenne* were incubated in 5 ml of distilled water for 1 h prior to loading with ^{14}C-paraquat.

5.2.2 Chemicals

The unlabelled and ^{14}C-paraquat solutions are described in Sections 2.2.5 and 4.2.2.

5.2.3 Loading and efflux of tissues

All tissues were incubated for 16 h in 5 ml of 72.5 or 725 µM ^{14}C-paraquat.

After loading, the incubation medium was removed and the tissues quickly washed in 5 ml of distilled water to remove the remaining ^{14}C-paraquat not associated with the tissues. The efflux process was

then initiated by the the addition of 5 ml of eluant and its regular replacement at intervals over 7 h. In general the elution times for the experiments were 2, 5, 7, 10, 15, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180, 210, 240, 270, 300, 330, 360 and 420 min. The eluants used in these experiments were water (commonly used in herbicide efflux studies in vascular plant tissue (Price, 1978; Orwick, Schreiber and Hodges, 1976; Pillmoor, Roberts and Gaunt, 1982)), and unlabelled paraquat. Eluant was removed and replaced using the technique described in Section 4.2.4. Throughout the loading and efflux period the vials were incubated at 20°C, received 40 μmol m^{-2} h^{-1} irradiance and were continuously shaken.

After elution, the moss tissues were washed three times in 5 ml of unlabelled paraquat, extracted with boiling distilled water and then solubilized using 'NCS' tissue solubilizer as described in Section 4.2.5.1. The *L. perenne* leaf slices were frozen and then thawed to disrupt the cell membranes and extracted three times with distilled water to remove the intracellular paraquat component. The leaf slices were then solubilized using the same procedure as for *R. squarrosus*.

Radioactivity measurements of all efflux extracts, incubation solutions and tissue extracts, were by liquid scintillation counting as described in Section 4.2.6.

5.2.4 Modifications to experimental procedure

Incorporation of nutrients and/or buffers into the solutions was avoided in the experiments described in this Chapter. Other users of the efflux analysis technique have used buffered solutions (Pillmoor *et al.*, 1982), salt/nutrient solutions (MacRobbie, 1969; Keck and Hodges, 1973), calcium solutions (Lüttge and Bauer, 1968) or simply a solution of the chemical under study (Pitman, 1963; Balke and Price, 1988). The study of cation uptake by moss tissues has generally involved the

use of dilute solutions of the appropriate salts only (Ruhling and Tyler, 1970; Brown and Bates, 1972; Brown and Beckett, 1985; Wells and Brown, 1987). As described by Bates and Brown (1974) and Bates (1976, 1982) the membrane permeability of moss cells, like those of vascular plants, is influenced by calcium availability. In the absence of calcium, the rates of influx and efflux of paraquat by the moss tissue might be expected to differ from those in the presence of calcium. However, comparisons are still possible for tissues analysed under the same experimental conditions. Calcium was therefore not included in the following experiments because (1) complications in the interpretation of paraquat uptake profile may occur due to competition between the two cations, (2) previous uptake experiments in this study did not involve the addition of calcium, (3) under field conditions additional calcium would not normally be supplied.

5.2.5 Analysis of data

The natural logarithms (ln) of the total disintegrations per minute (dpm) of radioactivity remaining in the tissue were plotted with respect to time. The data were analysed in two stages:

Stage 1. The traditional curve peeling technique (MacRobbie and Dainty, 1958) using linear regression of log-linear data in time (Balke and Price, 1988) was used to fit an equation of the form

$$y = A_0 + A_1 e^{-k1t} + A_2 e^{-k2t} + \ldots\ldots$$

where y = total solute in tissue at time t

A_i = solute content of ith cellular compartment

k_i = solute efflux rate of ith compartment.

to the eluted component (measured in dpm) of the efflux analysis. This technique involves plotting ln (Y) against time, and fitting a straight line to the linear portion of the curve (corresponding to the slowest

decaying component). The intercept gives ln(A) and the slope equals k, so that it is possible to subtract the calculated values, given by the analysis, from the observed counts. The process can be repeated by plotting the ln of these adjusted counts against time. It should be noted that in this type of analysis, A_0 is assumed to be zero.

Stage 2. Estimates of the efflux rates from Stage 1 were used as initial estimates in a non-linear regression program (MLP; Ross, 1987) for fitting the equation by least squares. This allowed the fitting of the additive constant A_0 (not available using the above method). The number of rate parameters required to describe each set of experimental data adequately was determined by analysis of variance. The analysis fitted the following models: two components, two components + a constant, three components, three components + a constant. For each linear component, the content at time zero (A_i) and the half life ($t_{\frac{1}{2}}$ = (ln2)/k) were calculated. Experiments were performed with two or four replicates per treatment. Compartmental analysis was performed on the individual replicates and the rates (k_i) and initial compartment contents (A_i) averaged.

5.2.6 Calculation of Nernst potential

The Nernst equation can be used to estimate the electrochemical potential concentrations of a cell where the internal and external concentration of an ion are in equilibrium. Using the calculated Nernst potential of the cell, an indication of whether active transport mechanisms are involved in an uptake procedure can be obtained.

To determine the transport mechanism for paraquat in *R. squarrosus* and leaf slices of *L. perenne*, an equilibrium between the tissue and the paraquat incubation medium was required. This was established prior to the elution of paraquat from the radioactively labelled tissue. The paraquat concentrations of the individual cellular pools

and of the remaining incubation solutions were used in the Nernst calculations of cell potential as follows:

$$E^{nj} = (RT/zF) \log C_{ij}/C_{oj} \text{ millivolts(mV)}$$

where E_{nj} = Nernst potential

R = Gas constant

T = temperature (absolute)

F = Faraday's constant

z = valency

C_{oj} = concentration of j in the incubation medium

C_{ij} = concentration of j in the cell

The Nernst equation calculates the electrical potential difference required to maintain an asymmetric distribution of an ion across a membrane at equilibrium. As described by Price (1978), divalent cations can accumulate within a cell at 1000x the external concentration. If the calculated Nernst potential resembles that of the measured electrochemical potential of the tissue, then transport passively across an electrochemical gradient will explain the uptake mechanism. A small Nernst potential implies an active removal of ions against the electrochemical potential of the cell. Conversely, a high Nernst potential indicates possible active accumulation of the ion above that due to the electrochemical potential of the cell. In addition, large deviations may also reflect an impermeable or selectively permeable membrane (Hall and Baker, 1977).

Without a direct measurement of the electrochemical potential of the cells, comparisons with the predicted Nernst potentials are not possible. However, comparisons with electrochemical potential values known for other species such as -86 mV for *Avena fatua* (Price, 1978) and between -58 and -116 mV for beetroot leaf discs (Pitman, 1963)

allow an assessment of the validity of the results. Electrochemical potentials of moss cells have not been extensively studied and are sometimes of unclear origin (Brown, 1984). However, excessive deviation of the calculated Nernst potential from -86 mV may indicate departure from a passive transport mechanism.

5.3 RESULTS AND DISCUSSION

The following results are based on the efflux of radioactivity from plant tissue preloaded with 14C-paraquat. The eluted molecules are assumed to be radioactive paraquat but could conceivably be labelled metabolic products of paraquat.

5.3.1 Efflux of paraquat from whole shoot segments of R. squarrosus into water

^{14}C-Paraquat was eluted from whole moss segments, pretreated (16 h) with paraquat (725 µM), into water. Water was used as the eluant in this study, rather than unlabelled paraquat, to reduce the exchange of ^{14}C-paraquat from the moss cell walls which could interfere in the detection of the efflux from the intracellular fraction. Efflux analysis using water or buffer as the eluant is a common procedure in the compartmental analysis of herbicides in vascular plant tissues (Price, 1978; Pillmoor, Roberts and Gaunt, 1982; Orwick, Schreiber and Hodges, 1976) where it is considered to be representative of the plant's normal environment. Using the curve peeling method described in Section 5.2.5, the linear portions of the logarithmic plot were extrapolated to time zero to obtain the initial quantity of paraquat in each compartment and the efflux time half life ($t_{\frac{1}{2}}$).

The model incorporating two linear components and a constant adequately described the paraquat efflux data (Table 5.3.1). The two linear components probably represent the cytoplasmic and vacuolar pools. The paraquat concentration of these pools and percentage

Table 5.3.1 Summary of efflux analysis of whole shoots of R. squarrosus. Shoots were incubated in 725 µM paraquat and eluted in water. Figures in parentheses are standard errors of means

Probable identity of compartment	Paraquat content at t=0 (µg g^{-1} dry wt)	% of total ^{14}C uptake	$t^{½}$
Vacuole	13585 (3339)	24 (5.7)	3.78 h (0.91)
Cytoplasm	5484 (1550)	9.8 (2.7)	13.35 min(2.26)
Cell wall (not exchangeable)	29602 (2146)	53.2 (4.6)	*
Sub-total	48671		
'Intercellular'(1)	7084	13 (0.85)	*
Total	55755 (902.2)		

n = 3
(4^{th} replicate did not converge)
* not rate determinable

(1) 'Intercellular' pool is derived from the difference between total ^{14}C-paraquat associated with R. squarrosus shoots and that accounted for in the compartmental analysis.

of the total 14C-paraquat in the tissue at the start of efflux were 5.48 and 13.58 mg g^{-1} dry weight representing 10% and 24%, respectively, with efflux half lives of 13.35min and 3.78 h. The constant was not rate-determinable (29.6 mg g^{-1} dry wt = 53%), and represents the exchangeably-bound or immobile paraquat fraction (Table 7.3.1). In this experiment a third rate determinable compartment equivalent to the fast inter- cellular pool/cell wall component was not found. However, radioactivity eluted from the tissue, but not accounted for in the compartmental analysis, can be used as an estimate of this intercellular compartment. In this experiment 13% of the ^{14}C-paraquat was unaccounted for in the compartmental analysis. The $t_{½}$ values normally attributable to the intercellular pool have values of approximately 1 min (Pillmoor, Roberts and Gaunt, 1982; Balke and Price, 1988). The very fast efflux time for the intercellular compartment requires a sufficiently large number of data points, over a short time period, for accurate calculation. Additionally, moss stem tissue contains very small intercellular spaces compared with leaf tissue of vascular plants (Duckett, 1986). This may account for the inability to determine a rate of efflux for this compartment. The calculated $t_{½}$ value for the predicted paraquat efflux across the tonoplast ($t_{½}$ = 3.78 h) was longer than for the plasmalemma ($t_{½}$ = 13.35 min) indicating a difference in their permeability to paraquat. The t½ values resemble those described by Price (1978) for the efflux of paraquat from wheat tissue (3.9 h and 15 min).

The compartmental analysis used in these experiments cannot distinguish between the non-eluted exchangeable and non-exchangeable residual paraquat components of the moss tissues. Consequently, these are referred to collectively as the total residual paraquat component. Differences were slight between calculated and measured total residual paraquat concentrations of the moss tissue (Table 5.3.2). The slightly

lower estimate for the predicted total residual paraquat concentration than that of the measured concentration indicates that some of this component can be eluted if the incubation in water is continued.

Table 5.3.2 Comparison of the computed with directly measured non-exchangeable ^{14}C-paraquat component in R. squarrosus with the predicted value from the compartmental analysis
Figures in parentheses are standard errors of the means

Component	dpm x 10^3	% of measured residual component
(a) Measured residual component (Σ non-effluxed paraquat)	31.8 (29.03)	
(b) Measured intracellular (soluble)	1.73 (4.18)	5.5
Observed non-exchangeable (a)-(b)	30.07	94.5
Predicted non-exchangeable	28.3 (40.65)	89.17

5.3.2 Efflux of paraquat from excised leaves and stems of R. squarrosus into water

Identical experiments were performed on separated leaves and stems of *R. squarrosus*, using 725 µM paraquat, for comparison with the results obtained for whole shoots.

Table 5.3.3 Summary of efflux analysis for separated leaves of R. squarrosus pre-incubated in 725 μM ^{14}C paraquat and eluted with water. Figures in parentheses are standard errors of means

Probable identity of compartment	Paraquat content at t=0 (μg g^{-1} dry wt)	% of total ^{14}C uptake	$t_{½}$
Vacuole	14495 (33)	25.5 (5.8)	4.06 h (0.59)
Cytoplasm	9604 (550)	16.7 (3.3)	29.3 min(3.46)
Cell wall (exchangeable)	8599 (2471)	13.8 (0.06)	3.26 min (1.04)
Cell wall (non-exchangeable)	12310 (12310)	21.2 (3.8)	*
Sub-total	45008		
'Intercellular'(1)	10667	22.7 (12.8)	*
Total	55675		

* not rate determinable; n = 2

(1) 'Intercellular' pool is derived from the differences between total ^{14}C-paraquat associated with R. squarrosus leaves and that accounted for in the compartmental analysis

Table 5.3.4 Comparison of directly measured non-exchangeable ^{14}C-paraquat component in R. squarrosus leaves with the predicted value from the compartmental analysis Figures in parentheses are standard errors of the means

Cellular Component	dpm	% of measured residual component
(a) Measured residual (Σ non-effluxed paraquat)	954 (81.5)	
(b) Measured intracellular (soluble)	139 (0.7)	14.6
Observed non-exchangeable (a)-(b)	815	85.4
Predicted non-exchangeable	697 (15.0)	73.1

The data were adequately described by the model utilising three linear and one constant component (Tables 5.3.3 and 5.3.5). In this experiment, the fast (intercellular/cell wall) component yielded $t_{\frac{1}{2}}$ values of 3.26 min and 1.18 min for leaves and stems, respectively. It is possible that the slightly greater $t_{\frac{1}{2}}$ value of the leaves compared to stems is due to slight loss of membrane integrity in the former, thereby increasing the level of paraquat in the intercellular/cell wall pool (*ca.* 14% in leaves, 7% in stems). Alternatively, the leaves may have a larger intercellular capacity. This is also reflected in the slightly larger amount of radioactivity (23%) in the intercellular component of the leaves, not accounted for in the analysis, compared with the intact moss shoots (13%) from the previous experiment. The distribution of paraquat in the leaves and stems shows similarity in vacuole content (25%, 21%) but significant differences in cytoplasmic concentrations (16.7%, 3.2%), and bound pools (21.2%, 48.7%). The significantly greater bound paraquat content of the stems agrees with previous results (Section 4.3.1.1). The predicted and measured non-elutable-total residual paraquat components of the stems (Table 5.3.6), as for the whole moss shoots (Table 5.3.2), are similar. However, the predicted value for the total residual paraquat component of the leaves of *R. squarrosus* was larger than that for the measured value (Table 5.3.4). This may be due to the release of a non-elutable soluble intracellular fraction of ^{14}C-paraquat during the boiling procedure for the extraction of the remaining intracellular component.

The low paraquat content of the stem cytoplasm could be due to greater vacuolar uptake. Amelioration mechanisms involving displacement of toxic molecules into the vacuole have often been hypothesised to explain plant tolerance to environments of high metal content (Fitter and Hay, 1981; Street and Opik, 1976). However, the paraquat content of the vacuole in stems (Table 5.3.5) did not differ significantly from that of the leaves (Table 5.3.3). No significant

Table 5.3.5 Summary of efflux analysis data for separated stems of R. squarrosus. Stems were incubated in 725 μM ^{14}C-paraquat and eluted in water

Probable identity of compartment	Paraquat content at t = 0 (μg g^{-1} dry wt)	% of total ^{14}C uptake	$t_{½}$
Vacuole	11407	20.7	5.06 h
Cytoplasm	1788	3.2	21.3 min
Cell wall (exchangeable)	3698	6.6	1.18 min
Cell wall (non-exchangeable)	27183	48.7	*

Sub-total	44076		
'Intercellular'(1)	11775	20.8	

Total	55851		

* not rate determinable; n = 1 (2nd replicate did not converge)

(1) 'Intercellular' pool is derived from the difference between ^{14}C-paraquat associated with R. squarrosus tissues and that accounted for in the compartmental analysis

Table 5.3.6 Comparison of the directly measured non-exchangeable ^{14}C-paraquat component in R. squarrosus stems, with the predicted value from the compartmental analysis.

Component	dpm	% of residual component
(a) Measured residual component (Σ non-effluxed paraquat)	2192	
(b) Measured intracellular (soluble)	1177	53.7
Observed non-exchangeable(a)-(b)	1015	46
Predicted non-exchangeable	1890	86.2

differences in the $t_{\frac{1}{2}}$ values were observed for the leaves and stems, confirming that there are no fundamental differences in membrane permeability between the two tissues.

A more plausible explanation for the difference in distributions of paraquat between the vacuole and cytoplasm of the leaves and stems are differences in their respective volumes. Measurements of Lüttge and Bauer (1968) indicated that the cytoplasm and vacuolar volumes are approximately equal following centrifugation of *Mnium cuspidatum* leaves, compared with a very thin protoplasmic layer in root cortical cells of *Zea mays.* The electron micrographs of *R. squarrosus* stem cells (Plate 1.5.7) show some cells to be multivacuolate with extensive cytoplasm, and others with reduced cytoplasm. The stems, with a major support function, may contribute less to photosynthesis than the leaves. The leaves may, therefore, possess a greater cytoplasmic volume supporting the chloroplasts than do the stems. Under these circumstances, a greater proportion of paraquat could be expected in the cytoplasmic phase of the leaves than in the stem cells of *R. squarrosus.*

Paraquat uptake (725 µM treatment) and distribution in the cells of leaves, stems and whole moss shoots of *R. squarrosus* from the efflux and kinetic (4 h uptake period) studies (Section 4.3.1.6) differed in 1) Total paraquat content. No significant differences were found in total paraquat content between the leaves (45.0 mg g^{-1} dry weight), stems (44.0 mg g^{-1} dry weight) and whole moss shoots (48.7 mg g^{-1} dry weight) from the efflux analysis. This contrasts with lower and more variable predicted maxima for total uptake derived from the kinetic analysis (Section 4.3.1.6) for the leaves (40.6 mg g^{-1} dry weight), stems (31.0 mg g^{-1} dry weight) and whole moss shoots (21.5 mg g^{-1} dry weight). Experimental conditions, source of *R. squarrosus* shoots, and the pretreatment environment were similar between experiments and were therefore unlikely to influence the results.

2) Intracellular paraquat content. The intracellular paraquat content for the leaves, stems and whole moss shoots from the efflux analysis were *ca.* 24.1, 13.2, and 19.1 mg g^{-1} dry weight, respectively, compared with the smaller 0.69, 1.54 and 2.51 mg g^{-1} dry weight from the kinetic study.

3) Exchangeable plus Residual paraquat content. In contrast to the intracellular paraquat concentrations, the total residual paraquat component from the efflux analysis of the leaves was significantly smaller with 20.9 mg g^{-1} dry weight compared with 39.86 mg g^{-1} dry weight from the kinetic analysis. Comparisons between the efflux and kinetic analysis determination of the total residual paraquat concentration for the whole moss and stem segments are difficult as the total uptake values differ between the techniques. However, the proportion of paraquat in the total residual plus extracellular, compared with the intracellular, pools are approximately 3:1 and 3:2 from the efflux analysis compared with 19:1 and 7:1 from the kinetic study for the stems and whole moss shoots respectively. The total bound paraquat concentration following the efflux study was, also, greater (30.88 and 29.60 mg/g^{-1} dry weight) than in the kinetic study (29.48 and 18.99 mg g^{-1} dry weight) for both the stems and whole moss shoots, respectively.

The differences observed in the compartmentation of paraquat in *R. squarrosus* following the efflux and kinetic methods of analysis may be attributable to:

a) Elution techniques. The kinetic study involved the initial displacement of bound paraquat from the tissue surface by incubation for a total of 1 h in unlabelled paraquat. Washing for 1 h with unlabelled paraquat corresponds to >2 times the $t_{\frac{1}{2}}$ of the "cytoplasm pool", resulting in an underestimate of paraquat uptake in the intracellular fraction.

b) Incubation time. The kinetic and efflux analyses differed in their ^{14}C-paraquat incubation times using a maximum 4 h and a 16 h period, respectively. As shown in Section 4.3.1.6 and by Wells and Brown (1987), redistribution of cations occurs from the extracellular sites to the intracellular and residual fractions. Therefore, the greater intracellular paraquat levels detected after the 16h incubation period may have been due to redistribution of paraquat from the extracellular to the intracellular pool.

c) Nutrient status. The physiological condition and nutrient status of the moss tissue may influence intracellular paraquat uptake between experiments. A low nutrient content of the tissue may lead to rapid accumulation of ions after immersion in the ^{14}C-paraquat. Pitman (1963) used starvation of beetroot leaf discs prior to incubation in isotopes, as a technique to encourage adequate uptake and therefore adequate elution levels. This can also relate to the calcium content and its availability prior to the efflux and kinetic studies. Bates and Brown (1974) described the importance of calcium in the integrity and permeability of *Schistidium maritimum* and *Grimmia pulvinata* membranes to K^+ and Na^+. Calcium has also been described as an essential component of efflux analyses (MacRobbie, 1971; Clarkson, 1974) in preventing increased permeability of the membranes. However, the absence of calcium from these studies still enables comparisons of parameters between tissues treated under similar conditions (Clarkson, 1974). The source and storage of *R. squarrosus* (Section 3.2.1) were kept constant throughout the project to minimise such differences.

d) Herbicidal activity. Paraquat has been described as affecting membrane permeability following the production of hydrogen peroxide and paraquat radicals in the presence of light (Dodge and Harris, 1970). Increasing the incubation time from 4 h to 16 h could encourage increased permeability of the moss tissue plasma membranes. However, membrane integrity still exists in the tissues used for efflux analysis, as independent rates were isolated for the different

intracellular pools.

5.3.3 Efflux of paraquat from whole segments of R. squarrosus using paraquat as an eluant

To determine the effects of paraquat concentration on its uptake and cellular distribution, moss tissue was incubated in a dilute paraquat solution (72.5 µM) and eluted into an equivalent concentration of unlabelled paraquat. A dilute paraquat solution was used for both the ^{14}C incubation and the unlabelled eluant to reduce the likelihood of membrane damage occurring. The use of unlabelled paraquat as the eluant also provided a substitution solute for the cell wall bound paraquat component. This aided in its detection as a rate determinable component of the efflux analysis. The results are presented in Tables 5.3.7 and 5.3.8.

The data were best described by the model with three linear components and a constant. The identification of a third rate determinable component, compared to only two when eluted in water (Section 5.3.1), was related to displacement of the exchangeable cell wall component by the unlabelled paraquat eluant. The elution of the exchangeable component was coupled with a corresponding decrease in the percentage of paraquat described by the constant term. The sum of the exchangeable components and the constant (1.92 mg g^{-1} dry weight) account for 41% of the total uptake compared with 65% from the 725 µM treatment. A reduction in the proportion of paraquat bound to the cell walls of *R. squarrosus* following a 72.5 µM ^{14}C-paraquat treatment was also observed in Section 4.3.1.3.

The vacuole (42%) contained more paraquat than the cytoplasm (17%), perhaps indicating an amelioration mechanism. The proportion of paraquat in the sum of the cytoplasmic and vacuolar pools was significantly greater in the 72.5 µM ^{14}C-paraquat treatment (59%) than in the 725 µM treatment (34%). A similar distribution of paraquat was observed between the vacuolar and cytoplasmic pools, following both the

Table 5.3.7 Summary of efflux analysis of whole moss shoots of R. squarrosus. Shoots were incubated in 72.5 µM ^{14}C-paraquat and eluted in an equivalent concentration of cold paraquat. Figures in parentheses are standard errors of means

Probable identity of compartment	Paraquat content at t=0 (µg g^{-1} dry wt)	% of total ^{14}C uptake	$t_{½}$
Vacuole	1939 (109)	41.8 (2.39)	3.65 h (0.24)
Cytoplasm	784 (19)	16.8 (0.4)	10.2 min (0.65)
Cell wall(exchanged)	856 (111)	18.4 (2.4)	3.1 min (0.29)
Cell wall (non-exchangeable)	1062 (121)	22.8 (2.6)	*
Total	4641		
'Intercellular'(1)	0		*

n = 4
* rate not determinable

(1) 'intercellular' pool is derived from the difference between total ^{14}C-paraquat associated with *R. squarrosus* tissue and that accounted for in the compartmental analysis

Table 5.3.8 Comparison of the directly measured non-exchangeable ^{14}C-paraquat component of R. squarrosus shoots, with the predicted value from the compartmental analysis. Shoots were incubated in 72.5 µM ^{14}C-paraquat and eluted in equivalent concentrations of cold paraquat. Figures in parentheses are standard errors of means

Cellular Component	dpm	% of measured residual
(a) Measured residual component (Σ non-effluxed paraquat)	3411 (176)	
(b) Measured intracellular (soluble)	277 (19)	8
Observed non-exchangeable (a)-(b)	3134	92
Predicted non-exchangeable	2149 (2899)	63

72.5 and 725 µM ^{14}C-paraquat treatments (Tables 5.3.1 and 5.3.7). Greater accumulation of cations by the vacuole of plant cells occurs when they are supplied as chlorides than when offered as sulphates (MacRobbie, 1971). This may be related to the greater ability of chloride anions to penetrate the plasmalemma countering the net transport of cations. Paraquat was presented as paraquat dichloride in these experiments and the presence of the chloride ions may have increased the permeability of the tonoplast to paraquat. The accumulation of cations by the vacuole is slower when accompanied by sulphates, probably because organic acid synthesis is required in the cytoplasm before both ions can enter the vacuole (Osmond and Laties, 1969; Torii and Laties, 1966). Maclennan, Beevers and Harley (1963) observed high levels of organic acids in the vacuole of *Bryophyllum calycinum* which were believed to generate a concentration gradient for cation movement from the cytoplasm to the vacuole. High levels of organic acids in the vacuole of *R. squarrosus* tissues and the supply of chloride ions from the paraquat salt may, therefore, cause the preferential accumulation of paraquat in the vacuole. However, total intracellular uptake of cadmium by *R. squarrosus* was not found by Wells and Brown (1987) to be influenced greatly by the accompanying anions.

Proportionally more paraquat entered the moss tissue from the 72.5 µM than the 725 µM paraquat solutions (Tables 5.3.1 and 5.3.7), indicating that intracellular uptake is not dependent on concentration of the treatment solution. An intercellular component was not identified in this experiment, presumably because the moss had absorbed the paraquat normally left in this fraction. The $t_{\frac{1}{2}}$ values for vacuolar and cytoplasmic fluxes (3.65 h and 10.2 min) did not significantly differ from those obtained from the higher paraquat concentration with water as an eluant (3.78 h and 13.3 min). The

elution of paraquat from these pools was therefore independent of intracellular or extracellular concentration and relates entirely to the permeability of the membrane to paraquat. This suggests that uptake is dependent on a finite number of carriers in the membrane and may resemble carrier-mediated ion transport described using the Michaelis-Menten equation (Hall and Baker, 1977).

The $t_{\frac{1}{2}}$ value obtained for the exchangeable fraction (3.1 min) may be larger than the true figure due to an initial inefficiency of ^{14}C-paraquat exchange. Incomplete saturation of the exchange sites following the 72.5 µM paraquat treatment may have led to further paraquat binding from the unlabelled paraquat washes, thereby increasing the t½ values.

The total paraquat uptake by *R. squarrosus* shoots from the efflux and kinetic analysis (Section 4.3.1.6), using 72.5 µM ^{14}C-paraquat, were 4.64 and 6.8 mg g^{-1} dry weight, respectively. The difference in the total paraquat uptake at low paraquat incubation concentrations may be attributable to exhaustion of the solution. However, the distribution of ^{14}C-paraquat in the intracellular and the exchangeable components following the 72.5 µM efflux experiment (Table 5.3.7) resembled those of the 725 µM paraquat efflux experiment (Section 5.3.1). The ratio of intracellular to exchangeable paraquat in the efflux and kinetic analysis were 1:1.5 and 1:32, respectively. Elution of ^{14}C-paraquat from the intracellular fraction of the moss tissue could occur during the 1 h displacement procedure used in the kinetic analysis (Section 4.2.5). This, however, based on the $t_{\frac{1}{2}}$ values for the cytoplasmic compartments, would not account fully for the differences observed between the two techniques.

5.3.4 Efflux of paraquat from Lolium perenne leaf slices

The slow onset of toxic effects in *R. squarrosus* after application of paraquat in the field (Sections 2.3.1.1 and 2.3.2) may be

attributable to the high cation binding capacity of the cell wall and/ or low plasma membrane permeability inhibiting intracellular uptake. Compartmental analysis of paraquat uptake, by efflux analysis, was also performed using leaf slices of *Lolium perenne*. *L. perenne* leaf slices (100 x 400 µm) were incubated for 16 h in 5 ml of 725 µM ^{14}C-paraquat, and water was used as the eluant. Comparisons of the compartmental concentrations and $t_{½}$ values were made between *L. perenne* and *R. squarrosus*.

The efflux data were described most adequately by the model with two linear components and a constant (Table 5.3.9). An intercellular rate was, again, not determinable due to its very fast efflux rate but a concentration equivalent to 54% of the total activity was associated with the intercellular compartment of the leaf slices (Table 5.3.9). This value is extremely high compared with the moss tissue, indicating a possible loss of membrane integrity. However, the successful extraction of linear components from the efflux curve suggests that the plasma membranes were still functional.

The $t_{½}$ value for efflux from the vacuolar compartment is approximately half that found for *R. squarrosus* leaves (Table 5.3.3). Either tonoplast permeability of *L. perenne* is greater than that of the moss or the paraquat has increased tonoplast permeability in the grass. Increased tonoplast permeability may be a general feature of paraquat damage to vascular plants. Dodge and Harris (1970) described increased tonoplast permeability following paraquat treatment of flax (*Linum usitatissimum*), and Price (1978) also found membrane damage in wheat tissue leading to reduced phloem mobility and increased xylem mobility.

The vacuole accounted for 23% of the paraquat associated with the *L. perenne* tissue (Table 5.3.9). This value increased to 53% if the intercellular component was not incorporated in the total paraquat

Table 5.3.9 Summary of efflux analysis data for Lolium perenne leaf slices pre-incubated in 725 μM ^{14}C-paraquat and eluted in water. Figures in parentheses are standard errors of means

Probable identity of compartment	Paraquat content at t=0 (μg g^{-1} dry wt)	% of total ^{14}C uptake	$t_{½}$
Vacuole	9386 (808)	23.6 (0.64)	1.52 h (0.27)
Cytoplasm	5349 (2088)	13.5 (6.14)	13.14 min (3.08)
Cell wall (non-exchangeable)	3536 (691)	8.4 (1.26)	*
Sub-total	18271		
'intercellular'(1)	14676	54.4 (4.9)	*
Total	32948 (5577)		

n = 3
(4th rep. did not converge)
* not rate determinable

(1) 'Intercellular' pool of derived from the difference between the total ^{14}C-paraquat associated with L. perenne leaf slices and that accounted for in the compartmental analysis

content. Thirteen per cent of the total paraquat was located in the cytoplasm. The proportions of paraquat in the cellular pools of *L. perenne*, therefore, resemble those found in *R. squarrosus*. However, the cell wall bound fraction of *L. perenne* leaf tissue was only 8% compared with 35% and 53%, respectively, in the leaves and intact shoots of *R. squarrosus* (Tables 5.3.3 and 5.3.1). This confirms the large cation exchange capacity of *R. squarrosus* tissues.

The vacuolar compartment of *L. perenne* (9.38 mg g^{-1} dry weight) contained significantly less paraquat than that of *R. squarrosus* leaves (14.49 mg g^{-1} dry weight). However, if the vacuole of *R. squarrosus* leaves accounts for *ca.* 50% of the intracellular fraction, as in the case of *Mnium cuspidatum* (Lüttge and Bauer, 1968), and *ca.* 80% in *L.*

perenne leaves (Bridges and Farrington, 1974), then the total concentration of paraquat in the vacuole will be greater for the moss tissue. The cytoplasmic volumes will, therefore, be larger in *R. squarrosus* implying a lower paraquat concentration than that in the smaller cytoplasm of *L. perenne*.

In contrast to the moss tissue, correlation was poor between the directly measured bound paraquat (2580 dpm) and the estimated bound paraquat (6877 dpm) in the *L. perenne* leaf slices (Table 5.3.10).

Table 5.3.10 Comparison of the directly measured non-exchangeable ^{14}C-paraquat component Lolium perenne leaf slices with the predicted values from the compartmental analysis. Leaf slices were incubated in 72.5 µM paraquat, eluted with water. Figures in parentheses are the standard errors of the means

Cellular Component	dpm	% of measured residual
(a) Measured residual component (Σ non-effluxed ^{14}C-paraquat)	8091 (1562)	
(b) Measured intracellular (soluble ^{14}C-paraquat)	5511 (854)	68.1
Observed non-exchangeable (a)-(b)	2580	31.9
Predicted non-exchangeable	6877 (1345)	85

Also, the intracellular paraquat content, free in solution after efflux from the *L. perenne* tissue, accounted for a larger proportion (68%) of the total residual fraction compared with leaves of *R. squarrosus* (14.6%). The results suggest the existence of a paraquat component bound to soluble molecules within the intracellular compartment of *L. perenne* tissue which is released by disruption of the cell membrane but which is not capable of movement across the plasma membranes. Price (1978) described reductions in chemical distribution in plants such as *Polygonum* spp. due to binding to tannin bodies concentrated in epidermal or perivascular cells. This is in complete contrast to the moss tissue where intracellular paraquat appeared to be free.

5.3.5 Calculation of Nernst potential

Estimates of the intracellular and external paraquat concentrations obtained in the efflux analyses enable calculation of the Nernst potentials for the various tissues.

These calculations require a knowledge of cell water content. Fresh and dry weight measurements of the moss tissue indicate this to be *ca.* 0.9 g H_2O g^{-1} dry weight at saturation. The water content was estimated to be 0.9 g H_2O g^{-1} dry weight of *L. perenne*.

To compare concentrations of intracellular paraquat with those of the bathing solution, paraquat must exist in passive equilibrium across the plasma membranes. Calculations of the Nernst electrochemical potentials for the intracellular compartments are presented in Table 5.3.11. For all *R. squarrosus* tissues and *L. perenne*, the calculated potentials are between -63 mV and -72 mV in equilibrium with 725 µM paraquat solutions. A lower value (-110mV) was obtained when the tissues were in equilibrium with 72.5 µM paraquat. Similar differences in the calculated Nernst potentials for oat coleoptiles and roots were observed by Higinbotham, Graves and Davies (1970) for potassium, over a

Table 5.3.11 Calculated Nernst potential for Rhytidiadelphus squarrosus and Lolium perenne using internal and external concentrations of ^{14}C-paraquat

Using the Nernst equation $E = RT/ZF (\log C_i/C_o)$ (Section 5.2.6)

Paraquat treatment 725 µM **Eluant = water**

Rhytidiadelphus squarrosus

Whole shoots

conc. inside (C_i)=19069.4 µg g^{-1} dry wt (ca. 90% water)

21.18 µg μl^{-1}

conc. outside(C_o)=214514.2 dpm/5 ml

0.04078 µg μl^{-1}

E = -78 mV

difference between vacuole and cytoplasm assuming equal volumes

E = -11.4 mV

Leaves (Moss)

conc. inside = 26.777 µg μl^{-1}

conc. outside = 0.115 µg μl^{-1}

E = -68 mV

Stems (Moss)

conc. inside = 14.66 µg μl^{-1}

conc. outside = 0.115 µg μl^{-1}

E = -63 mV

Lolium perenne

conc. inside = 16.872 µg μl^{-1} (ca. 90% water)

conc. outside = 0.101 µg μl^{-1}

E = -64 mV

Paraquat treatment 72.5 µM **Eluant = 72.5 µM paraquat**

Rhytidiadelphus squarrosus

Whole shoots

conc. inside = 3.026 µg μl^{-1}

conc. outside = 4.64×10^{-4} µg μl^{-1}

E = -110 mV

range of external concentrations. In the latter case, the variations were attributed to the existence of an electrogenic pump which was concentration sensitive. It is not possible to ascribe causes to variation in the present study. One cause may be high cation exchange properties of the moss tissue reducing the measured paraquat levels in solution close to the external surface of the plasmalemma.

The differences in paraquat content between the cytoplasm and vacuole of *R. squarrosus* following both the 72.5 µM and 725 µM paraquat treatments indicate a reduction of *ca.* 11.5 mV, assuming the volumes of the two compartments to be equal. The values determined for the leaf and stem tissue were -5 mV and -23 mV, respectively, based on equal cytoplasmic and vacuolar volumes. In *L. perenne*, the difference in concentration of paraquat between the vacuole and cytoplasm is equivalent to a reduction of 7 mV assuming that the volumes of the two compartments are equal, or 3 mV if the vacuole represents 70% of the intracellular volume.

Measurements of the resting potential difference of *R. squarrosus* and *L. perenne* tissues were not made in this study. Thus, the determination of whether paraquat accumulation was passive or active in both *R. squarrosus* and *L. perenne* cannot be made. A number of workers have measured resting potentials in moss tissues, as discussed by Brown (1984), although the values vary greatly according to species, experimental conditions and the techniques used. However, if *R. squarrosus* and *L. perenne* were to possess resting electrochemical potentials similar to those described for *Avena fatua* (-86 mV) and beetroot leaf tissue (-58 mV to -116 mV) then the calculated Nernst potentials for paraquat uptake would imply its passive accumulation.

5.4 CONCLUSIONS

The results of efflux analysis suggest that shoots of *R. squarrosus* and leaf slices of *L. perenne* differ little in their intracellular paraquat content following incubation in paraquat solutions. However, it is possible that the vacuole volumes are considerably smaller in mosses than vascular plant cells (Lüttge and Bauer, 1968). Assuming this, *R. squarrosus* probably contains a greater concentration of paraquat in solution in the vacuole per unit volume than does *L. perenne*.

Plasma membranes of *R. squarrosus* do not appear to be damaged by treatment with 72.5 µM or 725 µM paraquat, since in both cases rate determinable efflux curves were found which could be attributed to different cellular components. However, the *L. perenne* leaf slices showed signs of damage to the tonoplast, indicated by the relatively fast $t_{\frac{1}{2}}$ values in comparison with the moss tissue and with values quoted by Price (1978) for wheat leaf cells. *R. squarrosus* tissue was capable of binding approximately ten times more paraquat to its cell walls than *L. perenne*.

The calculated Nernst potentials for the moss and *L. perenne* leaf tissue did not differ substantially. However, estimates of the Nernst potentials for the vacuoles of the leaves and stems of *R. squarrosus* did differ, probably due to differences in their volumes. In contrast, the estimated Nernst potential for the vacuole of *L. perenne* showed little difference to that of the cytoplasm, indicating possible damage to the tonoplast and loss of transport selectivity as a result of the paraquat treatment.

Paraquat accumulation by *R. squarrosus* and *L. perenne* is likely to be passive if their resting electrochemical potentials were to resemble those of species such as *Avena fatua* (-86 mV) and beetroot (-58 to -116mV) and that of their calculated Nernst potential (*ca.* -70 mV).

CHAPTER 6

EFFECTS OF PARAQUAT, DICHLOROPHEN, FERROUS SULPHATE AND 2,4-D ON PHOTOSYNTHESIS AND RESPIRATION IN *RHYTIDIADELPHUS SQUARROSUS*

6.1 INTRODUCTION

Over half of the herbicides in current use act on the light reactions of photosynthesis (Corbett, Wright and Baillie, 1984). Photosynthesis in mosses has been shown to be sensitive to air pollutants (Inglis and Hill, 1974; Nash and Nash, 1974) but information on the effects of herbicides is limited. Photosynthesis and respiration are fundamental metabolic processes in green plants and it is therefore appropriate to include a study of the action of herbicides on these processes in mosses in the present investigation.

The macromolecular photosynthetic apparatus of mosses is generally considered to resemble that of vascular plants and green algae (Aro, Niemi and Valanne, 1981). Differences have been found but there are conflicting views on many of these (Valanne, 1984). One important difference is the reduced levels of the 32-34 kilodalton (kDa) polypeptide in photosystem II (PSII) of mosses (Aro, 1982). The 32-34 kDa polypeptide is a component of the protein shield covering the plastoquinone electron acceptor and carrier sites (QA and QB) of PSII (Figs. 6.1.1 and 6.1.2). Renger (1976) proposed that the protein shield regulated electron flow between QA and QB thus preventing reactions between redox components. In vascular plants this polypeptide has been shown to be responsible for the binding of several herbicide groups such as the ureas, triazines, triazones, anilides and uracils (Fedtke and Trebst, 1987; Renger, Hagemann and Dohnt, 1981; Vermaas and Govindjee, 1981). Insensitivity to these herbicides has also been shown in the pond weed *Spirodella oligorrhiza*, depleted specifically in the polypeptide by incubation in low levels of chloramphenicol (Gressel, 1982). Vermaas and Govindjee (1981) postulated that, in the absence of the PSII polypeptide, electron transport could be facilitated via another route which is diuron (N'-(3,4-dichlorophenyl)- N,N-dimethyl urea) insensitive. However, even in the absence of the 32-34 kDa polypeptide, the PSII of mosses

Fig. 6.1.1 Schematic description of the binding area for herbicides inhibited at the acceptor side of photosystem II (Trebst, Draber and Donner, 1983). Phenols and diuron bind to two different sub units of the membrane protein that cover photosystem II.

Abbreviations used:

P680, reaction centre chlorophyll of photosystem II; Q, primary electron acceptor for photosystem II; B_1, secondary acceptor of photosystem II; PQ, plastoquinone

Fig. 6.1.1

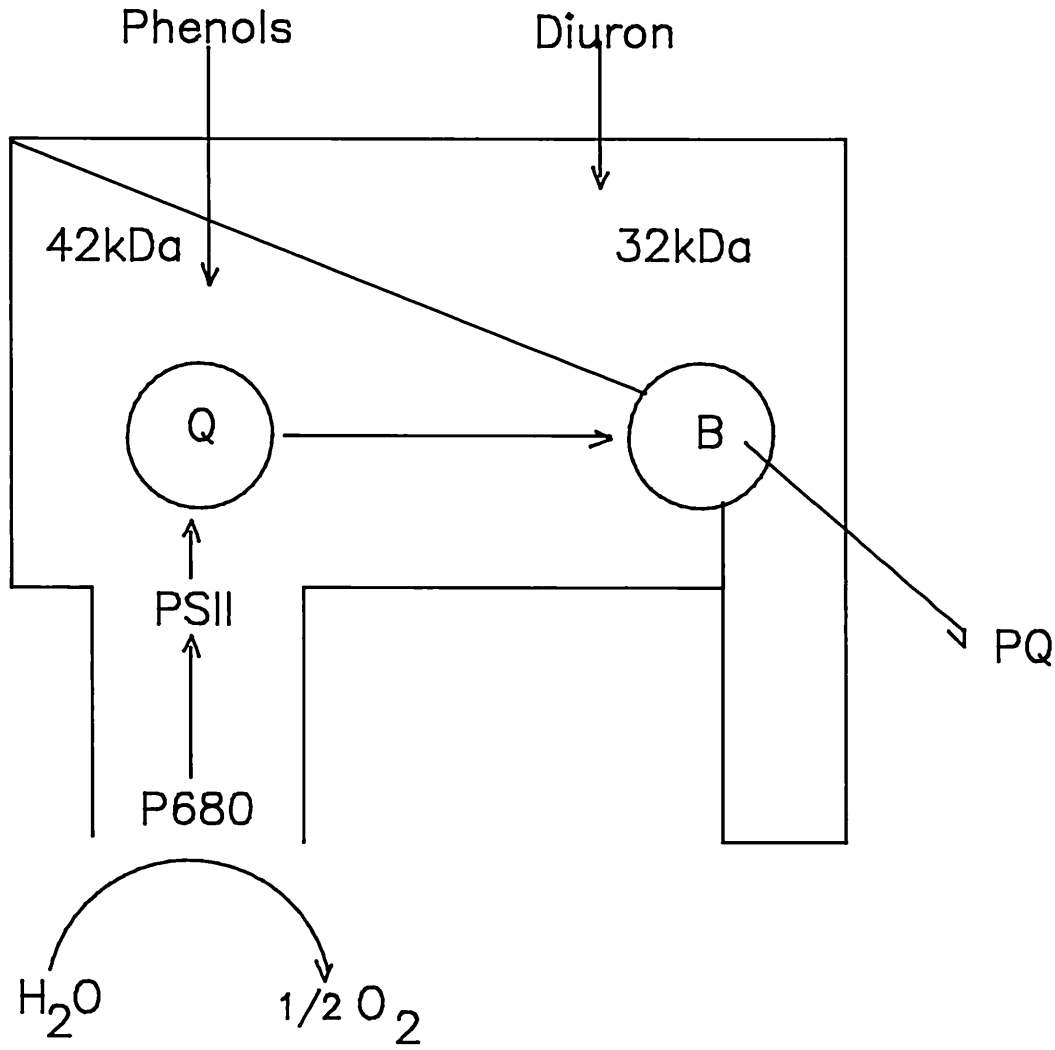


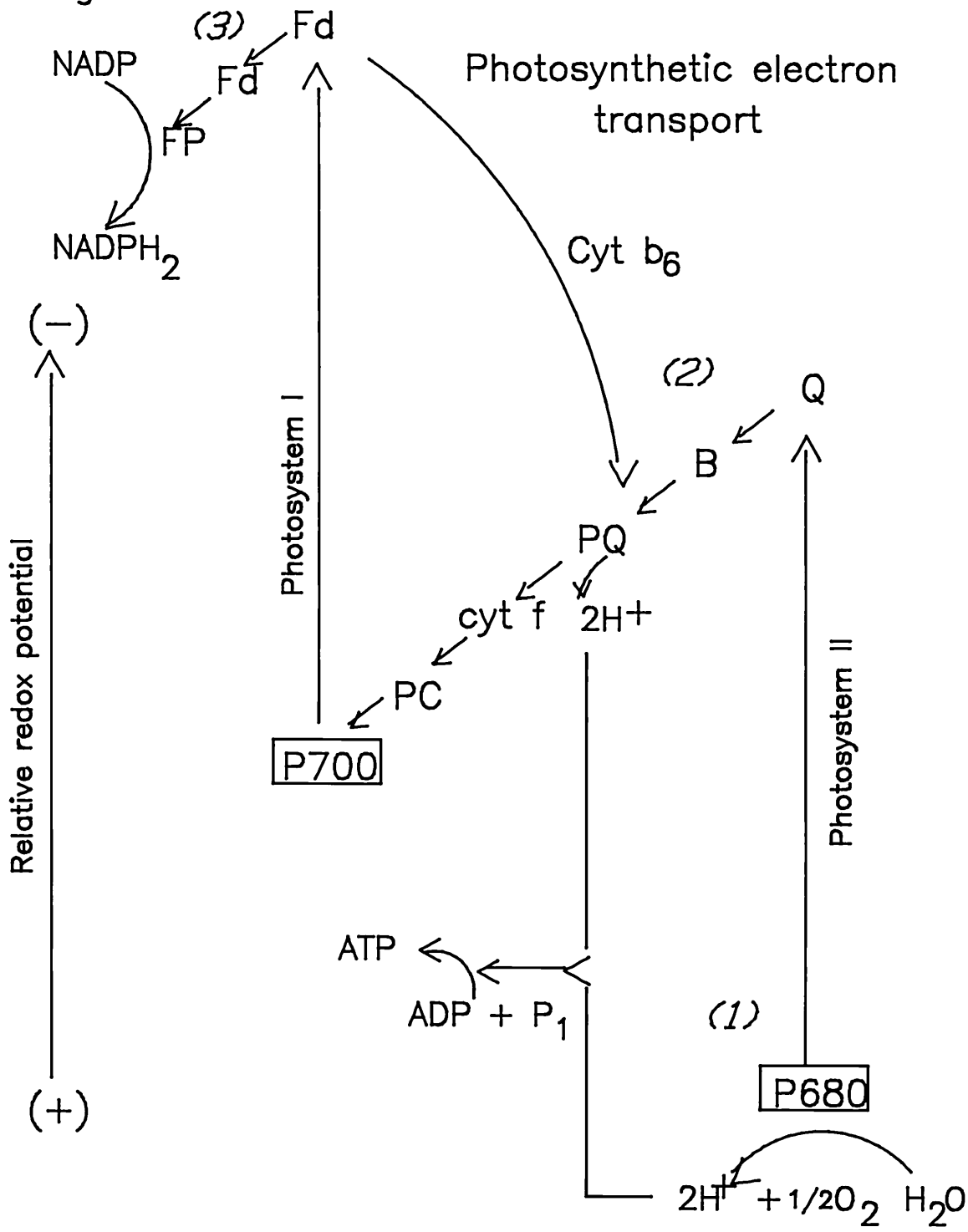
Fig. 6.1.2 Reactions in the chloroplast affected by phytotoxic compounds. (Non-cyclic electron transport in the chloroplasts, the arrows depicting major interactions (1) (2) (3).) The following numbers indicate sites of action by herbicides (1) inhibition of the reducing side of photosystem II (e.g. triazines and ureas), (2) inhibition of electron flow through cytochrome b (e.g. diphenyl ethers), (3) deviation of electrons from the reducing side of photosystem I (e.g. bipyridyliums), (4) dissipation of the proton gradient (e.g. diphenyl amines), (5) inhibition of ATP formation at the ATPase (e.g. nitrofen), (6) inhibition of carotenoid biosynthesis (e.g. oxadiazon), (8) peroxidative destruction of membranes (e.g. bipyridyliums).

Abbreviations used:

P680, reaction centre chlorophyll of photosystem II; Q, primary electron acceptor for photosystem II; B , secondary acceptor of photosystem II; PQ, plastoquinone; cyt b_6, b type cytochrome; cyt f, cytochrome f; PC, plastocyanin; P700, reaction centre chlorophyll of phtotosystem I; Fd, ferredoxin; FP, ferredoxin-NADP oxidoreductase.

Moreland, 1980; Sandmann and Böger, 1986

Fig. 6.1.2



Phosphorylation

Chloroplast components

Δ pH	ATP synthase	Carotenoid synthesis	Chlorophyll synthesis	Membrane lipids
(4)	(5)	(6)	(7)	(8)

is still inhibited by diuron (Aro, Niemi and Valanne, 1981) and the triazine herbicide atrazine (Valanne, 1984), although Atkinson, Abernathy and Crisp (1980), and Balcerkiewicz and Rusinska (1987) have observed limited effects of triazine herbicides on mosses. The lack of information on the effects of herbicides on mosses makes it necessary to rely heavily on information about modes of action in vascular plants. Attempts can then be made to compare observed biological responses in mosses with those shown by vascular plants. A summary of the modes of action in vascular plants of the four compounds investigated in the present study is given in Section 1.7.2.

The experiments described in this chapter measure the effects of dichlorophen, paraquat, 2,4-D and ferrous sulphate on *R. squarrosus* photosynthetic and respiratory carbon dioxide exchange. Chlorophyll content, chlorophyll *a* : chlorophyll *b* ratios and dry weights were also determined as they have previously been shown to be sensitive indicators of environmental stresses in mosses (Martin and Warner, 1984; Inglis and Hill,1974). Discussion of the results draws upon information available on the activity of these chemicals in vascular plants.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

Plant material as described in Section 3.2.1.

6.2.2 Herbicides

Herbicides were sprayed at the manufacturers' recommended rates, where applicable, for moss control; otherwise at rates recommended for agricultural purposes and without the addition of surfactants (Section 2.2.5). All formulations were made up in distilled water.

6.2.3 Herbicide application

Established trays of *R. squarrosus* were sprayed using a Birchier hand held sprayer. Applications were made in the open under still conditions, a temperature of 12°C and diffuse sunlight. The trays of *R. squarrosus* were left for 1 h before returning to the glasshouse. Direct misting was delayed for 8 h after herbicide application but humidity was maintained by under-surface watering of the sand trays during this period.

6.2.4 Measurement of photosynthesis and respiration

An Analytical Developments Type 225 Mk(2) infra-red gas analyser (IRGA) was used to detect changes in carbon dioxide in an assimilation chamber. The signal output was recorded on a Servoscribe chart recorder.

Carbon dioxide analysis was made by absolute measurement. The IRGA was set to zero with CO_2-free air and a known concentration of CO_2, from a cylinder, was used for calibration.

Photosynthesis and respiration were measured using the sample injection system described by Larson and Kershaw (1975), Clegg, Sullivan and Eastin (1978) and Snelgar, Brown and Green (1980). This involved the use of CO_2-free air passing through the analysis cell via the reference cell. A constant flow rate (500 cm^3 min^{-1}) through the IRGA ensured a linear response to CO_2 (Larson and Kershaw, 1975). Injection of a 5 ml sample of air with a known CO_2 concentration into the injection port, situated between the reference and the analysis cells, was used for calibration. The chart recorder was set so that cylinder air for the subsequent experimentation gave a deflection of 60%, thus enabling detection of both a reduced (photosynthesis) or increased (respiration) level of CO_2.

Apical segments of moss 3 cm long were removed from the herbicide-treated trays in the glasshouse and placed in Petri dishes containing a layer of moistened filter paper. Each dish containing 12 segments was preconditioned in a growth cabinet for 18 h at 15°C, with a 14 h photoperiod using fluorescent tube lighting (275 μmol m^{-2} s^{-1}). This procedure ensured a constant environment immediately prior to each experiment.

Before use, each sample of 12 segments was immersed in distilled water for 5 min, to ensure complete hydration and the removal of any associated debris. It was then gently tapped with a paper towel to remove excess water but without dehydrating or damaging the tissues. Samples were placed in cylindrical Perspex assimilation chambers of known volume (*ca.* 100 cm^3). Each chamber possessed an airtight removable top, an air inlet and outlet port sealable with small rubber bungs, and a sampling port with a Subaseal stopper (Gallenkamp).

Air of known CO_2 concentration (289 vpm) from a compressed air cylinder was used to flush through the assimilation chamber for 1 min at a flow rate of 1200 cm^3 min^{-1}. After 1 min, and with air still flushing through, a 5 cm^3 sample of air was then withdrawn by syringe and injected into the IRGA to establish the initial CO_2 concentration within the chamber. The air outlet port was stoppered and the air inlet tube removed and the port quickly stoppered, producing a closed system. The chamber was placed beneath a Perspex waterbath receiving overhead lighting from a bank of four tungsten lamps using a system previously designed by Sheahan (1985). The waterbath in this case functioned as a heat trap by its connection to a mains water supply and venting the overflow to the sink. Therefore, most of the radiant heat emitted from the tungsten lamps was absorbed by the water. This enabled chamber temperatures of 20°C to be maintained for the 10 min incubation period.

Light of different photon flux densities was obtained by different permutations of four tungsten lamps and by altering the chamber position. A photon flux density of 230 μmol m^{-2} s^{-1} was used, based on prior experimentation, to determine photosynthesis rates of *R. squarrosus* (see Section 6.3.1). On completion of the 10 min incubation time, the chamber was removed from the light source and a 5 ml air sample extracted with a syringe and injected into the IRGA. Prior to sampling the syringe was flushed with CO_2-free air to prevent carry over of CO_2 between samples.

To obtain respiration data from the same sample, the chamber was reflushed with air, following the above procedure, and incubated in the dark for 10 min.

6.2.5 Chlorophyll determination

Moss segments, taken from the assimilation chambers after photosynthesis and respiration measurements, were macerated in a pestle and mortar together with 10 cm^3 of a cold 80% acetone:water mixture acid-washed silver sand and magnesium carbonate. The macerate was then transferred to a glass scintillation vial and left to infuse for 16 h in darkness at 4°C.

The determination of chlorophyll in the extracts was based on the method of Arnon (1949). Absorbance was measured with a Cecil CE 588 Microcomputer Scanning Spectrophotometer at wavelengths of 645 nm and 663 nm against a reference of 80% acetone. Concentrations of total chl, chl*a*, chl*b* in the extracts were obtained using the following equations:

Total chl (mg l^{-1}) = 20.2D (645 nm) + 8.02D (663 nm)

chl*a* (mg l^{-1}) = 12.7D (663 nm) - 2.69D (645 nm)

chl*b* (mg l^{-1}) = 22.9D (645 nm) - 4.68D (663 nm)

where D is absorbance at the stated wavelengths.

6.2.6 Analysis of data

Net photosynthesis and respiration data were obtained for each treatment on eight replicate shoot samples. Four of these replicates were subsequently dried at 80°C, the remaining four being retained for the estimation of chlorophyll (chl) content. Samples were taken one day prior to herbicide application and at intervals of 1, 5, 9, 28, 56, and 82 days after spraying.

Treatments and replicates were randomly arranged in the glasshouse and in the growth cabinet and rerandomised at intervals of 7 days.

Net photosynthesis data are expressed and plotted against time as µl CO_2 g^{-1} dry weight h^{-1} and µl CO_2 mg^{-1} chl h^{-1}. Respiration is expressed in the same manner and was also subtracted from the net photosynthesis measurements to obtain gross photosynthesis. The expression of the data both in terms of dry weight and chlorophyll content is necessary if the exact cause of photosynthetic changes are to be identified (Prezelin, 1981).

6.3 RESULTS AND DISCUSSION

6.3.1 Determination of conditions for photosynthesis and respiration measurements

Photosynthesis in mosses is known to be dependent on temperature, tissue moisture content and light intensity (Snelgar, Brown and Green, 1980; Maberly, 1985). In the following experiments temperature was maintained at 20°C, within the optima described for temperate mosses by Kallio and Karenlampi (1975), and Longton (1979). Moisture content was kept high for experimental purposes, by equilibrating the shoots on wet filter paper in closed Petri dishes for 24 h prior to experimental use. Before measurements were taken, the shoots were rinsed again in distilled water and gently swabbed with tissue paper. The removal of excess water is necessary to minimise resistance to diffusion of CO_2 (Dilks and Proctor, 1979).

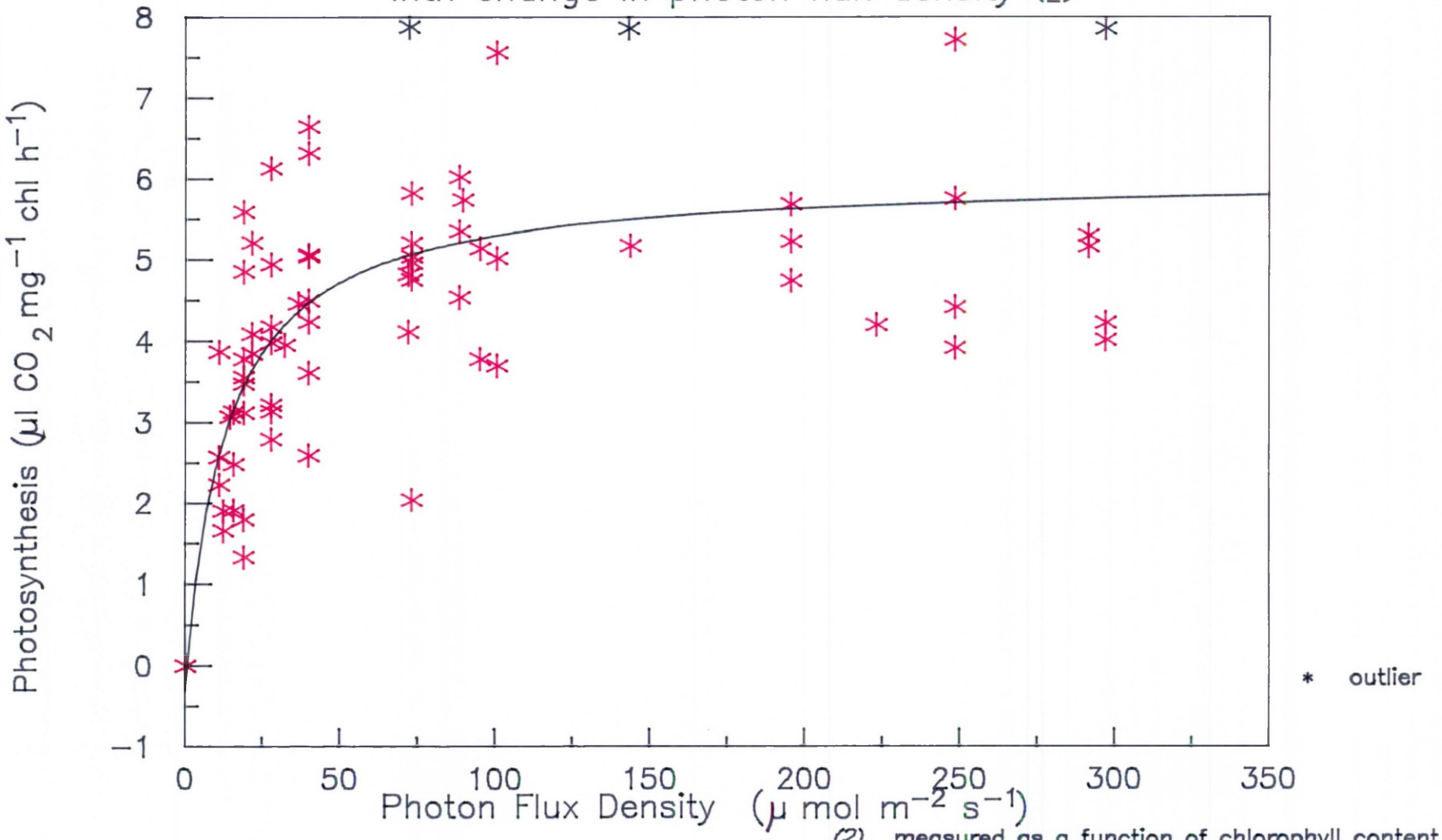
The relationship of photosynthesis to light intensity, as revealed by graphical analysis (PI curves), is influenced by several factors in mosses in addition to temperature and moisture content. Each species has a unique PI relationship and this changes with variation in habitat, season, latitude, nutritional status and previous light conditions (Kallio and Karenlampi, 1975; Valanne, 1984).

The PI response of *R. squarrosus* was determined over a 0-379 µmol m^{-2} s^{-1} photon flux density range. The photosynthetic response of 8 samples of moss (12 x 3 cm apical shoots) to increasing photon flux densities was measured (Section 6.2.4). The iterative Maximum Likelihood Program (Ross, 1987) was used to fit the PI equation of Baly (1935) (see Section 7.2.5), to the data for gross photosynthesis. Two PI curves were produced for *R. squarrosus* depicting photosynthesis as a factor of 1) dry weight (µl CO_2 g^{-1} h^{-1}) (Fig. 6.3.2), and 2) total chlorophyll (chl) content (µl CO_2 mg^{-1} chl h^{-1}) (Fig. 6.3.1). A photon flux density (230 µmol m^{-2} s^{-1}) producing an easily measurable photosynthetic response on the lower part of the asymptotic section of the PI curve was selected for use in the following experiments. This minimises differences between individual *R. squarrosus* samples and also prevents damage to the photosynthetic apparatus of the moss (Stalfelt, 1936 cited Kallio and Karenlampi, 1975; Kallio and Valanne, 1975) or overheating of the chambers.

6.3.2 Effects of herbicides on photosynthesis and respiration

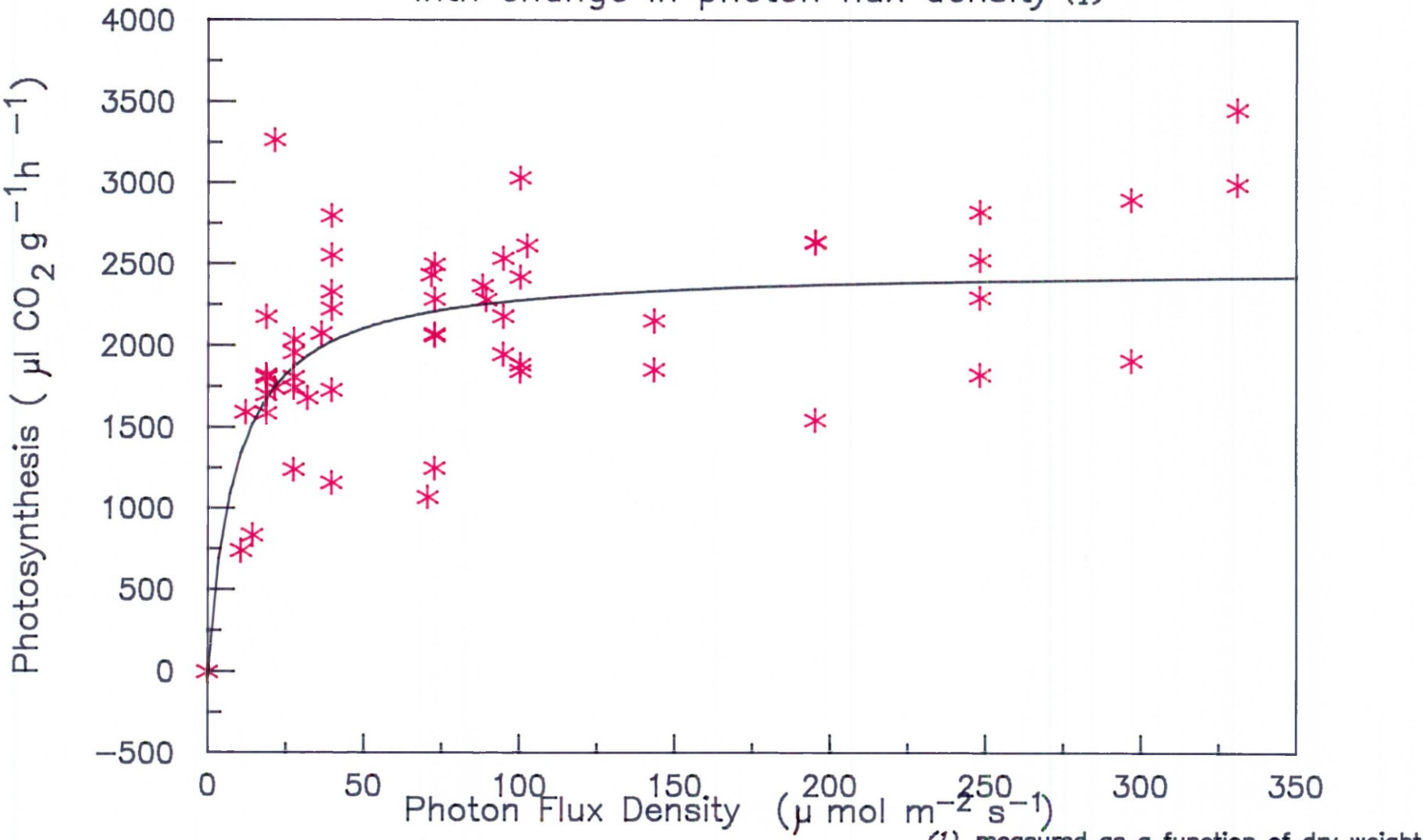
The effects of a single application of dichlorophen, $FeSO_4$, 2,4-D and paraquat on subsequent gross photosynthesis, respiration, chlorophyll content, ratio of chlorophyll *a* : *b* and dry weight of shoot apices of *R. squarrosus* are plotted in Figures 6.3.3 to 6.3.8.

Fig. 6.3.1 Gross CO_2 assimilation rate of *Rhytidiadelphus squarrosus* with change in photon flux density (2)



(2) measured as a function of chlorophyll content

Fig. 6.3.2 Gross CO_2 assimilation rate of *Rhytidiadelphus squarrosus* with change in photon flux density (1)



(1) measured as a function of dry weight

1. Dichlorophen

R. squarrosus became chlorotic within 1 day of dichlorophen application, as observed previously by Ougham (1983) and Anon (1982). However, regrowth from the stem base became apparent eight weeks after treatment. The new growth of dichlorophen-treated moss was slender and pale in colour compared to new growth of untreated moss.

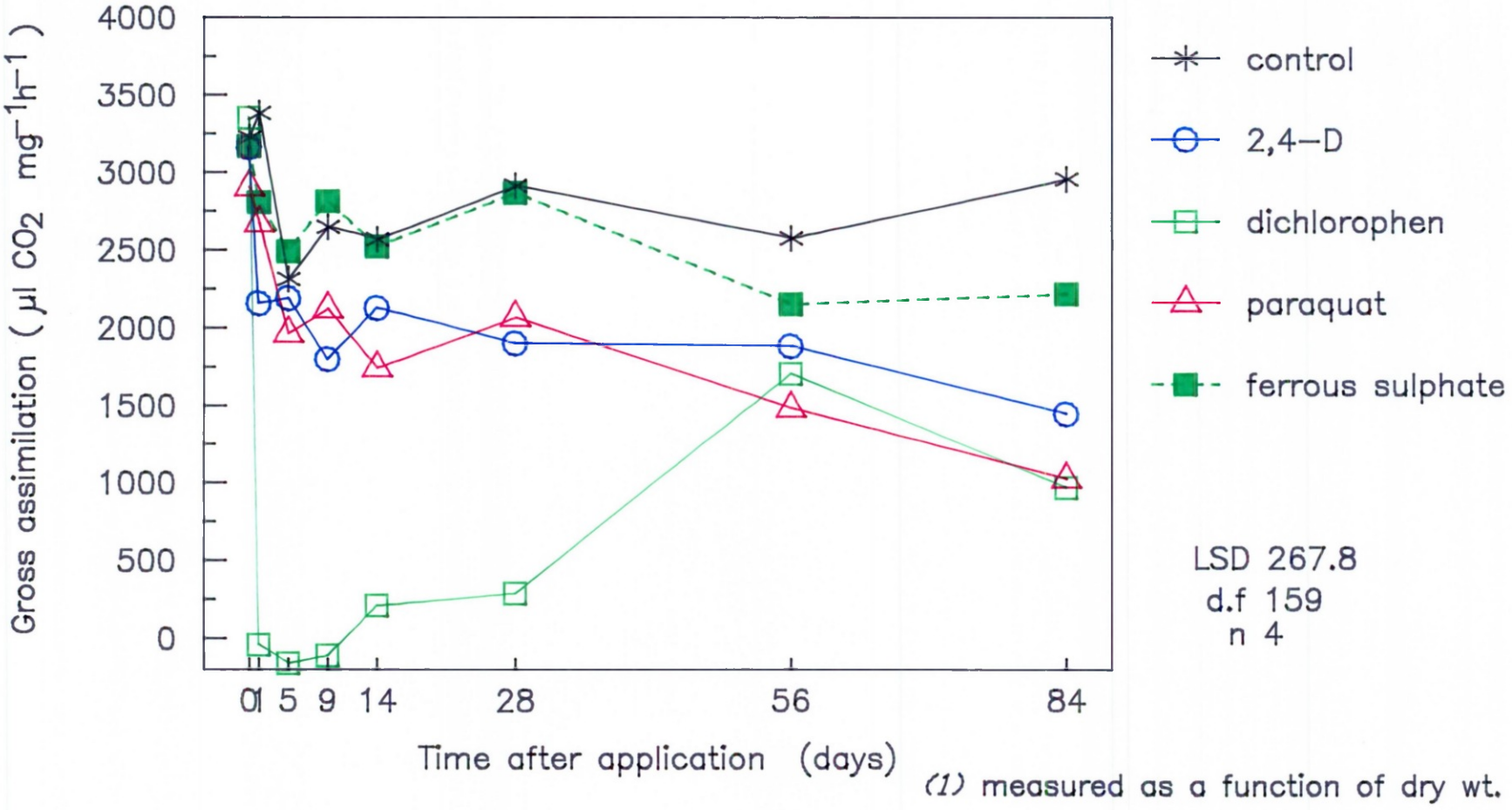
Dichlorophen treatment caused a rapid and significant decline in gross photosynthesis of *R. squarrosus* (Fig 6.3.3). Photosynthesis was undetectable one day after application of dichlorophen. Some recovery was apparent within five days so that gross photosynthetic rate was equal to 4% of the control rate. By week 12 this had increased to 33% of the control. A proportion of the photosynthetic activity measured 12 wk after treatment is attributable to new growth from the damaged moss tissue.

Dichlorophen caused a significant decline in the respiration rate of *R. squarrosus* (Fig. 6.3.4). A 60% reduction in respiration rate was observed one day after application. Recovery was rapid, and within five days of application the respiration rate was not significantly different from that of the controls. This cannot be explained by the inclusion of newly-grown shoots.

Dichlorophen is classified as an inhibitory uncoupler affecting electron transport and oxidative phosphorylation (Moreland, 1980) and would therefore be expected to affect respiration as well as photosynthesis at high doses. For an uncoupler, an initial increase in respiration rate is expected before a decline. In the present work such an increase was not detected, possibly because the first measurement was delayed until 24 h. The concentration of dichlorophen applied to the moss was sufficiently high to cause damage to the photosynthetic system. However, Moreland (1980) considered that photosynthetic damage occurred at doses greater than those required to inhibit respiration in vascular plants.

Fig. 6.3.3

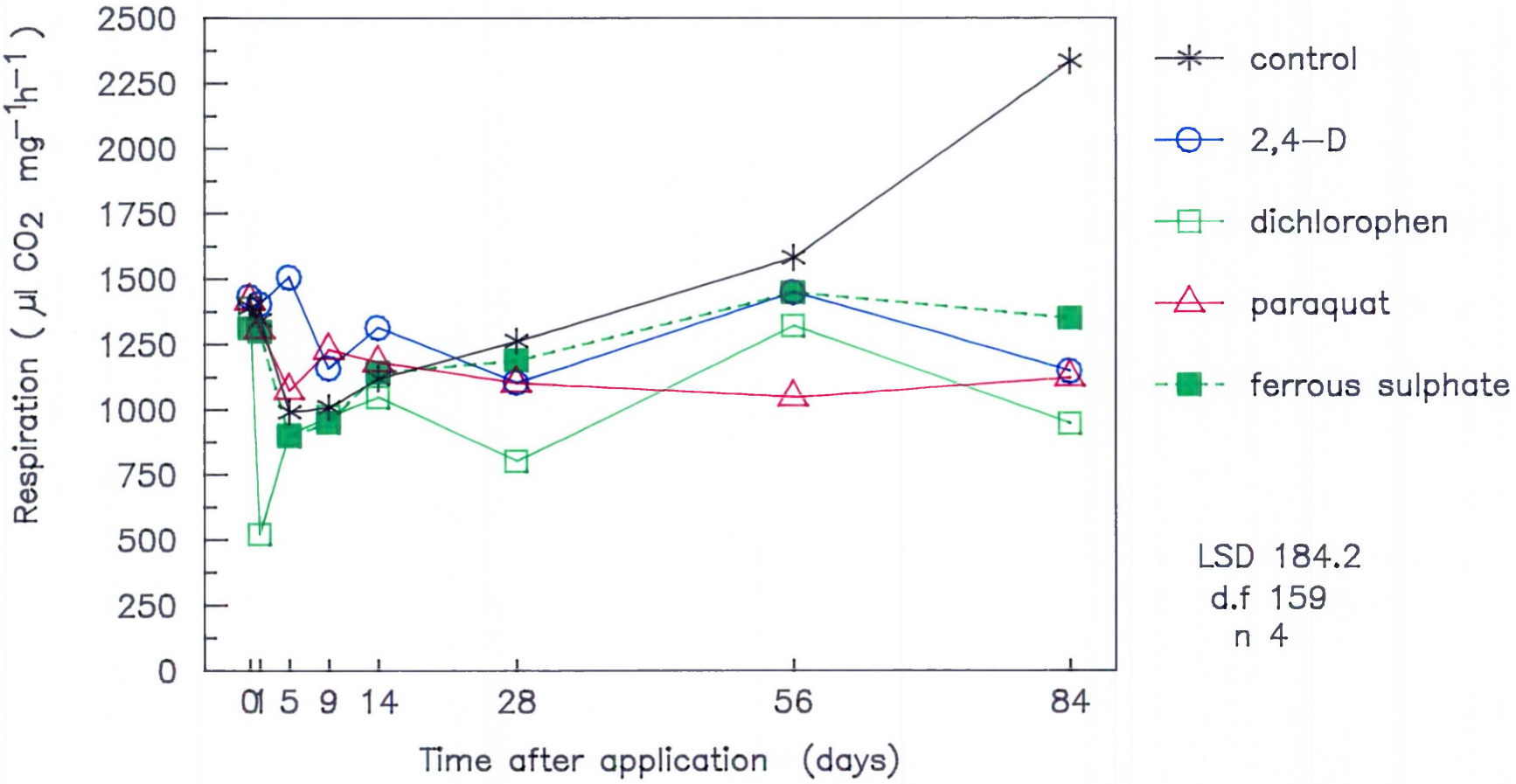
Changes in the gross CO_2 assimilation rate of *Rhytidiadelphus squarrosus* after treatment with dichlorophen, paraquat, 2,4–D and $FeSO_4$. *(1)*



(1) measured as a function of dry wt.

Fig. 6.3.4

Changes in the respiration rate of *Rhytidiadelphus squarrosus* after treatment with dichlorophen, paraquat, 2,4–D and $FeSO_4$.



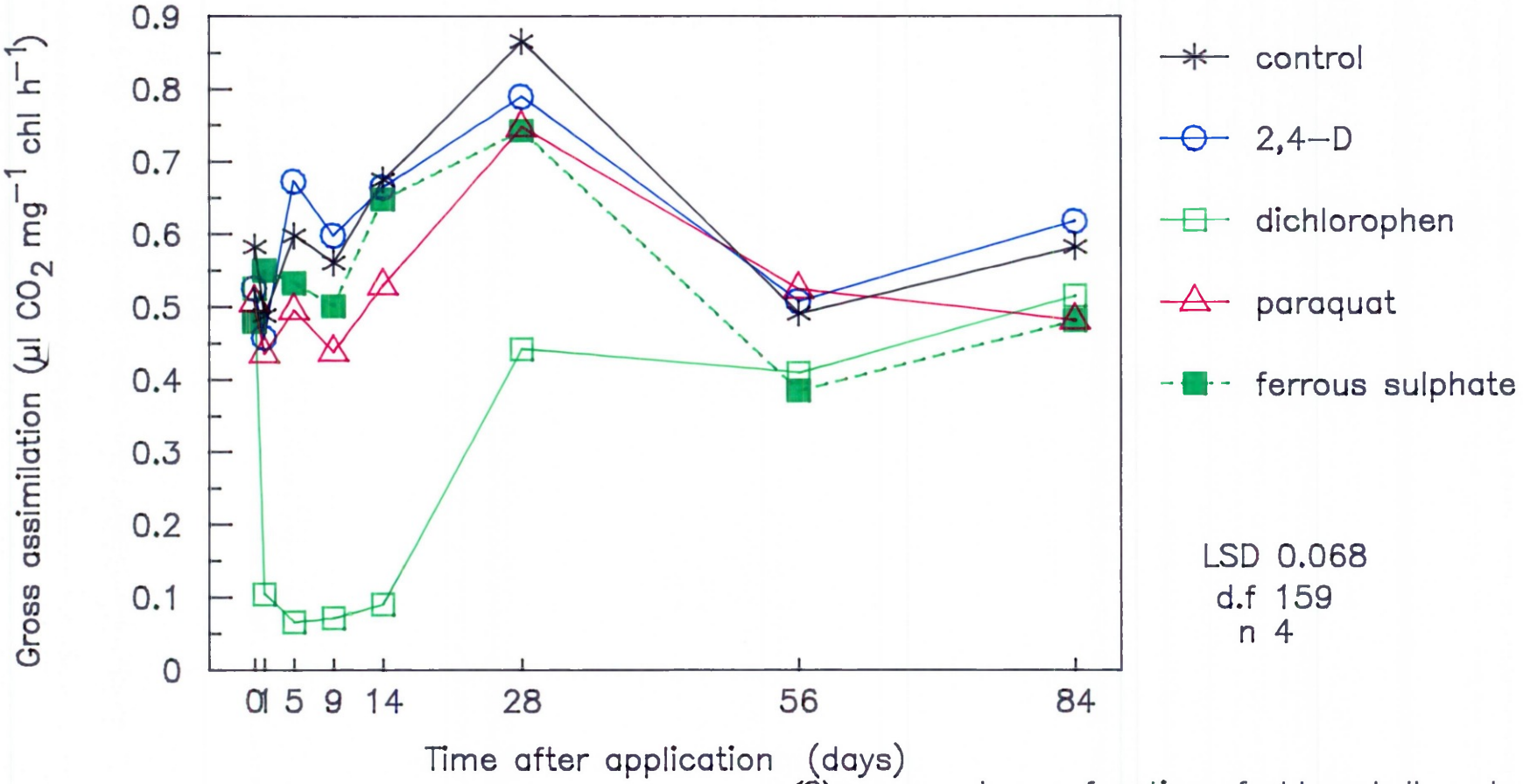
The lack of effect of dichlorophen on respiration was also noted by Brown, Ougham and Beckett (1986) in *R. squarrosus.* They attributed this to (1) the use of carbon dioxide release as the method of assessment, and (2) the possibility that carbon dioxide production might continue if mitochondrial membranes were damaged, but without the generation of ATP. In the case of photosynthesis, the production of ATP is essential for CO_2 uptake. Therefore, the results do not necessarily conflict with the uncoupler hypothesis.

An alternative explanation is that the density of *R. squarrosus* stems (Section 1.5) prevents adequate uptake of dichlorophen. The stems of *R. squarrosus* probably contain less chlorophyll than the leaves. It is likely that the photosynthetic potential of the stems is lower than that of the leaves on a dry weight basis. However, the stems probably make a larger contribution to respiration than the leaves. Thus the lack of inhibition of respiration may be a result of impaired uptake of dichlorophen into the stem due to its greater tissue density and protection by the surrounding leaves. Uptake by the leaves is likely to be considerable causing a rapid decline in photosynthesis and a slow recovery. This explanation, though, does not adequately account for the recovery of respiration.

Dichlorophen treatment of *R. squarrosus* also produced an 80% reduction in chlorophyll efficiency (µl CO_2 mg^{-1} chl h^{-1}) in comparison to controls (Fig 6.3.5). However, after 12 weeks no significant difference in the rate of CO_2 assimilation measured against chlorophyll content was apparent. The reduction in chlorophyll efficiency probably indicates inhibition of the photosynthetic pathway by dichlorophen.

Treatment of *R. squarrosus* with dichlorophen also caused a decline in total chlorophyll content (*ca.* -80%), five days after application (Fig 6.3.6). Afterwards, recovery of total chlorophyll content occurred and closely correlated with the recovery of photosynthesis.

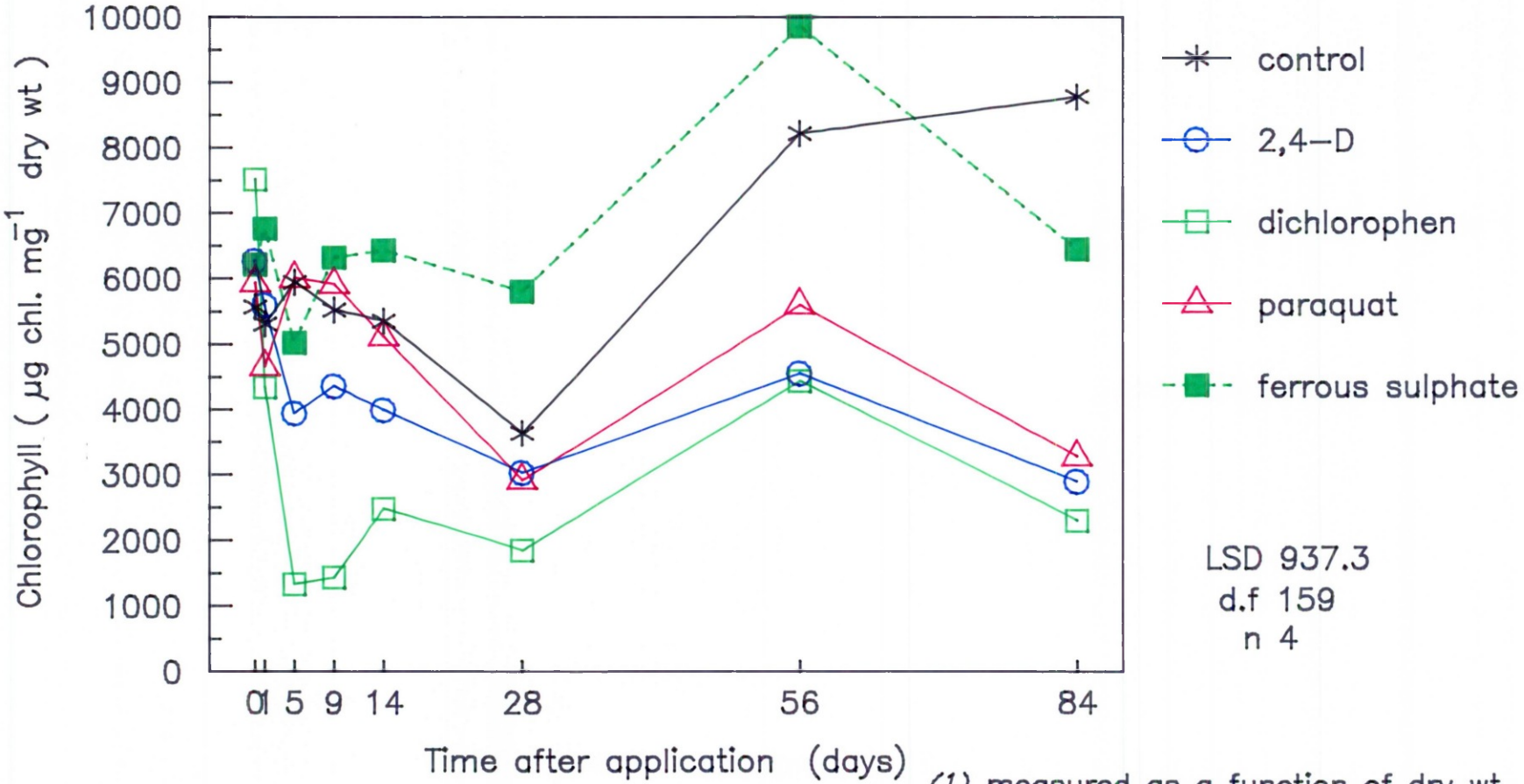
Fig. 6.3.5
Changes in the gross CO_2 assimilation rate of *Rhytidiadelphus squarrosus* after treatment with dichlorophen, paraquat, 2,4–D and $FeSO_4$. (2)



(2) measured as a function of chlorophyll content.

Fig. 6.3.6

Changes in the total chlorophyll content of *Rhytidiadelphus squarrosus* after treatment with dichlorophen, paraquat, 2,4–D and $FeSO_4$. *(1)*



(1) measured as a function of dry wt.

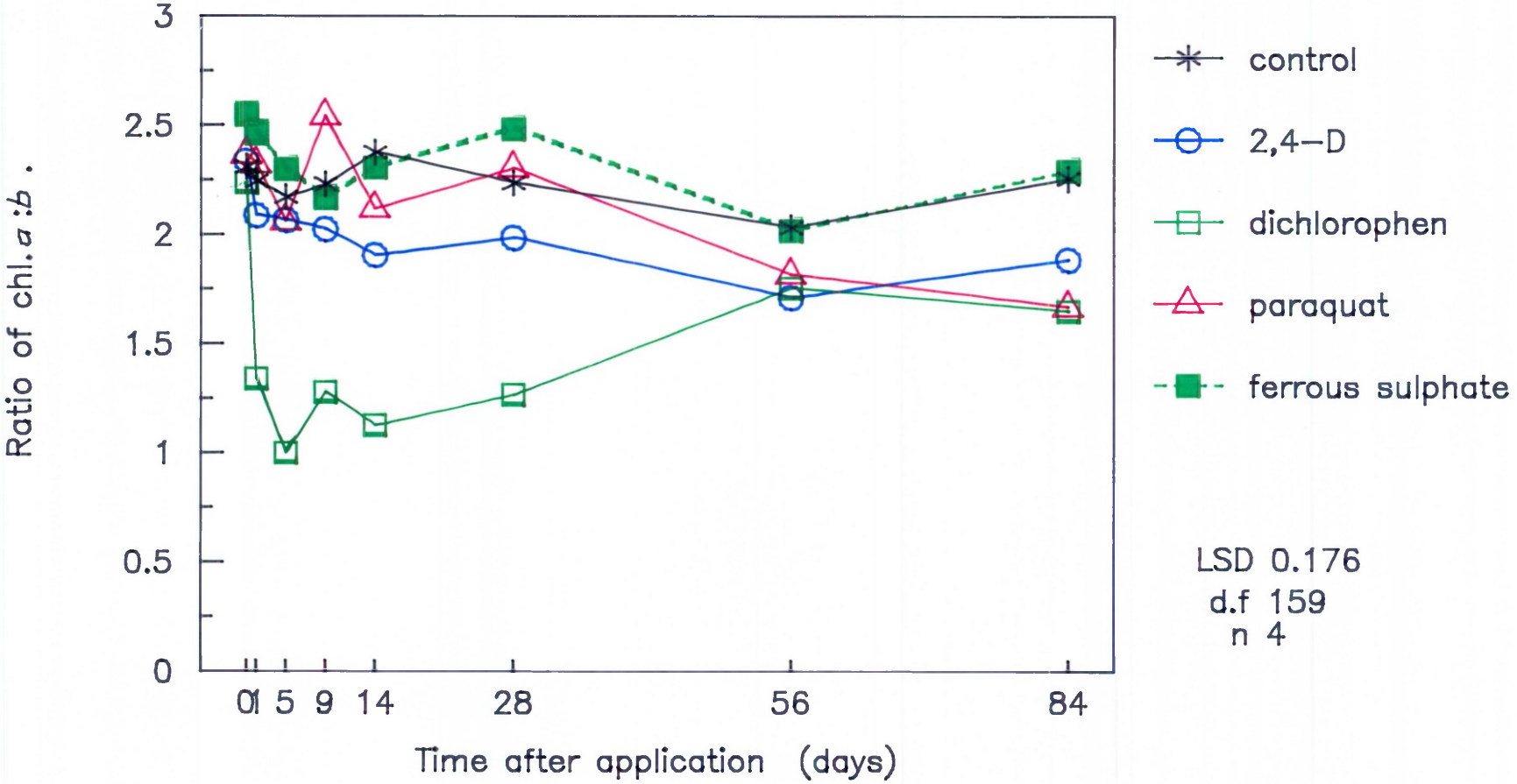
The chlorophyll *a* : *b* ratio of dichlorophen-treated tissue also declined significantly to 60% of the control after five days, recovering by week eight (Fig 6.3.7) and this suggests a greater sensitivity of chl*a* than chl*b* to dichlorophen.

The rapid reduction in photosynthesis, total chlorophyll content and the chlorophyll *a* : *b* ratio implies that dichlorophen affects the photosynthetic system of *R. squarrosus.* The larger reduction in chlorophyll *a* content compared to that of chlorophyll *b* may help identify the site of action of dichlorophen in the photosynthetic system of *R. squarrosus* (Fig. 6.3.7). Ridley (1977) studied the loss of photosynthetic pigments following diuron treatment of pea chloroplasts. He suggested that the inhibition of photosynthetic electron transport caused conformational changes to the membrane responsible for transferring excitation energy from chlorophyll pigments; and that the protection of chlorophyll pigments, normally via a mechanism involving carotenoids, is then no longer adequate and pigment breakdown occurs. The damaging species was described by Ridley (1977) as excited molecular oxygen. This is thought to be generated from excess light absorbed by chlorophyll which is dissipated by a reaction not with a carotenoid but with O_2. Ridley (1977) found that loss of the electron transport system serving chlorophyll *a* (photosystem I) (Fig. 6.1.2) led to the degradation of chlorophyll *a*. It is possible that the activity of dichlorophen may also centre in photosystem II, inhibiting the transfer of electrons to, and therefore causing degradation of chlorophyll *a* (PSI).

Evidence for this hypothesis is available in the literature on other phenolic herbicides and that dealing with herbicide resistance and selectivity in vascular plants, algae and mosses. Oettmeier, Masson and Johanningmeir (1982) observed that phenolic herbicides bind to a 41 kDa protein in the QA/QB region of the thylakoid membrane.

Fig. 6.3.7

Changes in the ratio of chlorophyll *a* : *b* in *Rhytidiadelphus squarrosus* after treatment with dichlorophen, paraquat, 2,4–D and $FeSO_4$.



They also suggested that the protein might be situated beneath the 32-34 kDa polypeptide in vascular plants because of the increased sensitivity of plants lacking in the 32-34 kDa polypeptide to phenolic herbicides (Fig. 6.1.1). Additionally, Aro (1982) recorded concentrations of the 41 kDa protein in mosses in excess of those found in vascular plants. Therefore, the reduced levels of the 32-34 kDa polypeptide but the presence of increased levels of the 41 kDa PS II polypeptide may explain the greater sensitivity of the mosses to dichlorophen.

This suggestion is further supported by comparison of the results of Atkinson, Abernathy and Crisp (1980) with those of Pfister, Radosevich and Arntzen (1979). Atkinson *et al.* (1980) observed little effect on mosses of the triazine herbicide simazine in comparison with the high toxicity of diuron to these plants. Pfister *et al.* (1979) observed similar responses to atrazine (a triazine herbicide) and diuron in isolated chloroplasts of the triazine-resistant vascular plant *Amaranthus retroflexus*. In addition, Pfister and Arntzen (1979) identified increased sensitivity of the triazine resistant vascular plant *Senecio vulgaris* to phenolic herbicides. Diuron and the triazine herbicides have been classified as inhibitors of Photosystem II at the site of the 32-34 kDa polypeptide (Trebst, Draber and Donner, 1983). Pfister and Arntzen (1979) concluded that the different groups of herbicides bind to distinct but overlapping binding sites of the protein in the PSII complex. A further physiological feature common to the triazine-resistant vascular plants and mosses, possibly supporting similarities in their PSII structures, is their reduced PSII electron transport efficiencies (Bowes, Crofts and Arntzen, 1980; Aro, 1981). The similarity in the response of the triazine-resistant vascular plants and mosses to triazine, urea and phenolic herbicides and their reduced PSII electron transport efficiency suggests similarities in their PSII protein complexes. The reduction of

photosynthesis and chl*a* content of *R. squarrosus* following dichlorophen treatment may indicate that it is active by inhibiting electron transport in PSII, as for other phenolic type herbicides in vascular plants. The sensitivity of *R. squarrosus* may relate to 1) a large quantity of the 41 kDa polypeptide in PSII (Aro, 1982b) to bind phenolic type herbicides in vascular plants (Oettmeier *et al.*, 1982) and 2) reduced levels of the 32 kDa shielding polypeptide (Aro *et al.*, 1981), believed to reduce the binding of phenolic herbicides to the 41 kDa polypeptide (Oettmeier *et al.*, 1982).

2. $FeSO_4$

The symptoms observed following $FeSO_4$ application to mosses have been well documented (Field, 1972; Anon, 1982; Kuttruff and Kleve-Kellen, 1980). A rapid blackening of the tissue occurs, sometimes referred to as scorch (Anon, 1982). However, in the present study, signs of recovery were obvious in *R. squarrosus* within one week of $FeSO_4$ application, prominent within four weeks, and by the eighth week little blackening was visible due to occlusion by the substantial new growth. Regrowth in *R. squarrosus* occurred from the apex and/or the lower stem, indicating little toxic effect of the $FeSO_4$ treatment on the stems.

The gross photosynthetic reponse of *R. squarrosus*, following $FeSO_4$ treatment, resembles that of the controls (Fig. 6.3.3). The extent of blackening does not correlate with the photosynthetic data or chlorophyll contents (Figs 6.3.3 and 6.3.6) indicating that it is misleading to use blackening as an indicator of damage. Plate 6.3.1 shows the effect of increasing $FeSO_4$ concentration on the intensity of the black coloration of *R. squarrosus* shoots.

Light microscopic examination of the blackened leaves of *R. squarrosus*, following $FeSO_4$ treatment, revealed no obvious structural differences from those of untreated plants. This, together with the lack of effect on photosynthetic response, suggests that the blackening



Plate 6.3.1 Blackening of Rhytidiadelphus squarrosus shoot apices in relation to ferrous sulphate concentration

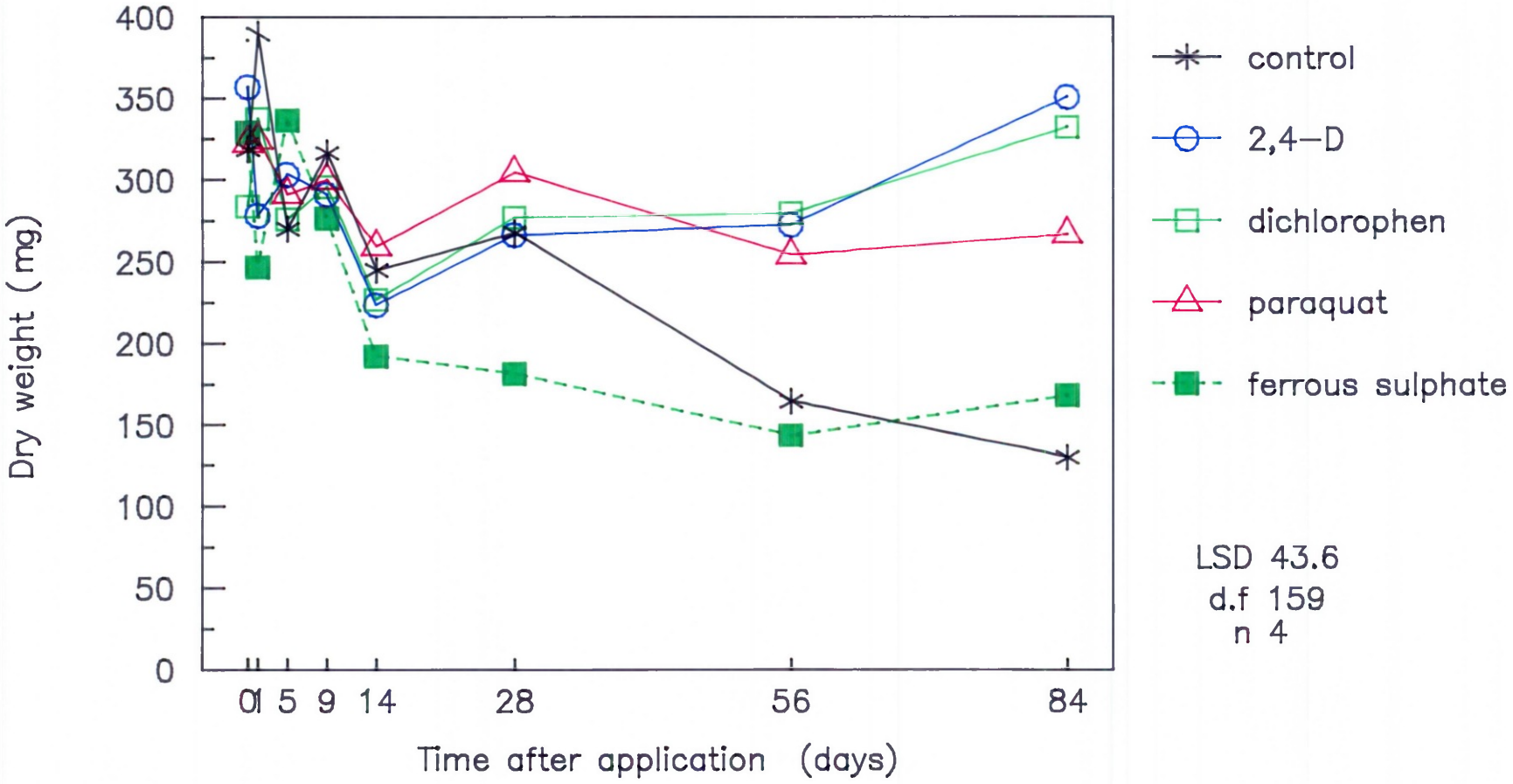
Fe concentration (from left to right) 0, 0.2, 1, 2, 10, 50, 100 mM

of the tissues is due to binding of Fe to the cell walls. The blackening effect is common to many moss species but absent in *Sphagnum* spp. (Field, 1972) and has not been described for the absorption of other cations by mosses. The formation of a pigmented complex with iron in moss cell walls could be attributable to adsorption to tannins (Field, 1969) and/or their phenolic derivatives, the deposition of $Fe(OH)_3$ deposits by oxidation (Armstrong and Boatman, 1967) or to the formation of insoluble sulphur compounds similar to those described by Satake and Miyasaka (1984) for mercury accumulation in the liverwort *Jungermannia vulcanicola.*

Respiration rate, chlorophyll efficiency, total chlorophyll content, and chlorophyll *a* : *b* ratio in *R. squarrosus* were unaffected by $FeSO_4$ application (Figs. 6.3.4 to 6.3.7). The fact that chlorophyll efficiency (µl CO_2 mg^{-1} chl h^{-1}) was not impaired by the $FeSO_4$ application suggests that the intense black coloration had not impeded light penetration under the experimental conditions used. An increase in chlorophyll content greater than the control was noted in the first six weeks following application of $FeSO_4$ (Fig. 6.3.6). This may indicate the existence of a deficit in internal iron content prior to application, or possibly the displacement of other nutrient ions from the cell wall by the $FeSO_4$ treatment and their subsequent uptake into the moss cells. Alternatively, the chlorophyll increase may be a response by the moss to a reduction in light penetrating the blackened tissue.

A major factor influencing the significance of measurements of total chlorophyll content in the moss after chemical treatments was the rapid growth shown by the control tissue during the course of the experiment. This new growth was of lower mass per unit length than the older tissue (Fig. 6.3.8). Consequently, control and $FeSO_4$ treated moss tissue may contain the same total amount of chlorophyll per

Fig. 6.3.8
Changes over time in dry weight of moss samples used for chlorophyll analysis after treatment with dichlorophen, paraquat, 2,4–D and $FeSO_4$.



unit length but when expressed as a function of dry weight, the value appears substantially greater in the former. Similar effects of tissue age were observed by Bates (1979) in *Pleurozium schreberi*, and Wells and Brown (1987) in *R. squarrosus.*

3. 2,4-D

The visible effects of 2,4-D on *R. squarrosus* were initially slight but chlorosis was visible at the stem apex 12 weeks after application. No new growth of *R. squarrosus* was observed following the 2,4-D application.

A gradual decline in gross CO_2 assimilation was observed in *R. squarrosus* following 2,4-D application (Fig. 6.3.3). However, no significant reduction was detected until 12 wk after treatment when gross assimilation had declined by 60% of that of the controls. Respiration rate, chlorophyll efficiency, chlorophyll concentration and chlorophyll *a* : *b* ratio all remained unaltered with respect to the control after 2,4-D application (Figs. 6.3.4 to 6.3.7). Total chlorophyll content did not significantly decline with time after 2,4-D treatment compared to the control (Fig. 6.3.6).

2,4-D is an anionic herbicide and is therefore not readily bound to the predominantly anionic sites of moss cell walls (Section 4.3.3.1). Consequently, it may remain in a form capable of uptake for a longer period of time than cations such as paraquat. The humidifier system of watering employed in the present experiments may have prolonged the availability of 2,4-D by maintaining a high moisture level at the moss surface without causing run-off and leaching of the chemical.

The activity of 2,4-D in dicotyledonous plants has been considered comparable with that of indole acetic acid. Symptoms of 2,4-D treatment in dicotyledonous plants are chlorosis, altered stomatal

function, stem tissue proliferation, disintegration of root tissue and abnormal apical growth (Corbett, Wright and Baillie, 1984). Chlorosis and a gradual decline in photosynthesis of *R. squarrosus* after treatment may be attributable to the auxin type activity of 2,4-D affecting cell growth (Section 1.7.2) and development. Visible growth abnormalities were not apparent, probably due to the absence of a translocation system in *R. squarrosus*. In dicotyledonous plants, 2,4-D interferes with auxin sensitive cells in their vascular tissue causing distorted growth. The absence of auxin sensitive cells in the vascular tissues of monocotyledonous plants is one of the factors contributing to their tolerance of 2,4-D.

4. Paraquat

Visible effects of paraquat on *R. squarrosus* tissue were much slower to develop than those of dichlorophen or $FeSO_4$ (Section 2.3.1 and 2.3.2). Chlorosis was detected during the fourth week after paraquat application. In general, chlorosis was initially detected at the stem apex but with time this was observed to spread basipetally. Unlike the response to dichlorophen (Section 2.3.1.1), leaf and stem tissue of *R. squarrosus* remained relatively turgid following paraquat treatment. However, no regrowth of *R. squarrosus* was visible after the application of paraquat.

Paraquat produced a progressive decline in photosynthetic rate with time (Fig. 6.3.3) but only after 12 wk was a significant reduction in photosynthesis detected, equivalent to a rate 65% of the control value. Chlorophyll efficiency was not impaired throughout the 12 wk experimental period (Fig. 6.3.5). However, the concentration of total chlorophyll present was significantly ($P<0.10$) reduced with time but was not significantly different from that of the control (Fig. 6.3.6). Chlorophyll *a* : *b* ratios declined slightly with time but this pattern

was also observed in the control (Fig. 6.3.7). Reductions in the chlorophyll content of the controls (Fig. 6.3.7) suggest that conditions within the glasshouse were not ideal or changed progressively during the course of the experiment.

The slow photosynthetic response and the limited effect of paraquat on chlorophyll content are not in accordance with the observed mode of action of paraquat in vascular plants. Harris and Dodge (1972) depicted the activity of paraquat in two phases: (1) the acceptance by paraquat of electrons from PS I, in the region of the ferredoxin molecule, leading to inhibition in $NADP^+$ reduction and a rapid cessation of CO_2 fixation, and (2) the production of activated oxygen species during auto-oxidation of paraquat radicals. Degradation of pigments and membranes therefore occurs, resulting in inhibition of PS II and of the electron transport components. The same authors also detected complete inhibition of photosynthesis in flax cotyledons, 5 h after application of paraquat. Interestingly, paraquat appears to have little effect on *R. squarrosus* during the first 4 wk following application but a significant reduction in photosynthesis was observed after 12 wk (Fig. 6.3.3).

In vascular plants paraquat usually acts rapidly when applied in the presence of oxygen and light. It would appear from the lack of immediate response in *R. squarrosus* that detoxification and/or a very slow uptake of paraquat into the cells occurs. The concept of slow uptake is unusual considering that paraquat is usually rapidly bound to anionically-charged surfaces (Section 4.3.1.6) and potentially deactivated soon after application. Consequently, it is extremely unlikely that paraquat will be freely available for uptake over a period of several weeks as suggested for 2,4-D (Section 6.3.2). However, strong evidence for transfer of exchangeably-bound paraquat to the protoplasts is presented in Chapter 4. This mode of cellular

uptake was also hypothesised for the pollutant cadmium in *R. squarrosus* (Wells and Brown, 1987) and the nutrient Mg in *Pseudoscleropodium purum* (Bates, 1989). The cation exchange capacity of bryophytes is generally higher than in vascular plants (Section 5.3.4) so that the slow uptake pathway for paraquat *via* the cell walls is likely to be of greater importance in the former.

R. squarrosus tissue may possess the ability to detoxify small quantities of paraquat. The moss cell membrane is not a completely impermeable barrier and a limited proportion of the applied paraquat can be expected to have permeated into the cells soon after application. Detoxification of the herbicide could occur by (1) limited binding to proteins or chelating agents within the moss tissue, and/or (2) the presence of protective mechanisms capable of minimising the effects of toxic oxygen such as those of superoxide dismutase, catalase, ascorbate peroxidase, carotenoids and α-tocopherol in vascular plants (Dodge, 1983). Such substances have not been studied in mosses although Valanne (1984) hypothesised that increased levels of protective substances (carotenoids or special lipids) may be present in mosses of sunny habitats to prevent photo-oxidation.

6.4 CONCLUSIONS

Photosynthesis, respiration and chlorophyll levels were determined in shoot segments of *R. squarrosus* before and after a single application of dichlorophen, $FeSO_4$, 2,4-D, and paraquat. The shoots were cultured in a glasshouse and measurements were made for 12 wk after herbicide application.

The reactions of *R. squarrosus* were different for each herbicide employed.

(1) Dichlorophen gave the most rapid reduction in the rates of photosynthesis and respiration. A reduction was also observed in the chlorophyll *a* : *b* ratio, due to the loss of chlorophyll *a*, and indicating a possible inhibition of electron transport in Photosystem II. The rapid effect of dichlorophen suggests easy uptake across the plasmalemma. Recovery was observed from the stem of *R. squarrosus* after dichlorophen treatment.

(2) The application of paraquat to *R. squarrosus* produced a slow decrease in the rates of photosynthesis and respiration. Chlorosis was observed following paraquat treatment but this took 8 wk to appear. In vascular plants, a substantial reduction in photosynthesis occurs soon after paraquat application due to its effects on membranes and the diverting of energy away from CO_2 fixation. The slow development of photosynthetic decline observed in *R. squarrosus* following paraquat treatment is probably attributable to binding of the herbicide to the cell wall and its slow relocation to the protoplast. It is possible that the moss can detoxify paraquat but no evidence was obtained for this suggestion.

(3) 2,4-D was not expected to have a detrimental effect on photosynthesis of *R. squarrosus* because its primary mode of action in vascular plants is as a plant growth regulator. However, a gradual reduction of photosynthesis was observed, but respiration and

chlorophyll levels were unaffected. This may reflect some auxin-type activity of 2,4-D in *R. squarrosus*. The classic symptoms of growth distortion may, however, not be apparent in *R. squarrosus* due to the absence of an internal translocatory system with auxin-sensitive cells, as found in dicotyledonous plants (Corbett, Wright and Baillie, 1984). (4) Treatment of *R. squarrosus* with $FeSO_4$, in contrast to the previous three compounds, produced a rapid blackening of the shoots but neither photosynthesis nor respiration rates were affected. Binding of iron to the cell walls of the moss may prevent it from influencing the photosynthesis and respiration of *R. squarrosus*. The blackening of the moss tissue is probably due to the formation of an insoluble, pigmented iron complex, perhaps with tannins, in the moss cell wall. Growth from the apex and the stem of *R. squarrosus* following $FeSO_4$ application was normal.

Spray applications of herbicides were used in this part of the study. These can be expected to give less satisfactory penetration of the moss tissue compared to immersion but have more relevance to the field situation. This factor may also have contributed to the limited damage observed following treatment with paraquat and 2,4-D.

CHAPTER 7

A STUDY OF THE MODE OF ACTION OF DICHLOROPHEN AND PARAQUAT ON *RHYTIDIADELPHUS SQUARROSUS* BY PHOTOSYNTHESIS-IRRADIANCE CURVE ANALYSIS

7.1 INTRODUCTION

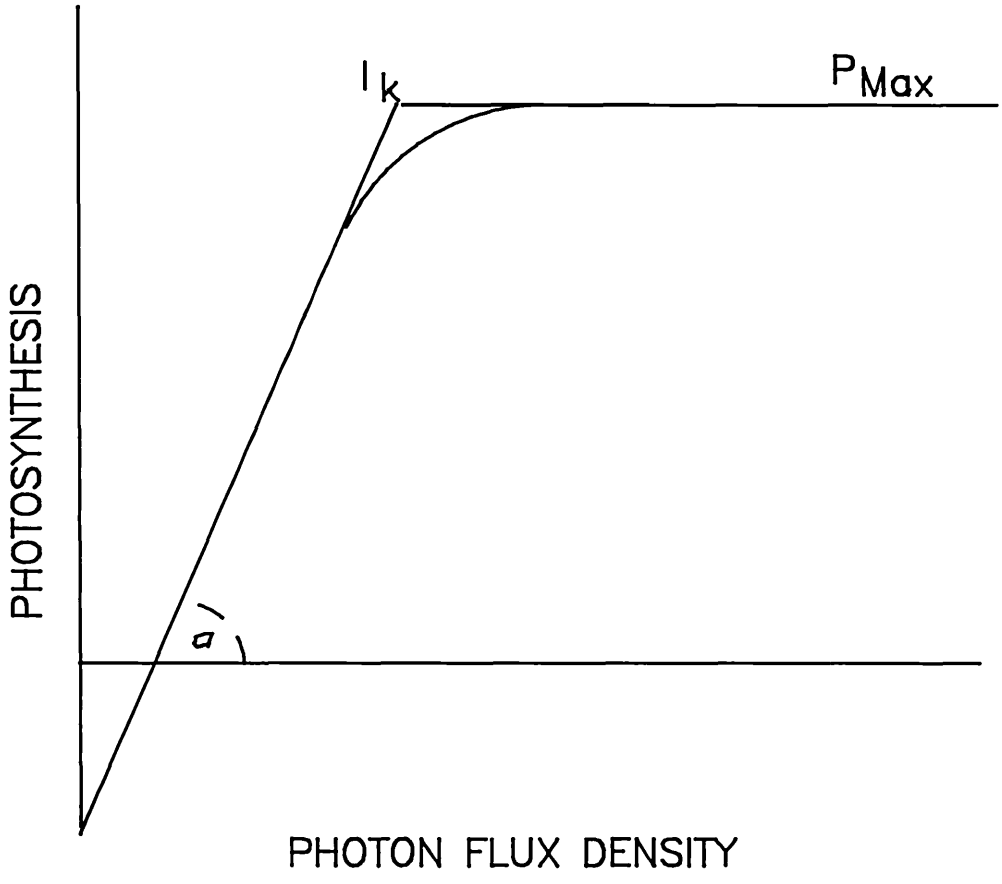
Photosynthesis-irradiance (PI) curve analysis is used in the following work as a method for determining the possible modes of herbicide inhibition in the photosynthetic system of *R. squarrosus*.

The analysis of PI relationships has been used by Prezelin (1981), Kershaw (1985), and Kershaw and Webber (1986), in studies of mosses, algae and lichens. In these cases, PI curve analysis was used as a method for investigating the biochemical and structural consequences of changes in environmental factors caused by seasonal changes. The analysis is based on changes in gross photosynthetic response to different photon flux densities, before and after environmental changes. The PI curve can be described by three parameters: 1) photosynthetic efficiency (α), 2) maximum rate of photosynthesis (P_{max}), and 3) light saturation point (I_k) (Fig. 7.1.1). To simplify the description of changes in the shape of these PI curves, Prezelin (1981) used the Thornber, Alberte, Hunter, Shiozawa and Kan (1977) model for characterizing the photosynthetic apparatus. This model describes the apparatus in terms of photosynthetic units (PSU) (Fig. 7.1.2). Each PSU is comprised of four chlorophyll proteins and each protein possesses a defined role:

1. The light harvesting pigment of photosystem II, essentially chlorophyll *b* and accessory pigments.
2. The reaction centre of photosystem II.
3. The P700 chlorophyll *a* protein = photosystem I
4. The light harvesting centre for photosystem I

The PSU acts as an antenna for light which it then focuses to traps. The traps in turn redistribute the energy to reaction centres (photosystem I and II) for the production of electrons. The resulting electron transport chain produces ATP and reduces NADP and carbon dioxide, and these enter the Calvin cycle for the production of sugars.

Fig. 7.1.1
Photosynthetic illumination (PI) curve described in terms of the Baly (1935) equation for photosynthetic rate as a function of photon flux density.

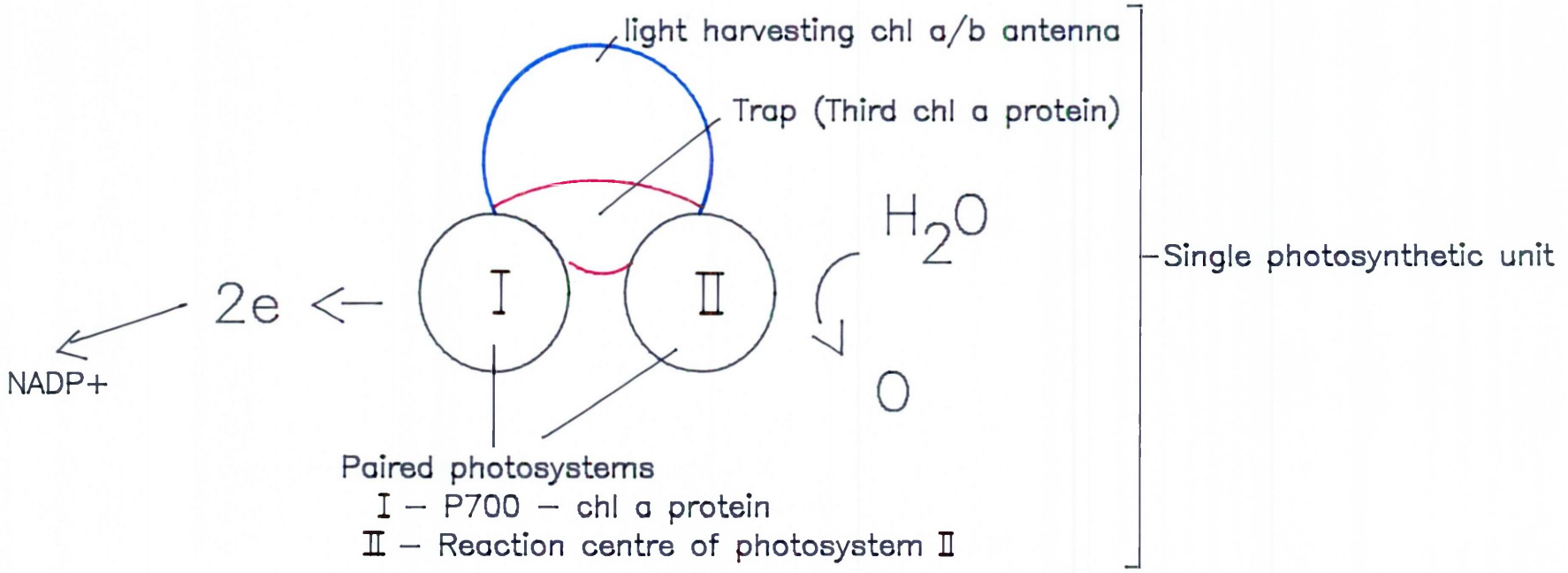


$$P = (P_{Max} \alpha I) / (P_{Max} + \alpha I) - R$$

P = rate of net photosynthesis
P_{Max} = maximum rate of gross photosynthesis
R = Rate of dark respiration
I = photon flux density
α = initial slope of P against I
(represents photosynthetic efficiency)
I_k = saturation point

Baly (1935), Kershaw (1985)

Fig. 7.1.2 Model of the photosynthetic unit of green plants. (proposed by Thornber *et al*, 1977)



Prezelin (1981) interpreted the form of the PI curves in terms of light absorption, and the size and number of the reaction centres in each tissue sample. Environmental changes to the photosynthetic apparatus were classified into four types:

1. A change in the PSU size or the average amount of light harvesting pigment per photosystem (Fig. 7.1.3).
2. Alteration of the PSU density (Fig. 7.1.3).
3. Variation in thylakoid membrane state, changing the extent of coupling/uncoupling of energy transduction and electron flow in discrete PSU (Fig. 7.1.4).
4. A reduction of dark enzyme activity and/or the rate of electron flow from one carrier to another in photosystem I and /or photosystem II (Fig. 7.1.4).

The experiments described in this chapter attempt, via the interpretation of PI curves, to define the area of photosynthetic inhibition exhibited following treatment of *R. squarrosus* with the two herbicides paraquat and dichlorophen. The herbicides were chosen on the basis of reductions in photosynthesis observed in experiments described in Chapter 6.

7.1.1 Interpretation of photosynthesis irradiance curves

Prezelin (1981) discussed the theoretical responses of the photosynthetic apparatus to endogenous and environmental changes using the photosynthetic unit (PSU) model derived by Thornber *et al.* (1977). The latter authors proposed the organisation of the PSU (Fig. 7.1.2) in terms of four chlorophyll proteins of differing roles. The spatial arrangement of the four chlorophyll proteins was related to the feed of excitation energy to the reaction centres. Prezelin (1981) described four basic ways in which the photosynthetic apparatus could alter in response to changes in internal or external factors:

Fig. 7.1.3 A schematic representation of the relationship between altered (a) photosynthetic unit size, and (b) photosynthetic unit density, and changes in the PI curves. Expressed as (1) dry weight, or (2) chlorophyll content basis (cited Kershaw, 1985).

Fig. 7.1.3

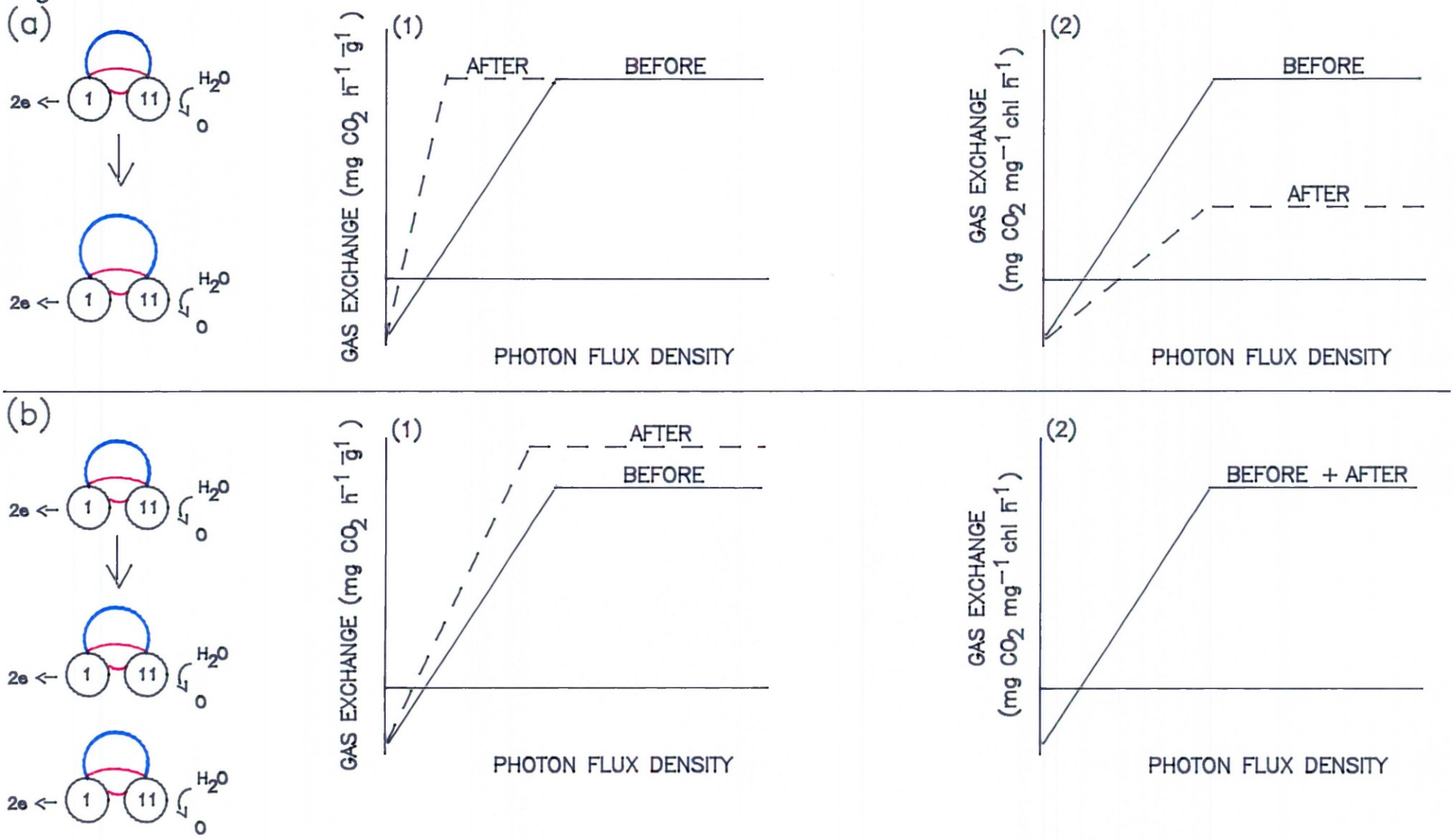
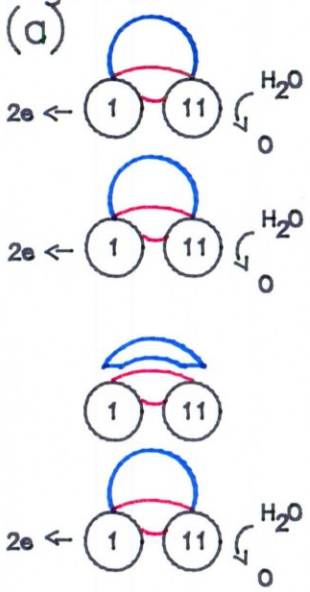
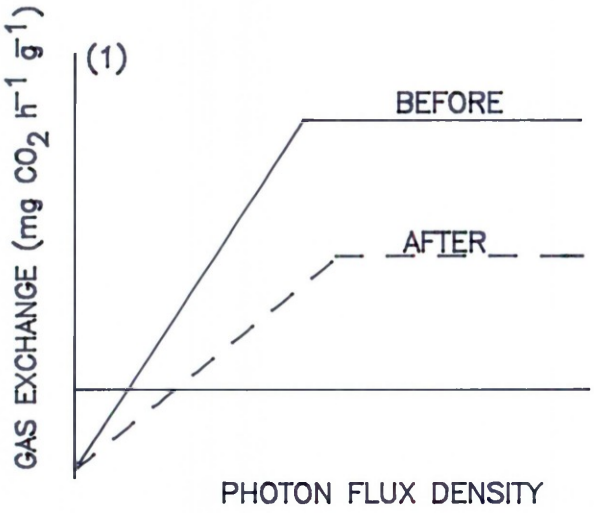
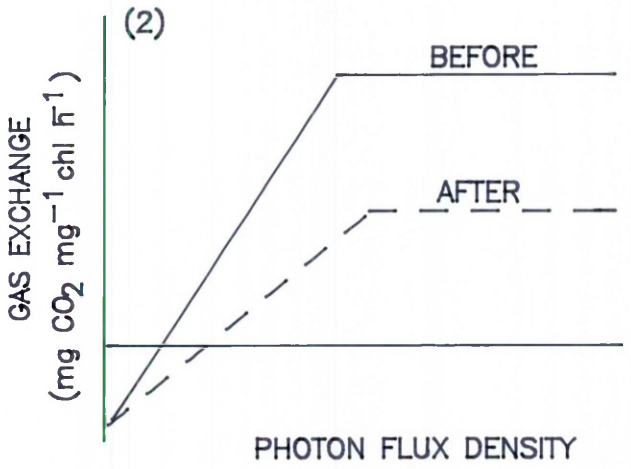

(a)
2e
1
11
H_2O
O
(1)
AFTER
BEFORE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
PHOTON FLUX DENSITY
(2)
BEFORE
AFTER
GAS EXCHANGE (mg CO_2 mg^{-1} chl h^{-1})
PHOTON FLUX DENSITY
(b)
(1)
AFTER
BEFORE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
PHOTON FLUX DENSITY
(2)
BEFORE + AFTER
GAS EXCHANGE (mg CO_2 mg^{-1} chl h^{-1})
PHOTON FLUX DENSITY

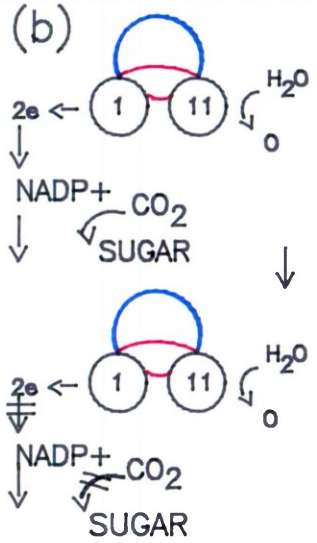
Fig. 7.1.4 A schematic representation of the relationship between altered (a) photosynthetic energy transduction, and (B) photosynthetic enzymatic reactions, and changes in the PI curves.
Expressed as (1) dry weight, or (2) chlorophyll content basis (cited Kershaw, 1985).

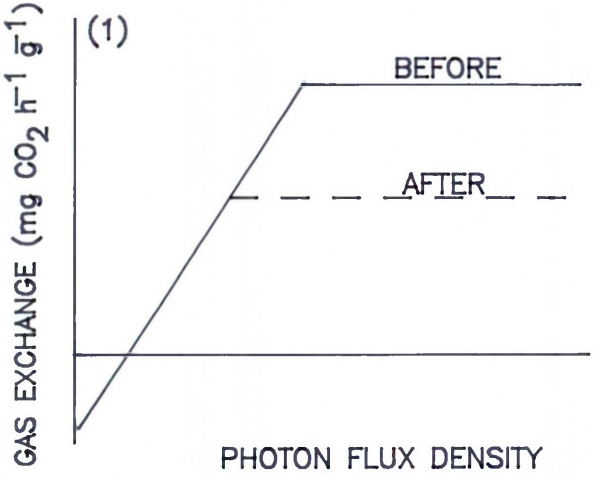
Fig. 7.1.4

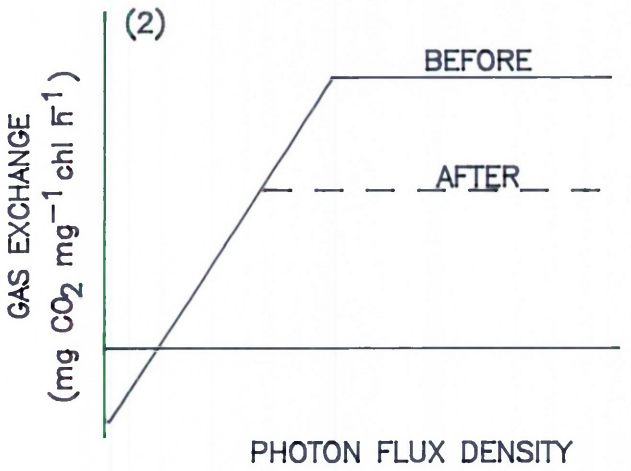

(a)
2e
1
11
H_2O
O
2e
1
11
H_2O
O
1
11
2e
1
11
H_2O
O


(1)
BEFORE
AFTER
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
PHOTON FLUX DENSITY


(2)
BEFORE
AFTER
GAS EXCHANGE
(mg CO_2 mg^{-1} chl h^{-1})
PHOTON FLUX DENSITY


(b)
2e
1
11
H_2O
O
NADP+
CO_2
SUGAR
2e
1
11
H_2O
O
NADP+
CO_2
SUGAR


(1)
BEFORE
AFTER
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
PHOTON FLUX DENSITY


(2)
BEFORE
AFTER
GAS EXCHANGE
(mg CO_2 mg^{-1} chl h^{-1})
PHOTON FLUX DENSITY

1. Size of the photosynthetic units

A change in PSU size relates to the number of light harvesting pigments per photosystem (Fig. 7.1.3). The chlorophyll core of the photosynthetic reaction centres is assumed to be constant. Therefore a change in the size of the PSU would be attributable to changes in the size of the light harvesting pigment component of the antenna and would lead to a change in the molar ratio of cell pigment. An increase in size of the light harvesting component with a uniform photosynthetic reaction centre will, when plotting photosynthesis in terms of chlorophyll *a* content, lead to a decrease in P_{max}. Photosynthesis expressed as a function of dry weight would reveal no change in P_{max}. However, the initial slope of the PI curve (α), will increase due to the increased efficiency of light capture from the enlarged light harvesting component.

2. PSU density

In this case the total amount of pigment per cell changes but this does not alter the pigment/PSU ratio. Therefore, no significant change in the chlorophyll *a*-based PI curves would be evident (Fig. 7.1.3). However, a change in the PSU density will be reflected on a dry weight basis by a change in P_{max}.

3. Coupling of energy transduction between PSI and PSII

The energy flow through a photosynthetic unit can be interrupted or uncoupled without altering the unit's size or physical density and, therefore, without changing the total pigmentation of the cell. In this case it is the number of functioning PSUs that are altered causing changes in both the light-limited and light-saturated rates (P_{max}) of photosynthesis, whether expressed in terms of dry weight or chlorophyll content (Fig. 7.1.4).

Many herbicides are believed to have uncoupling properties and might be expected to cause such changes.

4. Enzymic rates

These events are external to the PSUs and include changes in electron transport and CO_2 fixation, as well as chemical events which follow the Calvin cycle. These processes are mediated by enzyme activity and are therefore independent of PSU size, density and pigmentation. The light-limited rates of photosynthesis are thus not affected whilst P_{max} falls in proportion to decreased enzyme activity (Fig. 7.1.4).

7.2 MATERIALS AND METHODS

7.2.1 Plant material

R. squarrosus samples were collected and stored under standard conditions as described in Section 3.2.2. Supplementary lighting was not used because there was adequate sunlight available during the summer months when the work was carried out. The temperature range was 15-22°C during the day with a night temperature of 10°C. Misting with distilled water for 6 h per day (2 x 3 h) provided adequate moisture for growth without causing waterlogging.

7.2.2 Herbicides

Formulations were prepared in distilled water at the following concentrations:

Dichlorophen	(Supermosstox)	600 µM
Paraquat	(Technical)	7.25 mM

These concentrations were chosen as they proved to be effective in producing a reduction in photosynthesis of *R. squarrosus* without killing the shoots.

7.2.3 Incubation procedure

Apical segments of *R. squarrosus*, 3 cm long, were excised from trays of moss after glasshouse cultivation for 2 wk. The segments were placed in Petri dishes lined with filter paper and moistened with distilled water. A growth cabinet was used to provide a constant light and temperature source in which to incubate the moss samples before and after herbicide treatment.

Following a 24 h pre-treatment incubation in the growth cabinet the moss segments were washed three times in 200 ml distilled water to remove debris from the surface and also to ensure complete hydration. Samples of 12 moss segments were placed on paper towels to remove excess water from the surface of the tissue and were then transferred to glass scintillation vials. To each vial, 5 ml of the treatment herbicide solution was dispensed. Each vial was then incubated for 5 min at 20°C in a gently shaking water bath. On completion of the incubation, the herbicide solution was removed with a Pasteur pipette and the moss segments washed twice with 5 ml of distilled water. The moss segments were then returned to a Petri dish for a further 30 min incubation in the growth cabinet. An adequate incubation time was therefore provided for a limited amount of uptake to occur. This method was adopted because it gives superior penetration to spray application (Ch. 6).

7.2.4 Photosynthesis measurement

The Perspex assimilation chamber and IRGA system previously described (Section 6.2.4) was used for the photosynthesis and respiration measurements. The CO_2 concentration from the compressed air cylinder, used as the standard air supply, was 289 vpm.

The photosynthetic effects following herbicide treatment were monitored over a range of photon flux densities (0-379 μmol m^{-2} s^{-1}).

The photon flux densities were measured using a LI-185 Quantum sensor (Lambda Instruments Corporation, Nebraska). Only one measurement of photosynthesis and respiration was made for each sample of herbicide-treated moss.

Improvements were made to the experimental equipment from that of the previous photosynthesis experiment (Section 6.2.4). A uniform air temperature (15°C) within the assimilation chambers was achieved by submersion at a depth of 16 cm in a water bath (7°C). The bank of lights was positioned overhead *ca.* 35 cm above the water surface. This experimental system provided greater stability of the air temperatures of the assimilation chambers, over the range of photon flux densities.

7.2.5 Procedure for fitting PI curves

A number of empirical procedures exist for relating rate of photosynthesis to photon flux density. Mathematical models of gas exchange have often been used to study crop photosynthesis (Chartier, 1970; Ziegler-Jöns and Selinger, 1987). These studies have produced formulae describing photosynthetic responses in terms of CO_2 diffusion resistance, rates of electron transport and dissimilative electron transport in the leaves of vascular plants. However, in the present study only the basic parameters of the PI relationship are used with no reference to factors such as temperature, nutrients and adaptation.

Jassby and Platt (1976) compared eight different mathematical formulae describing PI curves of phytoplankton (Table 7.2.1). The equation of Baly (1935), which describes a rectangular hyperbola, gave the poorest description of their experimental data and the hyperbolic tangent function of Jassby and Platt (1976) gave the best fit. However, Maberly (1985) compared the similarly structured equations of Baly (1935) and Smith (1936) with the equation of Jassby and Platt (1976) (Table 7.2.1) in studies of the aquatic moss *Fontinalis antipyretica*.

Table 7.2.1 The different formulations of the light saturation curve tested by Jassby and Platt (1976)

Equation No.	Equation	Source
1	$P = \begin{cases} \alpha I, & I \leq P_m/\alpha \\ P_m, & I > P_m/\alpha \end{cases}$	Blackman (1905)
2	$P = P_m \alpha I/(P_m + \alpha I)$	Baly (1935)
3	$P = P_m \alpha I/[(P_m)^2 + (\alpha I)^2]^{\frac{1}{2}}$	Smith (1936)
4	$P = \alpha I \exp(-\alpha I/P_m e)$	Steele (1962)
5	$P = \begin{cases} \alpha I \exp(-\alpha I/P_m e), & I \leq P_m e/\alpha \\ P_m & I \leq P_m e/\alpha \end{cases}$	Steele (1962) modified
6	$P = P_m[1-\exp(-\alpha I/P_m)]$	Webb et al.(1974)
7	$P = \begin{cases} \alpha I - (\alpha I)^2/4P_m, & I \leq 2P_m/\alpha \\ P_m & I \leq 2P_m/\alpha \end{cases}$	Platt et al. (1975)
8	$P = P_m \tanh(\alpha I/P_m)$	Jassby and Platt (1976)

P = net photosynthesis per unit chlorophyll
I = irradiance
P_m = specific net photosynthesis at optimal illumination
α = slope of the light saturation curve at low light levels.

The hyperbolic tangent function of Jassby and Platt (1976) gave the poorest fit. In this case the Smith (1936) equation gave the closest fit with the Baly (1935) equation intermediate. Differences in the conclusions of Jassby and Platt (1976) and Maberly (1985) probably arise from the rather different experimental material studied. These differences are probably due to variation in photosynthetic pigments and ratios between the plants as discussed in detail by Thornber *et al.* (1977).

In the following experiments a comparison was made between the hyperbolic tangent function of Jassby and Platt (1976) with the simpler equation of Baly (1935) for the PI curves of *R. squarrosus*. Non-linear regression, using the Rothamsted Maximum Likelihood Program (Ross, 1987), was used to fit the curves to the data. Selection of the best fitting of the two alternative photosynthesis curves was by inspection of the residual sum of squares. For the 18 PI curves obtained from the treated and untreated moss samples (expressed as a function of dry weight, total chlorophyll content and chlorophyll *a* content) an equal number of best fits was obtained for the two equations. As discrepancies between the two methods were small, the Baly (1935) equation was thus chosen as the simplest model for the expression of the *R. squarrosus* PI curve data.

The Baly (1935) equation can be adapted for gross photosynthesis measurements by the addition of a term for dark respiration (Fig. 7.1.1). This adaptation is based on the assumption that the rate of respiration is not affected by light. However, there is little suggestion in the literature that this is a serious cause of error in studies of gas exchange in bryophytes (Maberly, 1985; Kershaw, 1985; Green, Snelgar and Wilkins, 1985).

7.3 RESULTS AND DISCUSSION

Segments of *R. squarrosus* were incubated in dichlorophen, paraquat or distilled water (control) for 5 min. Measurements of photosynthesis and respiration were made after a 30 min post-incubation period as previously described (Section 6.2.4).

The parameters for the fitted PI curves for each of the treatments, based on the Baly (1935) equation (Fig.7.1.1), are presented in Table 7.3.1. Parameters for the PI curves based on chlorophyll are presented both with respect to total chlorophyll and,

Table 7.3.1 Parameters (± SE) for PI curves using the Baly (1935) equation based on (a) dry weight; (b) total chlorophyll, and (c) chlorophyll a content of R. squarrosus

a)	R ($\mu l\ CO_2\ g^{-1}\ h^{-1}$)	α ($\mu l\ CO_2\ g^{-1}\ h^{-1}$ [$\mu mol\ m^{-2}\ s^{-1}$]$^{-1}$)	P_{max} ($\mu l\ CO_2\ g^{-1}\ h^{-1}$)	df
Control	-100 (242)	292.3 (154)	2498 (443)	43
Dichlorophen	-20 (407)	15 (15.4)	1035 (25)	26
Paraquat	*	*	*	

b)	R ($\mu l\ CO_2\ mg^{-1}$ chl h^{-1})	α ($\mu l\ CO_2\ mg^{-1}$ chl h^{-1} [$\mu mol\ m^{-2}\ s^{-1}$]$^{-1}$)	P_{max} ($\mu l\ CO_2\ mg^{-1}$ chl h^{-1})	df
Control	-0.0459 (0.93)	0.29 (1.216)	5.48 (0.71)	21
Dichlorophen	-0.00066(0.36)	0.32 (0.78)	1.14 (0.73)	26
Paraquat	-0.0545 (1.65)	0.11 (2.67)	0.67 (0.683)	22

c)	R ($\mu l\ CO_2\ mg^{-1}$ chl*a* h^{-1})	α ($\mu L\ CO_2\ mg^{-1}$ chl*a* h^{-1} [$\mu mol\ m^{-2}\ s^{-1}$]$^{-1}$)	P_{max} ($\mu L\ CO_2\ mg^{-1}$ chl*a* h^{-1})	df
Control	-0.0127 (0.55)	0.464 (0.16)	8.08 (0.81)	21
Dichlorophen	0.0037 (0.89)	0.488 (1.19)	1.65 (1.17)	26
Paraquat	0.084 (0.65)	0.146 (0.64)	0.96 (0.97)	22

R = respiration

α = photosynthetic efficiency

P_{max} = gross photosynthesis

* = curve did not converge

following Prezelin (1981), with respect to chlorophyll *a* content. In these experiments, no differences were found between the two methods of presenting the chlorophyll data. The PI curves based on total chlorophyll are presented, because the data were less variable than for chlorophyll *a*.

The significance of differences between the fitted parameters for the various herbicide treatments were examined by t-tests.

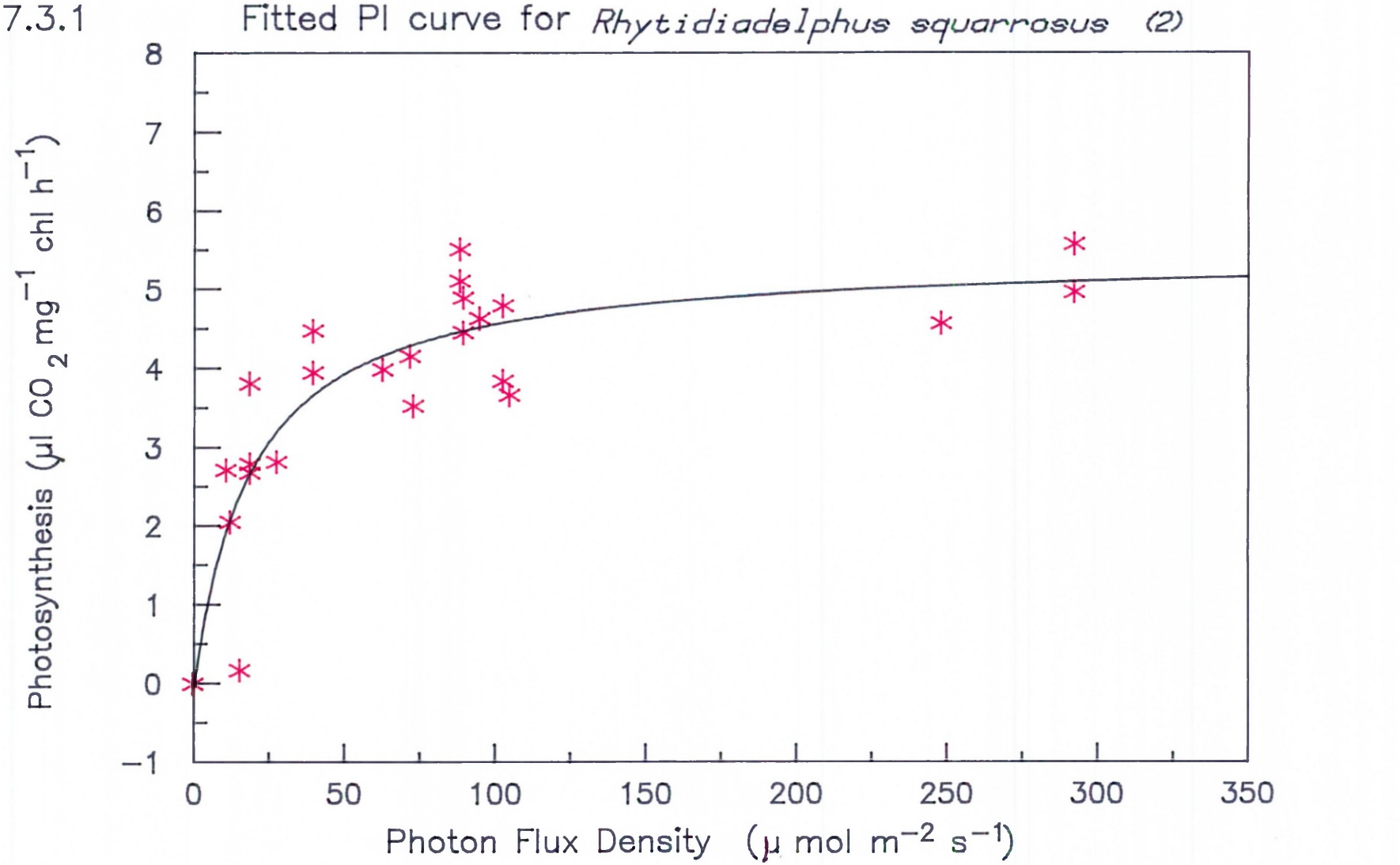
7.3.1 Effects of Dichlorophen on the PI curves of R. squarrosus

Significant reductions in P_{max} were obtained for the dichlorophen treatment, compared to the controls, in the PI curves based on chlorophyll content and those based on dry weight (Table 7.3.1, Figs 7.3.1 to 7.3.4). No significant difference in the photosynthetic efficiency (α) in the chlorophyll based PI curve was obtained with dichlorophen compared with the water control (Table 7.3.1, Fig. 7.3.3). Additionally, the initial slope representing photo- synthetic efficiency (α), in the dry weight-based PI curve, was also significantly lower following dichlorophen treatment than in the distilled water control (Fig. 7.3.4).

According to Prezelin (1981), a reduction in both P_{max} and (α) is indicative of reduced electron flow through the PSU. The presence of a significant reduction in (α) in the dry weight-based PI curve for dichlorophen suggests that dichlorophen acts as an electron transport inhibitor of PSII in *R. squarrosus* as hypothesised in Section 6.3.2.

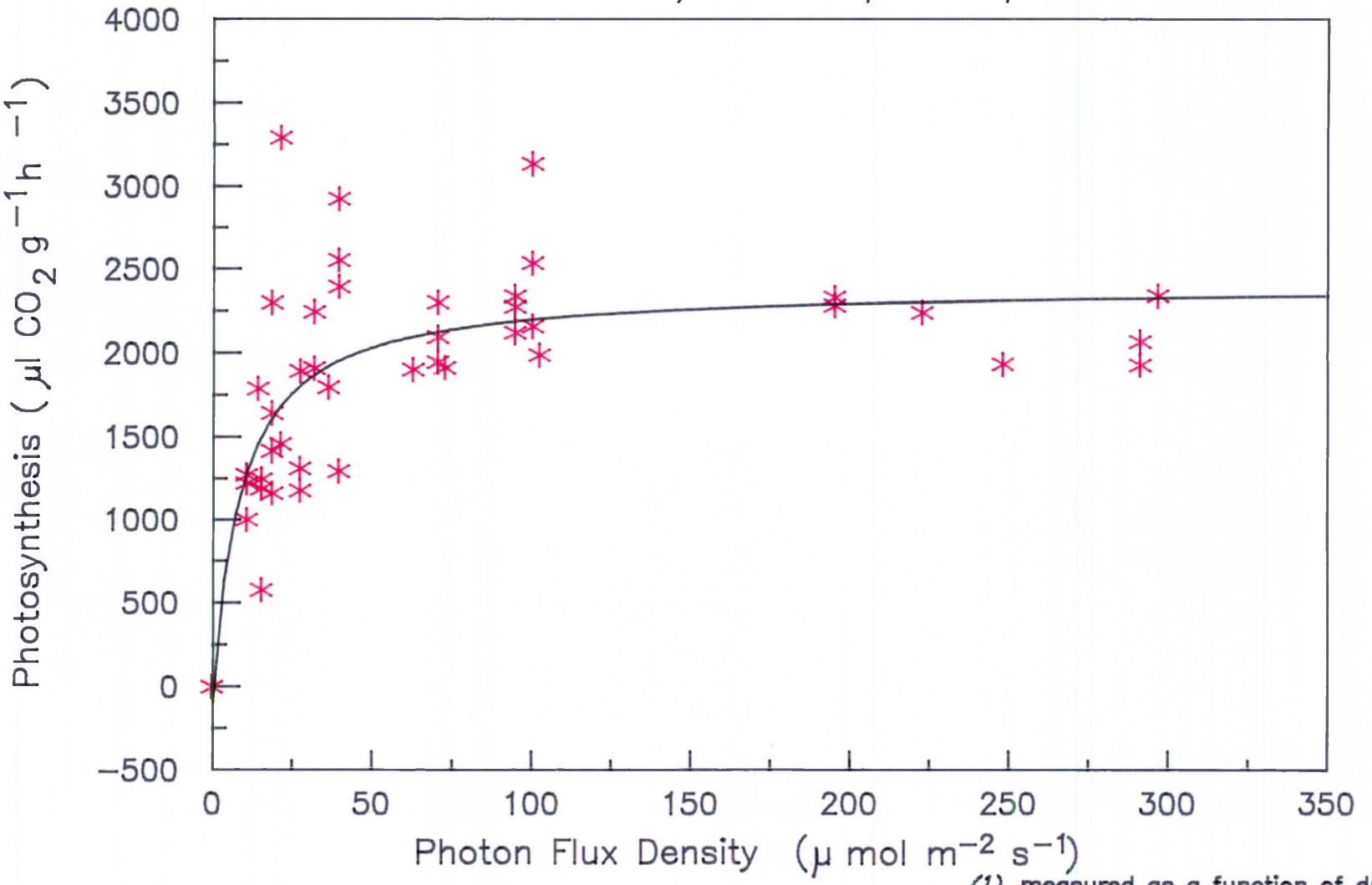
The apparent uptake and effects on photosynthesis of dichlorophen were rapid in *R. squarrosus* considering that the PI measurements were made only 30 min after application. It is not surprising therefore that no evidence was obtained for an effect on either pigmentation or PSU size or density.

Fig. 7.3.1



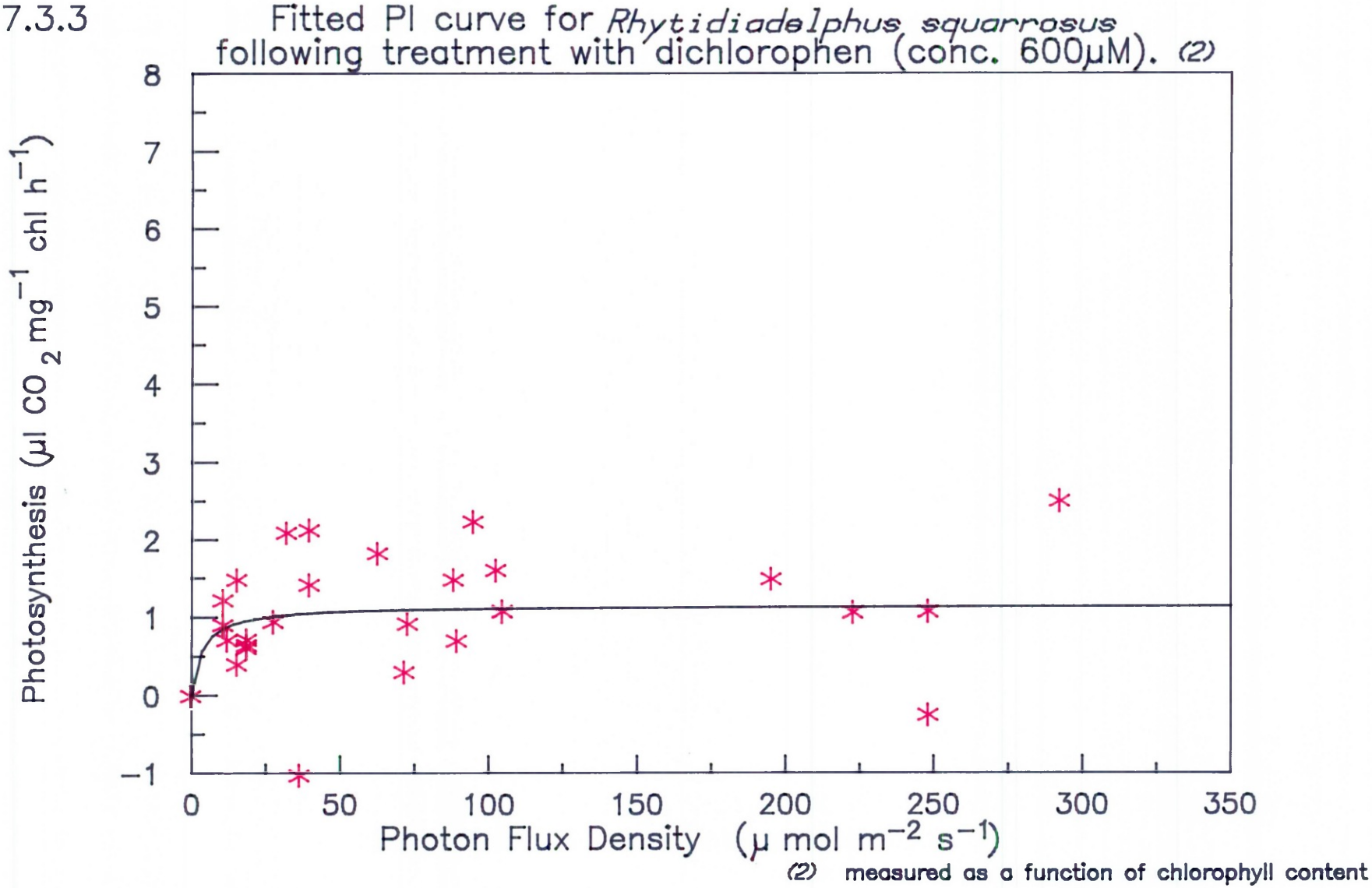
(2) measured as a function of chlorophyll content

Fig. 7.3.2 Fitted PI curve for *Rhytidiadelphus squarrosus* (1)



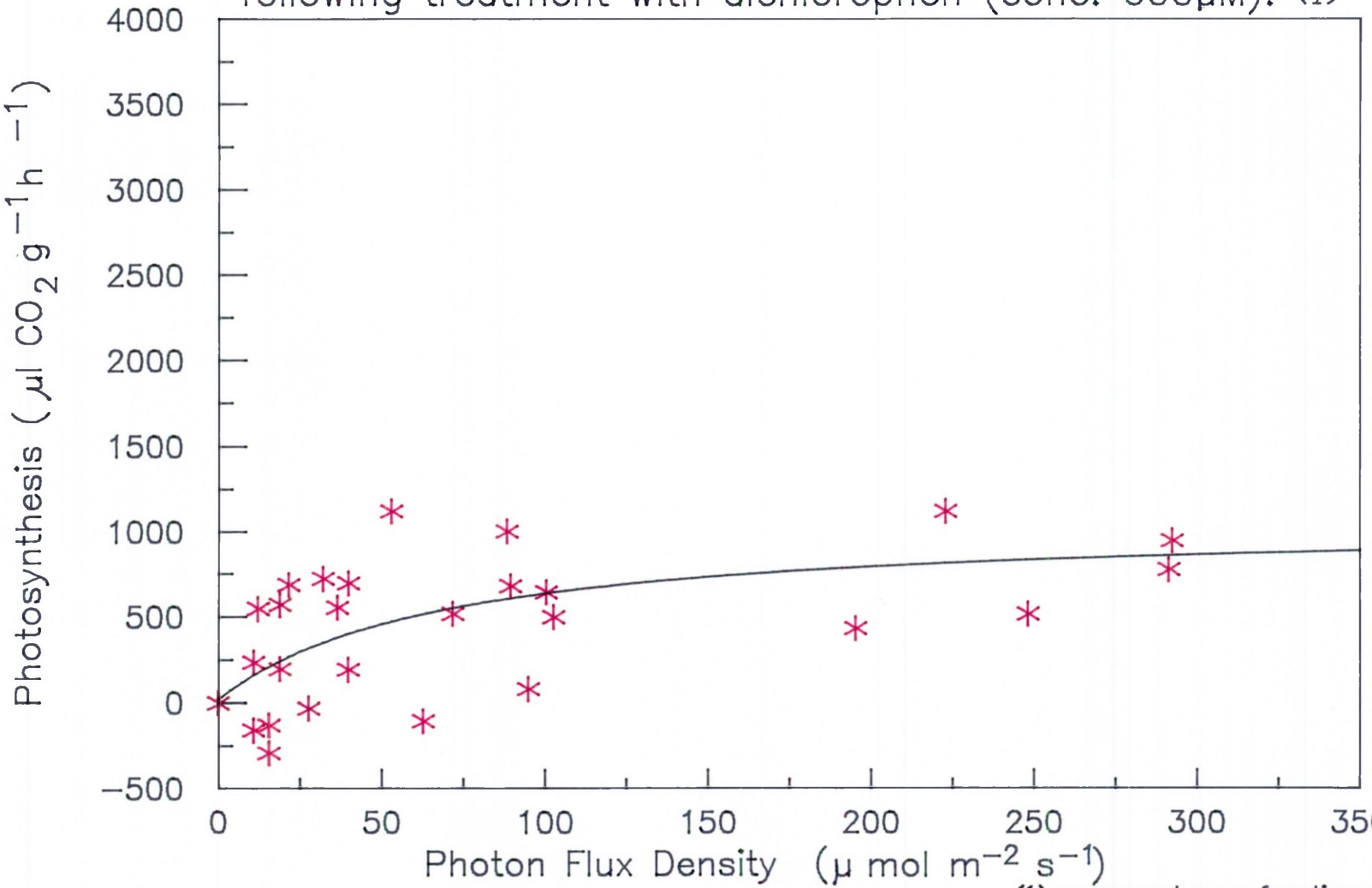
(1) measured as a function of dry weight

Fig. 7.3.3



(2) measured as a function of chlorophyll content

Fig. 7.3.4 Fitted PI curve for *Rhytidiadelphus squarrosus* following treatment with dichlorophen (conc. 600µM). *(1)*



(1) measured as a function of dry weight

As previously found (Ch. 6), the effect of dichlorophen on *R. squarrosus* respiration (R) was not significant. This indicates that the suggested uncoupling action of dichlorophen (Nakaue, Caldwell and Buhler, 1972) on oxidative phosphorylation is either inapplicable to *R. squarrosus* or requires higher concentrations (a low concentration was deliberately selected here) or greater treatment time. The lack of effect of dichlorophen on respiration might be associated with losses of the chemical by preferential binding within the photosynthetic system. Slight damage to the plasmalemma of *R. squarrosus* may have occurred at the 600 µM dichlorophen concentration employed (Section 8.3.5) which may also have caused a reduction in photosynthesis. A ten-fold dilution of the dichlorophen incubation solution (60 µM) and extending the post treatment period produced no significant difference in respiration or photosynthesis of *R. squarrosus* shoots (Table 7.3.1, Figs 7.3.5 and 7.3.6). Corbett, Wright and Baillie (1984) described the direct but non-specific interactions of inhibitory uncouplers with vascular plant mitochondrial and chloroplast membranes suggesting effects on other membranes may occur.

7.3.2 Effects of Paraquat on the PI curves of R. squarrosus

Treatment of *R. squarrosus* with paraquat also gave a significant reduction in the rate of maximum gross photosynthesis (P_{max}) for the PI relationship based on chlorophyll content (Fig. 7.3.7). In addition, no significant difference was found for the slope for photosynthetic efficiency (α) for the PI curve based on chlorophyll. The PI curve based on dry weight failed to converge during the non-linear regression computation due to excessive variability in the data. Inspection of the data points, and the fitted PI curve based on dry weight, shows a substantial decline in P_{max} (Fig.7.3.8).

Fig. 7.3.5

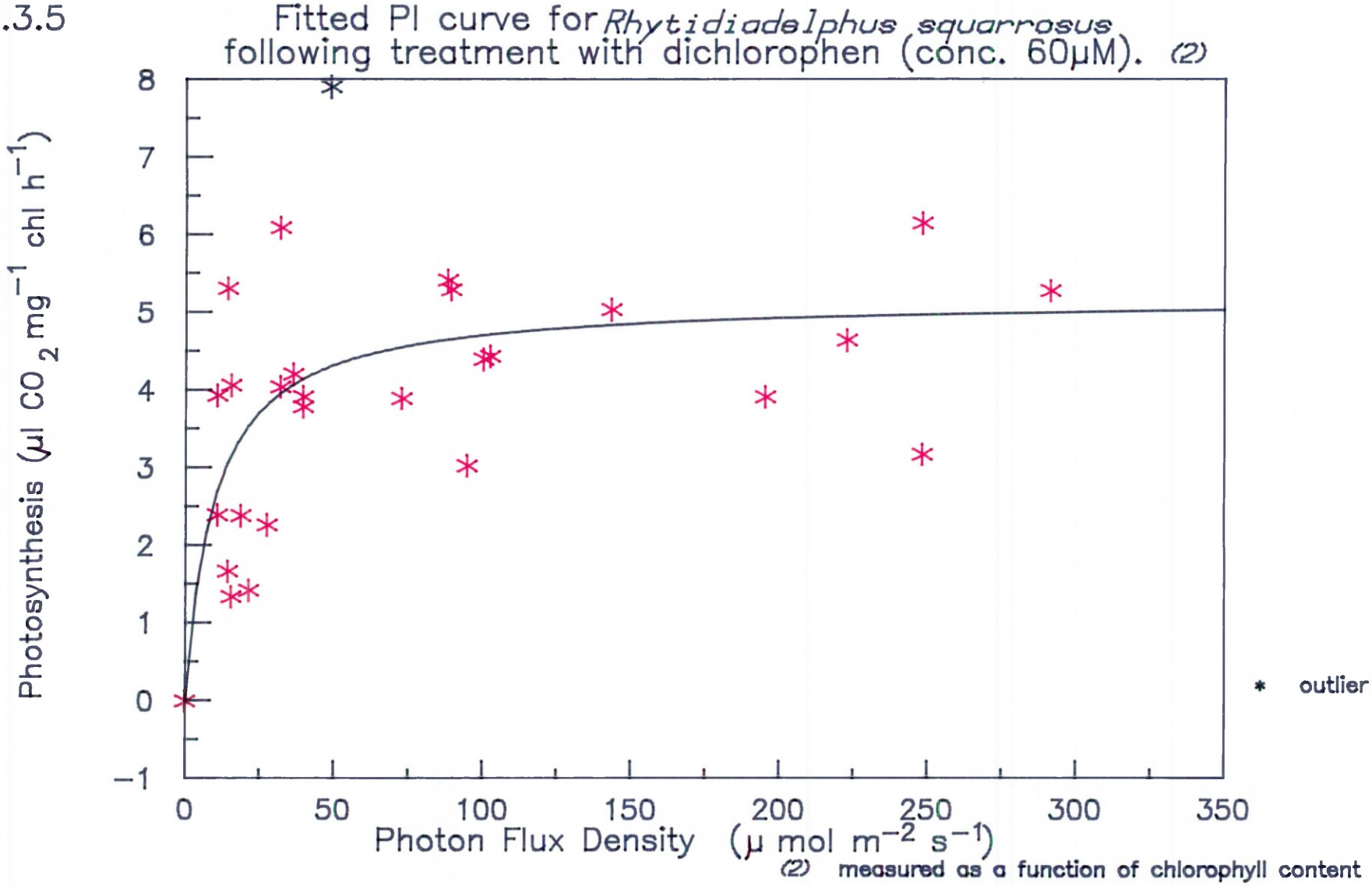
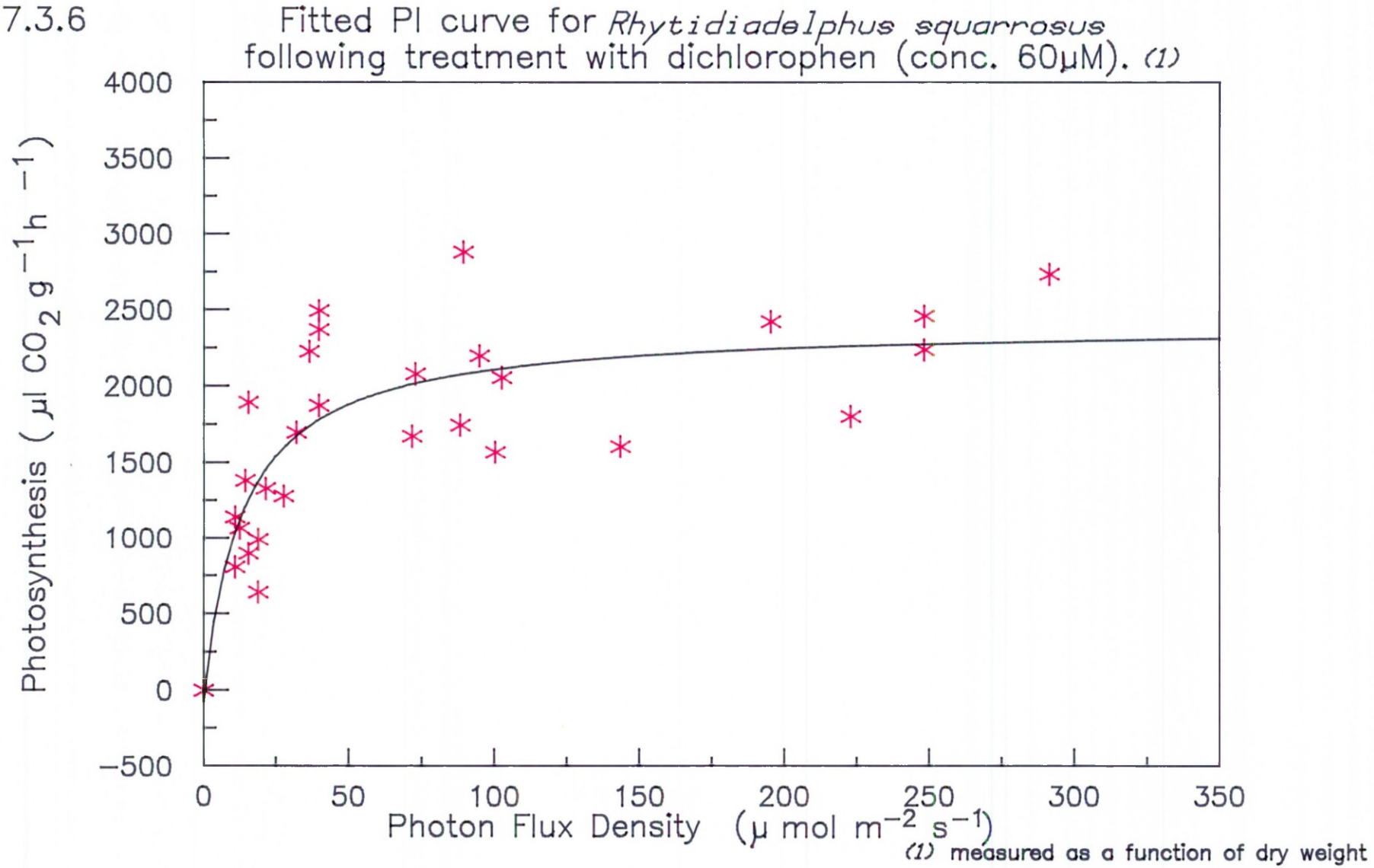
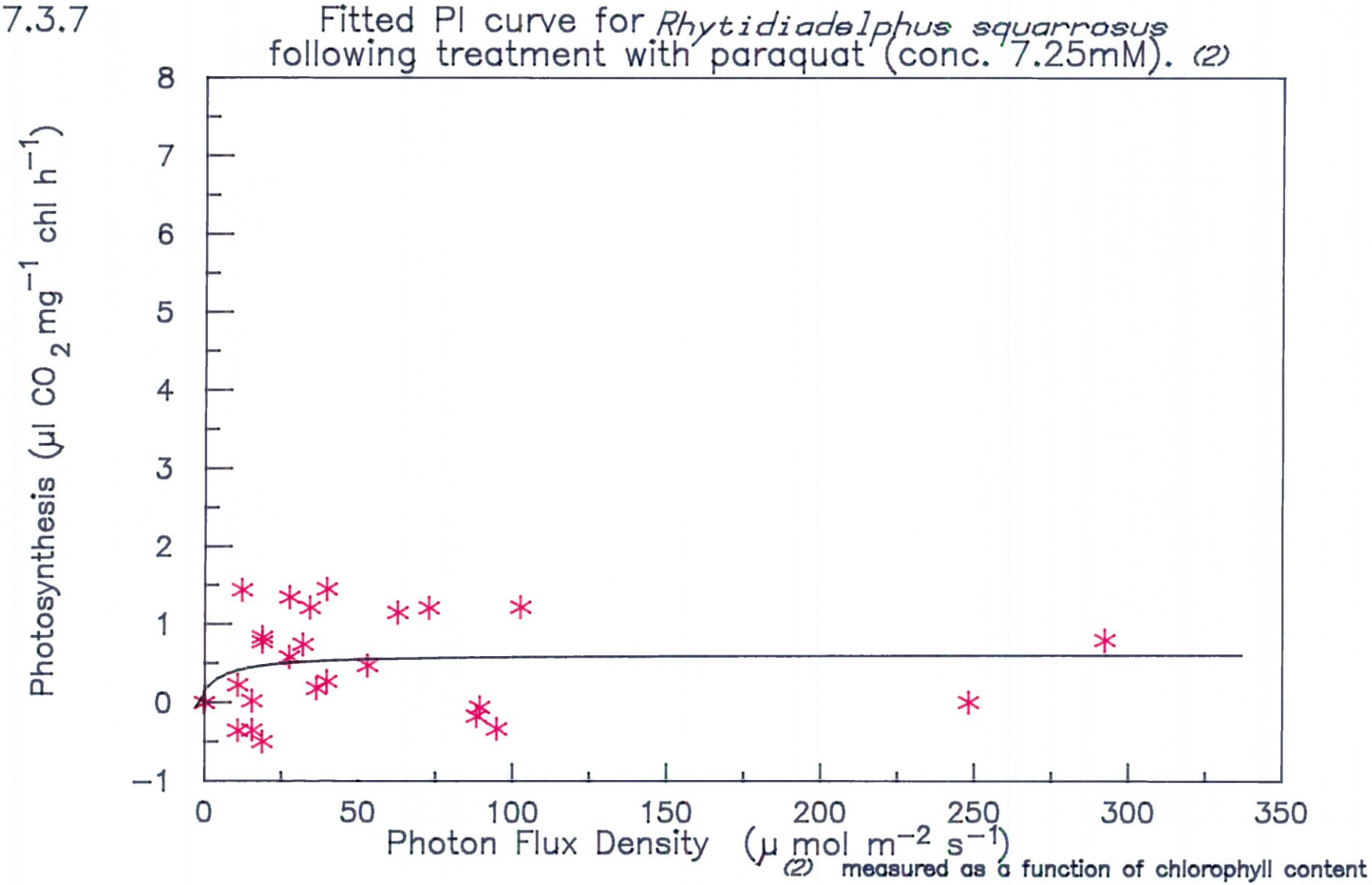

Fitted PI curve for Rhytidiadelphus squarrosus following treatment with dichlorophen (conc. 60µM). (2)
Photosynthesis (µl CO2 mg-1 chl h-1)
8
7
6
5
4
3
2
1
0
-1
0
50
100
150
200
250
300
350
Photon Flux Density (µ mol m-2 s-1)
* outlier
(2) measured as a function of chlorophyll content

Fig. 7.3.6



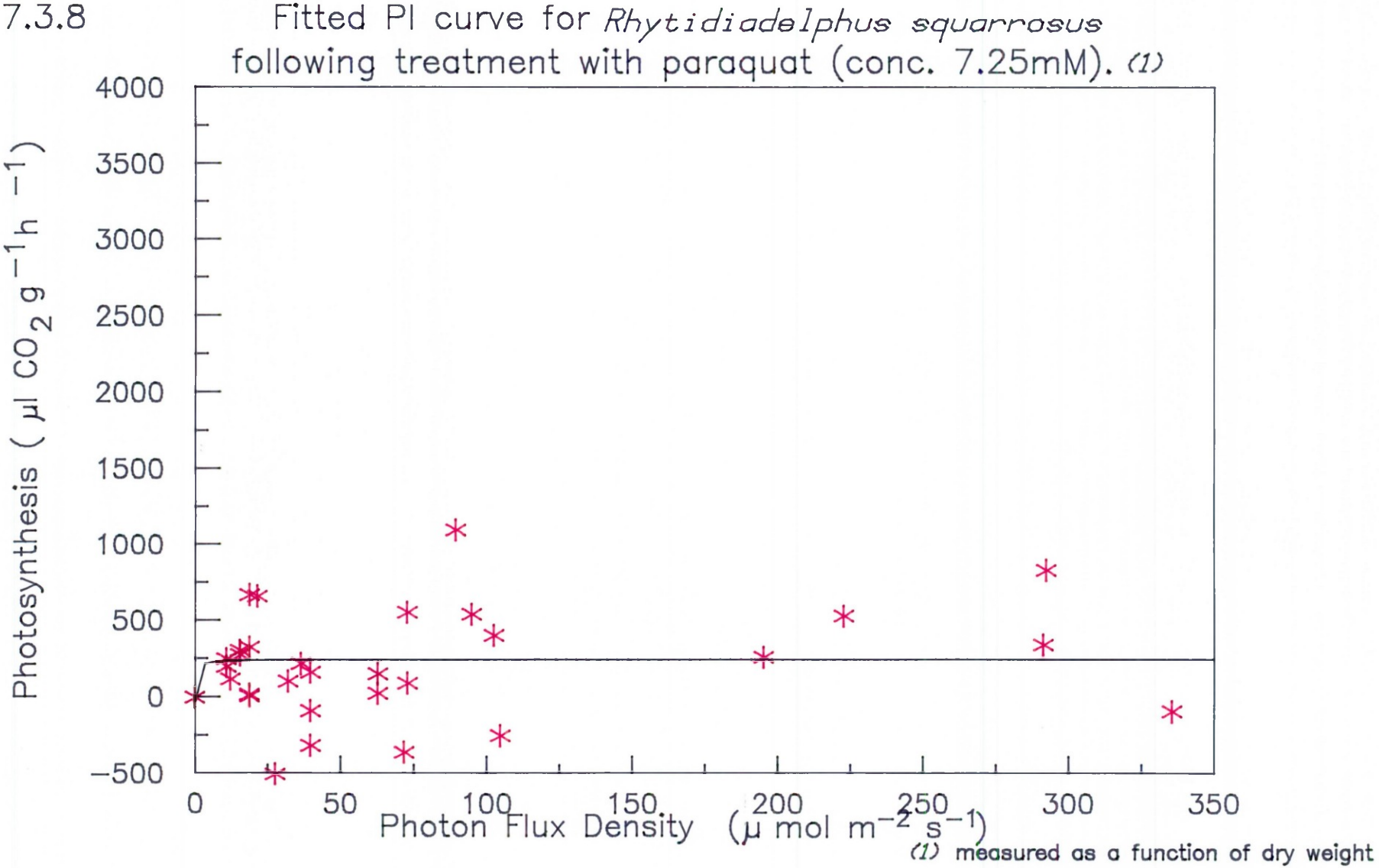
(1) measured as a function of dry weight

Fig. 7.3.7



(2) measured as a function of chlorophyll content

Fig. 7.3.8



(1) measured as a function of dry weight

The effect of paraquat on the form of the PI curves for *R. squarrosus* was similar to that of dichlorophen and signifies the uncoupling of electron transport; but high variability in the data at low photon flux densities prevented accurate determinations of the photosynthetic efficiencies (α). The results, therefore, are in broad agreement with the observed effects of paraquat inhibition of the photosynthetic electron transport system of vascular plants (Harris and Dodge, 1972). The high variability of the photosynthesis measurements may be due to membrane disruption or pigment degradation caused by the paraquat treatment.

The damaging effect of paraquat on the PI relationships of *R. squarrosus* is a clear indication of the entry of this herbicide into the cells. The lack of photosynthetic response to paraquat in earlier photosynthesis experiments (Ch. 6) is probably attributable to the spraying method of application, giving poor penetration compared to the total immersion used here.

7.4 CONCLUSIONS

Analysis of the form of fitted PI curves for *R. squarrosus* after herbicide treatment indicates that dichlorophen causes a reduction of electron transport rates and, therefore, of the rate of CO_2 assimilation. This observation supports the hypothesis that dichlorophen binds to the thylakoid membranes of *R. squarrosus*, inhibiting PSII (Section 6.3.2). However, this may not necessarily be a primary effect as plasmalemma damage also occurs following dichlorophen treatment (Section 8.3.5).

The effects of paraquat on the PI curves were superficially similar to those of dichlorophen, but only the curve for chlorophyll content was adequately described by the equation of Baly (1935). Variability in the data which prevented curve fitting for some paraquat data may have been a result of membrane damage caused by the herbicide.

The analysis of PI curves has proved useful in this study in supporting the hypothesis (Section 6.3.2) of dichlorophen inhibition of electron transport in the photosynthetic system of *R. squarrosus*. However, excessive variability in the measurements reduced the efficiency of the curve fitting procedure, making comparisons between the different herbicide treatments difficult.

CHAPTER 8

EFFECTS OF PARAQUAT, DICHLOROPHEN, IRON SALTS AND 2,4-D ON CELL PERMEABILITY OF *RHYTIDIADELPHUS SQUARROSUS*

8.1 INTRODUCTION

Normally herbicides must move through the cell membrane to be phytotoxic. Plant membranes are structural components of the cell and also function in the regulation of cell permeability, ion transport, electron transport and enzyme activity.

Plant membranes consist of lipids and proteins plus other constituents such as sterols, metal ions and water (Rivera and Penner, 1979; Clarkson, 1974). The lipids are considered to be arranged mainly in a bilayer configuration, with some globular proteins located in or partially submerged within it. The plasmalemma is involved in the process of cellular uptake of herbicides via active transport or diffusion (Rivera and Penner, 1979; Clarkson, 1974). The ability of a herbicide to permeate the plasmalemma is therefore influenced by the lipid composition of the membrane and the lipid solubility of the herbicide (Hall and Baker, 1977). Binding of herbicides to the plasmalemma may also influence cell permeability by disrupting membrane integrity. Herbicides can therefore interfere with both the function and structure of plant cell membranes (Rivera and Penner,1979).

Rivera and Penner (1979) related the speed at which loss of membrane integrity was observed to different types of herbicidal activity. A rapid reduction in membrane integrity may indicate binding of the herbicide to membrane lipids and modification of their permeability. Alternatively, a delay in the onset of membrane disruption suggests interference with lipid synthesis.

Potassium leakage has been used as a technique to assess membrane damage in bryophytes (Bates and Brown,1974; Brown, Ougham and Beckett, 1986; Brown and Whitehead, 1986; Bates, 1982). Potassium has been identified as a predominantly intracellular constituent of moss tissue (Bates and Brown, 1974; Brown, 1982, 1984) and is therefore suitable as a marker of intracellular solute retention. The technique of measuring

potassium leakage has been used for several different studies including pesticide damage (Harris and Dodge, 1972; Kirkwood, 1987; Brown, Ougham and Beckett, 1986; Brown and Whitehead, 1986), surfactant phytotoxicity (Horowitz and Givelberg, 1979; Silcox and Holloway, 1986), desiccation effects on mosses (Brown and Buck, 1979), control of cation levels in mosses (Bates and Brown, 1974; Bates, 1982) and anion accumulation and toxicity in lichens (Richardson, Nieboer, Lavoie and Padovan, 1984).

Both paraquat and 2,4-D have been identified as inducing loss of membrane integrity in vascular plants. Paraquat and other bipyridylium compounds have been observed to cause peroxidation of membrane lipids, resulting in loss of membrane integrity and the inactivation of membrane bound enzymes such as ATPase in *Linum usitatissimum* cotyledons (Dodge and Harris, 1970; Harris and Dodge, 1972). Further damage was attributed to the release of hydrolytic enzymes from the vacuole following the destruction of the tonoplast.

2,4-D was observed by White and Hemphill (1972) to rupture membranes of the tonoplast, plasmalemma, chloroplasts and mitochondria in tobacco leaf mesophyll cells.

Dichlorophen, sold commercially as a moss killer, has also been observed to cause loss of membrane integrity in *R. squarrosus* tissue by Brown, Ougham and Beckett (1986) although it is unknown whether this is a primary or secondary effect.

The experiments described in this chapter were designed to identify whether moss cell membranes were affected by selected herbicides. Potassium loss was measured from *R. squarrosus* tissues following incubation in solutions of paraquat, 2,4-D, dichlorophen and iron salts.

8.2 MATERIALS AND METHODS

Details of the collection and cultivation of *R. squarrosus* and of the herbicides used are given in Sections 3.2.1 and 2.2.5.

8.2.1 Incubation procedure

Intact shoot apices of *R. squarrosus* were prepared and treated with herbicide as decribed in Sections 3.2.2 and 4.3.2. The extraction procedure was as described in Section 4.2.5 except that the herbicide incubation sample was analysed for K content. After incubation the tissues were dried (80°C), weighed and digested in acid (Section 4.2.5.2) to obtain the total K content of the moss tissue.

Following incubation with dichlorophen only, the moss was shaken with distilled water for 24 h. This was to determine whether absorbed dichlorophen caused further membrane damage after the incubation solution had been removed and to see whether K loss during the 24 h wash was dependent on initial incubation times.

Control moss samples, for each experiment, were incubated in distilled water for the appropriate times.

8.2.2 Method for determining the distribution of potassium in R. squarrosus tissues

To determine the proportions of K 1) extracellularly bound to exchange sites on the cell wall, 2) in solution in the intracellular compartments and 3) in the residual fraction (neither exchangeable nor in solution) an elution sequence similar to that of Brown and Buck (1979, 1985) was used. This involved a similar elution sequence to that described in Section 4.2.5 except that no initial incubation in herbicide was necessary. Also, the exchangeable cell wall fraction was displaced by shaking the tissues twice, for one hour, in 5 ml 0.02M nickel chloride solution. On completion of the elution stages the shoots were digested in acid (Section 4.2.5.2).

All extractions, except the acid digests, were acidified with 0.3ml of a 1:1 mixture of concentrated HNO_3 and H_2SO_4 to prevent precipitation of salts.

8.2.3 Potassium determination

Potassium was determined by flame emission spectrophotometry (Section 4.2.6).

Measurements were recorded at 766.5 nm using a fuel lean air acetylene flame and a slit width of 320 µm (Instrumentation Laboratory Inc., 1975). Fresh calibration standards (1-20 mg l^{-1}) were prepared for each experiment by diluting a 'Spectrosol' potassium nitrate solution (10 mg K ml^{-1}; 256mmol K l^{-1}) (BDH) with a solution of 3% (v:v) HNO_3 in water.

8.2.4 Presentation and analysis of results

The data are presented as µg K g^{-1} dry weight and as the proportions of K in the different cellular fractions. K losses from moss tissue following herbicide treatments are presented as percentages of the total potassium content of the tissue. Corrections were made to all samples for the very small quantities of K detectable in the herbicide solutions.

Analyses of variance were used to compare the effects of herbicide concentration or incubation time on the extent of K leakage. The uneven distribution of the residual sums of squares of the data indicated that transformation was required. For consistency, logarithmic transformations were applied to the data. All tables are presented as the untransformed data and are accompanied by values for the logarithm of the standard error of the difference.

8.3 RESULTS AND DISCUSSION

8.3.1 Distribution of potassium in R. squarrosus tissue

The cellular distributions of potassium in leaves, stems and whole shoots of *R. squarrosus* are presented in Table 8.3.1.

Table 8.3.1 Potassium distribution within leaves, stems and whole shoots of Rhytidiadelphus squarrosus a)in μg K g^{-1} dry weight and b) percentage of total potassium content. Figures in parenthesis are standard errors of the mean

a)	Intercellular	Exchangeable Step 1	Exchangeable Step 2	Intracellular (soluble)	Residual (insoluble)
Leaves	124 (12)	288 (75)	296 (67)	8774 (243)	389 (42)
Stems	299 (19)	266 (72)	157 (37)	24444 (995)	1254 (17)
Whole shoots	39 (3)	96 (27)	109 (32)	13714 (828)	1805 (159)

n = 8

b)	Intercellular	Exchangeable Step 1	Exchangeable Step 2	Intracellular (soluble)	Residual (insoluble)
Leaves	1.3 (0.12)	2.8 (0.74)	2.9 (0.66)	89.6 (1.165)	3.9 (0.37)
Stems	1.2 (0.11)	1.5 (0.27)	0.631 (0.148)	91.4 (0.47)	5.11 (0.729)
Whole shoots	0.24 (0.02)	0.56 (0.15)	0.64 (0.18)	87.3 (1.06)	11.26 (1.33)

n = 8

Step 1 and Step 2 of the exchangeable fractions are 1 h incubations in 0.02M nickel chloride solution

The total K content of leaves, stems and whole shoots differed considerably (9.87, 24.0 and 16.0 mg g^{-1} dry weight, respectively) but percentages of total K found in the intracellular fraction were remarkably similar (90%, 91% and 87%, respectively) in the three sample types. The average value for total K content of the stems and leaves (*ca.* 17.0 mg g^{-1} dry weight) closely resembles the figure for the complete moss segments (16.0 mg g^{-1} dry weight) and, therefore, suggests that little damage was caused to the cells by removing the leaves. The exchangeable K displaced using $NiCl_2$ solutions, for all three tissue types, was between 1% and 3% of the total K content (Table 8.3.1).

Values obtained for the K content of *R. squarrosus* in the present study (Table 8.3.1) resemble those obtained by Brown, Ougham and Beckett (1986) for the same species. Additionally, the high intracellular values suggest that K could be useful as an indicator of membrane leakage as described by Brown and Bates (1972).

8.3.2 Effects of paraquat on potassium retention

Initial experiments involved the use of 725 µM and 7.25 mM paraquat treatments, the lower concentration corresponding to that used in the paraquat uptake experiment (Section 4.3.1.6). The higher paraquat concentration was included because no visible damage to the moss tissue was observed with the lower paraquat concentration in the uptake experiment. *R. squarrosus* shoots were incubated in the paraquat solutions for incubation times in a range from 5 mins to 96 h. The effects of these solutions on K retention by whole moss shoots, measured as K leakage into the herbicide incubation solutions, are presented in Table 8.3.2.

Progressive net loss of K from the moss shoots was detected following the 7.25 mM paraquat treatment, with 90% of the total K detectable in the incubation solution after 72 h. The 725 µM paraquat

Table 8.3.2 Percentage of the total potassium content lost from Rhytidiadelphus squarrosus shoots during incubation in 725 µM and 7.25 mM paraquat solutions

Incubation time (h)	Paraquat Concentration Control	725 µM	7.25 mM
5 min	0.1	8.0	19.3
30 min	0.7	4.7	11.1
1	0.1	11.2	22.3
2	1.4	7.1	15.0
4	0	5.5	12.4
24	0.1	11.7	63.2
48	0.2	5.2	68.7
72	0.9	40.9	90.5
96	0.4	6.6	90.7

ln SED = 0.402; df = 85; n = 4

(mean total K^+ content of untreated moss 6341 µg g^{-1} (SE 242.7)

treatment did not cause a marked K loss during the 96 h incubation period. However, the K loss of approximately 7% for most incubation periods was significantly greater than for the water controls which averaged 0.4% K loss. Probably, the K loss into the 725 µM paraquat solution represents K exchanged from the cell walls plus some loss from the protoplast, as the values are higher than the measured extracellular K content (1-3%).

As described by Rivera and Penner (1979), a rapid loss of membrane integrity may be attributed to structural interference with the membrane due to binding of herbicides to the plasmalemma. Therefore, the initial K loss (19%) following the 7.25 mM treatment may be due to paraquat binding at the plasmalemma inducing leakiness. Further losses in K were not evident following the 7.25 mM treatment until the 24 h incubation period when a 63% loss of K was detected. Harris and Dodge (1972) described similar delays in the detection of membrane damage in *Linum usitatissimum* from paraquat (100 µM) treatments. In *L. usitatissimum* chlorophyll reductions were not detectable for 20 h and K loss for 5-7 h. The K loss was related to increased levels of malondialdehyde, a breakdown product from peroxides of tri-unsaturated fatty acids. The sudden increase in K loss from the *L. usitatissimum* cotyledons was attributed to release of hydrolytic enzymes from the vacuole following damage to the tonoplast. These caused rapid breakdown of the cellular components (Harris and Dodge, 1972).

Increasing the light intensity might have enhanced the rate of K loss from the moss tissue if paraquat activity in moss resembled that in vascular plants. In vascular plants the electrons required for paraquat binding are generated from the light reaction of photosynthesis. Subsequent auto-oxidation yields the original paraquat ion and generates the potentially damaging free radicals, superoxide anion, singlet oxygen and hydrogen peroxide. The 7.25 mM paraquat solution also exceeds the 1 mM ionic concentration described by Lüttge and Bauer (1968) as causing loss of membrane function. Therefore, K loss due to paraquat may also be partly attributable to changes in the plasmalemma due to the ionic concentration of the incubation solution as well as specific binding effects. In general, it would appear that the moss tissue and *Linum usitatissimum* cotyledons (Harris and Dodge, 1972) behave in a similar manner following paraquat treatment.

8.3.3 Effects of iron salts on potassium retention

K leakage was measured from leaves, stems and whole shoots of *R. squarrosus* treated with a range of $FeSO_4$ concentrations (Table 8.3.3).

Table 8.3.3 Percentage of the total potassium content lost by leaves, stems and whole shoots of Rhytidiadelphus squarrosus during treatment in a range of ferrous sulphate solutions over a 24 hour period

$FeSO_4$ concentration µM	Tissue type		
	Leaves	Stems	Whole Shoots
0	4.3	0.2	0.1
20	2.4	3.8	0.2
200	2.6	1.4	1.5
1000	2.3	6.8	6.5
1100	2.9	18.4	1.3
2000	3.7	9.8	6.5
11000	11.2	23.1	26.6

ln SED = 0.6914; df = 60; n = 3

The greatest percentage K losses observed were 11%, 23% and 27% for leaves, stems and whole shoots, respectively, following the 11 mM $FeSO_4$ treatment. Except for the value for stems treated with 1 mM $FeSO_4$, the more dilute $FeSO_4$ solutions caused relatively little K loss (Table 8.3.3). The low levels of K detected in the more dilute $FeSO_4$ solutions are probably due to displacement of K from the cell walls (Brown and Buck, 1985).

The results of a further experiment incorporating a long incubation time (120 h) but $FeSO_4$ concentrations in a lower concentration range are presented in Table 8.3.4. In this experiment

Table 8.3.4 Percentage of the total potassium content lost by leaves, stems and whole shoots of Rhytidiadelphus squarrosus during treatment in ferrous sulphate solutions

Tissue Type	Incubation Time (h)	Fe Concentration (μM) 20	200	1000	2000
Leaves	24	5.2	2.2	9.1	2.4
	120	25.1	2.7	46.8	48.5
Stems	24	0.7	1.5	13.6	9.8
	120	1.5	1.4	32.1	39.1
Whole shoots	24	0.5	0.4	6.6	6.5
	120	0.4	0.1	2.0	24.8

ln SED = 0.7048; df = 59; n = 3

significant ($P<0.001$) main effects of time, concentration and tissue type were identified. High incubation concentration of $FeSO_4$ (>1000 μM) and long incubation times (120 h) leaves and stems both showed significantly greater K loss than from whole shoots of *R. squarrosus* but were not significantly different from each other. Greater K loss was detected for whole shoots during the 120 h treatment than in the 24 h incubation, particularly at the high Fe concentration (2000 μM). The observation that a greater Fe concentration is required to cause K loss in whole shoots, is probably a consequence of the greater mass of

tissue in relation to solution volume than in the leaf and stem preparations (Table 8.3.4).

The relatively slow induction of membrane damage by $FeSO_4$ treatment may indicate interference in lipid synthesis, as suggested by Rivera and Penner (1979). However, it is equally plausible that Fe binds to the cell membranes, disrupting their structure as has been proposed for copper in vascular plants (Fitter and Hay, 1981). The slow progress of K loss could be attributable to the time required for Fe ions to reach the plasmalemma in sufficient quantities to cause damage. Previously discussed results (Section 4.3.1.6) show slow relocation of externally bound paraquat to the intracellular pool over long incubation periods (96 h). Possibly, relocation of exchangeable iron also occurs over the 120 h period leading to progressive cellular damage.

The effect of different iron sources ($FeSO_4$, $FeCl_3$, $Fe(NO_3)_3$ and Fe^{3+}EDTA) on the loss of K from *R. squarrosus* were also studied during a 24 h incubation (Table 8.3.5). The corresponding Fe uptake values for the different moss tissues showed no significant differences except that uptake levels were significantly lower from Fe^{3+}EDTA (Table 8.3.5). The analysis of variance of the percentage K loss data indicates differences between the main effects for iron salts, concentrations and tissues ($p<0.01$).

K loss was greater in $FeCl_3$, $Fe(NO_3)_3$ and Fe^{3+}EDTA (*ca.* 20% loss) for leaves or stems than in $FeSO_4$ (*ca.* 4% leaves, 9% stems) at the highest concentrations employed (Table 8.3.5). Only the $FeCl_3$ and

Table 8.3.5 Percentage of the total potassium content lost by leaves, stems and whole shoots of Rhytidiadelphus squarrosus treated with different iron salt solutions of different concentrations for a 24 hour period

Concentration Fe(μM)	Iron Salt $FeCl_3$	$FeSO_4$	$Fe(NO_3)_3$	FeEDTA
Leaves				
20	2.3	4.7	7.4	1.9
200	1.3	2.4	14.2	1.1
1000	35.9	4.3	29.9	6.4
2000	23.8	4.6	19.8	23.6
Stems				
20	2.6	0.3	0.8	0.3
200	0.8	1.3	3.3	0.2
1000	12.2	12.9	20.8	5.5
2000	15.3	9.3	20.2	18.2
Whole Shoots				
20	0.1	0.5	0.4	0.2
200	0.2	0.2	0.4	0.1
1000	10.5	3.3	10.6	0.3
2000	12.4	6.1	9.8	1.6

ln SED = 0.785; df = 143; n = 3

$Fe(NO_3)_3$ treatments produced significant K loss following the 1mM treatment (*ca.* 30% leaves, 15% stems). The release of K into the incubating solutions does not correlate closely with the total iron uptake (Section 4.3.2.2), indicating that total iron uptake is not a valid indicator of toxicity of the iron salt. Greater K loss occurred in solutions containing ferric salts. However, the considerable percentage K losses induced by $FeCl_3$ and $Fe(NO_3)_3$ may be a result of their greater acidity compared to the other solutions (Table 4.2.1). Alternatively, if redistribution of the exchangeable Fe content from the cell wall to the protoplast occurs, as suggested for paraquat (Section 4.3.1.6), this may proceed more rapidly in the more acidic environment and accelerate internal displacement of potassium. It is unlikely that the different abilities of the salts to displace K from *R. squarrosus* are a function of the oxidation state of iron, as Fe^{2+} is rapidly oxidised to Fe^{3+}.

Iron-EDTA at high concentrations also caused K loss from leaves and stems but not from whole shoots. In this case high acidity was not a factor as the 2000 µM treatment had a pH of 5.2. Possibly, Fe^{3+}EDTA displaces calcium from membrane sites as described by Bates (1982) for Na EDTA. Calcium displacement from *Tortella tortuosa* and *Racomitrium lanuginosum* following treatment with Na EDTA solutions was accompanied by the loss of intracellular K. Marked K loss from *R. squarrosus* was not detected in the present work (Table 8.3.5) below a concentration of 2000 µM Fe^{3+}EDTA. This is similar to the threshold value described by Bates (1982) for the effects of Na EDTA on Ca and K displacement (1000 µM).

The loss of K following immersion in iron salt solutions can therefore be attributed to Fe binding to the cell membranes consequently altering permeability.

8.3.4 Effects of 2,4-D on potassium retention

K loss was measured following incubation of *R. squarrosus* leaves, stems and whole shoots in 7.5 mM 2,4-D solutions for various periods (Table 8.3.6). An analysis of variance of the data indicated no significant differences in K loss between tissue types, although a significant effect of incubation time on K loss was found.

Table 8.3.6 Percentage of the total potassium content lost from leaves, stems and whole shoots of Rhytidiadelphus squarrosus during incubation in 7.5 mM 2,4-D solution

Incubation time (h)	Treatment					
	Control			2,4-D		
	Leaves	Stems	Whole shoots	Leaves	Stems	Whole shoots
0.5	5.2	3.0	1.2	16.4	5.9	2.4
1	3.5	3.3	1.6	7.5	7.2	3.9
24	2.7	1.5	0.5	19.9	4.5	4.5
48	4.3	1.2	4.6	48.9	15.8	2.6
96	4.4	0.6	0.1	27.9	12.1	10.3

ln SED = 1.456; df = 112; n = 4

In general, the incubation periods producing marked K leakage for leaves and stems during 7.5 mM treatments caused less severe leakage in the whole shoots, where exhaustion of the 2,4-D may have occurred (Table 8.3.6).

A similar experiment using only intact moss segments gave significantly greater K loss following the 75 mM treatment than the 75 µM and control treatments (Table 8.3.7). The effect of 2,4-D on membrane integrity of *R. squarrosus* is, therefore, concentration-dependent.

Table 8.3.7 Percentage of the total potassium content lost from segments of Rhytidiadelphus squarrosus during incubation in 75 µM and 75 mM 2,4-D solution

Incubation time (h)	2,4-D Concentration Control	75 µM	75 mM
0.5	0.1	0.4	14.4
1	0.3	0.3	21.5
24	0.1	0.1	79.8
96	0.2	0.2	76.7

ln SED = 0.4639; df = 41; n = 4

The rapid leakage of K during the 75 mM treatment may indicate plasmalemma damage due to the high ionic concentration. In contrast, the gradual loss of membrane integrity observed during the 7.5 mM 2,4-D treatments (Table 8.3.7) is probably related to the effects of the 2,4-D on moss membranes. The slow K loss indicates that 2,4-D is not directly affecting the plasmalemma as reactions of this type are considered to be fast (Rivera and Penner,1979). Membrane damage may therefore relate to the effects described by Switzer (1957,cited Rivera and Penner,1979) who suggested that 2,4-D affects entire enzyme complexes bound to mitochondrial membranes, influencing both membrane structure and oxidative phosphorylation.

Ultrastructural studies of tobacco leaf mesophyll cells by White and Hemphill (1972) showed rapid structural breakdown of the tonoplast, plasmalemma, chloroplasts and mitochondria as a result of high 2,4-D concentrations. Greater damage was observed in older leaf tissues due to their reduced lipid synthesis and metabolism.

R. squarrosus appears to be very susceptible to membrane damage following high concentrations of 2,4-D. Leaf and stem cells both showed K loss although the former may be slightly more sensitive, possibly due to their greater accessibility. It is conceivable that differences exist in lipid synthesis rates in leaf and stem cells which might also yield differences in susceptibility to 2,4-D.

The very low levels of 2,4-D uptake (Section 4.3.2.2) and the corresponding gradual leakage of K during 7.5 mM treatments indicate that *R. squarrosus* is very sensitive to 2,4-D.

8.3.5 Effects of dichlorophen on potassium retention

R. squarrosus was incubated in a 65 µM dichlorophen solution for a range of times from 5 min to 48 h. Following each incubation period the tissues were incubated for a further 24 h in water to determine whether progressive loss of membrane integrity occurred (Table 8.3.8).

Net K loss into the treatment solution increased significantly from 0.4% to 13% of total tissue K over the 48 h incubation time. Approximately 20% of the total K was recovered in the secondary incubation in water (24 h) irrespective of the initial incubation time in dichlorophen. The results indicate that the membrane damaging

Table 8.3.8 Percentage of the total potassium content lost by Rhytidiadelphus squarrosus shoots treated in 65 µM dichlorophen solution (Initial K loss to incubation medium, additional loss following 24 h in distilled water)

Incubation time (min)	% K recovered Dichlorophen	water (24 h incubation)
5	0.4	17.0
10	0.6	14.9
30	0.03	16.4
120	1.2	25.9
1440	7.1	23.6
2880	12.6	20.5

ln SED = 0.3522; df = 23; n = 2

action of dichlorophen is rapid but that the extent of the damage is not fully revealed by short incubation times. Alternatively, the uptake of dichlorophen may be rapid but long incubation times may be required for extensive damage to occur. The results resemble those obtained by Brown, Ougham and Beckett (1986) for the post-incubation period in water where a treatment of 20 µl l^{-1} dichlorophen gave an initial (30 min) K loss of 25% followed by a further 21% after incubation for 3 h in water. This compares with 0.03% and 25.9% K loss, respectively, for the initial 30 min dichlorophen incubation and the 24 h water treatment in the present experiment. The low levels of K detected in the dichlorophen incubation solutions may be related to K eluted from the cell walls or intercellular spaces of the moss. The relatively slow loss of membrane integrity in both studies indicates that a direct binding effect of dichlorophen on the plasmalemma lipids (Rivera and Penner, 1979) is unlikely in *R. squarrosus*.

K losses, after a 24 h incubation period, were found to increase significantly with dichlorophen concentration over the range 6.5 - 6500 µM (Table 8.3.9). However, the maximum K loss was only 37% following the 6500 µM treatment, indicating that only partial membrane disruption had occurred. A similar result was obtained by Brown *et al.* (1986).

Slow loss of membrane integrity is not unexpected with dichlorophen which has been described as an uncoupler of oxidative phosphorylation in rat mitochondria (Nakue, Caldwell and Buhler, 1972). The affinity of dichlorophen for proteinaceous material (Purkiss, 1983) and its function as an uncoupler suggest that binding to proteins in the mitochondrial membranes may occur. Such binding has been found to cause conformational changes in the phosphorylating enzymes, loss of their coupling function and induced proton fluxes in rat mitochondria (Nakue *et al.*, 1972). Leakage of K into the incubating solutions soon after immersion of the moss shoots may possibly reflect some binding of dichlorophen to proteinaceous material in the plasmalemma causing slight changes in membrane structure and, therefore, membrane integrity.

Table 8.3.9 Percentage of the total potassium content lost by Rhytidiadelphus squarrosus shoots during incubation (24 h) in dichlorophen solution of different concentrations

Dichlorophen concentration (µM)	% Total K	SE
6500	37.3	1.06
650	29.3	2.28
65	9.9	0.39
6.5	12.2	11.95
0	0	0.01

n = 3

The normal field application concentration of dichlorophen is approximately 50 mM. As suggested by Brown *et al.* (1986) disruption of the plasmalemma of *R. squarrosus*, following application of dichlorophen at the high recommended field rates, may be a factor in its observed toxicity. However, the physiological effects of dichlorophen (reduced photosynthesis, temporary inhibition of respiration and selective reduction of chlorophyll *a* compared with that of chlorophyll *b*), would tend to suggest that electron transport inhibition in photosystem II and in respiration may be the primary modes of action. Membrane permeability changes are likely to be of secondary importance, possibly enhanced by the complete immersion of the shoots in the dichlorophen solutions. Further evidence to suggest that membrane damage is not the primary mode of action is the loss of chlorophyll *a* (but not chlorophyll *b*) in dichlorophen-treated *R. squarrosus* shoots. Severe loss of membrane integrity might be expected to reduce the chlorophyll *a* and *b* content of the moss shoots to an equal extent.

8.4 CONCLUSIONS

Solutions of paraquat, 2,4-D, dichlorophen and iron salts all reduced membrane integrity of *R. squarrosus* as determined by K leakage from intact shoot apices. However, rapid loss of membrane integrity was not detected following any of the treatments. This information casts some light on the mode of action of each of the chemicals. Slow effects of the herbicides on membrane function would suggest a functional rather than a structural effect of the herbicides on the moss plasma membrane based on Rivera and Penner's (1979) classification of herbicide effects on plant membranes. Alternatively, a delayed effect of herbicides on the moss plasma membranes may result from the cell wall impeding the penetration of herbicides. This is most likely to be true for cations such as paraquat and Fe that are rapidly exchanged to the cell walls of *R. squarrosus* tissues (Sections 4.3.1.1 and 4.3.2.1).

No differences in K leakage between leaves, stems and whole moss shoots for the different herbicides were found. This indicates that the plasma membranes of the leaves and stems of *R. squarrosus* do not differ in their sensitivity to the herbicide treatments.

K leakage from *R. squarrosus* was concentration-dependent for all herbicides. Generally, loss of membrane integrity was observed following treatments greater than 1 mM. Loss of K above this concentration may be related to damage to the plasmalemma due to ionic concentrations of the herbicide solutions (Lüttge and Bauer, 1968; Clarkson, 1974). However, K leakage from *R. squarrosus* did increase slowly during increased incubation times for solutions of all herbicides at concentrations less than 1 mM, suggesting concentration is irrelevant.

K leakage from *R. squarrosus* was found to be greater with $FeCl_3$, $Fe(NO_3)_3$ and Fe^{3+} EDTA solutions than with $FeSO_4$. The greater acidity of $FeCl_3$ and $Fe(NO_3)_3$ may account for the considerable loss of K from

these solutions compared with $FeSO_4$ solutions. In the case of Fe^{3+}EDTA, loss of membrane integrity might be due to displacement of calcium from membranes as described by Bates (1982) for the effects of Na EDTA on the membranes of *Tortella tortuosa* and *Racomitrium lanuginosum*.

CHAPTER 9

GENERAL CONCLUSIONS

9.1 GENERAL CONCLUSIONS

The objectives of this project were to examine the absorption of a range of herbicides, including proprietory moss killers, and to assess their effects on moss physiology. Knowledge of herbicide activity on mosses is limited and published work is often contradictory. For instance, resistance of mosses to highly phytotoxic substances, such as paraquat and simazine, has been described (Balcerkiewicz and Rusinska, 1987). The results presented in this thesis demonstrate that the moss *Rhytidiadelphus squarrosus*, a common weed in lawns, is susceptible to the herbicides tested. Field, glasshouse and laboratory studies show that the apparent resistance of mosses to herbicides described in published reports may be attributed to : 1) poor herbicide application and therefore contact with the moss shoots, 2) the morphology of the moss shoots such that the leaves prevent penetration of the herbicides to the stem tissue, 3) poor intracellular uptake and the high cation exchange capacity of moss cell walls, and 4) biochemical differences which may exist between mosses and vascular plants.

Initial field studies were designed to determine the sensitivity of mosses to a range of herbicides (Ch. 2). Undoubtedly the most economically important aspect of moss control is in the treatment of lawns. For this reason, infested turf was used for the initial herbicide comparisons. Lawn herbicide applications also have the advantage of enabling direct comparisons of herbicide sensitivity in mosses to be made with grasses and broad leaved weeds present in the plots. However, it should also be noted that field trials, in addition to providing authentic conditions in which to study the effects of herbicides on lawn mosses, also possess limitations. These include: 1) variation in the physiological state of the mosses depending on environmental conditions before and after herbicide application, 2) influence of the herbicide performance by the post application

meteorological conditions (Fryer and Makepeace, 1977), 3) changes in the micro-environment of the mosses following either death of the vascular plants leading to the subsequent desiccation and dislodging of the moss shoots (Sections 2.3.1.1 and 2.3.2), or increased competition from the vascular plants following fertiliser applications (Section 2.3.1), and 4) the inefficiency of spray application methods and variation in the height and density of the lawn grasses, influencing contact between the herbicide and moss shoots. The herbicide treatments used in the field trials included the commercially sold lawn moss killers, dichlorophen (Supermosstox), ferrous sulphate, chloroxuron with iron (Tumblemoss) and ammonium sulphate. Other herbicides tested were selected because of effects, or absence of effects, on moss control suggested by other workers (Section 2.1). These were oxadiazon, acifluorfen, oxyfluorfen, 2,4-D, paraquat, Gloquat C, and thiram.

From the field data, *R. squarrosus* was found to be sensitive to all herbicide treatments (Ch. 2). Chlorosis of the leaves, after herbicide application, was the most common toxic effect observed in *R. squarrosus* shoots. The proportions of the moss shoots showing toxic effects differed between treatments. Dichlorophen caused rapid chlorosis (*ca.* 2 days) of *R. squarrosus* shoots, affecting up to 90% of the tissues. In contrast, paraquat did not cause chlorosis of the moss shoots for at least 8 wk after treatment (Section 2.3.2). In addition to those treatments causing chlorosis in *R. squarrosus*, the chloroxuron preparations containing $FeSO_4$, and $FeSO_4$ alone, caused blackening of the shoots (Plate 2.3.1) (Field, 1969; Anon., 1985). The black appearance is believed to be due to the formation of a pigmented iron complex, possibly with tannins (Field, 1969) or their phenolic derivatives, located at the moss cell wall. Despite visible toxic effects of all the herbicide treatments, long term control (>17 wk), measured as changes in moss cover, was only achieved with treatments

of dichlorophen, chloroxuron with iron, oxadiazon, thiram and ammonium sulphate. Regrowth from herbicide treated shoots was observed a few centimetres below the shoot apex, following all treatments.

To minimise environmental variations and the competitive effects of vascular plants, a glasshouse trial with 4 selected herbicide treatments was performed on monocultures of *R. squarrosus* and two other moss species (Section 2.3.3). *Dicranum scoparium* and *Polytrichum juniperinum* were included to investigate the possibility that herbicide activity was species specific. The three chosen species encompass a range of types of water relations. The chemicals were selected on the basis of the field trial results and included two highly toxic (dichlorophen and ferrous sulphate) and two less effective (2,4-D and paraquat) herbicides. The results were similar for all three moss species and corroborate those obtained for *R. squarrosus* in the field experiments. These were rapid chlorosis or blackening of the moss with dichlorophen and $FeSO_4$ treatments, respectively, and a very slow (>8 wk) appearance of chlorosis following 2,4-D and paraquat applications (Plates 2.3.1 to 2.3.5). *P. juniperinum* alone exhibited apical growth distortions following 2,4-D application (Plate 2.3.4). This effect is a typical symptom of 2,4-D damage in dicotyledonous plants, and it is of interest that *P. juniperinum* is the only species investigated that possesses a well developed water conducting system. In dicotyledonous plants auxin sensitive cells in the vascular system are believed to initiate this response (Corbett, Wright and Baillie, 1984). Polytrichales may therefore possess similar auxin sensitive cells in connection with their water conducting system. The absence of growth distortions in *R. squarrosus* and *D. scoparium* may reflect the absence of similar auxin sensitive cells and a more primitive physiology. The sensitivity of mosses to growth regulator type herbicides such as

2,4-D, and therefore the presence or absence of auxin sensitive cells, may represent a useful criterion in bryophyte systematics.

The extent of penetration of paraquat, 2,4-D, dichlorophen and $FeSO_4$ into *R. squarrosus* shoots was found to differ in this study. This was measured by culturing previously treated shoot apices of *R. squarrosus* in herbicide free conditions (Ch. 3). Growth of the shoots following immersion in 2,4-D or dichlorophen was sensitive to increasing incubation time (5 min or 24 h) and concentration. A 24 h incubation time caused greater inhibition of moss shoot growth compared with a 5 min incubation. In the latter case, a 100-fold increase in concentration was required to produce a similar inhibitory effect on moss growth to that of the 24 h incubation (Section 3.2.1). This result suggests a slow but continuous uptake of these herbicides by the moss shoots. In contrast, paraquat inhibition of moss growth increased with increasing concentration (7.24 µM - 72.5 mM) but was independent of incubation time (Section 3.3.3). This is attributed, in the light of uptake experiments (Section 4.3.1), to poor intracellular uptake probably due to rapid exchange of paraquat to the cell walls. Similar low intracellular uptake of metallic cations with high affinities for the moss cell wall has been repeatedly observed (Brown and Beckett, 1985). Stimulation of moss shoot growth was observed following low concentrations (200 - 1000 µM Fe) of $FeSO_4$ and short incubation times (5 min) (Fig. 3.3.9). Inhibition of growth was noted following 24 h incubations in $FeSO_4$ (Fig. 3.3.9). Growth stimulation was attributed to either reversal of iron deficiency or etiolation of the moss in response to reduced light penetration (caused by the blackening of the tissues).

The herbicide uptake studies (Ch. 4) provided useful data to aid interpretation of the results of field, glasshouse and laboratory growth experiments. Using a method based on the differential

displacement technique of Brown and Buck (1979), rapid paraquat exchange to the moss cell walls and, consequently, poor intracellular uptake was observed. However, by increasing the moss incubation times in dilute paraquat solutions (72.5, 725 µM), previously observed (Section 4.3.1.4) to become rapidly exhausted in freely available paraquat (15 min), redistribution from the exchangeable cell wall sites to the intracellular fractions was detected (Section 4.3.1.6). Similar observations have been made for the redistribution of cadmium in *R. squarrosus* (Wells and Brown, 1987). Paraquat redistribution from extracellular to intracellular sites may account for the slow onset of visible toxicity in the field and glasshouse experiments (Figs 4.3.9 and 4.3.10).

Different quantities of paraquat were absorbed by the component leaves and stems of the whole moss shoots incubated in dilute paraquat solutions (72.5 µM and 725 µM). The leaves were found to exchange paraquat rapidly on their cell walls, possibly hindering penetration of paraquat to the stems (Section 4.3.1.2). Inhibition of paraquat penetration to the stems of moss shoots in a field situation would account for the ability of *R. squarrosus* to regrow from the stems of herbicide treated shoots (Sections 2.3.1.1 and 2.3.2). Kinetic studies of paraquat uptake by detached leaves and stems of *R. squarrosus* shoots (Section 4.3.6.1) showed a faster rate of exchange by the leaves than by stems with both tissues showing slow intracellular uptake. The very fast paraquat exchange rates and poor intracellular uptake accounts for the absence of an incubation time effect in the growth experiments (Section 3.3.3). The effect of increasing paraquat concentration on the inhibition of growth (Section 3.3.3) can be attributed to greater total uptake and eventual redistribution to the protoplast (Section 4.3.6.1). These results reveal that spray application of cationic herbicides to mosses provides poor stem penetration, and this may be a

major factor in the apparent resistance of mosses to herbicides as regrowth can occur from the unaffected stems.

Similar studies of iron uptake (Section 4.3.2) confirm the ability of *R. squarrosus* to exchange cations rapidly to the cell walls with subsequent low levels of intracellular uptake. Iron exchange was not affected by the initial valency or anionic component of the iron salt. However, iron supplied in chelated form (Fe^{3+}EDTA) gave poor total iron uptake, probably due to the stability of the chelated complex.

An important effect was observed when $FeCl_3$-pretreated *R. squarrosus* shoots were treated with ^{14}C-paraquat (Section 4.3.1.7). In this experiment it was found that extracellular and intracellular paraquat uptake were inhibited by the $FeCl_3$ pretreatment. The reduction in ^{14}C-paraquat uptake was greater with increasing pretreatment $FeCl_3$ concentration (Fig. 4.3.10). Presumably exchanged iron prevents later exchangeable uptake of paraquat. It is probable that in nutrient rich, calcareous or polluted habitats, the high availability of cations would seriously impair the efficiency of cationic herbicide uptake. This possibly warrants further investigation.

In contrast to the rapid uptake of paraquat and iron by *R. squarrosus*, uptake of ^{14}C-2,4-D was undetectable (Section 4.3.3). The absence of exchanged 2,4-D (anionic) on the cell walls was expected because of the low anion exchange capacities of mosses (Clymo, 1963). The high pH of the 2,4-D solutions (Table 4.2.1) and the extensive dissociation of its molecules may account for poor uptake through lipid membranes and its limited toxicity. Briggs, Rigitano and Bromilow (1987) and Rigitano, Bromilow, Briggs and Chamberlain (1987) have shown that the concentration of 2,4-D in the protoplasm of *Ricinus communis* and barley roots increases with decreasing pH of the incubating solution, probably a result of increased levels of the undissociated

2,4-D molecules in more acidic solutions. The cell membrane of *R. squarrosus* probably resembles those of vascular plants in being permeable to the lipid soluble undissociated 2,4-D molecule rather than the dissociated form.

In the present study, dichlorophen was found to be absorbed by *R. squarrosus* shoots (Section 4.3.4). However, membrane damage and loss of intracellular components, as observed by Brown, Ougham and Beckett (1986), interfered with the detection of dichlorophen using UV spectrophotometry (Clements and Newburger, 1954). The uptake of anionic dichlorophen, like that of 2,4-D, is attributed to undissociated dichlorophen (pK_a 7.6 and 11.5) which, in this case, was available in the incubation solution (pH 8.1). It is clear from these results that adequate uptake of anionic herbicides by moss shoots may require regulation of pH of the formulation to ensure adequate activity of undissociated molecules. The results obtained in Chapter 4 also have important implications for studies using moss tissues as indicators for pollution studies. The previous history of exposure to cations, the degree of dissociation of molecules, and the influence of pH could seriously affect subsequent uptake.

In this study, the technique of efflux analysis from ^{14}C-paraquat-loaded *R. squarrosus* tissue was used to determine the cellular location of the herbicide (Ch. 5). Comparisons were made between paraquat uptake by *R. squarrosus* tissues and by *Lolium perenne* leaf slices (Sections 5.3.1 - 5.3.4). No major differences in the total intracellular paraquat concentrations of the two species were found. Differences in the inferred rates of paraquat efflux across the plasmalemma and tonoplast were found. However, the rate of efflux across the tonoplast of *L. perenne* leaf slices was faster than in *R. squarrosus*; this was perhaps attributable to damage to the tonoplast of

the former. A similar loss of tonoplast integrity following paraquat treatment was observed in flax leaves by Dodge and Harris (1970). The total cytoplasmic and vacuolar ^{14}C-paraquat content did not differ between species in the present study. However, a smaller vacuolar volume in the moss, apparent from electron microscope studies (Section 1.4), suggests that paraquat present in the vacuole of *R. squarrosus* is at a higher concentration than in the cytoplasm of *R. squarrosus* or in either the cytoplasm or vacuole of *L. perenne*. From these results, it is possible that in the moss, selective accumulation of paraquat in the vacuole occurs, thus reducing its toxicity. The quantity of paraquat exchanged on to the cell walls of *R. squarrosus* was ten times greater than in *L. perenne* following a 725 µM paraquat treatment. This is probably a result of a higher cation exchange capacity in the cells of the moss than in the grass leaves.

The Nernst potentials for *R. squarrosus* and *L. perenne* cells were calculated using the data for intracellular and external paraquat concentrations, and were similar in two species (Table 5.3.11). It is predicted from these results that paraquat is accumulated passively by *R. squarrosus*.

It was observed in the present study that dichlorophen, 2,4-D, and paraquat inhibited photosynthesis in *R. squarrosus* whereas $FeSO_4$ did not (Ch. 6). This study involved the spray application of the herbicides to simulate field treatment, a procedure which is known to give inadequate contact between the herbicide solution and the moss tissues. Dichlorophen was found to inhibit respiration and photosynthesis rapidly (1 d) after treatment, although respiration recovered within 5 days of application. A greater reduction in the content of chlorophyll *a* compared to chlorophyll *b* (Fig. 6.3.7) suggests possible inhibition by dichlorophen of electron transport in

photosystem II. Ridley (1977) described a similar loss of chlorophyll *a* an diuron treatment of *Pisum sativum* leaves as being due to inhibition of electron transport in PSII. Paraquat and 2,4-D did not reduce photosynthesis significantly until 12 wk after application (Fig. 6.3.3). The long delay before 2,4-D and paraquat exert their effect is attributed to slow intracellular uptake (Sections 4.3.1.6 and 4.3.3.2). In the case of paraquat this is probably due to cell wall binding and, in the case of 2,4-D, due to the absence of undissociated molecules in the treatment solution. The delayed suppression of photosynthesis following 2,4-D application may also be due to its primary effects on cell elongation as in dicotyledonous plants (Section 1.7.2). The photosynthetic and respiration potential of the $FeSO_4$ blackened *R. squarrosus* tissues was not impaired (Figs 6.3.3 and 6.3.4). This supports the view that the blackening of the moss tissue is due to the formation of an external pigmented iron complex and not due to internal damage.

The effects of dichlorophen and paraquat on photosynthesis of *R. squarrosus* were also studied by analysis of PI curves to provide information on the sites of activity within the photosystems (Ch. 7). In these experiments, moss apices were immersed in the herbicide solution to ensure adequate uptake. Both chemicals were found to inhibit photosynthesis by preventing electron transport. The effects of dichlorophen on both the form of the curves (Figs 7.3.3 and 7.3.4, Table 7.3.1) and on chlorophyll *a* : *b* ratio (Fig. 6.3.7) are suggestive of inhibition of electron transport in PSII. Structurally, dichlorophen resembles the phenolic type herbicides which, in vascular plants, bind to the 41 kDa polypeptide in the thylakoid membrane (Oettmeier, Mason and Johanningmeir, 1982). The sensitivity of mosses to dichlorophen may be due to their possession of a large quantity of the 41 kDa polypeptide (Aro, 1982) and low levels of the shielding

(Oettmeier, Mason and Johanningmeir, 1982) 32-34 kDa polypeptide (Aro, 1982) compared to vascular plants. The effects of dichlorophen on membrane integrity, photosynthesis and respiration in *R. squarrosus* all support its description, using the herbicide classificatory system of Moreland (1980), as an inhibitory uncoupler herbicide. The effects of paraquat on the form of the PI curves (Figs 7.3.7 and 7.3.8, Table 7.3.1) are consistent with its known inhibition of electron transport in photosystem I of vascular plants (Harris and Dodge, 1972). Such a mode of action would not affect the chlorophyll *a* : *b* ratio (Fig. 6.3.7).

Although the PI curve analysis has been helpful in this study in locating the possible sites of paraquat and dichlorophen activity in *R. squarrosus*, technical problems were encountered. Variation in the data, possibly due to secondary activity of the herbicides, reduces the accuracy of the curve fitting. Increased replication of data points may have aided in the fitting and accuracy of the final PI curve. However, extreme variations in the individual replicates would still have led to large standard errors for the final curve parameters, influencing the significance of the findings. This approach may have applications in toxicological studies where the toxicant is unlikely to affect the cell membranes of the moss tissues, or perhaps in pollution studies where the accumulation of toxic substances is sometimes more gradual and where subtle changes in the PI relationship may occur.

Membrane integrity of *R. squarrosus* shoots during immersion in herbicide was assessed by the detection of potassium leakage (Ch. 8). Damage to the plasmalemma is often the first sign of herbicidal activity and can be expected to lead to uncontrolled loss and entry of solutes and uncoupling of electron flow. Membrane damage was detected during incubation in dichlorophen, 2,4-D, paraquat and $FeSO_4$. However, rapid loss of membrane integrity was not observed during incubation in

the herbicide solutions. The effects observed for paraquat, dichlorophen, 2,4-D and $FeSO_4$ on *R. squarrosus* were assessed using the classification system of Rivera and Penner (1979). For all four herbicides it is concluded that interference in the functioning of the membrane was caused rather than rapid structural membrane damage. Greater potassium loss was also observed during incubation in $Fe(NO_3)_3$ and $FeCl_3$ than in $FeSO_4$ (Section 8.3.3). This is attributed to the lower pH of the ferric iron solutions (Table 4.2.1). These results emphasise the greater sensitivity of *R. squarrosus* to the four herbicide treatments when treated by immersion compared to spray application.

To summarise, the present study has clarified the reasons for the apparent resistance of mosses, exemplified here by *R. squarrosus*, to some herbicides. Inefficient application is probably the major factor preventing the full activity of herbicides against mosses. The absence of an efficient internal translocation system in *R. squarrosus* coupled with its ability to regrow from small fragments of stem tissue, indicates the need for complete herbicide coverage of the shoots for effective control. In addition, the high cation exchange capacity of the moss cell walls reduces the activity, or delays the onset of activity, of cationic herbicides such as paraquat. These differences from vascular plants make direct comparisons of the two groups difficult and a number of unanswered questions remain:

1) Do mosses possess high levels of free radical scavengers, such as carotenoids and superoxide dismutase, thereby reducing the toxicity of bipyridylium and diphenyl ether herbicides ?
2) Do mosses possess a protein structure in PSII similar to that of triazine-resistant vascular plants ?
3) Is absorption to the cell walls of mosses a pre-requisite for intracellular uptake of herbicidal and nutrient cations ?

It is likely that some of these features are present in mosses and they may represent adaptations for their poikilohydric lifestyle. For instance, the presence of free radical scavengers has been hypothesised to prevent the destruction of nucleic acids, proteins and membrane lipids by toxic oyxgen species produced within the cells during drying of desiccation-tolerant moss species (Bewley, 1979).

A polypeptide structure in photosystem II of mosses, similar to that of triazine-resistant vascular plants, might be a general feature of mosses, enabling survival in shaded conditions (sciophytism). In triazine-resistant vascular plants, electron transport is low and limited by reduced levels of the *ca.* 32 kDa polypeptide. In mosses, low levels of electron transport carriers might lead to chlorophyll destruction at high light intensities. Radical scavengers such as carotenoids have been hypothesised as protective mechanisms preventing overloading in vascular plants (Ridley, 1977) and it is likely that they also occur in bryophytes. Carotenoid synthesis has been identified in *Polytrichum commune* during the germination of spores (Karunen, Valanne and Wilkinson, 1976; Bowers and Rule, 1982).

The paraquat uptake study (Section 4.3.1) showed binding to the cell walls and subsequent transfer to the protoplast of *R. squarrosus.* It is possible moss cell walls with their high cation exchange capacities, act as a sink for the accumulation of cationic nutrients from precipitations (Schofield, 1985; Ruhling and Tyler, 1970). Such a system might also increase sensitivity of mosses to cationic pollutants, both by encouraging their accumulation and by competitively lowering nutrient uptake. A greater understanding of cation accumulation processes is essential, especially in studies where quantitative measurements of heavy metal pollutants associated with tissues of indigenous mosses are made (Ruhling and Tyler, 1970; Hermann

and Hubner, 1984). Other cations exchanged on the moss cell wall could compete against and reduce the concentration of, the metal pollutants being studied.

Research into the physiological and biochemical effects of herbicides on mosses may appear to be of little biological importance. However, in vascular plants, studies on the mode of action of herbicides have improved our understanding of fundamental biology. Studies using photosynthetic electron transport inhibitor herbicides have been particularly important in elucidating the complexity of the photosynthesis pathway. Considerable research is still required to determine details of photosynthesis in bryophytes, such as the composition of electron carriers. Greater knowledge of the biochemistry of bryophytes may also be used to decipher the phylogeny of bryophytes. Suire and Asakawa (1979) discussed the importance of biochemical data in bryophytes systematics. They describe an excellent example of the value of biochemical differences in bryophyte taxonomy involving the distribution of lunularic acid and the varied metabolism of D-methionine in the five groups comprising the phylum Bryophyta. Investigations involving the use of herbicides of known mode of action may therefore help in determining details of bryophyte photosynthesis and lead to a greater understanding of sciophytic adaptations and phylogeny of the Bryophyta.

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