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The biological effects and mode of action of a chemical hybridising agent

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THE BIOLOGICAL EFFECTS AND MODE OF ACTION

OF A CHEMICAL HYBRIDISING AGENT.

by

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A thesis submitted for the degree of Doctor of Philosophy in the

University of Durham, England.

Department of Biological Sciences, November 1988

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- 4 OCT 1989

Thesis
1988/kEL

This thesis results entirely from my own work and has not previously been

offered in candidature for any other degree or diploma

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H.A. Kelly

November 1988

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We cannot suppose that all the breeds were suddenly produced as perfect and as useful as we now see them. The key is man's power of accumulative selection: nature gives successive variations; man adds them up in certain directions useful to him.

Charles Darwin, The Origin of Species, 1859.

ABSTRACT

The behaviour of the chemical hybridising agent, WL84811, was studied on diverse plant systems. Information about its effect on germinating seeds and on growth of the unicellular alga *Chlorella* was used to compliment information obtained from direct studies on pollen.

Although work with germinating seeds showed that WL84811 affects coleoptile and root growth somewhat differently, there were similarities between the responses of the seed and algal systems. In both cases, WL84811 has two separate effects: at low WL84811 concentrations, much higher concentrations of proline (the amino acid of which WL84811 is an analogue) cannot overcome its effect significantly; however, at high WL84811 concentrations the same concentration of proline significantly reduces the effect of WL84811. WL84811 causes the production of giant algal cells which fail to divide normally until the cells are returned to medium without WL84811. [inserted: implicated in determining pollen viability,]

Azetidine-2-carboxylic acid (A2C), the naturally-occuring isomer of WL84811, also inhibits growth in both systems but proline is unable to ameliorate its effect at any concentration.

In vivo pollination experiments showed that the gametocidal effect of WL84811 is expressed solely through the pollen, which is prevented from germinating. The development of wheat pollen is severely affected by stem-injection with WL84811 and is unable to germinate *in vitro*. No such effect is apparent with rye pollen. Addition of WL84811 to the germination medium has little effect on pollen germination of either the bi- or trinucleate pollen tested. In some cases pollen tube length is reduced by WL84811 and in other cases it is increased; some pollen may utilise the compound as a nitrogen source after germinating.

WL84811 does not appear to act in the same way as its isomer A2C, at least at higher concentrations. It may be involved in some aspect of wall formation common to the division of algae, root and coleoptile extension and pollen tube growth.

ABBREVIATIONS

s	second
min	minute
h	hour
nm	nanometre
μm	micrometre
mm	millimetre
cm	centimetre
m	metre
μg	microgram
mg	milligram
g	gram
μl	microlitre
ml	millilitre
l	litre
μmol	micromole
mmol	millimole
mol	mole
μM	micromolar
mM	millimolar
M	molar
°C	degrees Celsius
kPa	kiloPascal

kV	kiloVolt
W	Watt
UV	ultraviolet
P	probability
r	correlation coefficient
r^2	coefficient of determination
H_0	null hypothesis
K	Kruskal-Wallis test statistic
$\log_e$	natural logarithm
ND	not determined
TEM	Transmission electron micoscopy
SEM	Scanning electron microscopy
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
CMS	Cytoplasmic male sterility
NMS	Nuclear male sterility
monocot	monocotyledonous
dicot	dicotyledonous
cv.	cultivar

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1 Wheat and barley as crops

Cereals, particularly wheat and rice, are the major staple crops for most of the world, providing about half the total energy and protein intake in the west and more in many developing countries (Zwartz & Hautvast, 1979). All the cereals, along with the wild grasses, belong to the family Graminae; wheats to the genus *Triticum* and barley to the genus *Hordcum*.

The progenitors of our modern cereals, two-row barley and emmer and einkorn wheats, were important as crops some 10000 years ago. Their gradual domestication over a period of around one thousand years allowed hunter-gatherers in the Near East to settle in more or less permanent farming villages and subsequently led to rapid population growth and the development of the first civilizations (Harlan & Zohary. 1966). The diploid progenitors of wheat underwent sequential changes on domestication until cultivated forms, including the hexaploid *Triticum acstivum*, predominated by around 5800 B.C. (Helbaek, 1966). Since their early domestication, cereals have spread widely; wheat is now grown from latitudes of 67° N in Norway, Finland and Russia to 45° S in Argentina although it is restricted to elevated ground in the tropics and subtropics (Feldman, 1976). Around 14 million tons of winter wheat were grown in the UK in 1986; for the first time the area under wheat exceeded that used for barley. Despite an almost three-fold increase in the amount of wheat grown here since 1976 we still import around 0.8 million tons of high quality bread-making wheat from North America every year (Annual Report of the Plant Breeding Institute, 1986).

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1.11 Wheat

Wheat is important nutritionally mainly as a source of carbohydrates; these constitute 60 – 80 % of the grain's dry matter. However, the endosperm also contains significant amounts of protein (8 – 15 % of the dry weight), albeit with low levels of three essential amino acids; lysine, tryptophan and methionine (Feldman, 1976).

Most of the 17000 modern varieties of wheat are of the hexaploid *Triticum aestivum* var. *aestivum*, used in bread making because of the high gluten content of its endosperm. *T. turgidum* var. *durum* is the most important tetraploid wheat; its large, hard grains produce a flour low in gluten which is used for pasta products.

1.12 Barley

Modern cultivated barley, *Hordeum vulgare*, like its wild progenitors is diploid. It is now most important as animal food and as the source of malt for the brewing industry and is of only minor importance as a human food crop. Strains of barley from different sources vary widely in nutritional quality; some from Ethiopia produce a grain containing as much as 18 % protein, of which 44 % is lysine (Munck *et al.*, 1971). The short growing season of barley and its tolerance to various forms of stress allow this crop to be grown on the margins of agricultural areas where others are not well adapted (Harlan, 1976).

1.2 The flower

The flowers of both wheat and barley are borne in a spike which emerges from the upper leaf-sheath about a week before flowering. The spike consists of a jointed central axis or rachis to which spikelets are attached at the nodes; one per node in

wheat and three in barley. In the case of wheat, each spikelet is composed of up to five flowers or florets, the central one of which is usually sterile (Peterson, 1965). In barley, each spikelet contains a single floret, the outer two spikelets on each node being sterile in two-row types (Chapman & Carter, 1976).

The flower of wheat and barley is essentially the same; enclosed within a pair of glumes, the palea and lemma, it contains a whorl of three stamens and a single carpel. From the tip of the ovary arise two feathery styles which curve apart at flowering to provide a stigmatic surface for the pollen (Fig. 1.1).

Wheat and barley are both autogamous crops, that is, they usually self-pollinate before the flowers open. At flowering, the anthers split along their length and simultaneously the filaments lengthen rapidly, depositing pollen on the stigma as the anthers brush past. During this period the lodicules, two small scales at the base of the carpel, swell enormously, pushing the glumes apart and allowing the dehiscing anthers to be extruded. After pollination the lodicules collapse and the glumes close again, often leaving the anthers outside the flower (Percival, 1921). Flowering starts about two-thirds of the way down a spike and proceeds up- and downwards from this point; it may take three to five days to be completed (Peterson, 1965).

1.3 Breeding systems and breeding objectives

Although both wheat and barley normally self-pollinate, a low level of spontaneous outcrossing does occur (Leighty & Taylor, 1927). This, along with chromosome doubling to restore fertility, has led to the allopolyploid wheats of today (Peterson, 1965). In contrast to wheat, barley has remained a diploid like its progenitors.

Fig. 1.1a Components of the wheat flower (after Percival, 1921). Flower is approximately six millimetres from tip of anther to base of lodicule.

a = anther

s = style bearing stigmatic branches

l = lodicule

Fig. 1.1b Anther starting to dehisce by splitting along the length of one side.

Fig. 1.1c Further enlargement of a single stigmatic branch, with a transverse section. Each branch is 0.7 – 0.8 mm in length.

p = papillate cell

n = prominent nucleus

a.
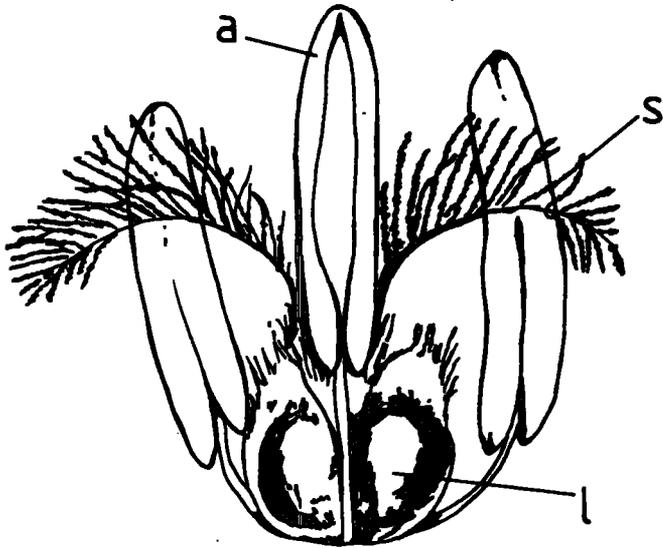
b.

c.
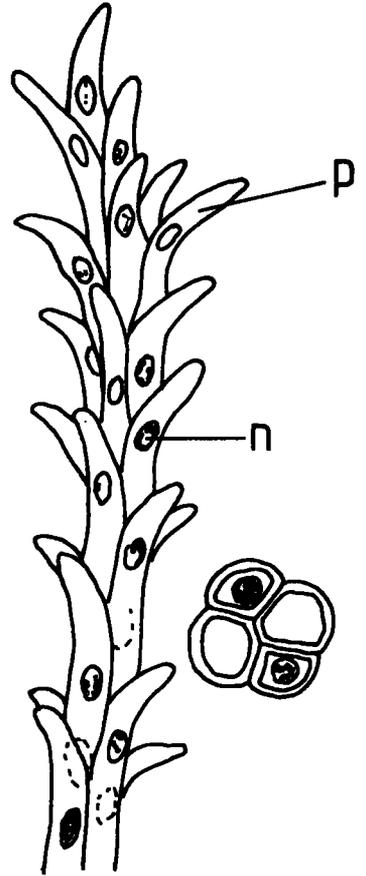
Wheat and barley are now grown as fields of pure-line cultivars and hybrids of these rather than as heterogeneous 'land races' (mixtures of inbred lines and hybrid segregates). This has led to a narrowing of the genetic base available for gene exchange (Harlan, 1976). Consequently, artificial methods must now be used in breeding to facilitate the gene exchange necessary to generate new varieties for selection.

Early selection in the development of both crops was for traits such as rapid and synchronous germination of seeds, the simultaneous ripening of grains, large seeds, a tough rachis to allow harvesting of complete ears and loose glumes to facilitate threshing (Evans *et al.*, 1975). The most consistent difference between cultivated plants and their wild progenitors according to Schwanitz (1966) is the gigantism of the cultivated forms, in particular the organs harvested by man. These have been encouraged to develop at the expense of other organs whose role was more important for survival in the wild. In the light of our modern understanding of the way in which all our crops have changed under domestication it is hard to understand Darwin writing in 1868 that "Although the principle of selection is so important, yet the little which man has effected, by incessant efforts during thousands of years, in rendering the plants more productive or the grains more nutritious than they were in the time of the old Egyptians, would seem to speak strongly against its efficacy."

More recent breeding has concentrated on improving yield by producing cultivars with more fertile florets per spikelet and longer or more dense spikes and also dwarf cultivars which respond well to artificial fertilizers without lodging (Peterson, 1965). The development of varieties with genetic resistance to various diseases and early-maturing varieties which escape adverse climatic conditions and disease late in the season has also been important in improving yields and their stability.

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In 1859, Darwin wrote "....the importance of crossing of varieties has, I believe, been greatly exaggerated both in regard to animals and to those plants which are propagated by seed." However, between 1947 and 1977, wheat yields in Britain increased annually by an average of three percent and barley by more than two percent, giving a total increase of 84 % for wheat and 63 % for barley. Of this total, 50 % in the case of wheat and 30 % in the case of barley was directly attributable to varietal improvements and the rest to technical improvements (Sylvey, 1978). This shows clearly the importance of plant breeding.

Aims for the future are slightly different and include increasing the protein content of grains and improving their amino acid composition, as well as continuing to breed for early maturation and disease resistance (Feldman, 1976).

1.4 The importance of hybrid cereals

Crop improvements depend largely on being able to carry out intraspecific hybridisations between different varieties to obtain novel combinations of genetic material, of which the best can be selected as new varieties. As wheat and barley are natural inbreeders, outcrossing must be carried out artificially. On a small scale, the prospective female parent is emasculated by removing the anthers with forceps and pollen from the desired male parent is applied to the stigmas using a brush. The seeds produced are collected, grown and the best plants selected either for repeated selfing to produce new, stable inbred lines or for back-crossing with parental types when only a few desired characteristics are to be incorporated from one parent (Peterson, 1965).

However, the ability to produce first generation (F_1) hybrids on a large scale is also important because of the phenomenon of heterosis. Outbreeding species in gen-

eral suffer from 'inbreeding depression' when they are forced to inbreed for several generations; the fixing of deliterious recessive genes in the genome leads to a decrease in yield and in tolerance to stress and diseases (Lawrence. 1967). When two such inbred lines are crossed with one another, the resultant F_1 generation shows an increase in yield and disease resistance relative to the offspring of inbreds. The increase in the mean grain yield of a hybrid relative to the mean yield of its two parents is known as 'heterosis' or 'hybrid vigour'. A similar effect can be seen when two lines of a crop such as wheat or barley which normally inbreeds are crossed with one another (Simmonds, 1979), the effect being most pronounced in the F_1 generation (Curtis & Johnston, 1969).

Increase in yield over that of the parents varies according to the varieties and growing conditions. A minimum of 10 % greater yield than the most productive inbred available is necessary to make large-scale hybrid production commercially viable because of the extra work involved (Carver & Nash. 1984). Zeven (1972) has suggested that the effect of heterosis may be smaller in wheat than other species due to the buffering effect of the hexaploid genome but Batch (1978) records increases in yield of 15 – 30 % over the parents for Hard Red Winter wheats grown in North America.

In addition to increased grain yield. F_1 hybrids often also exhibit desirable agronomic traits such as improved pasture yield (allowing the vegetative growth to be grazed for forage). increased stem strength (reducing the danger of lodging) and increased seedling vigour and tillering (allowing the seeding rate to be reduced by around 25 %; Batch. 1978). As an added bonus, hybrid seeds often have a higher protein content than seeds of inbreds.

1.5 Large scale hybrid production

As hybrid production on a large scale is necessary, a more efficient alternative to laborious emasculation and pollination by hand is required. These problems can be dealt with by producing male-sterile plants to use as the female parent. Male sterility is usually due to irregularities in microspore development which lead to the production of non-functional pollen (Lawrence, 1967). If the carpel is not fertilised successfully by its own pollen at anthesis, the stigma remains receptive for three to five days (Evans *et al.*, 1972); the floret will often reopen allowing the stigma to receive pollen from nearby flower spikes (Hoshikawa, 1961).

Male sterility for use in breeding programmes can be produced in two ways; by taking advantage of natural male sterility or by the use of chemical gametocides.

1.51 Natural male sterility

Natural male sterility is either nuclear (controlled by one or more *ms* genes), cytoplasmic (due to extrachromosomal hereditary particles) or a combination of the two (Sneep *et al.*, 1979). Both types of male sterility can be used as tools for hybrid production but each has its drawbacks.

The use of nuclear male sterility (NMS) is complicated by the fact that all useful *ms* genes must be recessive. The male-sterile line, *msms*, has to be propagated by crossing with a male-fertile maintainer. *MSms* and the 50 % of progeny which are male-fertile have have to be removed before they flower. This is laborious and, as selection against the fertile plants can only be carried out just before flowering, there is the risk that any which flower early may fertilize the male-sterile plants, reducing heterosis and causing heterogeneity in the seeds (Simmonds, 1979).

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The "balanced tertiary trisomic" system developed by Ramage (1965, 1975) allows early separation of sterile and fertile segregates but the plants are often weak with poor female fertility and low levels of pollen production. The latter further aggrevates the problem of poor pollen dispersal in normally self-pollinating plants. There is also a tendency for fertile plants to appear in the male-sterile population by transmission of the extra chromosome which causes fertility (Sneep *et al.*, 1979).

In the case of cytoplasmic male sterility (CMS), plasmagenes causing sterility are transferred from the mother to all her progeny; thus all offspring of a CMS parent crossed with a male-fertile plant will be male sterile. Producing a commercial CMS variety usually involves crossing distantly related plants (for example *Aegilops caudata* × *T. acstivum*; Kihara, 1951) then making a series of back-crosses to replace the genome of the wild CMS parent with that of the cultivated variety. It is also necessary to identify isogenic plants with fertile cytoplasm which can be used as a male parent to maintain the CMS line. Finally, to produce fertile hybrids, *Rf* (restorer) genes which overcome the effect of the plasmagenes must be incorporated into the pollinator by crossing with a restorer source then back-crossing repeatedly with the inbred pollinator (Simmonds, 1979). The production of seed using this method is complex and laborious and there is also the danger of introducing deliterious CMS-linked characteristics such as the susceptibility of maize with 'Texas' cytoplasm to the fungus *Helminthosporum maydis* which causes 'Southern Leaf Blight' (Tatum, 1971).

With both nuclear and cytoplasmic male sterility, maintaining a pure breeding line in seed production fields is difficult and, as the process of producing both types of female parent requires several generations of growth, any hybrids eventually produced are usually surpassed by inbreds bred conventionally during this time, even if

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techniques such as 'single seed descent' (*i.e.* taking one seed from each plant of the F_2 and subsequent generations to develop a reasonably homogeneous population before carrying out any selection; Simmonds, 1979) are employed.

1.52 Gametocides

An alternative way of producing a male-sterile parent is to employ a gametocide or chemical hybridising agent. This is a chemical capable of suppressing pollen formation or rendering the pollen inviable without any detrimental effect on female fertility. This method has many advantages; the male-sterile parent can be produced conveniently in bulk in one generation, there is no requirement for fertility-restoring lines and no maintenance of male-sterile stock and it also prevents reliance on a single cytoplasm (Batch, 1978).

The requirements for such a gametocidal compound are stringent (Batch, 1978; Carver & Nash, 1984):

1. it must reliably induce complete male sterility, with no reduction in female fertility or seed quality

2. it must be applicable over the range of crop growth stages found within a field at any given time and remain effective for a period of time to sterilise secondary tillers

3. it must not be affected by environmental conditions

4. it must not be affected by genotypic differences and must act on a range of parent cultivars

5. it must have no phytotoxic effect and not present any environmental hazard

6. it must be cost effective.

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Over the last 35 years a range of compounds have been tested on wheat and barley with varying degrees of success. The properties of some of these are summarised in Table 1.1. Most are applied by foliar spraying.

Many of the compounds which induce good levels of male sterility often also reduce female fertility and the level of outcrossing. For commercial purposes it is important that a gametocide does not reduce yield or the ability of flowers to open and cross-pollinate naturally; it is uneconomic to use hand pollination for large scale hybrid production.

1.6 Azetidine-3-carboxylic acid

It is hoped that the gametocide developed by Shell Research Ltd, azetidine-3-carboxylic acid (known hereafter as WL 84811), will meet all the criteria listed above. Wl 84811 induces high levels of male sterility with minimal effect on female fertility and can be used effectively over a wide range of developmental stages. It is reported to act not by preventing pollen formation but by affecting its viability, treated pollen being prevented from germinating on a receptive stigma (Jeffcoat *et al.*, 1982).

Preliminary studies at Durham University (Gates, unpubl.) suggested that treated pollen bursts on the stigma; this, however. conflicts with results from Nickerson RPB (unpubl.) who are field-testing the chemical. They observed treated pollen producing short tubes although these never penetrated the stigmatic branches.

Table 1.1

Compound	Tested on	Reference	Properties
Maleic hydrazide	T. aestivum cv. 591	Chopra et al. (1960)	Affects meiosis; maximum male-sterility (93.6 %) when applied at tillering. Crossing by hand with cv. NP790 gave 80 % hybrid seeds. Hybrids vigorous, with normal, fertile pollen. Effect dependent on environmental factors. Activity lasts around two weeks.
FW 450	T. aestivum cv. 591	Chopra et al. (1960)	Causes marked suppression of plant growth. Pollen appears normal in shape, size and staining.
Hybrex	T. aestivum	Alper (1983)	Blocks pollen formation and causes florets to gape.
RH 007	T. aestivum	Carver & Nash (1984)	Inhibits pollen formation. Only effective at some latitudes.
RH 531	H. vulgare cv. Midas, Mari	Colhoun & Steer (1983); Batch (1978)	Causes abortion of pollen if applied within 48 hours either side of meiosis. Produces abnormal, stunted ears.
	T. aestivum cv. Anza, Olaf, Yecora 70, Bonanza, Waldron	Jan et al. (1974, 1976); Miller & Lucken (1977)	Anza is most sensitive cultivar: 90 – 99 % male sterility induced when RH 531 applied before meiosis. Equal effect on female fertility. Causes low yield and level of outcrossing. Only 2 – 6 % of normal yield as hybrid seeds after open pollination.
RH 532	T. aestivum cv. Anza, Olaf, Yecora 70, Bonanza, Waldron	Jan et al. (1974, 1976); Miller & Lucken (1977)	More than 90 % male sterility induced in Anza, Bonanza and Waldron. Female fertility also reduced; yield only 1 – 21 % of normal, better if applied at booting stage. Decreases plant height.
RH 2956	T. aestivum cv. Anza, Olaf, Yecora 70, Bonanza, Waldron	Jan et al. (1974, 1976); Miller & Lucken (1977)	Induces more than 90 % male sterility in all except Waldron. Little effect on female fertility but low levels of outcrossing due to incomplete spike-emergence.
RH 4667	T. aestivum cv. Bonanza, Olaf, Waldron	Miller & Lucken (1977)	Unable to induce more than 90 % male sterility
Ethrel	H. vulgare cv. Midas, PL56, C164	Colhoun & Steer (1983); Verma & Kumar (1978); Sheep et al. (1979); Fairey & Stosskopf (1975)	Induces male sterility if applied within 48 hours of meiosis. Action varies with application time. No effect on female fertility but low levels outcrossing due poor spike emergence if applied too early. Granular ethrel applied through roots may be more reliable.
DPX 3778	H. vulgare cv. Mari	Batch (1978)	Must be applied around meiosis; no effect if applied later.
Methanoproline	T. aestivum	Kerr (1984)	Induced more than 90 % male sterility over several years field trials with about 55 % of control seed set, but very variable. Sometimes affects female fertility. Window of application time too short for practicability.

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WL 84811 is an isomer of the naturally-occuring analogue of proline, azetidine-2-carboxylic acid (A2C) (Fig. 1.2). A2C, which occurs as a soluble nitrogen compound (but is not incorporated into proteins) in members of the Liliaceae (Fowden & Steward, 1957), is incorporated into proteins in place of proline in a range of other species in which it does not occur naturally (Baum *et al.*, 1973; Fowden & Richmond, 1963; Takeuchi & Prockop, 1969; Troxler & Brown, 1974; Jacobson *et al.*, 1970). When A2C is incorporated into proteins this invariably leads to a reduction in growth rate, probably due to conformational changes imposed on proteins by the substitution of A2C for proline. Berman *et al.* (1969) estimate that A2C changes the direction of successive amide bonds by as much as 16° relative to proline, causing an α-helical structure to turn through an angle/smaller ~~than normal~~.
16°

It seems that A2C, at least in low concentrations, is not toxic to species in which it occurs naturally because of a highly specific prolyl-tRNA-synthetase enzyme which prevents the analogue being activated for protein synthesis; the equivalent enzyme in other species has a lower specificity (Peterson & Fowden, 1965; Papas & Mehler, 1970). In some cases, adding exogenous proline has been shown to ameliorate the effects of A2C, presumably by outcompeting the A2C for uptake or in binding to the prolyl-tRNA-synthetase (Vanlerberghe & Brown, 1986).

In order to use a gametocide efficiently it is important to understand how it has its effect; this would make clear its limitations and might suggest ways in which it could be used more effectively. Whether WL84811 acts in a similar manner to its isomer or in some totally different way must be determined.

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Fig. 1.2 The chemical structure of proline and its two analogues, azetidine-2-carboxylic acid (A2C) and azetidine-3-carboxylic acid (WL 84811).

Molecular weight of proline = 106.1

Molecular weight of A2C/WL84811 = 94.1
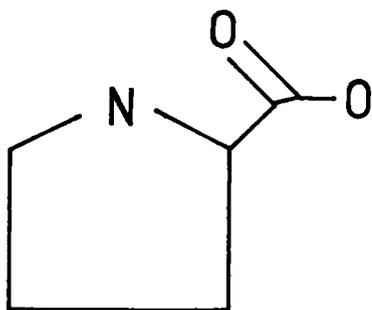
L-PROLINE

A 2 C

WL 84811

35
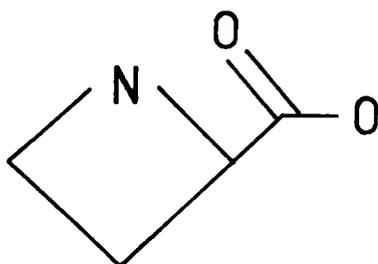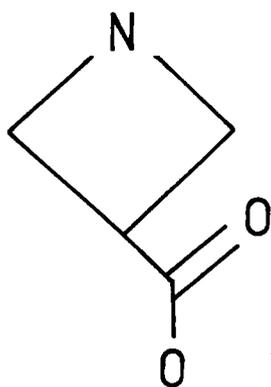
1.7 How can the mode of action of WL84811 be determined?

1.71 The effect of WL84811 on pollen

The obvious way to study a gametocide is to investigate its effects on pollen. Any direct effects on pollen development can be seen readily using light or electron microscopy (Colhoun & Steer, 1983). The behaviour of pollen on a stigma can also be easily observed and any growing pollen tubes followed down the stigma branches (Martin, 1959). However, to look in any more detail and to screen a large number of pollen grains it is necessary to germinate the pollen *in vitro*.

Many binucleate pollen types will germinate readily on filter paper soaked in a liquid medium (Brewbaker & Kwack, 1963) but the trinucleate pollen of the Graminae presents more of a problem (Mulcahy & Mulcahy, 1983). There are few reports in the literature of successful germination of *Hordeum* and *Triticum* pollen *in vitro* (Bar-Shalom & Mattsson, 1977; Loo & Hwang, 1944; Mathur, 1977). This has been attributed to the difficulty of controlling pollen hydration *in vitro* to mimic its slow, regulated hydration on a 'dry' stigma (*sensu* Heslop-Harrison *et al.*, 1975; Bar-Shalom & Mattsson, 1977).

To compound these problems with germinating pollen *in vitro*, there is the additional problem that wheat and barley grown outdoors will only flower during a short period each summer. For any attempt to germinate pollen it is essential to have healthy pollen; unfavourable light or temperature conditions lead rapidly to the production of sterile pollen (Knox & Heslop-Harrison, 1966). Storing the pollen is not practicable; wheat pollen is unable to germinate *in vivo* after being stored for only 24 hours in liquid air although the pollen of some other cereals such as rye can

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be stored for more than seven days (Collins *et al.*, 1973).

1.72 Other test systems

It is clearly difficult to maintain a supply of healthy cereal pollen throughout the year, even when a glasshouse and supplementary lighting are available. In view of these difficulties, it was thought desirable to develop other plant systems on which the effects of WL84811 could be studied.

The main questions to be answered in this way were;

1. Does WL84811 affect the behaviour of tissues other than pollen?

2. Is the effect of WL84811 limited to particular groups of plants *eg.* monocotyledonous species. the Graminae?

3. Does WL84811 affect cell expansion, cell division or both *i.e.* is it likely to affect pollen by preventing nuclear division during pollen development, by inhibiting cell expansion during pollen tube growth or by some combination of the two?

4. Can the effects of WL84811, like those of A2C. be overcome by proline?

In the light of these questions. two systems were chosen; one which allowed the effects of WL84811 to be studied over a wide range of species and one which allowed its effects on individual cells to be seen clearly.

1.721 The effect of WL84811 on seed germination and seedling growth

The seed germination system was chosen largely for its convenience. Most seeds. and certainly those of all the common cereals. will germinate within a few days at room temperature when supplied simply with water. Large numbers of seeds can be used conveniently to study the effect of compounds on percentage germination

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and the seedlings produced can be measured to determine the effects on cell growth and division. Because of their commercial importance, the germination of seeds and factors affecting seedling vigour have been much studied (see Bewley & Black, 1978); this background literature further enhances the appeal of seeds as a test system.

When seeds are first imbibed in water they take it up rapidly; there is then a lag before a second period of rapid uptake leading to emergence of the radicle which is the first visible sign of germination. During the lag phase, many metabolic processes ~~are~~ occur enhanced the initiation of ~~initiated~~ including respiration and ribonucleic acid (RNA) and protein synthesis. The latter is initially directed towards the synthesis of enzymes to mobilise food reserves (Villiers. 1975). These food reserves are stored either in the endosperm where this persists or in the cotyledons of seeds whose endosperm degenerates. Cereals fall into the first category although they are an unusual case; the endosperm cells, although persistent. are dead. The cotyledon in cereals is a highly modified structure known as the scutellum which acts as a transfer organ between the endosperm and embryo during germination (Bryant, 1985). The processes leading to mobilization of food reserves in barley are well understood: the embryo is believed to release gibberellins which cross the scutellum and act on the aleurone layer surrounding the endosperm causing it to produce hydrolytic enzymes which liquefy the endosperm (Cohen & Paleg. 1967; Paleg *et al.*. 1962: Varner, 1964).

Although the dispersal units of cereals will be referred to as seeds throughout this thesis. they should more properly be called grains or caryopses. Each 'seed' is. in fact, a single-seeded fruit whose rudimentary testa has fused with the ovary wall or pericarp (Bewley & Black, 1978).

A simple test system, imbibing seeds in tap water then germinating them on damp

filter paper, was used to study the effect of WL84811 on seeds of a diverse range of plants. One cultivar of barley was also chosen for a series of more detailed experiments where the ability of proline and IAA to overcome the effect of WL84811 was studied. This system was also used as a bioassay to look at the effects of temperature and storage on WL84811 activity.

1.722 The effect of WL84811 on algal growth

Unicellular algae, in particular *Chlorella*, were used to study the effects of WL84811 on growth and division of individual cells. As algal cultures are easily rendered sterile, these effects can be studied without fear of interference from bacteria. Because they are small in size (three to ten micrometers in diameter) and spherical, large numbers of *Chlorella* cells can be counted quickly and their size measured easily. The cells also grow rapidly. dividing once every two or three days by the formation of autospores which are released from the mother cell when the outer wall splits. The number of autospores produced varies both with species and environmental conditions: in actively growing cultures the two-cell stage of division is most conspicuous. whereas in older cultures daughter cells remain together for longer and eight cells may often be seen held together by a parental wall (Fogg. 1953).

Chlorella has the added advantage of being one of the most widely studied algae. having been used by Warburg (1919) for the earliest experiments on photosynthesis. An extensive literature covers its physiology and ultrastructure (*eg.* Dempsey *et al.*, 1980; Gergis. 1971: Silverberg, 1976: Thinh & Griffiths. 1972; Treharne *et al.*. 1964). The effect of various herbicides, for example atrazine (Ashton *et al.*. 1966), 2,4-D (Bertagnolli & Nadakavukaren. 1970) and diquat (Stokes *et al.*. 1970) on *Chlorella*

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has also already been tested.

Detailed studies were carried out to determine the effect of WL84811 on cell growth and division and material was embedded and stained for electron microscopy to study any ultrastructural effects.

1.8 Summary of aims

The aim of this project was to determine the mechanism by which WL84811 prevents self-pollination in wheat and barley in the hope of being able to improve its efficiency. Because of the short period for which grass pollen remains viable once shed from the anthers, other systems were developed in which the effects of WL84811 (and other gametocides) on cell growth and division could be studied. These results were then related to observations on the behaviour of pollen. The specific aims were as follows:

1. to determine whether the gametocidal effect of WL84811 is the result solely of its effect on pollen or whether it also affects the female parts;

2. to determine how late WL84811 may be applied to developing pollen to have its full effect, in order to make deductions about the pathway on which it may act;

3. to determine whether WL84811 has the same mode of action as its natural isomer, azetidine-2-carboxylic acid.

2. MATERIALS AND METHODS

2.1 Biological materials

Seeds of a number of cultivars of *Triticum aestivum* var. *aestivum, Hordeum vulgare* and *Secale cereale* were supplied by Nickerson RPB Ltd. These are listed, along with some of their characteristics as described by Nickersons, in Table 2.1.

Table 2.1 Cereal cultivars used for experiments with WL84811.

Species	Cultivar	Characteristics
H. vulgare	Sonja	2 row, pollen sensitive to WL84811 *in vivo*
	Protidor	2 row, pollen sensitive to WL84811 *in vivo*
	Natalie	2 row, pollen insensitive to WL84811 *in vivo*
T. aestivum	Banner	Good pollinator, pollen sensitive to WL84811 *in vivo*
	Hammer	Poor pollinator, pollen sensitive to WL84811 *in vivo*
	CWW 3547/1	Receptive female, pollen sensitive to WL84811 *in vivo*
Secale cereale	Dominant	Rye, pollen sensitive to WL84811 *in vivo*

Field-grown *T. aestivum* cv. Mercia was supplied by Durham College of Agriculture and Horticulture, Houghall.

Other seeds used for germination experiments are listed in Table 2.2.

Table 2.2 Seeds used for germination experiments.

Species	Variety	Source
Pennisetum typhoides (millet)	unknown	Dr P.J. Gates
Oryza sativa (rice)	IR 36	IRRI (International Rice Research Institute)
Beta vulgaris ssp. *vulgaris* (beetroot)	Globe	Suttons Seeds Ltd, Torquay
Lactuca sativa (lettuce)	Little Gem	Suttons Seeds Ltd, Torquay
Allium cepa (onion)	Ailsa Craig	Suttons Seeds Ltd, Torquay
Pisum sativum (pea)	Meteor	Suttons Seeds Ltd, Torquay
Daucas carota (carrot)	Early Nantes	R. & G. Cuthbert, Upper Dee Mills, Llangollen, Clwyd

Pteridium aquilinum (bracken) spores were collected by Dr P.J. Gates. Pollen of *Tropaeolum majus* and *Vicia faba* (cv. Sudanese Triple White) was obtained from plants growing in the glasshouses of Durham University Botanic Gardens.

The green algae *Chlorella zofingiensis* Donz. (CCAP 211/14; Durham 548) and *Closterium moniliferum* Ehr. (CCAP 611/1) were supplied from the Durham University culture collection from cultures originally held by the Cambridge Collection of Algae and Protozoa (now Culture Collection of Algae and Protozoa, The Freshwater Biological Association, Ambleside). These algae were chosen as representatives from each of the two groups of eukaryotes described by Pickett-Heaps and Marchant (1972), based on differences in orientation of microtubules during cell division. In cells of the "phycoplast" type such as *Chlorella*, microtubules are oriented in the plane of cell division during cytokinesis. However in cells of the more common "phragmoplast" type, which include *Closterium* along with bryophytes and higher plants, the microtubules are oriented perpendicular to the eventual plane of cytoplasmic division.

2.2 Chemicals

Supplied by Shell Research Ltd, Sittingbourne Research Centre, Kent:

L-Azetidine-3-carboxylic acid (WL84811)

L-Azetidine-[carboxyl-C^{14}]-3-carboxylic acid (^{14}C-WL84811)

Supplied by Sigma Chemical Co., St Louis, Mo., USA:

Agarose Type 1: Low EEO

Aniline blue

L-Azetidine-2-carboxylic acid (A2C)

Bisbenzimidazole

Chlorpromazine

Citric acid

Cyanocobalamin (Vitamin B_{12})

Herring sperm deoxyribonucleic acid (DNA)

Ethylenediaminetetraacetic acid (EDTA)

Fluorescein diacetate

N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)

Indole-3-acetic acid (IAA)

Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)

Ninhydrin

Piperazine-N,N'-bis[2-ethanesulfonic acid]; 1,4-Piperazinediethanesulfonic acid (PIPES)

Potassium phosphate (K_3PO_4)

L-Proline

Sodium phosphate (Na_2HPO_4)

5-Sulfosalicylic acid

Thiamine (Vitamin B_1)

Supplied by British Drug Houses Ltd, Poole, Dorset:

Acetic acid (glacial)

Acetone

Acridine orange

'Amberlite' resin IR-120 (H)

Boric acid (H_3BO_3)

Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$)

Ethanol

Iodine, resublimed

Methanol

Potassium dihydrogen orthophosphate (KH_2PO_4)

Potassium iodide (KI)

Potassiums nitrate (KNO_3)

Sodium hydroxide (NaOH)

Sucrose

Toluidine blue

Supplied by Hopkin and Williams, Chadwell Heath, Essex:

Orthophosphoric acid

Supplied by Boehringer Mannheim GmgH, W. Germany:

Tris (hydroxmethyl)-aminoethan

Supplied by Riedel-deHaën, AG.D-3016 Seelze 1:

Sodium chloride (NaCl)

Supplied by Packard Instrument Co. Ltd. 2200 Warrenville Rd. Downers Grove, Ill., USA:

Packard Carbo-sorb

Packard Permafluor V

Packard Spec-Chec ^{14}C

Supplied by Fisons plc. Equipment Division. Loughborough. LE11 0RG:

Optiphase 'Safe'

Supplied by TAAB Laboratories Equipment Ltd. Reading, Berks.:

Glutaraldehyde

Paraformaldehyde

Spurr resin

Supplied by Emmscope Laboratories Ltd. Kingsworth Industrial Estate, Ashford, kent:

Osmium tetroxide (OsO_4)

Supplied by the London Resin Co. Ltd. Woking, Surrey:

LR White resin

Supplied by Koch-Light Laboratories Ltd, Colnbrook:
Auramine O

Supplied by Gurr Products:
Carmine powder

2.3 Growth conditions

2.31 Cereals

Cereal seeds were surface-sterilised in two percent hypochlorite for 10 min, washed
and allowed to imbibe overnight in tap water. They were transferred onto damp
filter paper in a Petri dish, incubated in the dark at 25 °C for three days or until
the coleoptile appeared and then planted, three seeds per 15 cm pot, in Levington
compost. Plants were kept in a growth cabinet at 20 °C for three weeks before
being transferred to a glasshouse with an ambient temperature fluctuating between
13 and 26 °C. They were watered daily with tap water and, when the daylength was
less than 16 h, given supplementary light (high pressure 400 W sodium lamps type
SON 'T suspended one metre above the pots). Under these conditions Sonja, the
fastest growing barley cultivar, started flowering after 15 weeks.

In order to determine percentage seed set, sample ears were enclosed in bags made
of porous cellophane. These prevented cross-pollination whilst permitting photosyn-
thesis and transpiration, although high humidity within the bags encouraged the
growth of fungi such as *Erisiphc graminis* (powdery mildew).

Cereals were treated with a standard (to Shell) dose of 0.1 ml of 3000 mg l^{-1} WL84811 (0.03M)
This was injected into each tiller at the base of the internode directly below the ear,
just before the ear emerged.

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2.32 Algae

The algal growth medium used was a modification of recipe number 10 of Chu (1942), as described by Wehr *et al.* (1987) but with Silicon omitted. Hereafter, the medium will be referred to as Chu 10. Silicon is essential only for the growth of diatoms and chrysophytes and tends to precipitate out, possibly removing other nutrients with it. The medium is very 'soft' *i.e.* low in calcium and magnesium ions, but has a relatively high phosphate concentration. The nominal elemental composition of the medium before pH adjustment is given in Table 2.3.

The medium was buffered with HEPES (pKa=7.5) and adjusted to pH 7.5 using molar sodium hydroxide after adding any test compounds. Iron was chelated with EDTA to prevent its precipitation from solution. For the growth of *Closterium*, vitamins B_1 (200 μg l^{-1}) and B_{12} (20 μg l^{-1}) were also included.

25 ml of medium was decanted into 100 ml conical flasks and autoclaved for 15 minutes at 121 °C (10.33 kPa). Each flask of sterile medium was inoculated with 1.0 ml of a uni-algal, bacteria-free culture of *Chlorella* or *Closterium* under aseptic conditions. The flasks were incubated at 25 °C under continuous light (100 μmol photons m^{-2} s^{-1}) and 0.5 ml aliquots removed at intervals and fixed immediately with 0.05 ml of Lugols iodine (two grams potassium iodide plus one gram iodine crystals dissolved in 300 ml of water). The number and size of cells in each sample was measured using a 0.1 mm haemacytometer for *Chlorella* and a 0.2 mm haemacytometer for the larger *Closterium*. For *Chlorella* the parameter measured for size was cell diameter; as *Chlorella* cells are spherical, cell volume was readily calculated from this. For *Closterium*, the distance in a straight line between the two ends of the

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banana-shaped cells was measured. The effect of number of volumes counted on the mean count and its standard error was determined (Fig. 2.1) and a suitable sample size chosen accordingly. For *Chlorella*. the number of cells in 50 volumes of 0.05 × 0.05 × 0.1 mm^3 was counted for each replicate sample and for *Closterium*, the number of cells in 18 volumes of 1.0 × 1.0 × 0.2 mm^3.

Table 2.3 Composition and physico-chemical parameters of algal growth medium (Chu 10E). Conductivity and alkalinity measurements from Kelly (1986).

Element	Concentration (mg l^{-1})	(mM)
N	6.84	0.49
Mg	2.47	0.10
P	0.89	0.03
K	1.12	0.03
Ca	9.78	0.24
Fe	0.50	0.009
Cu	0.005	0.0001
Mo	0.003	0.00003
Na	2.18	0.097
S	3.27	0.10
Cl	1.13	0.03
Mn	0.012	0.0002
Co	0.002	0.0003
Zn	0.013	0.0002
B	0.125	0.01
HEPES		2.5
EDTA		3.15

Conductivity   144 μS cm^{-1}

Total alkalinity   2.5 meq l^{-1}

Fig. 2.1 The effect of total number of volumes counted on the mean number of *Chlorella* cells in each volume, and its standard error. On the basis of this, 50 volumes were counted from each replicate sample to ensure reliability.
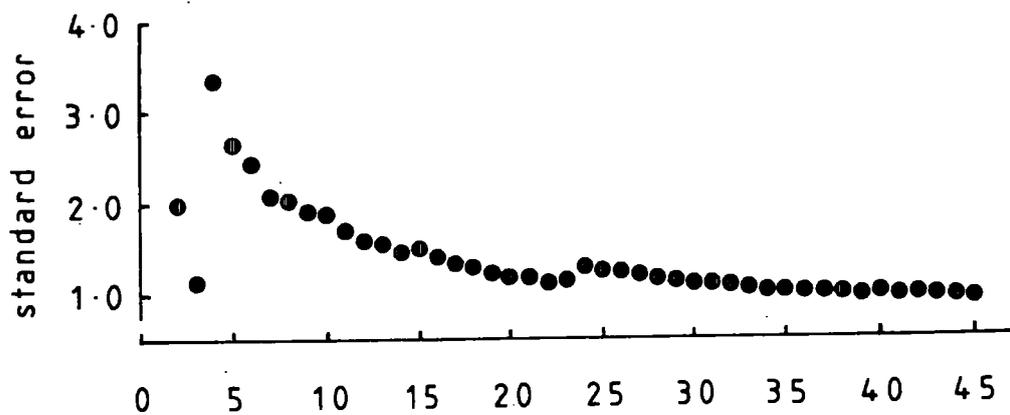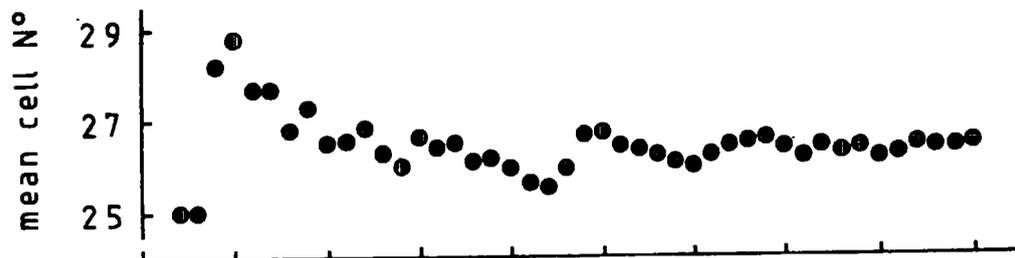
number of $2.5 \times 10^{-4} mm^3$ volumes counted

2.4 Microscopy

2.41 Light Microscopy

A Nikon Diaphot-TMD Inverted Microscope was used for light microscopy, with a high-pressure mercury lamp HBO 100W/2 and an epi-fluorescence attachment TMD-EF for fluorescence microscopy. Fresh material was studied after staining with fluorochromes or acetocarmine and material was also fixed and embedded for bright field microscopy in LR White resin.

2.411 Fluorescence microscopy

Various fluorochromes, together with filters of the appropriate wavelength, were used to stain different cellular components specifically.

Chlorpromazine (at a concentration of 0.001 M) was used with the UV excitation filter (365 nm) to observe pollen tubes growing *in vitro*. Chlorpromazine complexes irreversibly with the calcium-binding protein calmodulin and is photo-oxidised to a fluorescent derivative by UV light (Hausser *et al.*, 1984).

Decolourised aniline blue (a one percent solution of aniline blue in K_3PO_4) was used to show cereal pollen tubes growing *in vivo* (Kho & Baer. 1968). Fixation and maceration of the tissue as suggested by Kho and Baer (1968) was found to be unnecessary for this material. Aniline blue stains the callose laid down by pollen tubes as they grow (Mulcahy & Mulcahy, 1983), allowing them to be clearly differentiated from stigmatic tissue.

Auramine O and acridine orange were used to study stigmatic structure. Cell endomembranes, including those of the nucleus and golgi apparatus, fluoresce a bright

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yellow-green with the B excitation filter (480 nm) when stained with 0.01 % auramine (Harris & Gates, 1984). Acridine orange stains nucleic acid material. It exhibits the property of metachromasia *i.e.* can fluoresce with more than one colour. When bound to DNA it fluoresces bright green with the B filter as the ordered secondary structure of DNA prevents dye molecules from interacting with one another. However, when bound to randomly coiled RNA, denatured DNA or acid polysaccharides, it fluoresces red (Bertalanaffy, 1963).

The viability of pollen was assessed using the fluorescein di-acetate (FDA) method of Heslop-Harrison and Heslop-Harrison (1970). This depends on a non-polar, fatty acid ester of fluorescein (fluorescein di-acetate) readily entering living cells through the lipoprotein plasmalemma. It is then cleaved by intracellular esterases to acetate and polar fluorescein; the latter cannot leave cells with an intact plasmalemma and so is accumulated. This accumulation is observed with the B filter as bright fluorescence which develops as the FDA is broken down. This method can be used to indicate cell viability, if this is assumed to depend on membrane integrity. Shivanna and Heslop-Harrison (1981) found good correlation between fluorochromasia and ability of *Secale* pollen to germinate, although the latter was always slightly lower.

FDA for use was made up freshly each time by adding stock solution (two milligrams of FDA per millilitre of acetone) dropwise to solutions of 30 – 40 % sucrose until these became persistently milky (Shivanna & Heslop-Harrison, 1981). Sucrose was included to reduce the number of pollen grains bursting due to osmotic effects during the test.

2.412 Staining with acetocarmine

Acetocarmine was prepared by dissolving four percent acetocarmine in 45 % acetic acid, refluxing the mixture for 24 hours and filtering it through Whatman N° 1 paper. Acetocarmine stains nuclear material in particular and so was used to study developing pollen. However, it was also found to be useful for the study of pollen tubes growing down stigmatic branches *in vivo*.

2.413 Embedding for light microscopy

Excised stigmas and algal cells were fixed for two hours in 2.5 % glutaraldehyde in citric acid-Na_2HPO_4 buffer at pH 7.2. After washing twice in buffer, they were dehydrated in ethanol (two 30 min changes of 12.5, 25, 38, 50, 70 and 100 % ethanol, followed by four 30 min changes of 'dry' ethanol). The material was gently agitated overnight in 1:1 dry ethanol and LR White resin, then given three further changes of resin at 12 hour intervals before being embedded in fresh resin and polymerised at 70 °C. Semi-thin sections (1 – 5 μm) were cut and stained with toluidine blue.

2.414 Photography

All photographs were taken using a Nikon FE camera body mounted on the Nikon microscope. For black and white photography, 400 ASA XP1 or 100 ASA FP4 film was used and for colour photography, 100 ASA Fujichrome and Fujicolor film. All films were processed according to the manufacturers instructions.

2.42 Electron microscopy

2.421 Transmission electron microscopy

For the study of pollen, a modification of the method of Colhoun and Steer (1983)

was used. Anthers with the tip excised were fixed for five hours in five percent glutaraldehyde in 0.025 M phosphate buffer (pH 6.8), washed twice in buffer then treated with two percent OsO_4 for two hours. After washing again, the anthers were dehydrated in ethanol as for light microscopy and embedded in Spurr's low viscosity resin (Spurr, 1969).

Algal cultures were first concentrated by centrifugation at 5000 r.p.m. for 10 min. The pellet was then injected into hemispherical blobs (four millimetres in diameter) of one percent agar just on the point of setting. When these had solidified, $2 \times 2 \times 2$ mm^3 blocks containing algae were cut and fixed immediately in 2.5 % glutaraldehyde plus 1.5 % paraformaldehyde in 0.05 M PIPES buffer at pH 7.0. After two hours the blocks were washed, stained and embedded as described above.

Thin sections (90 - 150 nm) were cut using a glass knife on a Sorvall MTB-2 Ultra Microtome. These were stained with uranyl acetate and lead citrate before viewing using a Phillips EM 400 Transmission Electron Microscope.

2.422 Scanning electron microscopy

Fresh material was placed on a grid, frozen rapidly in liquid nitrogen and coated under vacuum with gold. A Phillips 501B Scanning Electron Microscope was used to view the samples.

2.43 X-ray microscopy

Fresh, intact wheat spikelets laid on X-ray film were subjected to low-energy X-rays (5.5 kV). The film was developed, examined under the light microscope and photographs taken of the magnified images.

2.5 Pollination experiments

2.51 *In vivo* pollination

In order to observe the behaviour of WL84811-treated and untreated pollen on the stigma, florets were either allowed to self-pollinate naturally with the spike bagged to prevent cross-contamination or pollinated by hand with the required pollen. The latter technique was employed to determine whether the gametocidal effect of WL84811 was due to it changing some property of the pollen, the stigma or a combination of the two. Pollen from untreated plants was used to pollinate emasculated florets of WL84811-treated plants and treated pollen to pollinate emasculated florets of untreated plants. This was carried out by shaking pollen from anthers on the point of dehiscing onto a glass slide and applying it to the stigmas with fine brush. As graminaceous pollen grains dehydrate and shrivel up within minutes with concommitant loss of viability when exposed to a relatively dry atmosphere (Shivanna & Heslop-Harrison, 1981), this was done as rapidly as possible.

After pollination by either method the whole pistil was immersed in decolourised aniline blue for one hour or stained with acetocarmine for a few minutes then the stigma lobes were cut away from the ovary and mounted in glycerol or water for observation.

2.52 Pollen germination *in vitro*

Many media and various methods were tested in an attempt to germinate the pollen of *T. aestivum*, *H.vulgare* and other cereals *in vitro* (see Appendix 1). Of these, the only ones to prove successful were the semi-solid medium used by Heslop-

Harrison (1979a) to germinate *Secale* pollen. and an adaptation of Mathur's medium (1977) containing one percent. agarose.

Heslop-Harrison's medium (1979a) proved the best for germination of barley pollen *in vitro*. Percentage germination on this medium was variable but some pollen tubes over 1.2 mm long were recorded. The composition of this medium is given in Table 2.4.

Table 2.4 Compostition of germination medium for barley pollen.

Constituent	Concentration (g l^{-1})
Agarose	10.0
Sucrose	205.2
H$_3$BO$_3$	0.062
Ca(NO$_3$)$_2$.4H$_2$O	0.590

Wheat pollen, however, produced only short pollen tubes on this medium (generally less than 150 μm long). The adaptation of Mathur's medium (1977) proved more successful; although percentage germination was relatively low, pollen tubes up to 230 μm long were obtained. The composition of this medium is given in Table 2.5.

Table 2.5 Composition of germination medium for wheat pollen.

Constituent	Concentration (g l^{-1})
Agarose	10.0
Sucrose	250.0
H$_3$BO$_3$	0.10
Ca(NO$_3$)$_2$.4H$_2$O	0.30
KNO$_3$	0.10
MgSO$_4$.7H$_2$O	0.20

Mathur's basic medium is derived from the standard germination medium of Brew-

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baker and Kwack (1963), the only difference being that Brewbaker and Kwack use a lower sucrose concentration (100 g l^{-1}). Other, binucleate pollen germinated successfully on Brewbaker and Kwack's (1963) medium unadapted.

In all cases, pollen was used fron anthers on the point of dehiscing and was scattered in clumps on the medium because of the enhancement of growth known as the "population effect" which this produces (Savelli & Caruso, 1940). This effect is believed to be due to the rapid diffusion of calcium ions out of pollen on a germination medium; a high enough equilibrium concentration to allow pollen tube growth may only be created where there is a high density of pollen (Brewbaker & Kwack, 1963).

Pollen tubes growing *in vitro* are difficult to measure directly under the microscope as they rarely grow straight. To overcome this problem, a number of fields of view were photographed, the negatives enlarged and pollen tube lengths measured using a piece of fine string.

2.53 Determination of free proline content of pollen

The method of Bates *et al.* (1973) was modified for use in determining the free proline content of both untreated and WL84811-treated wheat pollen. The method depends on the synthesis of a red product when proline reacts with ninhydrin at pH 1.0.

Pollen from one ear of wheat (approximately seven milligrams dry weight) was collected from dehiscing anthers, weighed and allowed to burst in two millilitres of three percent aqueous sulphosalicylic acid to precipitate out proteins. After 20 minutes the suspension was centrifuged for five minutes at 5000 r.p.m. and the supernatant decanted and shaken with 0.15 g of 'Amberlite' resin. This removes basic amino acids

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such as lysine and ornithine which might otherwise interfere with the reaction. The mixture was heated for one hour at 100 °C with two millilitres of glacial acetic acid and two millilitres of acid ninhydrin reagent (1.25 g ninhydrin dissolved in 30 ml of glacial acetic acid plus 20 ml of six molar orthophosphoric acid). Four millilitres of this was extracted with four millilitres of toluene, the toluene phase removed and its absorbance at 520 nm (A^{520}) read on an LKB Ultrospec 4050 Spectrophotometer.

Standards were prepared by adding one millilitre of 0, 10^{-5}, 10^{-4}, 5 x 10^{-4} and 10^{-3} M proline to one millilitre of three percent sulphosalicylic acid. These were then treated in the same way as the samples and a calibration curve produced relating A^{520} to free proline concentration in the final solution (Fig. 2.2). From this, the amount of proline in the original samples was calculated.

A modification of this method was also used to check the stability of proline in solution over a period of seven days. Proline was made up as 2.5 x 10^{-3}, 2.5 x 10^{-2} and 0.25 M solutions which were stored at 4 °C. Each was diluted 250 fold before use. Three millilitres of solution were reacted with three millilitres of glacial acetic acid and three millilitres of acid ninhydrin at 80 °C for one hour. Each sample was extracted with nine millilitres of toluene and the A^{520} of the toluene phase read.

This showed that there was no significant change in the free proline content of solutions over a period of seven days (Fig. 2.3).

Fig. 2.2 Standard curve relating free proline concentration in the final solution to absorbance at 520 nm when using the colorimetric method of Bates *et al.* (1973). Each value is mean of two readings.

Fig. 2.3 Change in free proline concentration of solutions over seven day period as determined by absorbance at 520 nm.

● $= 10^{-3}$ M proline

○ $= 10^{-4}$ M proline

■ $= 10^{-5}$ M proline
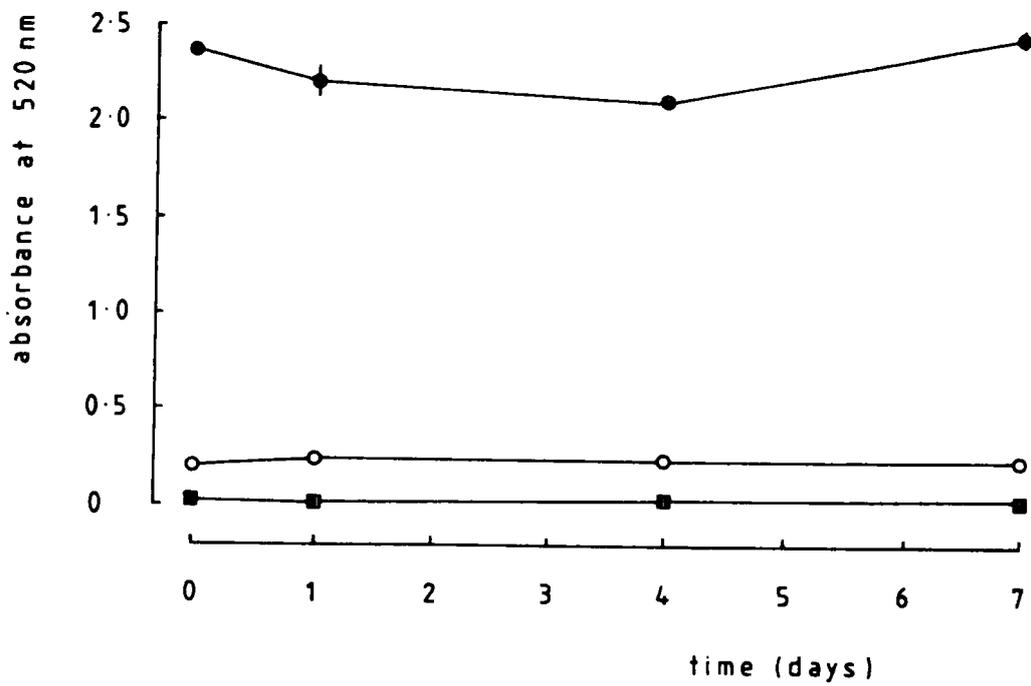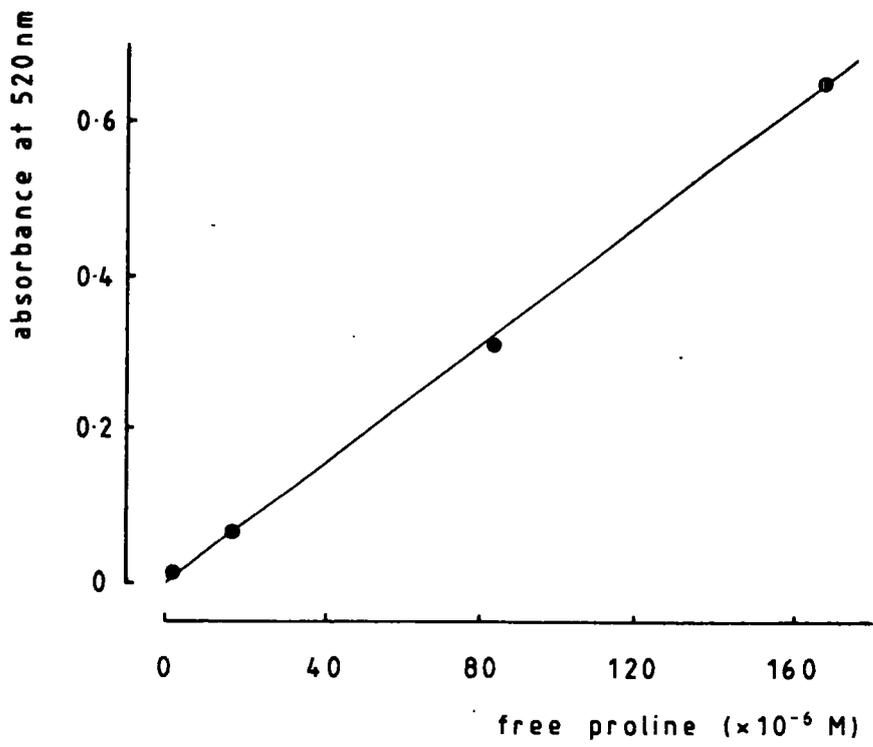
2.6 Localisation of injected ^{14}C-WL84811

Batch 571 ^{14}C-WL84811, with a specific activity of 12.5 μCi mg^{-1} was made up as 30 and 3 mg l^{-1} solutions. 0.1 ml of ^{14}C-WL84811 was injected into the internode below the ear when this was still enclosed within two leaves, the ear was bagged and the plant returned to the glasshouse for two weeks.

After two weeks the treated ears were harvested and each separated into 'pistil' and 'anther' fractions. Each fraction was wrapped in paper, compressed into a pellet and combusted in a Packard Tricarb Sample Oxidiser B306. The $^{14}CO_2$ produced from each sample was taken up by seven millilitres of 'Carbo-sorb'. To calculate the percentage recovery from this operation, standards consisting of paper with five microlitres of 'Spec-Chec' of known specific activity ($8.4 \times 10^5 \pm 3$ % decays per minute per millilitre were also combusted. 12 ml of 'Permafluor V' scintillation cocktail were added to each sample before counting the number of ~~decays~~ disintegrations per minute (DPM) on a LKB Wallace 1215 Rackbeta Liquid Scintillation Counter. The specific activity of the solutions used for injection was checked by adding different volumes to 15 ml of 'Optiphase' scintillant for counting.

2.7 Seed and spore germination experiments

2.71 Seed germination and seedling growth

The effect of WL84811 on both seed germination and seedling growth of a wide range of species. including both monocotyledonous and dicotyledonous types, was tested. 80 seeds per treatment were surface-sterilised with two percent hypochlorite, washed and imbibed for at least eight hours in the test solution. WL84811 concen-

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trations ranging from 50 to 6000 mg l^{-1} were tested. The seeds were then plated out on filter paper moistened with four millilitres of solution, in four replicate dishes each containing 20 seeds. A further one millilitre of solution, stored at 4 °C but allowed to equilibriate to room temperature before use, was added each day for four days and on the fifth day the seedlings were harvested. Percentage germination for each replicate dish and length of the shoot and the longest root of each germinated seedling were measured.

The standard procedure used in toxicity tests where seeds are imbibed, placed on filter paper soaked in say five millilitres of solution then left for five days before being measured was not used. At the high temperatures in this laboratory (up to 30 °C), the liquid evaporated rapidly and the seedlings dried up if Petri dishes were left unsealed. If the dishes were sealed, the seeds went mouldy and growth was again poor (see Appendix 3).

This system was used to determine the effect of repeated stem-injection with 3000 mg l^{-1} WL84811 on the germination of seeds subsequently produced. Spikes of field-grown *T. aestivum* cv. Mercia were marked at pollination and injected with 0.1 ml of WL84811 at 2, 4, 7, 10, 14 and 21 days after pollination. The ears were allowed to ripen for 12 weeks before being harvested then allowed to dry out for a further three weeks before using in germination experiments as described above. The treatments being tested were:

1. Control (no WL84811)

2. WL84811 injected 2 days after pollination

3. WL84811 injected at 2 and 4 days

4. WL84811 injected at 2, 4 and 7 days

5. WL84811 injected at 2, 4, 7 and 10 days

6. WL84811 injected at 2, 4, 7, 10 and 14 days

7. WL84811 injected at 2, 4, 7, 10, 14 and 21 days.

Seeds of one barley cultivar, Sonja, which showed particularly consistent results with WL84811 were used to test the stability of WL84811 during storage and when subjected to high temperatures (see Appendix 2). Sonja was also used to check the correlation between length of the longest root and total root dry weight at a range of WL84811 concentrations to ensure that the former was a reliable indicator of the latter; cereals generally have five seminal roots which emerge from the germinating seed within the first few days (Peterson, 1965), although in the presence of high concentrations of WL84811 only one short root may emerge. Seeds were grown as usual for five days and length of the longest root and coleoptile measured before excising all the roots and the coleoptile as separate fractions. All roots or coleoptiles (plus leaves) from a replicate dish were combined and dried at 100 °C for 24 hours before weighing. The total root or coleoptile dry weight of each replicate dish was plotted against total root or coleoptile length for that replicate (Fig. 2.4) and a close relationship found between them (coefficient of determination, $r^2 = 0.993$ for coleoptiles, 0.956 for roots; $P < 0.001$ for both). Root and coleoptile length may be measured far more rapidly than dry weight and so were measured in preference in all subsequent experiments.

Sonja was also used to determine the effect of WL84811 on rate of seed germination and seedling growth. Seeds were imbibed and plated out on 0, 300 and 3000 mg l^{-1} WL84811 as described above and growth measured at daily intervals for five days. Median values of root and coleoptile length were calculated at each sampling time and growth in each treatment expressed as a percentage of the control.

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Fig. 2.4a Relationship between total coleoptile dry weight and total coleoptile length for each replicate set of seedlings.

● = water only

○ = 150 mg l^{-1} WL84811

■ = 300 mg l^{-1} WL84811

□ = 1000 mg l^{-1} WL84811

▲ = 3000 mg l^{-1} WL84811

Fig. 2.4b Relationship between total root dry weight and total length of the longest roots for each replicate set of seedlings. Key as for Fig. 2.4a.

61
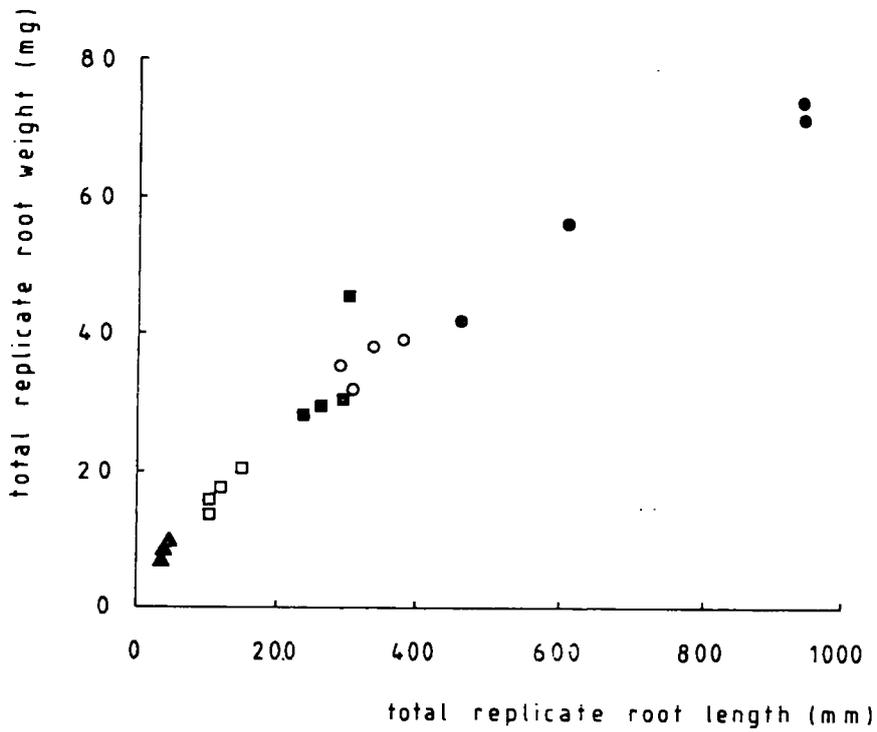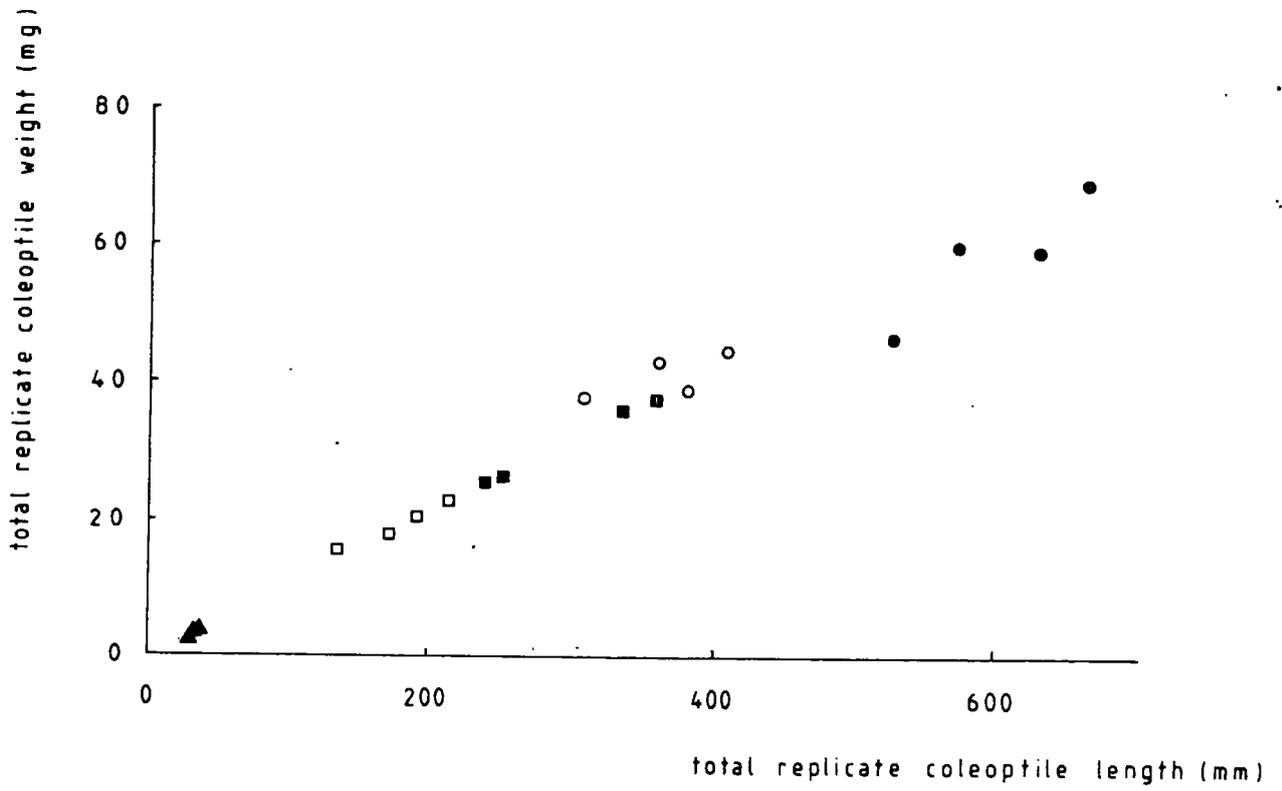
The effect of adding WL84811 to Sonja seeds germinated first on water was studied by imbibing and plating out seeds on water as described above, adding one millilitre of water after one day and measuring percentage germination, root and coleoptile length after two days. Seedlings were then transferred onto fresh filter paper, with three millilitres of water or 6000 mg l^{-1} WL84811 added per dish. A further one millilitre of the appropriate solution was added daily and the seedlings measured again after four days on the fresh medium.

Sonja seeds were also used to determine whether adding exogenous proline or indole-3-acetic acid (IAA) could overcome the effect of WL84811. Proline was made up as aqueous solutions of 50, 150, 300, 1000 and 3000 mg l^{-1} and the effect of these was tested on percentage germination of the seeds and root and coleoptile growth after five days as described above.

Proline was also tested for its effect in conjunction with WL84811. The treatments used initially are listed below:

1. Distilled water

2. 50 mg l^{-1} WL84811

3. 50 mg l^{-1} WL84811 + 57 mg l^{-1} proline

4. 150 mg l^{-1} WL84811

5. 150 mg l^{-1} WL84811 + 171 mg l^{-1} proline

6. 300 mg l^{-1} WL84811

7. 300 mg l^{-1} WL84811 + 342 mg l^{-1} proline

8. 1000 mg l^{-1} WL84811

9. 1000 mg l^{-1} WL84811 + 1138 mg l^{-1} proline

10. 3000 mg l^{-1} WL84811

11. 3000 mg l^{-1} WL84811 + 3416 mg l^{-1} proline

12. 6000 mg l^{-1} WL84811

13. 6000 mg l^{-1} WL84811 + 6832 mg l^{-1} proline

The concentrations of proline used were calculated to provide equimolar concentrations of proline and WL84811 in solution as the latter has a slightly lower molecular weight.

In a subsequent experiment, the effect of high concentrations of proline on the activity of low concentrations of WL84811 was determined. The treatments used were:

1. Distilled water

2. 50 mg l^{-1} WL84811

3. 50 mg l^{-1} WL84811 + 500 mg l^{-1} proline

4. 50 mg l^{-1} WL84811 + 1000 mg l^{-1} proline

5. 150 mg l^{-1} WL84811

6. 150 mg l^{-1} WL84811 + 1000 mg l^{-1} proline

IAA is extremely insoluble in water and so was first dissolved in a drop of methanol then diluted to produce a 10^{-2} M stock solution. This was diluted with distilled water before use to give 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M solutions. Seeds were imbibed, plated out and measured after five days as usual. Subsequently, the effects of 10^{-7}, 10^{-5}, 10^{-3} and 10^{-2} M IAA supplied in conjunction with 1000 mg l^{-1} WL84811 (approximately 10^{-2} M) were studied.

Azetidine-2-carboxylic acid (A2C), an isomer of WL84811 occurring naturally in the *Liliaceae* (Fowden & Steward, 1957), was also tested for its effect on Sonja seed

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germination and growth. 0, 50, 300 and 1000 mg l^{-1} solutions were applied to the seeds and growth measured after five days. The ability of proline to overcome the inhibition caused by high concentrations of A2C was also tested as it has been shown to ameliorate the effect of A2C on the marine green alga *Nannochloris* (Vanlerberghe & Brown, 1986). The treatments used were:

1. Distilled water

2. 300 mg l^{-1} A2C

3. 300 mg l^{-1} A2C + 342 mg l^{-1} proline

4. 300 mg l^{-1} A2C + 1000 mg l^{-1} proline

5. 1000 mg l^{-1} A2C

6. 1000 mg l^{-1} A2C − 1138 mg l^{-1} proline.

2.72 Fern spore germination

Bracken (*Pteridium aquilinum*) spores were germinated on cellophane overlaying filter paper soaked in three millilitres of modified Knop's solution (Knop, 1865; Table 2.6) with added WL84811 of 0, 50, 150, 300 and 1000 mg l^{-1}. An additional 0.5 ml of solution was added to all replicates every second day to keep the cellophane moist.

Table 2.6 Composition of Knop's solution.

Constituent	Concentration (g l^{-1})
(CaNO$_3$)$_2$.4H$_2$O	0.8
KNO$_3$	0.2
KH$_2$PO$_4$	0.2
MgSO$_4$.7H$_2$O	0.2

Percentage germination of the spores was measured after four and seven days,

scoring at least 300 spores on each of two replicate plates per treatment.

2.8 Algal experiments

Initially a range of WL84811 concentrations (0, 10, 20, 50, 150, 300 and 1000 mg l^{-1}) were made up in the growth medium and their effect on cell number and size of both *Chlorella* and *Closterium* measured at intervals after inoculation. Subsequently, *Chlorella* cells initially grown up in WL84811 were inoculated back into control Chu 10 and the resultant change in cell number and size measured.

The effect of WL84811 on biomass of *Chlorella* was also determined. Chu 10, with and without 300 mg l^{-1} WL84811, was inoculated with *Chlorella* and the dry weight of cells in each of three replicate flasks determined at intervals. 0.5 ml samples were removed for counting before the contents of each flask were collected on a Whatman glass fibre filter (GF/C), previously oven-dried and weighed. The filters plus cells were dried overnight before weighing to determine the dry weight of cells present. This was expressed both as total dry weight of culture and dry weight per cell.

The effect of WL84811 on DNA content of cells was determined using the method of Labarca and Paigen (1980). One millilitre samples of Chlorella grown in Chu 10 with and without 300 mg l^{-1} WL84811 were taken for counting and DNA analysis at intervals after inoculation. For DNA determination, one millilitre of culture was added to one millilitre of 0.1 M $NaPO_4$ / 4 M NaCl buffer, pH 7.4 and two microlitres of 1.0 mg ml^{-1} bisbenzimidazole and emission measured at 458 nm on a Baird FP100 Fluoripoint Spectrofluorimeter. A calibration curve was produced using 10 mg ml^{-1} herring sperm DNA diluted to 20, 2 and 0.2 μg ml^{-1} (Fig. 2.5). DNA content was expressed both as total amount per millilitre of culture and amount per cell.

Fig. 2.5 Standard curve relating the DNA content of a solution to emission at 458 nm when using the method of Labarca and Paigen (1980).
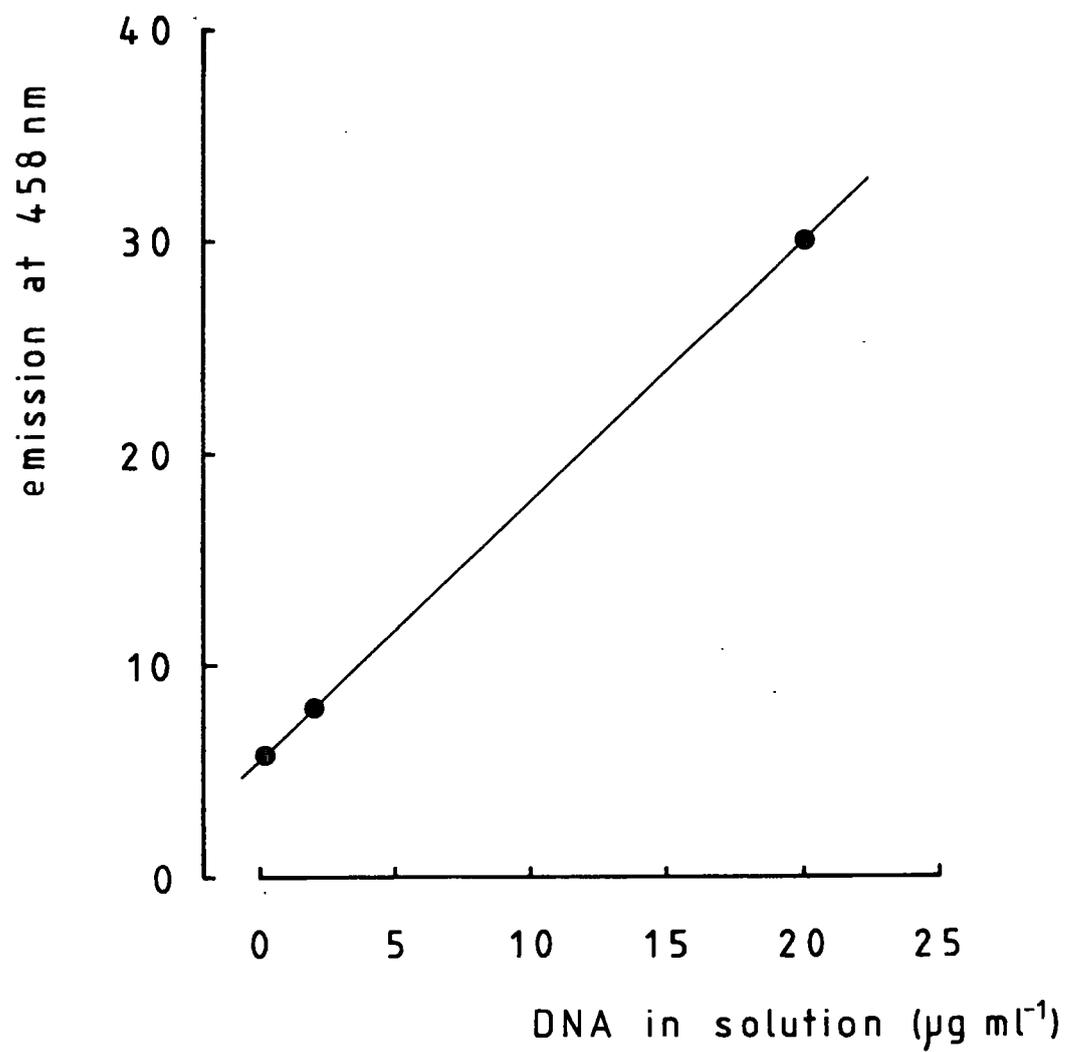
Fig. 2.5 Standard curve relating the DNA content of a solution to emission at 458 nm when using the method of Labarca and Paigen (1980).
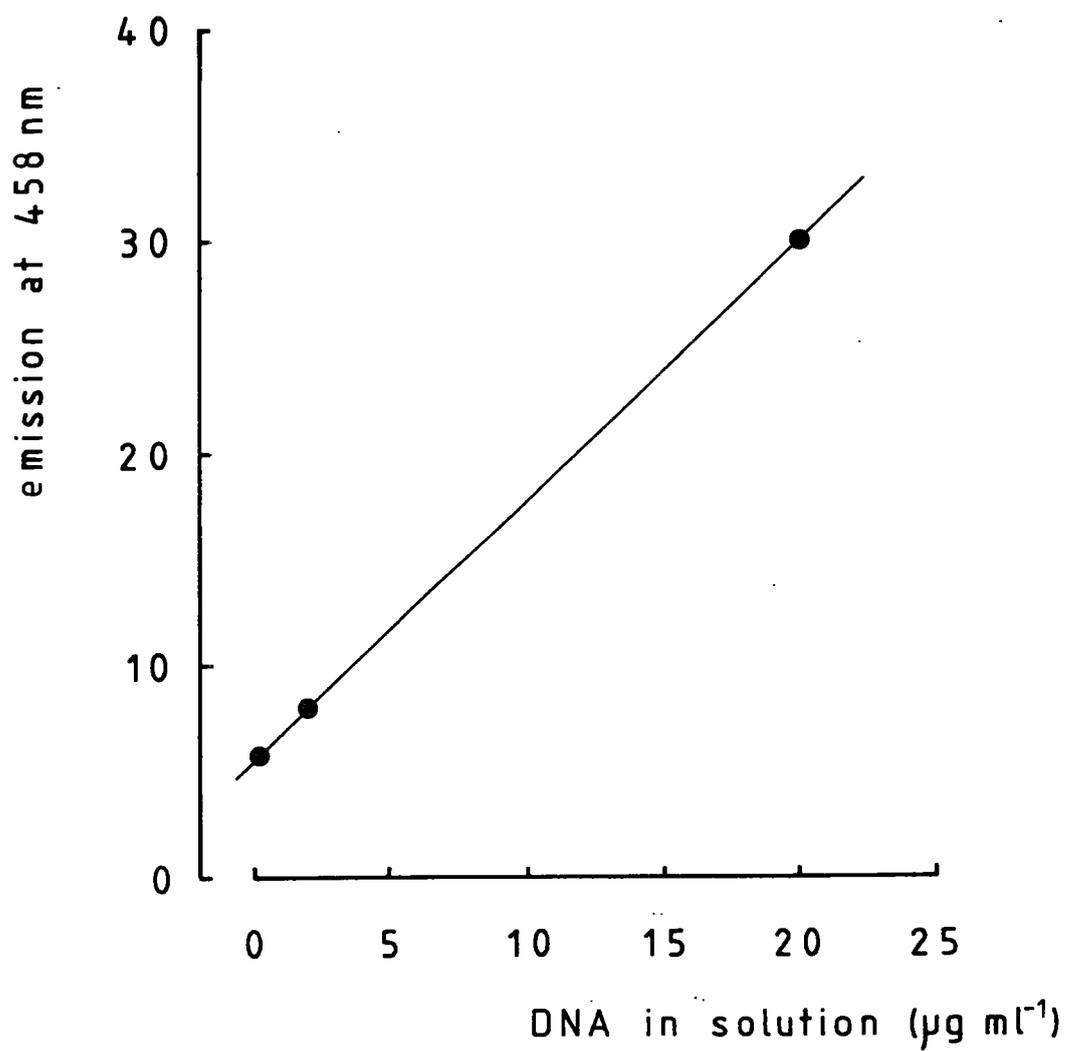
This method depends on the large enhancement of fluoresence of bisbenzimidazole which occurs when this dye binds to DNA, after the chromatin has been dissociated using a high salt buffer. Enhancement of fluorescence is linear over the range 0 – 15 μg DNA per ml and RNA does not interfere. The method is also sensitive to as little as 10 ng of DNA.

Proline was tested for its effect on *Chlorella* growth, alone and in conjunction with WL84811, to determine whether, as a possible functional analogue, it could overcome the inhibitory effects of WL84811. The following treatments (in Chu 10) were used:

1. Control

2. 1000 mg l^{-1} WL84811

3. 1138 mg l^{-1} Proline

4. 1000 mg l^{-1} WL84811 + 1138 mg l^{-1} Proline.

The effect of azetidine-2-carboxylic acid on *Chlorella* growth was also tested. alone and with proline. The treatments used (all made up in Chu 10) were:

1. Control

2. 50 mg l^{-1} A2C

3. 57 mg l^{-1} Proline

4. 50 mg l^{-1} A2C + 57 mg l^{-1} Proline

5. 1000 mg l^{-1} A2C

6. 1138 mg l^{-1} Proline

7. 1000 mg l^{-1} A2C + 1138 mg l^{-1} Proline.

The effect of algal growth on WL84811 content of the medium was determined by growing up *Chlorella* on various concentrations of WL84811, removing the cells

by centrifugation and using the supernatant to germinate seeds of barley cv. Sonja. Seeds were also germinated on freshly prepared WL84811 of the same concentrations and growth on the fresh and used media compared. The treatments used are listed below (all made up in Chu 10):

1. Fresh control

2. Control after algal growth

3. Fresh 50 mg l^{-1} WL84811

4. 50 mg l^{-1} WL84811 after algal growth

5. Fresh 150 mg l^{-1} WL84811

6. 150 mg l^{-1} WL84811 after algal growth

7. Fresh 300 mg l^{-1} WL84811

8. 300 mg l^{-1} WL84811 after algal growth.

2.9 Statistical analyses and computing

Data were analysed on the Northumbrian Universities Multiple Access Computer (NUMAC) at Durham University, an Ahmdahl 470/V8 running under the Michigan Terminal System (MTS). The statistical packages MINITAB (Ryan *et al.*, 1982) and SAS (SAS Institute Inc., 1985) were used. This thesis was prepared using the T$_E$X word processing package (Knuth, 1984).

2.91 Seed germination data

Many of the data for root and shoot length of germinating seeds were not normally distributed. Logarithmic transformations were applied and also a modification of the calculation based on Taylor's power law given in Elliott (1977; M.G. Kelly, pers.

comm.) but these did not normalise the data sufficiently (Fig. 2.6). To overcome this difficulty, non-parametric statistics which do not assume a normal distribution were used; median values for each treatment were calculated with upper and lower quartiles and treatment medians were compared using the Mann-Whitney U-test instead of Student's t-test and the Kruskal-Wallis test instead of an analysis of variance.

The Mann-Whitney U-test has much high power efficiency than is achieved using parametric methods with non-normal data (Siegel, 1956) and is particularly suitable for small samples. It tests the null hypothesis (H_0) that the two independent samples are drawn from populations with the same parent distribution (the form of which is not specified) and the same median, and is a test of rank order $i.e.$ counts are replaced by their rank values in a single combined sequence and test statistics calculated from the sum of ranks for each treatment. These are referred to Table 14 in Elliott (1977).

The Kruskal-Wallis test is a one-way analysis by ranks with a power efficiency of about 96 % (Seigel, 1956). The H_0 that all samples come from the same population and that there is therefore no difference in the mean rank of the samples is tested (Elliott, 1977). This test may be used where the number of counts in each sample is different. The test statistic K is distributed approximately as χ^2 unless there are only three treatments each containing less than five samples; then it must be referred to special tables (Siegel, 1956; Table O). The procedure is detailed by Elliott (1977).

2.92 Pollen germination data

Many of the data for pollen germination and tube growth were also not normally distributed (Fig. 2.7). For the reasons given above, these results were again expressed as median values and analysed using non-parametric statistics.

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Fig. 2.6a Distribution of lengths of *Daucas carota* seedlings germinated on water. Untransformed data.

Fig. 2.6b Distribution of lengths of *Daucas carota* seedlings germinated on water. Data transformed using a modification of Taylor's power law on replicate means.

Fig. 2.6c Distribution of lengths of *Daucas carota* seedlings germinated on water after $\log_{10}$ transformation.

Fig. 2.6d Distribution of lengths of *Daucas carota* seedlings germinated on water. Data transformed using a modification of Taylor's power law on treatment means.

a.

b.

c.

d.
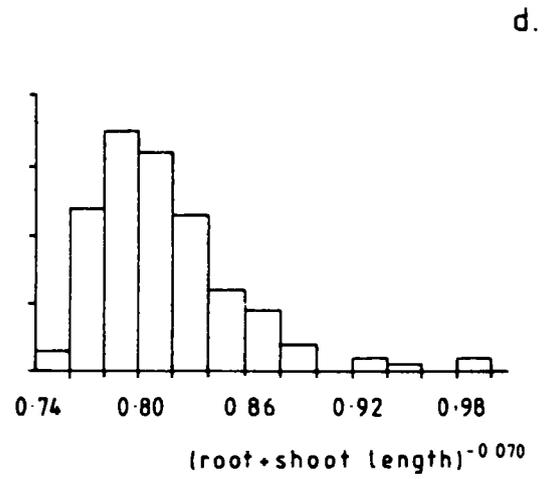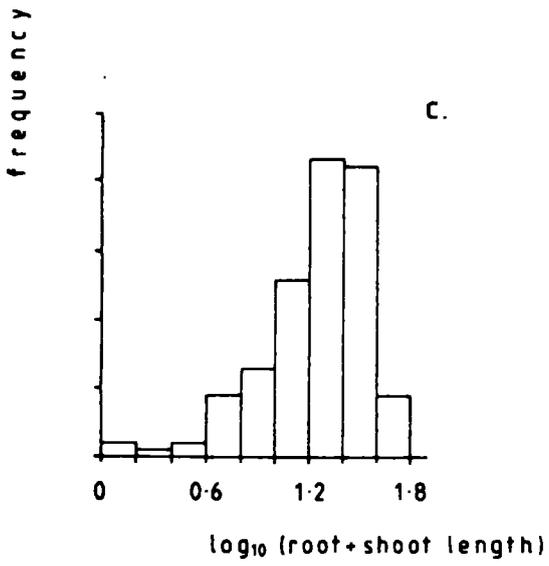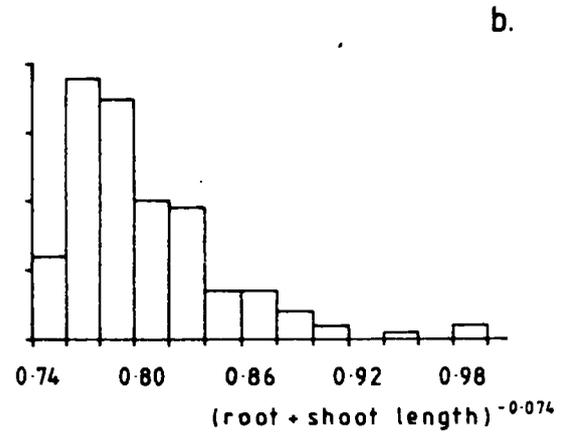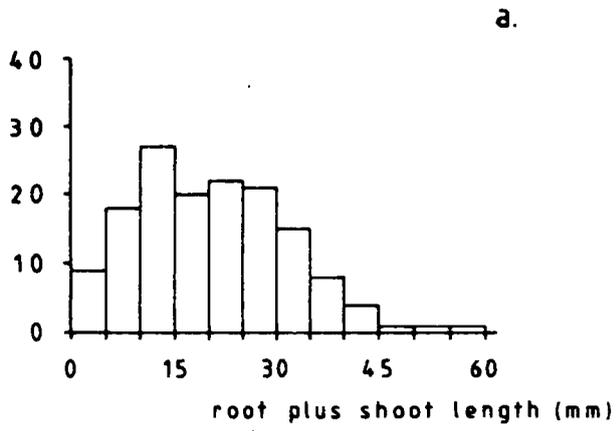
Fig 2.7a Distribution of percentage germination in samples of *H. vulgare* cv. Sonja pollen germinating *in vitro*.

Fig 2.7b Distribution of percentage germination in samples of *H. vulgare* cv. Sonja pollen germinating *in vitro* in the presence of 6000 mg l^{-1} WL84811.

Fig 2.7c Distribution of pollen tube lengths of *H. vulgare* cv. Sonja pollen germinating *in vitro*.

Fig 2.7d Distribution of pollen tube lengths of *H. vulgare* cv. Sonja pollen germinating *in vitro* in the presence of 6000 mg l^{-1} WL84811.

a.

b.

c.

d.

percentage germination

pollen tube length (µm)

71
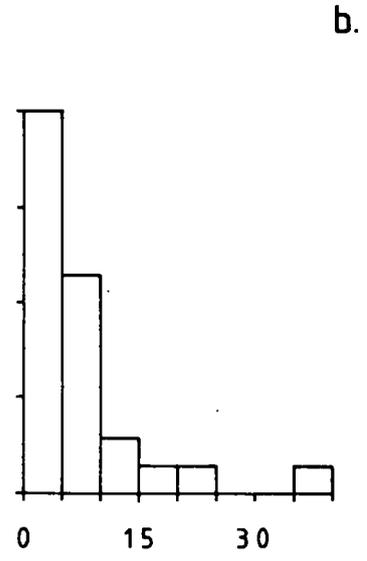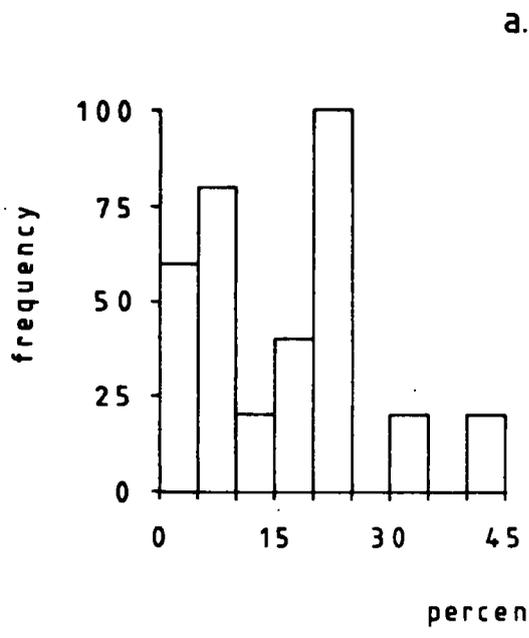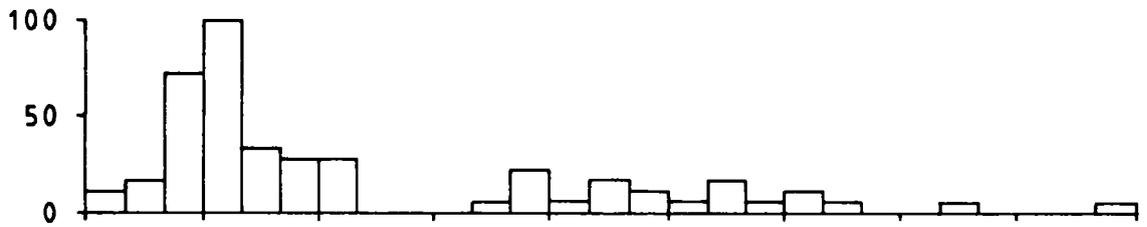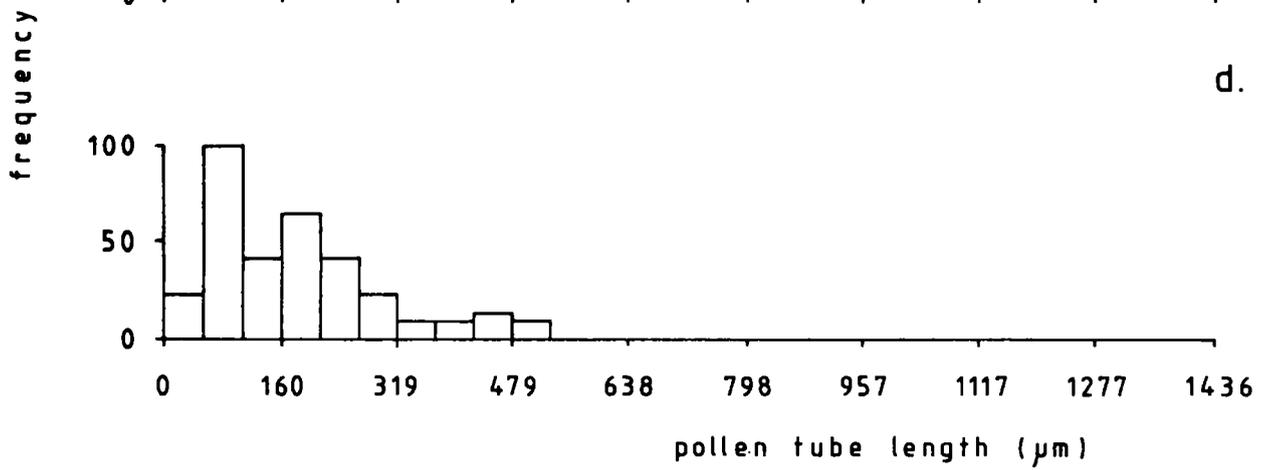
2.93 Algal data

The algal data for cell size was displayed as histograms to show changes in size distribution. Counts of cell number followed a Poisson distribution so 95 % confidence limits for this distribution were calculated using the expression:

$$X - \frac{1.96^2}{2} \pm 1.96\sqrt{X + \frac{1.96^2}{4}}$$

where X is the total number of cells counted per treatment (Parker, 1979). When the total number of samples was the same in each case, treatments were compared using the expression:

$$d = \frac{|X_1 - (\frac{X_1+X_2}{2})| - 0.5}{\sqrt{(\frac{X_1+X_2}{4})}}$$

where X_1 and X_2 are the total number of cells counted in the treatments to be compared. When the sample sizes of treatments to be compared were different the expression used was:

$$d = \frac{|X_1 - (X_1 + X_2)(\frac{Y_1}{Y_1+Y_2})| - 0.5}{\sqrt{(X_1 + X_2)(\frac{Y_1}{Y_1+Y_2})(\frac{Y_2}{Y_1+Y_2})}}$$

where Y_1 and Y_2 are the number of counts taken in the two samples. The values of d calculated in this way were compared with the tabulated value of 1.96 for P=0.05 and, when the calculated value exceeded 1.96, were deemed to be significant at P=0.05.

RESULTS

3. EFFECTS OF WL84811 ON FLORAL DEVELOPMENT IN CEREALS

3.1 Introduction

The destination of WL84811 applied to wheat plants by stem-injection was studied using ^{14}C-WL84811. As it was found to be accumulated equally by male and female parts of the flower, the effect of WL84811 on both stigma and pollen development was studied using a variety of microscopic techniques to ascertain whether it caused any morphological changes.

3.2 Transport and accumulation of ^{14}C-WL84811 after stem injection

each $(3.75 \times 10^{-2} \text{ or } 3.75 \times 10^{-3} \mu Ci)$

Four wheat plants were injected with 0.1 ml of 30 or 3 mg l^{-1} ^{14}C-WL84811 at the stage when the ear was still enclosed within two leaves and returned to the glasshouse

pooled

for 14 days. At the end of this period, just before flowering, anther and pistil fractions were harvested from each treated ear, dried and their specific activity determined (see section 2.6). The median values are summarised in Tables 3.1 and 3.2.

Table 3.1 Median activity in anther and pistil fractions after injection with WL84811. Quartiles are given in brackets.

WL84811 conc.	Activity in anthers (μCi mg^{-1})	Activity in pistils (μCi mg^{-1})
30 mg l^{-1}	1.36×10^{-4} ($0.97 - 1.93 \times 10^{-4}$)	1.13×10^{-4} ($1.02 - 1.36 \times 10^{-4}$)
3 mg l^{-1}	1.02×10^{-5} ($0.92 - 1.30 \times 10^{-5}$)	1.45×10^{-5} ($1.04 - 1.55 \times 10^{-5}$)

Table 3.2 Median percentage of applied WL84811 in anther and pistil fractions. Quartiles are given in brackets.

WL84811 conc.	% of applied WL84811 in anthers	% of applied WL84811 in pistils
30 mg l^{-1}	2.44 (1.63 – 2.90)	1.03 (0.79 – 1.40)
3 mg l^{-1}	2.10 (1.98 – 2.51)	0.99 (0.91 – 1.23)

Using the Mann-Whitney U-test it was shown that there was no significant difference (P = 0.05) between anther and pistil fractions in the amount of WL84811 accumulated per milligram dry weight. This was true whether WL84811 was applied at a concentration of 30 or 3 mg l^{-1}. The percentage of applied WL84811 ending up in the anther and pistil fractions was also unaffected by the concentration of the WL84811 applied although the percentage found in the anthers was always approximately twice that found in the pistils due to the greater mass of this tissue present.

In view of these results, both stigma and pollen development were studied for any visible effects resulting from the presence of WL84811 in the tissues.

3.3 Stigma development

At maturity, the grass stigma is a plumose structure consisting of two tapering styles three to four mm long, each covered in multiseriate, papillate stigmatic branches (see Fig. 1.1). At the earliest stage observed, however, the styles were fairly smooth with very few short stigmatic branches, these differentiating during development. For study, this developmental process was arbitrarily divided into four stages:

1. Styles about one millimetre long, smooth stigmatic branches starting to differentiate.

2. Styles three millimetres long with more and longer branches on which individual papillae are starting to bend out at the tip, giving a multiseriate structure. The most mature florets were at this stage when ears were injected with WL84811 *i.e.* just before the boot began to split.

3. Many fully developed stigmatic branches on styles which are starting to bend apart at their tips.

4. Fully mature stigma with styles completely bent apart from one another at their tips to provide the maximum surface area for pollen capture.

Excised pistils at each stage were stained for 15 mins with auramine or acridine orange (2.411) or embedded for light microscopy (2.412). In some ears of glasshouse-grown material the stigmas did not develop normally; this occurred in both WL84811-treated and untreated plants and was probably due to unfavourable light or temperature conditions. No consistent differences were observed in stigma development between treated and untreated plants so the normal process will be described.

3.31 Fluorescence microscopy

After staining with auramine or acridine orange, the styles were cut away from the ovary and mounted in glycerol or water for observation. With both stains there was much variation in the staining of neighbouring cells, particularly in more mature stigmas. This may have been due, in part, to differential permeability of the cells but was probably also due to differences in the degree of contact with the stain: air bubbles tended to become trapped between the stigmatic branches. The latter explanation is likely to account for a number of cells where only the cuticle fluoresced when stained with auramine, the stain apparently having failed to penetrate this.

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Pictures to illustrate these results are located at the back of this thesis; they have been presented as transparencies because of the extreme difficulty of making colour prints which show details of fluorescence.

Branches on the youngest stigmas examined (stage 1, above) were composed largely of cells which fluoresced a uniform, dull yellow with auramine (Fig. 3.1) and whose nuclei and, in some cases endomembranes, fluoresced bright green with acridine orange (Fig. 3.2). A few cells containing discreet areas of red fluorescence were located at the tip of some branches stained with acridine orange.

At stage 2, with papillae starting to differentiate, most branches were composed largely of cells showing some red fluorescence with acridine orange (Fig. 3.3). At the base of each branch were cells whose nucleus and golgi apparatus fluoresced bright green with auramine, while the cytoplasm remained largely unstained.

In more mature stigmas, variability in response to the stain was more pronounced; adjacent cells in a stigmatic branch often stained differently (Fig. 3.4). With acridine orange, some cells were observed where the cytoplasmic contents stained red but the nucleus failed to stain at all, as well as others where the nucleus stained bright green or red (Figs 3.5, 3.6). Overall, the greatest amount of red fluoresence was present at this stage (Fig. 3.7).

In fully mature stigmas, cells fluorescing red with acridine orange were located mainly at the tips of stigmatic branches (Fig. 3.8). Some of these cells had aggregated endomembranes and corresponded to cells whose endomembranes stained bright yellow with auramine at this stage (Fig. 3.9). The aggregation of membranes in these cells may indicate damage or degeneration, in which case the red fluorescence might indicate not active RNA synthesis but DNA being denatured (section 2.411).

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3.32 Light microscopy

Thin sections of material embedded at various stages of development were studied after staining with toluidine blue (2.413 ̭section). At all stages, the cells of stigmatic branches had prominent nuclei, occupying up to 10 percent of the total cell volume. The nuclei often appeared granular, with clear nucleoli (Fig. 3.10). In the younger stigmas, chromosomes were visible in cells undergoing mitosis (Fig. 3.11). Cells were highly vacuolate, the cytoplasm often restricted to strands connecting the nucleus and cell membrane.

3.4 Pollen development

Pollen development from the stage of WL84811 injection to anther dehiscence was studied both by squashing anthers in acetocarmine (2.412 ̭section) and using transmission electron microscopy (2.421 ̭section). As acetocarmine stains nuclear material, the various stages can be easily identified. Pollen development, as observed in untreated material of *T. aestivum* cv. Mercia, may be divided into a series of arbitrary stages.

1. Anthers green, around three mm long. The microspores have a large vacuole and a single nucleus opposite the previously-formed pore (Fig. 3.12). This is the stage at which ears are treated with WL84811.

2. Anthers green, four mm long. The single nucleus has now divided (Fig. 3.12). The daughter nuclei move apart: the large vegetative nucleus rests under the pore and the smaller generative nucleus (in its hemispherical cell cut off after cytokinesis) lies at the opposite side of the pollen grain (Fig. 3.13). The grain has just started to fill with starch. The ear is fully emerged from its sheath by this stage.

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Fig. 3.10 Oblique section through mature stigmatic branches of *T. aestivum* stained with toluidine blue. Note the prominent, granular nuclei. Scale bar = 20 μm.

n = nucleoli

Fig. 3.11 Transverse section through immature stigmatic branches of *T. aestivum* stained with toluidine blue. Scale bar = 12 μm.

n =nucleus undergoing mitosis
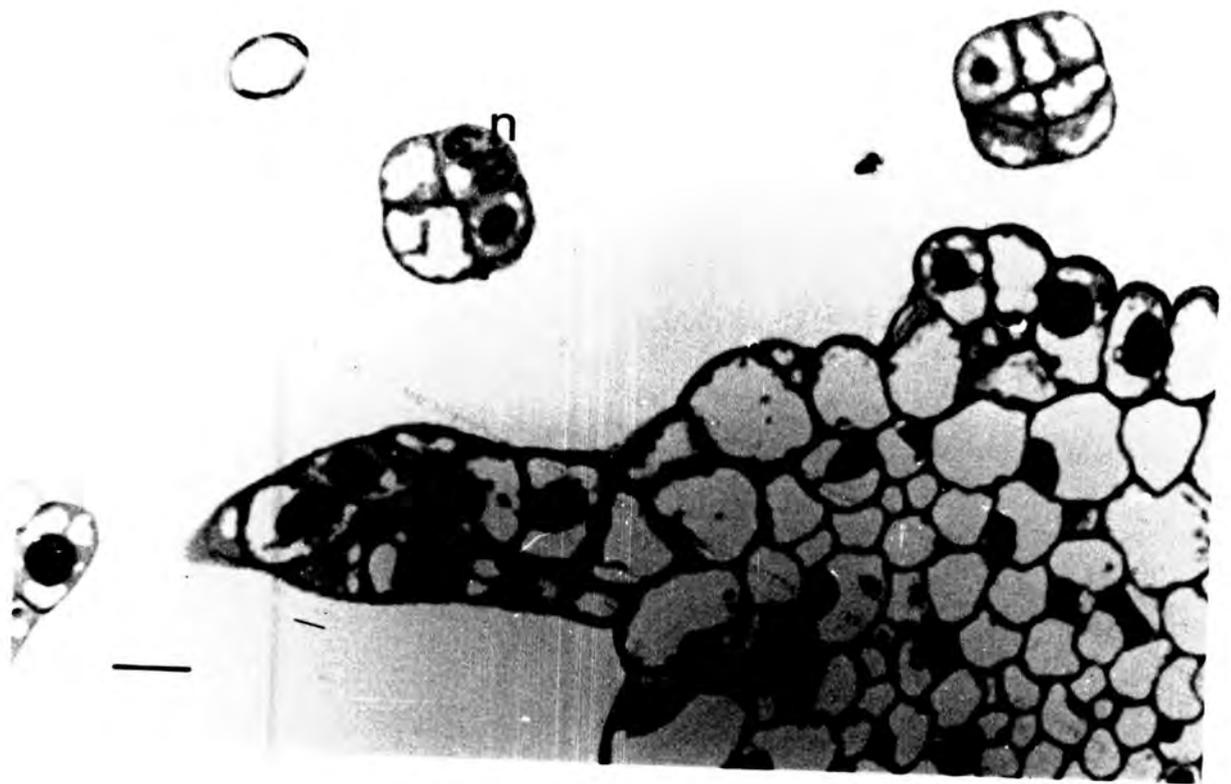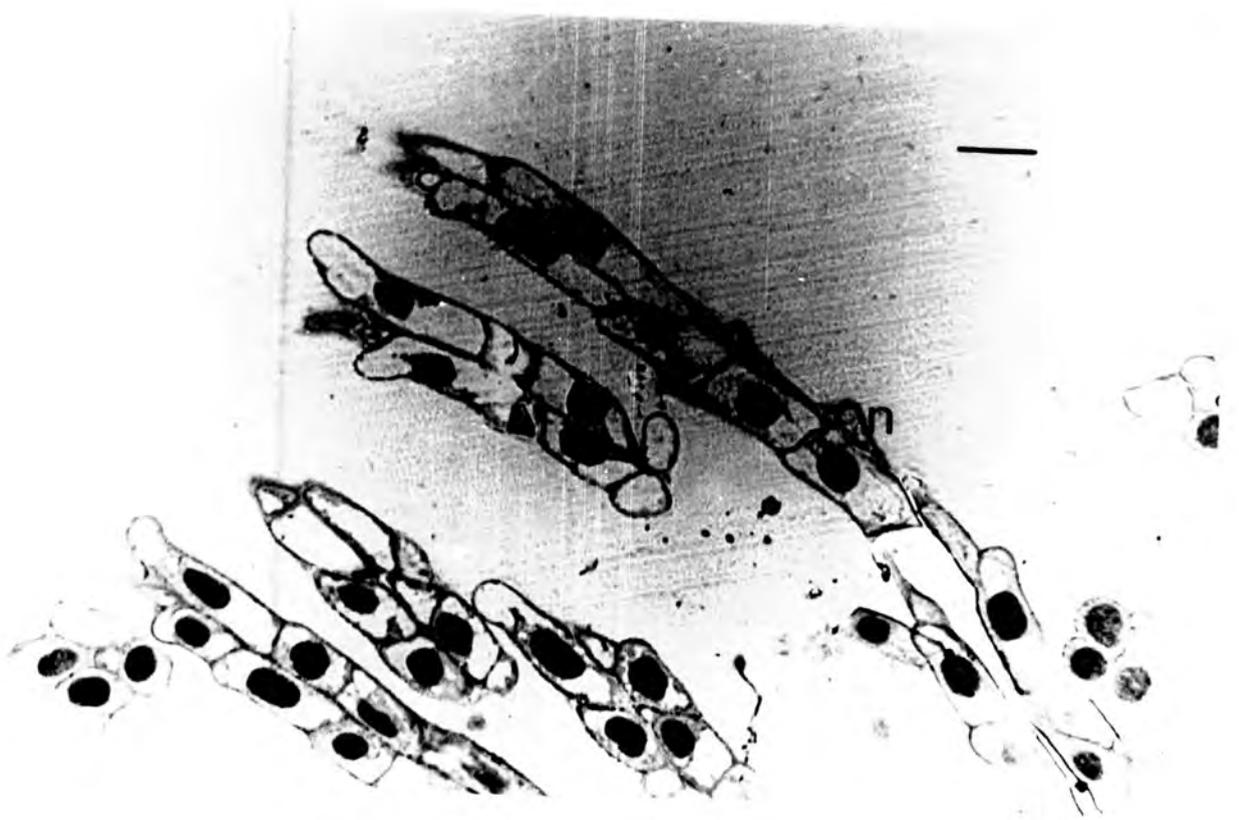
Fig. 3.12 Two grains of *T. acstivum* cv. Mercia pollen stained with acetocarmine. The grain at bottom right is at the stage, before pollen mitosis. when ears are normally injected with WL84811 (stage 1). The nucleus has just divided in the upper grain. Scale bar = 25 μm.

n = single, granular nucleus just before division

v = vegetative nucleus just after pollen mitosis

g = generative nucleus just after pollen mitosis

Fig. 3.13 Two grains of *T. acstivum* cv. Mercia pollen stained with acetocarmine. The nucleus of the grain on the right is just about to divide: chromosomes can be made out. The grain on the left has undergone mitosis: the larger, vegetative nucleus has moved away from the generative nucleus and rests under the pore (stage 2). Scale bar = 25 μm.

g = generative nucleus

v = vegetative nucleus
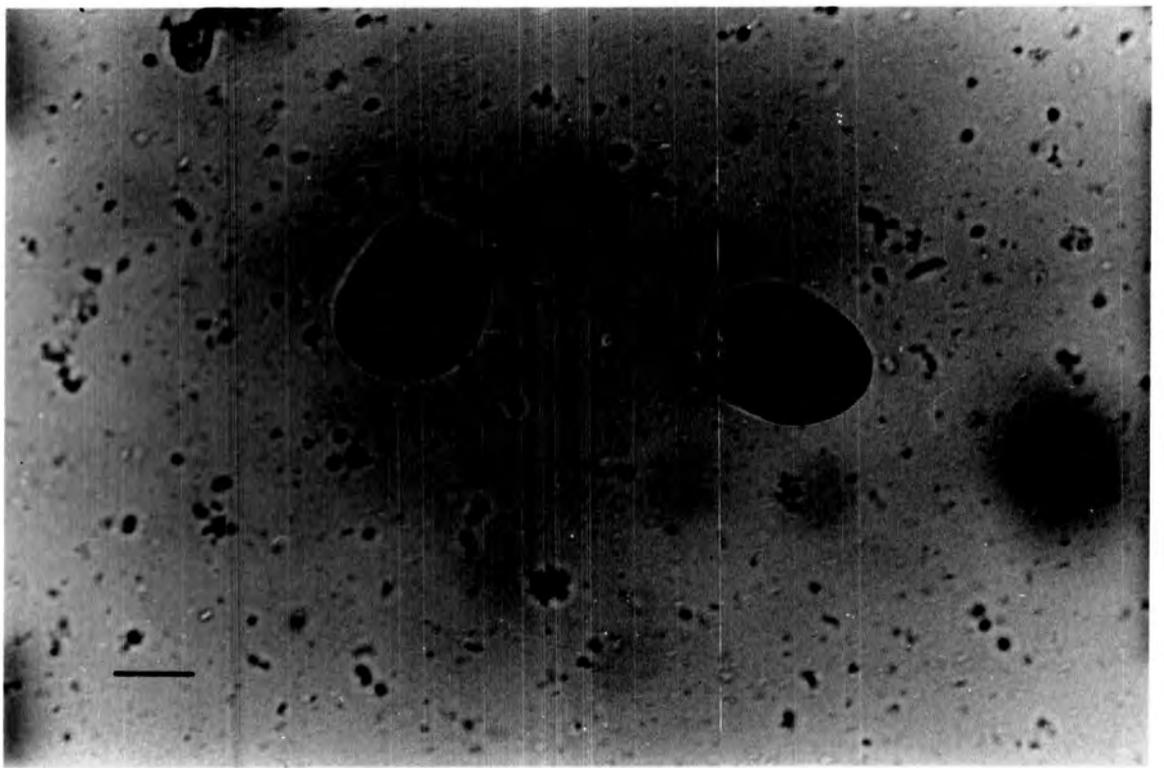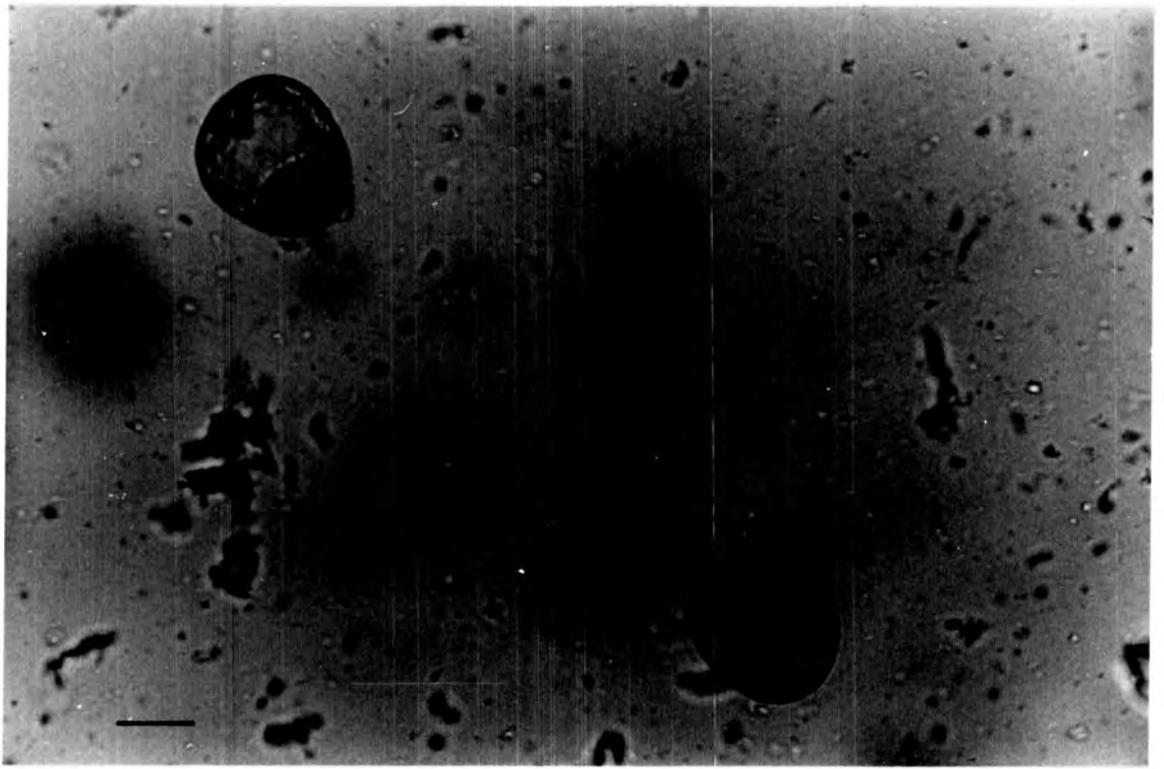
3. Anthers yellow, starting to dehisce. The generative cell has now separated from

   the parental wall and lies within the much larger vegetative cell. The generative

   nucleus has divided into two sperm cells although all the nuclei are now obscured

   by starch grains and polysaccharide particles with which the pollen is packed.

3.41 Light microscopy

No differences were apparent between fresh, WL84811-treated and untreated *T.*
aestivum pollen / from stem-injected plants when stained with acetocarmine under the light microscope. WL84811-
treated pollen appeared to be developing normally and to be filling with starch (Fig.

3.14). No difference was apparent at maturity; in both cases the grains appeared to

be fully engorged with starch at the time of dehiscence (Figs 3.15, 3.16). However,

it was noticeable that anthers from treated ears burst less readily than untreated

anthers during filament elongation, so shedding less pollen onto the stigma.

When *T. aestivum* anthers were fixed and embedded for light microscopy (2.413)

at dehiscence, clear differences were seen between WL84811-treated and untreated

pollen. Whereas untreated pollen was spherical and fully engorged (Fig. 3.17), pollen

from plants treated by stem injection with 3000 mg l^{-1} WL84811 was badly mishapen

(Fig. 3.18). This appears to have been hidden by the fact that pollen takes up water

rapidly from an aqueous stain such as acetocarmine and appears normal.

No such difference was observed in anthers of *S. cereale* embedded and sectioned

in the same way. Pollen from plants treated with WL84811 looked perfectly normal

(Fig. 3.19).

Fig. 3.14 Grain of *T. aestivum* cv. Mercia pollen from a WL84811-treated plant stained with acetocarmine. The nucleus has divided into vegetative and generative nuclei and the grain appears to be starting to fill normally. Scale bar = 25 μm.

g = generative nucleus

v = vegetavive nucleus

Fig. 3.15 Mature (stage 3) *T. aestivum* cv. Mercia pollen from an untreated plant, stained with acetocarmine. The nuclei can no longer be seen clearly as the grains are full of starch. Scale bar = 25 μm.
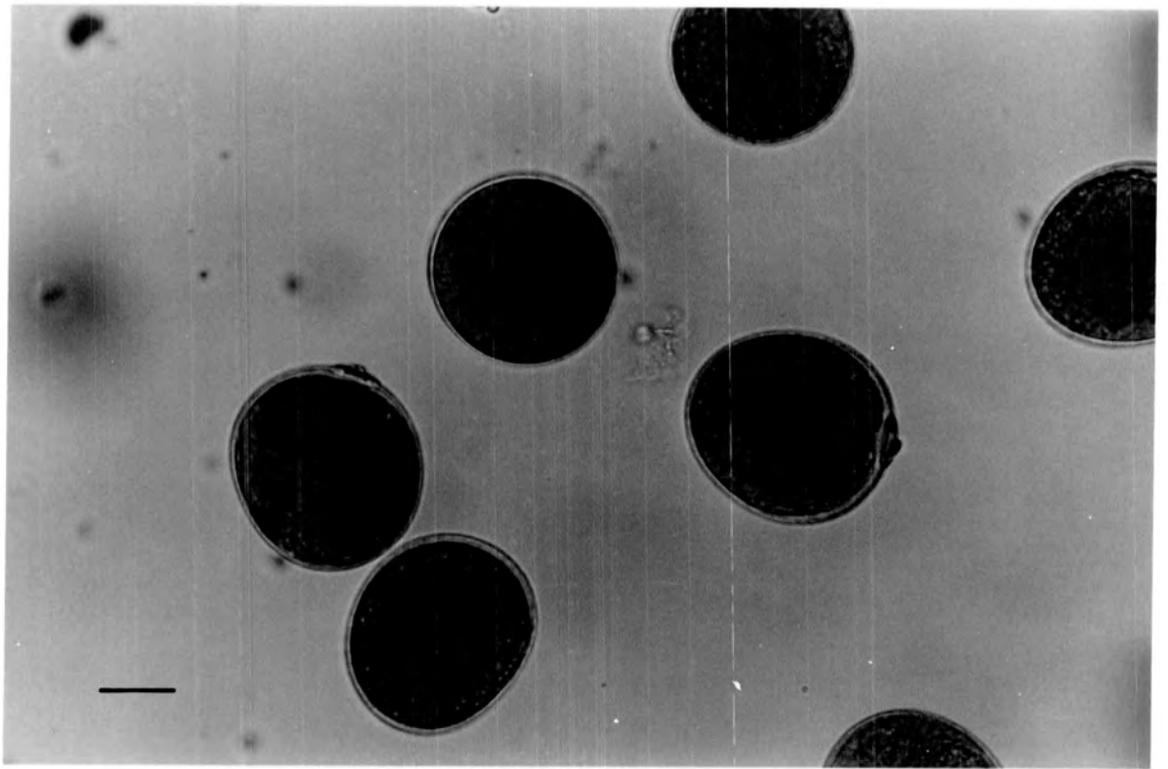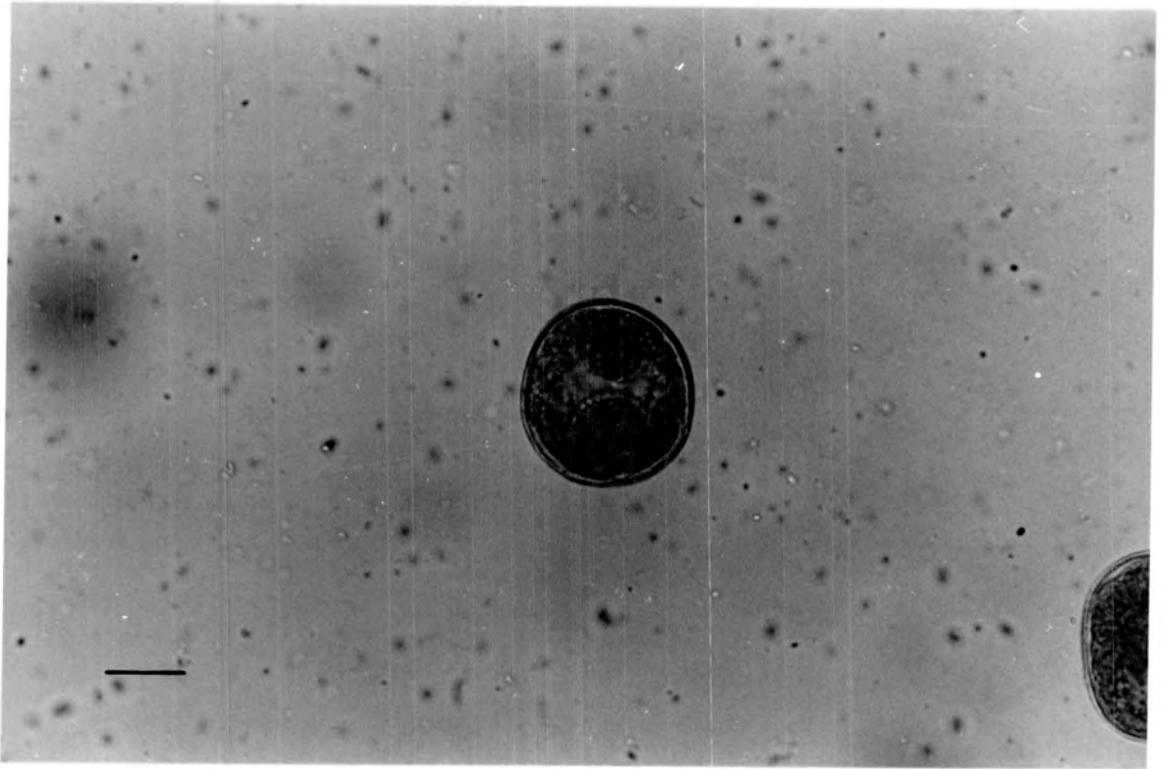
Fig. 3.16 Mature *T. aestivum* cv. Mercia pollen from a plant treated with WL84811, stained with acetocarmine. It appears perfectly normal. The operculum has been lifted by the intine bulging out. Scale bar = 25 μm.

o = operculum

Fig. 3.17 Section through a mature *T. aestivum* cv. Mercia anther from an untreated plant. stained with toluidine blue. The pollen grains are spherical and filled with starch. Scale bar = 20 μm.

a = anther wall
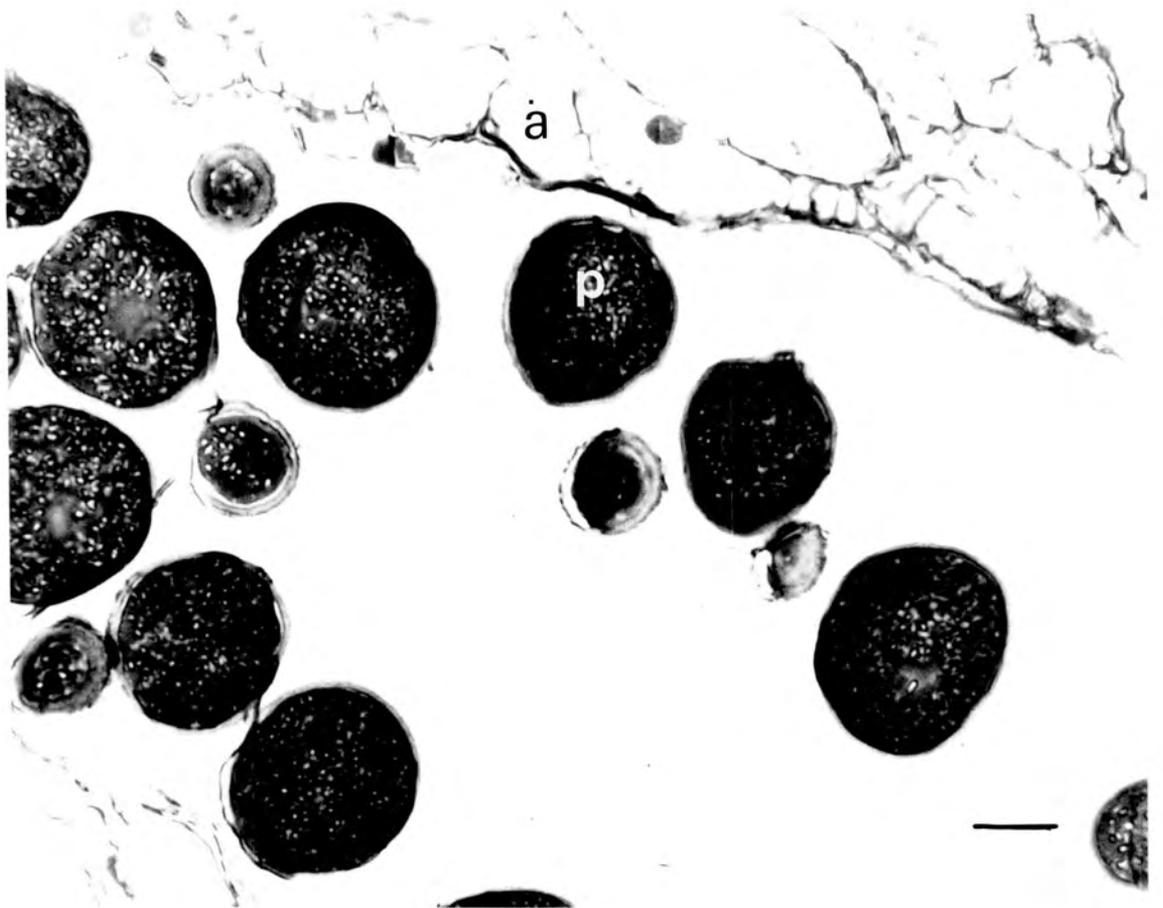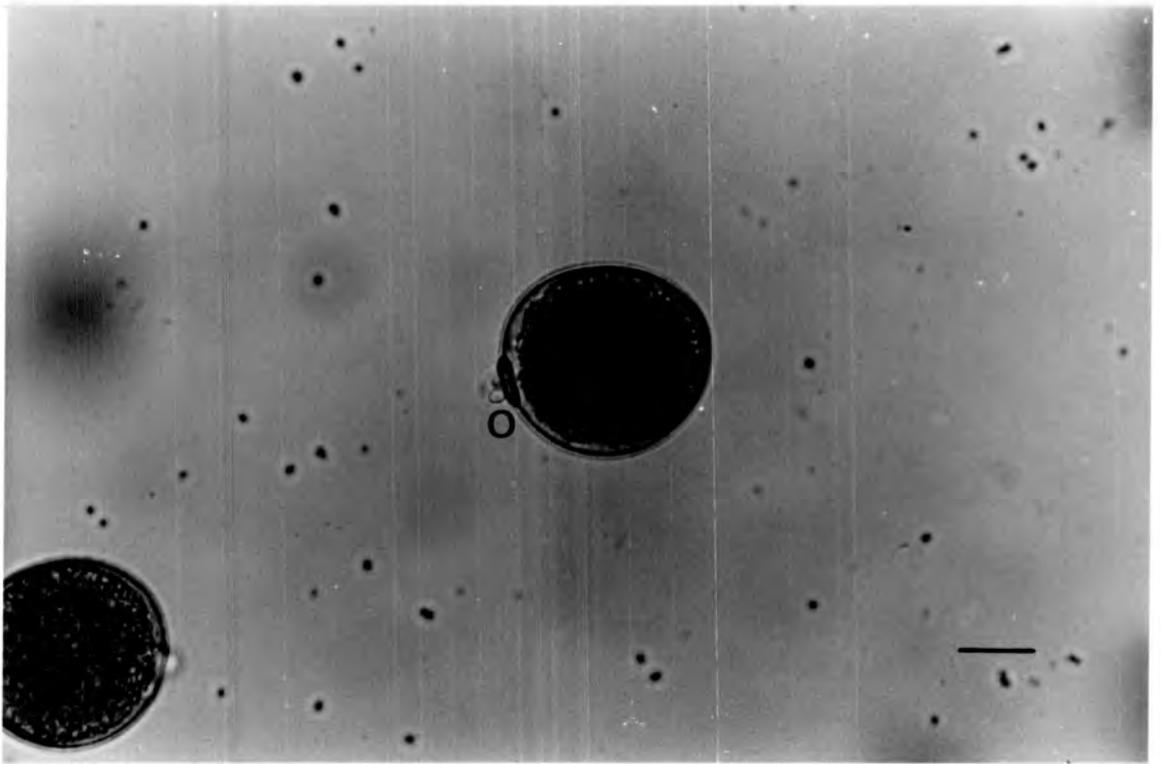
p = pollen grain

Fig. 3.18 Section through a mature *T. aestivum* cv. Mercia anther from a WL84811-treated plant, stained with toluidine blue. The pollen grains are mishapen with variable amounts of patchy cytoplasm. Scale bar = 20 μm.

a = anther wall

p = pollen grain

Fig. 3.19 Section through a mature *S. cereale* cv. Dominant anther from a WL84811-treated plant, stained with toluidine blue. In this case the pollen appears to be unaffected by WL84811; the pollen is spherical and fully filled with starch. Scale bar = 18 μm.
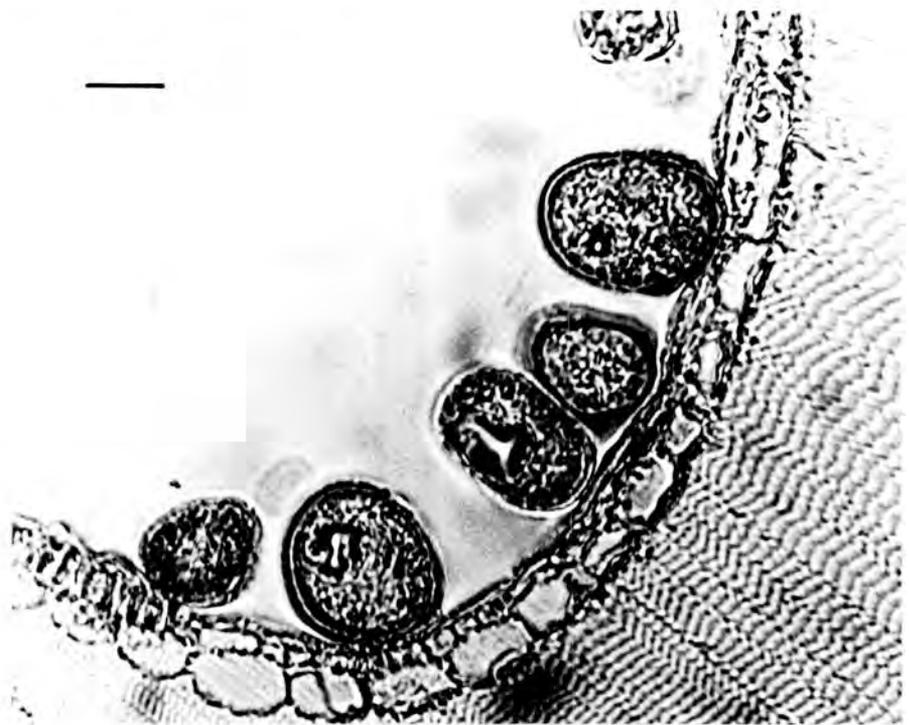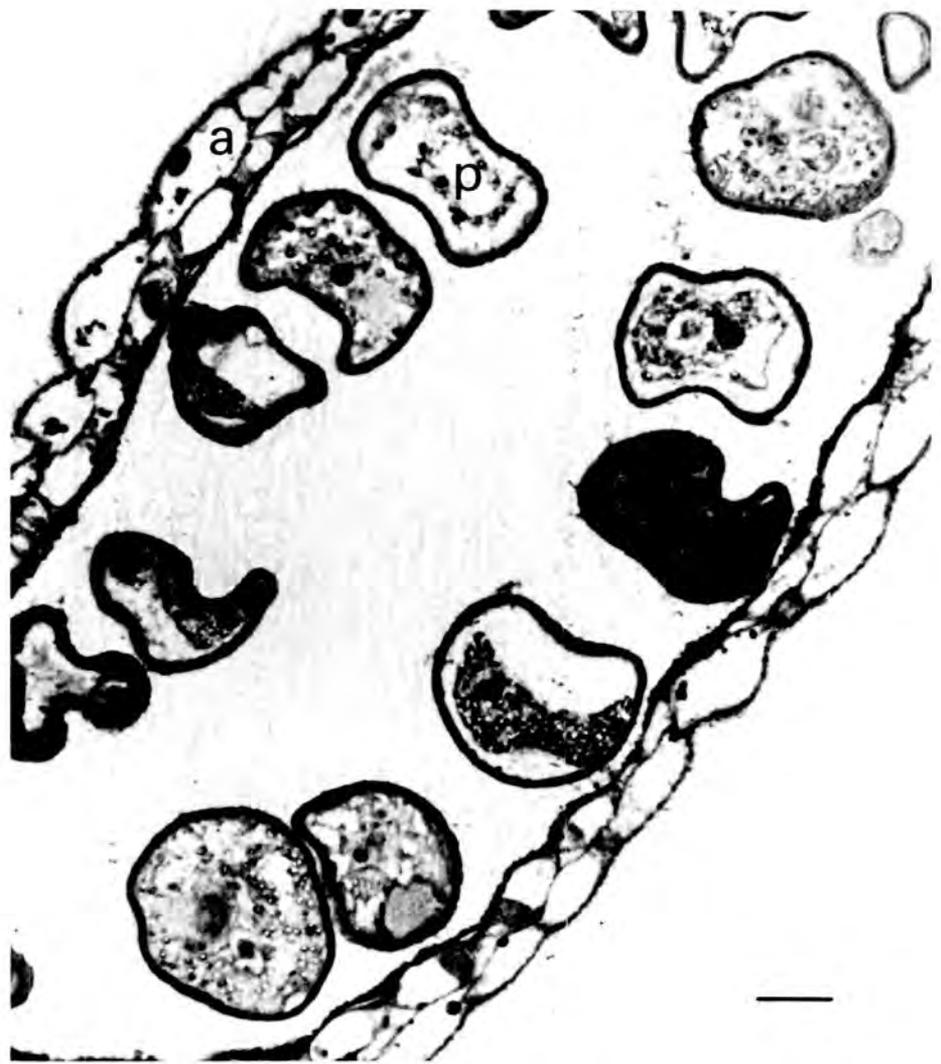
3.42 Transmission electron microscopy (TEM)

Material fixed and embedded for TEM also showed differences between WL84811-treated and untreated wheat pollen. The cytoplasm of untreated pollen was packed with starch and bounded by a wall consisting of an intine and exine of approximately equal thickness (Fig. 3.20). The intine of untreated pollen had a fibrillar appearance, the fibrils running perpendicular to the exine (Fig. 3.21). In contrast, the cytoplasm of WL84811-treated pollen was less densely packed and a large volume of the cell was occupied by electron-lucent material with a net-like appearance which appeared to replace the intine. The cytoplasm was invaginated into this intine-type material (Fig. 3.22).

3.43 Scanning electron microscopy (SEM)

SEM allowed the surface structure of wheat pollen to be clearly seen. The grain has a single prominent pore (approximately 10 μm in diameter) surrounded by a raised lip of exine material, the annulus. The pore has a central operculum which rests on a thin sporopollenin lamella overlying the intine (Fig. 3.23).

The only difference apparent between WL84811-treated and untreated pollen was that after self-pollination much, but not all, of the treated pollen on stigmas was dehydrated and folded (Fig. 3.24). Dehydrated pollen was also present on untreated stigmas but, for some time after pollination, most of the pollen remained approximately spherical. Although no pollen tubes could be seen clearly, pollen grains were observed with the intine bulging out at the aperture (Fig 3.25).

Fig. 3.20 Transmission electron micrograph of section through a mature *T. aestivum.* cv. Mercia pollen grain from an untreated plant. The exine and intine layers of approximately equal thickness can be seen clearly and the interior of the grain is characterised by many holes where starch grains have fallen out during sectioning. Scale bar = 1.5 μm.

e = pollen exine

i = pollen intine

Fig. 3.21 Enlargement of pollen wall area from Fig. 5.20. Fibrils can be seen throughout the intine, running perpendicular to the pollen wall. Scale bar = 280 nm.

e = exine

i = intine

85
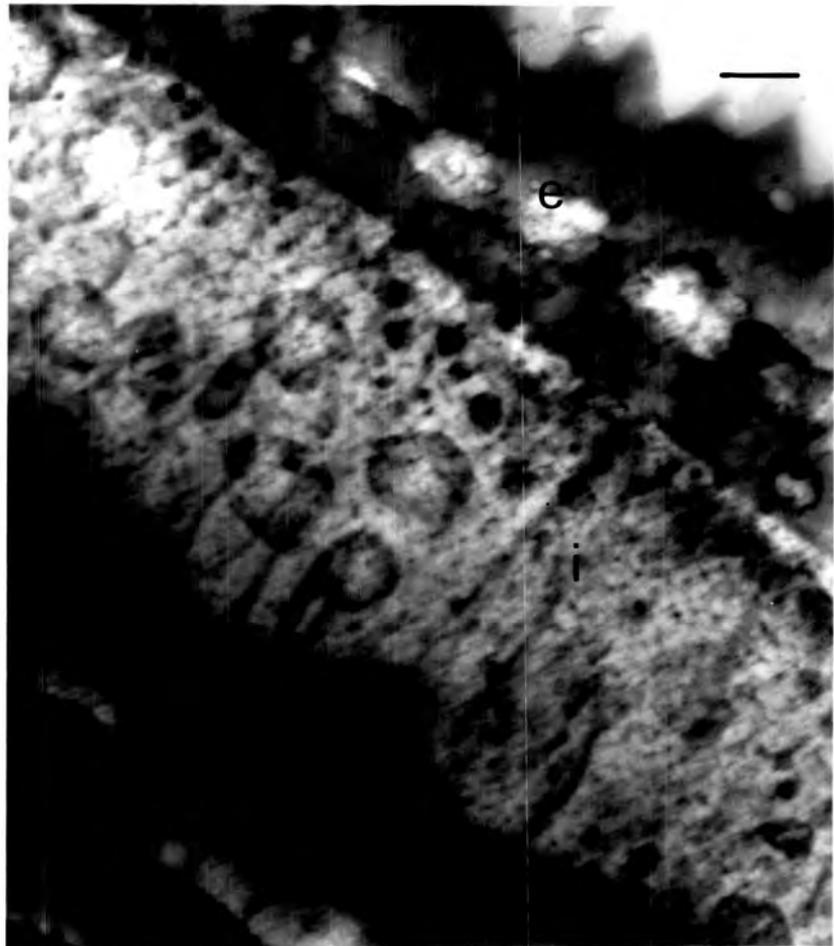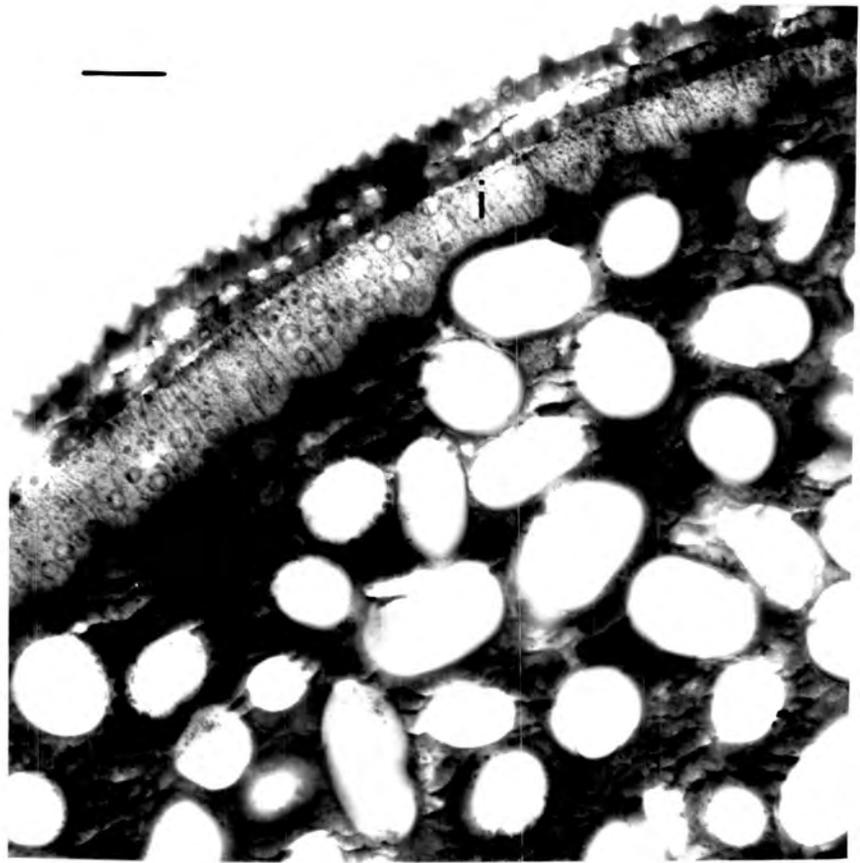
Fig. 3.22 Transmission electron micrograph of a section through a mature *T. aestivum* cv. Mercia anther from a plant treated with WL84811. The pollen exine appears normal but the intine is of very variable thickness with the pollen cytoplasm invaginated into it. The intine has also lost its fibrillar structure and has a net-like appearance. Starch grains in this case have not been lost on sectioning and remain in place in the cytoplasm. Scale bar = 3 μm.

t = tapetal cell

e = pollen exine

i = replacement for intine

c = pollen cytoplasm.
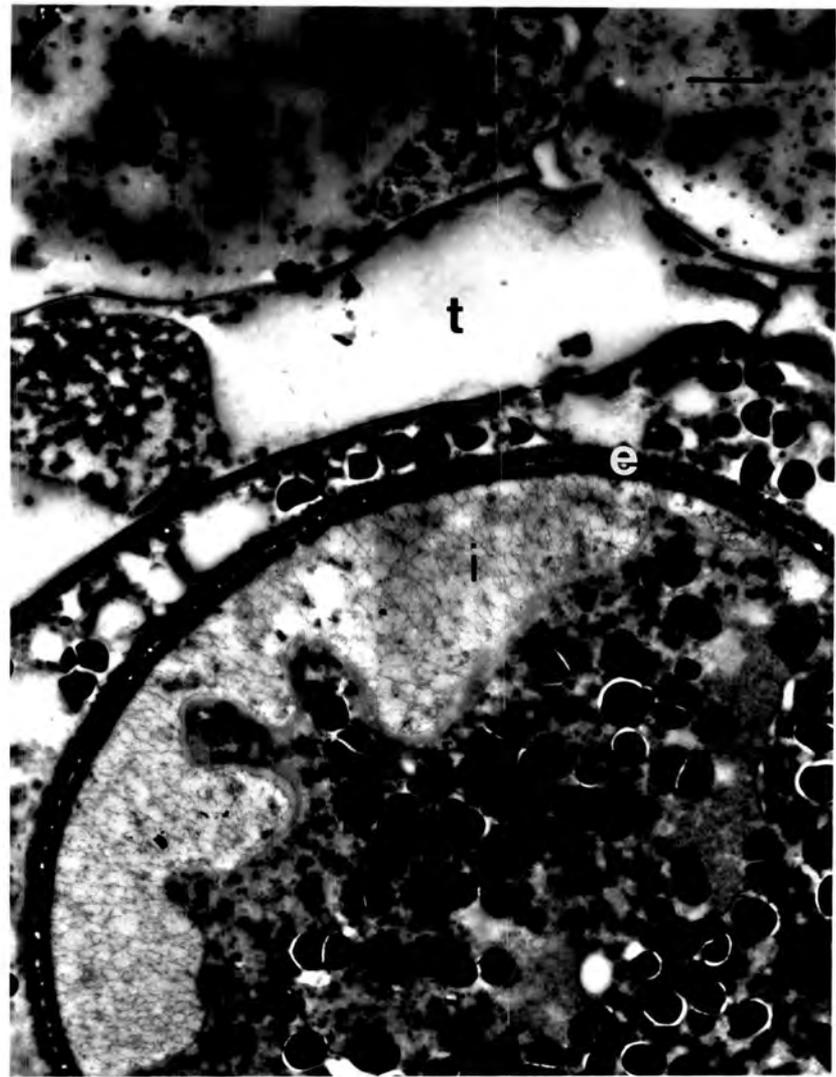
Fig. 3.23 Scanning electron micrograph of crumpled pollen on the stigma of a WL84811-treated *T. aestivum* plant after self-pollination. Scale bar = 10 μm.

p = pollen

s = stigmatic papilla
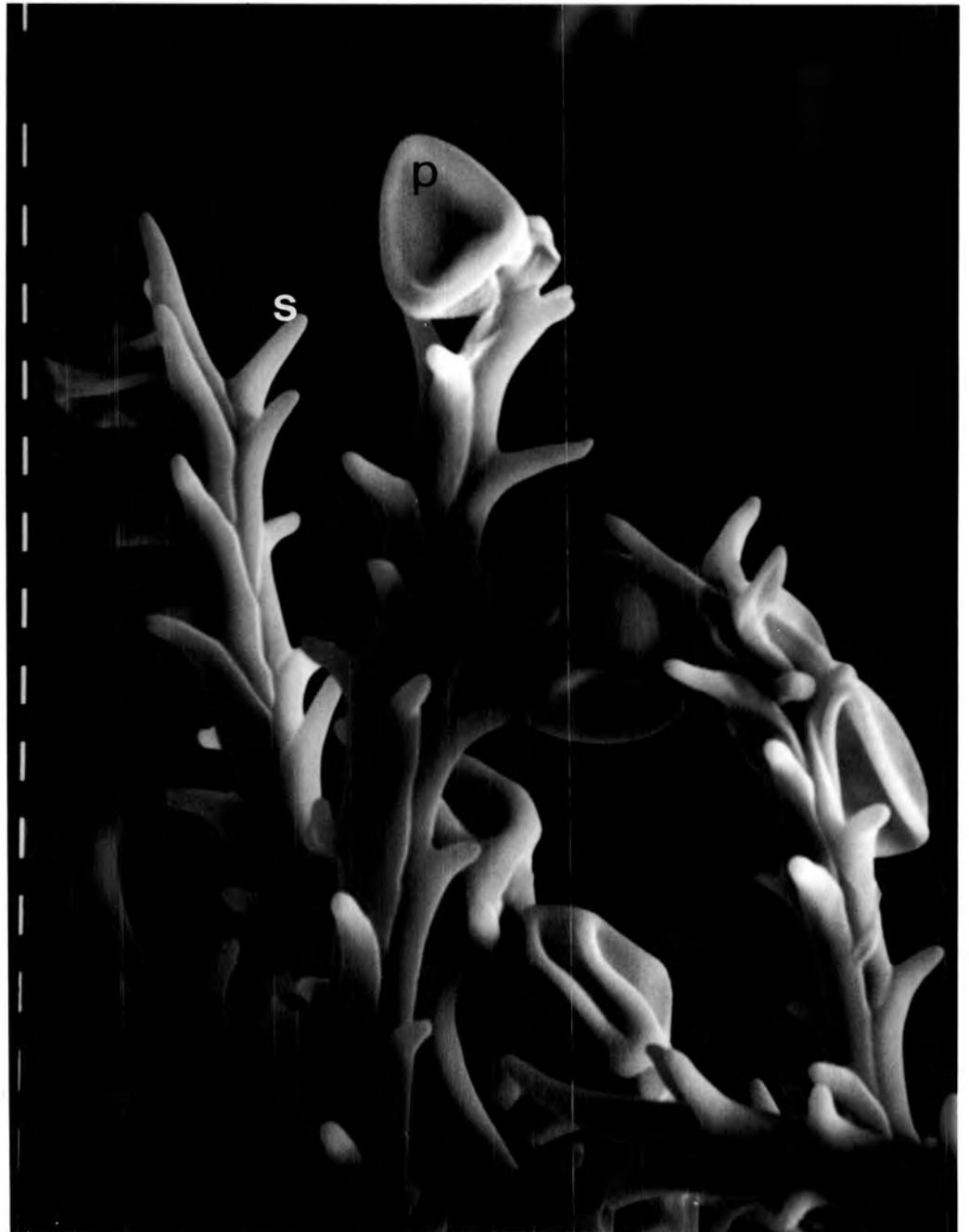
Fig. 3.24 Scanning electron micrograph of a pollen grain on the stigma of an untreated *T.aestivum* plant after self-pollination. The intine is bulging out at the aperture. Scale bar = 10 μm.

o = operculum

i = intine

a = annulus
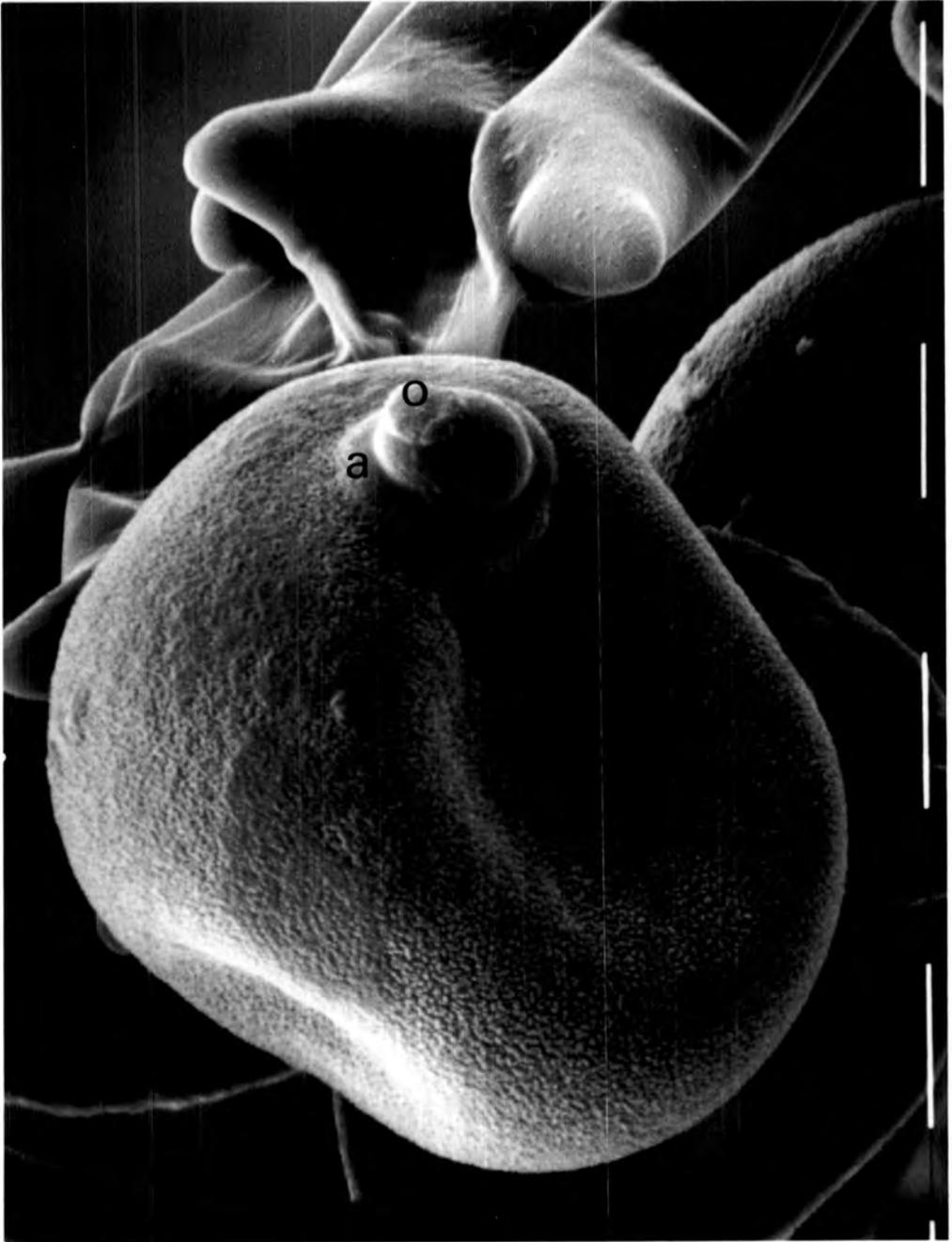
3.44 Proline content of pollen

The free proline content of dried samples of T. aestivum cv. Banner, both un-treated and treated with WL84811 by stem injection, was determined as described in section 2.53; this amino acid is believed to be very important in determining pollen viability. Proline content was expressed as a percentage of the pollen dry weight. The median values are presented in Table 3.3.

Table 3.3 Median proline content of pollen as percentage of dry weight. Quartiles in brackets.

Sample	Proline content (%)
Control	0.86 (0.53 – 1.05)
WL84811 treated	0.75 (0.69 – 0.93)

Comparison of the median values using the Mann-Whitney U-test showed that there was no significant difference ($P > 0.9$) between the proline content of treated and untreated pollen.

3.5 Effect of WL84811 on plant morphology

There was no difference between WL84811 treated and untreated plants until relatively late in flowering, when the florets of treated plants characteristically gaped to ~~encourage~~ permit the outcrossing necessary for seed set after the failure of self-pollination (Fig. 3.25).

Fig. 3.25 Ear of *T. aestivum* emerging after the plant has been treated
with WL84811.

f = gaping floret

4. EFFECTS OF WL84811 ON SEED AND SPORE GERMINATION

4.1 Introduction

Germinating seeds were chosen as another plant system on which to test the effects of WL84811. This system, chosen partly for its convenience and partly because of the abundant background literature available, yielded some interesting results. The measurable effects of WL84811 on seed germination and seedling growth allowed deductions to be made about its effects on cell division and cell expansion in general.

4.2 Effects of WL84811 on seed germination and seedling growth

This was studied using several types of seed and a range of concentrations of aqueous WL84811. Seeds were imbibed in WL84811, plated out on filter paper soaked in solution and allowed to germinate. Solution was added daily in order to keep the paper moist and the seedlings harvested and measured, usually after five days growth.

As most of the data were not normally distributed, median values with upper and lower quartiles were calculated and Kruskal-Wallis one-way analyses performed to show the significance of differences between treatments. The significance of the difference between individual pairs of treatments was determined using the Mann-Whitney U-test: treatments not significantly different from one another at $P=0.05$ are bracketed together.

As seeds were being germinated in water alone, the effect of various concentrations of WL84811 on pH was determined to assess how large a part pH plays in the effect of WL84811 in this situation (Fig. 4.1). 50 mg l^{-1} WL84811 caused the pH to fall

It has been shown that even an external pH of 3.0, more than one unit lower than the pH of 3000 mg l^{-1} WL84811, has no significant effect on seed germination and seedling growth (Appendix 4).

by 0.7 pH units and 300 mg l^{-1} WL84811 or more caused it to fall by 1.3 units.⅄

4.21 *Daucas carota* (carrot)

Carrot seeds were germinated in WL84811 at concentrations ranging from 0 to 6000 mg l^{-1} and the seedlings harvested after five days. Percentage germination and total length of the root plus shoot of germinated seeds were measured and median values calculated for each treatment (Fig. 4.2).

WL84811 reduced total root plus shoot length of germinated seeds significantly ($P < 0.001$) and also percentage germination ($0.01 < P < 0.025$).

Root + shoot length: $0 > 300 > 1000 > (3000, 6000)$ mg l^{-1} WL84811

Percentage germination: $(0, 300, 1000) > (1000, 3000, 6000)$ mg l^{-1} WL84811.

High concentrations of WL84811 reduced both percentage germination and growth of germinated seedlings of carrot significantly, while lower concentrations affected only growth of germinated seedlings.

4.22 *Beta vulgaris* ssp. *vulgaris* (beetroot)

Beetroot seeds were germinated in WL84811, at concentrations ranging from 0 to 6000 mg l^{-1}. The seeds were slow to germinate in all treatments so seedling growth was measured after 10 days. Percentage germination and total root plus shoot length were measured and median values calculated for each treatment (Fig. 4.3).

WL84811 reduced total root plus shoot length of germinated seeds significantly ($P < 0.01$) but had no significant effect on percentage germination ($0.5 > P > 0.1$).

Root + shoot length: $(50, 150, 0) > (300, 1000) > 6000$ mg l^{-1} WL84811.

Fig. 4.1 Effect of WL84811 on pH of water. Values given are the mean of two readings, with ranges.

Fig. 4.2a Effect of WL84811 on median root plus shoot length of germinated *Daucas carota* seedlings after five days growth, with upper and lower quartiles.

Fig. 4.2b Effect of WL84811 on median percentage germination of *Daucas carota* seeds after five days, with upper and lower quartiles.

94
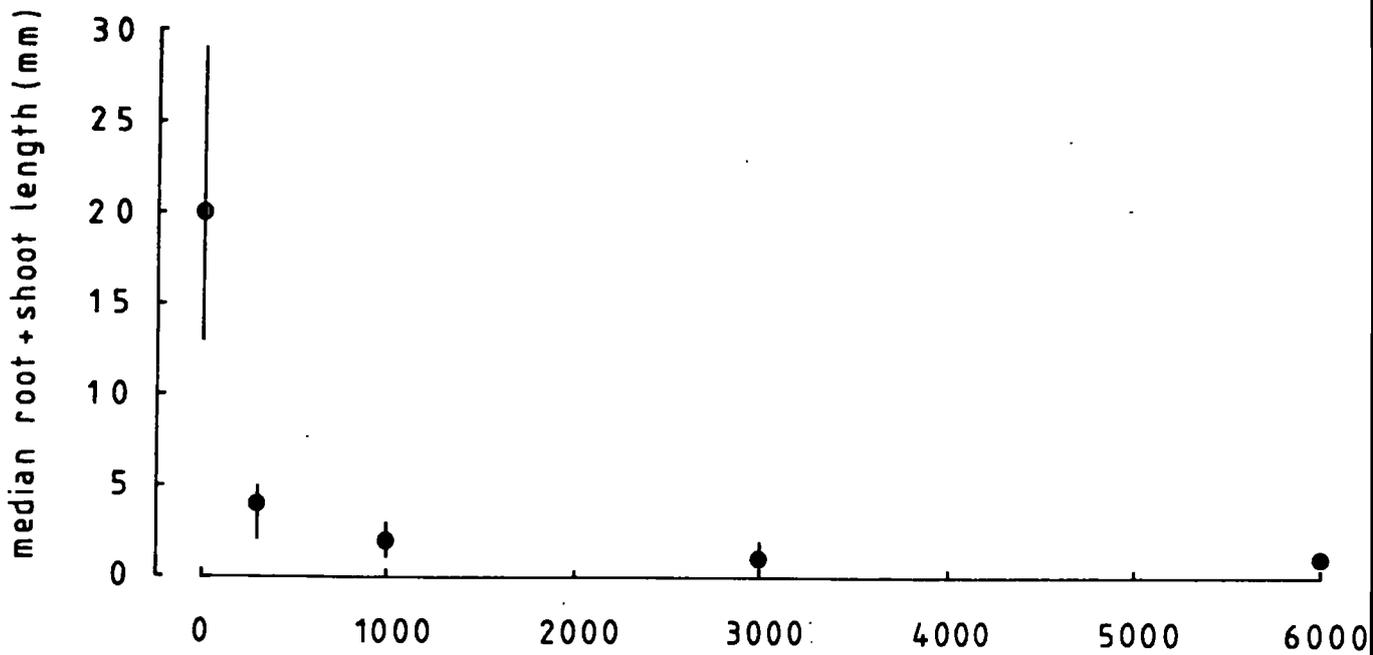
Fig. **4.3a** Effect of WL84811 on median root plus shoot length of germinated *Beta vulgaris* seedlings after 10 days growth, with upper and lower quartiles.

Fig. **4.3b** Effect of WL84811 on median percentage germination of *Beta vulgaris* seeds after 10 days, with upper and lower quartiles.
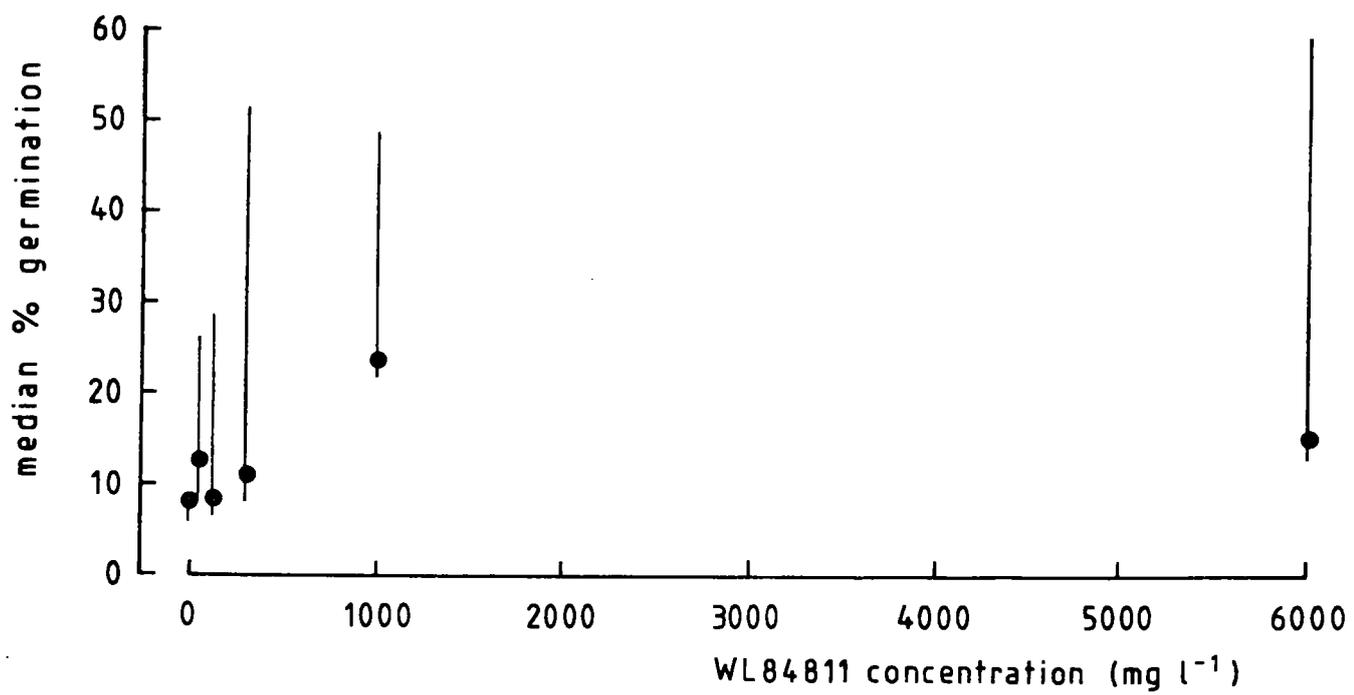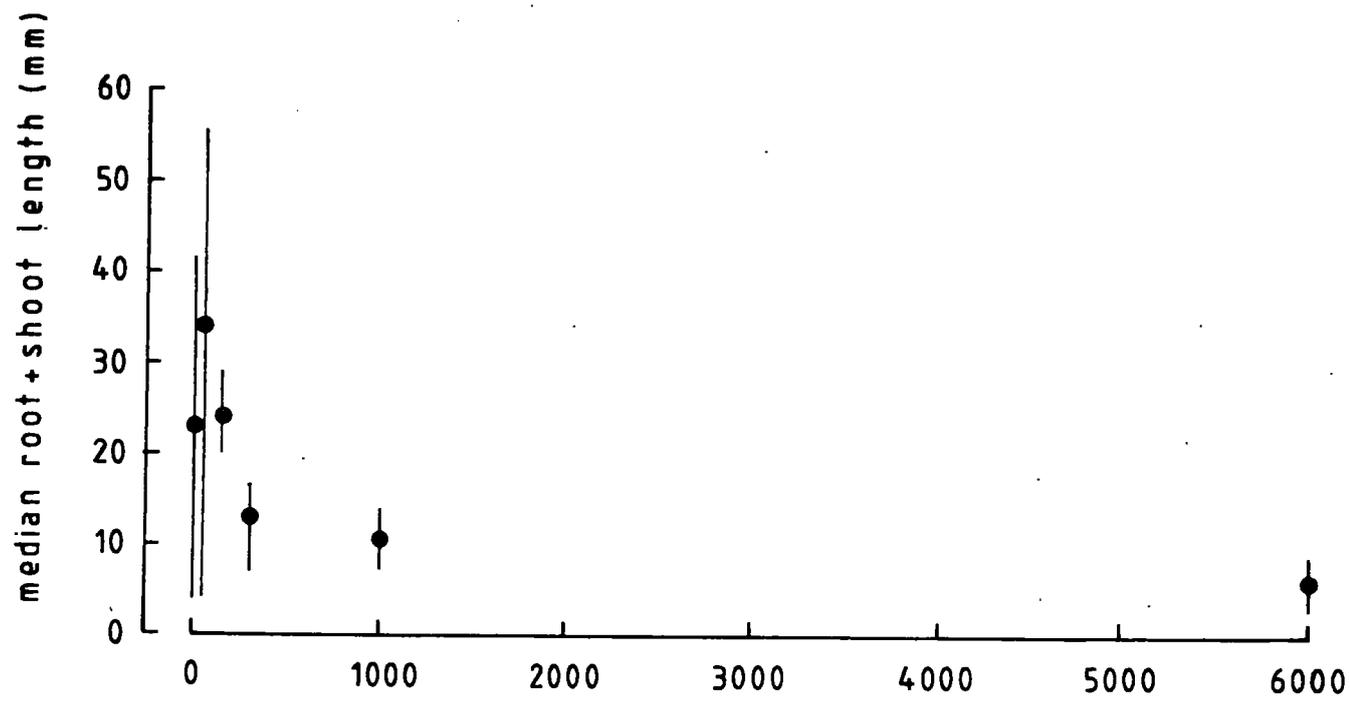
Only WL84811 concentrations of 300 mg l^{-1} or more inhibited seedling growth significantly although a reduction in the normal pink pigmentation of seedlings was noticed at all WL84811 concentrations greater than 50 mg l^{-1}.

4.23 *Lactuca sativa* (lettuce)

Lettuce seeds were germinated in WL84811, at concentrations ranging from 0 to 6000 mg l^{-1}. Percentage germination, root and shoot length were measured after five days growth and median values calculated for each treatment (Fig. 4.4).

WL84811 reduced both shoot and root length of germinated seedlings significantly (P < 0.001 for both) and also percentage germination (0.01 > P > 0.001).

Shoot length: 0 > 50 > 150 > 300 > 1000 > (3000, 6000) mg l^{-1} WL84811

Root length: 0 > 50 > 150 > (300, 1000, 3000) > 6000 mg l^{-1} WL84811

Percentage germination: (150, 50, 0, 300, 1000) > 3000 > 6000 mg l^{-1} WL84811.

All concentrations of WL84811 tested inhibited root and shoot growth of germinated lettuce seeds significantly (Figs 4.5, 4.6) but only the highest concentrations inhibited seed germination. All concentrations of WL84811 caused browning of the root tips and 3000 or 6000 mg l^{-1} WL84811 caused the roots to become totally brown and quite soft.

4.24 *Pisum sativum* (pea)

Pea seeds were germinated in WL84811 concentrations of 0 to 6000 mg l^{-1}. After four days, root plus shoot length and percentage germination were measured and median values calculated for each treatment (Fig. 4.7).

WL84811 had a significant effect on root plus shoot length of germinated seeds (P < 0.001) but not on percentage germination (0.5 > P > 0.1).

Fig. 4.4a Effect of WL84811 on median shoot length of germinated *Lactuca sativa* seedlings after five days growth, with upper and lower quartiles.

Fig. 4.4b Effect of WL84811 on median root length of germinated *Lactuca sativa* seedlings after five days growth, with upper and lower quartiles.

Fig. 4.4c Effect of WL84811 on median percentage germination of *Lactuca sativa* seeds after five days, with upper and lower quartiles.

Fig. 4.5 Seedling of *Lactuca sativa* after five days growth on water. Scale bar = 5 mm.

c = cotyledons

Fig. 4.6 Seedling of *Lactuca sativa* after five days growth on 3000 mg l^{-1} WL84811. Scale bar = 1 mm. Cells appear to have been prevented from elongating.

98
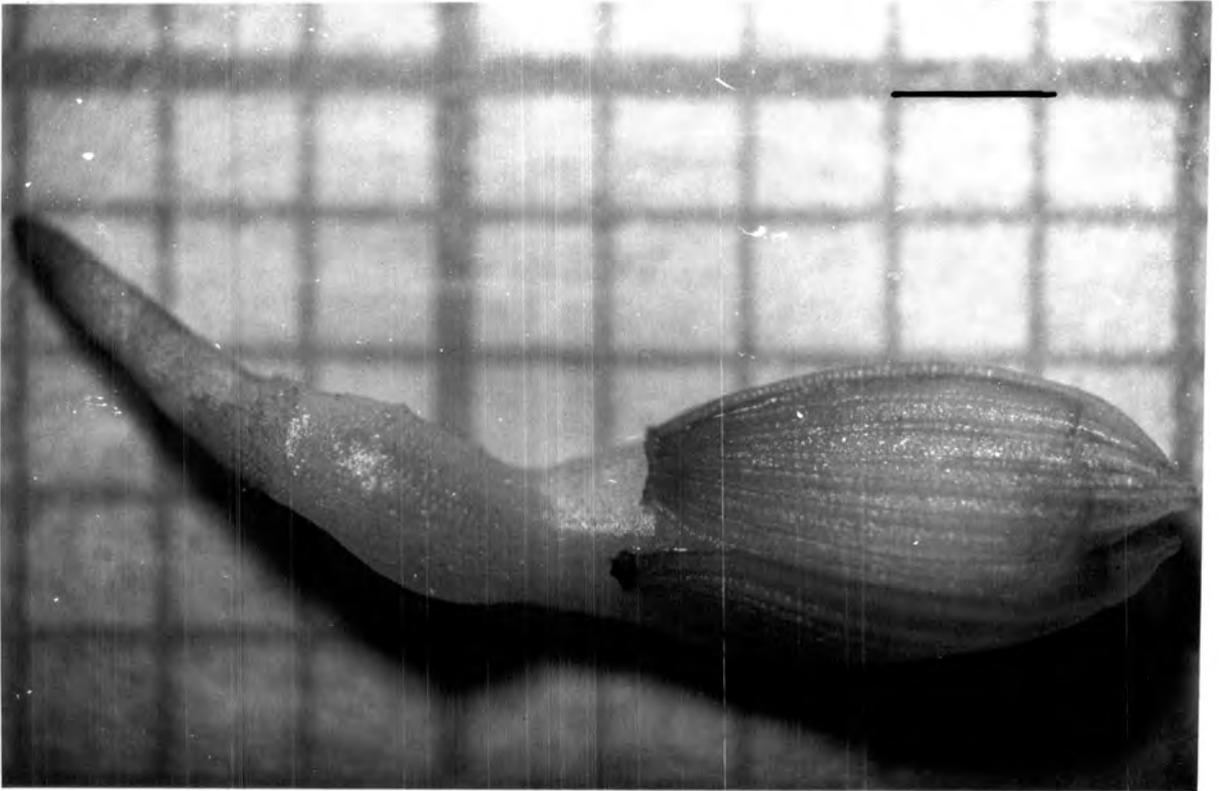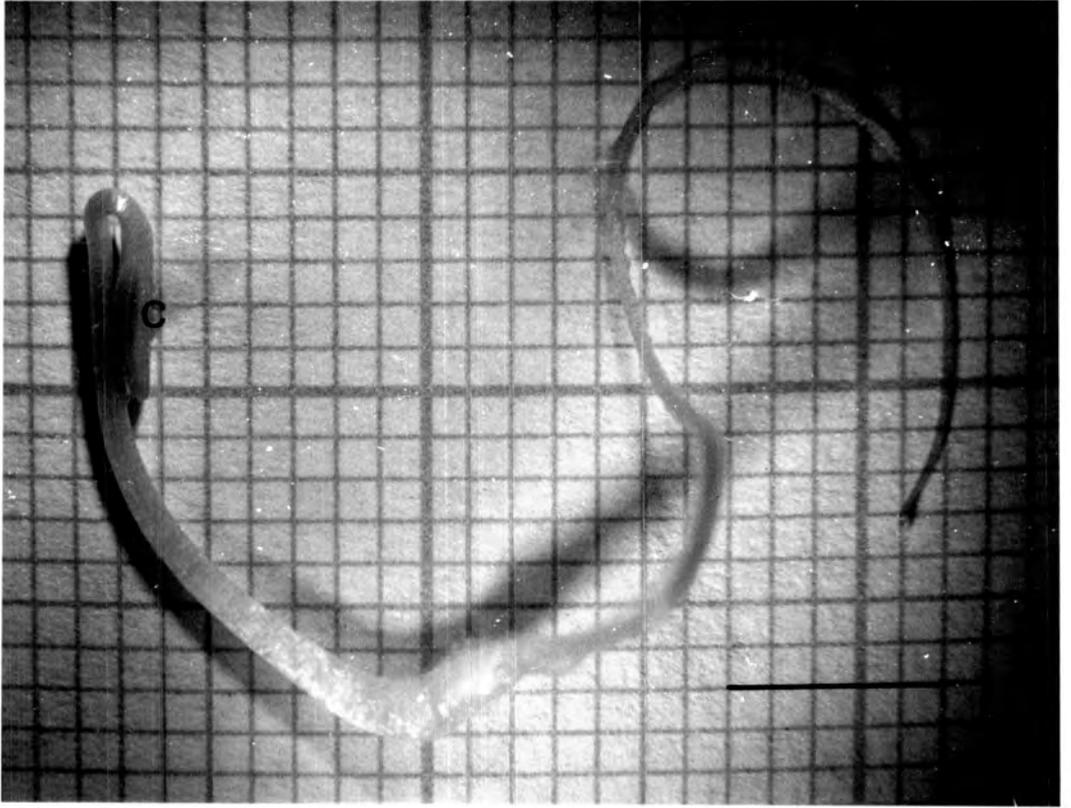
Fig. 4.7a Effect of WL84811 on median root plus shoot length of germi-
nated *Pisum sativum* seedlings after four days growth. with upper and lower
quartiles.

Fig. 4.7b Effect of WL84811 on median percentage germination of *Pisum
sativum* seeds after four days, with upper and lower quartiles.

99
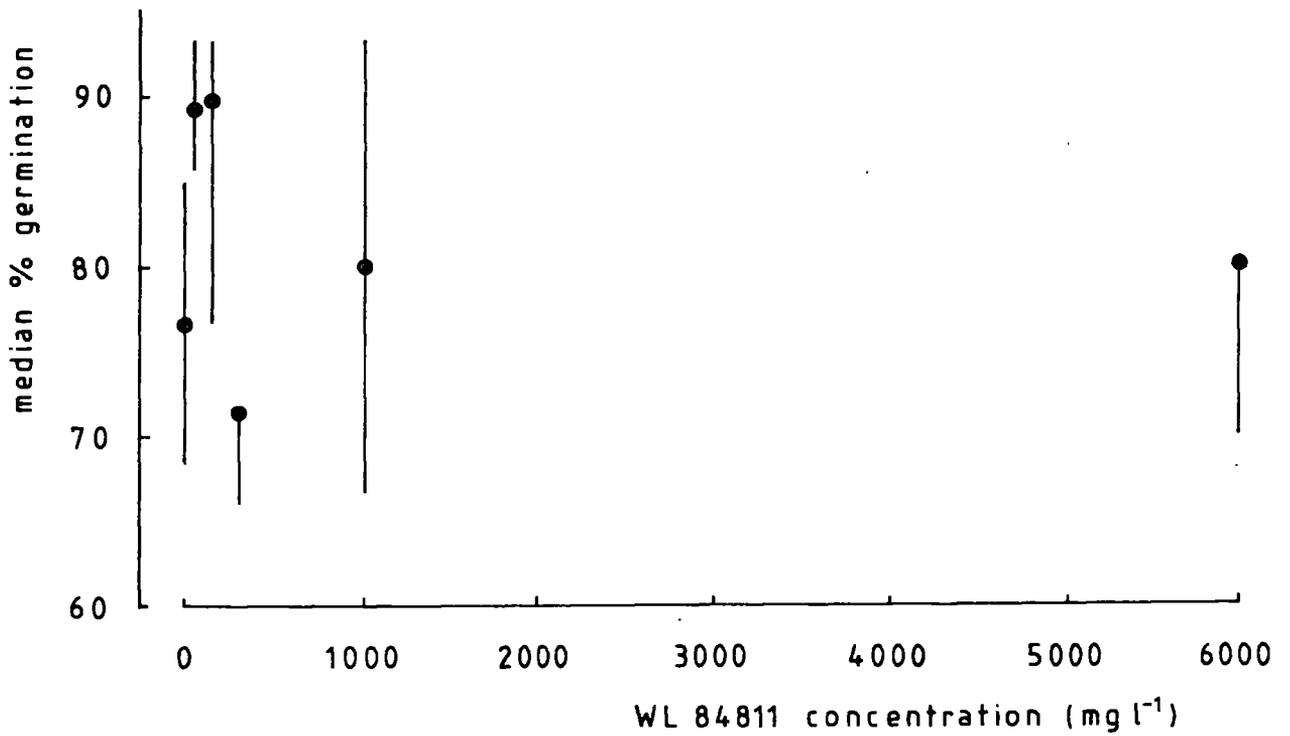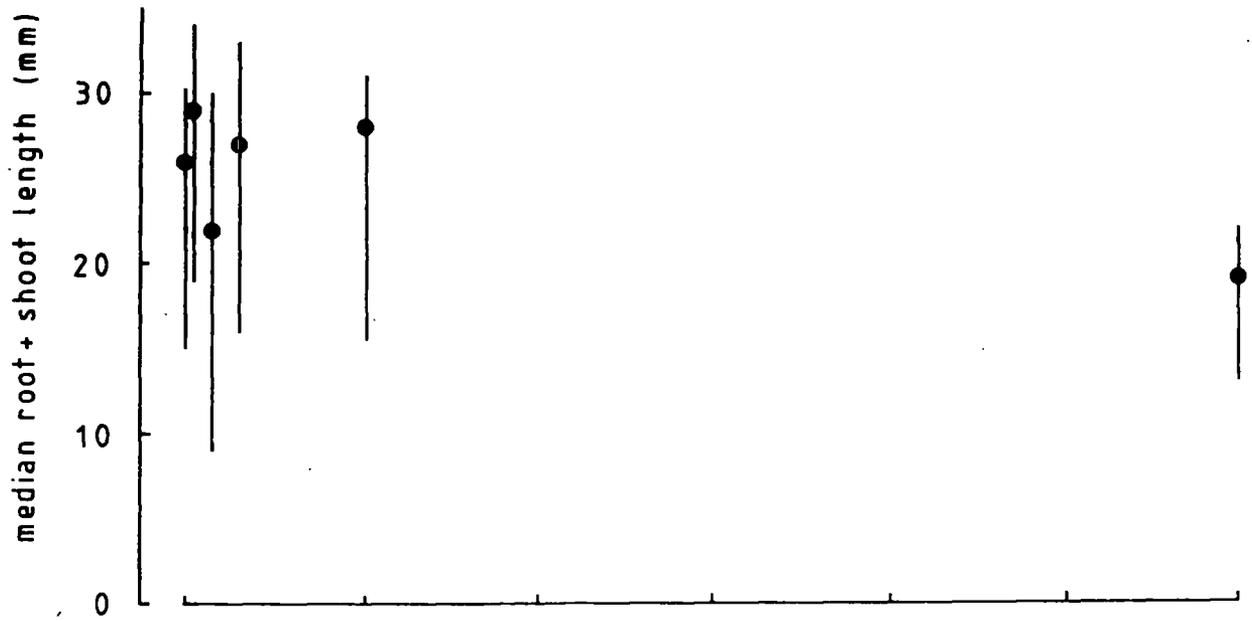
Root + shoot length: $(50, 1000, 300) > (1000, 300, 0, 150) > 6000$ mg l^{-1} WL84811.

Only the highest concentration of WL84811 reduced seedling growth of germinated pea seeds significantly; the lowest concentration actually enhanced growth significantly and all other concentrations had no significant effect.

4.25 *Allium cepa* (onion)

Onion seeds were germinated in WL84811, at concentrations ranging from 0 to 3000 mg l^{-1}. Percentage germination, root and shoot length were measured after eight days growth and median values calculated for each treatment (Fig. 4.8).

WL84811 reduced significantly both shoot and root length of those seeds which had germinated ($P < 0.001$ in both cases) but did not have a significant effect on percentage germination ($0.5 > P > 0.1$).

Root and shoot length: $0 > 50 > 150 > (1000, 3000)$.

All concentrations of WL84811 tested inhibited shoot and root length of germinated seeds significantly. Coleoptiles also failed to green up at WL84811 concentrations greater than 150 mg l^{-1} and at 1000 mg l^{-1} and 3000 mg l^{-1} both roots and coleoptiles were often soft and brown in colour.

4.26 *Hordeum vulgare* (barley) cv. Natalie, Protidor and Sonja

cv. Natalie (Fig. 4.9): WL84811 inhibited coleoptile and root growth of germinated seeds significantly ($P < 0.001$ for both) but had no significant effect on percentage germination ($0.9 > P > 0.5$).

Coleoptile length: $(0, 50) > 150 > 300 > 1000 > 3000$ mg l^{-1} WL84811

Root length: $0 > 50 > (150, 300) > 1000 > 3000$ mg l^{-1} WL84811.

100

Fig. **4.8a** Effect of WL84811 on median shoot length of germinated *Allium cepa* seedlings after eight days growth, with upper and lower quartiles.

Fig. **4.8b** Effect of WL84811 on median root length of germinated *Allium cepa* seedlings after eight days growth, with upper and lower quartiles.

Fig. **4.8c** Effect of WL84811 on median percentage germination of *Allium cepa* seeds after eight days, with upper and lower quartiles.

Fig. 4.9a Effect of WL84811 on median coleoptile length of germinated *H. vulgare* cv. Natalie seedlings after five days growth, with upper and lower quartiles.

Fig. 4.9b Effect of WL84811 on median root length of germinated *H. vulgare* cv. Natalie seedlings after five days growth, with upper and lower quartiles.

Fig. 4.9c Effect of WL84811 on median percentage germination of *H. vulgare* cv. Natalie seeds after five days, with upper and lower quartiles.
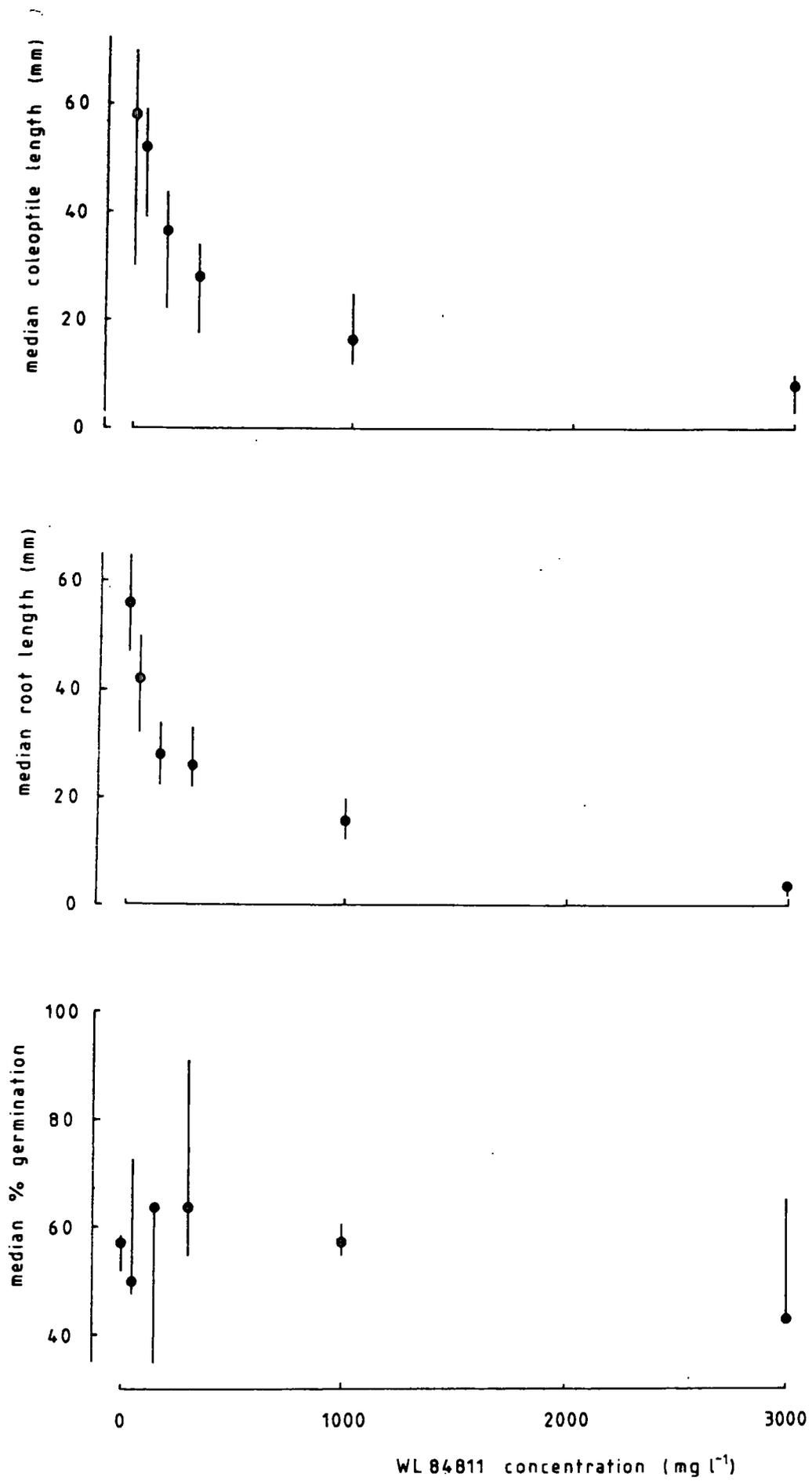
All concentrations of WL84811 used inhibited root growth of germinated Natalie seedlings significantly and all except for the lowest concentration also inhibited coleoptile growth significantly.

cv. Protidor (Fig. 4.10): WL84811 inhibited coleoptile and root growth of germinated seeds significantly ($P < 0.001$ for both) but had no significant effect on percentage germination ($0.1 > P > 0.05$).

Coleoptile length: $0 > 150 > 300 > 1000 > (3000, 6000)$ mg l^{-1} WL84811

Root length: $0 > 150 > 300 > 1000 > 3000 > 6000$ mg l^{-1} WL84811.

All concentrations of WL84811 used inhibited both coleoptile and root growth of germinated Protidor seedlings significantly.

cv. Sonja (Fig. 4.11): WL84811 inhibited coleoptile and root growth of germinated seeds significantly ($P < 0.001$ for both) but had no significant effect on percentage germination ($0.5 > P > 0.1$).

Coleoptile length: $0 > (150, 300) > 1000 > (3000, 6000)$ mg l^{-1} WL84811

Root length: $0 > 150 > 300 > 1000 > 3000 > 6000$ mg l^{-1} WL84811.

All concentrations of WL84811 used inhibited both coleoptile and root growth of germinated Sonja seedlings significantly.

4.27 *Triticum aestivum* (wheat) cv. Banner, CWW 3547/1 and Hammer

cv. Banner (Fig. 4.12): WL84811 inhibited coleoptile and root growth of germinated seeds significantly ($P < 0.001$ for both) but had no significant effect on percentage germination ($0.1 > P > 0.05$).

Coleoptile and root length: $0 > 150 > 300 > 1000 > 3000 > 6000$ mg l^{-1} WL84811.

Fig. **4.10a** Effect of WL84811 on median coleoptile length of germinated *H. vulgare* cv. Protidor seedlings after five days growth, with upper and lower quartiles.

Fig. **4.10b** Effect of WL84811 on median root length of germinated *H. vulgare* cv. Protidor seedlings after five days growth, with upper and lower quartiles.

Fig. **4.10c** Effect of WL84811 on median percentage germination of *H. vulgare* cv. Protidor seeds after five days, with upper and lower quartiles.
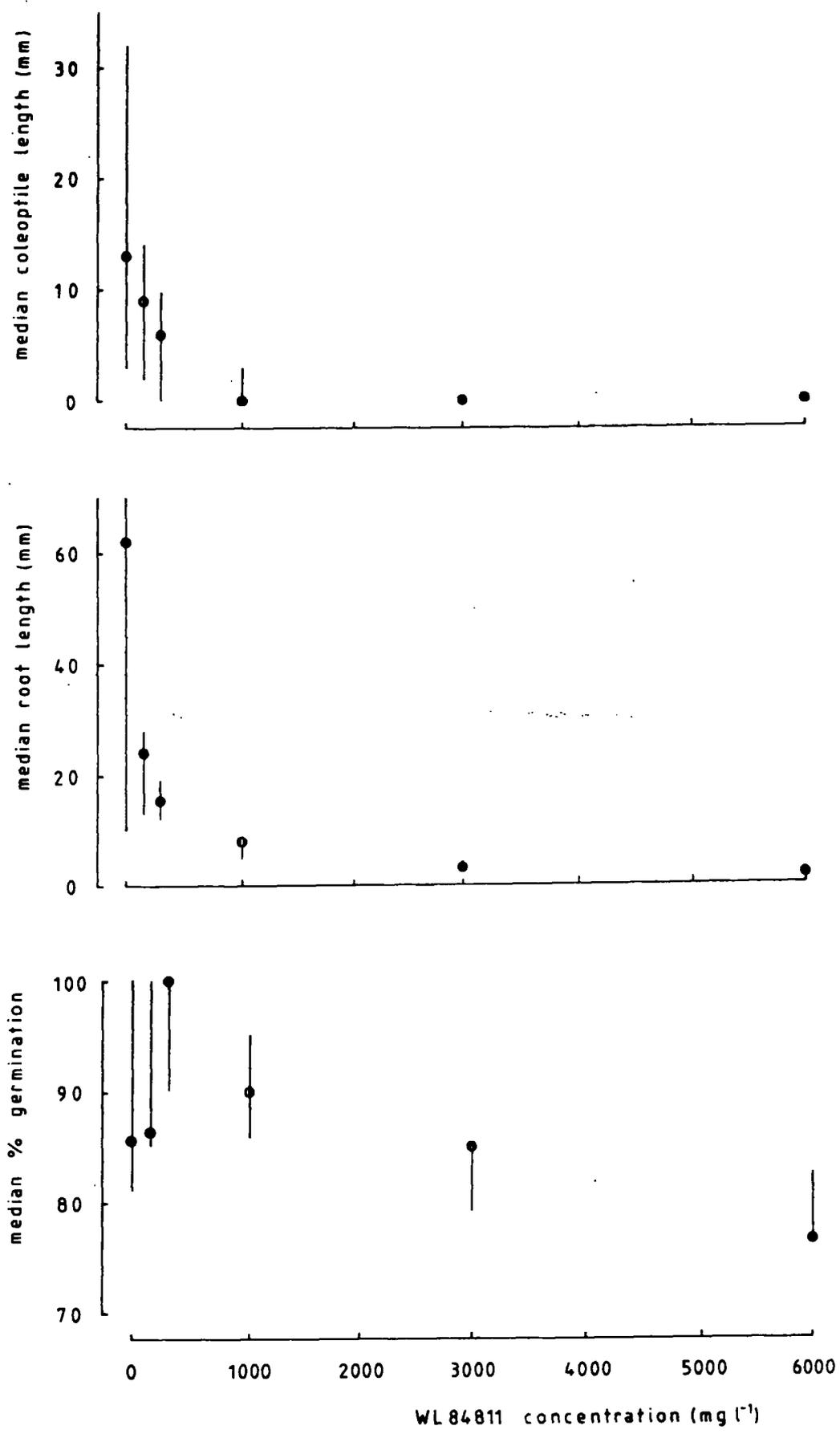
Fig. 4.11a Effect of WL84811 on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.
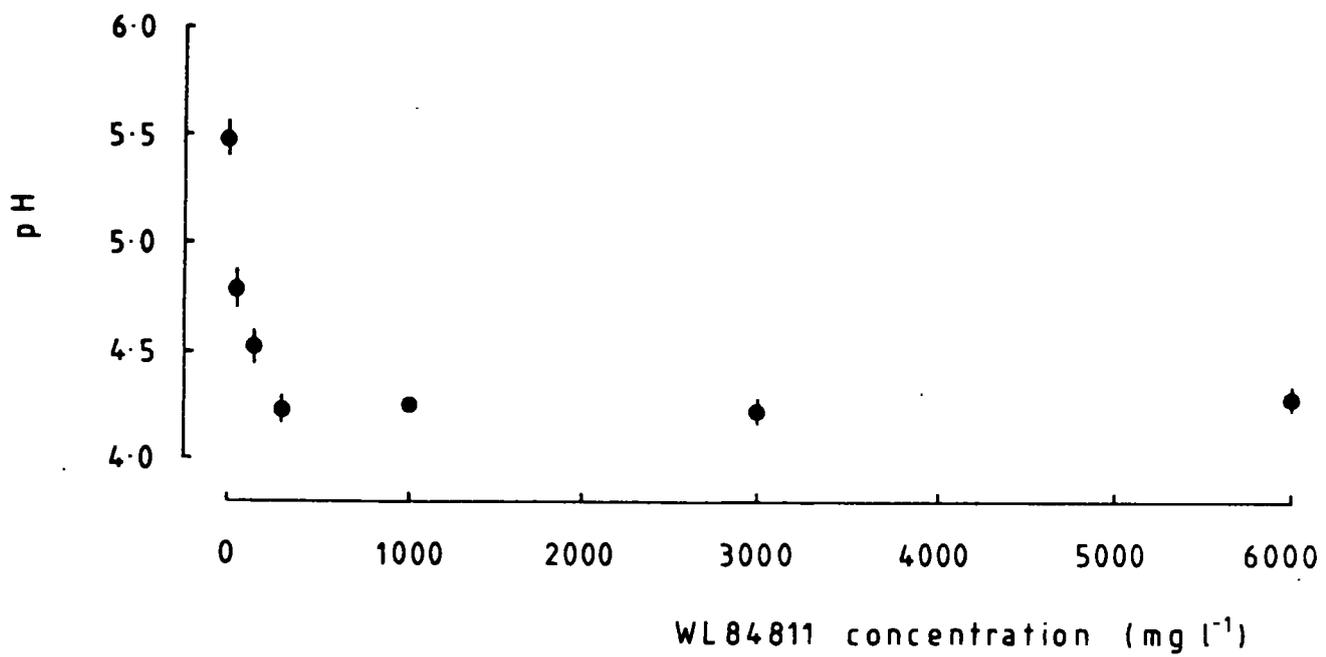
Fig. 4.11b Effect of WL84811 on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.11c Effect of WL84811 on median percentage germination of *H. vulgare* cv. Sonja seeds after five days, with upper and lower quartiles.
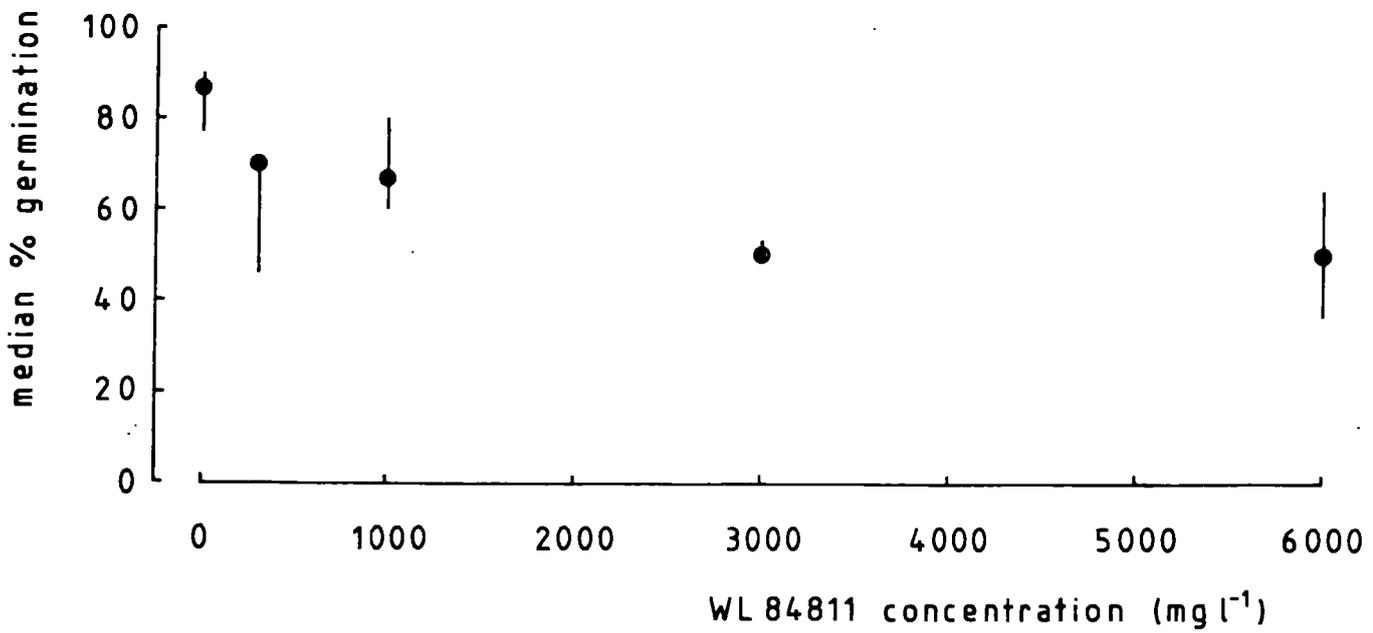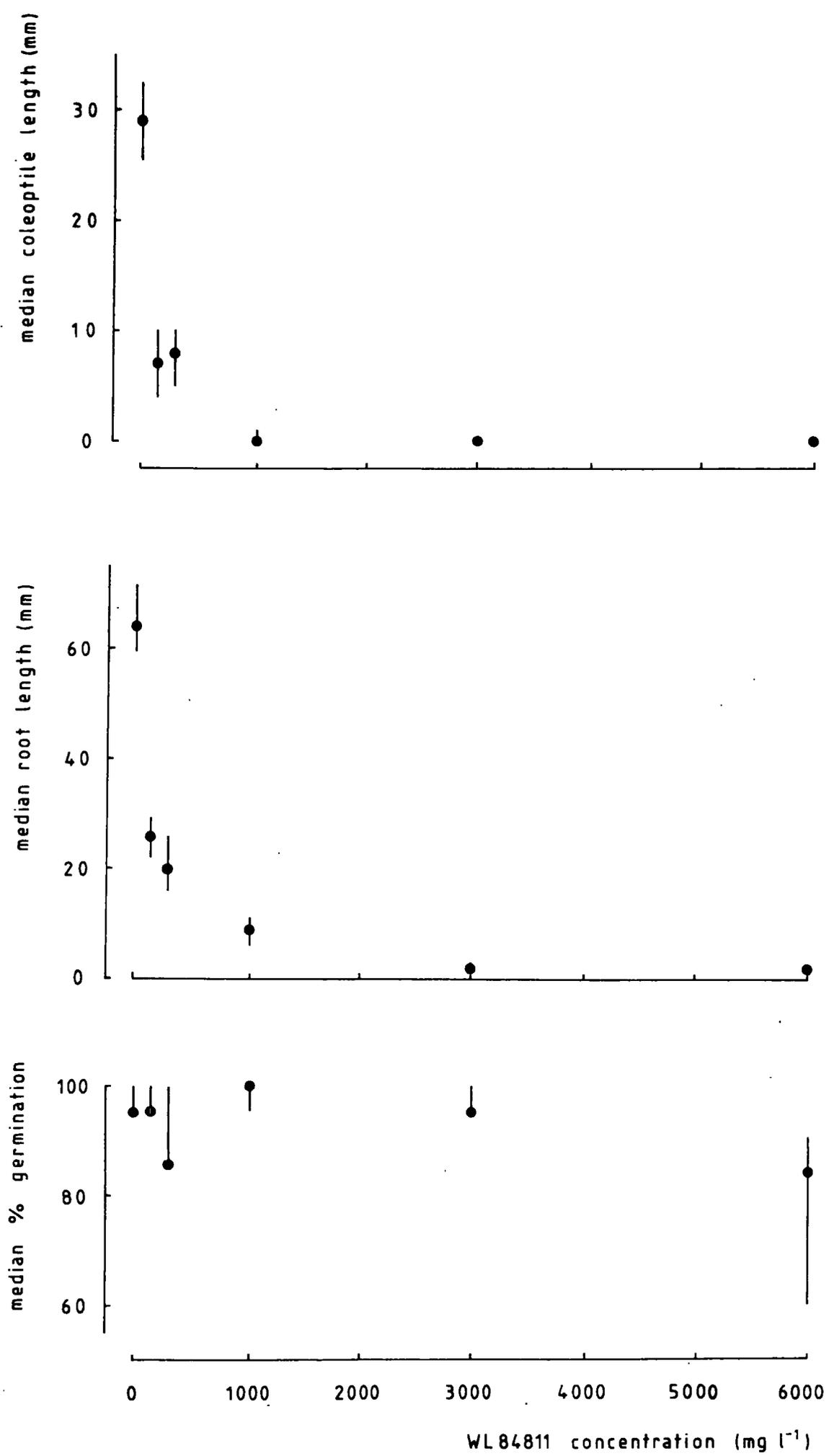
Fig. 4.12a Effect of WL84811 on median coleoptile length of germinated *T. aestivum* cv. Banner seedlings after five days growth, with upper and lower quartiles.

Fig. 4.12b Effect of WL84811 on median root length of germinated *T. aestivum* cv. Banner seedlings after five days growth, with upper and lower quartiles.

Fig. 4.12c Effect of WL84811 on median percentage germination of *T. aestivum* cv. Banner seeds after five days. with upper and lower quartiles.
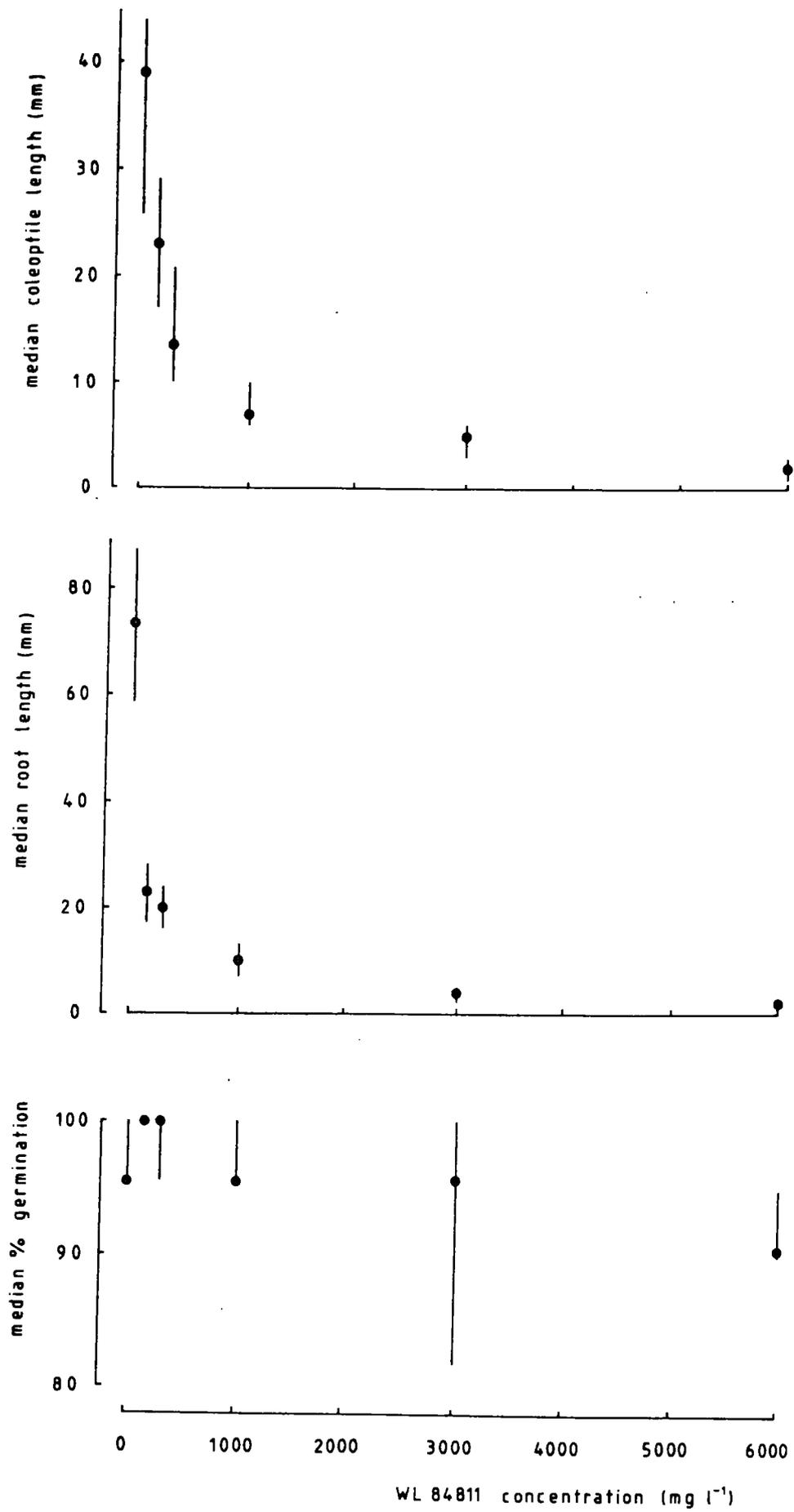
All concentrations of WL84811 used inhibited both coleoptile and root growth of germinated Banner seedlings significantly.

cv. CWW 3547/1 (Fig. 4.13): WL84811 inhibited coleoptile and root growth of germinated seeds significantly (P < 0.001 for both) and also percentage germination (0.05 > P > 0.001).

Coleoptile length: $0 > 150 > 300 > 1000 > 3000 > 6000$ mg 1^{-1} WL84811

Root length: $0 > (150, 300) > 1000 > 3000 > 6000$ mg 1^{-1} WL84811

Percentage germination: $0 > (300, 1000. 3000) > (3000, 150) > 6000$ mg 1^{-1} WL84811.

All concentrations of WL84811 used inhibited coleoptile and root growth of germinated CWW seedlings and percentage germination of seeds significantly.

cv. Hammer (Fig. 4.14): WL84811 inhibited coleoptile and root growth of germinated seeds significantly (P < 0.001 for both) but had no significant effect on percentage germination (0.5 > P > 0.1).

Coleoptile and root length: $0 > 150 > 300 > 1000 > 3000 > 6000$ mg 1^{-1} WL84811.

All concentrations of WL84811 used inhibited both coleoptile and root growth of germinated Hammer seedlings significantly.

4.28 *Secale cereale* (rye)

(Fig. 4.15): WL84811 inhibited coleoptile and root growth of germinated seeds significantly (P < 0.001 for both) but had no significant effect on percentage germination (0.5 > P > 0.1).

Coleoptile length: $(0, 50) > 150 > 300 > 1000 > 3000 > 6000$ mg 1^{-1} WL84811

Root length: $0 > 50 > 150 > 300 > 1000 > 3000 > 6000$ mg 1^{-1} WL84811.

Fig. 4.13a Effect of WL84811 on median coleoptile length of germinated *T. aestivum* cv. CWW 3547/1 seedlings after five days growth, with upper and lower quartiles.

Fig. 4.13b Effect of WL84811 on median root length of germinated *T. acstivum* cv. CWW 3547/1 seedlings after five days growth, with upper and lower quartiles.

Fig. 4.13c Effect of WL84811 on median percentage germination of *T. aestivum* cv. CWW 3547/1 seeds after five days, with upper and lower quartiles.
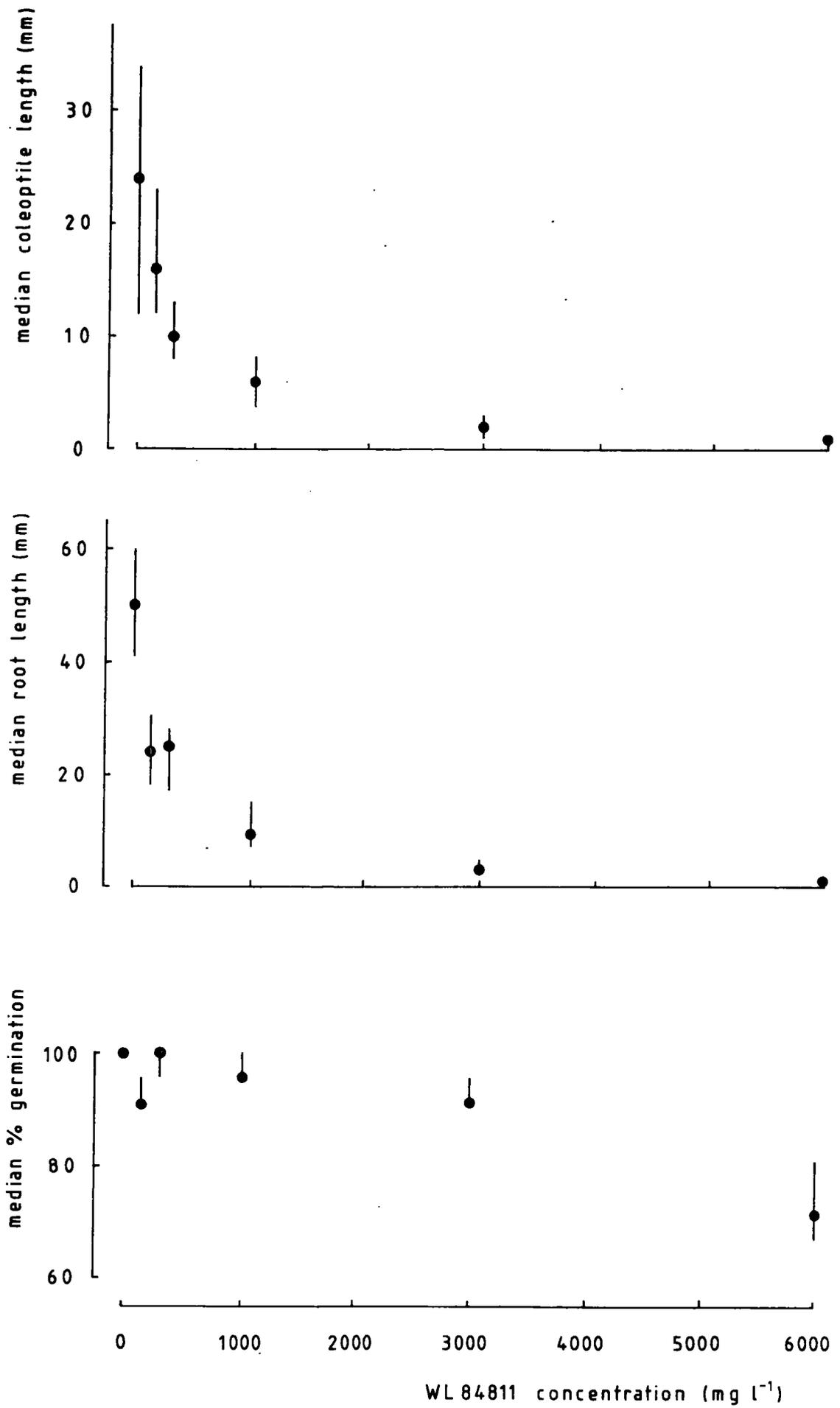
Fig. 4.14a Effect of WL84811 on median coleoptile length of germinated *T. aestivum* cv. Hammer seedlings after five days growth, with upper and lower quartiles.

Fig. 4.14b Effect of WL84811 on median root length of germinated *T. aestivum* cv. Hammer seedlings after five days growth, with upper and lower quartiles.

Fig. 4.14c Effect of WL84811 on median percentage germination of *T. aestivum* cv. Hammer seeds after five days, with upper and lower quartiles.
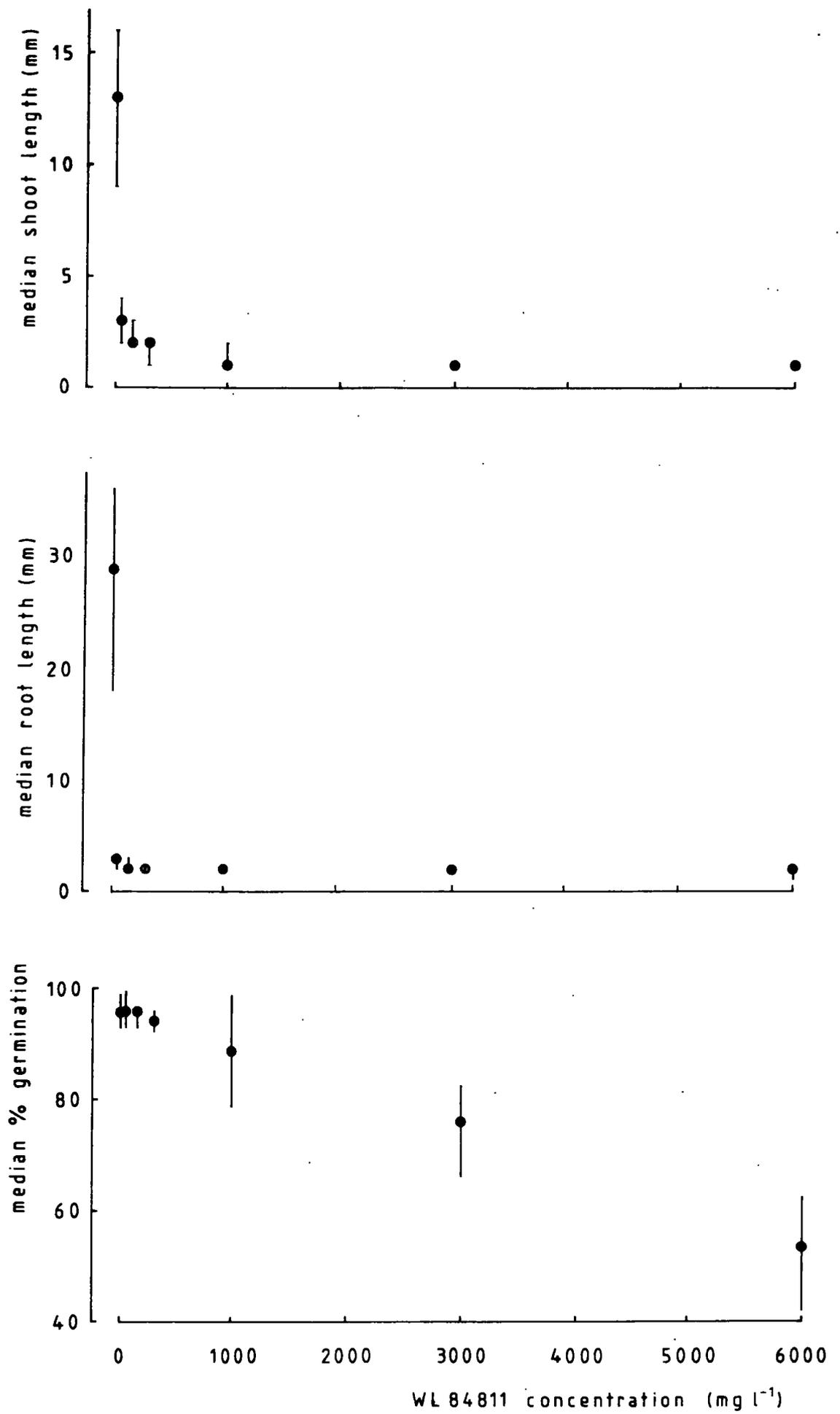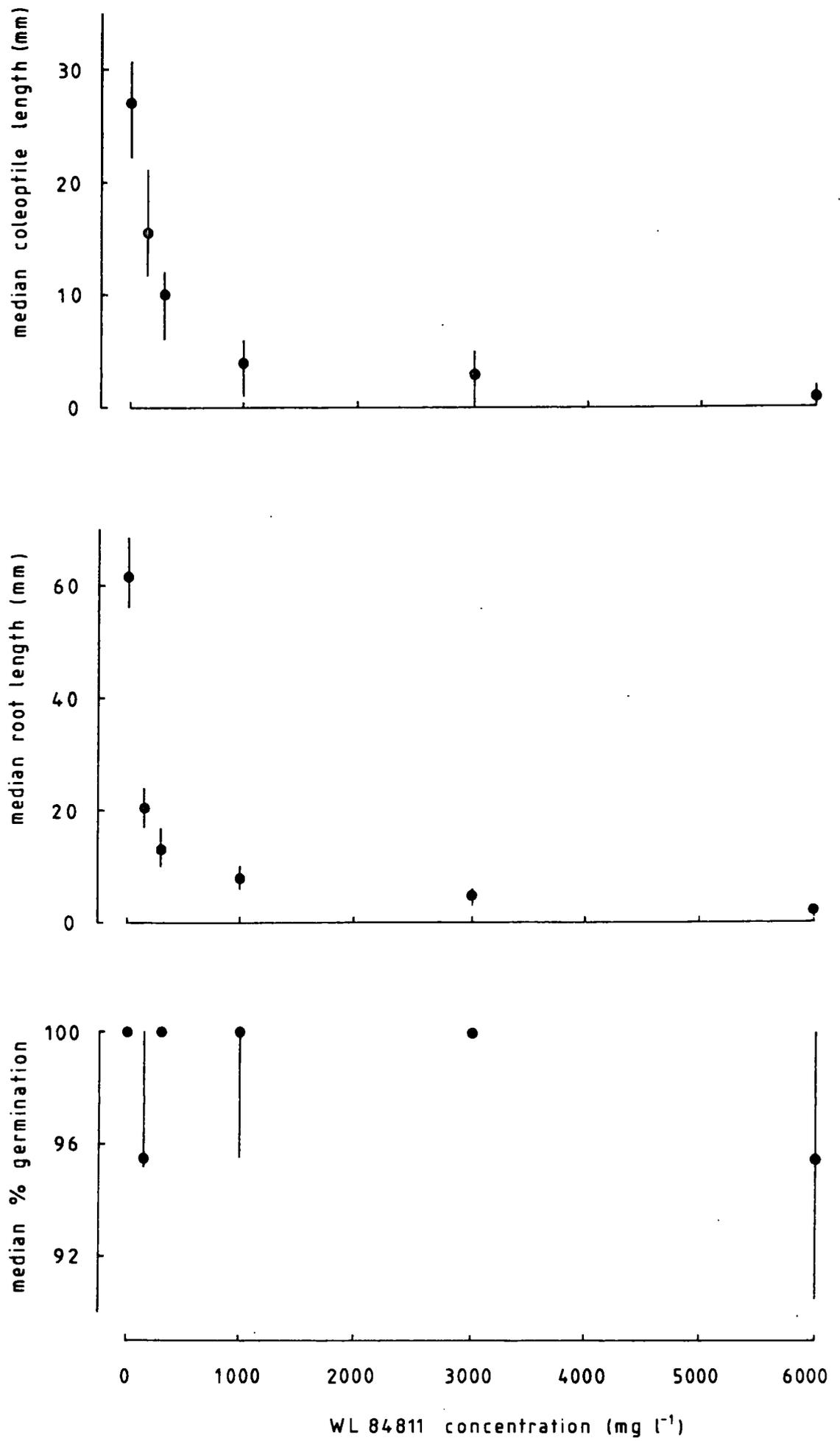
Fig. 4.15a Effect of WL84811 on median coleoptile length of germinated *Secale cereale* seedlings after five days growth, with upper and lower quartiles.

Fig. 4.15b Effect of WL84811 on median root length of germinated *Secale cereale* seedlings after five days growth, with upper and lower quartiles.

Fig. 4.15c Effect of WL84811 on median percentage germination of *Secale cereale* seeds after five days, with upper and lower quartiles.
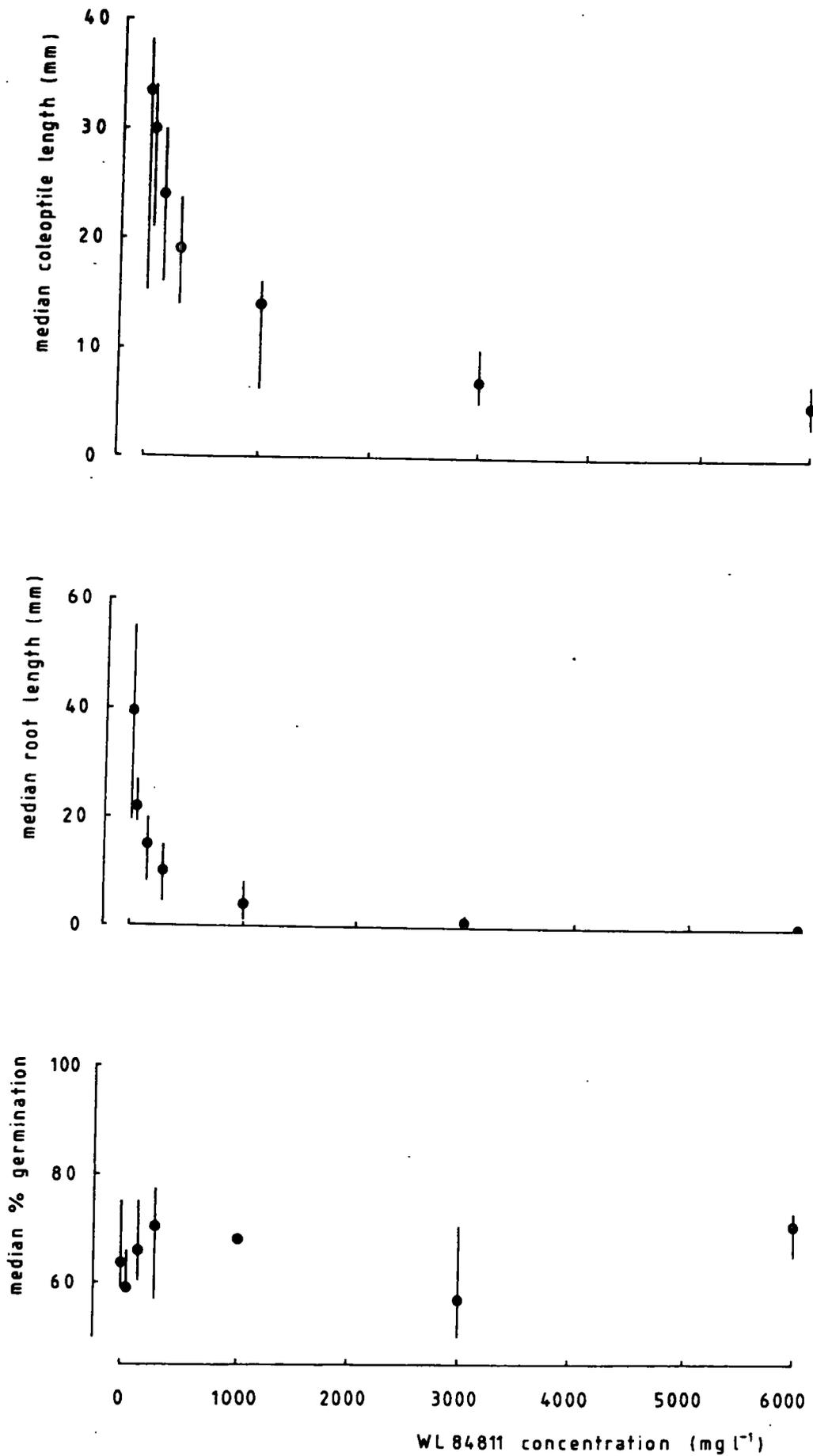
All concentrations of WL84811 used inhibited root growth of germinated rye seedlings significantly and all concentrations greater than 50 mg l^{-1} also inhibited coleoptile growth significantly.

4.29 *Oryza sativa* (rice)

cv. IR36 (Fig. 4.16): WL84811 inhibited coleoptile and root growth of germinated seeds significantly ($P < 0.001$ for both) and also percentage germination of seeds ($0.05 > P > 0.01$).

> **Coleoptile length**: (50, 0) > 150 > 300 > 1000 mg l^{-1} WL84811
>
> **Root length**: 0 > 50 > (150, 300, 1000) mg l^{-1} WL84811
>
> **Percentage germination**: (50, 300, 150, 0) > (300, 150, 0, 1000) mg l^{-1} WL84811.

All concentrations of WL84811 used inhibited root growth of germinated rice seedlings significantly and all concentrations greater than 50 mg l^{-1} also inhibited coleoptile growth significantly. The effect on percentage germination was not so clear; only the 50 mg l^{-1} and 1000 mg l^{-1} WL84811 treatments were significantly different from one another and neither of these differed significantly from the control.

WL84811 affected seed germination significantly in only two cereals, wheat cultivar CWW 3547/1 and rice cv. IR36. The barley cultivar Sonja was used for most subsequent experiments because of the highly significant differences it exhibited under different treatments and the lack of variability under a given treatment.

Fig. 4.16a Effect of WL84811 on median coleoptile length of germinated *Oryza sativa* cv. IR36 seedlings after five days growth, with upper and lower quartiles.
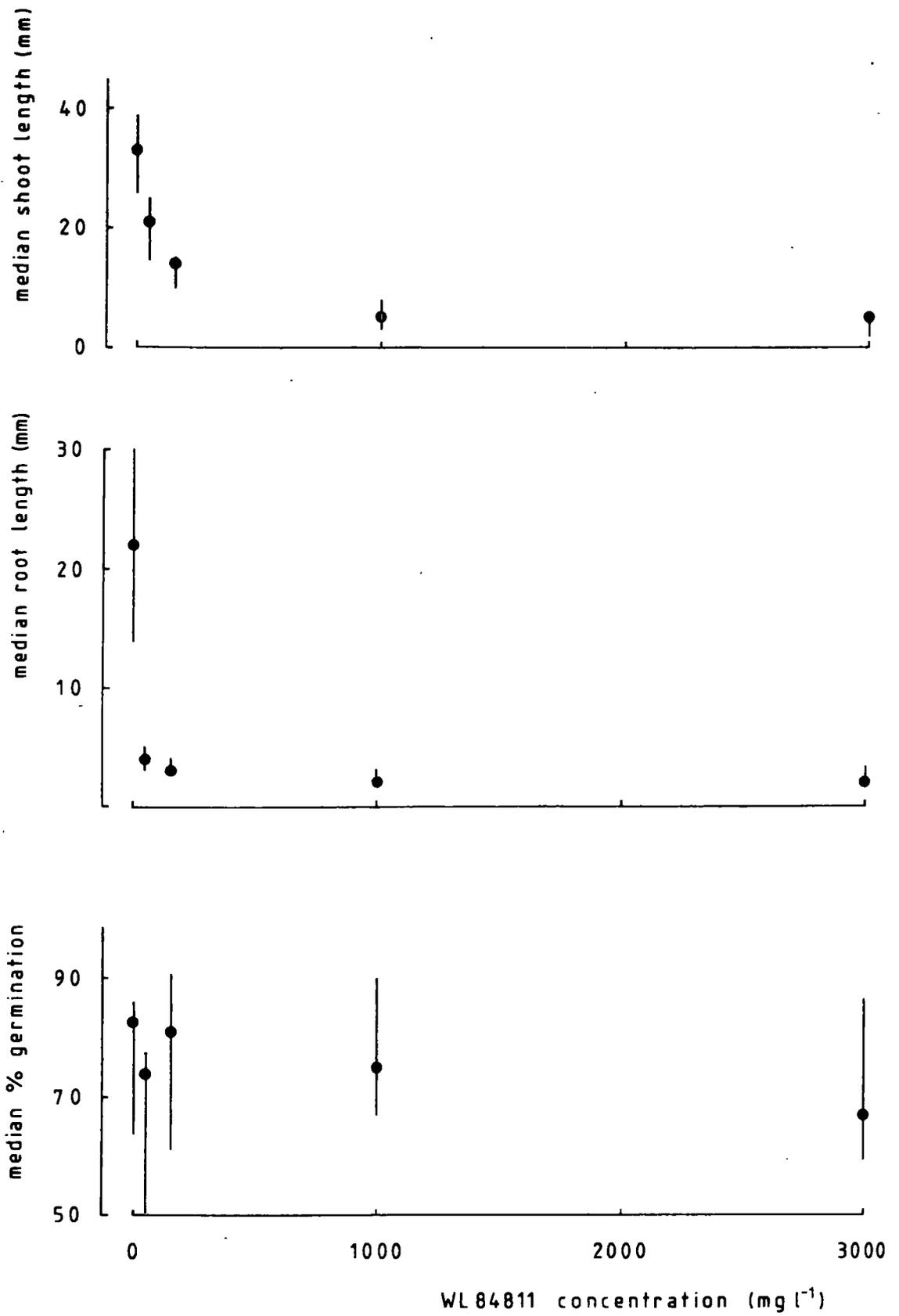
Fig. 4.16b Effect of WL84811 on median root length of germinated *Oryza sativa* cv. IR36 seedlings after five days growth, with upper and lower quartiles.

Fig. 4.16c Effect of WL84811 on median percentage germination of *Oryza sativa* cv. IR36 seeds after five days, with upper and lower quartiles.
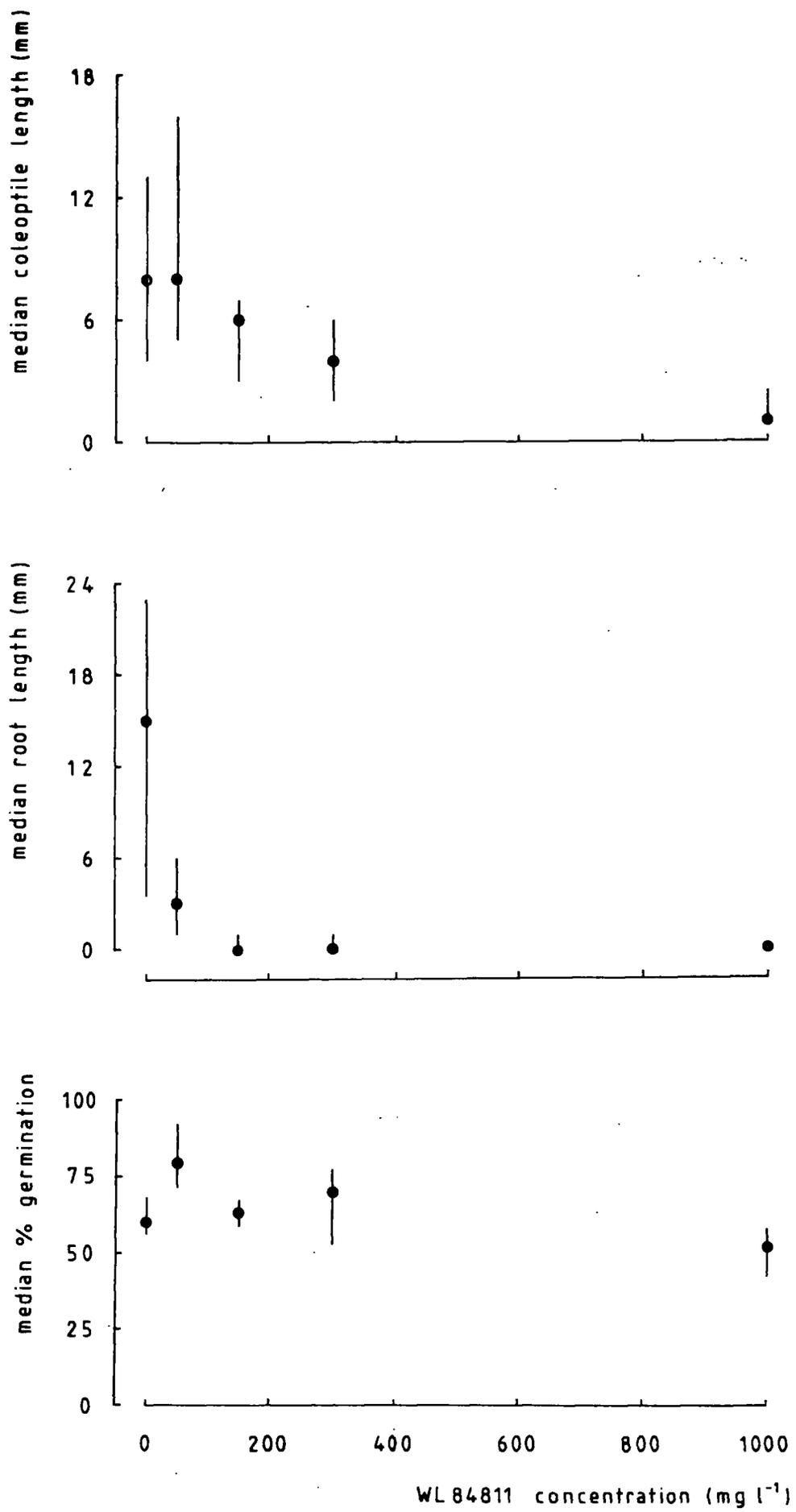
4.3 The effect of WL84811 on rate of germination of barley cv. Sonja seeds

The effect of WL84811 on rate of germination. root and coleoptile growth was studied using the barley cultivar Sonja. Percentage germination, root and coleoptile length at three WL84811 concentrations were measured at daily intervals after plating out the imbibed seeds.

WL84811 affected only the rate of root growth and not the onset of growth (Fig. 4.17c). producing a series of lines with different gradients radiating from the same point. After 36 hours growth. only 3000 mg l^{-1} WL84811 reduced root growth significantly but after two days. 300 mg l^{-1} WL84811 also had a significant effect. With all treatments the rate of growth was greatest initially. levelling off slightly after three to four days.

WL84811 had an additional effect on coleoptile growth, delaying its onset (Fig. 4.17b). There was no significant difference between treatments after two days growth but after three days both 300 and 3000 mg l^{-1} WL84811 reduced growth significantly. The rate of coleoptile growth, unlike the rate of root growth, increased over the five days of the experiment at all concentrations of WL84811.

Mann-Whitney U-tests showed that whereas 300 mg l^{-1} WL84811 did not affect percentage germination significantly at any time. 3000 mg l^{-1} WL84811 did. but only during the first 36 hours of growth (Fig. 4.17a). After two, three, four and five days there was no significant difference between treatments.

Fig. 4.17a Effect of WL84811 on rate of germination of *H. vulgare* cv. Sonja seeds, with upper and lower quartiles.

● = 0 mg l^{-1} WL84811

O = 300 mg l^{-1} WL84811

■ = 3000 mg l^{-1} WL84811

Fig. 4.17b Effect of WL84811 on rate of coleoptile growth of germinated *H. vulgare* cv. Sonja seedlings, with upper and lower quartiles. Key as for Fig. 4.17a.

Fig. 4.17c Effect of WL84811 on rate of root growth of germinated *H. vulgare* cv. Sonja seedlings, with upper and lower quartiles. Key as for Fig. 4.17a.
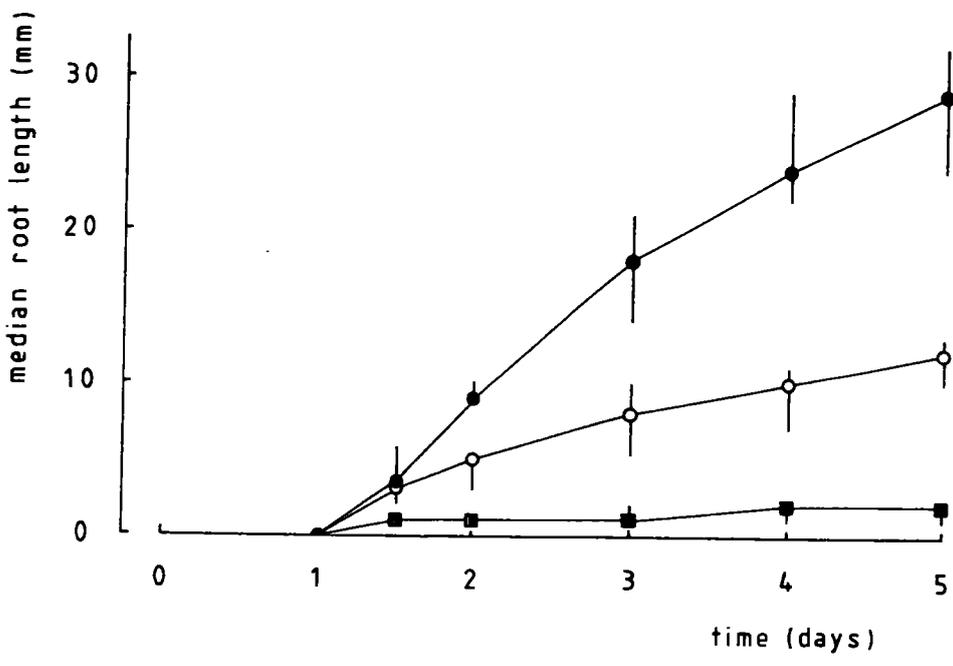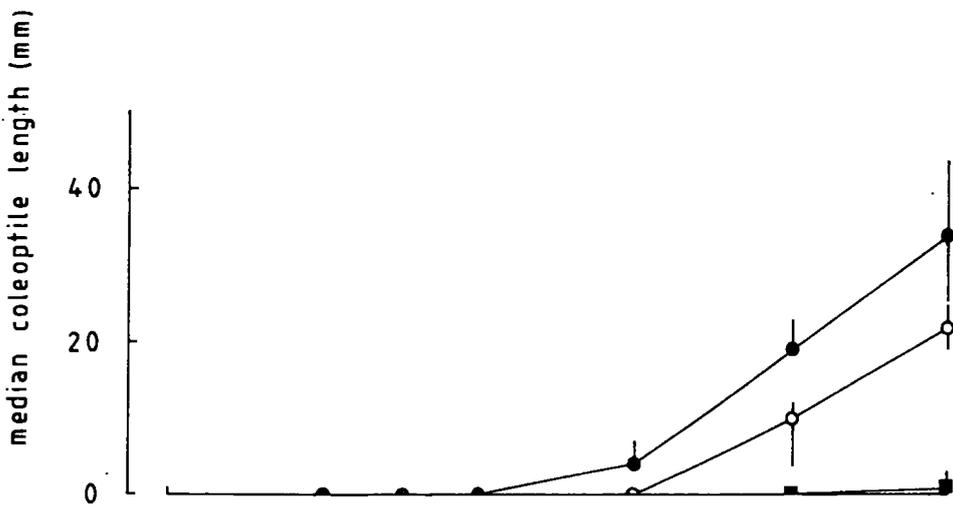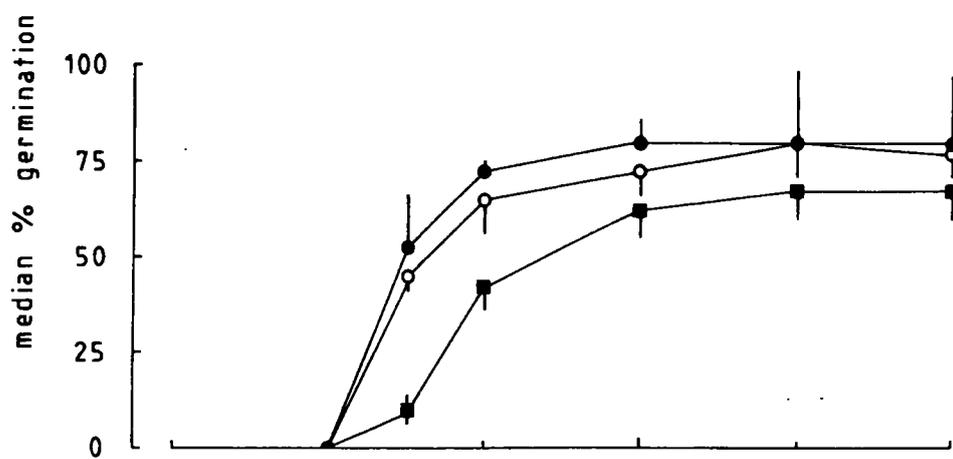
Plotting the coleoptile or root length at different concentrations of WL84811 as a percentage of the control growth (Fig. 4.18) allowed the estimation of that WL84811 concentration which reduced growth by 50 %. For root growth, values around 260 mg l^{-1} were obtained whether growth after three, four or five days was considered although much higher concentrations were required to cause similar inhibition at the earliest stages (1980 mg l^{-1} at 36 hours, 650 mg l^{-1} at 2 days). The effect of a given WL84811 concentration thus increased during the early stages of growth.

For coleoptile growth however, the effect of a given WL84811 concentration decreased with time during the experiment; after three days 150 mg l^{-1} WL84811 reduced growth by 50 %, after four days 440 mg l^{-1} was required and after five days, 940 mg l^{-1}.

4.4 The effect of adding WL84811 to Sonja seedlings after two days growth

Sonja seeds were imbibed and plated out on filter paper soaked in distilled water in the usual way. After two days, percentage germination and seedling growth were measured. Half the seedlings were then replaced on filter paper soaked in water and the other half placed on filter paper soaked in 6000 mg l^{-1} WL84811. Germination and growth were measured after a further three days and the two sub-sets of seedlings compared.

Fig. 4.18a Coleoptile growth of *H. vulgare* cv. Sonja seeds germinating in the presence of WL84811 as a percentage of growth in the absence of WL84811.

● = after 5 days

O = after 4 days

■ = after 3 days

Fig. 4.18b Root growth of *H. vulgare* cv. Sonja seeds germinating in the presence of WL84811 as a percentage of growth in the absence of WL84811.

● = after 5 days

O = after 4 days

■ = after 3 days

□ = after 2 days

▲ = after 36 hours

WL 84811 concentration (mg l⁻¹)
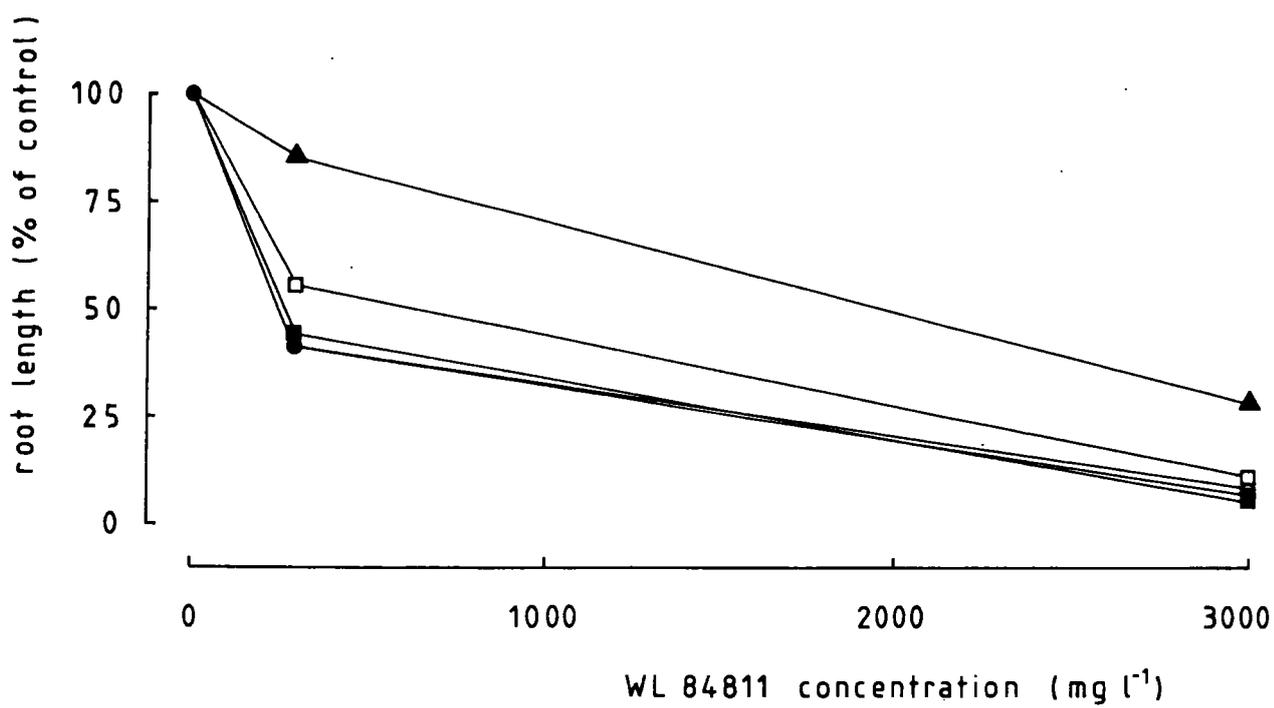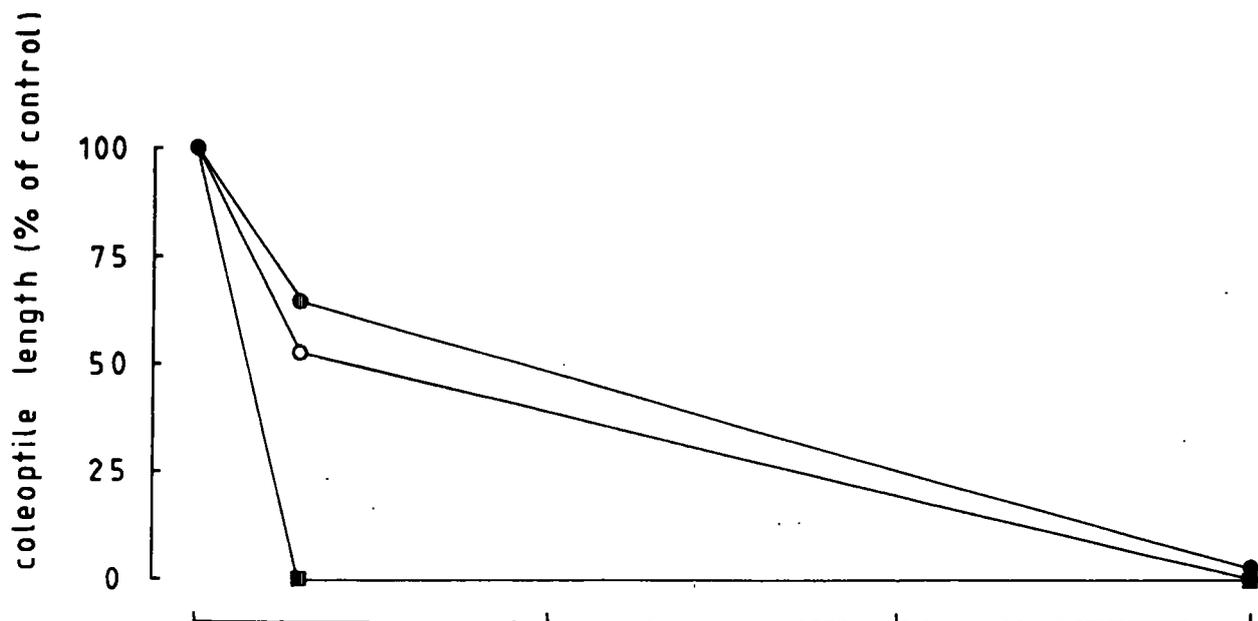
After the initial two days on water. median percentage germination, root and coleoptile length of the two sets of seedlings were not significantly different. After three further days with half the seedlings on WL84811 however, there were significant differences in root and coleoptile length of germinated seeds between treatments. The seeds which had remained on water throughout had significantly longer coleoptiles and roots.

Coleoptile length: 0 mg l^{-1}/5 days > 6000 mg l^{-1}/5 days > 0 mg l^{-1}/2 days.

Root length: 0 mg l^{-1}/5 days > (0 mg l^{-1}/2 days, 6000 mg l^{-1}/5 days).

6000 mg l^{-1} WL84811 thus prevented the roots from growing significantly over the latter three days of the experiment.

Percentage germination of the two groups was not significantly different after five days (0.5 > P > 0.1).

4.5 The effect of repeated stem injection with WL84811 on seed viability

Ears of *T. acstivum* cv. Mercia were injected repeatedly with WL84811 at intervals after pollination: 30 ears were injected with 0.1 ml of 3000 mg l^{-1} WL84811 two days after pollination, 25 of these also at four days. 20 at seven days. 15 at 10 days. 10 at 14 days and five at 21 days. The ears were allowed to ripen for 12 weeks before harvesting and dried in the laboratory for a further three weeks before being germinated in the usual way. After five days. percentage germination. root and coleoptile length were measured and median values calculated for each treatment (Fig. 4.19).

Repeated injection of ears with WL84811 during seed set had a significant effect on the growth of seedlings from those seeds (P < 0.001 for both coleoptile and root growth) but not on their germination (0.5 > P > 0.1).

Fig. 4.19a Effect of repeated injection of *T. aestivum* cv. Mercia plants with 0.1 ml of 3000 mg l^{-1} WL84811 (after pollination) on median coleoptile length of seeds produced, after five days growth. Upper and lower quartiles are attached.

Fig. 4.19b Effect of repeated injection of *T. aestivum* cv. Mercia plants with 0.1 ml of 3000 mg l^{-1} WL84811 (after pollination) on median root length of seeds produced, after five days growth. Upper and lower quartiles are attached.

Fig. 4.19c Effect of repeated injection of *T. aestivum* cv. Mercia plants with 0.1 ml of 3000 mg l^{-1} WL84811 (after pollination) on median percentage germination of seeds produced, after five days. Upper and lower quartiles are attached.
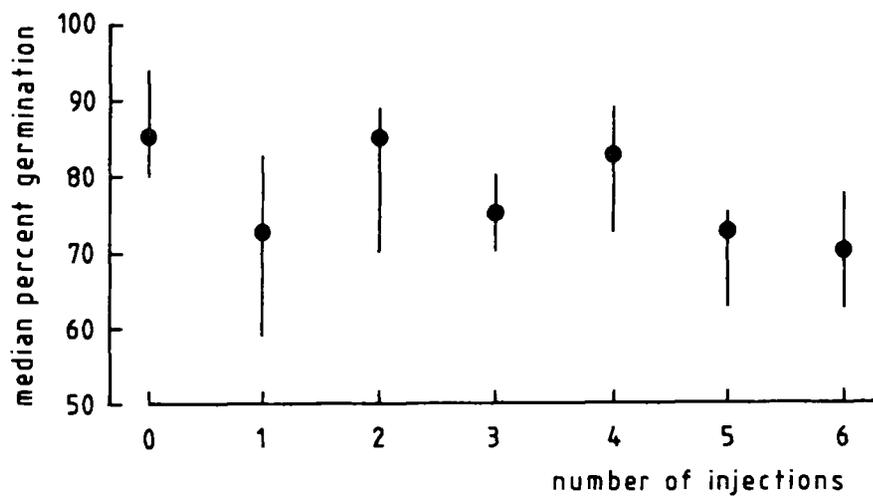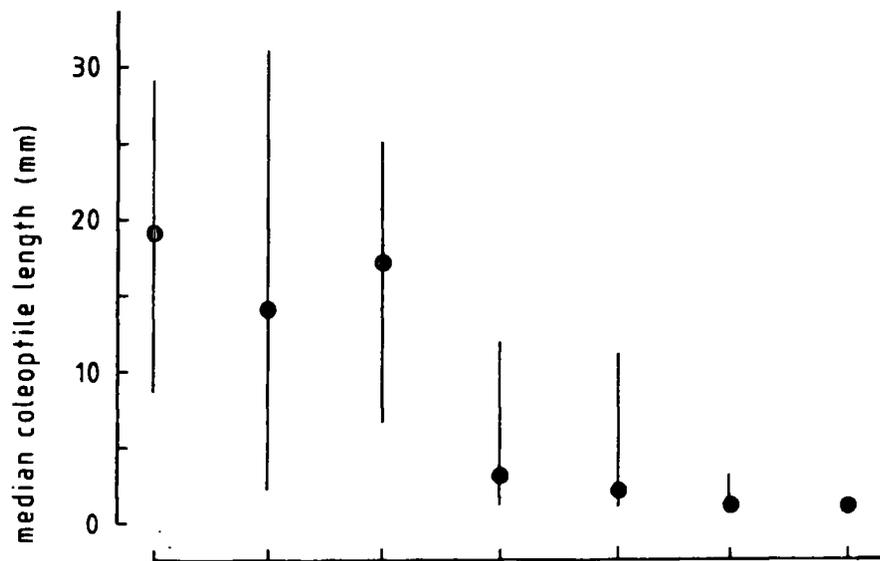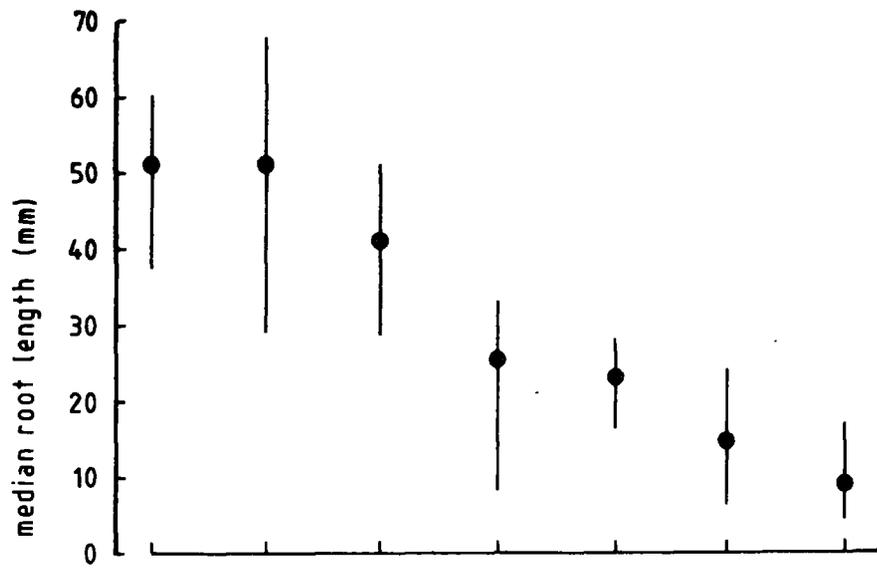
Coleoptile length: (0,1,2) > (3,4) > 5 > 6 injections

Root length: (1,0) > 2 > (3,4) > 5 > 6 injections.

(0·1ml of 3000mg l⁻¹)

Only twice the standard dose of WL84811 applied at this stage subsequently inhibited the root growth of seedlings significantly and three times the standard dose also inhibited coleoptile growth. More work is necessary to ascertain whether this problem only occurs with WL84811 applied after pollination. If this dose applied at the normal time (booting) also inhibits seedling growth, it could pose a serious problem; when WL84811 is applied by foliar spraying it is not unlikely that some plants will receive twice the standard dose.

4.6 The effect of proline, with and without WL84811, on Sonja seeds

The effect of a range of concentrations of proline on Sonja germination and growth was studied prior to determining whether it could alleviate the effects of WL84811 on seedling growth. Seeds were germinated on 0 to 3000 mg l⁻¹ proline, the seedlings harvested and measured after five days. Median values for coleoptile and root length and percentage germination were calculated for each treatment (Fig. 4.20).

Proline affected both coleoptile and root growth of germinated seeds significantly (P < 0.001 for both) but had no significant effect on percentage germination of seeds (0.5 > P > 0.1).

Coleoptile length: 50 > (150, 0, 3000) > (0, 3000, 1000) > (3000, 1000, 300) mg l⁻¹ proline

Root length: 50 > (0, 150) > (3000, 1000, 300) mg l⁻¹ proline.

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Fig. 4.20a Effect of proline on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.20b Effect of proline on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.20c Effect of proline on median percentage germination of *H. vulgare* cv. Sonja seeds after five days, with upper and lower quartiles.
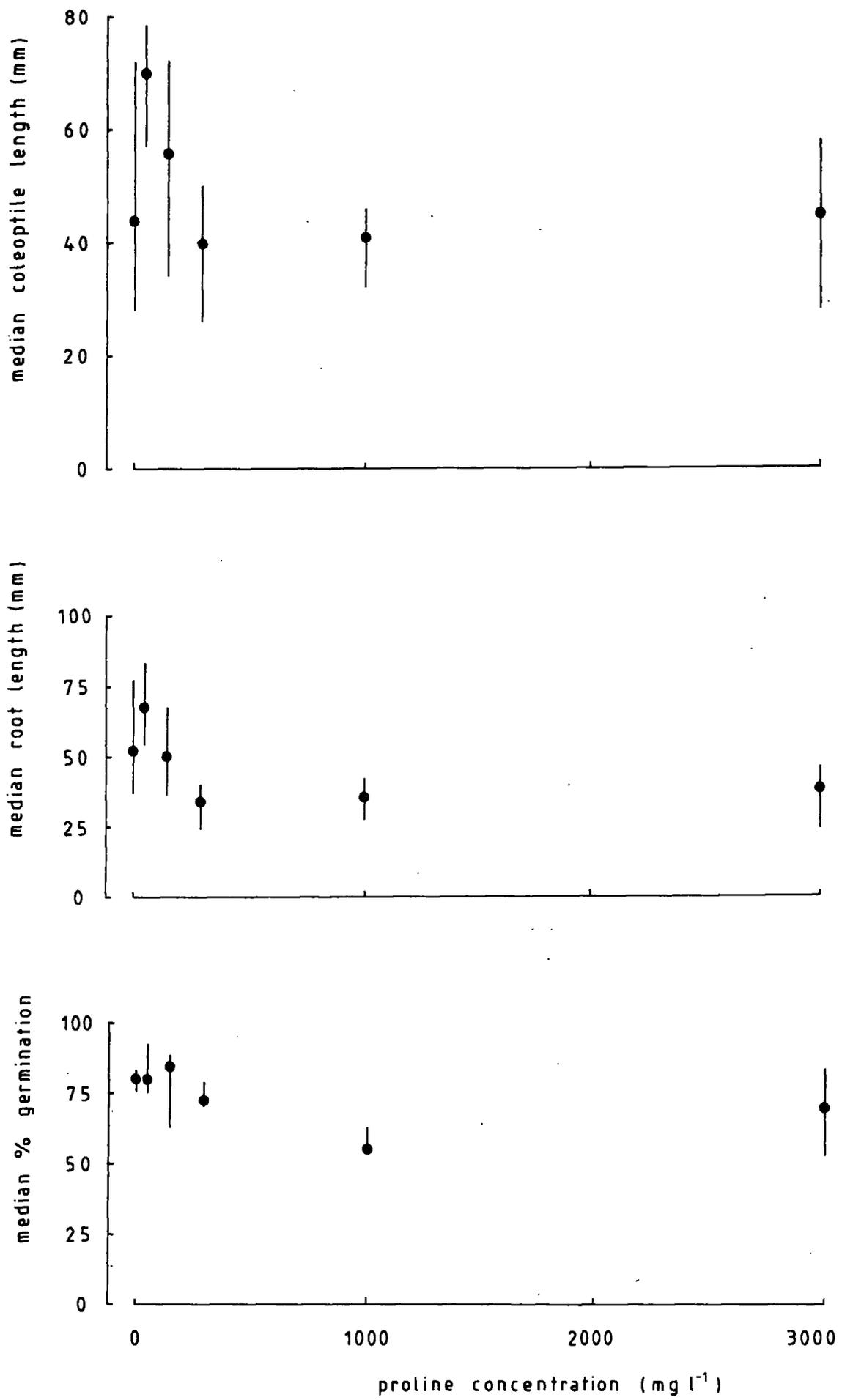
Although proline affected both coleoptile and root growth significantly, the magnitude of that effect was not directly related to the proline concentration. 50 mg l^{-1} proline enhanced both coleoptile and root growth of germinated seedlings relative to the control while higher concentrations (300 mg l^{-1} or more) reduced root growth significantly. Root growth appeared to be more sensitive than coleoptile growth to high concentrations of proline.

Proline was also supplied in conjunction with equimolar concentrations of WL84811 ranging from 0 to 6000 mg l^{-1} and the effect compared with that of WL84811 alone. Median values for coleoptile and root length and percentage germination were calculated after five days growth (Fig. 4.21).

WL84811 reduced coleoptile and root growth of germinated seeds significantly, whether or not equimolar proline was present (P < 0.001 in all cases). In addition, WL84811 had no significant effect on percentage germination, irrespective of the presence or absence of proline.

Coleoptile length: (0, 150 W+P) > (150 W+P, 50 W) > (50 W, 300 W+P, 50 W+P, 150 W, 300 W) > 1000 W+P > 1000 W > 3000 W+P > 3000 W > 6000 W+P > 6000 W

Root length: 0 > (50 W, 50 W+P, 300 W) > (50 W+P, 300 W, 150 W, 150 W+P) > (150 W+P, 300 W+P) > 1000 W+P > 1000 W > 3000 W+P > 3000 W > 6000 W+P > 6000 W

(all concentrations in mg l^{-1}; W=WL84811, P=proline).

At low concentrations of WL84811, equimolar proline did not affect its activity against coleoptile growth significantly and at 300 mg l^{-1} appeared to act synergistically with WL84811 on root growth, increasing the inhibition. At higher WL84811 concentrations however (1000 – 6000 mg l^{-1}), equimolar proline did significantly reduce the inhibition of both coleoptile and root growth by WL84811.

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Fig. 4.21a Effect of WL84811 plus equimolar proline on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

● = WL84811 alone

○ = WL84811 + equimolar proline

Fig. 4.21b Effect of WL84811 plus equimolar proline on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles. Key as for Fig. 4.21a.

Fig. 4.21c Effect of WL84811 plus equimolar proline on median percentage germination of *H. vulgare* cv. Sonja seeds after five days, with upper and lower quartiles. Key as for Fig. 4.21a.
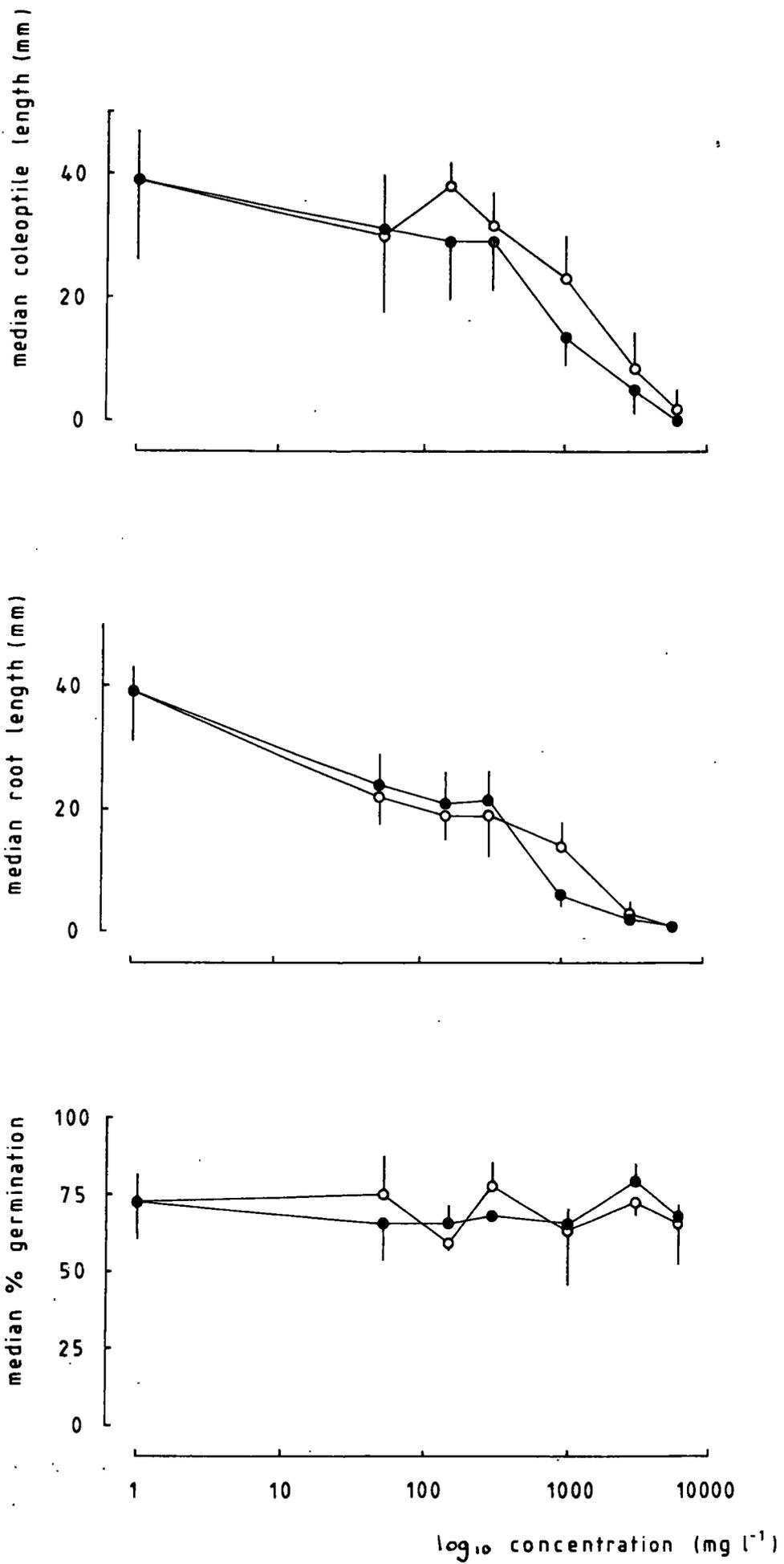
High concentrations of proline were also supplied in conjunction with low concentrations of WL84811 and median values of coleoptile and root length and percentage germination determined after five days growth. The treatments used affected both coleoptile and root length significantly but had no effect on percentage germination.

Coleoptile length: 0 > (50 W + 1000 P, 50 W + 500 P, 50 W) > 150 W + 1000 P > 150 W

Root length: (50 W, 0, 50 W + 1000 P) > (50 W + 500 P, 150 W) > (150 W, 150 W + 1000 P).

Even 1000 mg l^{-1} proline was unable to alleviate the effect of 50 mg l^{-1} WL84811 on coleoptile growth significantly but the same concentration was able to significantly reduce the effect of 150 mg l^{-1} WL84811. The effect on root growth was also complex; 50 mg l^{-1} WL84811 reduced growth relative to the control significantly only when present in conjunction with 500 mg l^{-1} proline. At higher concentrations (150 mg l^{-1}) WL84811 did reduce root growth significantly and this inhibition was not significantly altered by the presence of 1000 mg l^{-1} proline.

4.7 The effect of IAA, with and without WL84811, on Sonja seeds

The effect of a range of IAA concentrations on Sonja germination and growth was studied before determining whether it could overcome, wholly or in part, the effect of WL84811. Seeds were germinated in 10^{-9} to 10^{-3} M IAA, harvested and measured after five days growth. Median coleoptile and root lengths and percentage germination were calculated (Fig. 4.22).

IAA had a significant effect on both coleoptile and root growth of germinated seeds (P < 0.001 for both) but not on percentage germination (0.9 > P > 0.5).

Coleoptile length: $(10^{-4}, 10^{-5}, 10^{-9}) > (0, 10^{-3}, 10^{-8}, 10^{-7}) > 10^{-6}$ M IAA

Root length: $(10^{-8}, 10^{-5}, 10^{-7}) > (10^{-7}, 10^{-6}, 10^{-9}, 0, 10^{-4}) > (0, 10^{-4}, 10^{-3})$ M IAA.

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Fig. 4.22a Effect of IAA on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.22b Effect of IAA on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.22c Effect of IAA on median percentage germination of *H. vulgare* cv. Sonja seeds after five days, with upper and lower quartiles.
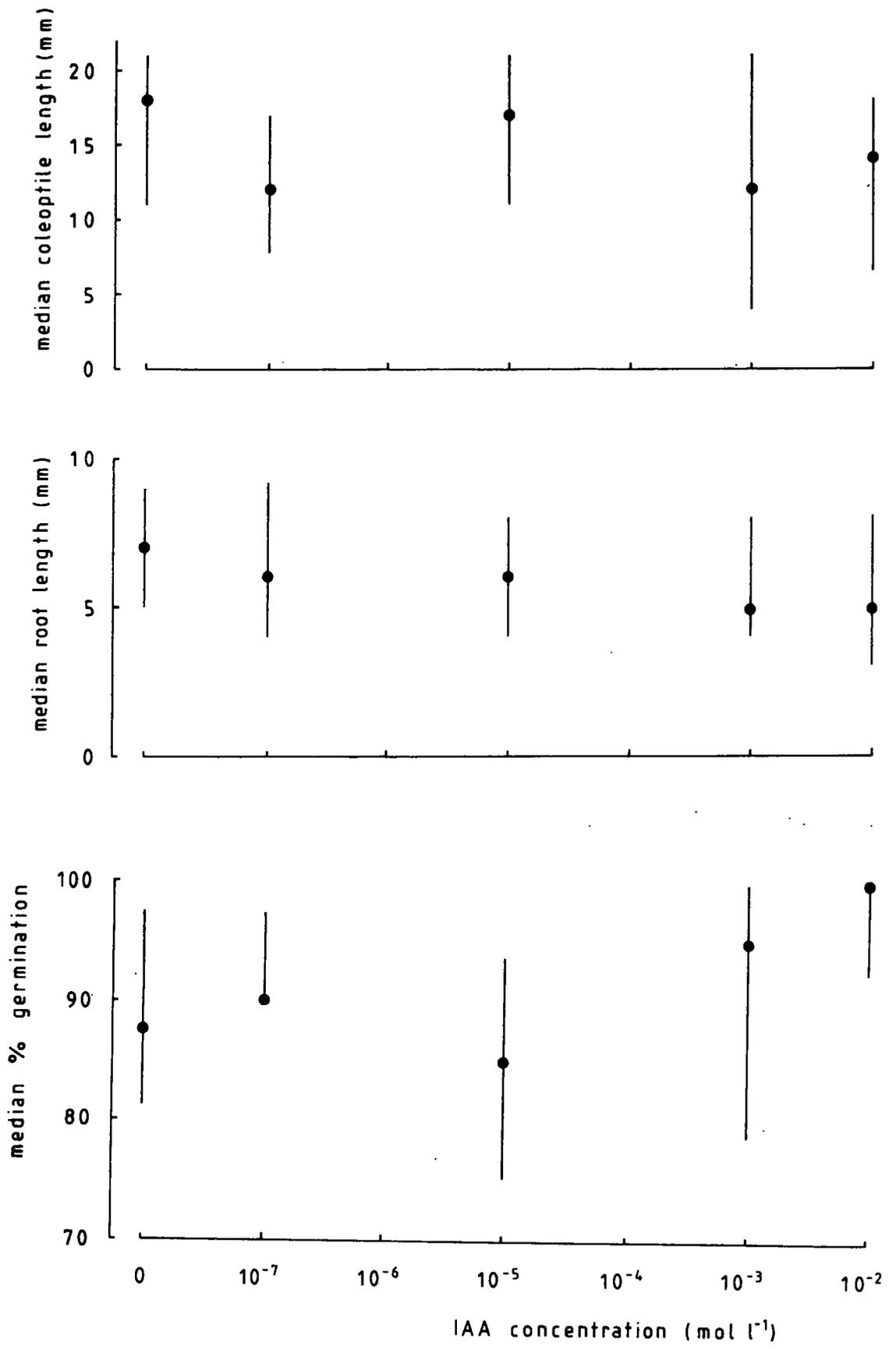
In this experiment, IAA does not show the enhancement or reduction of root and coleoptile growth expected according to concentration. This may have been due to diurnal temperature fluctuations in the laboratory; as the action of IAA is sensitive to temperature, a given concentration might have both a stimulatory and an inhibitory effect on growth at different times during the day, with no net effect.

The results were not straightforward; only 10^{-6} M IAA significantly reduced coleoptile growth relative to the control. Some concentrations, both higher and lower than this, enhanced coleoptile growth significantly. No concentration of IAA significantly reduced root growth; 10^{-8} and 10^{-5} M IAA actually enhanced it. λ

IAA was also supplied, at concentrations of 10^{-7} to 10^{-2} M, in conjunction with 1000 mg l^{-1} WL84811 (approximately 10^{-2} M) and median coleoptile and root length of germinated seeds and percentage germination measured after five days (Fig. 4.23).

The presence of IAA affected both coleoptile and root growth of germinated seeds in WL84811 significantly ($0.01 > P > 0.001$ and $0.05 > P > 0.01$ respectively) but had no significant effect on percentage germination ($0.5 > P > 0.1$).

Coleoptile length: $(W, W + 10^{-5}$ M IAA$) > (W + 10^{-2}$ M IAA, $W + 10^{-7}$ M IAA, $W + 10^{-3}$ M IAA$)$

Root length: $(W, W + 10^{-7}$ M IAA, $W + 10^{-5}$ M IAA$) > (W + 10^{-7}$ M IAA, $W + 10^{-5}$ M IAA, $W + 10^{-3}$ M IAA$) > (W + 10^{-5}$ M IAA, $W + 10^{-3}$ M IAA, $W + 10^{-2}$ M IAA$)$

$(W = 1000$ mg l^{-1} WL84811$)$.

Where IAA had a significant effect on growth it always reduced this further than WL84811 alone. In the case of roots, this reduction in growth increased with increasing concentration of IAA but no such pattern was evident for coleoptile growth.

4.8 The effect of L-azetidine-2-carboxylic acid (A2C) on Sonja seeds

To determine whether the isomer of WL84811, azetidine-2-carboxylic acid, had a similar effect on seedling growth seeds were germinated on 50, 300 or 1000 mg l^{-1} A2C. After five days, the seedlings were harvested, measured and median coleoptile length, root length and percentage germination calculated (Fig. 4.24).

Fig. **4.23a** Effect of WL84811 plus IAA on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. **4.23b** Effect of WL84811 plus IAA on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. **4.23c** Effect of WL84811 plus IAA on median percentage germination of *H. vulgare* cv. Sonja seeds after five days. with upper and lower quartiles.
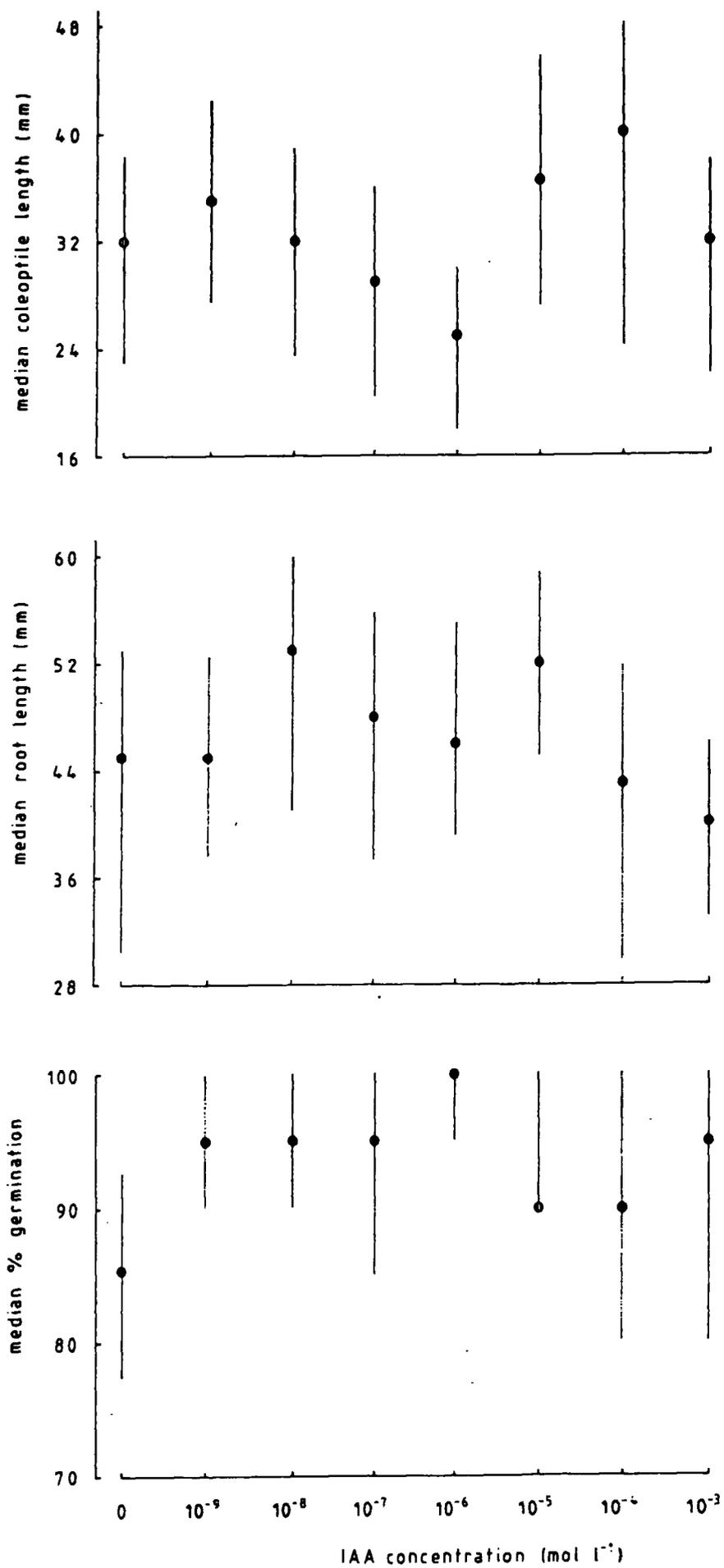
Fig. 4.24a Effect of L-azetidine-2-carboxylic acid (A2C) on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.24b Effect of A2C on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

Fig. 4.24c Effect of A2C on median percentage germination of *H. vulgare* cv. Sonja seeds after five days, with upper and lower quartiles.
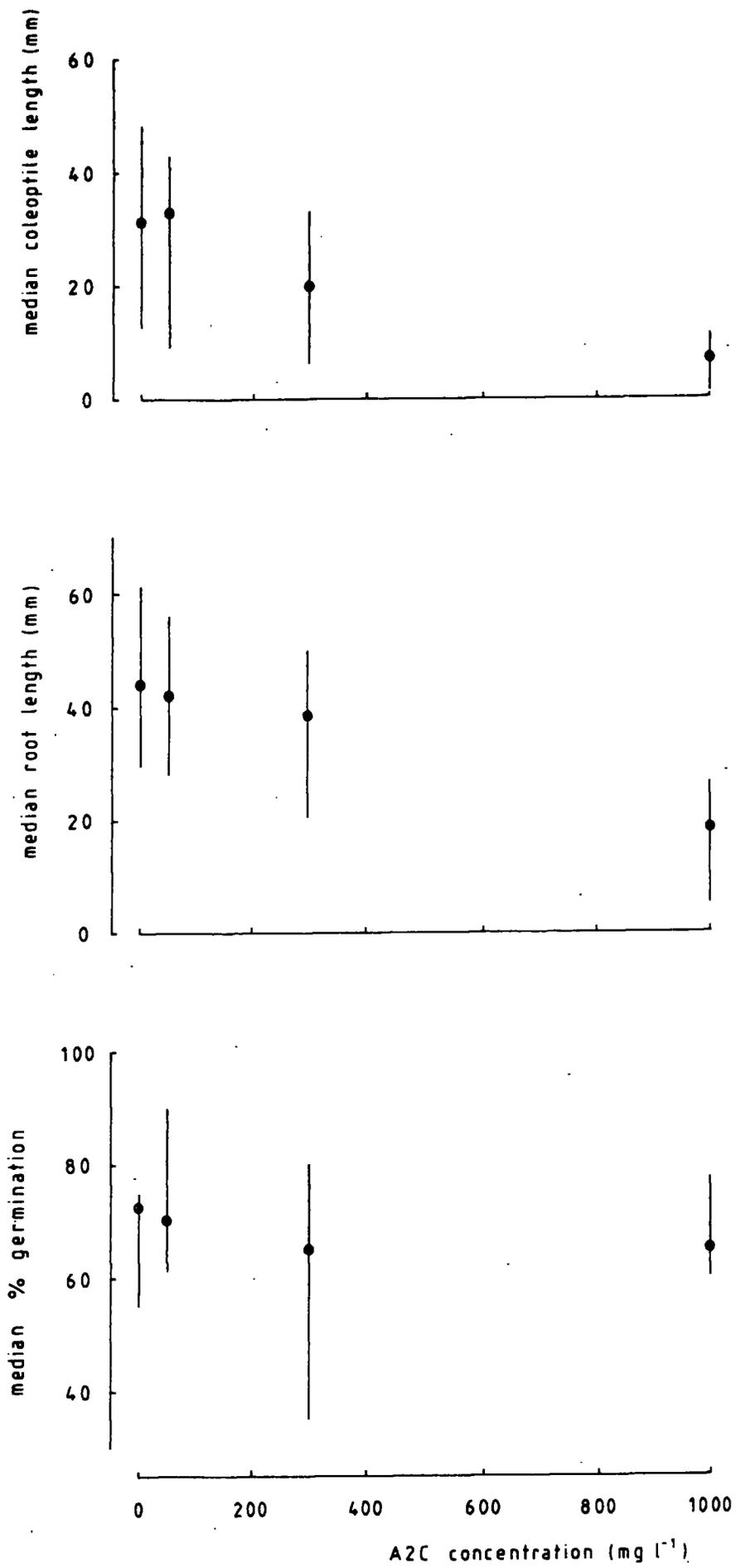
A2C had a significant effect on both root and coleoptile growth of germinated seeds ($P < 0.001$ for both) but had no effect on percentage germination ($P > 0.9$).

Coleoptile length: $(0, 50) > 300 > 1000$ mg l^{-1} A2C

Root length: $(0, 50, 300) > 1000$ mg l^{-1} A2C.

Whereas only the highest concentration of A2C tested inhibited root growth significantly, coleoptile growth was more sensitive. Unlike WL84811 however (see 4.6 above), 50 mg l^{-1} A2C had no effect on either root or coleoptile growth.

The ability of proline to ameliorate the effect of A2C was tested by supplying it in equimolar or higher concentrations along with A2C. Seedlings were again measured after five days and median values calculated (Fig. 4.25).

Coleoptile length: $0 > (300$ A+P, 300 A $+ 1000$ P, 300 A$) > (1000$ A, 1000 A+P$)$

Root length: $(0, 300$ A, 300 A $+1000$ P, 300 A+P$) > (1000$ A+P, 1000 A$)$

Percentage germination: $(300$ A, $0, 1000$ A$) > (0, 1000$ A, 1000 A+P, 300 A+P$) >$
$(1000$ A, 1000 A+P, 300 A$-$P, 300 A $+ 1000$ P$)$

(all concentrations in mg l^{-1}; A=A2C, P=proline).

Proline was not able to overcome the inhibition of root and coleoptile growth of germinated seedlings caused by A2C, even when present at three times its concentration. Proline also inhibited seed germination relative to the control or A2C alone.

Fig. 4.25a Effect of L-azetidine-2-carboxylic acid (A2C) plus equimolar proline on median coleoptile length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles.

● = A2C alone

O = A2C + equimolar proline

**Fig. 4.25b** Effect of A2C plus equimolar proline on median root length of germinated *H. vulgare* cv. Sonja seedlings after five days growth, with upper and lower quartiles. Key as for Fig. 4.25a.

Fig. 4.25c Effect of A2C plus equimolar proline on median percentage germination of *H. vulgare* cv. Sonja seeds after five days, with upper and lower quartiles. Key as for Fig. 4.25a.
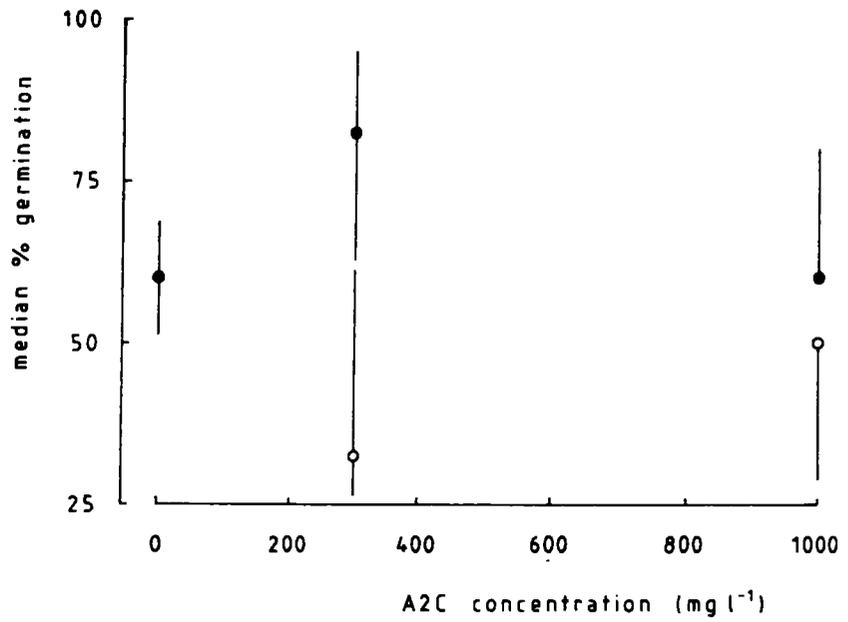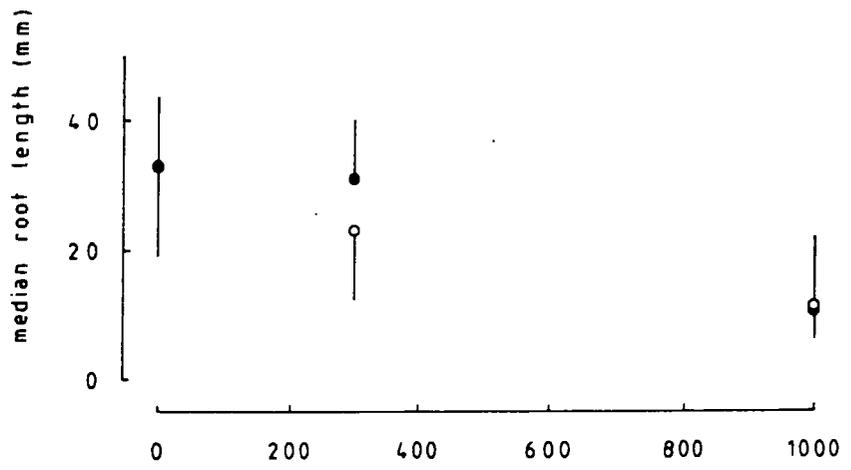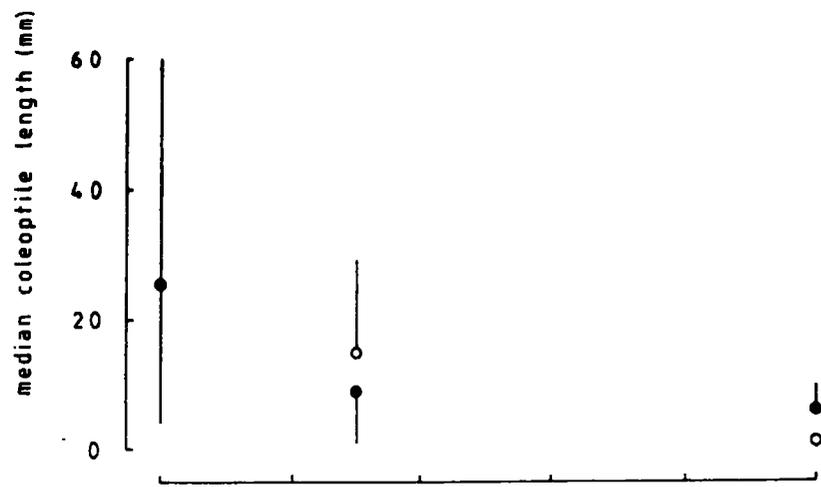
4.9 The effect of WL84811 on germination of bracken spores

The effect of a range of WL84811 concentrations on germination of bracken spores in Knop's solution was measured. At least 300 spores were counted on each of two replicate plates per treatment after four and seven days on the medium. Mean percentage germination for each treatment, with the range, is given in Fig. 4.26.

Kruskal-Wallis analyses appeared to suggest that the effect of WL84811 on percentage germination was not significant at either four or seven days ($0.1 > P > 0.05$ for both). However, Mann-Whitney tests showed that;

At four days: $0 > 50 > 150 > (300, 1000)$ mg 1^{-1} WL84811

At seven days: $0 > 50 > 150 > 300 > 1000$ mg 1^{-1} WL84811.

Part of the effect of WL84811 seemed to be to reduce the initial rate of germination of the spores, preventing it altogether initially at the highest concentrations. Fig. 4.26 shows that the rate of germination of spores was greater over the last three days of the experiment than the first four days when WL84811 was present. This contrasts with the control situation where the rate of germination decreased with time.

As Knop's medium is unbuffered, an experiment was carried out to determine how much the concentrations of WL84811 used affected pH of the medium. The results are shown in Fig. 4.27. 50 mg 1^{-1} WL84811 reduced the pH by around 0.3 pH units and 1000 mg 1^{-1} WL84811 only reduced it by 0.5 pH units. In view of the difference in magnitude of the effects of these two treatments on spore germination it seems unlikely that pH is responsible.

Fig. 4.26 Effect of WL84811 on rate of germination of *Pteridium aquilinum* (bracken) spores, with upper and lower quartiles.

● $= 0$ mg l^{-1} WL84811

○ $= 50$ mg l^{-1} WL84811

■ $= 150$ mg l^{-1} WL84811

□ $= 300$ mg l^{-1} WL84811

▲ $= 3000$ mg l^{-1} WL84811

Fig. 4.27 Effect of WL84811 on pH of Knop's solution for fern spore germination. Each value is mean of two readings.
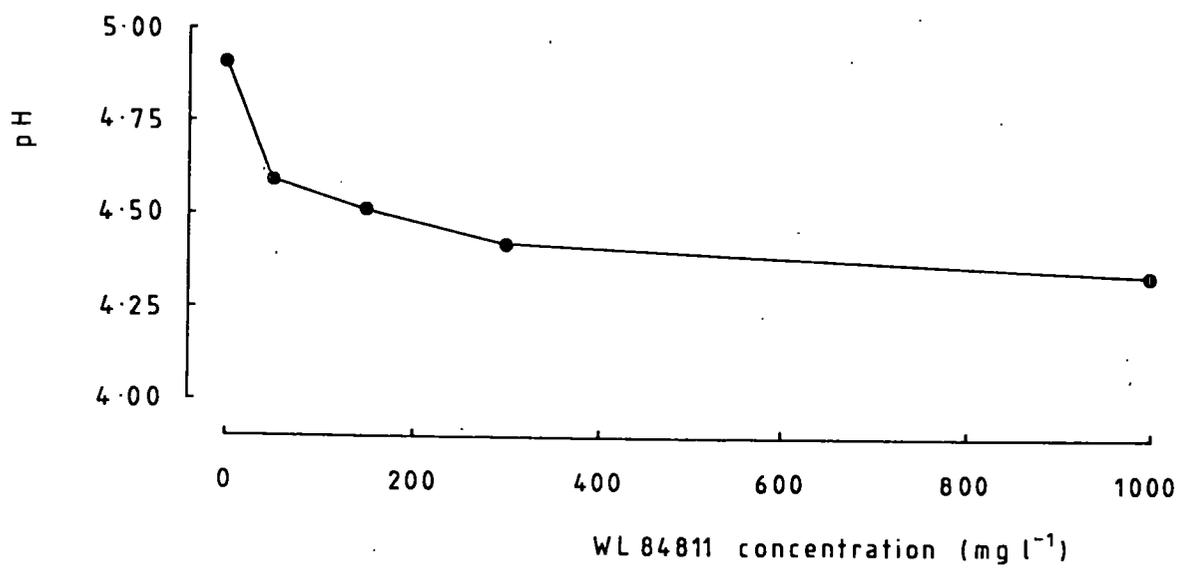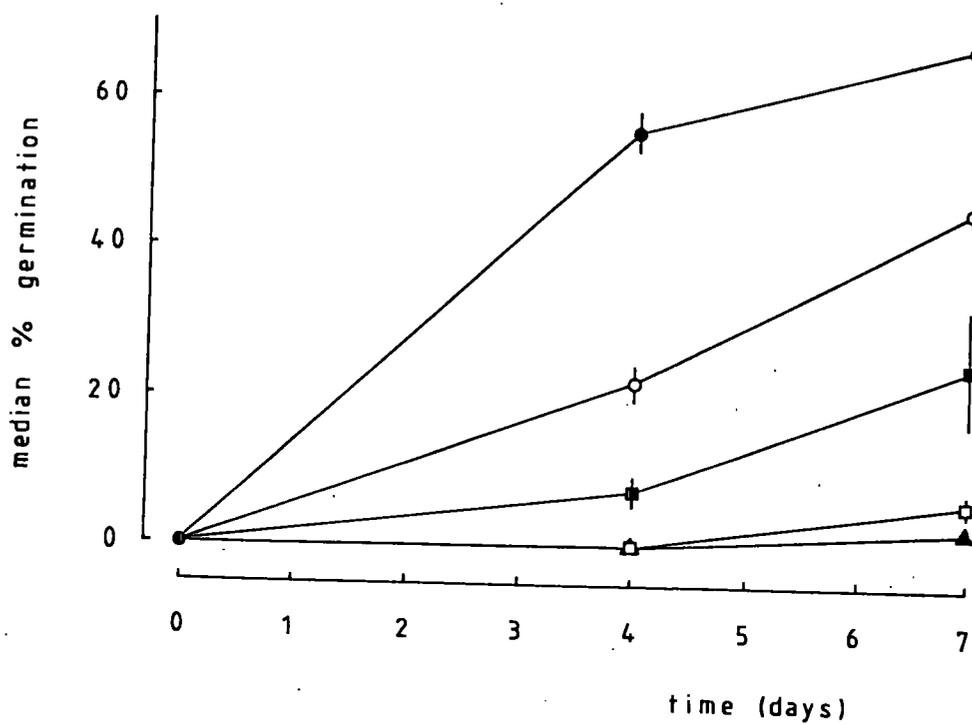
5. EFFECTS OF WL 84811 ON UNICELLULAR GREEN ALGAE

5.1 Introduction

The effects of WL84811 on growth and cell division of unicellular algae, *Chlorella* in particular, were studied to gain further information about the action of WL84811 on individual cells. Because of their small size, large numbers of cells were readily measured and counted to determine whether WL84811 was affecting cell expansion or cell divsion. These results were confirmed and by clarified by electron microscopy.

5.2 The effects of WL84811 on growth of two algae

5.21 *Chlorella zofingiensis* Donz.

5.211 Effect of WL84811 on cell growth and cell division

In order to determine the effect of WL84811 on cell growth and division, *Chlorella* cells were grown in Chu 10 medium (section 2.32) containing various concentrations of WL84811 in addition to the standard constituents. The cell concentration was calculated from measurements taken on inoculation and after three, six and ten days growth. The number of cells in 50 squares of the haemacytometer (each $0.05 \times 0.05 \times 0.1$ mm^3) was counted for a sample from each replicate flask and the total of four replicates extrapolated to give a cell count per millilitre. 1000 mg l^{-1} WL84811 was not tested during this experiment but results from a later experiment are included here for comparison (Fig. 5.1).

Fig. 5.1 Effect of WL84811 on cell division of *Chlorella*.

● = 0 mg l^{-1} WL84811

○ = 50 mg l^{-1} WL84811
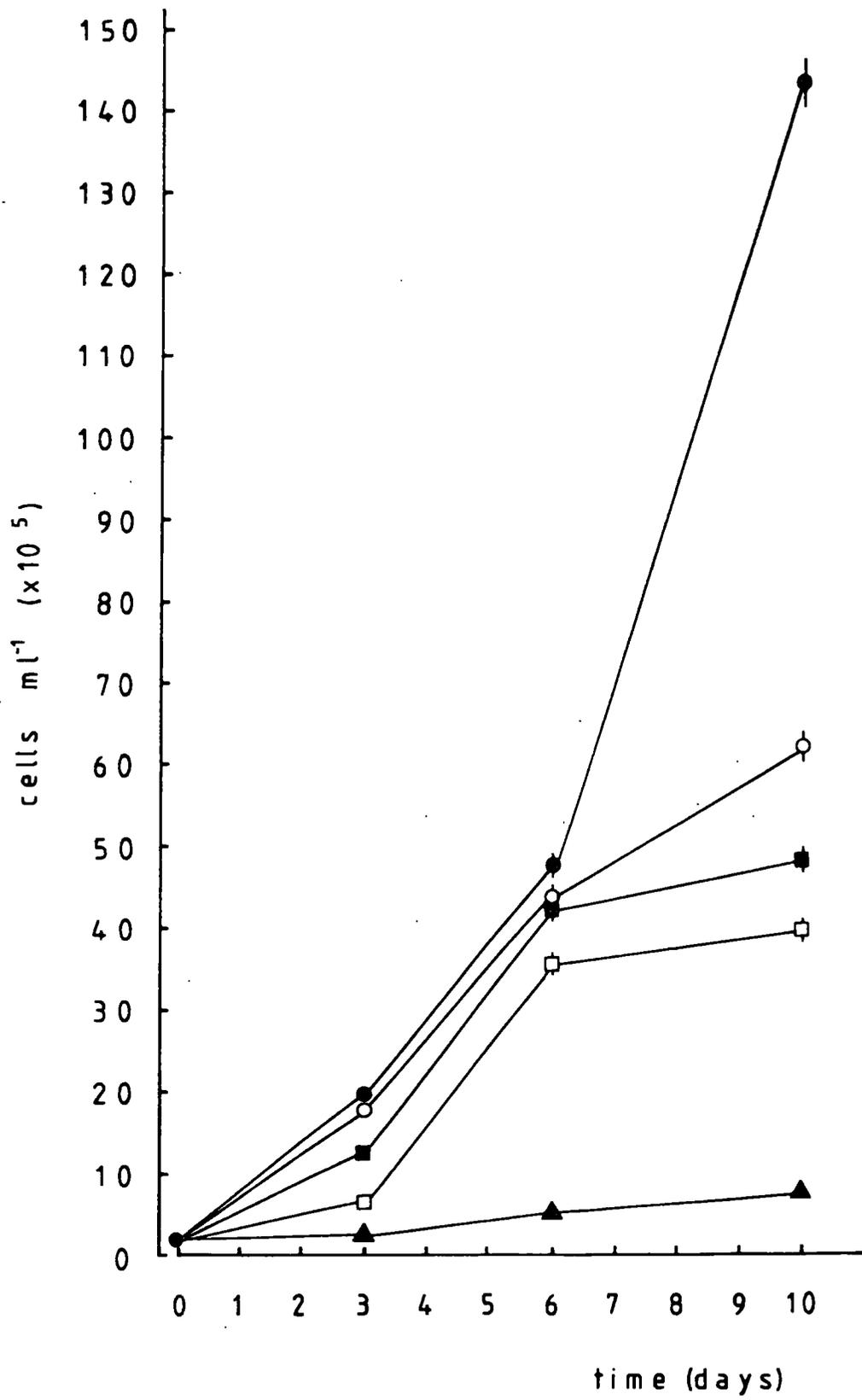
■ = 150 mg l^{-1} WL84811

□ = 300 mg l^{-1} WL84811

▲ = 1000 mg l^{-1} WL84811

The significance of differences between treatments at P=0.05 was determined as described in section 2.93. Treatments not significantly different from one another are bracketed together:

Day 0: (1000, 150, 50, 0) > (150, 50, 0, 300) mg l^{-1} WL84811

Day 3: (0, 50) > 150 > 300 > 1000 mg l^{-1} WL84811

Day 6: 0 > (50, 150) > 300 > 1000 mg l^{-1} WL84811

Day 10: 0 > 50 > 150 > 300 > 1000 mg l^{-1} WL84811.

All concentrations of WL84811 tested reduced cell division significantly after six days and cells subjected to 1000 mg l^{-1} WL84811 only increased four-fold in number over 10 days. At lower concentrations, however, cells underwent an extended lag phase, followed by a period of increased growth at a rate equivalent to cells in the control medium. This lasted for only three days before the increase in cell number levelled off in the presence of all concentrations of WL84811, while in the control medium cell numbers continued to increase exponentially.

A second experiment was carried out using much lower concentrations of WL84811 (Fig. 5.2). Treatments not significantly different from one another are again bracketed together:

Day 0: (0, 20, 10) mg l^{-1} WL84811

Day 3: (0, 10, 20) mg l^{-1} WL84811

Day 6: (20, 10) > 0 mg l^{-1} WL84811

Day 10: 0 > 10 > 20 mg l^{-1} WL84811.

Fig. 5.2 Effect of low concentrations of WL84811 on cell division of

Chlorella.

● = 0 mg l^{-1} WL84811

O = 10 mg l^{-1} WL84811

■ = 20 mg l^{-1} WL84811
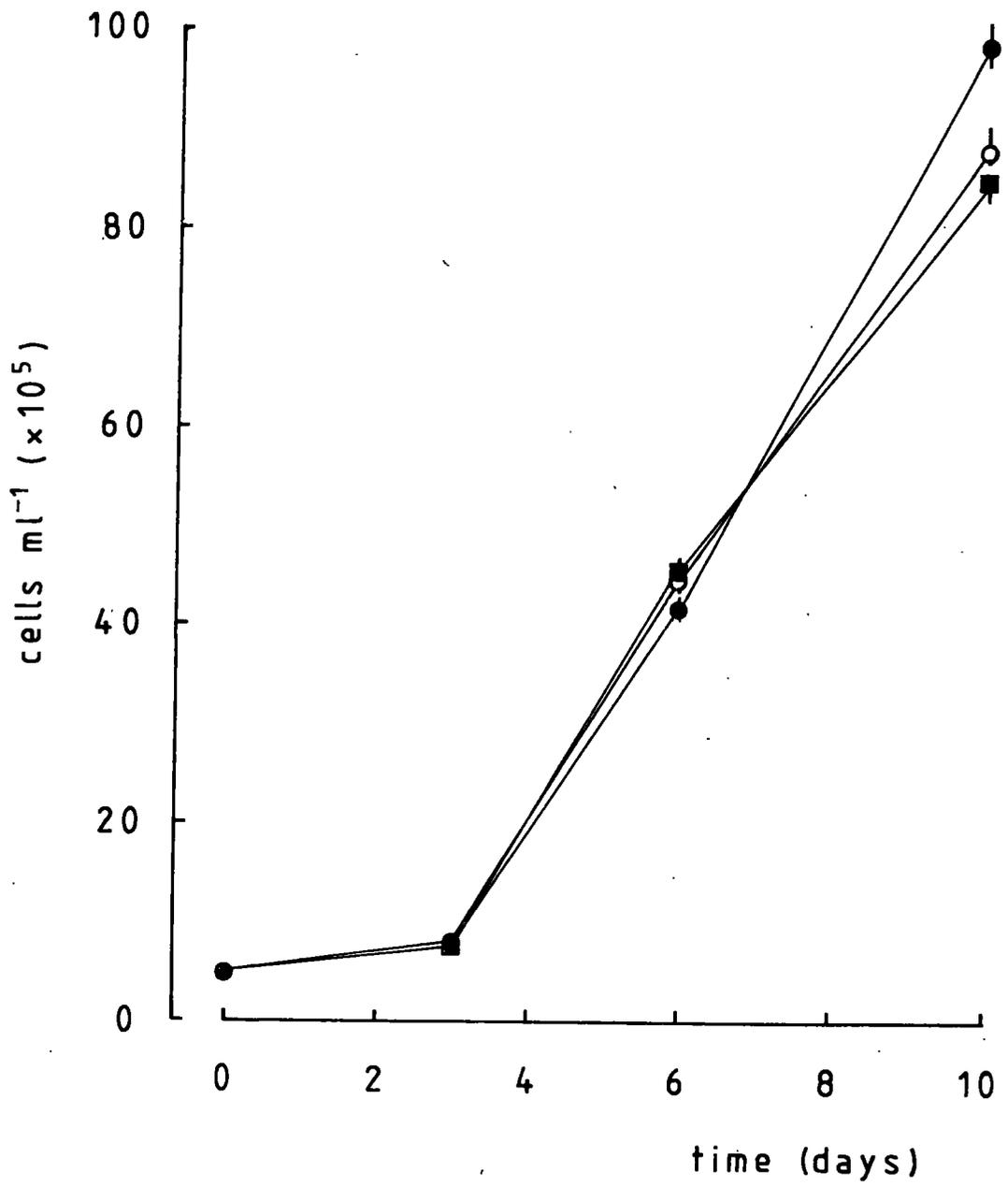
These low concentrations of WL84811 had very little effect on cell division initially; after six days growth both concentrations had enhanced division relative to the control and only after ten days did they reduce the rate of cell division significantly.

The distribution of cell size was also markedly affected by the presence of WL84811 (Fig. 5.3). High concentrations of WL84811 in particular produced a small 'population' of very large cells ($8 - 15 \mu m$ in diameter), in addition to cells of the normal size ($2 - 7 \mu m$), making the distribution highly skewed. After 10 days growth, cells in the control medium ranged in volume from 4.8 to $82.4 \mu m^3$, while cells grown in 1000 mg l^{-1} WL84811 ranged from 4.8 to 2145 μm^3. The largest cells from the medium containing WL84811 had a volume approximately 26 times that of the largest cells from the control medium (Figs 5.4, 5.5).

5.212 Effect of WL84811 on biomass of *Chlorella*

In order to determine the effect of WL84811 on cell biomass, *Chlorella* cells were grown in Chu 10 with or without 300 mg l^{-1} WL84811, cell concentration calculated and dry weight determined from samples taken at intervals after inoculation (Fig. 5.6).

The significance of differences in cell number between treatments was determined as above and the differences in biomass of samples using the Mann-Whitney U-test (Table 5.1).

· **Fig. 5.3** Effect of WL84811 on size distribution of *Chlorella* cells.
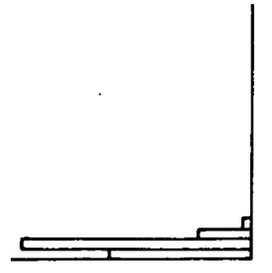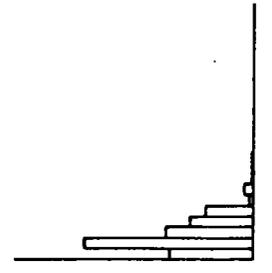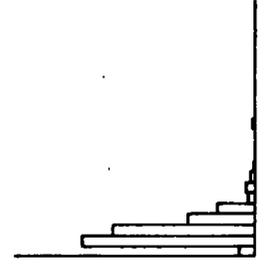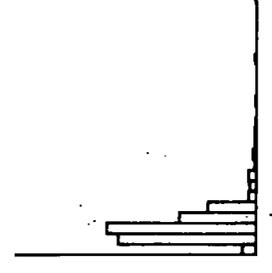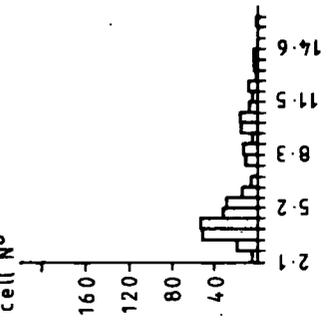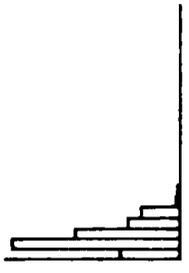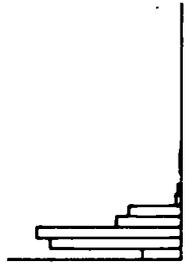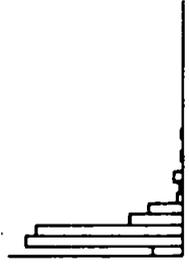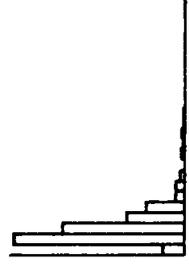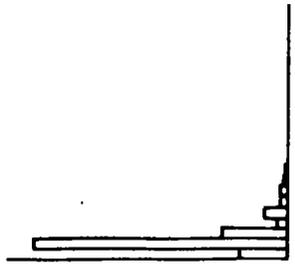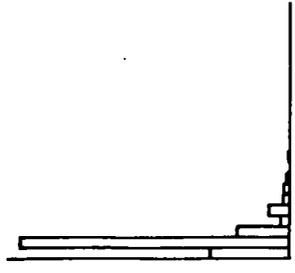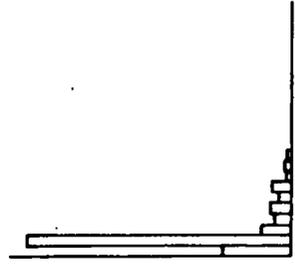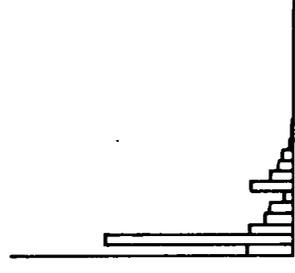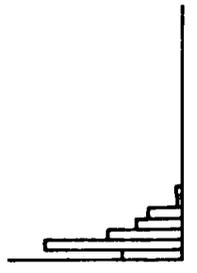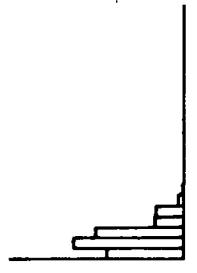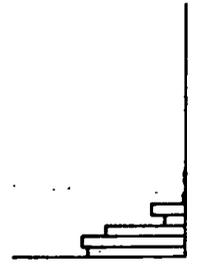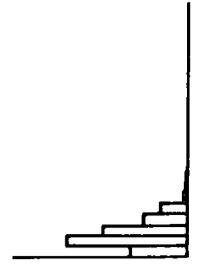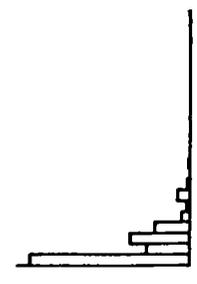
Fig. 5.4 *Chlorella* cells just after division, grown in control Chu 10 medium. Scale bar = 7 μm.

Fig. 5.5 *Chlorella* cells grown in the presence of 1000 mg l^{-1} WL84811. Scale bar = 7 μm.
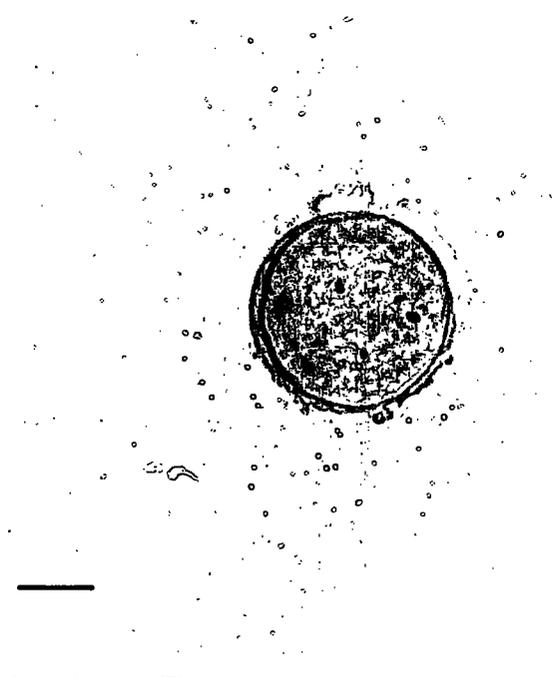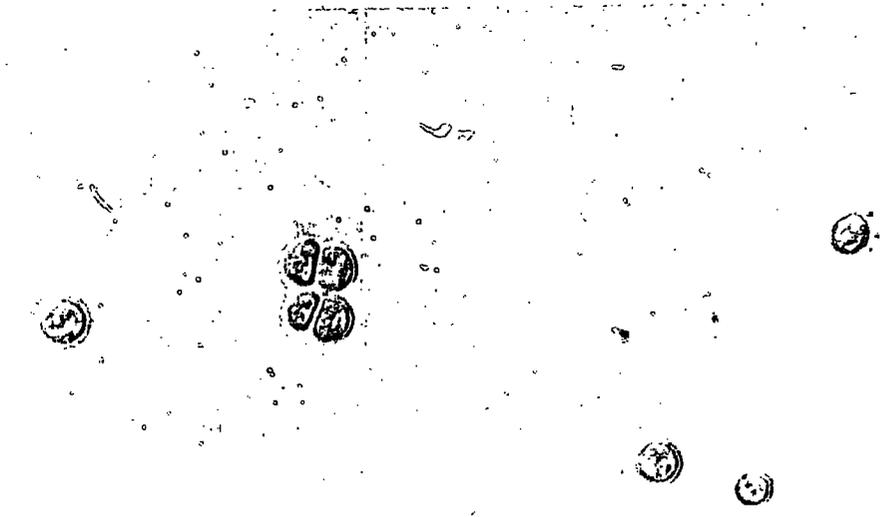
Fig. **5.6a** Effect of WL84811 on total dry weight of a *Chlorella* culture.

Each value is median of three replicates, with upper and lower quartiles.

● = control

○ = 300 mg l^{-1} WL84811

Fig. **5.6b** Effect of WL84811 on median dry weight per *Chlorella* cell.

Key as for Fig. 5.6a.

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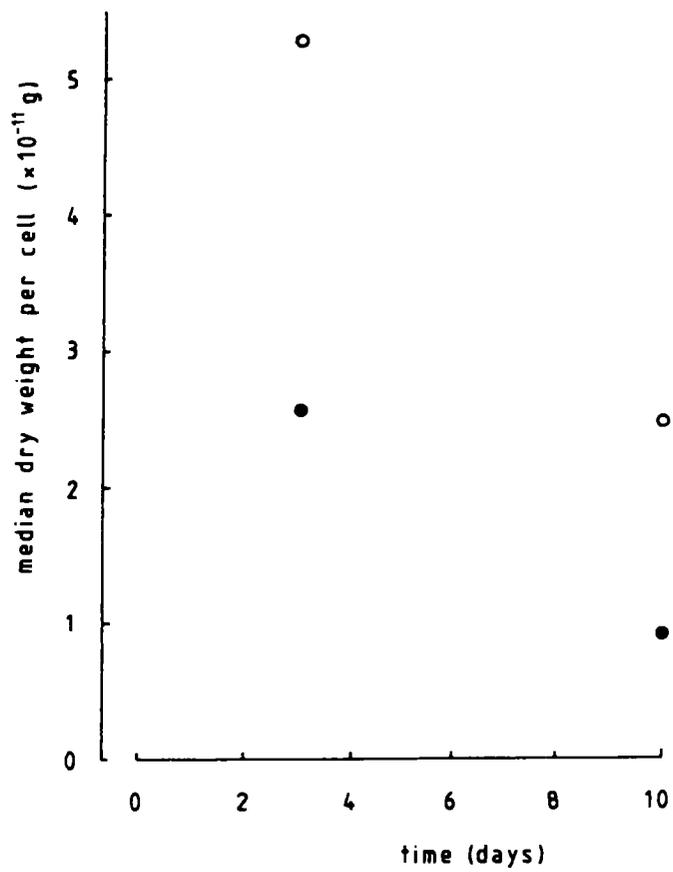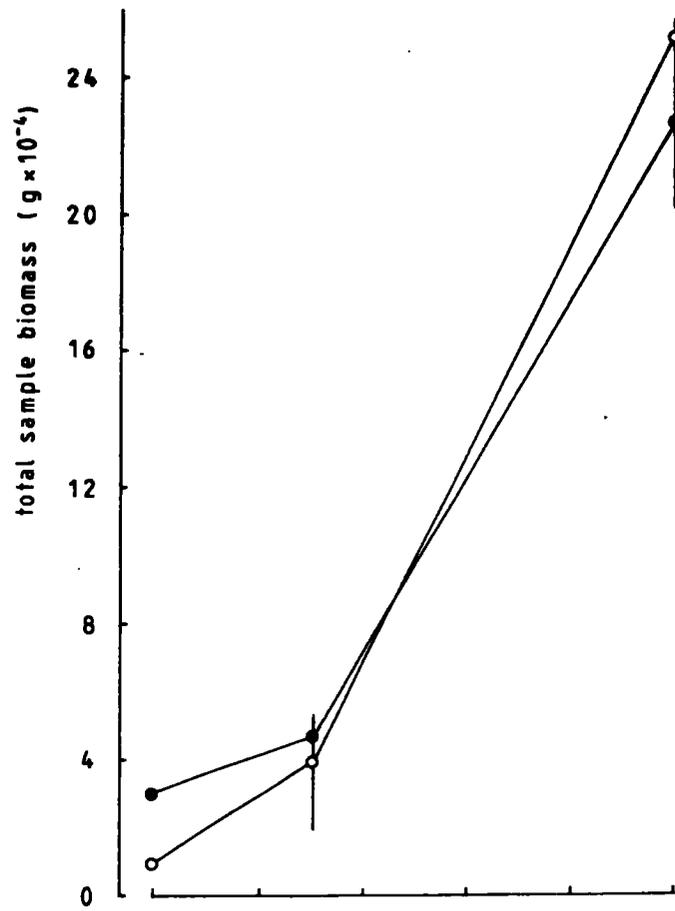
Table 5.1 Significance of differences in cell number, sample and median cell weight of *Chlorella* **treated with WL84811.**

Time (days)	Cells per ml	Sample dry weight	Dry weight per cell
0	$P > 0.1$	ND	ND
3	$P < 0.001$	$P > 0.05$	$P > 0.05$
10	$P < 0.001$	$P > 0.05$	$P < 0.05$

The total sample dry weight (from 25.5 ml of culture) was not affected significantly by the presence of 300 mg l^{-1} WL84811 in the culture medium. However, after 10 days growth the median dry weight per cell in the presence of WL84811 was more than double that of cells in the control medium although, in both cases, this value was lower after 10 days growth than after three.

5.213 Effect of WL84811 on DNA synthesis of *Chlorella*

To determine the effect of WL84811 on DNA synthesis in *Chlorella*, cells were again grown in the presence or absence of 300 mg l^{-1} WL84811 and cell concentration calculated from samples taken at intervals after inoculation. The DNA content of one millilitre samples after the cells had been fragmented by sonication was determined using the method of Labarca and Paigen (1980) and median DNA content per cell calculated (Fig. 5.7).

The significance of differences in cell number between the treatments was calculated as before and differences in DNA content using the Mann-Whitney U-test (Table 5.2).

Fig. 5.7a Effect of WL84811 on total DNA content of a *Chlorella* culture.

● = control

○ = 300 mg l^{-1} WL84811.

Fig. 5.7b Effect of WL84811 on median DNA content per *Chlorella* cell.
Key as for Fig. 5.7a.
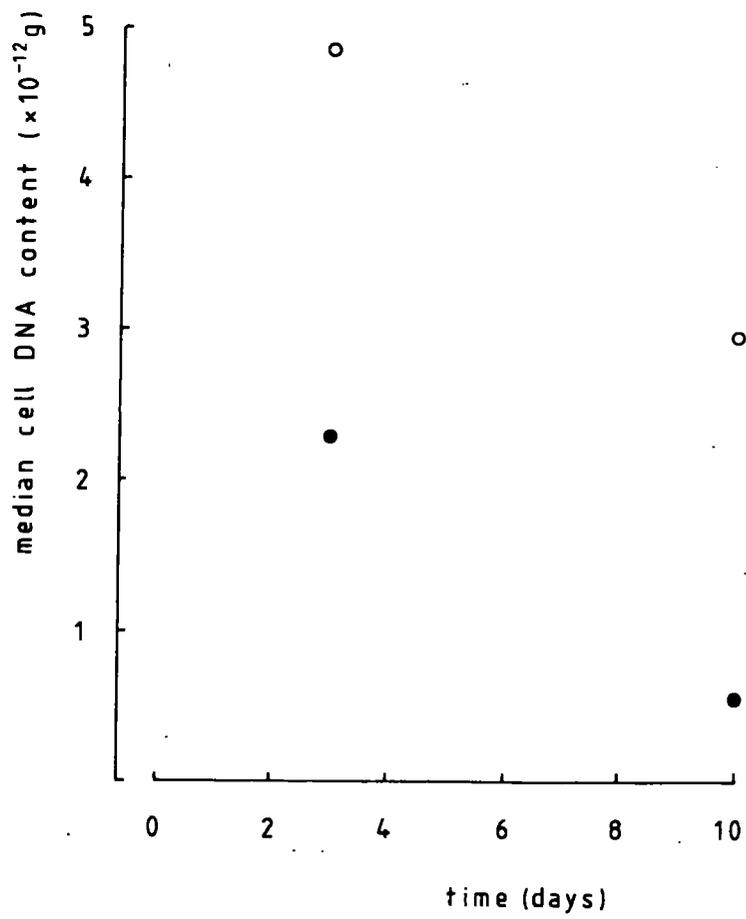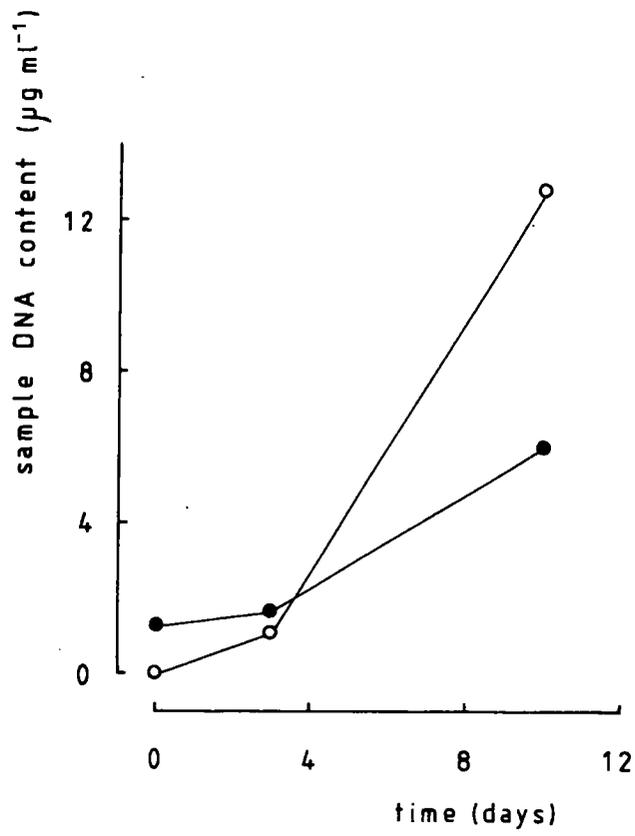
Table 5.2 Significance of differences in cell number, sample and median cell DNA content of *Chlorella* treated with WL84811.

Time (days)	Cells per ml	Sample DNA content	Cell DNA content
0	$P > 0.1$	$P > 0.05$	ND
3	$P < 0.001$	$P > 0.05$	$P > 0.05$
10	$P < 0.001$	$P < 0.05$	$P < 0.05$

Samples from media containing 300 mg l^{-1} WL84811 contained approximately twice as much DNA as those from control media after 10 days growth. When DNA content per cell was considered however, cells grown in the presence of WL84811 contained approximately six times as much DNA as control cells after 10 days. Under both treatments DNA content per cell was lower after 10 days growth than after three.

5.22 *Closterium moniliferum* Ehr.

In order to look at the effect of WL84811 on another type of cell division, *Closterium* was grown in Chu 10 with added vitamins (section 2.32) and a range of concentrations of WL84811 Cell concentration was calculated from samples taken at intervals after inoculation (Fig. 5.8).

The significance of differences between treatments at $P=0.05$ was determined as above. Those not significantly different from one another are bracketed together:

Day 0: (50, 300, 150, 0) mg l^{-1} WL84811

Day 3: (300, 50, 150, 0) mg l^{-1} WL84811

Day 6: (150, 300) > (300, 0) > (0, 50) mg l^{-1} WL84811

Day 10: (0, 50, 300, 150) mg l^{-1} WL84811

Day 18: 0 > 300 > (150, 50) mg l^{-1} WL84811.

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Fig. 5.8 Effect of WL84811 on cell division of *Closterium*.

● = control

O = 50 mg l^{-1} WL84811

■ = 150 mg l^{-1} WL84811

□ = 300 mg l^{-1} WL84811.
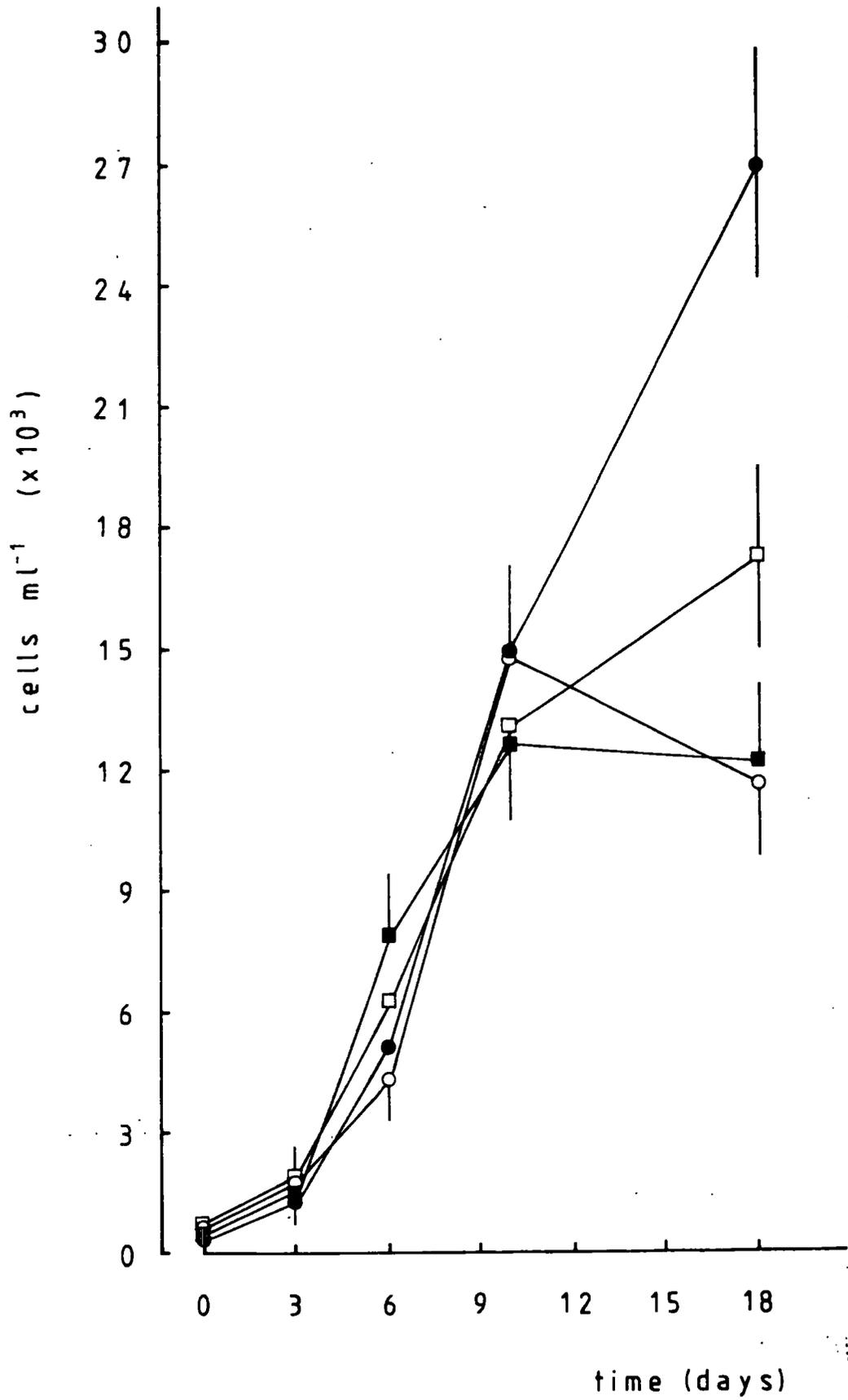
Only after 18 days did WL84811 reduce cell division significantly; even this inhibition was not directly proportional to the concentration of WL84811. In addition, WL84811 had little effect on the distribution of cell size (Fig. 5.9).

5.3 The effect of returning *Chlorella* treated with WL84811 to fresh medium

In order to see whether WL84811 was algicidal or merely algistatic. *i.e.* whether or not its inhibitory effect on cell division was reversible, cells grown for 11 days in the presence of various concentrations of WL84811 were returned to fresh Chu 10 containing no WL84811. Cell concentration was calculated from samples taken at time zero, one day and eight days after inoculation into fresh medium. The results, with 95 % confidence limits. are given in Table 5.3.

Table 5.3 Effect of WL84811 in original medium on cell division of *Chlorella* on return to control Chu 10.

WL84811 conc. (initially)	Cells per ml at time 0	Cells per ml after 1 day	Cells per ml after 8 days
0 mg l^{-1}	500000 ± 51300	558000 ± 54200	7791000 ± 202800
50 mg l^{-1}	283000 ± 38600	ND	5323000 ± 167700
150 mg l^{-1}	191000 ± 31700	ND	5813000 ± 175200
300 mg l^{-1}	165000 ± 29400	ND	7840000 ± 203500
1000 mg l^{-1}	44000 ± 17200	57000 ± 19700	3901000 ± 165700

Cells previously grown in high concentrations of WL84811 increased in number more rapidly over the eight day period than cells from the medium without WL84811. The number of doublings undergone by cells in this time was closely related (coefficient of determination. $r^2 = 0.998$; P < 0.001) to $\log_e$ [WL84811 concentration of the initial medium in mg l^{-1}]. when the initial medium contained WL84811 (Fig. 5.10).

Fig. 5.9 Effect of WL84511 on size distribution of *Closterium* cells.
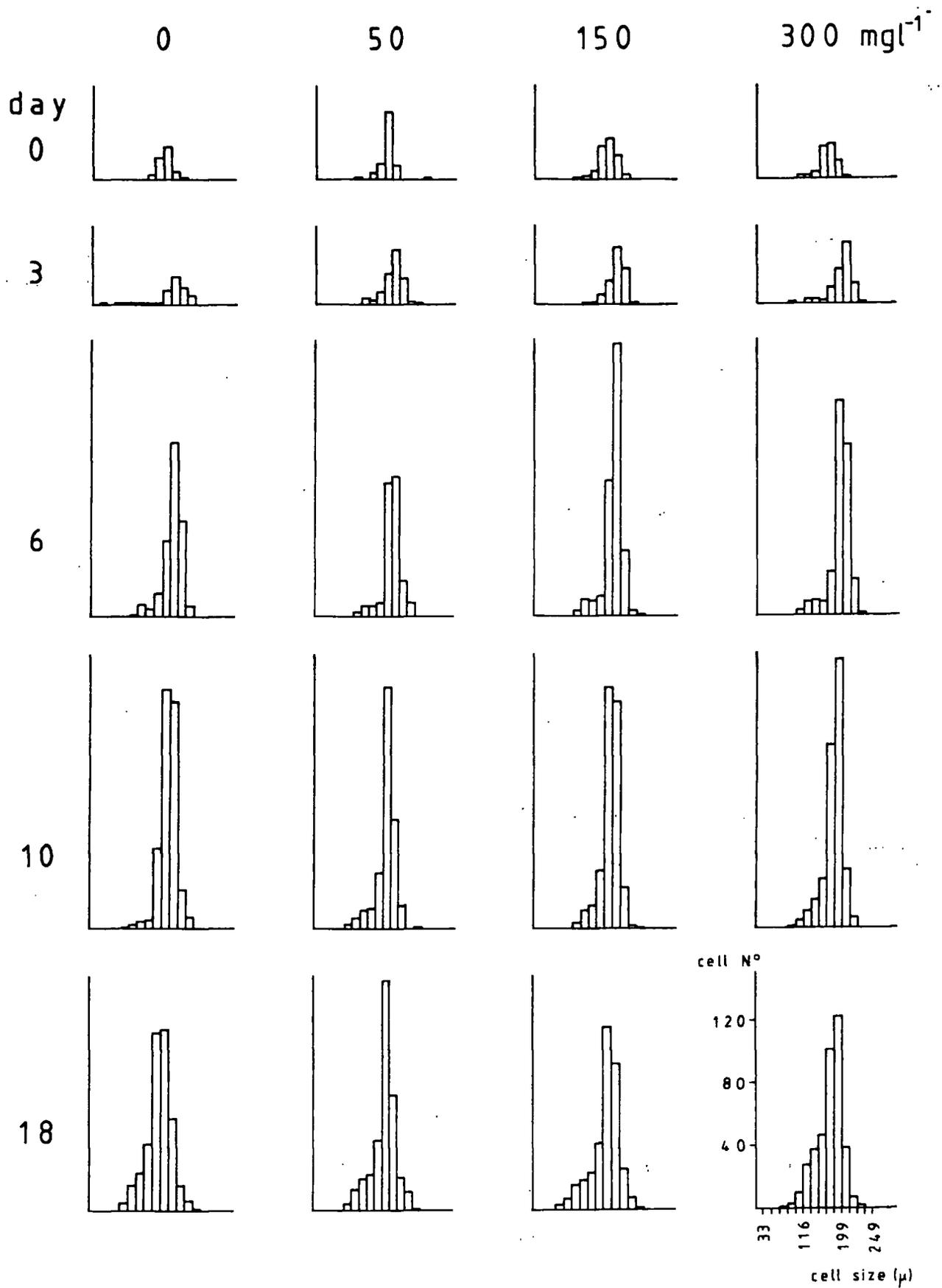
Fig. 5.10 Relationship between number of doublings undergone in eight days by WL84811-treated *Chlorella* cells returned to the control medium and $\log_e$ [WL84811 concentration of the initial medium in mg l^{-1}].
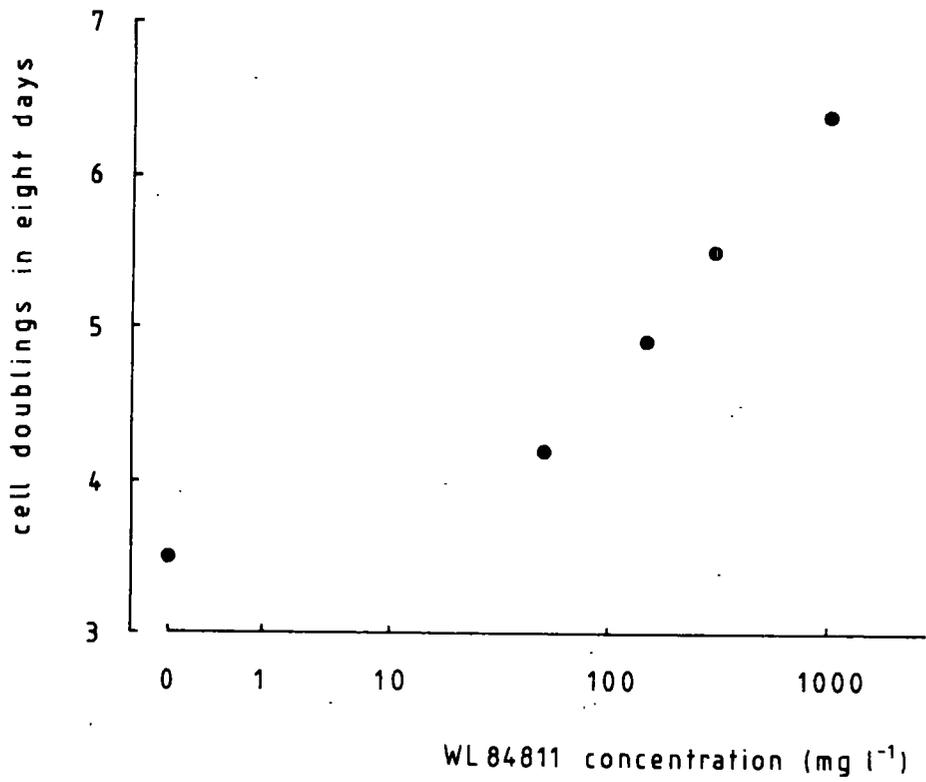
The number of doublings should not be equated directly with number of cell divisions as *Chlorella*, when it divides, forms a variable number of autospores within each parent cell rather than dividing by binary fission. The distribution of cell size also changed after cells grown up in WL84811 were transferred to control Chu 10, although some large cells remained (Fig. 5.11).

5.4 The effect of proline on inhibition of growth of *Chlorella* by WL84811

Initially *Chlorella* cells were grown in media containing 1000 mg l^{-1} WL84811, alone or in conjunction with 1138 mg l^{-1} proline (an equimolar concentration) in order to determine whether equimolar proline could overcome the inhibitory effect of WL84811 on cell division. The cell concentration was again calculated at intervals after inoculation (Fig. 5.12).

The significance of differences between these counts was determined as above. Treatments not significantly different from one another at $P=0.05$ are bracketed together:

Day 0: (Proline, WL84811, Control, WL84811 − Proline)

Day 3: (Proline, Control) > WL84811 > WL84811 + Proline

Day 6: Control > Proline > (WL84811, WL84811 + Proline)

Day 10: Control > Proline > WL84811 + Proline > WL84811.

Equimolar proline showed a very limited ability to ameliorate the inhibitory effect of WL84811 on cell division after 10 days. although high concentrations of proline alone inhibited cell division slightly. Proline alone had little effect on the distribution of cell size (Fig. 5.13) and when supplied in conjunction with WL84811 the proportion of smaller cells present was increased only slightly compared with WL84811 alone.

Fig. 5.11 Change in distribution of cell size after returning *Chlorella* cells grown up in 50, 150, 300 or 1000 mg l^{-1} WL84811 to the control medium.

0—0 mgl^{-1} 50—0 mgl^{-1} 150—0 mgl^{-1} 300—0 mgl^{-1}

day

0

cell N°

120

60

0

2·1   5·2   8·3   11·5

cell size (µm)

8

0 mgl^{-1}

⟶ 0 mgl^{-1}

cell N°

80

40

0

0·8   4·0   7·1   9·6   12·1   15·2

cell size (µm)
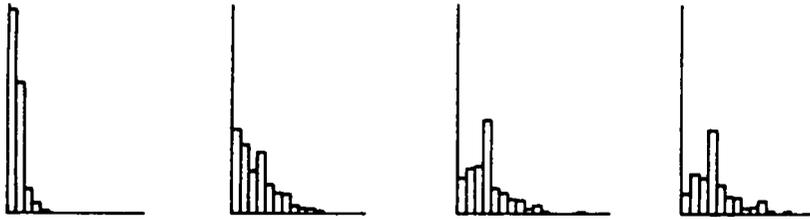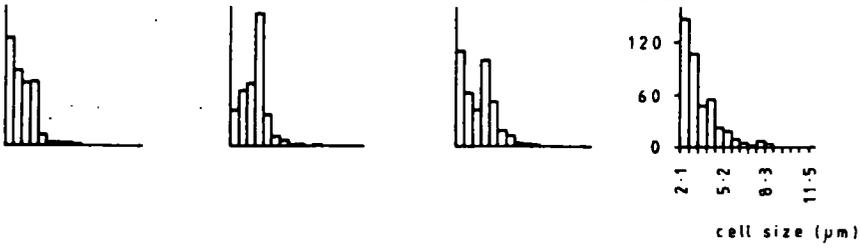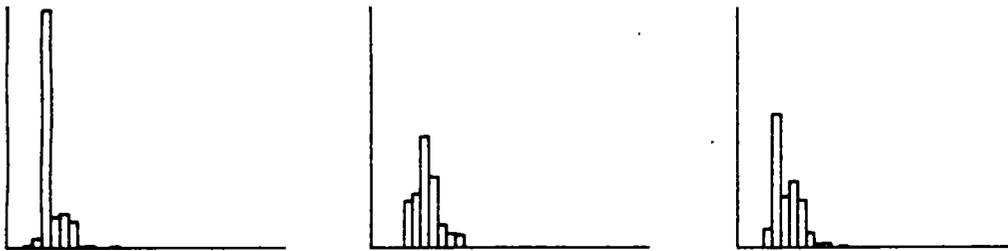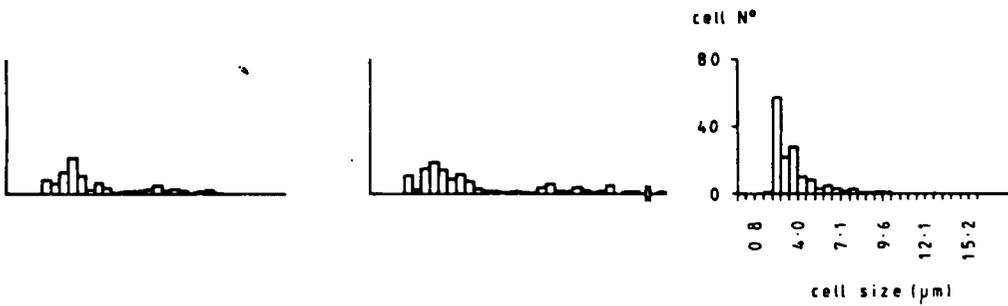
1000 mgl^{-1}

⟶ 0 mgl^{-1}

day   0   1   8

148

Fig. 5.12 Effect of WL84811 and proline, alone or combined at equimolar

concentrations, on cell division of *Chlorella*.

● = control

○ = 1138 mg l^{-1} proline

■ = 1000 mg l^{-1} WL84811
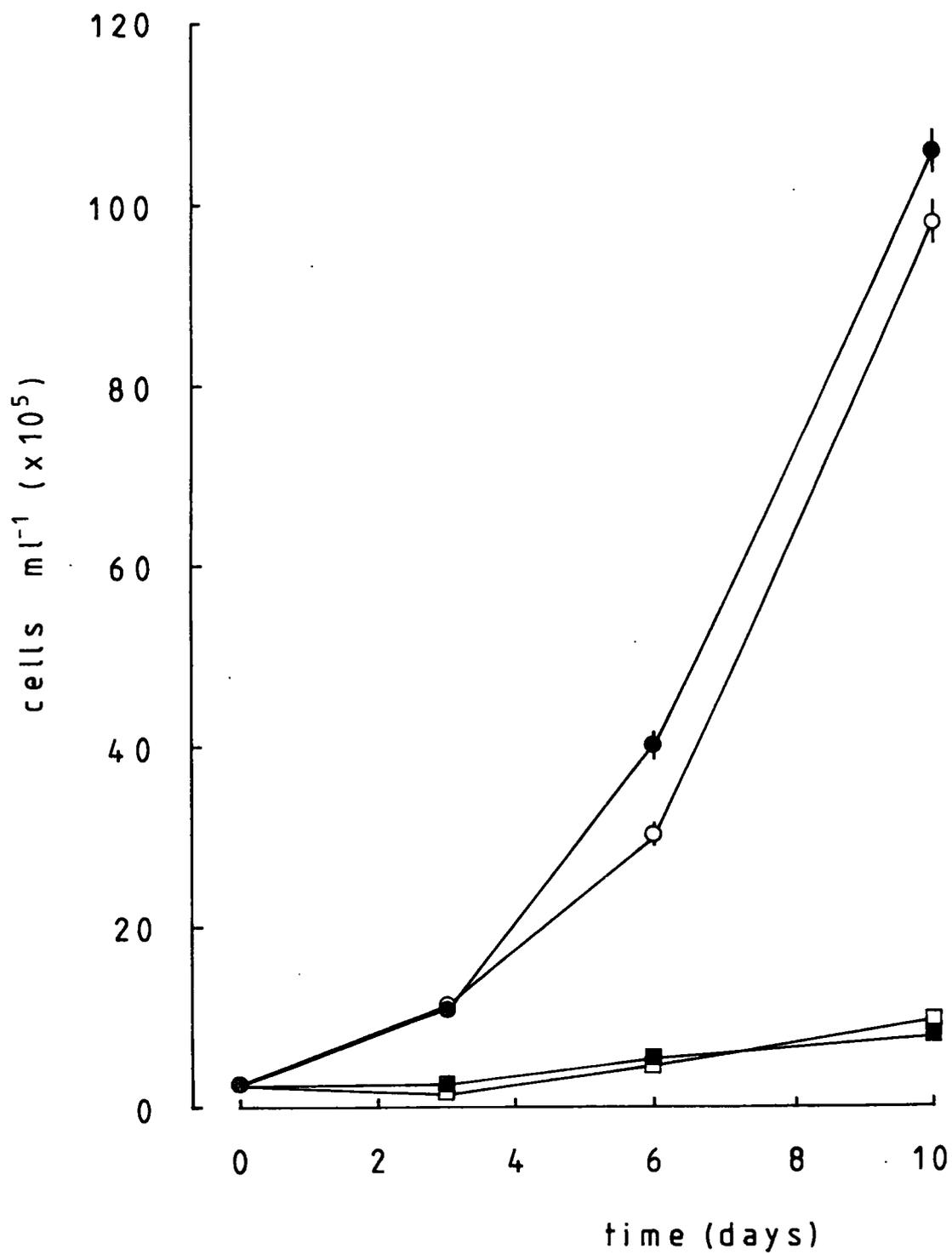
□ = 1000 mg l^{-1} WL84811 + 1138 mg l^{-1} proline

Fig. 5.13 Effect of WL84811 and proline, alone or combined at equimolar concentrations, on size distribution of *Chlorella* cells.

control

1000 mg l^{-1}
WL 84811

1138 mg l^{-1}
proline

WL 84811
+proline

cell N°

160

80

2·1   5·2   8·3   11·5   14·6

cell diameter (μm)

day 0   day 10

150
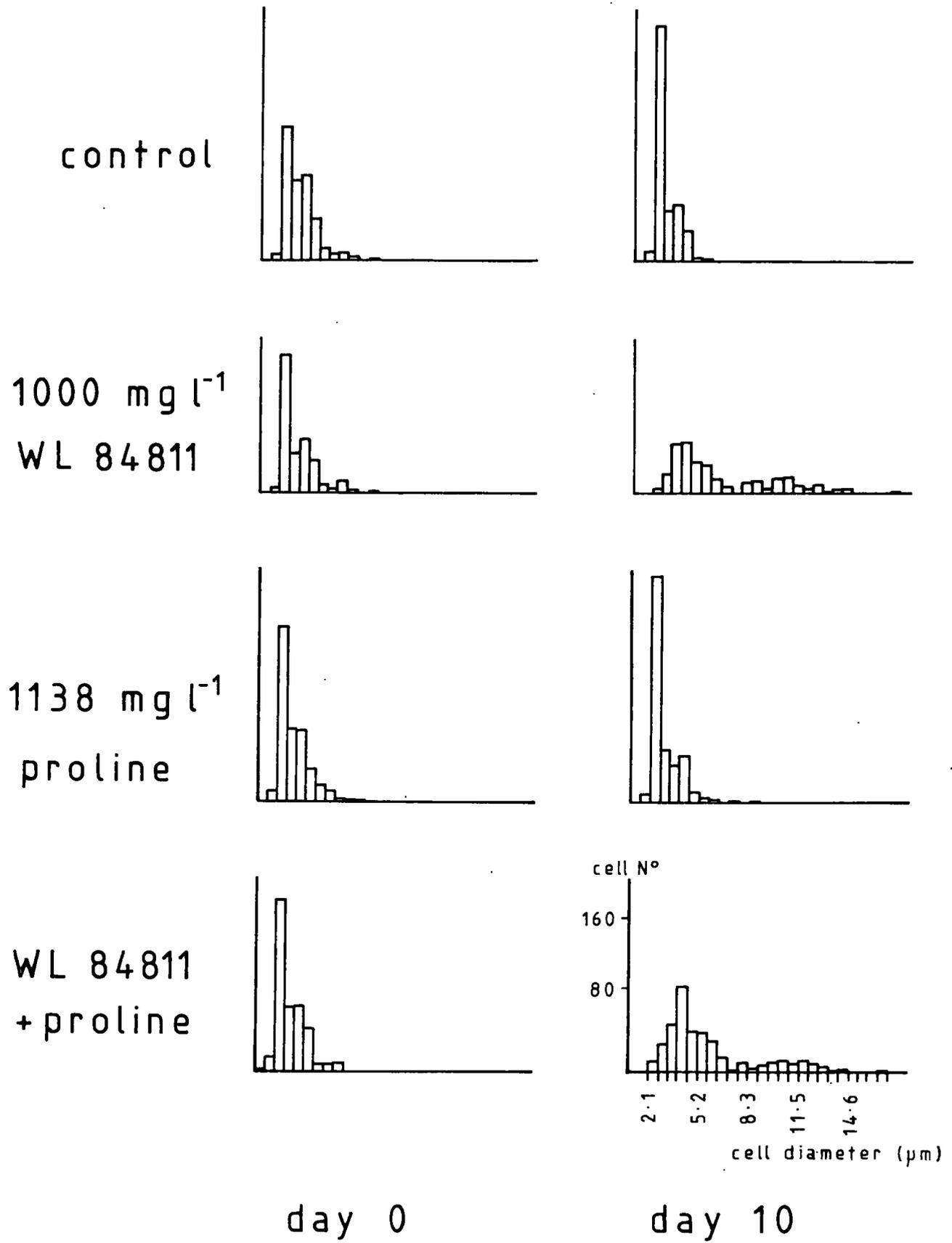
In a second experiment, the ability of high concentrations of proline to overcome the effect of much lower concentrations of WL84811 was tested (Fig. 5.14). Treatments not significantly different from one another at $P=0.05$ are bracketed together ($W=WL84811$, $P=Proline$; all concentrations in mg l^{-1}):

Day 0: (Control, 50 W) > (50 W, 50 W + 500 P) > 50 W + 1000 P

Day 3: (50 W + 1000 P, 50 W + 500 P, Control) > 50 W

Day 6: Control > 50 W > (50 W + 1000 P, 50 W + 500 P)

Day 10: Control > (50 W + 500 P, 50 W + 1000 P, 50 W).

Even proline concentrations twenty times greater than the WL84811 could not overcome the limited inhibition of cell division caused by 50 mg l^{-1} WL84811 to a significant degree.

5.5 The effect of L-Azetidine-2-carboxylic acid (A2C) on growth of *Chlorella*

Cells were grown in media containing 50 or 1000 mg l^{-1} A2C, alone or in conjunction with equimolar proline, to determine whether this naturally-occurring isomer of WL84811 had a similar inhibitory effect on cell division. The cell concentration at intervals after inoculation was calculated (Fig. 5.15) and the significance of differences between counts determined at $P=0.05$. Those not significantly different from one another are bracketed together (A=Azetidine-2-carboxylic acid, P=Proline; all concentrations in mg l^{-1}):

Fig. 5.14 Effect of high concentrations of proline supplied in conjunction with low concentrations of WL84811 on cell division of *Chlorella*.

● = 0 mg l^{-1} WL84811

O = 50 mg l^{-1} WL84811

■ = 50 mg l^{-1} WL84811 + 500 mg l^{-1} proline

□ = 50 mg l^{-1} WL84811 + 1000 mg l^{-1} proline
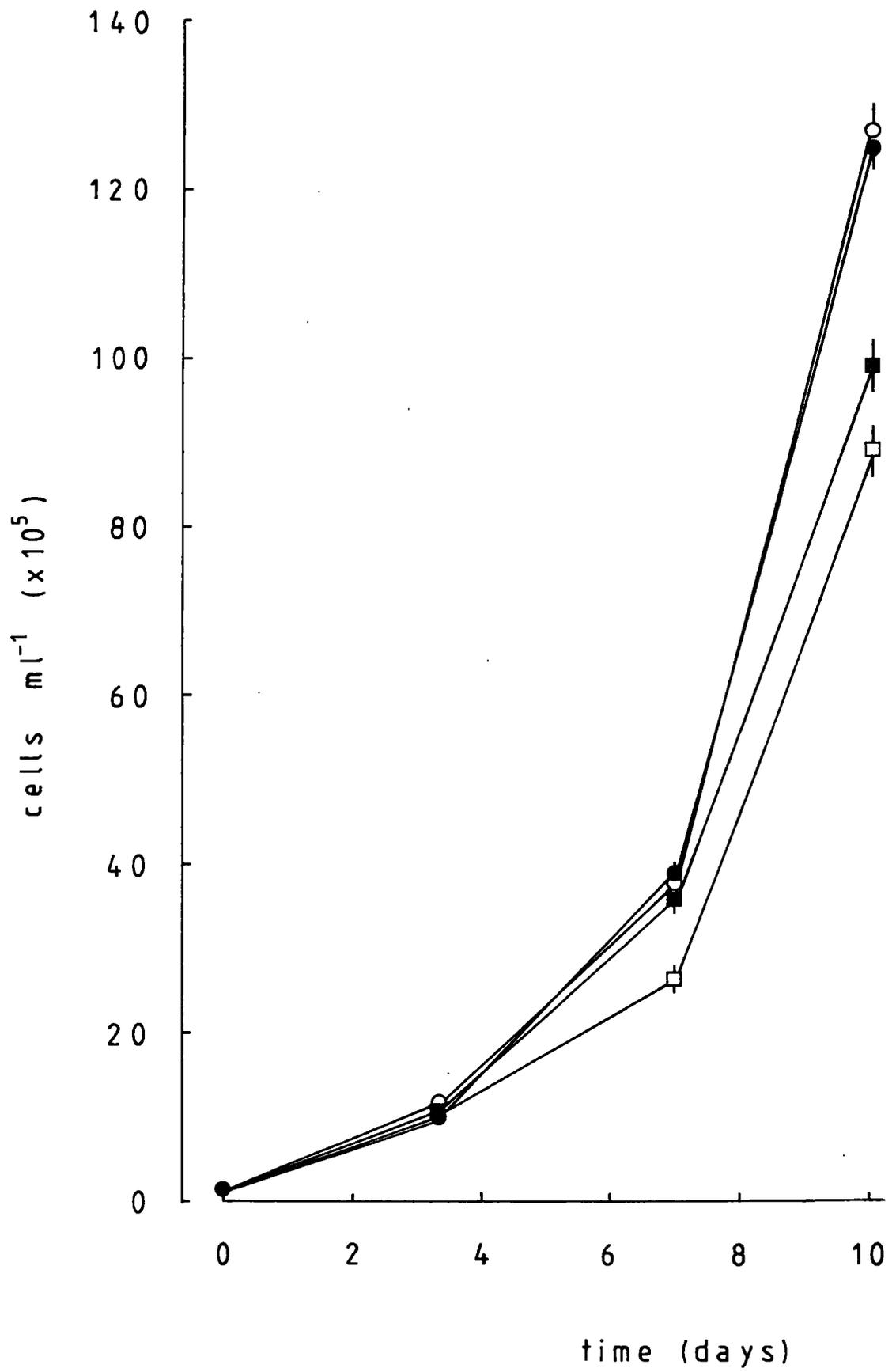
Fig. 5.15a Effect of proline alone on cell division of *Chlorella*.

● = 0 mg l^{-1} proline

○ = 57 mg l^{-1} proline

■ = 1138 mg l^{-1} proline

Fig. 5.15b Effect of L-azetidine-2-carboxylic acid (A2C), with or without equimolar proline on cell division of *Chlorella*.

● = 0 mg l^{-1} A2C

○ = 50 mg l^{-1} A2C

■ = 1000 mg l^{-1} A2C

□ = 1000 mg l^{-1} A2C + 1138 mg l^{-1} proline
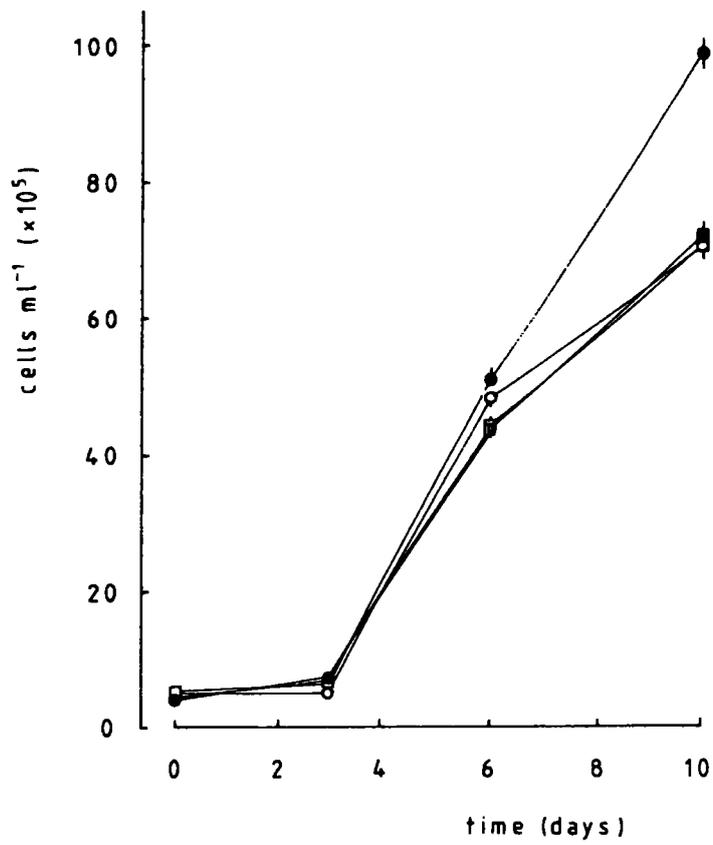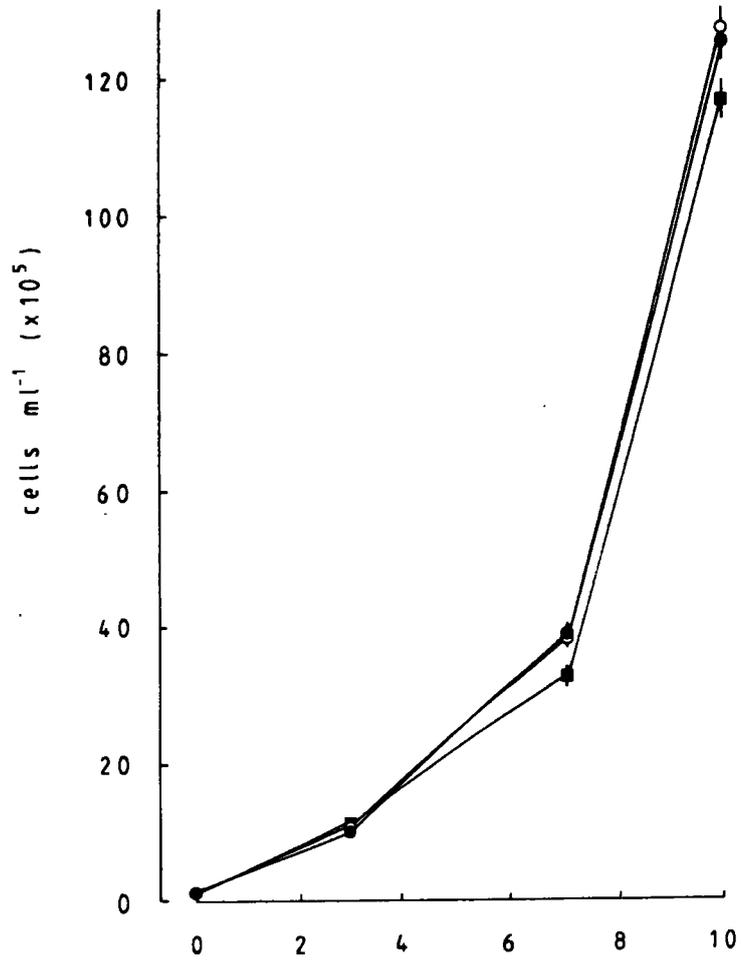
Day 0: (57 P, Control, 1000 A + P, 1000 A, 1138 P, 50 A, 50 A + P)

Day 3: (50 A, 1138 P, 50 A + P, 1000 A, 57 P, 1000 A + P) > (50 A + P, 1000 A, 57 P, 1000 A + P, Control)

Day 6: (Control, 57 P, 50 A) > (57 P, 50 A, 1000 A) > (1000 A, 50 A + P) > (50 A + P, 1138 P) > 1000 A + P

Day 10: (50 A, 57 P, 50 A + P, Control) > 1138 P > 1000 A > 1000 A + P.

High concentrations of both A2C and Proline inhibited cell division, the effect being greater when both were present. In low concentrations however they had no significant effect. None of the treatments affected distribution of cell size markedly (Fig. 5.16).

5.6 Effect of *Chlorella* growth on WL84811 content of medium

Chlorella was grown for 11 days in Chu 10 containing a range of WL84811 concentrations, the cells removed by centrifugation and the supernatant used to germinate seeds of barley (cv Sonja; section 2.7). Seeds were also germinated in freshly prepared solutions of WL84811 of the same concentrations and the root and coleoptile lengths and percentage germination after five days measured and compared. Median values of root and coleoptile length are used as percentage germination of the seeds in Chu 10 was poor (Table 5.4).

Fig. 5.16 Effect of A2C and proline, alone or combined at equimolar concentrations, on size distribution of *Chlorella* cells.
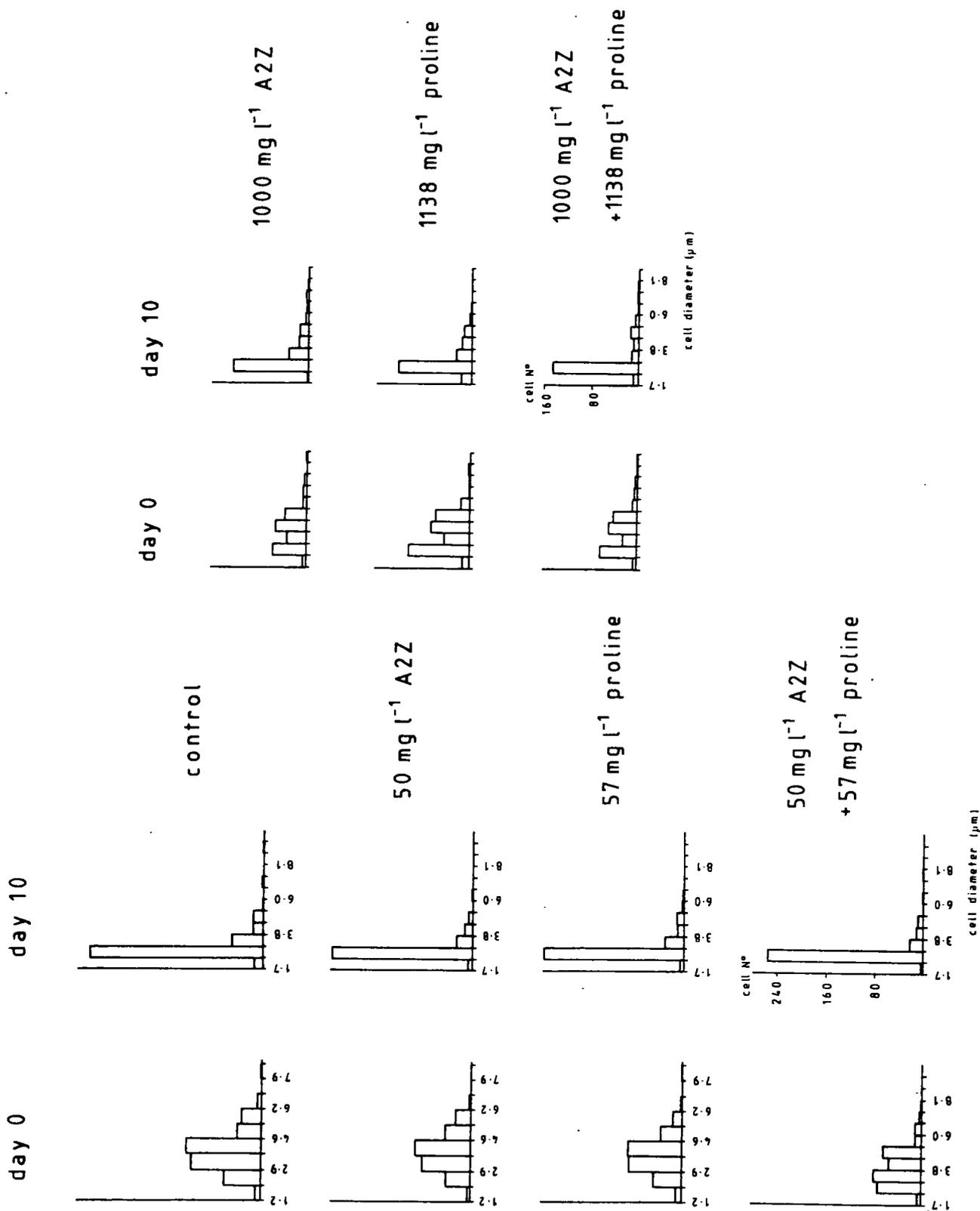
Table 5.4 Effect of fresh and used media on Sonja seed germination and growth after 5 days. F=freshly made up WL84811 in Chu 10: C=WL84811 in Chu 10 after algal growth.

Medium	Median coleoptile length (mm)	Median root length (mm)	% germination
Control (F)	32.0	27.0	8.8
Control (C)	15.0	41.0	11.7
50 mg l^{-1} (F)	6.5	11.5	7.5
50 mg l^{-1} (C)	10.0	20.5	12.5
150 mg l^{-1} (F)	13.5	18.5	7.5
150 mg l^{-1} (C)	22.5	17.0	12.5
300 mg l^{-1} (F)	0	8.0	11.25
300 mg l^{-1} (C)	0	5.5	20.0

Each set of root and coleoptile data for a given WL84811 concentration was compared using the Mann-Whitney U-test and no significant differences between fresh and used media were found although. in most cases. median root and coleoptile length as well as percentage germination were smaller in the fresh medium of a given WL84811 concentration. Caution should be used in interpreting these results as some other constituent of the Chu 10 is obviously also inhibiting seed germination; these values are far lower than usual (see Fig. 4.11). This may be due to pH being buffered at 7.5.

5.7 Ultrastructural observations on *Chlorella*

Chlorella cells were prepared for transmission electron microscopy as described in the section 4.241. Thin sections were cut, mounted on formvar-coated grids and stained.

Some cells, both WL84811-treated and untreated, contained well-formed autospores (Fig. 5.17). Other untreated cells were undivided with a single, prominent nucleus (Fig. 5.18). The exceptionally large cells found after WL84811 treatment were not composed of many autospores which had failed to separate. Instead they contained numerous large starch grains in the chloropast(s) of a single undivided cell (Fig. 5.19).

Fig. 5.17 Transmission electron micrograph of a *Chlorella* cell containing

autospores after growth in plain Chu 10. Scale bar = 250 nm.

a = autospore

Fig. 5.18 Transmission electron micrograph of an undivided *Chlorella*

cell after growth in plain Chu 10, showing cup-shaped chloroplast around the

edge of the cell. Scale bar = 250 nm.

n = nucleus

l = chloroplast lamellae .

158
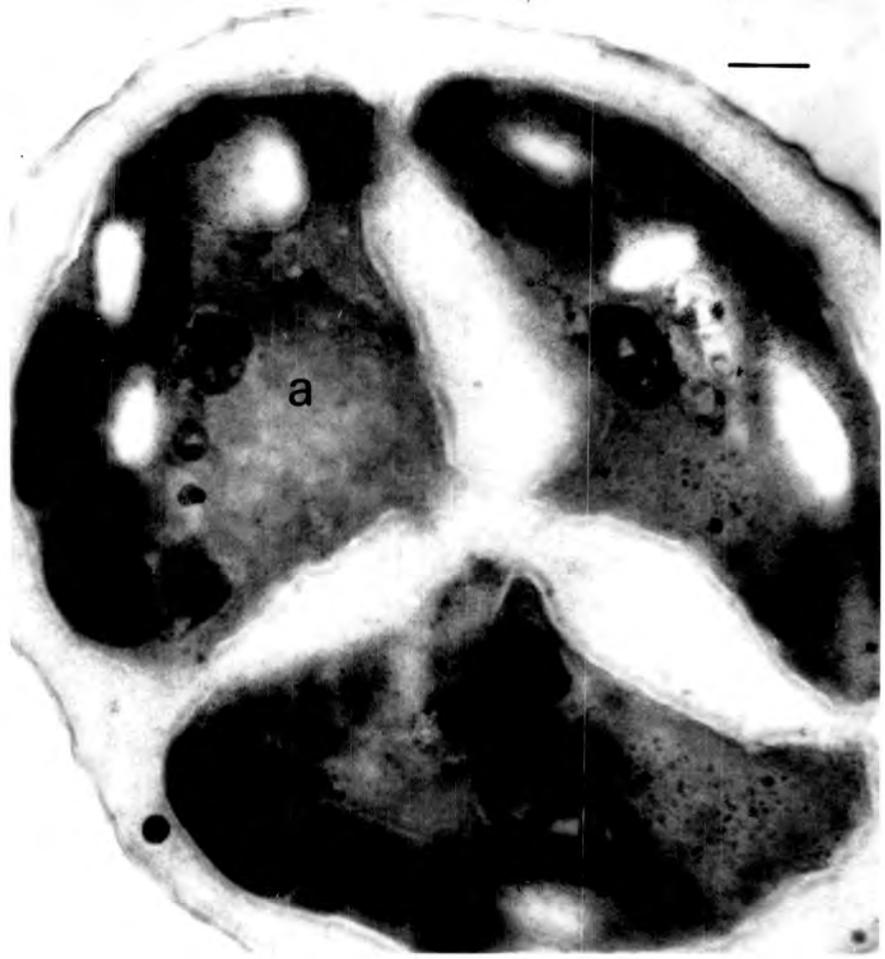
Fig. 5.19 Transmission electron micrograph of an undivided *Chlorella* cell after growth in the presence of 300 mg l^{-1} WL84811. Large amounts of starch can be seen in the chloroplast. Scale bar = 430 nm.

s = starch granule
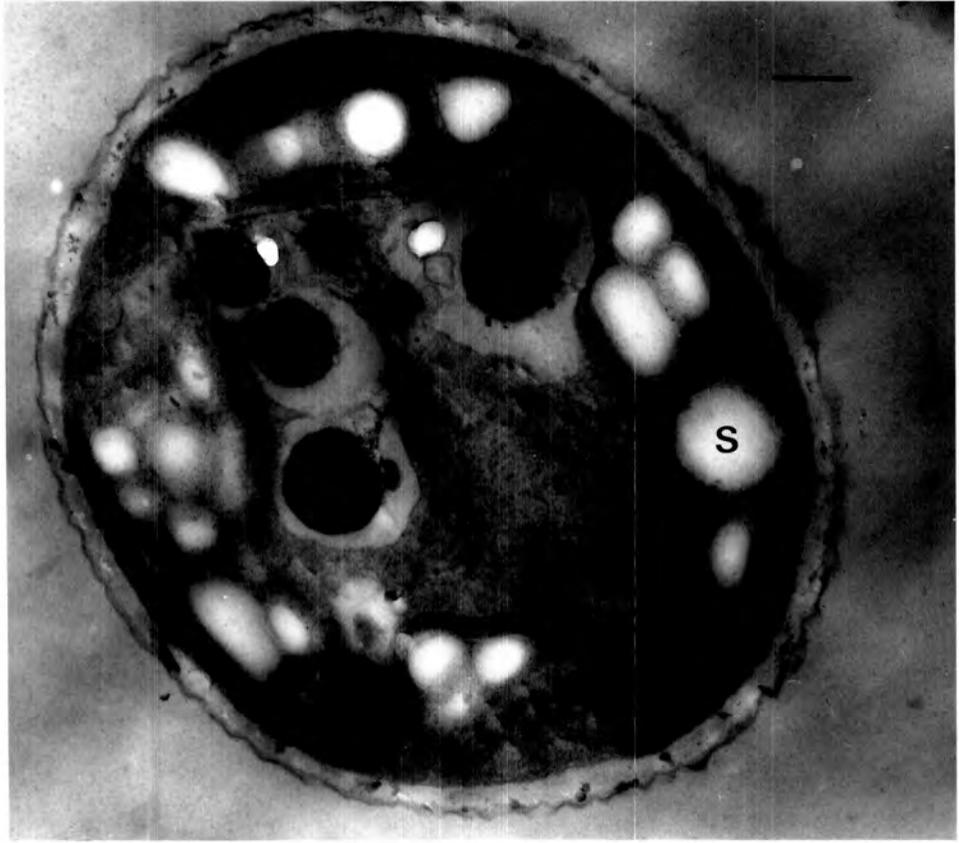
6. EFFECTS OF WL84811 ON POLLEN GERMINATION AND GROWTH

6.1 Introduction

The effects of WL84811 on pollen germination were studied both *in vivo*, after the parent plant had been injected with WL84811 and *in vitro*, when the compound was included in the germination medium used for untreated pollen. The ability of pollen from WL84811-treated plants to germinate *in vitro* was also determined.

6.2 *In vivo* germination and growth of *T. aestivum* pollen

A series of experiments were carried out in which the effect of WL84811 on germination of *T. aestivum* cv. Mercia pollen *in vivo* was studied. Florets were either allowed to self-pollinate naturally or were pollinated by hand. Styles were excised approximately one hour after pollination and stained with acetocarmine.

Pollen from control plants germinated rapidly after self-pollination (Fig. 6.1) with many tubes reaching the base of stigmatic branches one hour after pollination. In some cases, several pollen grains germinated on one branch. However, an hour after self-pollination of WL84811-treated plants, no pollen tubes were visible on the stigma. Most of the pollen had burst (Fig. 6.2).

To determine whether treated pollen was bursting as a result of some property of the stigma or of the pollen itself, two experiments were carried out:

i. florets from untreated plants were emasculated before self-pollination and the stigmas hand-pollinated with pollen from WL84811-treated plants;

Fig. 6.1 Pollen germinating on the stigma of untreated *T. aestivum* cv. Mercia after self-pollination, stained with acetocarmine. The gametes can be seen still inside the pollen grain. Scale bar = 25 μm.

t = pollen tube

g = gametes (sperm cells)

Fig. 6.2 Burst pollen grain on the stigma of WL84811-treated *T. aestivum* cv. Mercia after self-pollination, stained with acetocarmine. Scale bar = 25 μm.
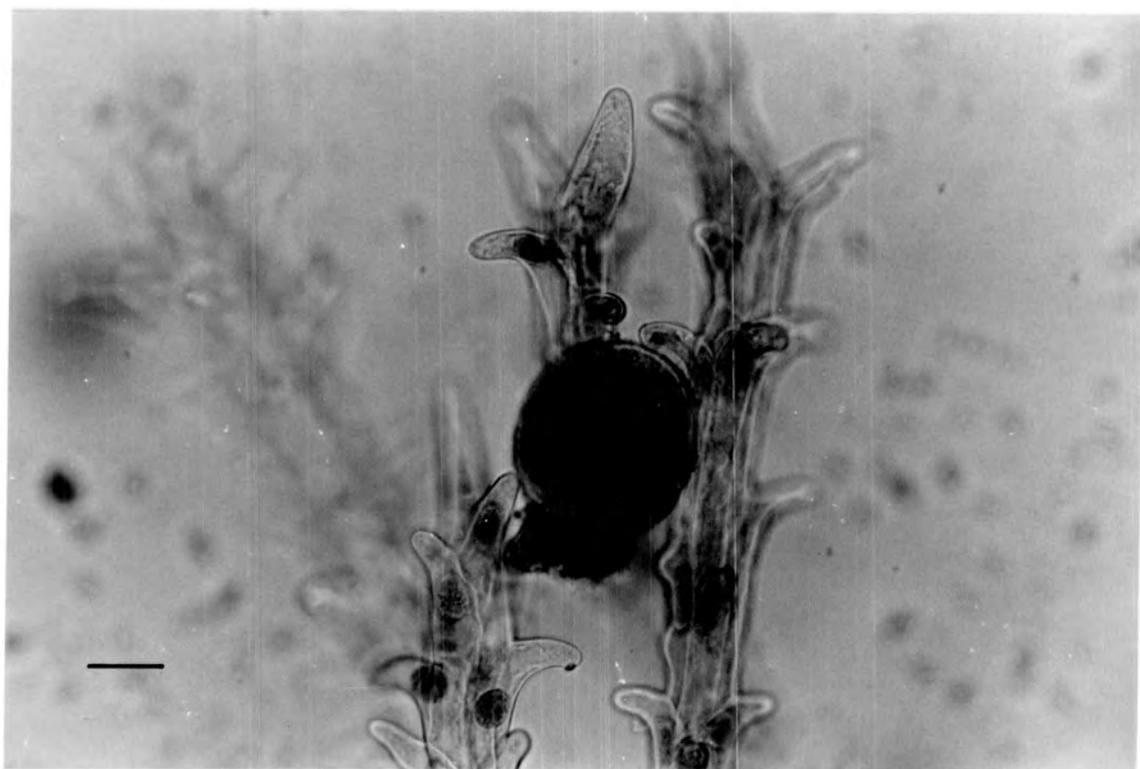
ii. florets from WL84811-treated plants were emasculated and the stigmas hand-pollinated with pollen from untreated plants.

If the effect of WL84811 was solely on pollen, untreated pollen would germinate normally on the stigmas of WL84811-treated plants but treated pollen would not germinate on untreated stigmas. If, however, the effect was mediated through the stigma alone, WL84811-treated pollen would germinate on an untreated stigma but untreated pollen would not germinate on the stigma of a treated plant. If the effect was on both pollen and stigma, untreated pollen would not germinate on the stigma of a WL84811-treated plant or treated pollen on the stigma of an untreated plant.

In fact, untreated pollen germinated normally on the stigmas of treated plants (Fig. 6.3) whereas pollen from treated plants failed to germinate on untreated stigmas (Fig. 6.4). The effect of WL84811 clearly lies in some altered property of the pollen alone which prevents its germination.

Fresh, untreated pollen was killed by shaking it from the anthers onto a microscope slide and exposing it to a dry atmosphere for five minutes. When this pollen was applied by hand to the stigma of an emasculated, untreated plant it failed to germinate (Fig. 6.5). In order to determine whether some exine-held fraction of this dead pollen could overcome the effects of WL84811, it was applied by hand to the stigmas of treated plants at anthesis. No germinating pollen was seen on these stigmas (Fig. 6.6), implying that it is not a lack of some externally-held recognition factor which prevents treated pollen from germinating.

Fig. 6.3 Pollen from an untreated plant germinating on the stigma of WL84811-treated *T. aestivum* cv. Mercia after hand-pollination, stained with acetocarmine. Scale bar = 25 μm.

t = pollen tubes

Fig. 6.4 Pollen from a WL84811-treated plant on the stigma of untreated *T. aestivum* cv. Mercia after hand-pollination, stained with acetocarmine. The.pollen has failed to germinate. Scale bar = 25 μm.
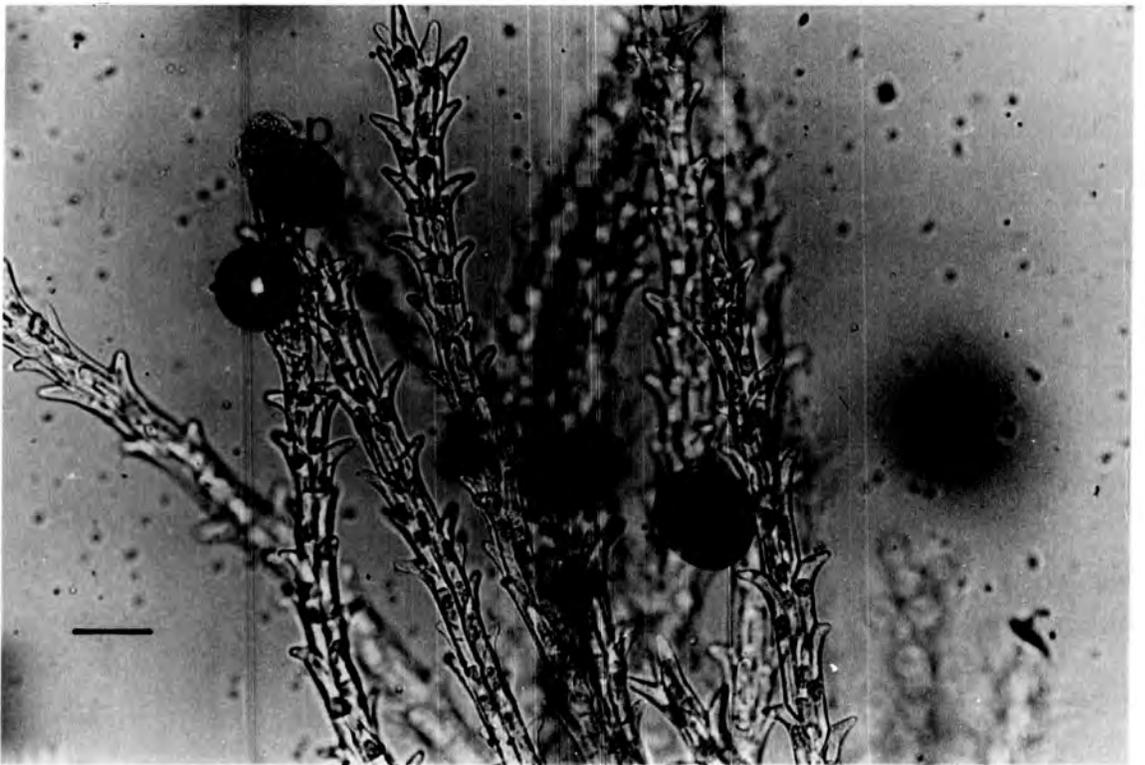
p = pollen

Fig. 6.5 Pollen from untreated *T. aestivum* cv. Mercia killed by dehydration and applied to the stigma of an emasculated, untreated plant; stained with acetocarmine. The pollen failed to germinate. Scale bar = 25 μm.

u = untreated pollen grain

Fig. 6.6 A mixture of dead, untreated *T. aestivum* cv. Mercia pollen applied by hand and WL84811-treated pollen on the stigma of a WL84811-treated plant at anthesis. stained with acetocarmine. No pollen has germinated. Scale bar = 12 μm.
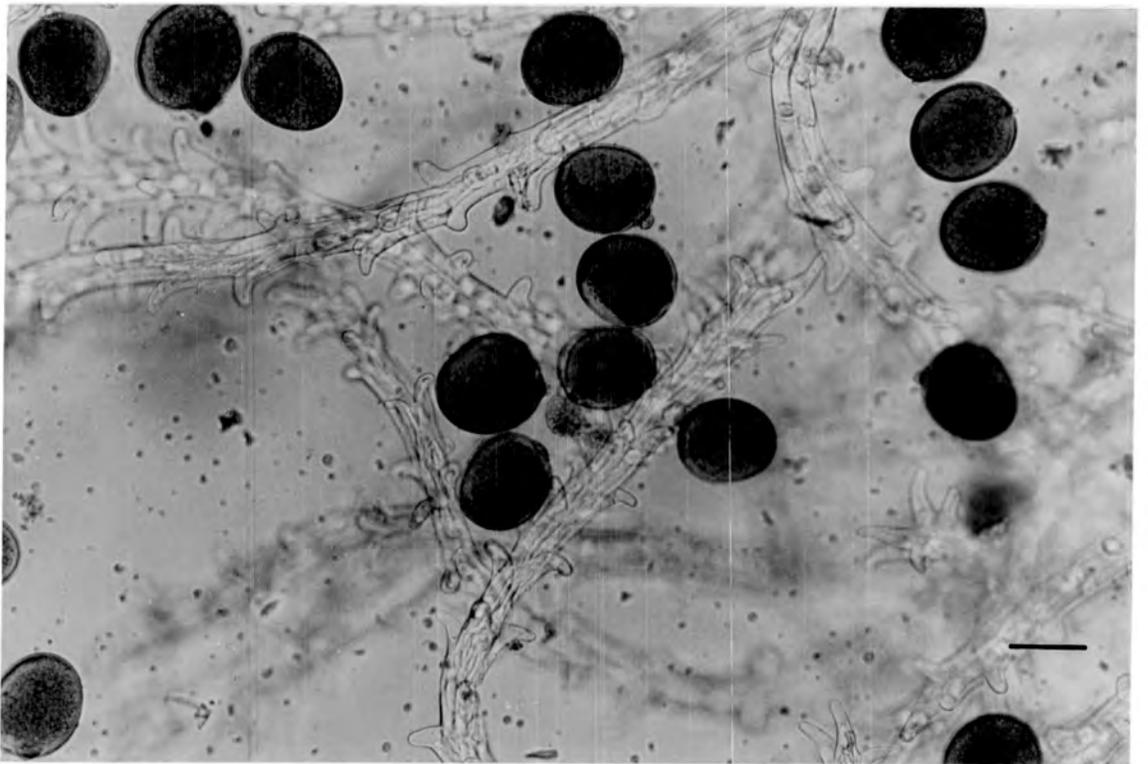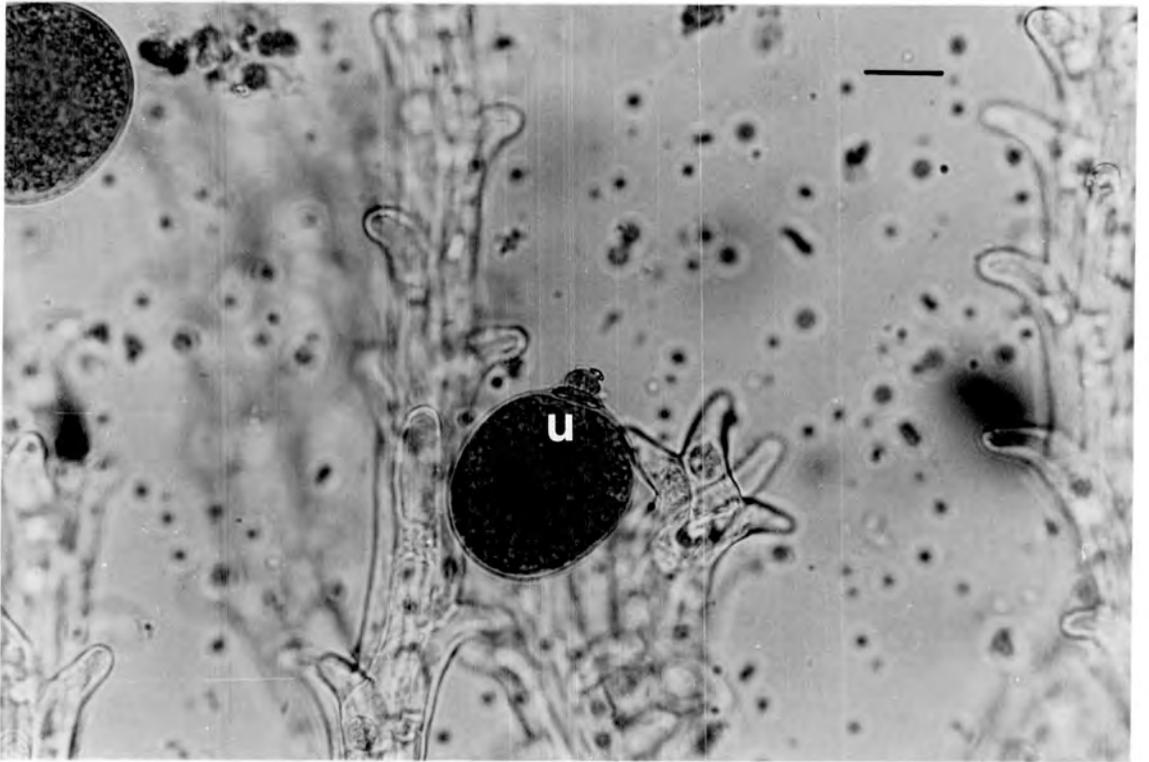
The technique of X-ray microscopy was tested in the hope that it would allow the study of pollen germination and pollen tube growth in intact florets of live material (2.43). The stigma and anthers of each floret could be seen clearly through the palea and lemma (Fig. 6.7), along with pollen from anthers that had dehisced. It was not, however, possible to resolve individual pollen grains on the stigma or to see pollen tube growth at the maximum magnification available on this occasion.

6.3 Effect of WL84811 on pollen germination *in vitro*

The effect of adding WL84811 to pollen germination media was tested to determine how late WL84811 could be applied in order to have its gametocidal effect. This was studied with two species having binucleate pollen which was easy to germinate (*Vicia faba* and *Tropaeolum majus*) as well as four cereals with trinucleate pollen (*Hordeum vulgare, Secale cereale, Triticum aestivum* and *Pennisetum typhoides*).

As, like the seed data, most of these data were not normally distributed, median values with upper and lower quartiles were calculated and Kruskal-Wallis one-way analyses performed to show the significance of differences between treatments. The significance of the difference between particular treatments was determined using the Mann-Whitney U-test. A range of concentrations of WL84811 were tested to determine the effect of concentration on its action.

Fig. 6.7 X-ray micrograph of a *T. aestivum* floret at anthesis showing pollen being shed onto the stigma. Scale bar = 2 cm.

A = anther

S = stigma

6.31 *Vicia faba* (field bean) cv. Sudanese Triple White

Vicia faba pollen germinated readily on the basic medium of Brewbaker and Kwack (1963). Percentage germination and pollen tube length on this medium, with and without added WL84811, were measured from photographs taken of at least seven fields of view per treatment after six hours incubation (Fig. 6.8). Median percentage germination and pollen tube length of germinated pollen after this time are given in Table 6.1.

Table 6.1 Germination and growth of *Vicia faba* pollen. Quartiles given in brackets.

WL84811 concentration	Median % germination	Median tube length (μm)
0 mg l^{-1}	60.0 (53.4 – 70.7)	333 (173 – 907)
500 mg l^{-1}	65.5 (46.0 – 69.2)	560 (277 – 1070)
1000 mg l^{-1}	43.9 (18.6 – 58.8)	613 (340 – 800)

WL84811 had no significant effect on percentage germination of *V. faba* pollen ($0.05 < P < 0.1$) although the highest concentration tested did cause some reduction. However it did have a significant effect ($0.001 < P < 0.001$) on tube length of germinated pollen; both concentrations of WL84811 tested increased the pollen tube length of germinated pollen relative to the control, with 500 mg l^{-1} WL84811 having the greater effect.

Fig. 6.8 *Vicia faba* cv. Sudanese Triple White pollen germinating *in vitro*
after six hours incubation. Scale bar = 170 μm.

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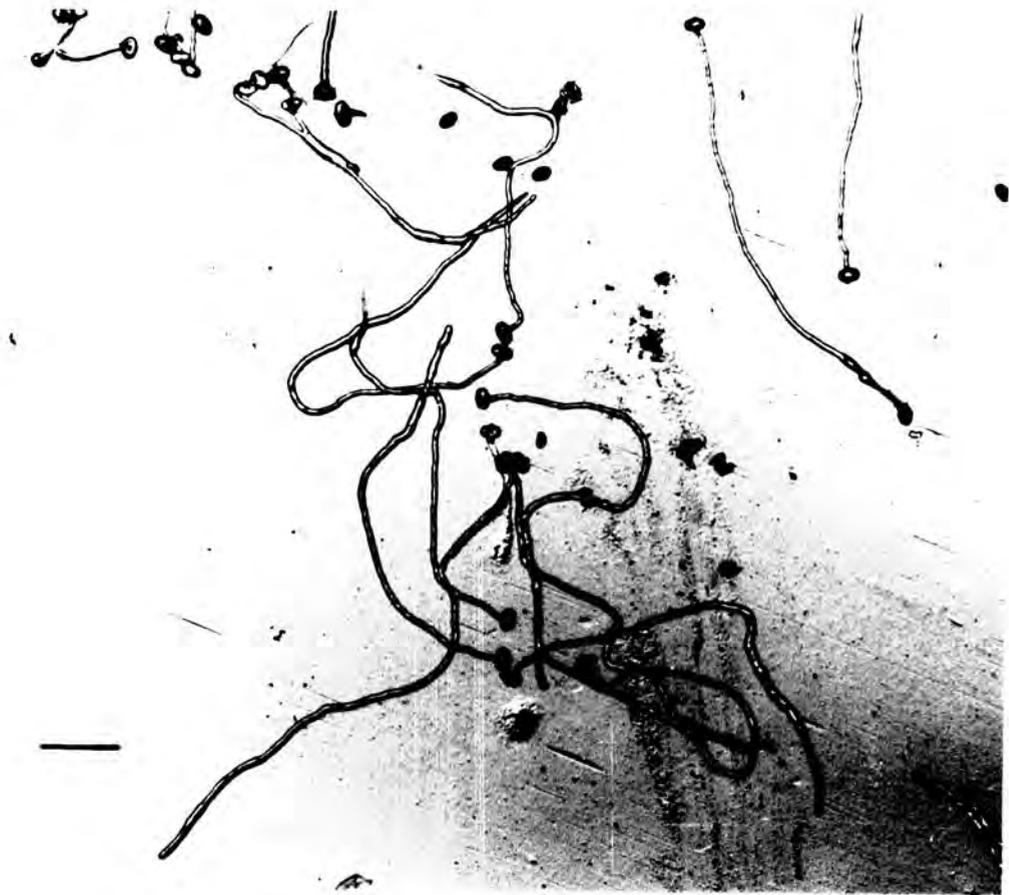
6.32 *Tropaeolum majus* (nasturtium)

Nasturtium pollen also germinated readily on cellophane overlaying filter paper soaked in Brewbaker and Kwack's (1963) medium, with or without WL84811 present. Percentage germination and pollen tube lengths of germinated pollen were measured for at least 15 fields of view per treatment after three hours. Median values are presented in Table 6.2.

Table 6.2 Germination and growth of *T. majus* pollen. Quartiles given in brackets.

WL84811 concentration	Median % germination	Median tube length (μm)
0 mg l^{-1}	44.9 (30.6 – 64.8)	362 (176 – 492)
300 mg l^{-1}	42.9 (18.4 – 65.0)	428 (294 – 559)
3000 mg l^{-1}	26.2 (10.0 – 53.6)	394 (201 – 545)

WL84811 had no significant effect on percentage germination of *T. majus* pollen *in vitro* ($0.05 < P < 0.1$) although a reduction was apparent at the highest concentration used. As for *V. faba* pollen, WL84811 did have a significant effect ($0.001 < P < 0.01$) on the length of tubes produced by germinating pollen; the higher concentration had no effect but the lower concentration increased pollen tube length significantly relative to the control.

6.33 *Hordeum vulgare* (barley) cv. Sonja

H. vulgare cv. Sonja pollen germinated on the medium of Heslop-Harrison (1979a) with one percent agarose added to partially solidify it. Moderate germination was obtained on this medium but some pollen tubes over one millimetre long were measured. Percentage germination and tube length of germinated pollen were measured over at least 18 fields of view per treatment after three hours growth in the presence

or absence of WL84811 (Fig 6.9). Median values are presented in Table 6.3.

Table 6.3 Germination and growth of *H. vulgare* pollen. Quartiles given in brackets.

WL84811 concentration	Median % germination	Median tube length (μm)
0 mg l^{-1}	17.2 (6.6 – 25.0)	229 (162 – 630)
6000 mg l^{-1}	4.5 (2.6 – 8.6)	162 (90 – 255)

This high concentration of WL84811 reduced both percentage germination and tube length of germinated pollen significantly ($P < 0.01$ for both).

6.34 *Pennisetum typhoides* (millet)

P. typhoides pollen germinated successfully on the same medium as used for barley pollen: although percentage germination was generally poor, some long pollen tubes were produced (Fig. 6.10). Median values of percentage germination and pollen tube length from at least four fields of view after 90 minutes incubation are given in Table 6.4.

Table 6.4 Germination and growth of *P. typhoides* pollen. Quartiles given in brackets.

WL84811 concentration	Median % germination	Median tube length (μm)
0 mg l^{-1}	4.6 (2.2 – 9.0)	376 (350 – 575)
6000 mg l^{-1}	3.3 (2.0 – 4.4)	137 (88 – 186)

6000 mg l^{-1} WL84811 had no significant effect on percentage germination of *Pennisetum* pollen *in vitro* ($P > 0.1$) although it did appear to reduce this slightly. WL84811 did, however, reduce the tube length of those pollen grains which germinated ($P < 0.001$) significantly.

Fig. 6.9 *H. vulgare* cv. Sonja pollen after three hours germinating *in vitro*. Scale bar = 70 μm.

Fig. 6.10 *Pennisetum typhoides* pollen after three hours germinating *in vitro*. Scale bar = 40 μm.
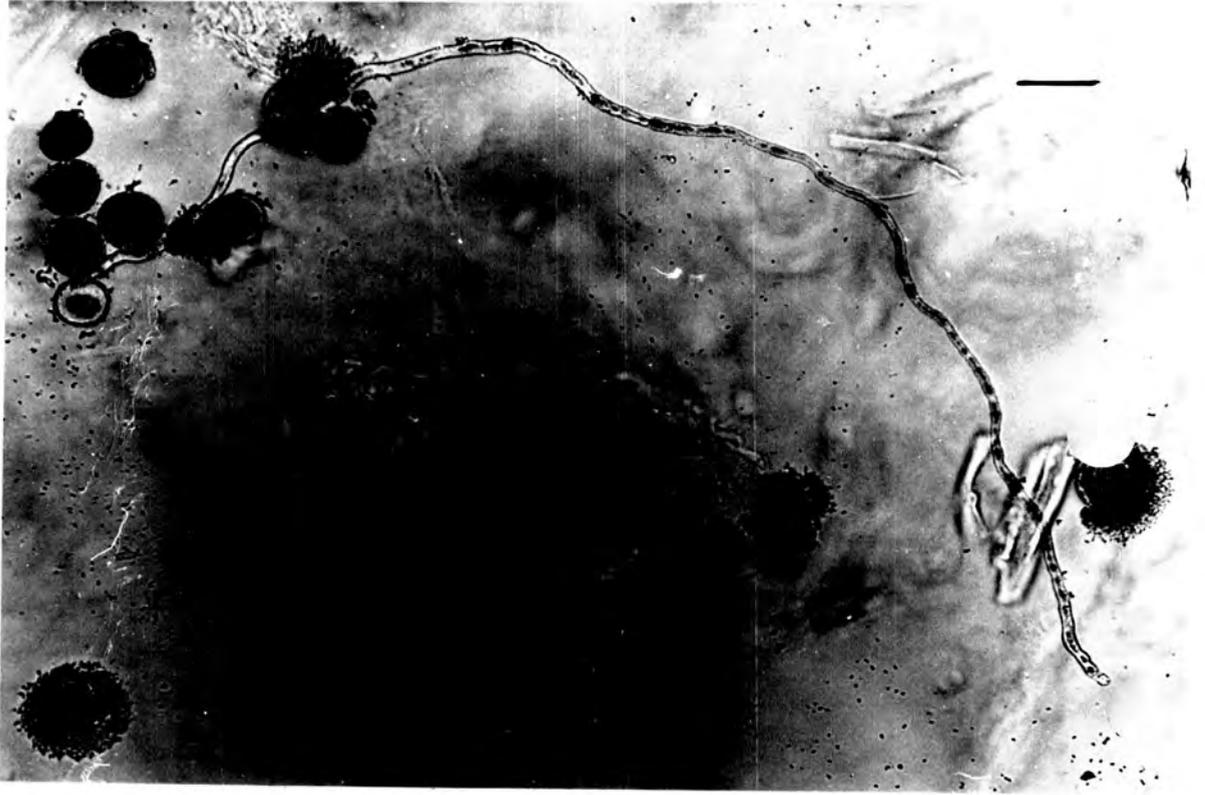
6.35 *Secale cereale* (rye) cv. Dominant

S. cereale pollen germinated successfully on the same medium as used for barley and millet; percentage germination was generally poor but some very long pollen tubes were measured (Fig. 6.11). The behaviour of untreated pollen on media with and without WL84811 was compared, along with the behaviour of pollen from plants treated by stem injection with WL84811. Median values of percentage germination and pollen tube length from at least 10 fields of view after 90 minutes incubation are given in Table 6.5.

Table 6.5 Germination and growth of *S. cereale* pollen. Quartiles given in brackets.

WL84811 concentration	Median % germination	Median tube length (μm)
0 mg l^{-1}	8.0 (4.1 – 21.7)	270 (182 – 467)
6000 mg l^{-1} in medium	4.4 (3.2 – 7.1)	604 (319 – 825)
3000 mg l^{-1} (stem injected)	4.4 (3.6 – 9.5)	227 (142 – 402)

Neither 6000 mg l^{-1} WL84811 in the medium or 3000 mg l^{-1} WL84811 applied by stem-injection had any significant effect on percentage germination of pollen although both appeared to reduce it relative to the control. In addition, WL84811 applied by stem-injection did not affect pollen tube length significantly. However, the presence of WL84811 in the medium increased the tube length of germinated pollen significantly ($P = 0.012$).

The apparent lack of effect of WL84811 applied by stem-injection on the germination and tube growth of rye pollen is presumably related its lack of effect on the morphology of this pollen (Fig. 3.19). More work is required to show conclusively whether this species is indeed not susceptible to the gametocidal effects of WL84811.

Fig. 6.11 *Secale cereale* cv. Dominant pollen grain germinating *in vitro* after 90 minutes incubation. The three nuclei can be seen clearly; one sperm nucleus has entered the pollen tube and the other two remain inside the pollen grain. Scale bar = 20 μm.

v = vegetative nucleus

▲ = sperm nuclei

6.36 *Triticum acstivum* (wheat) cv. Mercia

Mercia pollen was germinated on the medium used by Mathur (1977), modified by the addition of one percent agarose (2.52). Untreated pollen was germinated on this medium, with or without 3000 mg l^{-1} WL84811 present, and the percentage germination and length of germinated pollen tubes measured over at least six fields of view after 30 minutes and 16 hours incubation (Fig. 6.12). Median percentage germination and pollen tube length of germinated pollen are given in Table 6.6.

Table 6.6 Germination and growth of *T. aestivum* pollen. Quartiles given in brackets.

WL84811 conc.	Time	Median % germination	Median tube length (μm)
0 mg l^{-1}	30 min.	7.1 (0.0 – 16.7)	116 (45 – 138)
	16 hours	6.2 (4.0 – 13.0)	63 (47 - 137)
3000 mg l^{-1}	30 min.	13.4 (8.4 – 24.2)	187 (137 – 224)
	16 hours	3.7 (1.4 – 15.6)	163 (75 – 288)

The Mann-Whitney U-test showed that differences between percentage germination and pollen tube length at 30 minutes and 16 hours were not significant ($P = 0.05$) on either medium; all pollen competent to do so had germinated after 30 minutes. In addition, the difference between percentage germination on the medium containing WL84811 and the control medium was not significant at either time. However, the presence of WL84811 in the medium did affect pollen tube length highly significantly ($P = 0.001$ at 30 minutes, $P = 0.014$ at 16 hours).

Fig. 6.12 Untreated *T. aestivum* cv. Mercia pollen germinating *in vitro* after 90 minutes incubation. Scale bar = 70 μm.

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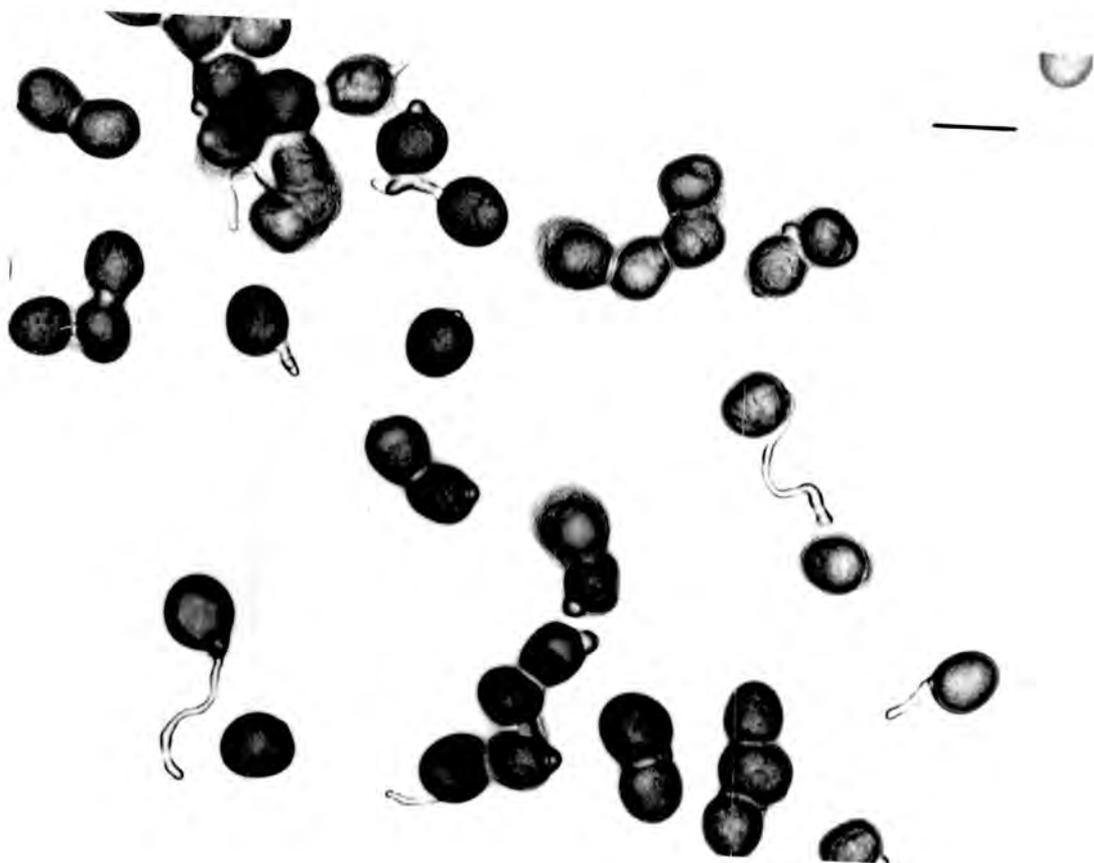
As for all pollen tested, with the exception of *P. typhoides* and *H. vulgare*, WL84811 in the medium enhanced pollen tube length significantly rather than reducing it as might perhaps have been expected. When pollen from plants previously treated with WL84811 by stem injection was scattered on medium without WL84811 it totally failed to germinate (Fig. 6.13).

These results show that the compound must be taken up into the pollen late in its development (after meiosis but before the first mitosis) in order to have a gametocidal effect: no such effect was observed when the chemical was supplied exogenously to mature pollen.

Fig. 6.13 Pollen from WL84811-treated *T. acstivum* cv. Mercia failing to germinate *in vitro* after 16 hours incubation. Scale bar = 70 μm.
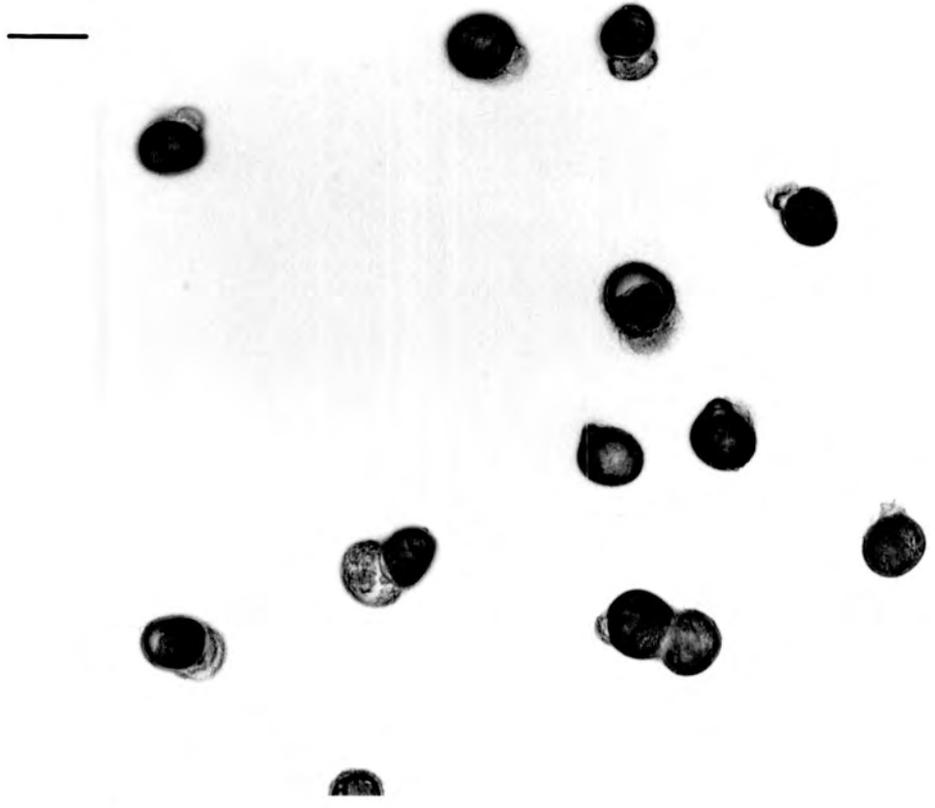
7. DISCUSSION

7.1 Introduction

This chapter discusses the effects of WL84811 observed on the various systems studied and, from these results, constructs a general hypothesis as to its mode of action. Initial studies of the effects of WL84811 on floral development were extended to investigate its effects on cell division and expansion in other plant systems. These results were, in turn, related to observations about the effect of WL84811 on germination of pollen, both *in vivo* and *in vitro*, and conclusions drawn.

7.2 The effect of WL84811 on floral development

WL84811 is believed to exert its gametocidal effect by altering the interaction between pollen and stigma, so preventing normal pollen tube growth and fertilization (Jeffcoat *et al.*, 1982). Results using ^{14}C-WL84811 showed that the compound is taken up in equal amounts per milligram of dry tissue by male and female parts of the flower (3.2) and is not accumulated selectively by the anther tissue. Does this mean that WL84811 affects the morphology, and hence behaviour, of both pollen and stigma? To ascertain this, the development of both tissues was studied using *Triticum aestivum* (3.3, 3.4).

WL84811 has a visible effect only on pollen development in *T. aestivum* (3.41, 3.42); it appears to have no effect on stigma development. This is important as a potential gametocide must not affect female fertility if it is to be used for large-scale F_1 hybrid production (Batch, 1978). Grains of WL84811-treated wheat pollen appeared

perfectly normal when hydrated in an aqueous stain such as acetocarmine. However, when semi-thin sections of embedded material were stained with toluidine blue, clear differences were seen between WL84811-treated and untreated wheat pollen. Whereas untreated pollen was spherical and packed with starch grains (Fig. 3.17), treated pollen was irregular in shape with a less densely-packed cytoplasm which often did not fill the entire grain (Fig. 3.18). Such a difference was not apparent in the case of *S. cereale* pollen; this appeared normal after the parent plant had been injected with WL84811 (Fig. 3.19). This should be repeated before a definitive statement is made, but it may indicate that rye is not susceptible to the gametocide.

A good supply of starch and presynthesised polysaccharide particles (P-particles) is necessary for pollen germination (Heslop-Harrison, 1979a). The inability of WL84811-treated pollen to germinate might be due, in part, to its lack of reserves. The slight reluctance of WL84811-treated wheat anthers to dehisce is a phenomenon common to cytoplasmic male sterile barley (Ahokas, 1978).

Further differences were observed using transmission and scanning electron microscopy. Untreated pollen had a thin intine layer of regular thickness (Fig. 3.20) with a fibrillar structure (Fig. 3.21), surrounding a densely packed cytoplasm. In WL84811-treated pollen, however, the intine layer was replaced by a layer of variable thickness with a net-like structure into which the cytoplasm was invaginated (Fig. 3.22). In addition, the cytoplasm of treated pollen was less densely packed with inclusions. This lack of a normally-structured intine may play a large part in the inability of WL84811-treated pollen to germinate; the intine layer is continuous with the pollen tube wall during its initiation (Heslop-Harrison, 1979a).

Few previous studies have looked in detail at the effects of gametocides on pollen

development; most reports of gametocidal action concentrate on detailing efficiency and any possible side-effects. However, the cytological effects of the gametocides ethrel and RH-531 on barley have been studied (Colhoun & Steer, 1983). Colhoun and Steer (1983) found differences in the initial effects of ethrel depending on the time of its application; premeiotic application caused additional mitoses and tangential wall formation whereas post-meiotic application led to exine malformation and microspore degeneration. The effects of RH-531 also differed with time of application. For both chemicals the visible effects were limited to the pollen itself, with tapetal cells apparently unaffected.

Many more TEM studies have been carried out comparing fertile and cytoplasmic male sterile (CMS) lines of wheat and barley (e.g. Ahokas, 1978; De Vries & Ie, 1970; Hu et al., 1977). These show a diverse range of effects associated with CMS which, like those produced by the gametocides, probably reflect the primary results of male sterility rather than its cause.

Scanning electron micrographs show that most WL84811-treated pollen collapses and folds up shortly after being shed onto the stigma (Fig. 3.23), whereas untreated pollen retains its shape for some time (Fig. 3.24). This rapid collapse of WL84811-treated pollen may be due to failure of the osmoregulatory system which maintains turgor. ~~related to the lack of cytoplasm observed in TEM sections~~.

Proline accumulates in many types of cells when subjected to water-stress and may be important in maintaining the complete hydration of biopolymers under these conditions (Schobert, 1977). Consequently, it is abundant in pollen which has a very low water content (10 – 15 % of the weight) by the time it is released from the anther (Knowlton, 1921). It may account for more than one percent of the dry weight (Bathhurst, 1954) and has been implicated as one factor determining pollen

viability (Pálfi *et al.*, 1981). No difference was found between the proline content (as a percentage of the total pollen dry weight) of the two types of pollen; this may have been partly due to insensitivity of the colorimetric method employed. However, the proline concentrations measured were in general agreement with the concentrations measured by Bathurst (1954).

The only difference observed in the morphology of the ear of WL84811-treated plants was a characteristic gaping of florets after self-pollination (Fig. 3.25). This response is common in situations where for some reason self-pollen is unable to effect fertilization; the stigma remains receptive to other pollen for several days (Evans *et al.*, 1972).

7.3 The effect of WL84811 on seed germination and seedling growth

If WL84811 is accumulated by both anthers and pistils, why does it only affect development of the former? Does it affect any other plant systems and if so, how? The seed germination system was developed as a convenient way of answering these questions by studying the physiological effects of WL84811.

Despite differences in structure, size and composition, the initial stages in germination and growth of the dispersal units of a wide range of angiosperms, be they true seeds, achenes (single-seeded fruits such as lettuce) or caryopses (achenes where the testa and pericarp are fused together as in wheat and barley), are remarkably similar. These dispersal units (Fig. 7.1) will collectively be described as 'seeds' in the rest of this chapter. In order to understand the effects of WL84811 on germinating seeds, it is necessary to know something of the germination process in different types of seed.

Fig. 7.1a Structure of a *T. aestivum* caryopsis. Scale bar = 1 cm.

Fig. 7.1b Structure of a *Beta vulgaris* seed. Scale bar = 1cm.

Fig. 7.1c Structure of a *Lactuca sativa* achene. Scale bar = 1cm.

a.

pericarp and testa

aleurone

endosperm

scutellum

coleoptile and leaves

radicle

b.

cotyledons

testa

perisperm

plumule

endosperm

radicle

c.

pericarp and testa

endosperm

plumule

radicle
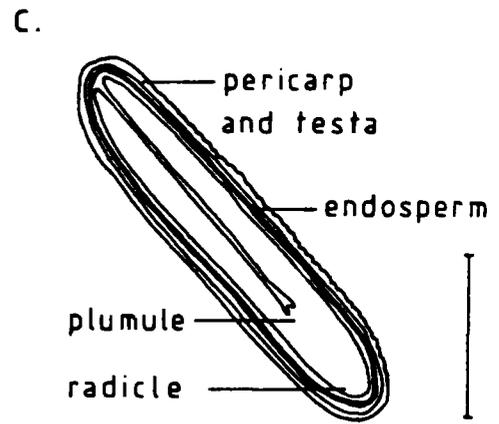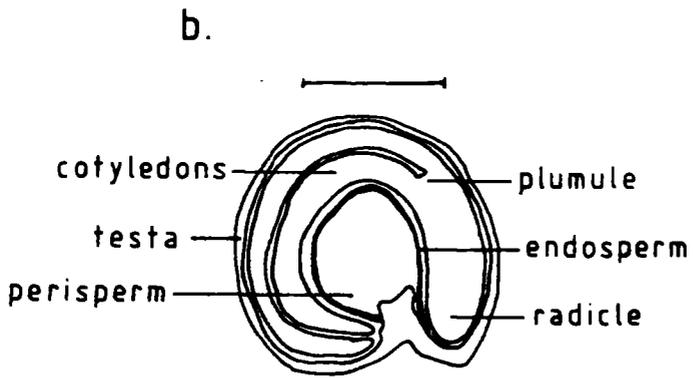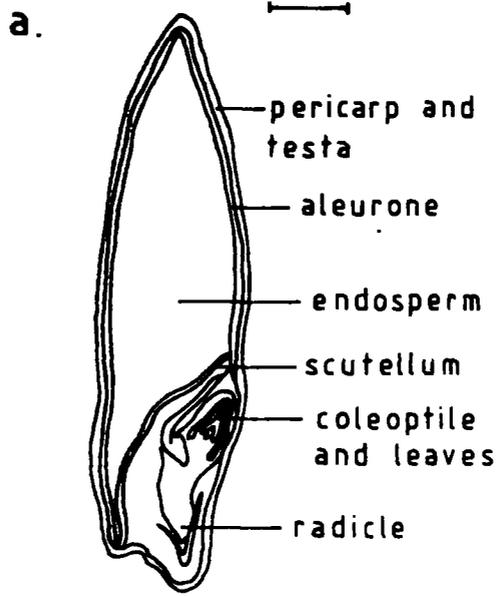
Seeds can be divided into two broad categories; endospermic (mostly monocots) and non-endospermic (mostly dicots). Non-endospermic seeds such as the legumes store the reserves necessary for early seedling growth in large cotyledons, whereas endospermic seeds such as the cereals store most of their reserves in the persistent endosperm. The latter have a single cotyledon (known as the scutellum in cereals) which is highly modified to aid in the mobilization of reserves (Bewley & Black, 1978). Both types of seed have an embryonic axis consisting of two meristems connected by a rod of non-vacuolate cells; these are formed during seed development by a series of rapid divisions which prevent them from expanding (Brown, 1972).

During imbibition and germination of all seeds there is an initial period of non-metabolic water absorption until the seeds become saturated; this lasts up to 48 hours. During this period, cells of the embryonic axis expand rapidly, increasing the length and fresh weight of the axis. No increase in dry weight occurs until the onset of a second period of water uptake linked with metabolic activity. This is the point at which the radicle emerges and, in most seeds, it also marks the onset of cell division (Brown, 1972; Haber & Luippold, 1960). In barley, however, the radicle emerges several hours before the onset of division (Caldecott & Smith, 1952). Haber and Luippold (1960) have shown that cell expansion and division are initiated independently in lettuce seeds; mitosis is neither necessary for, nor sufficient alone to initiate, germination. Cell expansion, on the other hand, is necessary and, in the absence of cell division, can allow germination to proceed up to the point where the radicle emerges.

WL84811 had broadly similar effects on germination and seedling growth of a wide range of species. both monocots and dicots. but the magnitude of the effect varied.

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For all the dicot seeds studied, WL84811 reduced seedling growth of germinated seeds significantly. This effect was greatest in the case of *Lactuca sativa* (Fig. 4.4), where 50 mg l^{-1} WL84811 reduced growth significantly and least in the case of *Pisum sativum* (Fig. 4.7) where 6000 mg l^{-1} WL84811 was required. Some of the lower WL84811 concentrations actually enhanced the growth of pea seedlings significantly. This difference may be due, in part, to the sizes of these particular seeds. Whereas lettuce seeds are tiny (around three millimetres long), dry pea seeds may be up to 10 millimetres in diameter. Assuming a similar rate of uptake over their respective surfaces, the concentration of WL84811 in lettuce tissues might be more than three times the concentration in pea tissues because of the latter's much smaller surface area/volume ratio. However, this does not account for all the difference in their sensitivity. The fact that low WL84811 concentrations actually enhance the growth of pea seedlings suggests that there may also be a difference in the two species' ability to metabolise WL84811.

Only with *Daucas carota* (Fig. 4.2) and *Lactuca sativa* (Fig. 4.4) did WL84811 affect percentage germination of seeds significantly (where germination is defined as the process which "culminates in radicle emergence" (Bewley & Black, 1978)). In both cases germination was only reduced significantly by very high concentrations of WL84811. Onion (Fig. 4.8) and the graminaceous monocots (Figs 4.9 – 4.16) were affected similarly to the dicot seeds by WL84811. In all cases, 50 or 150 mg l^{-1} WL84811 reduced coleoptile and root growth significantly. In only two cereals, the *T. aestivum* cultivar CWW 3547/1 and *Oryza sativa* cv. IR36, did WL84811 affect percentage germination significantly (Figs 4.13, 4.16).

These results suggest that WL84811 has most of its effect on cell division, or on the wall formation associated with cell division or expansion

the mobilization of stored reserves, processes which are initiated after the embryonic axis has elongated sufficiently for the radicle to emerge from the seed coat.

Further experiments with the *H. vulgare* cultivar Sonja showed that WL84811 affects coleoptile and root growth of germinated seeds differently. It affected only the rate of root growth and not its onset (Fig. 4.17c), adding weight to the hypothesis that WL84811 is having ~~some~~ its initial effect on cell division rather than cell expansion. WL84811 had an additional effect on coleoptile growth, delaying its onset as well as reducing its rate (Fig. 4.17b). This may be due, in part, to the dependency of shoot growth on root function; as plumule growth almost invariably starts after the radicle has emerged (Brown, 1972), the inhibition of root growth caused by WL84811 presumably impaired their ability to supply growth substances required for shoot development (Evans *et al.*, 1975).

The concentration of WL84811 required to inhibit root growth by 50 % relative to the control was constant at around 260 mg l^{-1} after three or more days (Fig. 4.18b) but much higher during the earliest stages (up to 48 hours). This may reflect the period during which cell expansion in the embryo is not dependent on metabolic activity in the seed. However, the effect of a particular WL84811 concentration on coleoptile growth decreased throughout the experiment (Fig. 4.18a). This may explain the lack of effect of WL84811 on vegetative parts of the plant when it is applied at booting to act as a gametocide. At this stage, its transport from the internode or leaves is most likely to be with assimilates going to the developing inflorescence rather than to the roots; these, although more sensitive to WL84811, would only come into contact with very low concentrations of the chemical.

The highest concentration of WL84811 tested (3000 mg l^{-1}) reduced percentage

germination significantly but only during the earliest stages of growth (Fig. 4.17a). As germination has been defined as culminating in emergence of the radicle its onset, like root growth, was not delayed by WL84811.

As WL84811 affects seedling growth more than germination, it was not surprising that it reduced the growth of seedlings germinated and grown for two days on water significantly. In fact, 6000 mg l^{-1} WL84811 added after two days prevented any further significant growth of the roots and reduced coleoptile growth relative to the control (4.3).

The fact that WL84811, applied at only twice the standard dose (but after rather than before pollination), inhibits seedling growth in the subsequent generation may be important; it must be ascertained whether a similar dose applied at the normal time during booting has the same detrimental effect on seedling vigour. If this were so, any advantages gained from heterosis (see 1.4) might be nullified.

7.31 The effect of proline on inhibition of seedling growth by WL84811

Can proline, the amino acid of which WL84811 is an analogue, alleviate its effects on cell division?

Proline alone reduced coleoptile growth significantly at only one, intermediate concentration. Low concentrations enhanced both coleoptile and root growth significantly while all concentrations greater than 300 mg l^{-1} reduced root growth (Fig. 4.20). At low concentrations, proline supplied with equimolar WL84811 did not reduce its effect on coleoptile or root growth and at 300 mg l^{-1}, the two compounds had a synergistic effect. At higher concentrations however, equimolar proline did reduce the inhibition of both coleoptile and root growth caused by WL84811 significantly.

but only partially (Fig. 4.21). 1000 mg l^{-1} proline, supplied in conjunction with 50 mg l^{-1} WL84811, overcame the effect of WL84811 on coleoptile growth slightly but not significantly. The same concentration did overcome the effect of 150 mg l^{-1} WL84811 significantly but was not able to ameliorate the effect of either WL84811 concentration on root growth (4.6).

When barley seeds germinate, storage proteins in the endosperm are broken down and transported to the embryo. Here, transaminations are carried out to convert amino acids such as glutamic acid and proline to aspartic acid, alanine and glycine for the synthesis of enzymes and structural membrane proteins (Folkes & Yamm, 1958). Low levels of exogenous proline may speed up this process by reducing the requirement for proteolysis. The inhibitory effect of higher proline concentrations on root growth may be connected with its unusual properties in solution; proline behaves more like a hydrophilic colloid than a low molecular weight amino acid. It has been proposed (Schobert & Tschesche, 1978) that proline molecules in water form aggregates by hydrophobic interaction of their pyrrolidine rings, the hydrophilic groups exposed on the surface forming strong hydrogen bonds with water molecules. At high proline concentrations, the increased viscosity might affect the ability of roots to absorb water. The osmotic effect of high proline concentrations might also cause cells to lose water to the solution and hence to lose turgor.

As proline can only relieve the effects of WL84811 significantly when the latter is present at high concentrations, it seems unlikely that WL84811 has its primary effect by acting as a functional analogue of proline. If WL84811 was competing with proline and being incorporated into proteins in its place, exogenous proline should be more able to overcome low than high concentrations of WL84811; in fact the reverse is true. WL84811 may have two separate effects; at low concentrations it has its main,

as yet unknown. effect and at high concentrations it has an additional effect, perhaps involving its incorporation into proteins.

7.32 The effect of IAA on inhibition of seedling growth by WL84811

From the results so far it appears that WL84811 has its effect by preventing cell division. To confirm that WL84811 does not inhibit cell expansion, the ability of IAA to alleviate its effects was tested.

IAA, when supplied alone, affected both coleoptile and root growth of germinated Sonja seeds significantly but not percentage germination. The effects were complex: one, intermediate concentration of IAA inhibited coleoptile growth while others, higher and lower, enhanced growth significantly. No concentration of IAA tested inhibited root growth significantly and two concentrations enhanced it (Fig. 4.22). This may have been due to diurnal temperature fluctuations (section 4.7)

IAA also affected the growth of seedlings in WL84811: the highest concentrations used both acted synergistically to enhance the effect of WL84811 on root growth. All except one concentration of IAA also acted synergistically to further inhibit coleoptile growth (Fig. 4.23). The fact that at no concentration does IAA ameliorate the effect of WL84811 on seedling growth is further support for the hypothesis that WL84811 has its effect on cell division, rather than cell extension (it has been believed for many years that the softening of cell walls to allow expansion is one of the major rôles of this auxin (Cleland, 1958)).

7.33 The effect of azetidine-2-carboxylic acid (A2C) on seedling growth

A2C (the natural isomer of WL84811) like WL84811, reduced coleoptile and root

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growth of germinated Sonja seedlings (Fig. 4.24). However, greater concentrations of A2C were required to inhibit growth, particularly of roots, significantly. More than 50 mg l^{-1} A2C was required to inhibit coleoptile growth and more than 300 mg l^{-1} to inhibit root growth whereas 50 mg l^{-1} WL84811 inhibited both significantly in at least one case (4.6).

Proline was unable to alleviate the inhibition of coleoptile growth caused by 300 mg l^{-1} A2C significantly, even when present at 1000 mg l^{-1} (Fig. 4.25). In addition, high concentrations of proline could not reduce the inhibition of coleoptile and root growth caused by equimolar A2C as with WL84811 (4.6). This is unexpected; proline is thought to compete with A2C and prevent its incorporation into proteins in animal cell lines, plants in which A2C does not occur naturally and bacteria (Baum *et al.*, 1973; Fowden & Richmond, 1963). In these cases, A2C incorporation invariably reduces the growth rate, probably because of changes in tertiary structure imposed on proteins; this effect is relieved by exogenous proline (Fowden & Richmond, 1963).

It is clear from these results that, at least at high concentrations where its effects can be alleviated by proline, WL84811 is not acting in the same way as A2C on this system.

7.34 The effect of WL84811 on bracken spore germination

WL84811 reduced the initial rate of germination of bracken spores, preventing it altogether at first at the highest concentrations (Fig. 4.26). The rate of germination was greater in the latter part of the experiment than over the first three days when WL84811 was present.

7.4 The effect of WL84811 on algal growth

The results using the seed system suggested that WL84811 is having its effect on some process associated with cell division. The effect was further isolated by considering a single-cell system: unicellular algae.

The effect of WL84811 on growth and cell division of the two algae chosen, *Chlorella zofingiensis* Donz. and *Closterium moniliferum* Ehr., was quite different. Whereas low concentrations of WL84811 reduced cell number of *Chlorella* after six days (Fig. 5.1) the effect on *Closterium* was significant only after 18 days (Fig. 5.8); even then the reduction in cell division was not directly proportional to the WL84811 concentration applied. Furthermore, whilst all concentrations of WL84811 led to growth of some very large *Chlorella* cells (up to 2145 μm^3 in volume) giving a highly skewed distribution of cell size in the population (Fig. 5.3), very little change in size distribution of *Closterium* was observed (Fig. 5.9) even with higher WL84811 concentrations.

That WL84811 affects cell division rather than growth *per se* is confirmed by the ability of *Chlorella* in the presence of WL84811 to produce cells larger than the size at which division normally occurs. Some difference in the effect of WL84811 on cell division of the two algae is to be expected as their mode of division is very different; *Chlorella* divides by the formation of a variable number of autospores within a mother cell (Fogg, 1953), whilst *Closterium* divides by binary fission (Brook, 1981). Something of these differences must be understood in order to interpret the effects of WL84811.

The wall of a *Chlorella* cell has two layers; a trilaminar outer layer containing

sporopollenin and a more diffuse inner one composed largely of cellulose. The outer layer remains intact until the daughter cells are released after division (Dempsey *et al.*, 1980). After the first nuclear division, a partition membrane forms, dividing the cell in two. A second partition branches off perpendicular to this before the nuclei have finished their second division and this may be repeated once or twice more depending on the number of autospores being formed. Microtubules are absent from *Chlorella* cells for much of the cell cycle but are conspicuous after mitosis when they form a cortical band at the site where the partition membrane will join the parental plasma-membrane; they are also present in the plane of the developing membrane (Atkinson *et al.*, 1971). Cell wall material is deposited on these membranes, possibly by closely-associated dictyosomes (Bisalputra *et al.*, 1966).

Cell division in the desmid *Closterium* is very different. After nuclear division a septum forms between the parent semicells. Microtubules then approach the thickening septum and orientate themselves perpendicular to it. A furrow develops between the semicells and, after these separate, the microtubules associated with the septum take up a hoop-like configuration along the region of new wall. This arrangement is maintained until the wall has fully expanded to give symmetrical semicells again and the nucleus moves to the centre of the new cell (Pickett-Heaps & Fowke, 1970).

The differences between cell division in *Closterium* and *Chlorella* relate to the taxonomic divide proposed by Pickett-Heaps and Marchant (1972); *Chlorella* cells are of the "phycoplast" type where microtubules orientate themselves in the plane of the new cell walls whereas *Closterium* cells, like those of higher plants, are of the "phragmoplast" type where microtubules are oriented perpendicular to the plane of wall formation.

From the results with seeds (7.3) its seems odd that cells which divide in the same way as higher plant cells should be unaffected by WL84811. Other factors may be involved; for instance, because of their much larger size, *Closterium* cells have a smaller surface area/volume ratio than *Chorella* cells. Assuming that the rate of WL84811 uptake is constant over the cell surface, the concentration inside a *Chlorella* cell will be many times greater than that inside a *Closterium* cell subjected to the same external WL84811 concentration.

When *Chlorella* cells grown up in WL84811 were returned to a medium without it, they divided more rapidly over a period of eight days than cells not previously treated with WL84811 (Table 5.3). This implies that many of the processes necessary for cell division can go on in the presence of the chemical and that blockage of one crucial step does not prevent the accumulation of many of the precursors. However, release from inhibition was not instantaneous; after one day in the fresh medium, cell numbers showed relatively little increase. Electron micrographs of large *Chlorella* cells grown in WL84811 (Fig. 5.18) show that the cells do not contain preformed autospores: division must be blocked prior to partition membrane and wall formation. These large *Chlorella* cells differ in this respect from the "giant" cells observed by Thinh and Griffiths (1972) after heterotrophic growth in the dark. In this case cells contained more or less complete autospores and divided into as many as 30 new cells each within 24 hours of their return to more favourable conditions.

The total dry weight of a culture after 10 days was not affected significantly by the presence of 300 mg l^{-1} WL84811 (Table 5.1). However, the median dry weight per cell did increase around 2.7-fold (Fig. 5.6). Over the same period, median cell volume increased by around 3.7-fold; most, if not all, of this increase in cell volume

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is thus accounted for by true growth where cell dry weight increases in proportion to the change in volume. The lack of effect of WL84811 on total dry weight of cultures suggests that all the processes concerned with photosynthesis and assimilate accumulation function normally in the presence of relatively high concentrations of WL84811. This would appear to be confirmed by electron micrographs of WL84811-treated cells (Fig. 5.18) which show cells filled with abundant starch grains. However, massive accumulation of starch in the chloroplasts is a characteristic response of *Chlorella* cells to heterotrophic conditions where organelle growth cannot keep pace with the enormous increase in cell volume (Griffiths & Griffiths, 1969).

The total DNA content of each flask and the DNA content per cell were both significantly greater in the presence of 300 mg l^{-1} WL84811 (Table 5.2). DNA content per flask was increased by around 2-fold and DNA content per cell around 6-fold (Fig. 5.7). These results seem to indicate increased DNA synthesis in the presence of WL84811, presumably leading to polyploid or multinucleate cells. It is not surprising to find that DNA content per cell is greater in the presence of WL84811; although the large cells produced are not composed of complete autospores (Fig. 5.18), they do divide relatively quickly on return to medium without WL84811 (Table 5.3). Nuclear material may be one of the precursors for division which can be synthesised in the presence of WL84811. This ties in with the observation that nuclear division in pollen is not inhibited in WL84811-treated plants. The apparent increase in total sample DNA content is more difficult to explain; perhaps it is a form of compensatory growth, utilizing energy normally used in those processes inhibited by WL84811.

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7.41 The effect of proline and A2C on inhibition of algal growth

If WL84811 is affecting cell division in the same way in germinating seeds and unicellular algae, proline should be able to alleviate its effects on algae as it did with the seed system. This was tested, along with the ability of proline to overcome the inhibitory effects of A2C.

Proline has been shown to be a functional analogue of A2C able, at equimolar concentrations, to overcome the inhibitory effects of this compound (at 2000 mg l^{-1}) on the green alga *Nannochloris bacillaris*, like *Chlorella* a member of the Chlorococcales (Vanlerberghe & Brown, 1985). However, low concentrations of either A2C or proline had no significant effect on cell number of *Chlorella* and high concentrations of either inhibited cell division. When high concentrations of A2C and proline were supplied together they had a synergistic effect, the inhibition of cell division being greater than with either one alone (Fig. 5.15).

The apparent difference in the behaviour of *Nannochloris* and *Chlorella* is interesting. *Nannochloris*, unlike *Chlorella zofingiensis*, is a marine alga which accumulates free proline in its cytoplasm to balance the external water potential of sea water. This large internal pool of proline may mean that, even at high exogenous concentrations, only limited amounts of A2C are incorporated into proteins. It was found that the inhibitory effects of A2C on *Nannochloris* were overcome naturally after cells had grown for around 15 days in its presence (Vanlerberghe & Brown, 1985).

High concentrations of proline did, however show a significant, but very limited, ability to ameliorate the effect of equimolar WL84811 on *Chlorella*, despite having an inhibitory effect when supplied alone at this concentration (Fig. 5.12). The same

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concentration of proline alleviated slightly, but not significantly, the effect of 50 mg l^{-1} WL84811 (Fig. 5.14). These results parallel observations on germinating seeds (7.4) that only at high WL84811 concentrations does proline seem able to overcome its effect significantly although, again, this reversal of inhibition is far from complete.

7.5 The effects of WL84811 on pollen germination and pollen tube growth

In the light of these results showing that in two other plant systems WL84811 inhibits cell division, what can be said about its effect on pollen germination? Must WL84811 be applied before division of the microspore nucleus in order to exert its gametocidal action? It does not prevent division of the nucleus but does it prevent separation or separate development of the vegetative and generative cells?

7.51 Pollen germination *in vivo*

Studies on the effect of WL84811 on pollen germination *in vivo* were carried out on the wheat cultivar Mercia. By staining with acetocarmine, the behaviour of pollen on the stigma was easily observed. When untreated plants self-pollinate, pollen germinates in a matter of minutes on the stigma (Fig. 6.1) and pollen tubes quickly penetrate the stigmatic branches; when a WL84811-treated plant self-pollinates, however, the pollen does not germinate and bursts rapidly (Fig. 6.2). There was no evidence of even short pollen tubes ever being produced by WL84811-treated pollen.

In general, germination of pollen of small-grain cereals *in vivo* takes place in less than five minutes (Chandra & Bhatnagar, 1974; Luxova, 1967; Watanabe, 1961). Often, many pollen grains will germinate on one stigma and it has been suggested that those pollen tubes which do not succeed in fertilizing the ovule may be involved

in supplying nutrients to the developing embryo (Klyuchaneva, 1963). It is believed that a complex dialogue takes place between pollen and stigma in the few minutes between pollen landing on the stigma and, in the case of viable, compatible pollen, the pollen tube starting to grow.

Watanabe (1955) was the first to recognise that during hydration on the stigma, grass pollen does not simply take up water and dilate until it reaches the point where germination is possible. When viable pollen lands on the stigma, a meniscus forms between the grain and stigmatic papilla and the grain then dilates. After this initial enlargement, the pollen exudes liquid back onto the stigma, both from the aperture and through the non-aperturate exine. This is an essential prelude to germination of pollen. This exudate will contain, among other things, those wall-held fractions responsible for sporophytic self-incompatibility in, for example, the Cruciferae (Roggen. 1974). As inbreeders, wheat and barley do not have a self-incompatibility system but they do have an incompatibility system to prevent inter-specific crossing. For germination to proceed, enzymes such as "cutinase" in the pollen exudate must be recognised and activated by stigmatic enzymes (Kroh, 1964). The activated exudate is then reabsorbed by the pollen, the tube tip emerges and the pollen germinates. Pollen may abort at any point in this process; it may fail to dilate, it may burst as a result of uncontrolled dilation, it may fail to reabsorb the exudate or burst after doing so, the pollen tube tip may burst or the tube fail to elongate (Heslop-Harrison, 1979a).

Pollen is known to 'leak' solutes during imbibition (Heslop-Harrison, 1979b). It has been proposed from work on isolated membranes that this may be due to the lack of a continuous plasmalemma in ungerminated pollen (Heslop-Harrison, 1979b; Shiv-

anna & Heslop-Harrison, 1981). This is not, however, supported by freeze-fracture observations on pollen membranes (Platt-Aloia *et al.*, 1986) or by ^{31}P-nuclear magnetic resonance studies on membrane lipids (Priestly & de Kruijff, 1982). The leakage may simply be due to disruption of the membrane by the rapid influx of water (Larson, 1968) or to physical diffusion (Powell & Matthews, 1981). If the membrane cannot initially act as an efficient osmotic barrier, it may be that precise control by the stigma of the rate at which water enters the pollen is necessary to prevent it from bursting.

Cereal pollen has a relatively simple structure; the grain is monoporate. the single aperture is surrounded by an thickened annulus of exine material (Fig. 3.22) and the central operculum rests on a thin sporopollenin lamella overlying the Zwischenkörper (Rowley, 1964). The Zwischenkörper is a zone of gel-forming, pectic polysaccharides, dehydrated at the time of pollen dispersal to seal the aperture firmly (Heslop-Harrison & Heslop-Harrison. 1979). Early in the hydration of a pollen grain, the sporopollenin membrane on which the operculum rests is ruptured and the Zwischenkörper gelatinized. As the pectic material swells it pushes aside the operculum. and the intine emerges. Dead pollen may hydrate in a similar way; in this case the intine balloons out in an isodiametric swelling rather than a true pollen tube and this protrusion will eventually burst, as seen with WL84811-treated pollen. This is simply the result of plastic expansion and does not involve any wall growth (Heslop-Harrison. 1979a).

When treated pollen was applied by hand to the stigma of an untreated plant it again failed to germinate (Fig. 6.4), whereas untreated pollen germinated normally on the stigma of a treated plant (Fig. 6.3). Clearly, despite the fact that WL84811 is taken up by the stigmatic tissue and that it affects cell growth and division in

systems as diverse as unicellular algae and germinating seeds, its gametocidal effect is expressed solely through its effect on pollen and not via any effect on the receptiveness of the stigma. This is very important; if the effect of WL84811 was mediated in part through a change in the stigma, the ability of untreated pollen to germinate on it would be impaired and female fertility presumably reduced. This would severely restrict the potential of WL84811 as a gametocide.

To determine whether a lack of some externally-held recognition factor was preventing WL84811-treated pollen from germinating, untreated pollen was killed by dehydration and applied to the stigmas of treated plants at anthesis. However, no pollen germinated on these stigmas. This technique, utilising mentor pollen, has been used successfully in apple to overcome self-incompatibility (Dayton, 1974; Sansavini *et al.*, 1984) and in other species to effect interspecific crosses (Knox *et al.*, 1972; Stettler & Ager, 1984). Generally, compatible mentor pollen is irradiated with a dose which damages its chromosomes and prevents the gametes from fertilizing ovules but which permits pollen tube growth. The situation discussed above where the mentor pollen was unable to germinate at all would only be of use in overcoming incompatibility of the sporophytic type where pollen is inhibited at the stigma surface, not for gametophytic inhibition where pollen tubes grow for some distance down the style before stopping (Stettler & Ager, 1984).

7.52 Pollen germination *in vitro*

If WL84811 prevents pollen germination when applied at booting, can it also do so when included in a germination medium for untreated pollen?

WL84811 was added to the basic germination medium used for the germination

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of several types of pollen and also, in the case of wheat and rye, the behaviour of pollen from untreated plants and from plants previously treated with WL84811 was compared. Pollen germination, *in vitro* as *in vivo*, is a complex process and to interpret the effects of WL84811 it is necessary to understand something about this process and about the subsequent pollen tube growth.

The cytoplasm of ungerminated grass pollen contains, as well as abundant amyloplasts, a dense population of small polysaccharide-containing particles known as 'P-particles' (see 7.2). When the pollen tube starts to elongate, these move into the tube and are the most abundant cytoplasmic components in the region immediately behind the growing tip. They are believed to contain wall precursors which they release into the inner callose lining of the growing tube. (Heslop-Harrison, 1979a). During this early tube growth proline is utilised both for protein synthesis and, via the citric acid cycle and gluconeogenesis, for the synthesis of carbohydrates and wall materials. It may also have a small role as a respiratory substrate but is probably not normally used as a nitrogen source as Britikov *et al.* (1965) suggested (Zhang & Croes, 1983).

The binucleate pollen of many plants germinates readily on the liquid medium of Brewbaker and Kwack (1963). When 1000 and 3000 mg l^{-1} WL84811 were included in this medium germination of pollen of two such plants, *Vicia faba* and *Tropaeolum majus* respectively, was reduced slightly but not significantly (Tables 6.1, 6.2). Lower concentrations of WL84811 increased significantly the length of pollen tubes produced by that pollen which did germinate.

Trinucleate grass pollen is much more difficult to germinate *in vitro* (Gotoh, 1931; Mulcahy & Mulcahy, 1983). After many attempts. it was found that adding one

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percent agarose to partially solidify a nutrient medium improved germination and pollen tube growth greatly (Appendix 1). Pollen of *H. vulgarc, P. typhoides* and *S. cereale* was germinated on the medium used by Heslop-Harrison (1979a), with agarose added (Table 2.4). Although percentage germination was generally poor some long pollen tubes were obtained. It seems that controlling the rate of pollen hydration carefully as would happen on a "dry" stigma (*sensu* Heslop-Harrison *et al.*, 1975) like those of the Graminae is more important than the particular mineral concentration of the medium.

When 6000 mg l^{-1} WL84811 was added to this germination medium, both percentage germination and the tube length of germinated barley pollen were reduced significantly (Table 6.3); the same concentration reduced only the pollen tube length of millet pollen (Table 6.4). In contrast, 6000 mg l^{-1} WL84811 increased the length of pollen tubes produced by rye significantly.

T. aestivum pollen was germinated successfully on a slightly different medium (Table 2.5). There was no significant difference in either percentage germination or pollen tube length between measurements taken after 30 minutes on the medium and after 16 hours (Table 6.6); any pollen competent to germinate obviously does so very rapidly. This is presumably related to the fact that healthy wheat pollen germinates within minutes *in vivo* (6.2). Adding 3000 mg l^{-1} WL84811 to the medium had no effect on pollen germination but it did significantly increase the length of pollen tubes produced (Table 6.6).

Because of its potential usefulness as a selection system for plant breeders (Mulcahy, 1984) and as an experimental growth system for carrying out bioassays (Williams *et al.*, 1985), much effort has been put into achieving optimal germination and pollen

tube growth *in vitro* (see Stanley, 1971). The effect of adding various trace elements, plant growth regulators, vitamins, inhibitors of protein synthesis and free amino acids to the basic germination medium has been studied on pollen of a wide range of species (Brewbaker & Kwack, 1963; Jahr & Gottschalk, 1973; Loo & Hwang, 1944; Shivanna *et al.*, 1974; Wolff, 1974). In their study on the effects of free amino acids on the growth of *Pisum* pollen tubes, Jahr and Gottschalk (1973) point out that mutants with different genotypes respond differently to the presence of exogenous amino acids.

In the light of this, it is not surprising that pollen of different species, even within the same family, responds very differently to WL84811. With the exception of barley, adding WL84811 to the medium has little effect on pollen germination. The gametocidal effect clearly lies in its affecting some process in pollen development. However, WL84811 has a much more variable effect on pollen tube length; moderate concentrations increase pollen tube length in field bean and nasturtium and much higher concentrations have the same effect on rye and wheat pollen. High WL84811 concentrations, however, reduce pollen tube length in germinating barley and millet pollen. Once the pollen has germinated, some types appear to gain positive benefit from its presence; some pollen may be able metabolise the proline analogue in some way.

Rye pollen from plants injected with 3000 mg l^{-1} WL84811 in the usual way (2.31) behaved no differently to untreated pollen on the plain medium; percentage germination and pollen tube length were unaffected. However, wheat pollen from plants treated in the same way totally failed to germinate (Fig. 6.14) and much of the pollen burst as noted *in vivo* (Fig. 6.2).

This difference between the behaviour of rye and wheat pollen after injection is

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presumably a result of the lack of effect of WL84811 on the morphology of rye pollen (Fig. 3.19). It appears that this pollen is not sensitive to the gametocide under the standard conditions.

7.6 Conclusions

This work has established several important facts about the way in which WL84811 has its gametocidal effect:

1. The effect of WL84811 is exerted solely on pollen which is prevented from filling normally during maturation and subsequently bursts on the stigma.

2. WL84811 does not prevent germination when added to an *in vitro* germination medium; it actually enhances germination and growth of some types of pollen from both monocotyledonous and dicotyledonous families which may be able to metabolise it.

3. Its effect on other systems is chiefly on cell division rather than cell expansion and can be separated into two parts: one can be alleviated by proline and the other cannot.

The common link between the effect of WL84811 on pollen and other systems seems to be that it prevents wall formation. The fact that WL84811 must be applied during pollen development and has little effect when added to a germination medium for previously untreated pollen can be interpreted in at least two ways. Either WL84811 inhibits separation of the generative and vegetative cells after pollen mitosis and thus halts normal pollen development or it acts directly to inhibit the accumulation of some precursor (perhaps 'P-particles' or microtubule arrays) necessary for formation of the pollen tube wall. The effect on pollen filling could simply

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be a symptom of microspore degeneration caused by failure of cytokinesis after the first pollen mitosis.

If WL84811 is inhibiting separation of the generative and vegetative cells it should be possible to detect this by cutting serial sections of embedded pollen grains and comparing the wall in untreated and WL84811-treated material using the transmission electron microscope. An alternative approach would be to use the technique of freeze-fracture microscopy to compare the wall structures. A detailed electron microscopy study might also allow any differences in cytoplasmic organelles to be detected but would not show whether these were the cause or result of the induced male sterility.

It is for this reason that a multidisciplinary approach has been employed throughout this project. Information about the effect of WL84811 on pollen ultrastructure is not presented in a vacuum but is related to observations of its effects on pollen germination *in vivo* and *in vitro* and on cell growth and division in other systems.

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APPENDIX 1.

Germination media for cereal pollen

In vitro germination of grass pollen has been reported on liquid (Brewbaker & Kwack, 1963; Mathur, 1977), semi-solid (Pfahler, 1967; Loo & Hwang, 1944; Heslop-Harrison, 1979a) and solid media (Bar-Shalom & Mattsson, 1977), so media of all three types were tested with wheat and barley pollen. All media were prepared by heating the constituents together to dissolve them, then cooling to room temperature before use.

1. Liquid media

1.1

> 10 % sucrose
>
> 100 mg 1^{-1} H_3BO_3
>
> 300 mg 1^{-1} $Ca(NO_3)_2.4H_2O$
>
> 200 mg 1^{-1} $MgSO_4.7H_2O$
>
> 100 mg 1^{-1} KNO_3

Used by Brewbaker and Kwack (1963) to germinate pollen of *Pennisetum* and *Zea in vitro*. Pollen was scattered on porous cellophane overlying filter paper soaked in the medium, in the base of a petri dish.

Wheat and barley pollen burst very rapidly in this liquid medium.

1.2

Pure sucrose solutions at a range of concentrations (30 – 50 %), with fluorescein diacetate added to indicate pollen viability (see 2.411).

Pollen was scattered on the surface of a drop of medium on a microscope slide. *T. aestivum* cv. CWW 3547/1 pollen burst rapidly at low sucrose concentrations and plasmolysed at high sucrose concentrations.

1.3

25 % sucrose

100 mg l^{-1} H_3BO_3

100 mg l^{-1} KNO_3

200 mg l^{-1} $MgSO_4.7H_2O$

300 mg l^{-1} $Ca(NO_3)_2.4H_2O$

Used by Mathur (1977) to germinate pollen of *Zea* and *Triticum in vitro* - he claimed 50 % germination for wheat pollen. Pollen was scattered onto drops of medium on glass slides and these were supported in an inverted position in a petri dish lined with moist filter paper for three hours (hanging drop technique).

Most *T. aestivum* cv. Mercia pollen burst under these conditions although a few short pollen tubes were produced. When the medium was solidified with 0.1 % agarose however, germination of this pollen was greatly improved. In this case, the medium was prepared by heating the constiuents together and pouring a thin film into a petri dish to cool. For use, a piece approximately 1.0 $\times$ 1.0 cm^2 was cut using a glass coverslip and inverted onto a microscope slide. The pollen was scattered on this surface which has an even layer of surface water (Heslop-Harrison, 1979a) and the slide incubated in a petri dish lined with damp filter paper.

2. Semi-solid media

2.1

15 % sucrose

100 mg l^{-1} H_3BO_3

300 mg l^{-1} $Ca(NO_3)_2.4H_2O$

0.6 % bactoagar

Used by Pfahler (1967) to germinate *Zea* pollen *in vitro*.

To produce a humid atmosphere above the medium the open plate was placed in a sealed container along with a dish containing a saturated solution of KH_2PO_4. This has an equilibrium vapour pressure equivalent to a relative humidity of 96.5 % (Winston & Bates, 1960). After inoculation with *Triticum* pollen, the plates were incubated at 27 °C for up to 12 hours. No proper pollen tubes were seen although the intine bulged out at the aperture of many grains. When lower sucrose concentrations were tested, much of the pollen burst.

2.2

A modified version of Pfahler's medium (1967), designed to aid observation of the pollen: one percent agarose replaced the bacto agar. The medium was poured onto microscope slides in a thin film.

Bulging of the intine of wheat pollen was again observed but no pollen tubes. The effect of removing calcium from Pfahler's medium was also tested in case it was making the pollen membrane too rigid to allow germination. However, in the absence of calcium, most pollen grains burst very rapidly.

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2.3

20 % sucrose

62 mg l^{-1} H$_3$BO$_3$

590 mg l^{-1} Ca(NO$_3$)$_2$.4H$_2$O

0.1 % agarose

Used by Heslop-Harrison (1979a) to germinate *Secale cereale* pollen. The medium was prepared as described for Mathur's medium (1977).

H. vulgare cv. Sonja, *Pennisetum typhoides* and *Secale cereale* cv. Dominant pollen were all germinated successfully on this medium, producing some pollen tubes over one millimetre long. The medium was not so successful for *T. aestivum* pollen which produced only very short pollen tubes which burst within a few minutes.

2.4

5 % gelatin

20 % sucrose

Used by Loo and Hwang (1944) to germinate *T. vulgare* and *H. sativum* pollen *in vitro* using the hanging drop technique.

This medium was supplemented with 100 mg l^{-1} H$_3$BO$_3$ and 300 mg l^{-1} Ca(NO$_3$)$_2$. A drop of medium was placed on a cover slip, pollen scattered on the surface or mixed in with this and the cover slip inverted over a cavity slide. Pollen of *T. aestivum* cultivars Mission and Hammer burst rapidly on this medium. Increasing the sucrose concentration to 40 % prevented pollen from bursting but did not permit germination.

3. Solid media

3.1

A range of concentrations of potato malt agar (up to 4 %) were used.

No germination was observed.

3.2

A medium used by Bar-Shalom and Mattsson (1977) to germinate *H. bulbosum* pollen *in vitro*. The composition is the same as Brewbaker and Kwack's medium (1963) but with 2 % agar added to solidify it. Higher concentrations of sucrose were also tested in this basic medium.

The pollen of both *T. aestivum* cv. CWW 3547/1 and *H. vulgare* cv. Sonja failed to germinate on this medium. With 15 % sucrose, the pollen burst; with 30 % sucose the pollen plasmolysed; with intermediate concentrations the pollen hydrated but still failed to germinate.

4. Conclusions

In view of these results, the method of Heslop-Harrison (1979a) was used to test the effect of WL84811 on germination of *H. vulgare*, *P. typhoides* and *S. cereale* pollen *in vitro* and the method of Mathur (1977) to test its effects on *T. aestivum* pollen.

APPENDIX 2.

Physical properties of WL84811

Seeds of one barley cultivar, Sonja, which showed particularly consistent results with WL84811 were used to test the stability of WL84811 in solution during storage and when subjected to high temperatures.

As for other data involving seed germination and seedling growth, median values with upper and lower quartiles were calculated for each treatment and Kruskal-Wallis analyses performed to show the significance of differences between them. The significance of the difference between individual pairs of treatments was determined using the Mann-Whitney U-test; treatments not significantly different from one another at $P=0.05$ are bracketed together.

1. Effect of storage time and temperature on activity of WL84811 in solution

A range of treatments were used to study the effect of time and temperature on storage of the chemical:

1. Fresh WL84811 at start of experiment, stored at 4 °C during use

2. WL84811 stored at room temperature for 48 hours before, and during, use

3. WL84811 stored at 4 °C for 48 hours before, and during, use

4. WL84811 stored at room temperature for 96 hours before, and during, use

5. WL84811 stored at 4 °C for 96 hours before, and during, use

6. WL84811 stored at room temperature for one week before, and during, use

7. WL84811 stored at 4 °C for one week before, and during, use

8. WL84811 stored at room temperature for two weeks before, and during, use

9. WL84811 stored at 4 °C for two weeks before, and during, use

10. WL84811 stored at room temperature for four weeks before, and during, use

11. WL84811 stored at 4 °C for four weeks before, and during, use.

Seeds were germinated in WL84811 samples treated as described above and the seedlings harvested after five days. Percentage germination, coleoptile and root length were measured and median values calculated for each treatment (Fig. A.1).

There was no significant difference between the treatments with regard to their effect on percentage germination $(0.5 > P > 0.1)$. There were, however, highly significant differences in coleoptile and root length of germinated seedlings between the different treatments $(P < 0.001$ for both).

Coleoptile length: $(11, 8, 9, 10) > (7, 1, 6) > (3, 2, 4) > (4, 5)$

Root length: $(6, 7) > (7, 8, 9) > (8, 9, 1, 4) > (1, 4, 10, 5, 11) > (4, 10, 5, 11, 2) > (10, 5, 11, 2, 3)$.

Storage of solutions for two weeks or more at either temperature tested reduced the effect of WL84811 on coleoptile growth of germinating seedlings significantly. Shorter storage periods, however, (48 or 96 hours) actually enhanced the effect of WL84811 significantly.

The effect of WL84811 on root growth appears to be affected rather differently by storage. After one week at either temperature, the effect of WL84811 was reduced significantly; however after two or four weeks the effect did not differ significantly from that caused by fresh material. As for coleoptile growth, storage for 48 hours before use enhanced the effect of WL84811 significantly.

Fig. A.1a Effect of storage time and temperature on inhibition of *H. vulgare* cv. Sonja coleoptile growth by WL84811 over five days. Median values are given, with upper and lower quartiles.

● = room temperature

○ = 4 °C

Fig. A.1b Effect of storage time and temperature on inhibition of *H. vulgare* cv. Sonja root growth by WL84811 over five days. Median values are given, with upper and lower quartiles. Key as for Fig. A.1a.
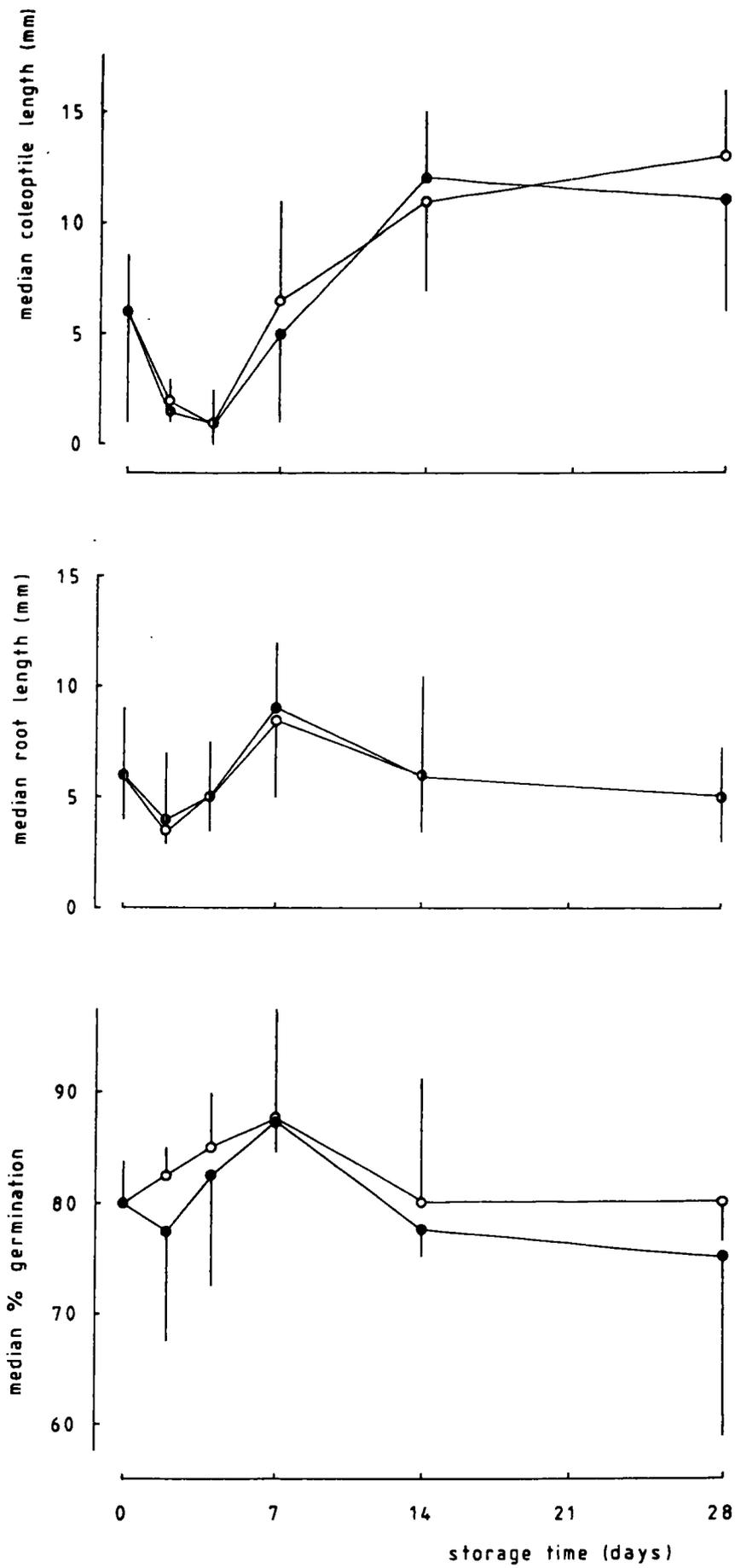
Fig. A.1c Effect of storage time and temperature on inhibition of *H. vulgare* cv. Sonja seed germination by WL84811 over five days. Median values are given, with upper and lower quartiles. Key as for Fig. A.1a.

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2. Effect of temperature on WL84811 activity

To look at the effect of extreme temperatures on the gametocide, fresh WL84811 (1000 mg l^{-1}) was treated for one hour at 4, 45, 60, 80 or 100 °C before being applied to the seeds as normal. During the course of the experiment, all solutions were stored at 4 °C. After five days growth percentage germination, and coleoptile and root length of germinated seeds were measured and median values calculated (Fig. A.2).

There was no significant difference between the temperature treatments with regard to their effect on percentage germination or coleoptile length of germinated seedlings ($0.5 > P > 0.1$ for both). There was, however, a highly significant difference in root length under the various treatments.

Root length: (80 °C, 45 °C, 60 °C) > (60 °C, 100 °C, 4 °C).

These results seem somewhat inconsistent; whereas 80 °C and 45 °C treatments appeared to reduce the effect of WL84811 relative to the same concentration kept at 4 °C, the 60 °C and 100 °C treatments had no significant effect.

3. Conclusions

In view of these results, WL84811 was stored at 4 °C once in solution and any unused solution was discarded after two weeks.

Fig. A.2a Effect of temperature on inhibition of *H. vulgare* cv. Sonja coleoptile growth by WL84811 over five days. Median values are given, with upper and lower quartiles.

Fig. A.2b Effect of temperature on inhibition of *H. vulgare* cv. Sonja root growth by WL84811 over five days. Median values are given, with upper and lower quartiles.

Fig. A.2c Effect of temperature on inhibition of *H. vulgare* cv. Sonja seed germination by WL84811 over five days. Median values are given, with upper and lower quartiles.
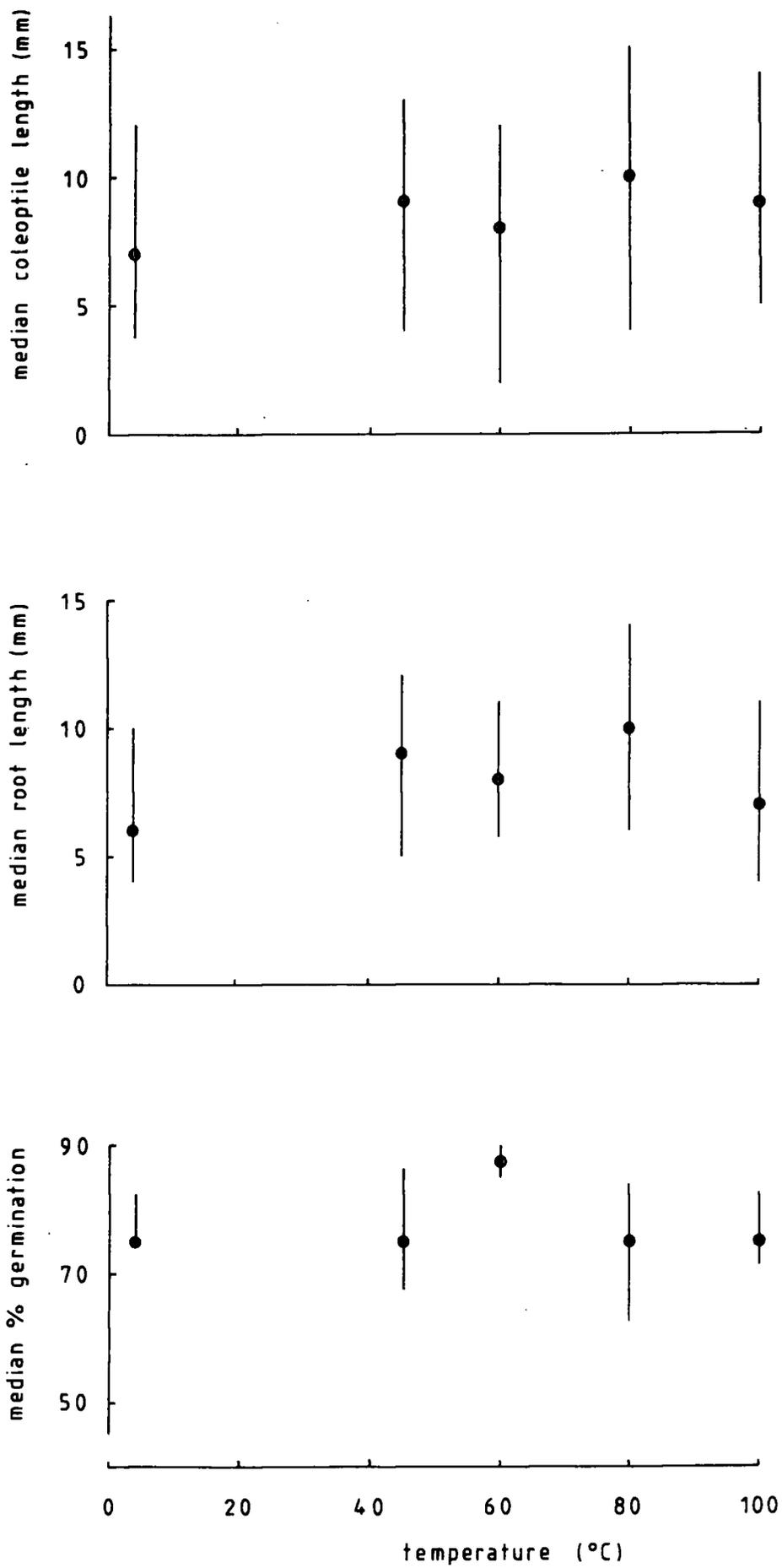
APPENDIX 3.

Comparison of methods for seed germination experiments

The barley cultivar Sonja was used in two experiments to determine the effect of IAA on seed germination and seeding growth. One experiment involved adding five millilitres of test solution to imbibed seeds on filter paper in a petri dish. sealing the dish with 'Nescofilm' and measuring germination and seedling growth after five days (if the dish was not sealed, the liquid evaporated rapidly under the conditions in this laboratory). This method is the standard one employed by toxicologists (method 1).

In the other experiment, the method described in section 2.71 was used. Imbibed seeds were placed on filter paper soaked in four millilitres of test solution and a further one millilitre of solution was added daily throughout the experiment. Germination and seedling growth were again measured after five days (method 2).

For the reasons described in section 2.91, the results are presented as median values with upper and lower quartiles.

Table A1 Effect of IAA on seed germination and seedling growth using method 1 (median values. with quartiles given in brackets).

IAA conc. (mol l^{-1})	Coleoptile length (mm)	Root length (mm)	% germination
0	7.0 (2.0 – 16.0)	21.0 (18.0 – 26.0)	65.0 (56.3 – 85.0)
10^{-8}	3.5 (1.0 – 7.5)	17.0 (15.0 – 19.0)	67.5 (61.3 – 73.8)
10^{-7}	4.0 (1.0 – 8.0)	19.5 (15.0 – 21.0)	67.5 (60.0 – 82.5)
10^{-6}	3.0 (1.0 – 8.8)	19.0 (16.3 – 21.0)	80.0 (76.3 – 83.8)
10^{-4}	3.5 (1.0 – 11.5)	20.5 (13.8 – 26.0)	77.5 (67.5 – 87.5)

Table A2 Effect of IAA on seed germination and seedling growth using method 2 (median values, with quartiles given in brackets).

IAA conc. (mol l^{-1})	Coleoptile length (mm)	Root length (mm)	% germination
0	32.0 (23.0 – 38.4)	45.1 (30.5 – 53.0)	85.5 (77.5 – 92.7)
10^{-8}	32.0 (23.5 – 39.0)	53.0 (40.9 – 60.0)	95.0 (90.0 – 100.0)
10^{-7}	29.0 (20.5 – 36.0)	47.8 (37.2 – 55.7)	95.0 (85.0 – 100.0)
10^{-5}	25.0 (18.0 – 30.0)	46.0 (39.0 – 55.0)	100.0 (95.0 – 100.0)
10^{-4}	40.0 (24.2 – 48.2)	43.0 (29.8 – 51.8)	90.0 (80.0 – 100.0)

Conclusions

Comparing these tables it is obvious that, under the conditions in this laboratory, the second method gives far better results, both in terms of percentage germination and the growth of seedlings. Many seeds rotted in the sealed petri dishes of method 1, some after producing short coleoptiles and roots. For this reason, the second method was employed throughout this work.

APPENDIX 4.

## The effect of pH on seed germination and seedling growth.

The barley cultivar Sonja was used to observe the effect of pH on seed germination and seedling growth in order to determine whether the effect of WL84811 on these processes was simply due to its behaviour as an acid.

The pH of distilled water was lowered (from 5.0) to 4.5, 4.0, 3.5 or 3.0 pH units at the start of the experiment by adding a few drops of hydrochloric acid and the seeds were germinated and grown on this acidified water as described in section 2.71. Percentage germination and seedling growth were measured after five days and median values calculated for each treatment (Fig. A.3). The significance of differences between treatments was determined using Kruskal-Wallis analyses.

pH was shown to have no significant effect on percentage germination of seeds. $(0.1 > P > 0.05)$ or on coleoptile or root growth of germinated seeds $(0.5 > P > 0.1$ for both).

Conclusion

6000 mg l^{-1} WL84811 only reduces the pH of distilled water to 4.25; in the experiment described above, pH values as low as 3.0 were shown to have no significant effect on seed germination and seedling growth. The inhibition of these processes by WL84811 is clearly not simply due to the acid nature of the compound.

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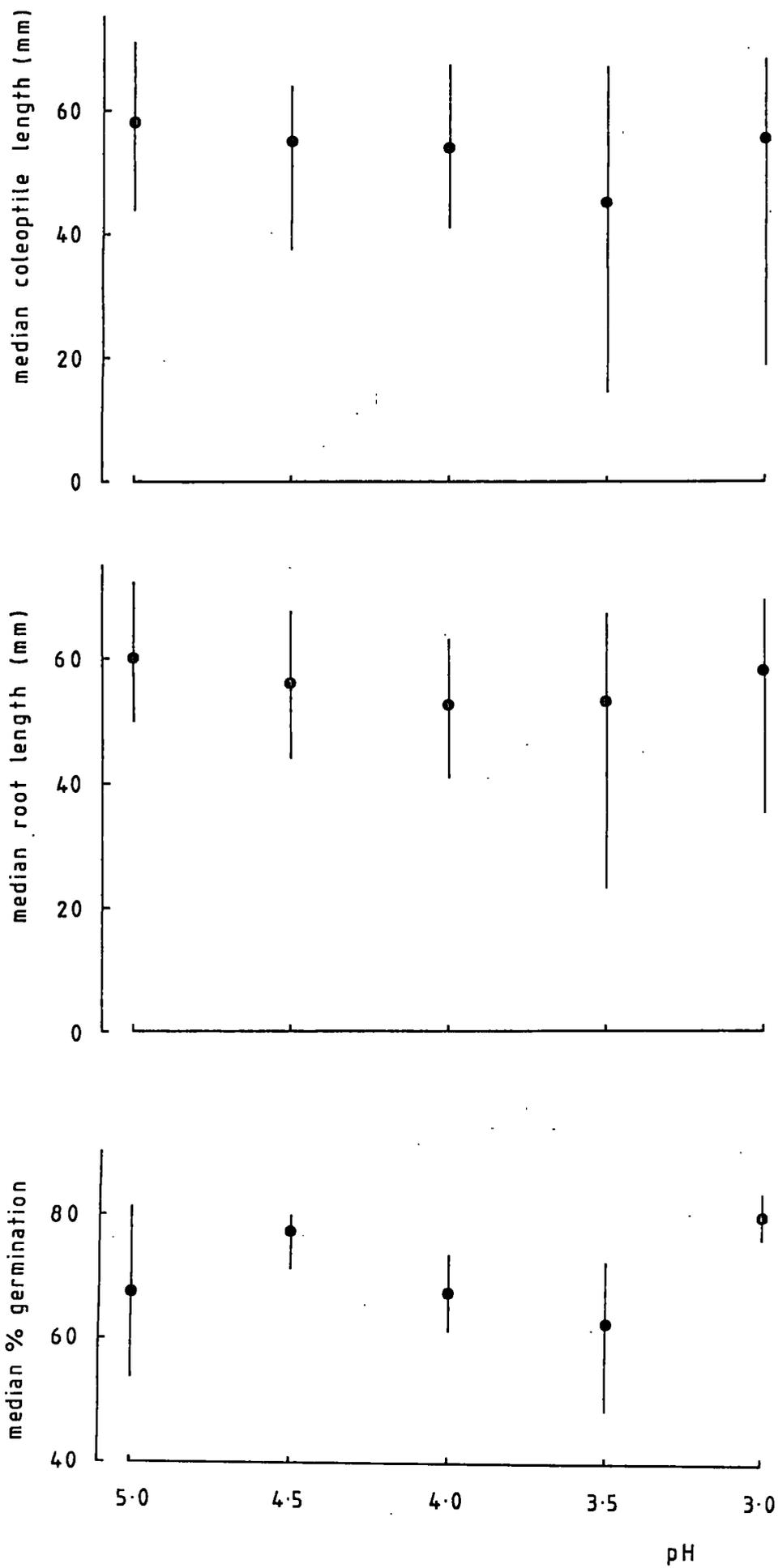
LIST OF SLIDES

3.1 Stage 1 stigma of *T. aestivum* stained with auramine. Papillae have not yet started to differentiate and some cells fluoresce a uniform, dull yellow. × 100

3.2 Stage 1 stigma of *T. aestivum* stained with acridine orange. The nuclei fluoresce bright green. × 100

3.3 Stage 2 stigma of *T. aestivum* stained with acridine orange. Papillae have started to differentiate on the stigmatic branches and there is red fluorescence in cells at the tip of some branches. × 50

3.4 Stage 3 stigma of *T. aestivum* stained with auramine. The differential staining of adjacent papillae can be seen clearly; in some cells only the nucleus fluoresces, in others the Golgi and endomembranes also. × 100

3.5 Branch of stage 3 stigma of *T. aestivum* stained with acridine orange. Most cells fluoresce red indicating high levels of RNA. Only some nuclei fluoresce green. × 100

3.6 Branches of stage 3 stigma of *T. aestivum* stained with acridine orange. Most cells contain some red fluorescence. × 100

3.7 Stage 3 stigma of *T. aestivum* stained with acridine orange. Overall, there is most red fluorescence at this stage of development, probably indicating active RNA synthesis. × 25

3.8 Mature stigma of *T. aestivum* stained with acridine orange. The style is covered in long, feathery stigmatic branches. × 25

3.9 Mature stigmatic branch of *T. aestivum* stained with auramine. auramine. The yellow-fluorescing cells at the tip of the branch have aggregated endomembranes. × 100