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JULY 1989

Non Photosynthetic Interactions of Bentazone with Plant Growth and Development

by

Paul Robert Miller

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

Collaborating Establishment: BASF (FRG)

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Signed Paul R. Miller
(Candidate)

Signed A. H. Cobb
(Director of Studies)

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ABSTRACT

Non Photosynthetic Interactions of Bentazone with Plant Growth and Development

Paul R. Miller

A range of bioassays were performed to establish the non photosynthetic PGR activity of the herbicide bentazone. In bioassays for auxin activity bentazone stimulated growth of primary leaf petioles, cotyledonary internode and first lateral of light-grown *Phaseolus vulgaris* over a 12d period, and stimulated elongation of etiolated *Avena sativa* coleoptile segments within 1h. These responses each had similar concentration dependence to, but were smaller in magnitude, than responses of the same tissues to exogenous IAA. IAA and bentazone-enhanced coleoptile elongation was similarly sensitive to a range of inhibitors. In *Phaseolus vulgaris* tissues, *Avena sativa* coleoptile segments, and *Lepidium sativum* roots, IAA and bentazone produced significant interactions, bentazone reducing IAA responses at high (optimum) concentrations, but enhancing IAA responses when both compounds were simultaneously applied at low concentrations. In a bioassay for gibberellin activity, bentazone inhibited elongation of juvenile internodes of intact, light-grown *Pisum sativum* similarly to IAA, and inhibited exogenous GA3-enhanced internode elongation, over 5d, again similarly to IAA. Investigation of a proposed plasmalemma mediated mechanism of action, the same as, or similar to that of IAA, found bentazone active in promoting H^+ efflux from etiolated *Avena* coleoptile segments, again similar to, but smaller in magnitude than IAA responses, and with clear interaction between the two compounds, particularly when bentazone was at low concentration. It is proposed that IAA and bentazone are active at the same, or a closely associated, plasmalemma bound auxin receptor, and that short and long term observed interactions may be accounted for by considering interactions at the initial binding site. Speculative models for these interactions are presented. The value of considerations of interactions at binding sites in herbicide action and interaction studies are discussed.

PUBLICATIONS ARISING FROM THIS STUDY

COBB, A.H., REES, R.T., NICHOLS, K.J., MILLER, P.R. and PALLETT, K.E. (1985). The cell membrane as a site for bentazone action. Proceedings of 1985 British Crop Protection Conference - Weeds 3, 1187-1193.

MILLER, P.R. and COBB, A.H. (1986). Rapid and long-term non-photosynthetic interactions of bentazone with plant growth and development. Aspects of Applied Biology 11, 111-119.

MILLER, P.R. and COBB, A.H. (1987). Growth regulator activity of the herbicide bentazone. Poster Presentation, SEB Spring Conference, York.

FITZSIMONS, P.J., MILLER, P.R. and COBB, A.H. (1987). Auxin-induced H^+ efflux: Herbicide activity and antagonism. Proceedings of 1987 British Crop Protection Conference - Weeds 1, 179-186.

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Chapter 1. INTRODUCTION

1.1 The Herbicide Industry

Plants have been cultivated since ancient times to provide materials for many uses including food, clothing and shelter. A common feature of the most powerful historic civilisations has been, and continues to be, a strong agriculture.

The successful cultivation of crop plants involves the provision of optimum conditions for the growth of the crop species, which has traditionally meant maintaining it free of insects and other pests and diseases, and by attempting to culture the desired species in exclusion of other plant species, i.e. weeds. While a reduction in crop yield is perhaps the most obvious effect of weeds, brought about by competition for light, water and nutrients, weeds may be undesirable because they reduce the value of a crop e.g. through seed contamination, or they may hamper cultural operations such as harvesting.

Many attempts were made in ancient times to control pests and diseases by chemical methods (BAA leaflet No.1), but control of weed problems has traditionally been based on four principles:-

1. Prevention of dispersal.
2. Well planned crop rotations.
3. Well timed cultural operations.
4. Hand weeding.

Only since the 1930s has a fifth contributing method been added to this list, i.e.

5. Chemical control.

Important early chemical herbicides, introduced in the 1940s, are 2,4-dichlorophenoxyacetic acid (2,4-D), methylchlorophenoxy-acetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and propham (Makepeace, 1979). The increased availability of

these chemicals coincided with increasing labour costs and new technology in agriculture, and as such helped to drive forward fundamental changes in agricultural practice. Method 5 (chemical control) has now almost totally superceded 4 in control of weeds. However, methods 1-3 can and should retain important places in weed control on well managed, modern farms. Potential problems arising from indiscriminate use of herbicides are the evolution of herbicide resistant weeds, and environmental build up of residues of persistent herbicides.

The following twenty five or so years saw a rapid increase in the number of herbicides and products becoming available world wide (figure 1.1), this being paralleled by a general increase in use of pesticides of all types. In 1957 herbicides accounted for 16% of the total value of pesticide sales. In 1986 herbicides took 44%, insecticides 31% and fungicides 19% of a pesticide market worth $17.4 bn worldwide (BAA Annual Report 1986/7).

The increased adoption of pesticides in general as a cost effective input has led to some marked changes in western agriculture. There now exist vast tracts of land, e.g. the USA grain belt and similar smaller areas in the UK, where monoculture is practiced. There is now an almost total reliance on the use of chemicals in some agricultural systems; having to cope without them would be anathema to many of today's producers. The benefits of pesticide use are given as preventing crop loss, improving food quality, reducing food prices, maintaining public health, promoting animal welfare, banishing drudgery, aiding habitat management, helping gardeners and groundsmen, controlling aquatic weeds and earning export revenue (BAA leaflet No.2).

There is however at present growing public concern at the increased use of pesticides and other chemical inputs in modern agricultural systems, and a general increased awareness of what we are eating. Such concerns, whether founded or not, are of the possibilities of chemical residues in the food chain and their accumulation therein, and of their effect on ecosystems, and are

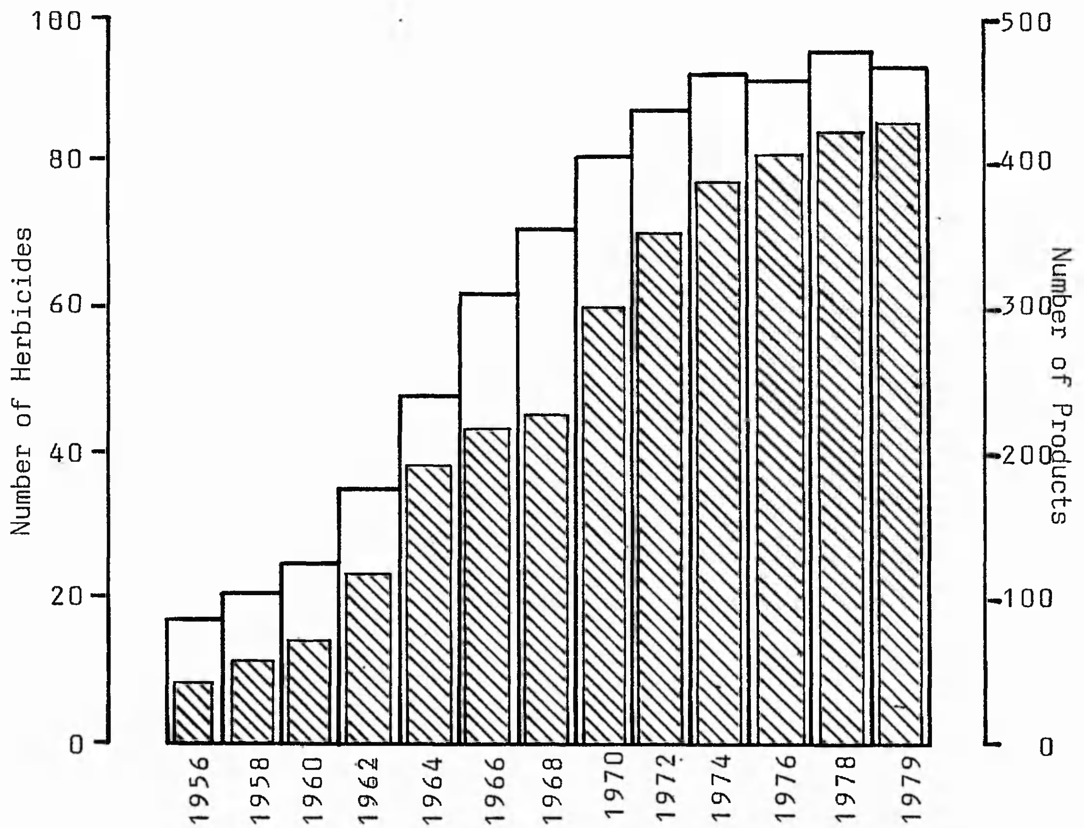


Figure 1.1 Number of herbicides (shaded) and number of herbicidal products, worldwide, 1956-1979. (Makepeace, R.J., in Hurd *et al*, 1979).

voiced through pressure groups such as Friends Of The Earth and Greenpeace. There has been a rise in "green" politics in the UK and the rest of Europe in recent years, and there is clearly an increase in demand for organically grown produce, as witnessed by the major supermarket chains all now carrying ranges of organic fruit and vegetables. The industry is clearly aware of these concerns, and is endeavouring to synthesise compounds of short persistence, fast breakdown, low mammalian toxicities and low residues in food (BAA leaflet No.3). There is still the opinion that pesticides will always be required to maintain quality of crop yield.

Disadvantages of modern monocultures are increased risk of major losses to pests and diseases, and in some areas e.g. USA grain belt, the land is losing productivity as lack of return of organic matter and overuse of agrochemicals is causing imbalance in the soil ecosystem.

Figure 1.1 illustrates how the rapid growth in number of herbicides and herbicidal products was declining by the 1980s. It is likely that the introduction of new herbicides will continue to decline, for a number of reasons.

It is now extremely expensive to research and develop a new compound, there being a 1 in 10000 success rate, costing £20 - £30 m per compound, taking ten years or more from initial synthesis and screening to commercial launch. The industry is finding it difficult to afford such research and development, a situation which is likely to continue as income from old patented compounds is reduced. However, as more information is gathered on structure/activity relationships of certain molecules it is increasingly likely that design of active molecules will become possible, taking some of the hit and miss out of synthesis and initial screenings.

If development and production costs of new chemicals remain high the use of pesticides may become relatively more expensive. Changes by and for the grower will probably be in multiple applications of more than one compound, new application methods including ultra low volume and controlled droplet application techniques, and improved timing of applications using information

on best growth stage for application and maximum cost/benefit interactions. It is also likely that growing more than one crop species simultaneously will suppress growth of different weed spectra, thereby negating the need for herbicidal sprays.

The pesticide industry appears to view the Third World and its developing agricultures as a huge potential market for their products. Doubtless there are successes in the use of pesticides in developing countries, e.g. control of the mosquito, the vector of malaria, but there are also serious implications inherent in the use of our technology in developing agricultures. The BAA lists "banishing drudgery" as a benefit of herbicides, yet if developing countries are short of one thing it is not labour. Is it appropriate fo these people to have their jobs taken from them by pesticides? Another listed benefit is the earning of export revenue. However, credit for a western chemical company is debt for a developing country. It is the servicing of huge foreign debts which draws off much of the earnings of developing nations, and which forces them to grow cash crops as opposed to staple crops which would be of more benefit to their populations. The use of modern pesticides requires the use of modern application technology. Thus the debt grows with the purchase of tractors and spraying equipment. It is doubtful whether the Third World has the infrastructure or skills to efficiently use such technology. It is impossible for them to call out the mechanic if the tractor breaks down. Expensive equipment lies idle and the budget is spent. It is also unlikely that modern pesticides could be used safely by people ignorant of the risks and short of training and safety equipment. Relief agencies such as Oxfam and Intermediate Technology would contend that the use of modern pesticides in developing countries is entirely inappropriate and is symptomatic of the views of western powers to the Third World situation.

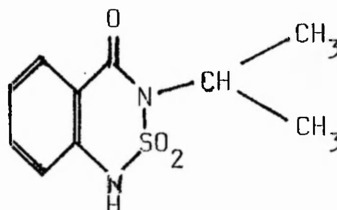
It is clear that the profit motive drives western thinking, yet we contend that use of these technologies is for the benefit of developing countries. It could be argued that this is not so.

1.2 Physical, Herbicidal and Physiological Properties of Bentazone

Technical Information

Chemical name: 3-isopropyl-1H 2,1,3 benzothiadiazin -(4)3H one 2,2-dioxide

Structural formula:



Empirical formula: $C_{10}H_{12}N_2O_3S$

Molecular weight: 240

Melting point: 137-139°C

Physical state: Crystalline Solid

Colour: White

Odour: Odourless

Solubility (gm bentazone per 100g solvent at 20°C)

Acetone	150.7	Ethyl Acetate	65.0
Benzene	3.3	Cyclohexane	0.02
Chloroform	18.0	Olive Oil	2.7
Ethanol	86.1	Water	0.05
Ether	61.6		

Vapour pressure: Very low, less than 10^{-5}Torr at 20°C

Stability:

a) To light: 94% active ingredient available after exposure to light for 24h at 30°C. 83% available after 48h.

b) To UV light: 85% a.i. available after 16h at 20°C.

c) To air: Unlimited

Source: Technical Study Project No.26, 1974. BASF United Kingdom Ltd.

Bentazone (3-isopropyl-1H 2,1,3 benzothiadiazin-(4)3H one 2,2-dioxide) is a post-emergent contact herbicide with some residual action for control of dicotyledonous weeds in several major crops including maize, rice, soybean and other graminaceous and large seeded legume crops. There is a broad spectrum of weeds susceptible to bentazone, including Chickweed (*Stellaria media*), Cleavers (*Galium aparine*) and Mayweeds (*Matricaria* species). If bentazone is applied together with a "hormone" type herbicide such as methylchlorophenoxybutyric acid (MCPB) a particularly wide spectrum of weed control can be achieved (Bentazone Technical Study Project, 1974).

Results of field trials with bentazone were presented at the 1972 British Crop Protection Conference. Luib and van der Weerd (1972) reported that bentazone at 1kg a.i. ha^{-1} effectively controlled annual weeds in soybean, taking effect as a contact herbicide. Crop tolerance was reported as being very good. Bentazone/MCPB mixtures (each at 1.5kg a.i. ha^{-1}) were similarly recommended for use in peas (Taylor and May, 1972), and bentazone/dichlorprop mixtures (4kg a.i. ha^{-1} bentazone) were recommended for use in cereals (Menck and Behrendt, 1972).

Mine, Miyakado and Matsunaka (1975) found that absorption and translocation of bentazone did not differ markedly between resistant rice and susceptible *Cyperus serotinus*. A rapid metabolism (80% within 24h) of bentazone to water soluble fractions e.g. 6-OH bentazone occurred in rice, but in *C. serotinus* 50-75% bentazone was unchanged after 7d, and this was reported as being the mechanism of selectivity. Furthermore, other resistant species' produced large amounts of water soluble metabolites e.g. 6-OH bentazone, while susceptible species' did not (Mine *et al*, 1975). Retzlaff and Hamm (1976) also reported hydroxylation as a detoxification step of bentazone.

Mine and Matsunaka (1975) found bentazone to inhibit photosynthetic CO_2 fixation in rice and *C.scrotinus*, and Retzlaff and Hamm (1976) reported inhibition of CO_2 uptake by bentazone which is reversible in resistant plants. Inhibition of CO_2 assimilation was correlated with uptake of bentazone into leaf tissue,

but recovered as bentazone was hydroxylated in the 6- or 8-position, in resistant species (Retzlaff and Hamm, 1976).

Bentazone was also shown to be an inhibitor of photosynthetic electron flow at photosystem 11 in isolated thylakoids (Pfister, Buschmann and Lichtenthaler, 1974; Mine and Matsunaka, 1975), and in intact leaves (Retzlaff and Hamm, 1976; Potter, 1977). Apart from its direct effect on the photosynthetic process, bentazone was also found to have physiological effects on the photosynthetic apparatus. Seedlings grown in the presence of bentazone develop shade type chloroplasts, these having more granal stacks and increased chlorophyll b and lutein content, with decreased content of plastidic prenylquinones and β-carotene, as compared to controls (Lichtenthaler, Burkhard, Grumbach and Meier, 1980). Lichtenthaler, Meier, Retzlaff and Hamm (1982) summarised the distribution and effects of bentazone in crop plants and weeds. They concluded that susceptible plants absorb bentazone rapidly and metabolise it only very slowly, with depression of photosynthetic CO_2 fixation being irreversible. In contrast, resistant plants absorb bentazone only slowly, and this coupled with rapid metabolism to 6-OH bentazone and glucose conjugates permits reversal of inhibition of CO_2 fixation. The effect of bentazone on photosynthetic apparatus as described (Lichtenthaler _et al_, 1980) occurs more readily in susceptible species and reduces their photosynthetic efficiency, contributing further to the phytotoxic effect of this compound.

In practical use however it was discovered that bentazone efficacy was particularly susceptible to environmental factors, noticably in reduced efficacy in the warm, dry summers of 1975 and 1976 in the UK (Davies, pers. comm.). Doran and Andersen (1976) reported that control of common cocklebur (_Xanthium pensylvanicum_) and velvetleaf (_Abutilon theophasti_) by bentazone could be reduced for both growth chamber and field grown plants if application was made in late evening, at night, or in early morning. The reductions in weed control under such treatments were of sufficient magnitude to be equated with success or failure of the operation in practical terms, and therefore of considerable significance. Doran and Andersen (1976) were only

able to speculate as to possible roles of temperature, photosynthate levels, dew (humidity), leaf orientation and degree of stomatal opening, but they were unable to provide a satisfactory explanation (Doran and Andersen, 1976). Dannigkeit (1977) reported that bentazone penetration into all plants investigated was favoured by high temperature and humidity, observations supported by Retzlaff (1983). Taylor, Davies and Cobb (1981) investigated the possibility that changes in epicuticular waxes of plants grown under different temperature and humidity regimes could account for changes in penetration and subsequent efficacy of bentazone. Although differences in response of such plants to bentazone were found there was a lack of correlation between environment and wax composition (Taylor *et al*, 1981). It was reported therein (Taylor *et al*, 1981) that bentazone had been shown to induce stomatal movements in *Chenopodium album*, both in epidermal peels and in intact plants, but these findings remained unpublished at that time. Also mentioned by Taylor *et al* (1981), in their summarising paragraph, was that the stomatal apparatus was thought to be an important site for bentazone penetration. Cobb, Dunleavy and Davies (1983) reported on the effect of bentazone on stomatal movement in *C.album*. Using infra-red gas analysis (IRGA), it had been demonstrated that intact leaf transpiration and net photosynthesis correlated strongly with CO_2 assimilation via changes in stomatal aperture (Cobb *et al*, 1983). Therefore bentazone could be reducing CO_2 assimilation simply by an effect on stomatal movement, and that this contributed significantly to its herbicidal properties. Stomata as a site of entry of bentazone had been discounted by the observation that the oil based adjuvant, "Actipron", enhanced absorption of bentazone when stomata were fully closed (Dunleavy, 1983). Continued work in this laboratory further characterised bentazone induced stomatal movement in *C.album*, both in whole plants (Dunleavy, 1983) and in epidermal peels (Dunleavy, 1983; Dunleavy and Cobb, 1984a and 1984b). It was reported that stomata which had been pre-incubated to close subsequently reopened in response to KCl, and in response to bentazone, in conditions of darkness and high CO_2. However, when the incubation medium contained both KCl and bentazone stomata failed to open. In conditions favouring

opening of stomata (low CO_2, light, KCl) KCl was required to maintain wide aperture. The addition of bentazone with KCl abolished KCl-dependent opening (Dunleavy and Cobb, 1984a). Dunleavy and Cobb (1984b) subsequently characterised the effect of different concentrations of KCl and CO_2 on bentazone induced stomatal movement. Relatively high KCl concentration (>25mM) was required to open stomata in conditions suited to their closure (high CO_2, darkness). However, bentazone could open stomata at KCl concentration less than 25mM, and at KCl concentrations which opened stomata in the absence of bentazone (i.e. 25mM), bentazone was able to override this and stomata remained closed. Light-enhanced stomatal opening in the presence of bentazone, with low KCl concentration, was inhibited by CO_2 concentration greater than $50\mu l.l^{-1}$. It was suggested therein that bentazone may effect stomatal movement in C.album by disrupting guard cell ion transport, and that CO_2 could directly control the process (Dunleavy and Cobb, 1984b).

Further work in this laboratory by Rees (1986) investigated the effects of bentazone on cellular processes other than photosynthesis, employing isolated Glycine max cells. He reported that bentazone could inhibit mitochondrial oxygen uptake within 25 min, depending on concentration. Bentazone also affected macromolecular syntheses, inhibiting the uptake of ^{14}C thymidine into DNA, ^{14}C uridine into RNA, acetate into lipids, and MVA into lactone, depending on concentration, and enhanced the uptake of leucine into protein, again in a concentration dependent manner (Rees, 1986). As all responses occurred prior to the total inhibition of photosynthesis it was concluded that bentazone "undoubtedly has time to interfere elsewhere in the plant prior to the inhibition of photosynthesis" (Rees, 1986). Al-Mendoufi and Ashton (1984) had similarly discovered that bentazone could inhibit photosynthesis, RNA synthesis, protein synthesis and lipid synthesis all with major effects within 30 min in a concentration dependent manner. Rees (1986) also suggested a study of bentazone on PGR metabolism and function as being worthwhile, and suggested further work into bentazone effects in other pivotal metabolic systems.

In view of the observed poor efficacy of bentazone in the field under warm and dry conditions, and the subsequent work in this laboratory investigating effect of bentazone on stomatal movement as a possible explanation for this (Cobb, Dunleavy and Davies, 1983; Dunleavy, 1983; Dunleavy and Cobb, 1984 a and b), a full investigation of the effect of bentazone on stomatal movement was undertaken by Nichols (1988). The aim of the study was to examine the effects of bentazone on stomatal aperture, guard cell metabolism, and guard cell membrane function (through ion fluxes), i.e. to probe mechanisms of effects on stomata. It was found that in conditions of low K^+ concentration and light, with bentazone present, stomata of *Commelina communis* opened. However, this was dependent on K^+ concentration, as stomata remained closed when potassium concentration was high. In darkness, bentazone induced stomatal opening independently of potassium concentration. The effect of IAA on stomata was similar, and was pH dependent. Furthermore, there was an interaction of IAA and bentazone at low pH in this system. It was therefore proposed that the two compounds could possibly affect and interact at a guard cell plasmalemma ATPase, which was responsible for K^+ movements, possibly through an opposite flux of protons (Nichols, 1988). There is a clear similarity here in that primary auxin action in stimulating cell elongation is proposed to act via a similar mechanism (Hager, Menzel and Krauss, 1971; Cleland, 1982). It was therefore considered worthwhile to investigate the auxin action and interaction and other PGR activities and interactions of bentazone.

1.3 Plant Growth Regulation: A Historical Perspective

From the end of the 19th century to the 1930s research into animal physiology was proceeding apace. Chemical messengers (given the name "hormones") had been discovered in mammals, and a theory concerning their mode of action was formulated. The theory incorporated three major features:

1. Discrete organs (glands) synthesise hormones in response to a given stimulus.
2. Hormones are released into the blood and transported to target cells and organs.
3. Change in hormone concentration at the target organ modifies the biochemistry and physiology of that organ.

At around the same time research into plant growth and development and its control was commencing. The basic findings of these studies in the 1920s and 1930s provided a conceptual framework based on findings from animal systems upon which much of the ensuing fifty years' work was built. However there is now emerging an increasingly loud voice of dissatisfaction with this situation, and current research into plant growth and development is in turmoil. The principal reason for this disquiet has been the adoption by plant physiologists of the hormone theory as developed in mammalian systems. Several current workers are making important basic and fundamental criticisms of this situation, and these academic arguments will now be addressed in some detail.

Much of the early work into plant growth and development concerned the phenomena of phototropism and geotropism, experiments often having been performed using coleoptiles of *Triticum* and *Avena.* The basic findings in such phototropism experiments were:

a) If illuminated from one side only, coleoptiles would bend towards the light source.
b) If the coleoptile tip was covered, phototropic bending did not occur.

c) If the coleoptile tip was excised and then replaced on top of a gelatine barrier, on the cut stump, phototropism did occur.
d) If an excised tip was replaced laterally on the cut stump bending could occur even in the absence of light.
e) If an excised tip was placed on an agar block, and this block was placed laterally on the stump, bending was induced.

The interpretation of these findings was as follows. The coleoptile tip perceives light, it then accelerates synthesis of a "growth substance" (auxin), which is transported down the shoot in unequal quantity to the shaded and illuminated side, where (in the shaded side) its increased concentration stimulates cells in the elongation zone to expand more rapidly and hence curvature towards a unidirectional light source is observed.

With geotropism (the upward bending of a horizontally laid shoot) essentially the same interpretations were made, i.e. the organ perceives gravity, and a modified synthesis and/or unequal redistribution of growth substance causes changes in growth rate of the lower side, and the shoot returns to the vertical. Inherent in these interpretations (the Cholodny-Went model of tropic responses) are:

i) Perception of a stimulus and modified synthesis of a specific compound by a specific organ.
ii) Transport and/or redistribution of "growth substance" causing change in concentration in a specific tissue.
iii) Modification of physiology i.e. change in growth rate of the tissue in response to the changing concentration of "growth substance".

It is important to recognise the three major features of the model of hormone action in mammals in these interpretations.

Trewavas (1981) and Weyers (1984) consider that these interpretations would never have been made in the absence of the hormone theory for mammals, and suggest that there is no good reason why it should have been applied to plants, and they have reapproached the problem from "first principles".

As taxonomic classes, plants and animals divided early during evolution, and subsequently evolved independently to their multitude of present forms. There are therefore several unique features of each which are entirely inapplicable to the other. While it is possible to predict that a 27 year old human will possess two eyes, four limbs and thirty-two teeth (barring accidents!), it is impossible to predict the final number of branches or leaves, or their relative positions, on a tree of 27 years. This is of course due to the regenerative, organisational and developmental plasticity of plant meristems. The activity of these meristems determines the plant's form, which may constantly change as environmental conditions change. Animals possess no meristems, indeed at birth they possess all their required parts, albeit at differing stages of maturity. Animals possess clearly defined tissue types (organs) each of which are suited to and serve a particular function. By contrast, in plants various cell types form functional organs but with less distinct boundaries between them. Plant cells also overlap in function, and are capable of many biochemical and physiological processes. Another clear and quite fundamental difference between animals is their lifestyle. Most animals are capable of locomotion , and use this in order to find food, to escape predation, and to migrate in response to a changing environment. However since most plants are rooted to the spot adaptation to their environment must be more subtle. If the environment is dry they cannot "search" for water, they must conserve it; if part of them is eaten they must renew it; if conditions are favourable they must exploit it by e.g. reproduction. In many of these instances the integration of activity of meristems determines the success or otherwise of the response.

It is against this background of fundamental dissimilarities that Trewavas (1981) and Weyers (1984) discuss the suitability of the classic hormone theory to plants. At the time of its adoption by plant physiologists there was, as already mentioned, much fruitful research into mammalian hormone systems. The hormonal model as formulated for mammals has an elegant simplicity, and this, coupled with a

sense of being "left behind" by animal research, proved irresistable to the plant physiologists of the time. Trewavas (1981) felt that its adoption was unduly hasty and naive, and extremely uncritical bearing in mind the fundamental differences between plants and animals.

However, armed with a classic model, the plant 'hormone' community set about providing data to support the theory. They had, according to Weyers (1984), become "conditioned to think... in hormonal terms", and the "straitjacket of the hormone concept has forced plant physiologists to design and perform their experiments in a biased fashion.".

As mentioned earlier, much of the early data was obtained from experiments studying tropisms in grass coleoptiles. Trewavas (1981) concluded that the bending process in coleoptiles was not sufficiently well characterised for such interpretations to be made. There exists a zone where cells elongate more rapidly, this is 6-9mm below the etiolated coleoptile tip. On either side of this region growth rates decline rapidly. Consequently, the observation that a wave of bending apparently goes down the plant would occur even if growth in all regions commenced at the same time. Firn and Digby (1980) were similarly critical. Differential growth can occur through five major ways, which are:

1) Increasing growth rate of one side.
2) Decreasing growth rate of one side.
3) Differential acceleration of the two sides.
4) Differential deceleration of the two sides.
5) Acceleration of one side and deceleration of the other.

For a meaningful study of the particular tropism the nature of the response must be fully characterised. Factors to be considered are when and where the differential growth is induced, and is it dependent upon other parts? Firn and Digby (1980) commented on the lack of such basic information, but of course without it the whole exercise misses the point.

Several workers have recently re-examined the evidence which has supported the hormone theory for the past half century. Using the examples of coleoptile phototropism, seed germination and stomatal movement these re-examinations will now be decribed, the conclusions being basic and central to this particular field of research, and therefore deserving of description in some detail.

Coleoptile Phototropism

The basic findings which gave rise to this theory have been mentioned earlier. Evidence which convinced early workers that the tip was the site of auxin synthesis was of two types. If excised tips were placed on an agar block auxin release into the block showed two phases. The first was diffusion of existing auxin, the second being a renewed synthesis. The second piece of evidence in favour was that an auxin gradient exists within the coleoptile. This evidence is, however, flawed. Comparison of diffused auxin is rather a dubious exercise because large losses may be incurred (up to 50%, see Trewavas, 1981). More importantly, however, are several other pieces of evidence. The original auxin extractions were not performed in sterile conditions. Repeats of these experiments under sterile conditions did not produce large amounts of diffusing auxin, indicating that in the original experiments it was probably bacterial in origin. It is also now considered theoretically impossible for such large amounts of auxin to be derived from the sterile coleoptile. Similarly, there is insufficient free tryptophan present to support such auxin synthesis; the tryptophan to IAA catalysing enzymes are of very low activity; hydrolysis of bound IAA (IAA esters) in excised tips would occur rapidly and therefore not be a source of prolonged auxin synthesis.

There is also compelling evidence that the seed is the source of auxin. The coleoptile possesses two vascular bundles, one running up either side and terminating about 0.5mm from the tip. Nutrients are supplied from the seed to the area of high division and expansion activity i.e. the tip, through these vascular bundles, and the tip then acts merely as a distribution centre.

The seed possesses about 100-200 times the auxin of the coleoptile (as IAA -myo-inositol conjugates), and it would be difficult to imagine that none of these would be carried into the vascular stream. However, more hard evidence is provided by the observations that deseeding of the plants leads to a rapid decline in coleoptile tip IAA, and radiolabelled IAA injected into the seed subsequently appears at the tip. The endospermic origin of coleoptile IAA was demonstrated as early as 1935 (Cholodny, 1935; Pohl, 1935), but the evidence was discounted because subsequent experiments failed to collect auxin by diffusion into agar blocks placed on the cut stump. These collection experiments have been more recently repeated, and using radiolabelled IAA, it was found that auxin accumulated beneath the agar block.

It has therefore been shown and calculated that the tip is probably not the biosynthetic source of IAA, and the auxin gradient down the coleoptile can be more satisfactorily explained.

The second essential feature of a truly hormonal model is a changing concentration of 'hormone' in the tissue in which the response occurs. In the case of the Cholodny-Went model for phototropism this would involve an increase in auxin in the shaded side and/or a decrease in the illuminated side, depending on what type of growth modification is observed (see Firn and Digby, 1980). This would, in the coleoptile, involve increased diffusion to the shaded side of the tip. Early workers purported to have found this, with approximately twice the auxin content in the shaded than in the illuminated side (Trewavas, 1981). (These experiments suffered from difficulties in quantification of auxin, often relying on bioassay. For a discussion of bioassays, their use and suitability, see section 1.4). With the advent of radio-labelled auxin there became available a less questionable method for determination of lateral auxin movements. Experimental evidence from the 1960's to date has been conflicting and Trewavas has concluded that "mechanisms other than lateral transport of IAA may exist for establishing differential growth

rates on the lighted and shaded side of the coleoptile." (Trewavas, 1981). Firn and Digby (1980) criticised existing models for phototropism. They dismantled the phenomenon of phototropism into several components, but in generally describing the Cholodny-Went model they reached a similar conclusion to Trewavas (1981). The role of the apex is overemphasised. Evidence for this was also presented by Weyers (1984) who demonstrated that photostimulation of a region distant from the tip could also induce phototropism, and positive phototropic curvatures could also occur in the absence of significant IAA movement. In cases where lateral redistribution of IAA does occur it is of small magnitude, and the question is asked as to whether this would be sufficient to induce the responses observed (Firn and Digby, 1980). This raises an important point in the consideration of the hormone model. If we assume for the time being that changes in "hormone" concentration are entirely responsible for observed responses then there have to be several basic features. Correlation must exist in time of changing concentration and the observed response. This means that for phototropism, with a lag period of as little as five minutes, there must be an IAA accumulation within that time. Considering cell extension studies, where there is a ten minute lag in an auxin response, it is unlikely that auxin could cause phototropism within 5 minutes, even if it did accumulate in sufficient quantity. Firn and Digby (1980) concluded that there is no convincing evidence that sufficiently large gradients are established during a lag period.

A further necessary criterion, if changing concentration produces an increase in growth rate, is that endogenous levels of auxin are growth-limiting. The obvious test of this is to apply auxin exogenously and observe the response. When this was done there was no substantial increase in growth rate (Trewavas, 1981).

Clearly then, the model of hormonal control of phototropism is in need of revision, with there being compelling evidence against the tip as a biosynthetic site, and against transport and changing concentration causing modification of the response.

Barley Germination

Another supposedly incontrovertable instance of a truly hormonal system at work in plants is the control of germination in barley seeds by endogenous gibberellins. The widely taught model states that embryo synthesised gibberellin diffuses to the aleurone layer where it activates amylases and proteases, these in turn commence breakdown of stored products required to drive germination. This model has also come under attack from Trewavas (1981), who presented the following pieces of evidence against it.

1. The embryo commences growth prior to any increase in α-amylase production i.e. increase in α-amylase doesn't drive germination.
2. The production of α-amylase by the aleurone in response to gibberellin requires the imbibition process to be well progressed i.e. the physiological state of the tissue is important.
3. There is sufficient free gibberellin in immature barley to drive amylase synthesis. This cannot occur however before the processes of drying and rehydration have occurred.
4. The aleurone doesn't require embryo gibberellin, it can synthesise its own.
5. Endogenous gibberellin concentrations are not limiting to the process of germination.
6. Amylase synthesis is all but finished when increases in gibberellin are detectable i.e. the correlation of the events in time is incorrect.

Trewavas (1981) concluded that there is no need even to invoke any hormonal role in this system!

Stomatal Movement

One further system widely held to concur with a hormonal system (also dicussed by Trewavas, 1981) is stomatal movement in wilting leaves, and its control by abscisic acid. The hormonal

model suggests that plants subjected to water stress increase abscisic acid (ABA) synthesis in the leaf mesophyll chloroplasts. The ABA then moves to stomata where it induces closure. Evidence does at first sight appear to support this model. In water stressed plants the stomata close. They also accumulate ABA in the leaves, and epidermal strips floated on solutions of ABA do indeed show stomatal closure. There are several experiments which may be performed to test this hypothesis, and Trewavas (1981) supplied the following evidence to refute the hormonal model.

One fundamental test again involves the correlation of hormone (ABA) accumulation with the response (stomatal closure) in the correct time sequence. Trewavas (1981) quoted that in several species (corn, sorghum, bean), in data from several workers, this does not occur. In fact the reverse is true, stomata close <u>before</u> an accumulation of ABA occurs, and often remain closed for a considerable period after decline in its concentration.

In explanation of this it has been suggested that ABA is held in isolated compartments in mesophyll cells until water stress occurs. It is then released and migrates to stomata where it effects their closure (Loveys, 1977). Trewavas (1981) challenges this with four pieces of evidence.

1. Concentration of ABA in the epidermis does not increase on water stress.
2. The half life of ABA is about 4 minutes, which is suggested as being contrary to a long-term storage strategy.
3. If stored ABA is sufficient why do its levels rise after closure?
4. If ABA is stored a lag would be observed between water stress and stomatal closure, but this is not usually the case.

Trewavas (1981) discounts the hormone theory for this system as he does for the other two systems described (coleoptile phototropism and barley germination). These examples are however

probably the best characterised "hormone" systems in plant physiology, yet they are remarkably poorly characterised as being truly hormonal in nature, and as described there exists consider-able evidence against the application of such theories to plant physiology. After fifty years effort there is still no conclusive proof of the control of a relatively simple phenomenon, modification of cell elongation rate, being under strictly hormonal control. The literature is littered with contradictory evidence (e.g. Brummell and Hall, 1987). Imagine then the difficulties in attempting to account for a complex process e.g. change of a grass meristem from vegetative to reproductive phases, by such a model.

It is therefore becoming increasingly widely held that now is the time for a radical rethink and reformulation of ideas about the roles of low molecular weight, physiologically active compounds found endogenously in many plants, i.e. PGRs.

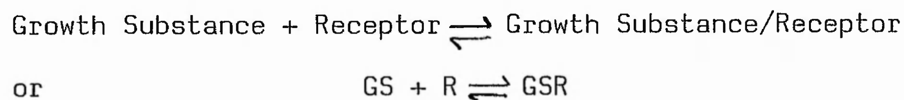
Trewavas is one of the more prominent and vociferous of the "new theorists", and as such is beginning to formulate ideas about differences in sensitivity of plant tissues to physiologically active substances. Again using the three examples cited earlier, these new ideas will now be outlined, along with thinking behind them.

There is no doubt that plants do possess low molecular weight compounds of high physiological activity. This is probably the parallel to the mammalian systems which led to the ill-fated analogy described earlier. If new theories are to be presented, and experimental data to be scrutinised to confirm or refute them, then these compounds and their activities must be considered independently of the hormone "straitjacket". The first and most fundamental question is, therefore, "what is the likely function of these substances?". To answer this the basic structural and physiological aspect of plants must be considered. Plants absorb minerals through their roots, and synthesise organic products (via photosynthesis) mainly in the leaves. These required nutrients are then transported in the vascular

system. Careful consideration of the vascular system of a plant reveals it to be quite different to that of a mammal. Not all plant cells are served by a direct vascular link; some epidermal cells of e.g. a stem, may be some distance from a vascular bundle. It would be inappropriate therefore to make growth directly dependent upon nutrient supply as it is in some single celled organisms. There has to be a mechanism to permit peripheral cells to receive sufficient nutrition, i.e. growth must be integrated. Trewavas (1981) proposed that the solution which plants have developed is to make growth dependent on the presence of the physiologically active compounds. Plant are not sufficiently sensitive to the small concentration differences that would occur as these compounds diffused through plant tissue, having evolved a logarithmic growth response relationship to growth substance concentration. Plants have also evolved the ability for all cells to synthesise these compounds, thereby helping to "iron out" further any small concentration gradients in terms of nutrient supply. The effect is that no cell can use more than its complement of nutrients; the organism grows and develops in an integrated manner. The different groups of physiologically active compounds co-act to ensure this integrated development, their function being one of coordination of behaviour of parts.

The next question then becomes, "if all cells possess a relatively constant quotient of plant growth regulatory compounds (PGRs), how is modification of their activity brought about?".

Trewavas (1981) proposed that a change in sensitivity of the cell or organ to a particular compound facilitates such modification. Sensitivity is broadly defined as the ability of the tissue to respond to the particular compound. Degrees of sensitivity also occur, and may be assessed by measuring the strength of a response to a particular stimulus. Trewavas (1981) assumes the molecular basis of sensitivity to be dependent upon the presence and concentration of specific receptor proteins, with the interaction of growth substance and receptor assessed simply as:



$$\text{Growth Substance} + \text{Receptor} \rightleftharpoons \text{Growth Substance/Receptor}$$

or $$GS + R \rightleftharpoons GSR$$

The formation of GSR initiates the given response, and the rate of this formation is dependent on the concentration of GS and R. The exact mechanisms controlling the equilibrium are not described, but it is clear that a simple increase in concentration of GS has little effect on the rate of procedure of the response. In fact a logarithmic increase in GS concentration is generally required to induce a measurable modification of response. Trewavas (1981) suggests a more productive mechanism of increasing concentration of GSR would be an increase in concentration of R i.e. an increased tissue receptiveness.

Trewavas (1981) cited several examples to illustrate this idea. One example is the variation in auxin sensitivity, with developmental age, of wheat coleoptiles. The system from which data was obtained was this:

Wheat seedlings were grown for up to three days, throughout which time measurements of growth rate were made. At regular intervals throughout this period coleoptiles were harvested from the population and subjected to bioassay, where their percentage increase in length, in response to a constant auxin concentration, was measured. The excised coleoptiles responded differently to the constant auxin treatment, depending on their age, i.e. there was a change in sensitivity depending upon growth stage. This change in sensitivity correlated extremely well in time with growth rate measured on the intact seedlings. Trewavas (1981) therefore proposed that growth rate in these seedlings is controlled by changes in tissue sensitivity (maximum responsiveness) to a constant endogenous auxin concentration. Several other examples were cited, and essentially the same conclusions drawn (Trewavas, 1981).

Trewavas (1981) proposed that in coleoptiles, for example, the young tissues possessed a higher density of receptor proteins, are thereby more sensitive, and hence showed increased activity (more rapid elongation) to exogenous auxin. As the tissue aged the concentration of receptor protein reduced, and with it the responsiveness of the tissue. This is obviously a simple model but Trewavas (1981) hoped that it will at least provide the necessary stimulus for an increased research effort in this area.

Another good example of the correlation between degree of binding and responsiveness was provided by Sanders, Smith and Hall (1986). These workers demonstrated that the region of pea epicotyl most responsive to ethylene was the hook and just below. This sensitivity was closely correlated to binding of ethylene and to the degree to which ethylene was incorporated into the tissue. Regions of low ethylene incorporation and sensitivity also showed reduced ethylene binding (Sanders et al, 1986). If an observed response to an environmental stimulus is the consequence of a change in tissue sensitivity the question must be asked, "how does the given stimulus alter sensitivity?". Trewavas (1981) argued that control of sensitivity is relatively straightforward.

In conditions of adequate nutrition the rate of protein synthesis is high, and protein degradation proceeds slowly, i.e. there exists an equilibrium of high protein concentration. In conditions of poor nutrition the reverse is the case, breakdown is more rapid than synthesis. As all known receptors in plants being proteins (Venis, 1985), it is clear that in a plant which is well nourished the maintained high number of protein receptors leads to high GSR concentration and thus increased response, i.e. sensitivity is high as a direct function of environmental status. From this idea it must also be assumed that factors such as light and gravity (and their roles in tropisms) affect concentration of receptor. There is clearly the need for considerable input to integrate all these ideas into new models of developmemtal control by plant growth substances. However, the aim of Trewavas in his 1981 review, to be provocative and to promote discussion and rethinking, is being achieved in part.

However, all is not simple. Firn (1986a) recognised the ambiguity of the term "sensitivity", and promoted the need for "clearer ideas, precise terms and purposeful experiments". He put forward five possible parameters by which sensitivity could be judged.

The five parameters are:-

1. Magnitude of maximum response.
2. Maximum response as a ratio of control.
3. The minimum concentration of GS required to invoke a significant response.
4. The concentration of GS required to invoke maximum response.
5. The concentration of GS required to give half maximum response.

The parameters are important when assessing different dose response curves obtained from bioassay. This is because bioassay curves can shift depending on experimental conditions imposed. Firn (1986a) illustrated how ranking of sensitivity of five plant tissues, from their bioassay curves, can change dramatically, depending upon which of the definitions of sensitivity (1-5 above) is applied. There is clearly a need for specific terminology.

Fundamentally different changes in physiology of the responding tissue can also dramatically alter sensitivity ranking depending on definition of sensitivity.

Applying the simple equation from Trewavas (1981) i.e. equation 1, it is clear that the equilibrium of GSR formation is dependent upon three basic factors, these being concentrations of GS and R, and the affinity of GS for R. Trewavas (1981) proposed that a shift in equilibrium is weighted towards a change in concentration of R, and that a change in concentration of GS has relatively little effect. However, this is largely beside the point for this appraisal. Firn (1986a) incorporated other physiological factors into the considerations, these being the response capacity of the subsequent events which produce the observed response, the efficiency of growth substance uptake systems under experimental conditions, and the possible metabolism of growth substance. The nature of the response will therefore be dependent upon and variable with any of the above factors.

Changes in these factors will affect the dose response curve, as eloquently illustrated by Firn (1986). Weyers, Patterson and A'Brook (1987) have recently proposed a system whereby mathematical values are assigned to each of three "sensitivity parameters", by which any dose response curve can be described. The sensitivity parameters were derived by Weyers *et al* (1987) by comparison of dose response curves to simple Michaelis-Menten enzyme kinetics, with the incorporation of an interaction coefficient. There are several important assumptions inherent in these ideas, of which Weyers and co-workers (1987) are aware and discuss regarding future experimental approach. The major experimental considerations are these.

Assay conditions *in vitro* must as closely as possible represent those *in vivo*. This requirement must however be balanced against that of isolation of the system under study, in order to eliminate interference from other plant parts. It must be assumed that growth substance concentration at the site of action is equal to that in the bathing medium, or at least holds a linear relationship with it. In the absence of bathing medium, e.g. in droplet applications to dwarf pea nodes, the relationship between PGR applied and that penetrating to site of action must be fully characterised. Working to the Weyers *et al* model (1987) it is important to gather information about initial rates of response. A good knowledge of molecular events underlying any response is desireable if meaningful analyses are to be made. Firn (1986) also described the need for purposeful experiments. The experiments must be designed and conducted specifically to provide definitive data regarding sensitivity. He also suggests greater knowledge of PGR mode of action to be fundamentally important, along with further characterisations of PGR receptors.

The molecular basis for PGR mode of action remains unclear, with the majority of work having been directed towards charactersation of end physiological effects. There has been some work to identify PGR induced changes in gene expression, and a small amount in characterising the nature of PGR receptor interaction (Venis, 1985). Venis (1985) suggested that a further decade of

fruitful research is required until molecular bases are better understood.

However, significant support for the currently developing ideas of Trewavas (1981 and in Trewavas and Cleland, 1983), that changes in plant sensitivity, rather than changes in "hormone" concentration, are responsible for changing plant responses to such compounds, is provided by the more recent data analyses by Nissen (1985 and 1988), regarding dose responses of plant tissues to auxins (Nissen, 1985) and gibberellins (1988).

Employing data from a formidable number of earlier publications, which had obtained this data largely from use of bioassays, Nissen (1985) assessed whether the responses are ultrasensitive, hyperbolic or subsensitive to auxin i.e. whether the change from 10 to 90% of maximal response requires less than, equal to or more than an 81-fold increase in exogenous auxin concentration. (The derivation and significance of an 81-fold increase in external PGR concentration are explained fully by Weyers _et al_ (1987)). To summarise, and to explain the significance in physiological terms, an ultrasensitive response i.e. one requiring a less than 81-fold increase in PGR concentration to shift from 10% to 90% of maximum response, indicates that a change in PGR concentration is an important and major factor in causing change in response. A hyperbolic response, i.e. one displaying straightforward Michaelis-Menten kinetics, requires a change in PGR concentration of 81-fold. A subsensitive response requires a change in PGR concentration of greater than 81-fold to shift response from 10% to 90% of maximum observed, and physiologically this indicates that a change in PGR concentration is an unimportant factor in control of response to the PGR, rather it suggests that a change in tissue sensitivity is the principal controlling factor.

In his analysis of the historical data for auxin sensitive responses, Nissen (1985) reported that auxin dependent callus growth is the only ultrasensitive response, with a few hyperbolic responses, but with the majority of cases exhibiting often

markedly subsensitive dose responses. Systems with subsensitive responses include elongation of coleoptile segments of both wheat and oats. Elongation of dicotyledonous tissues including pea epicotyls are also subsensitive in dose response, as is the inhibition of root growth by IAA (Nissen, 1985).

In his study of dose responses to gibberellins Nissen (1988) found similarly. Dose responses to gibberellins including enhanced elongation of rice leaf sheaths and of cucumber hypocotyls, as well as other elongation responses and production of α-amylase are assessed as being almost universally subsensitive.

The findings of these studies (Nissen 1985 and 1988) are that the majority of plant responses to a concentration range of PGRs are subsensitive, and as such they offer considerable support to the proposal and developing theories of Trewavas (see Trewavas, 1981), that responses of plants to PGRs are controlled principally by changes in tissue sensitivity rather than by the changing "hormone" concentration, as has been traditionally considered.

Until such time as molecular bases for PGR activity are elucidated the main thrust of PGR research will probably continue to be into physiological changes employing bioassay. This, along with attempted correlation of endogenous PGR concentrations, has produced the bulk of data providing current knowledge and theories about PGRs and their roles. This approach is not without its shortcomings, and several fundamental difficulties remain.

However, many workers within the PGR community do not hold with the ideas recently proposed by Trewavas, namely that sensitivity change is the main controlling factor of plant development. Trewavas and Cleland (1983) discuss both viewpoints, Trewavas arguing for sensitivity, Cleland for changes in concentration. Trewavas cites evidence and ideas previously discussed herein. In addition he suggested that PGRs are only one of a large number of factors that modify structure and

function of the plasmalemma, thereby affecting cellular function. Other factors affecting plasmalemma function include minerals, water, light regimes, temperature, osmotic status, genetic factors, biotic factors, and age and condition of the tissue. Cell and thereby tissue growth are directly affected via changes in any of these factors and their effect at the plasmalemma (Trewavas in Trewavas and Cleland, 1983). Robert Cleland, a prominent figure in auxin mode of action research since the 1960s, argued that changes in PGR concentration cannot be dismissed, and that such changes do contribute to control of plant growth and development. He suggested that a combination of the two regulatory processes are probably responsible for the overall response. Cleland (in Trewavas and Cleland, 1983), dicussed the application of the term "hormone", and while acknowledging the unique features of plants, and the innappropriate application of the term "hormone" in plant science, cited three examples of control of a process by plant hormones in cells distant from sites of synthesis. (Interestingly, two of the examples cited by Cleland (1983) are from 1957 and 1969 publications. These are prior to the emergence of the ideas of Trewavas, and it must be asked whether the design and execution of these experiments was subject to the biased attitudes touched upon by Weyers, (1984). However , if it is assumed that they were not then they can be accepted as part of the argument of Cleland (in Trewavas and Cleland, 1983) that these compounds do indeed behave as hormones, in the strictest sense).

To involve changes in concentration of "hormone" as a controlling factor, Cleland (in Trewavas and Cleland, 1983) cited examples in which there is no physiological response when "hormone" is absent, this being achieved using single gene mutants. This is rather a crude and cumbersome method of demonstrating a control mechanism by change in "hormone" concentration, and in fact it contributes little to the overall argument against the ideas of Trewavas. This is because at no time does Trewavas suggest that a particular response in which a PGR is implicated occurs in the total absence of that PGR. He does in fact argue the reverse i.e. that the reason for the presence of PGRs is to ensure integrated development (Trewavas, 1981).

His argument is simply that fine differences in PGR concentration are not the controlling factor of the response. If sensitivity changes are the explanation then the compound to which the tissue is sensitive must be present.

Cleland (in Trewavas and Cleland, 1983) also presented evidence where smaller changes in concentration of a PGR "appear to" control patterns of development. He cited the examples of phototropism and gravitropism in maize shoots and sunflower stems respectively. The auxin redistribution exactly correlates with the observed differential growth. A further example shows correlation of cytokinin : auxin ratios in tobacco callus with the type of differential growth observed (Cleland, in Trewavas and Cleland, 1983).

Cleland suggested in the discussion paper (Trewavas and Cleland, 1983) that the sensitivity concept is firmly established and is almost accepted without the requirement for discussion. However, if this is so, asked Trewavas (in Trewavas and Cleland, 1983) why is there virtually no mention of it in the literature and also, importantly, in textbooks which are shaping the thinking of the next generation of plant physiologists?

It is clear that this particular field of plant physiology is the forum for considerable debate at present, and there is an obvious need for open minds and meaningful discussion if our knowledge and understanding are to advance.

1.4 Aims of the Investigation

As already mentioned, there are two historical approaches to PGR research. The first is the attempt at correlation of observed physiological changes with changes in concentration of endogenous "hormone". The second is use of bioassay, in which changes in physiology are observed following application of exogenous "hormone". Both approaches have their drawbacks. As discussed earlier (section 1.3) correlation of events must concur in time, and if changing PGR concentration is the controlling factor in causing physiological changes then changes in concentration must be of sufficient magnitude to elicit the observed response. Trewavas (1981) criticised this approach in concluding that events often do not concur well in time, and he feels that measured changes in concentration of PGR are often insufficient to account for the observed physiological change. This assertion is however based on the proposal that responses occur proportionally to the logarithm of PGR concentration, a theory formulated from the use of bioassay.

Weyers (1984) discussed the difficulties which have existed throughout the history of PGR work in accurately assessing the concentration of "hormone" which may be present at nanograms per gram fresh weight. If by traditional separation techniques, such as thin layer chromatography, an active "spot" is obtained, it is impossible to be confident that this contains only one active compound. There is also the problem of different forms of the particular compound e.g. conjugates, and that of many members of a group of compounds e.g. the gibberellins, each of which may have different physiological activity. Such difficulties of traditional practice in PGR research are acknowledged by Cleland (in Trewavas and Cleland, 1983). More modern techniques such as HPLC should lead to more satisfactory identification and quantification of these compounds. Brenner (1981) reviewed procedures for PGR analysis and concluded that physicochemical and immunological techniques offered potential for improving PGR analysis, but suggested that bioassays will continue to be of value.

The second major approach which has provided much of our knowledge of effects of PGRs has been the use of bioassays. The basis of the bioassay is the exogenous application of a compound followed by observation of changes in physiology. Bioassays present several anomalies to the plant physiologist which are difficult to reconcile.

It must be assumed in bioassay that exogenous compounds, even if naturally occurring, behave in the same way in the plant as their endogenous counterparts. It is often found however that exogenous application to an intact plant does not induce a response. This could be for a number of reasons. It is possible that the compound does not enter the plant. Perhaps the exogenous compound enters the plant but is rapidly metabolised to an inactive form. If changing "hormone" concentration is the endogenous response control mechanism perhaps internal concentration is already saturating the response. If sensitivity is important perhaps intactness controls the sensitivity and the plant is physiologically unable to respond to the exogenous application. Whatever the reason, intact plants rarely respond to exogenous applications, and as such bioassays commonly employ excised plant tissues. An important assumption from work using excised sections is that the responses seen in the excised tissue are the same as those which occur in the intact plant in response to endogenous PGRs. This extrapolation is a major assumption and may not be true. The excised tissue commonly shows little or no response to concentrations of PGR detected endogenously. A far higher exogenous than endogenous concentration is required to elicit a response of similar magnitude. There are serious implications in this. Either there are large losses of endogenous compound on its extraction and quantification, or there is a fundamental change in the physiological state of the tissue accompanying its excision. Losses of compound on handling can be detected using known quantities of e.g. radiolabelled compound, and if losses were large then the method would be assessed as lacking sufficient accuracy to be of value. We are therefore compelled to accept that excised tissue is basically different to intact, and therefore to question the interpretation of data collected from excised tissue systems in relation to the intact plant.

Trewavas (1981) quoted that protein degradation occurs rapidly in excised plant tissues. All known "hormone" receptors are proteins (Venis, 1985) and it is unlikely that these escape degradation on excision. Perhaps therefore the loss of response of excised tissue is due to a reduction in number and/or affinity of receptors, and their consequent effects on the reaction equilibrium discussed earlier (section 1.3, and Weyers, Paterson and A'Brook, 1987).

As mentioned previously, bioassays have commonly provided curves showing a linear response relationship to changes in order of concentration of exogenous PGR (i.e. a log-linear relationship). The shortcomings of bioassay systems should be carefully considered when attaching significance to results obtained from them.

The arguments of Trewavas (1981, 1983) are largely concerned with the function or role of physiologically active compounds within the intact plant. A vast amount of work has attempted to discover what these compounds actually do i.e. what processes do they effect? The use of bioassays has provided a lot of information regarding this, but in some cases it is not known whether or not these compounds similarly affect these same processes in the intact plant as they do exogenously with tissue segments. Despite this the known effects of physiologically active PGRs in well characterised exogenous systems can be employed in order to determine the physiological activity of an unknown compound. This is largely the approach taken in this study.

Recent work in this laboratory on bentazone (Rees, 1987; Nichols, 1989) clearly suggests bentazone action at the plasmalemma. Briefly, this is an effect on protoplast physiology, and an effect on stomatal movement. To further the proposals that bentazone is active at the plasmalemma, and to establish further its similar action to IAA in a number of situations, it was desirable to assess its activity in a number of auxin sensitive bioassay systems. The aims of this study are therefore to provide data on PGR activity of bentazone, and to suggest mechanisms of its interactions.

Chapter 2. BENTAZONE ACTIVITY IN AUXIN BIOASSAYS

2.1 Occurrence, Synthesis and Metabolism of IAA

The greatest body of knowledge regarding the major groups of plant growth regulators (PGRs) exists for the auxins. Auxins were first characterised early in the 20th century as causing tropic bending of plant organs, and were proposed to exert such effects through changing concentration, a situation analagous to the theories concerning animal hormone function, as discussed in Chapter 1. While the debate over role and mode of action continues, there is no doubt of the ubiquitous nature of auxins, these being found in seeds and in both shoot and root tissues of many species (Cohen and Bandurski, 1982). IAA can exist in both free (assumed to be active) and conjugated forms within plant tissues, and interconversion between the two and their rates of biosynthesis and degradation, will determine the the concentration of free IAA. Cohen and Bialek (1984) suggest however that there is still a lack of basic information about the processes which lead to the *in vivo* production of IAA, but suggest that two possible routes exist.

Firstly, *de novo* synthesis of IAA can contribute to endogenous free IAA, but while most IAA exists as conjugates of either esters (mainly IAA-glucose), acyl anhydrides or amides, the release of IAA from conjugated forms is considered another major source of free IAA (Cohen and Bandurski, 1982; Cohen and Bialek, 1984). *De novo* synthesis of IAA occurs predominantly from tryptophan as a precursor, and could occur via several theoretical pathways described fully by Cohen and Bialek (1984). Release of IAA from conjugates occurs through hydrolysis from the esters, acyl anhydrides of amides, whose proportions vary depending on tissue type and age (Cohen and Bialek, 1984). While synthesis and release from conjugates contribute to maintain IAA levels there are parallel but opposite processes occurring to prevent concentrations becoming too high, and these have been reviewed by Bandurski (1984).

The two main routes for removal of free IAA are via conjugate formation to glucose, amino acids and myoinositol, and oxidation. Oxidation is a method of IAA catabolism, and this, along with the other processes for reduction of IAA concentrations, are described fully by Bandurski (1984). Unfortunately, as Cohen and Bialek (1982) freely admit, there is still a lack of basic knowledge about the processes involved in auxin metabolism, and a lack of sound, reproducible experimental systems to assist in our further understanding.

While relatively little is known regarding auxin biosynthesis and metabolism there exists a greater body of knowledge regarding the physiological effects of exogenous IAA. While not without limitations (Chapter 1), bioassays using exogenous compounds can yield potentially important information regarding the action of their endogenous counterparts. From use of such systems plant physiologists have attributed to IAA an involvement in stimulation of cell division, stimulation of shoot growth, concentration-dependent effects on root growth, control of apical dominance, control of abscission, and the effects on flowering. In the current study, the effects of the herbicide bentazone have been studied in several of these systems, both alone and in combination with exogenous IAA. The experimental procedures and findings will now be described.

2.2 Auxin in Experimental Systems

2.2.1 Auxins and Apical Dominance

One process in which endogenous auxins are thought to have an influence is apical dominance. Apical dominance, or correlative inhibition, is the phenomenon by which outgrowth of lateral, often axillary, buds is inhibited by the presence of the shoot apical bud. The presence or absence of the apical bud can also modify developmental patterns in other lateral organs including leaves, branches, rhizomes and stolons. The removal of the apical bud commonly leads to outgrowth of laterals, and considerable work over the last 100 years has been directed in attempts to elucidate the mechanisms by which the apical buds exert such control. From this input there have emerged two major ideas of how the correlative signal is transmitted from the apical to the lateral shoots, and these have been reviewed by Phillips (1975). The first is that nutritive control is the principal mechanism in lateral bud outgrowth. In this case the bulk of absorbed water, nutrients and photosynthetic products are presumed to be exploited by the apical bud which develops successfully at the expense of the laterals. This idea incorporates the supposition that nutrients and water are normally limiting to whole plant development. Support for this was provided by McIntyre (1973), who demonstrated that lateral bud outgrowth could occur even in the presence of the apical bud if the plants were provided with favourable conditions of nutrition and light, and were grown without water stress.

The second idea regarding control of apical dominance is that PGRs are responsible. Exogenous auxin (IAA) is able to substitute, wholly or partly, for the decapitated apex in several species including *Phaseolus vulgaris*, under certain conditions. The observation that the apex is a natural site for auxin synthesis would appear to support this proposal (Hillman, 1970; Phillips, 1975). Although IAA is strongly implicated as a controlling factor in apical dominance several of the other PGRs have been reported as showing activity in such systems. Phillips (1975) reported that exogenous gibberellin applied to the decapitated stump usually resulted in an enhancement of lateral bud outgrowth, but that the reverse was observed if

cytokinin was applied to the cut stump. Ethylene usually inhibits growth of both apical and lateral buds when applied continuously, and leads to outgrowth of axillary buds if applied briefly and then removed. These observations are interpreted as ethylene affecting auxin metabolism and transport, and subsequent disruption of auxin mediated processes of control (Phillips, 1975). Cytokinins are reported to break apical dominance and so lead to lateral bud outgrowth, in both intact and decapitated plants (Phillips, 1975; Tomar, 1985), these observations being consistent with the reported ability of both endogenous and exogenous cytokinins to give "sink" status to tissues in which their concentration is high (see 3.1.2). Abscisic acid (ABA) is reported to inhibit lateral bud outgrowth, due to a relatively high concentration in non growing lateral buds (Phillips, 1975).

Despite there being two major schools of thought on the control of apical dominance, it would not be unreasonable to suggest that the two are linked. The nutrition of the plant, while having an effect on development, must surely do so by changing the biochemical and/or physiological state of the plant. Put simply, sub-optimal (normal) nutrition enforces the plant to prioritise which bud is going to develop. However, the mechanism of such a choice must surely be through plant biochemistry and physiology, in which PGRs, while perhaps not exerting overall control, certainly have a role. Perhaps the differing PGRs, in their differing concentrations and ratios, control the sink status of the various competing buds.

In his 1975 review Phillips concluded that knowledge and understanding of the mechanisms by which apical dominance is controlled is poor. He called for more detailed kinetic data on cytohistological and PGR changes during the whole process of release of buds from apical dominance. As with the study of tropic curvature of grass coleoptiles as assessed by Firn and Digby (1980), it is apparent that at the time (1975) there had not been full characterisation of the process under study, and so it made assessment of methods and mechanisms of control of apical dominance all the more difficult.

2.2.2 Auxins and Root Growth

Auxins are known to be active in roots as well as in the aerial portions of the plant. Auxin was reported as being present in roots of numerous species, but Scott (1972) stopped short of suggesting that IAA was found universally in roots. Assessed as being worthy of note were the numerous mentions of tryptophan, the auxin precursor, in the roots of many species, and the fact that sterilised tryptophan could promote growth in wheat roots, suggesting that IAA biosynthesis could occur, using tryptophan as the precursor, in roots. Such synthesis was thought to occur in the root tip (Scott, 1972). It was considered at the time that the role of endogenous auxin was to promote growth. Exogenous IAA at varying concentrations had been found to significantly increase elongation rate of intact roots and isolated root segments, a stimulation which could be reversed by the addition of auxin antagonists. It was also reported that root growth would cease in the absence of auxin. It could therefore be summarised that auxin was an essential factor for root growth which also had a role in controlling growth rate. This was largely thought to occur via a change in auxin concentration. IAA thought to be synthesised in the root tip stimulated elongation by the greatest degree in the region immediately behind the tip. It was also noted however that root cells could vary in response to IAA with chronological age. (Although not recognised as such at the time this is clearly an example of changing tissue sensitivity, supporting the later thesis of Trewavas (1981, 1983). The concentration of PGR is not the sole factor in determination of cell elongation rate). Despite this observation the assumption that IAA concentration was in a linear relationship with root growth rate was adopted by Scott (1972) and the references therein, indicating how this concept was central to the thinking of plant physiologists at that time.

Prior to 1972 work had been directed towards an elucidation of a mechanism of primary action in shoot tissue, without the discovery of any single definite mechanism. The picture was largely the same in root research, with Scott (1972) concluding that the cell wall was the most likely site for primary auxin action.

As with shoot tissue (see e.g. Davies, 1973) a model had to be constructed which incorporated various possible factors within the time scale of the observed responses. Scott (1972) reviewed that auxin could evoke almost immediate responses in roots, and that auxin induced increased extensibility of cell walls could be detected within 15 minutes in the region where IAA was thought to promote elongation. Scott (1972) summarised by saying that the primary mechanism of auxin action remained obscure although it probably affected mechanical properties of the cell wall with possible involvment of the plasmalemma. This has obvious parallels with the model of auxin involvement in rapid shoot elongation as proposed by e.g. Hager, Menzel and Krauss (1971). Despite these parallels the differences in optimum IAA concentration for promotion of elongation in shoots and roots have been demonstrated as being widely different. For shoot tissue the optimum exogenous IAA concentration is in the region of 10μM (Evans, 1985), whereas this concentration is considered supra-optimal in roots of Pisum sativum (Evans, 1976a). Mulkey, Kuzmanoff and Evans (1982) reported that 1μM IAA is inhibitory to maize root elongation, with Evans (1976b) reporting that 10nM IAA was inhibitory to elongation of lentil (Lens culinaris) roots. Concentrations as low as 0.1nM IAA (Zea mays roots; Evans, Mulkey and Vesper, 1980) were apparently required for stimulation of root elongation by IAA. The use of ethylene biosynthesis inhibitors enabled Mulkey et al (1982) to detect stimulation of maize root elongation by 0.1nM - 10nM IAA, and by manipulation of pH Edwards and Scott (1977) were able to promote root growth of maize by 0.1μM IAA.

The subject was comprehensively reviewed by Evans (1985) who concluded that auxin stimulation of growth of intact roots had not been demonstrated regularly or consistently for any concentration of IAA. Evans (1985) suggested that there is a possibility that endogenous IAA concentration is supraoptimal, with partial support in that exogenous IAA can stimulate elongation of auxin depleted roots or root segments. However, these observations are also poorly reproducible and inconsistent. A major possibility is that endogenous auxin stimulates ethylene production, a compound inhibitory to root growth, and thereby indirectly negates its own growth stimulating ability (see Evans, 1985).

As mentioned previously the acid growth theory of auxin action in shoot growth became established in the early 1970s (Hager *et al*, 1971; Davies, 1973), and since then considerable evidence has accumulated that the acid induced growth system is also operative in roots. Evidence cited by Evans (1985) indicates that the acid efflux occurs from the elongating zones of intact roots, and that acidic solutions stimulate elongation in both excised root segments and intact roots. Also, compounds inducing proton efflux from roots are also stimulatory to growth (e.g. fusicoccin; Lado, De Michelis, Cerena and Marré, 1976), and changes in growth patterns are accompanied by comparable changes in proton efflux.

Evans (1985) also concluded that, generally speaking, concentrations of auxin which stimulate elongation also stimulate proton efflux, while inhibitory IAA concentrations inhibit proton efflux, these phenomena having been shown for both root segments and intact roots. As Evans (1985) freely admits, this data is all correlative and as such does not indicate a causal relationship between proton efflux and root elongation. On closer examination it would be rather hasty to assume a causal relationship as, for example, Lado *et al* (1976) concluded that although fusicoccin enhanced both elongation of excised segments of *Pisum*, *Phaseolus* and *Zea* roots and proton efflux from them, the degree of proton efflux from them could not theoretically account completely for the responses observed, according to their calculations. Despite such discrepancies, there is a close relationship in roots as in shoots, between the ability of auxins to stimulate elongation and correlative proton efflux, although the relationship may not be causal.

In this study, the elongation of both shoots and roots of cress (*Lepidium sativum*) was assessed in response to a wide range of bentazone concentrations, both alone and in all combinations with a range of IAA concentrations.

2.2.3 Auxins, Cell Elongation and H^+ Efflux in *Avena* Coleoptiles

Studies prior to 1940 established that exogenous auxin could cause elongation of excised plant tissue segments. The work was primarily concerned with the physiological mechanisms by which tropisms and growth patterns in plants were controlled, and was used to provide evidence for the hormone like role of auxin in plants. The hormonal theories are at present under criticism and review, as described in Chapter 1, but the observations that auxins can cause elongation in several systems are still valid. The mechanisms by which auxin could induce such elongation have generated much interest, and have received considerable experimental input over the last 30 years.

Early theories implicated changes in cell wall properties and protein synthesis as controlling elongation growth, and to attempt to correlate these to observed growth changes it was important to characterise the growth changes. Using etiolated *Avena* coleoptile segments it was found that, after exposure to IAA, a lag of around 10 min was normal before elongation rate increased (Ray and Ruesink, 1962; Evans and Ray, 1969), that the response was O_2 dependent within the first hour (Ray and Ruesink, 1962), that the lag period was temperature and KCN sensitive (Ray and Ruesink, 1962; Evans and Ray, 1969), and that the response was sensitive to various inhibitors of protein synthesis (Evans and Ray, 1969). Penny, Penny, Marshall and Hayes (1972) studied the elongation response of two dicot tissues to auxin, these being *Lupinus* hypocotyl and *Pisum* stem segments. They detected a lag of 15-20 min, and characterised the elongation rate as reaching a maximum at around 30 min (supported by Dela Fuente and Leopold, 1970), which slowed at 45 min and then increased to a second high rate, sustained from 70 min until the end of the experiment at 90 min (Penny *et al*, 1972). An interpretation of the dip in rate at 45 min was that the elongation response to auxin consisted of 2 independent effects (Penny *et al*, 1972).

Clearly there must be changes in cell wall properties in order to facilitate cell expansion. Bonner (1934) reported such

wall loosening of *Avena* coleoptiles in response to auxin, which was subsequently demonstrated by Cleland (1965) in the presence of actinomycin D, an inhibitor of RNA synthesis. Cleland (1967a) reported that cell turgor drove expansion within the loosened wall. IAA was demonstrated to loosen cell walls only in living tissue (Cleland, 1967b). This evidence clearly indicated that IAA itself was not the wall loosening factor, rather that it acted on a preformed system in metabolically active tissue. Meanwhile David Rayle had been reinvestigating the phenomenon earlier reported by Bonner (1934) that low pH could loosen the cell wall. Rayle, Haughton and Cleland (1970) reported that frozen-thawed *Avena* coleoptiles responded to low pH by cell wall loosening, with a reported pH optimum of 3.0-3.6. In a later publication (Rayle, 1973) it was reported that an error had been made in establishing the pH optimum at 3.0-3.6. The authors had failed to recognise the impermeable nature of the coleoptile cuticle to protons, and the pH optimum in peeled tissue was nearer to pH 5.0 (Rayle, 1973). This revised pH value was much more relevant physiologically. Rayle and Cleland (1970) further characterised the acid and auxin responses of *Avena* coleoptile cell elongation and wall loosening. The optimum pH and IAA concentration for the response were found to produce very similar maximum wall extensibilty and growth rate. Both Rayle and Cleland (1970) and Hager *et al* (1971) independently suggested that auxin induced a rise in cell wall proton concentration which, either directly or via an enzyme system, caused cell wall loosening. Hager *et al* (1971) proposed that the mechanism by which this occurred was an auxin activation of a plasmalemma bound, ATP requiring, electrogenic proton pump. In order to substantiate the new theory it was necessary to demonstrate that IAA caused cells to excrete protons with suitable kinetics to match the growth responses.

Cleland (1973) reported that peeled *Avena* coleoptile segments, *Pisum* epicotyl and *Helianthus* hypocotyl sections lowered the pH of their bathing medium in response to exogenous IAA, but data was presented only for *Avena* coleoptiles. Following a lag of 20 min medium acidification due to auxin continued for 150 min. The kinetics of the response correlated well with the elongation

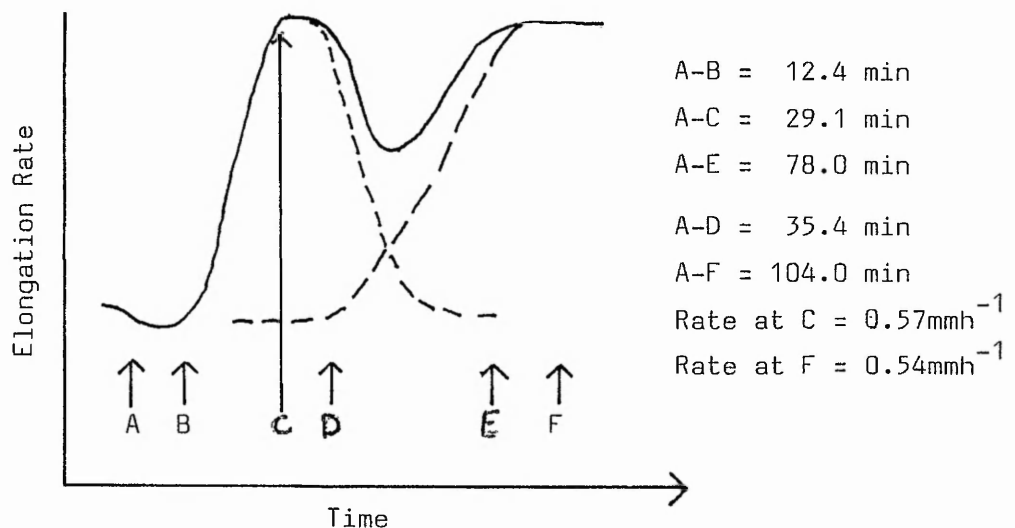
response (Cleland, 1973). Rayle (1973) further characterised the proton efflux response to auxin. (He also corrected the misrepresentation of optimum pH as described earlier). It was reported that only growth active auxins caused proton efflux, that such efflux was calculated to be sufficient to lower cell wall pH by the required degree, and that efflux stopped about 3h after application of IAA (Rayle, 1973). Marré, Rasi-Caldogno and Columbo (1973), using Pisum internode segments, and Rayle (1973), with peeled Avena coleoptile segments, both reported that inhibitors of auxin induced elongation (including dinitrophenol, ABA, cycloheximide, CCCP) also inhibited proton efflux. This led Rayle (1973) and Cleland (1973) to propose that a part of the elongation response to auxin could involve proton efflux.

Despite the close correlations between auxin induced proton efflux and elongation, all the data was for short term responses (<3h). It was therefore difficult to conceive that auxin induced proton efflux was responsible for long term auxin enhanced growth. Other ongoing work in the 1960s had implicated auxin induced RNA biosynthesis in coleoptile cells. Masuda, Tanimoto and Wada (1967) measured a stimulation by auxin of radiolabelled uracil incorporation into coleoptile cell RNA, detectable with a lag of 20 min. Masuda and Kamisaka (1969) reported this phenomenon in 10 min and suggested, as was widely held at the time, that RNA synthesis was required even for early auxin action. The acid growth workers (Rayle, Cleland and others) considered that it was not.

Davies (1973) reviewed the then current theories on mode of action of auxin. Among the important conclusions from this were that stimulation of protein synthesis probably occurred too slowly and as such was probably not required for early auxin stimulation of growth. On the other hand, the acid effect was considered sufficiently rapid, but lacking in duration (usually recorded at 45-90 min) to support sustained growth. Davies (1973) supported the ideas of Hager et al (1971), that auxin action at a plasmalemmal ATPase was likely, that acid growth could account for early growth promotion, but that continued growth was dependent upon enhanced RNA and protein syntheses,

which were required to maintain cell wall structure and function. The biochemical mechanisms by which these processes occurred had yet to be elucidated, but the hypothesis that the auxin induced elongation response consisted of two phases provided the structure for further work.

Vanderhoef, Stahl, Siegel and Zeigler (1973) showed that cytokinin inhibited the second, but not the first, response to auxin in soybean hypocotyl. Low pH mimicked the first of these two seperable responses (Vanderhoef et al, 1973). Vanderhoef, Stahl, Brinkmann and Greenfield (1976) provided further evidence that the two responses were separable by use of the auxin analogue 4-azido-2-chloro-phenoxyacetic acid. This compound increased the lag time of the second response but did not affect the first, so that the two phases became temporally separated. From their earlier work (Vanderhoef et al, 1973) and this (Vanderhoef et al, 1976) the following model of auxin response was formulated:-



Characterisation of the biphasic response to auxin of Soybean hypocotyls. From Vanderhoef et al, 1976.

Pearce and Penny (1983) suggested an alternative explanation for the biphasic nature of the auxin response, implicating tissue interactions. They suggested that in Lupinus hypocotyls the first phase was due to epidermal relaxation, followed by cortical control of the second phase. A major observation was that removal of the epidermis inhibited the second phase (Pearce and Penny, 1983). Evans (1985) concluded that the exact nature of the biphasic growth response remained unknown.

Since the early 1970s the acid growth theory has dominated theories on rapid auxin action. However, to retain credibility all theories must stand up to criticism, and the acid growth theory is no exception. Some important supportive evidence for the acid growth theory is that proton efflux and cell elongation are suitably correlated in time (Cleland, 1973; Rayle, 1973), but these observations were made on different tissue samples on different occasions. Developments in technique have subsequently enabled both elongation and proton efflux to be measured simultaneously on the same tissue sample. By such a technique Penny, Dunlop, Perley and Penny (1975), and Kutschera and Schopfer (1985) reported that proton efflux and elongation are not suitably correlated temporally, that elongation begins prior to any detectable pH drop (Penny et al, 1975), and that responses of these processes to other exogenous factors e.g. cations do not correlate in support of the acid growth theory (Kutschera and Schopfer, 1985). Pope (1977), employing various pH values as pre-treatments to IAA or acid exposure, demonstrated that IAA induced and acid induced elongations were separable and therefore occurred independently. He concluded that IAA does not stimulate the extension growth by the same mechanism as acid (Pope, 1977). Pope (1978) subsequently demonstrated that the IAA induced elongation could occur even at low pH (pH 3.4), suggesting that the action of IAA in causing proton efflux is not that which is responsible for growth. Kutschera and Schopfer (1985) experimentally investigated other predictions which could be tested for relevance to the acid growth theory. They demonstrated that pH 4.5-5.0 (that pH thought to occur endogenously in the cell wall) was insufficient to induce elongation. The concentration of fusicoccin, a fungal

toxin with known proton pumping activity, which pumped the equivalent number of protons to optimal IAA concentration, had no effect on elongation. Neutral or alkali buffers infiltrated into the cell wall had no effect on auxin induced elongation (Kutschera and Schopfer, 1985). It was demonstrated therefore, that although IAA could cause proton efflux, it was not this aspect of its activity which was responsible for increasing cell elongation.

Criticism was also levelled at the methodology used in experiments which had established the acid growth theory. Pope (1982) questioned the value of studies using peeled coleoptile segments. Peeling inhibited the IAA elongation proportionally to the degree of peeling (Pope, 1982). This criticism is justified in the light of work purporting to show that the IAA receptor pretein is located in outer epidermal cells (Lobler and Klambt, 1985 a and b). Brummell and Hall (1987) reviewed the evidence against a link between acid extrusion and elongation. Additional evidence cited includes the observation that there is a lack of correlation between degree of acidification and magnitude of elongation response. Tissues which show no elongation response to IAA are capable of auxin induced proton efflux, and tissues from which the auxin sensitive cells have been all but removed also excrete protons in response to IAA (Brummell and Hall, 1987). An experimental difficulty high-lighted by those authors is that accurate assessment of pH within the cell wall is technically difficult, and that cell wall pH can only be estimated by inference. A further problem exists in relating all the contributory data, mostly obtained from tissue segment experiments. Brummell and Hall (1987) suggest that, although proton efflux may be observed as a response to IAA, it is possible that experimental conditions of imposed external pH, employed in typical experiments, may have produced proton efflux artefactually as the tissue segments attempted to reach an equilibrium situation (Brummell and Hall, 1987).

The acid growth theory has provided a useful model for experimentation into effect of auxin upon cell elongation, but there now exists a considerable body of evidence which is apparently irreconcilable with it. It now seems that although proton efflux may be required for elongation to occur, it is probably not responsible nor regulatory for growth in the intact plant. Proton efflux may simply be an effect of auxin occurring in parallel to elongation but having no regulatory function.

The construction of a model for auxin action in causing cell elongation by Hager et al, (1971), and its endorsement by Davies (1973), strongly implicated an electrogenic PM associated proton translocating ATPase. Possible other mechanisms of auxin induced proton efflux were reviewed a decade later (Cleland, 1982). Having considered the available evidence Cleland (1982) also concluded in favour of such a model for the following reasons:-

1. Auxin causes hyperpolarisation of the membrane with a lag of 5-10 min.
2. Auxin decreases the ATP:ADP ratio, and
3. Increases respiration, both within 5 min.
4. Specific inhibitors of PM ATPases (DCCD and vanadate) both inhibit auxin induced proton efflux.
5. A system as proposed would exhibit sensitivity to external pH; this is demonstrated to occur in practice.
6. Auxin binding sites have been found on the plasmalemma of cells in several responsive tissues.

For a full description of other possible mechanisms of proton efflux, and discussions of their suitability in view of the available evidence, the reader is referred to Cleland (1982).

Cleland (1982) discussed whether auxin directly interacts with the ATPase. The in vitro work in this area has proved inconsistent and confusing, with small responses over a limited IAA concentration range, and auxin had not been demonstrated to directly activate a PM-ATPase (Cleland, 1982), but technical difficulties in this work are many.

The conclusions of Cleland (1982) were these:-

1. Auxin induced proton efflux is mediated by a vanadate-sensitive and therefore, presumably, PM-associated ATPase.
2. The process does not require any specific counter-ion, and may well be electrogenic.
3. Auxin action does not seem to involve PM electron transport, or a vesicle-mediated proton efflux mechanism.
4. Auxin does not apparently directly activate an ATPase.
5. Protein synthesis is required for proton efflux, probably the process rather than any specific protein.

Venis (1985) concluded similarly; binding of auxin to proteins of maize membranes is separable from ATPase activity. However, several pieces of both correlative and direct evidence suggest that the binding proteins do have a receptor function.

1. Benzoxazolinines similarly interact with binding site events and auxin induced growth.
2. Auxin binding and auxin induced elongation decrease in parallel along the length of maize coleoptile mesocotyl.
3. Growing coleoptiles exhibit good correlation between degree of auxin binding and responsiveness.
4. Apical *Avena* coleoptile segments, which are more auxin sensitive than basal sections, also exhibit more binding.
5. Maize coleoptile sections show the reverse; basal sections are more responsive and also bind more auxin.

This evidence is all correlative, but Venis (1985) cited two pieces of direct evidence to support the plasmalemma mediated binding site and proton efflux theory of auxin action. The first is that, in a membrane reconstruction experiment, the three elements of binding protein, ATP and auxin were all essential to elicit an electrochemical response resulting in transmembrane current. Secondly, experiments using immunological techniques produced an antiserum, directed against the membrane associated binding protein of maize coleoptiles, which blocked auxin binding and auxin induced elongation growth. The

auxin "receptor" was found to be located at the PM of the outer eidermal cells of the coleoptiles (Lobler and Klambt, 1985a and b; and in Venis, 1985).

Despite this evidence that auxin does indeed bind at the plasmalemma, and that this protein apparently has receptor function, it seems likely that the binding protein is not an ATPase proton pump. This implies a second messenger system linking the binding event to the measurable response. If it was known what the initial event was following binding this could become the measurable response. However the function of the binding event must also be known. If we suggest that it is to cause proton efflux then events between binding and response will become the "second messenger" events and assume that function.

Plant physiologists, as described earlier in this section, historically attached themselves too uncritically to the hormonal models and theories emanating from from research into mammalian hormones. The main tenets of these models were discrete site of synthesis, transport to sensitive tissues, where a change in hormone concentration, and therefore a change in hormone/receptor interactions, would induce a modification of physiological activity of the receptor tissue. These principles, as discussed in Chapter 1, which have recently received considerable criticism as to their applicability in plant systems, all deal with pre-binding events. Post binding events in mammalian systems were also well characterised and the findings also found their way into the thinking of plant physiologists. The justification for the consideration of parallels of post binding events in plants will now be addressed.

Venis (1985) has briefly reviewed the field of secondary messengers in plants. By 1970 cyclic AMP (cAMP) had been identified as a second messenger in many peptide and catecholamine mediated systems in mammals, and had also been implicated in gene regulation in bacteria. In rather the same style in which plant physiologists adopted the hormonal models from animal systems, it was attempted to find cAMP in plants. Early

work purported to show an effect of hormonal stimuli on cAMP in synthesis or degradation, or to show exogenous cAMP inducing a response normally mediated by the "hormone". This early work suffered from several deficiencies, which when identified led to a decline in interest in plant cAMP. These were that cAMP was uncritically identified, and that other endogenous compounds were shown to interfere in cAMP identification assays (Venis, 1985). New identification techniques including GCMS gave good characterisation of cAMP in plants, at concentrations comparable with animal cAMP, but by 1985 no firm evidence of cAMP as a second messenger had been achieved (Venis, 1985).

A further group of compounds implicated in physiological responses, including those induced by PGRs, is the polyamines. It is clear that polyamines can modify many regulatory processes in plants, but direct evidence of their link from stimulation to response as second messengers had not yet (by early 1985) been provided (Venis, 1985).

There is now increasing evidence implicating calcium (Ca^{2+}) as a second messenger involved in a plethora of stimulus/response coupling in plants. A number of recent articles have appeared describing this evidence and its implications. Here again plant physiology has borrowed a concept from the equivalent animal research field.

Animal cells have an internal Ca^{2+} concentration of approx 0.1µM, which is maintained at this low concentration by an active Ca^{2+} extrusion. Receipt of a suitable stimulus disrupts the Ca^{2+} extrusion leading to a 10-100 fold rise in internal concentration. This rapidly amplifies the initial signal, and the additional internal Ca^{2+} binds with calmodulin, a Ca^{2+} activated protein. The Ca^{2+}/calmodulin complex in turn binds to other intracellular proteins, thereby modifying activity of several enzymes and alters physiology (see Venis, 1985). Brummell and Hall (1987) were tempted to speculate that there exists an analagous system in plants despite the lack of direct evidence. One of the major drawbacks are the technical problems of measurement of cytoplasmic and cell wall Ca^{2+} concentrations,

measurements which are central to the advancement of the theory. There are however several compelling pieces of cicumstantial evidence that a similar system exists in plants. Calmodulin has been found in all plants in which it has been looked for, and several Ca-calmodulin complex regulated enzymes have been identified (Dieter, 1984). Suggested as a second messenger in PGR systems, Ca^{2+} can produce similar effects to IAA in the abscence of IAA, including assimilation of radiolabelled precursors into the cell wall, the stimulation of β-glucan synthase, and a stimulation of proton extrusion (Brummell and Hall, 1987). These authors also reported that experiments in which either Ca^{2+} -calmodulin binding, or calmodulin-enzyme interaction, is blocked, reduce the response to exogenous IAA, thereby implicating a Ca^{2+} -calmodulin system as some form of second messenger (Brummell and Hall, 1987). The interest in calmodulin as a second messenger system in plants is a relativly new phenomenon, and there are several fundamental and important areas into which future work will be directed. Accurate measurements of cytosolic and cell wall Ca^{2+} concentrations must be made, and changes, if any, on receipt of a stimulus must be carefully monitored. Possible Ca^{2+}-calmodulin interactions must be investigated. The response elements which bind Ca-calmodulin complex (if found) must be identified as effectors of a response. The mechanisms by which a cell modifies the Ca^{2+} signal in order to maintain a response must be characterised. The differences between metabolism in adjacent Ca^{2+} sensitive and Ca^{2+} in-sensitive cells must be characterised. Despite the infancy of the concept of Ca^{2+} as a key regulatory metabolite in plant systems the research community is predicting great things for it. Hepler and Wayne (1985) stated that, "it seems evident that Ca^{2+} will be found to participate as a second messenger", while Venis (1985) concluded, "it will be surprising if connections (of Ca^{2+}) with hormone action are not more firmly established." Such attitudes resound with those of some 50 years ago relating to the concept of plant "hormones". With these enthusiasms it is quite plausible that the historic mistakes will be repeated. Experiments will be subjectively designed, performed and analysed in order to support the popular theories of the day. Obviously the desired route is to thoughtfully and carefully design objective experiments, and to formulate theories in light of results obtained.

As discussed earlier, the acid growth theory states that cell elongation occurs initially as a consequence of turgor driven cell expansion within a loosened cell wall. Such cell wall loosening is considered a result of reduced pH caused by increase in proton concentration within the cell wall, the protons having been pumped across the plasmalemma from the cytoplasm. It is widely held that such proton pumping is stimulated by auxins via a membrane bound, ATP requiring proton pump, and that it is via this mechanism that auxins stimulate early cell and tissue elongation (detectable within 15 min). There are however those who suggest that auxin does not act by this mechanism, rather that increased elongation and proton pumping are simultaneous but independent responses to exogenous auxin. Despite this argument, proton extrusion by some plant cells is stimulated by auxin, and it represents a response one step closer to the primary action of auxin than does stimulation of elongation. It should therefore be advantageous to study proton efflux rather than elongation in order to gain knowledge of the characteristics of an early auxin mediated response, and this was a logical experiment to perform.

Proton efflux can conveniently be detected as a reduction in pH of an aqueous bathing medium, but for detection of such pH changes to be successful several aspects of the experimental procedure must receive careful attention.

For protons to be detected as a pH change they must escape the tissue and enter the bathing medium. For this to occur any barriers to proton movement must be removed, which in this instance necessitated the partial removal of the proton impermeable cuticle by gentle abrasion with a carborundum slurry. Secondly, it is important that excreted protons should cause a detectable reduction in pH of the bathing medium. This is achieved by use of a medium of low buffering capacity and a high tissue:volume ratio. It must also be established that only excreted protons should contribute to pH change. A possible cause of pH drop other than excreted protons is the increase in concentration of respiratory CO_2, which results in formation of carbonic acid and subsequent pH drop. To overcome this the medium should be aerated to maintain constant CO_2 concentration. Bearing these points in mind the protocol described in 2.3.4 was employed.

2.3 Materials and Methods

2.3.1 Apical Development in *Phaseolus vulgaris*

Dwarf French Bean (*Phaseolus vulgaris* L., cv. Masterpiece) seeds were sown, 2 to a pot, in 12.5 cm diameter pots, in J. Arthur Bowers potting compost, and grown on under 16h photoperiod, 27.5±2.5°C day, 16.5±1.5°C night, for 13-14 days. At this age the plants had produced 2 expanded leaves and about 15mm shoot above the node (Figure 2.1).

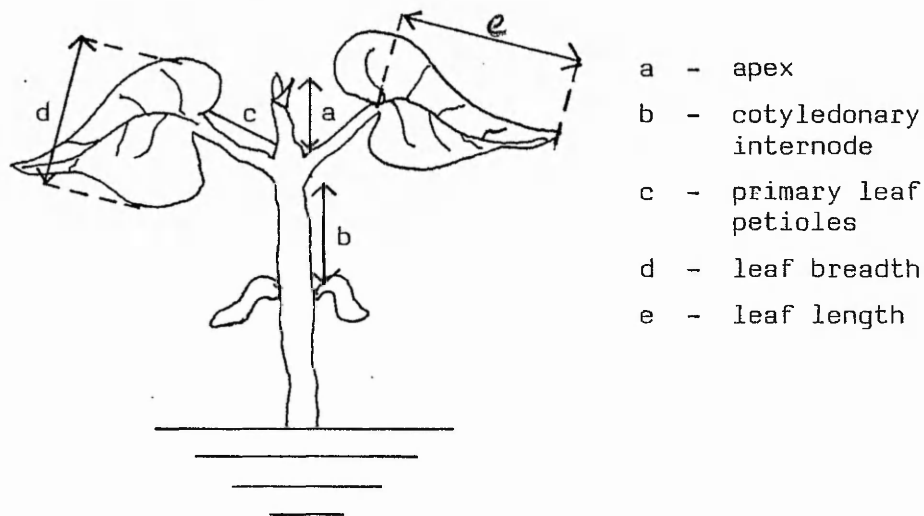


Figure 2.1 Aerial portion of *Phaseolus vulgaris* cv. Masterpiece after 13-14d under the growing conditions described.

At this time the shoot apices were decapitated with a sharp blade, Approx 5mm above the first node, and the appropriate PGR pastes, alone or in combination (see Chapter 2.4.1), were applied to the cut stump in prepared lanolin based formulations. For each treatment 10 plants were selected, and measurements were made and recorded of length of cotyledonary internode, primary leaf petioles, length and breadth of the primary leaves, and when appropriate, of the length of the dominant lateral upon

its expansion. Measurements were made at 2 day intervals until day 12, the plants having developed under the conditions above. Results are subsequently expressed as difference between treated and control plants measured as percentage of day-zero values

i.e. $\Delta\% = [\%d0 \text{ treated}] - [\%d0 \text{ control}]$

Comparison of results was made using Student's t-test.

Preparation of Lanolin Pastes:- Auxin (IAA) and bentazone* were initially dissolved in acetone and made up to give final concentrations of 1.5mM in 5mM K_2HPO_4/KH_2PO_4 buffer pH6.0 (1% aq v/v acetone) from which serial dilutions were prepared. Such aqueous solutions of IAA and bentazone were mixed 1 part to 2 parts (by weight) anhydrous lanolin (BDH) to give the concentrations stated in the resulting pastes.

*Bentazone used throughout this study was a 95% pure sample, a gift to the laboratory by BASF (FRG).

2.3.2 Cress Seed Germination Experiment

Approx 400 *Lepidium sativum* (cress) seeds were germinated in a Petri dish, between two Whatman No. 1 9cm diameter filter papers moistened with 5ml distilled water, for 24h at 25°C in darkness. After this time the testa of many seeds had split, indicating that germination had commenced, and fifteen of such seeds were placed in each experimental Petri dish containing a Whatman No.1 9cm filter paper moistened with various combinations of IAA and bentazone in 5mM K_2HPO_4/KH_2PO_4 buffer pH 6.0. IAA and bentazone stock solutions were prepared by dissolving the required quantity of IAA or bentazone in acetone (final concentration 0.1%), adjusting pH with dilute NaOH, and making volume up with 5mM phosphate buffer. For details of individual experimental treatments see results (2.4.2). The Petri dishes were incubated for 5d at 25°C in darkness, after which the lengths of shoots and roots were measured and mean length of each calculated. The mean lengths were compared to controls at 5d using Student's t-test. The experiment was repeated on nine occasions, with each treatment replicated at least three times, i.e. $n \geqslant 45$.

2.3.3 Avena sativa Coleoptile Elongation

Spring oat (Avena sativa cv. Maris Tabard) seeds were germinated on tap watered vermiculite in darkness at 25°C, for 70-75h. Coleoptiles were then prepared as follows. Segments (1cm) (approx 150) were cut from decapitated coleoptiles (0.5mm from the tip), in dim green light, and were stored in darkness in a covered Petri dish, this taking approx 30 min. The population of segments was sub-divided into groups of 15, in dim green light, and were pre-incubated in 5mM K_2HPO_4 /KH_2PO_4 buffer pH 6.0, at 25°C, in darkness for 45 min. Pre-incubation medium was strained off and segments were transferred to the appropriate incubation medium (IAA and/or bentazone and/or other compounds, in phosphate buffer, see 2.4.3 for details). Incubation followed, lasting 5-60 min, in 5mM K_2HPO_4/KH_2PO_4 buffer pH 6.0, at 25°C, in darkness. The incubation medium was strained off, and the lengths of the segments were measured using a shadowgraphic technique which gave a magnification factor of 5. Mean length of coleoptiles was calculated, and lengths compared using Student's t-test. Each experimental treatment was repeated on at least three occasions, giving $n \geqslant 45$.

Several of the compounds required to be dissolved prior to incorporation into aqueous medium. The solvents used were as follows

Acetone	Dilute NaOH	K-Phosphate Buffer
IAA	BAP	Ethrel C (Liquid Preparation)
Bentazone	Kinetin	
GA3		
GA4/7		
ABA		
DES		
NaVO4		

If required, adjustments of pH were made using dilute HCl and NaOH. Final concentrations of solvents were $\leqslant$ 0.1% aq. v/v.

2.3.4 Proton Efflux from *Avena* Coleoptile Segments

Spring oat (*Avena sativa* cv. Maris Tabard) were sown in tap watered vermiculite, and germinated in darkness at 25$\pm$1°C under high humidity, for 88-94h. All subsequent operations were performed in normal laboratory light. Approx. 150 seedlings were individually harvested, lightly abraded between thumb and forefinger with a carborundum slurry (800-grit silicon carbide powder in distilled water), and stored and washed in distilled water. From each coleoptile, commencing 5mm from the tip, a 15mm segment was cut, then placed in c.50mls 0.3mM MES/TRIS buffer pH 6.2-6.3, and washed by vigourous aeration for 20-30 minutes. Seventeen segments were placed in each of 8 small polythene vials containing 1700µl 0.3mM MES/TRIS + 1mM KCl, pH 6.2-6.3, 25$\pm$1°C. Aeration commenced (50cm^3/vial min^{-1}, normal laboratory air) and a pre-incubation of 30-40 minutes was administered. The following procedure was then applied in turn to each vial. Approx. 1500µl of bathing medium was removed, using a Pasteur pippette, and was placed in the cuvette of a water jacketted (25$\pm$1°C) O_2- electrode. The appropriate PGR additions were made (total final volume 1700µl), and the pH was recorded to 2 decimal places (Corning 140 pH meter, Russell semi-micro pH electrode), and the solution was returned to the incubation vial. pH was similarly recorded for each vial sequentially at 15, 30, 45, 60, 90, 120 and 150 minutes. Over the pH range 6.3-5.5 the buffering capacity was linear, permitting direct assessment of change in proton concentration. Outside this pH range calculations of change in proton concentration were made, permitting a plot of pH as if buffering capacity had remained linear. Each experimental treatment was repeated on at least three separate occasions, and mean ΔpH for each time value was calculated. Such "mean curves" of ΔpH with time were used to calculate rate of change of pH and all other calculations.

The experimental apparatus is illustrated in Figure 2.3.

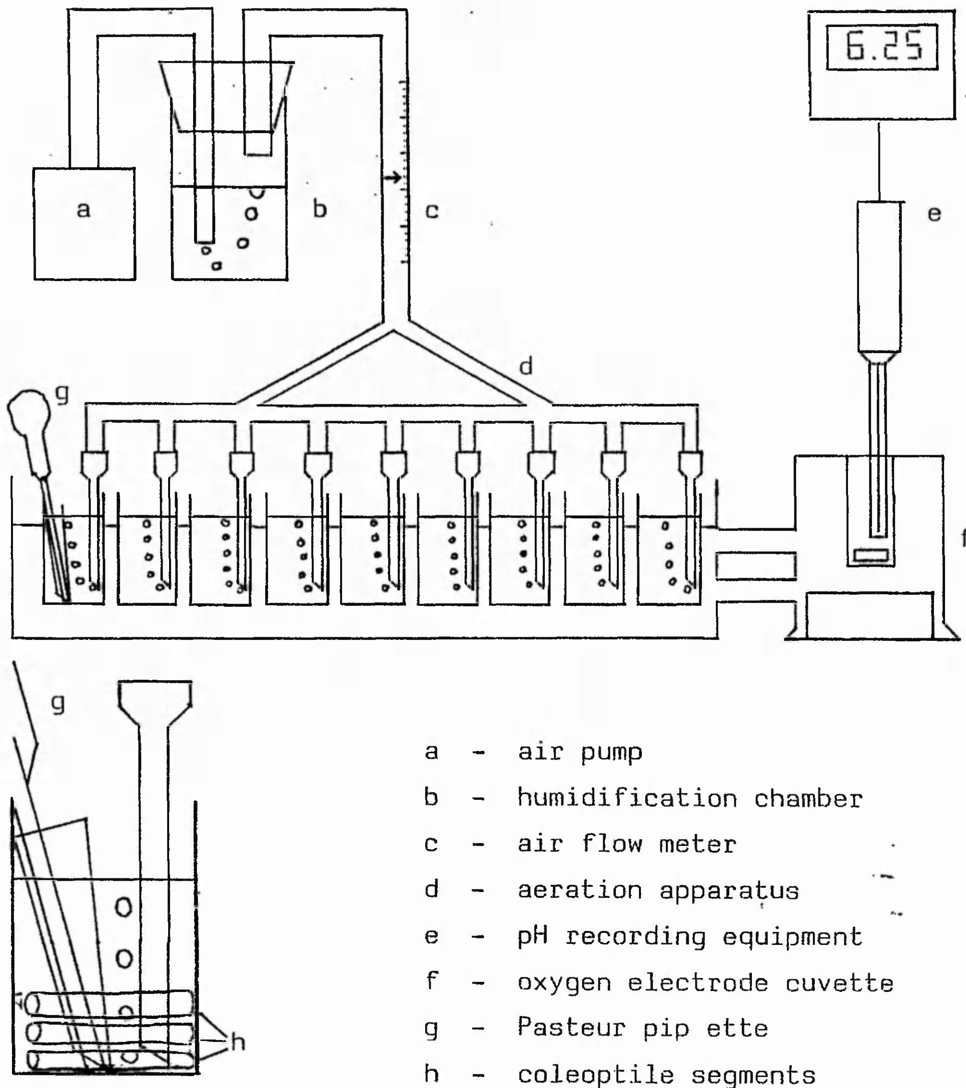


Figure 2.3 Experimental apparatus for measurement of medium acidification by *Avena* coleoptile segments.

2.4 Results

2.4.1 Apical Development in *Phaseolus vulgaris*

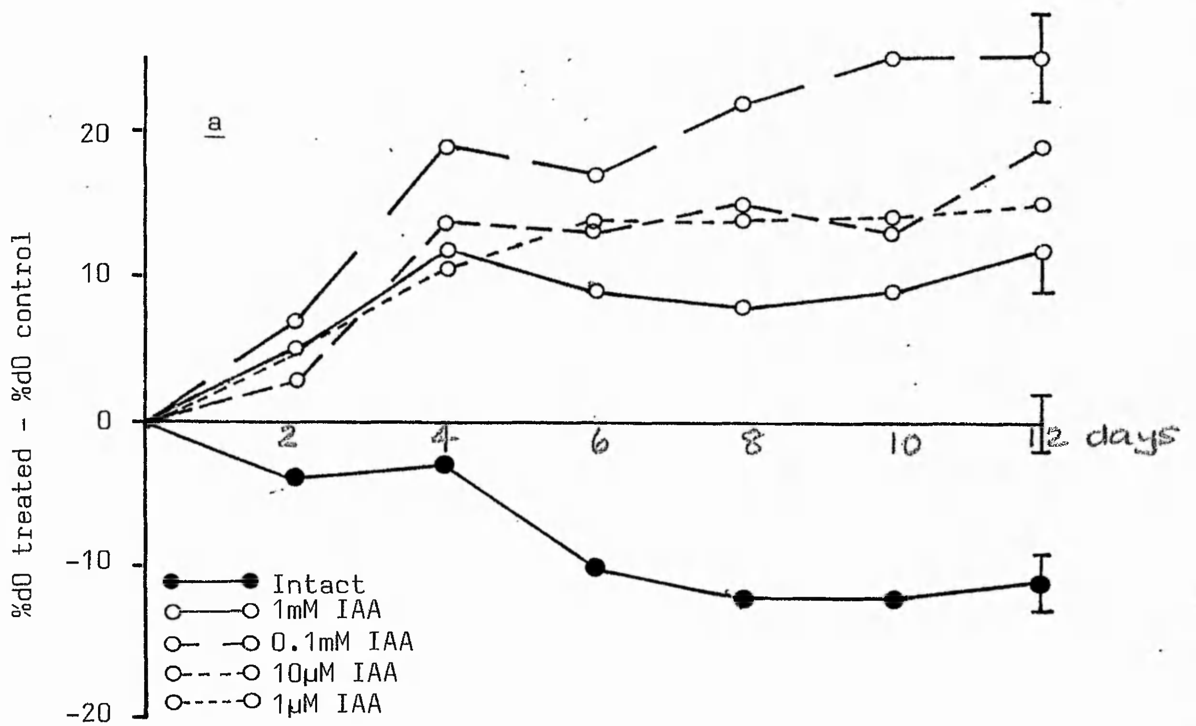
No differences were observed in leaf dimensions between control and any other treatment, or between appropriate comparative treatments, at any stage of the experiment, i.e. neither IAA nor bentazone nor any combination of the two had any effect on leaf size.

The data obtained for petioles is presented in Figure 2.4, which shows that apical intactness was inhibitory to subsequent petiole elongation, until about 8 days after treatment (d.a.t.). The IAA concentrations used herein all stimulated petiole elongation, until a maximum difference had been reached at around d10 (Figure 2.4a). Bentazone at 0.1mM and 1mM had a similar effect to IAA (Figure 2.4b), 1μM bentazone had no effect, but 10μM bentazone inhibited petiole outgrowth compared to control plants in a manner similar to, but smaller in magnitude than, apical intactness.

(d = day)

There was marked interaction in petiole elongation between IAA and bentazone. IAA and bentazone both at 1mM caused a modest increase in petiole length, but when applied together this was translated into a stimulation of almost twice the magnitude (Figure 2.5a). The effect of 1mM bentazone on the other concentrations was the reverse, the petiole growth in combination treatments being almost the same as that of 1mM bentazone with 0.1mM IAA (Figure 2.5b), and the compounds proving mutually antagonistic with both 10μM IAA or 1μM IAA combined with 1mM (Figure 2.5c and d), when the resultant growth was virtually as control plants.

Bentazone at 0.1mM with IAA proved mutually antagonistic to petiole elongation (Figure 2.6) except with 1mM IAA in which instance the effect of the combination was as that for IAA alone (Figure 2.6a).



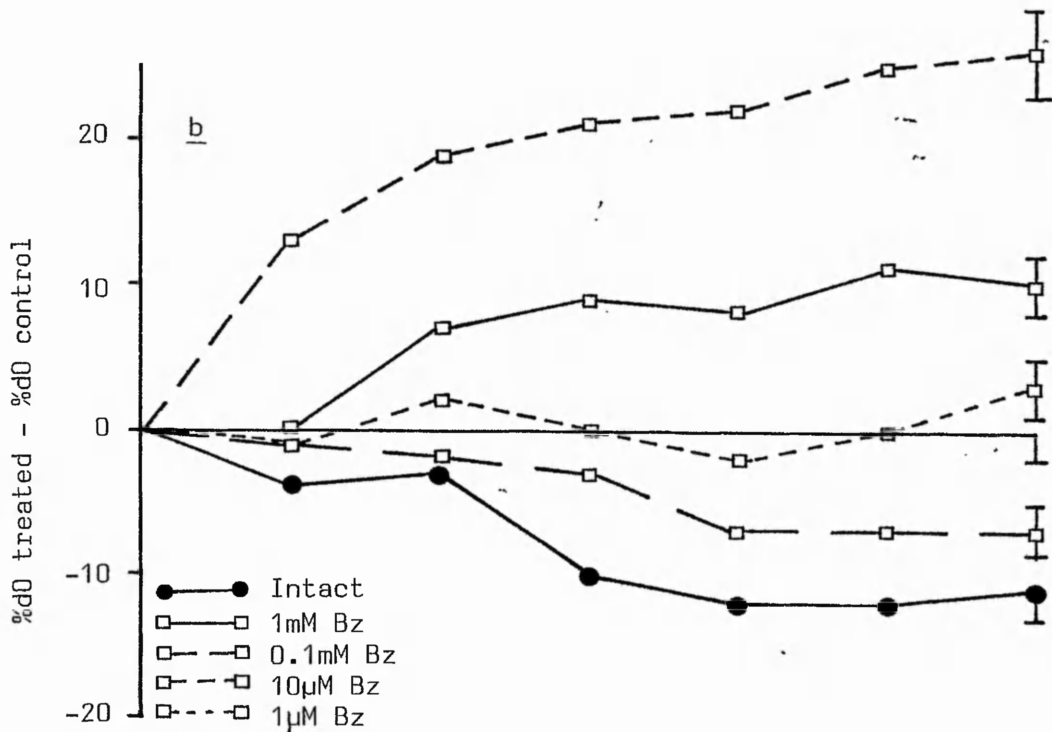


Figure 2.4 Effect of IAA (a) and bentazone (b) on primary leaf petiole growth of *Phaseolus vulgaris*. n=10 plants (20 petioles), 25°C, 16h photoperiod.

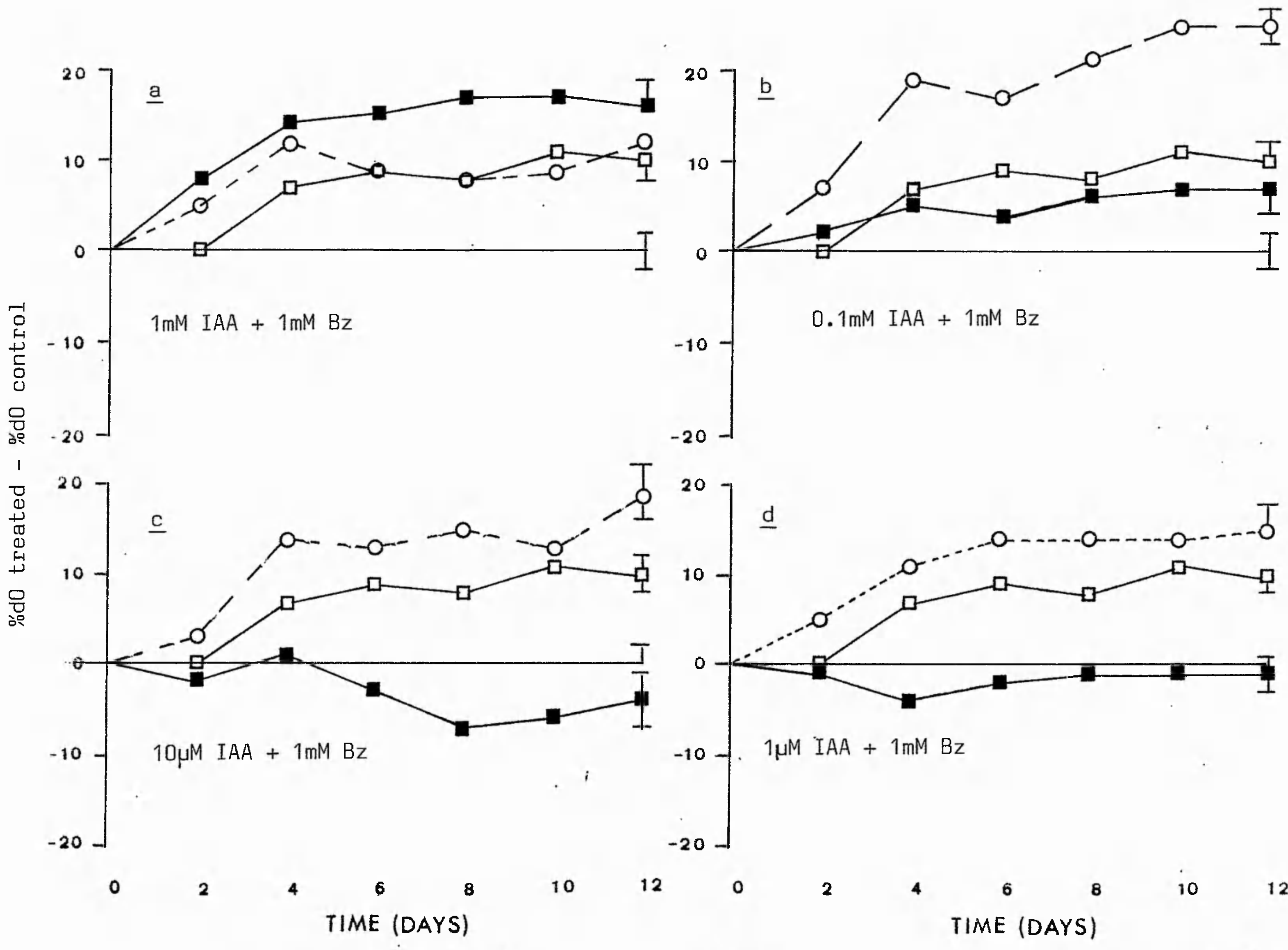
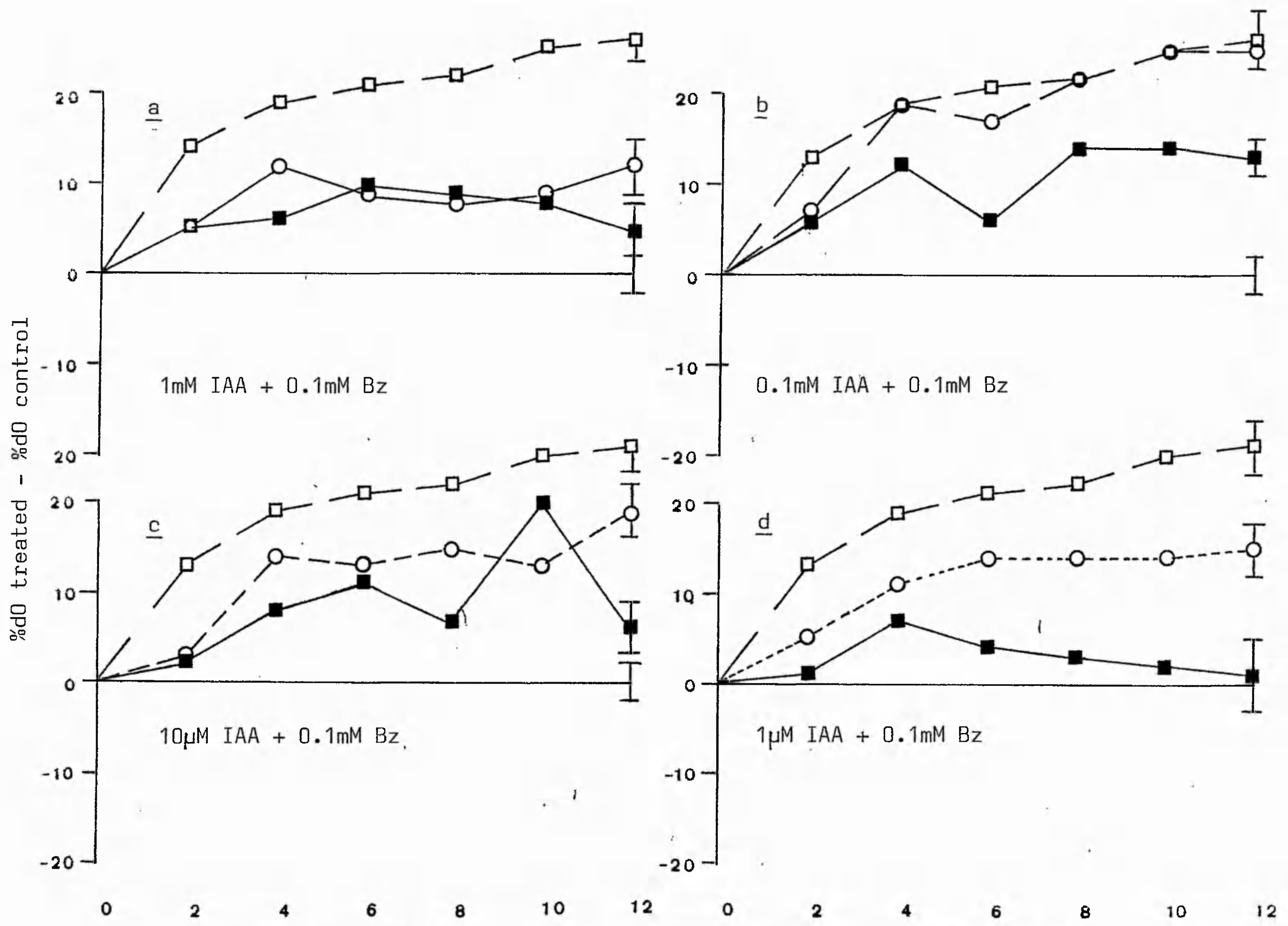


Figure 2.5 Effects of IAA (○--○) and bentazone (□—□) and IAA/bentazone combinations (■—■) on petiole growth of 13d old, decapitated *Phaseolus vulgaris*. n=10, 16h photoperiod.



<u>Figure 2.6</u> Effects of IAA (○—○), bentazone (□—□) and IAA/bentazone combinations (■—■) on petiole growth of 13d old, decapitated *Phaseolus vulgaris*. n=10, 16h photoperiod.

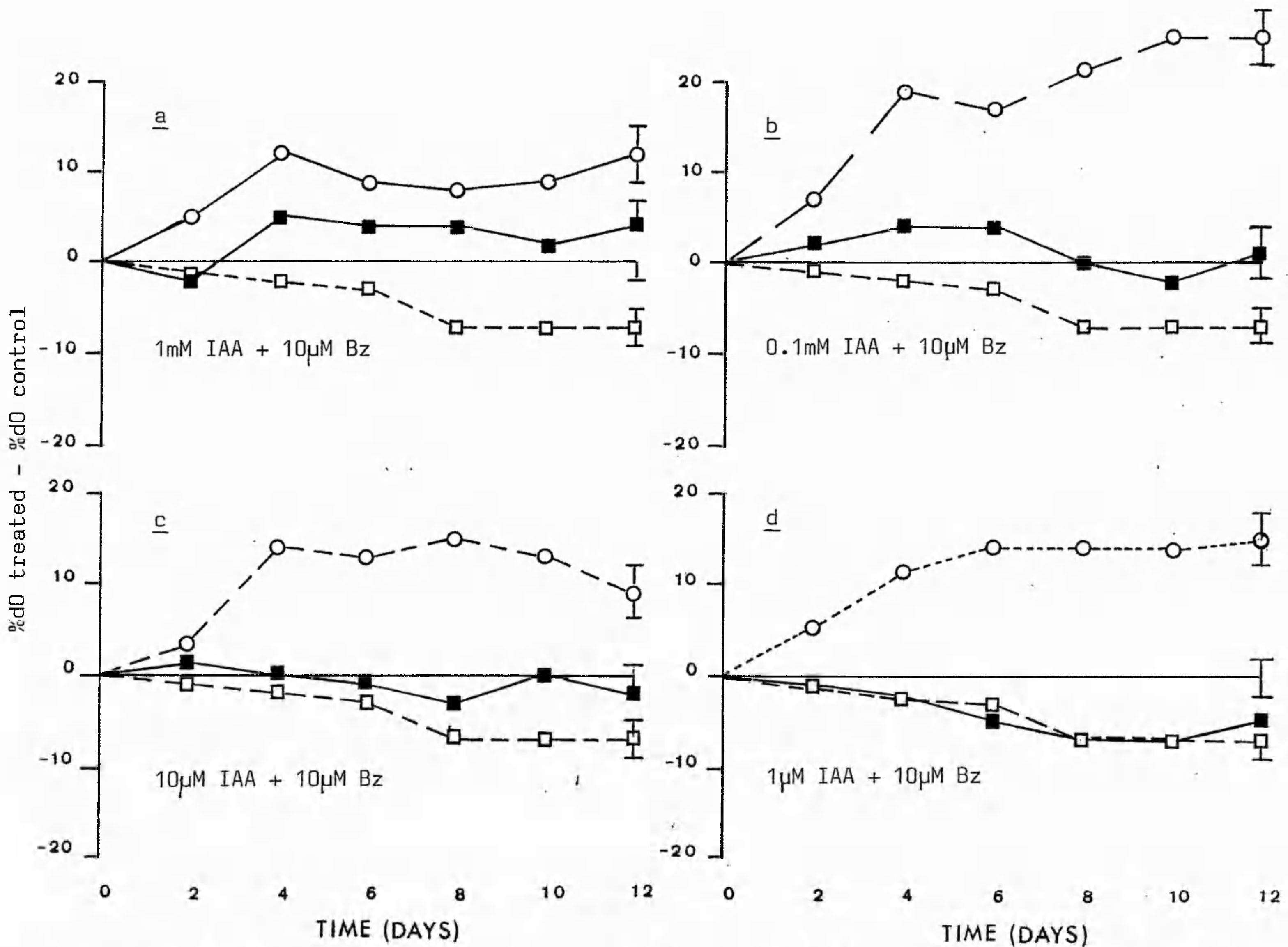


Figure 2.7 Effects of IAA (○--○) and bentazone (□—□) and IAA/bentazone combinations (■—■) on petiole growth of 13d old Phaseolus vulgaris. n= 10, 16h photoperiod,

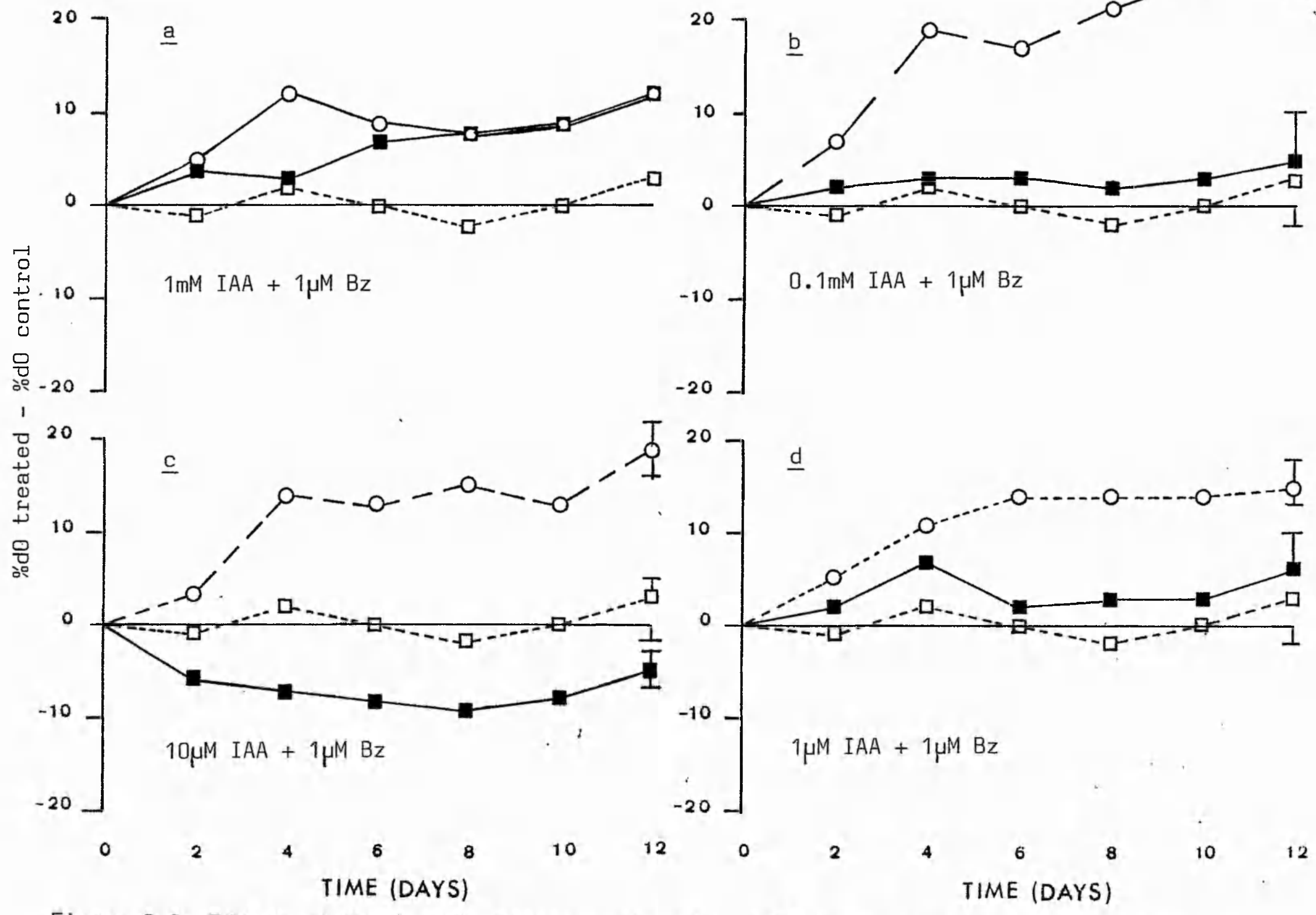


Figure 2.8 Effect of IAA (○--○), bentazone (□—□) and IAA/bentazone combinations (■—■) on petiole growth of 13 d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

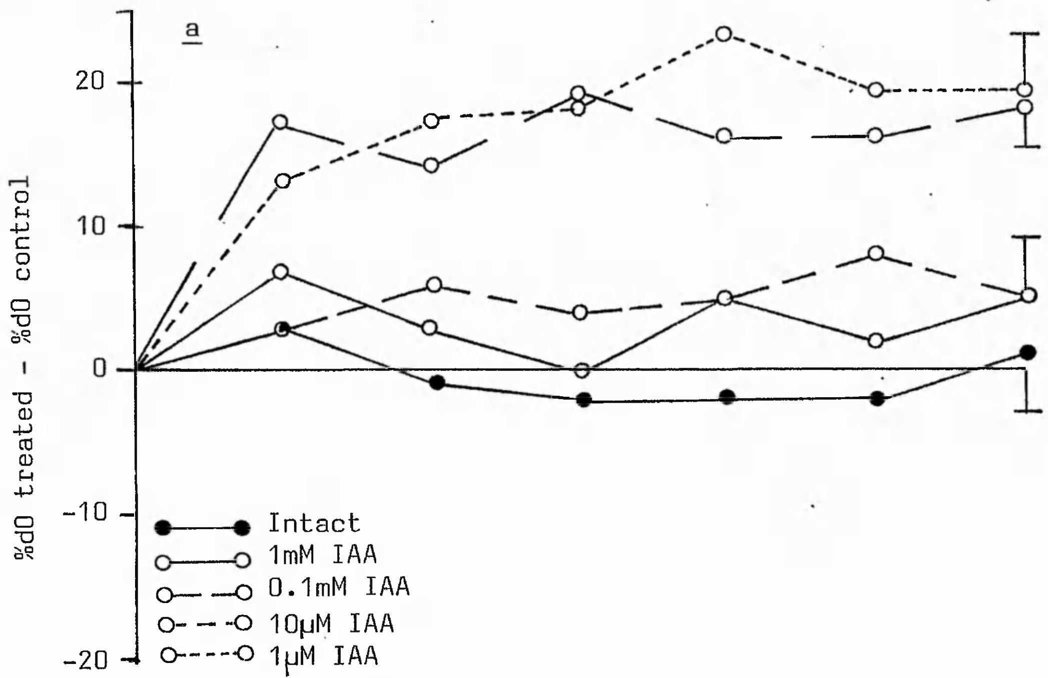
Ten micromolar bentazone reduced the IAA response in petioles in all cases (Figure 2.7) but in no instance was the combination treatment inhibitory compared to both bentazone and IAA i.e. there was no mutual antagonism in this case. This is also true for 1µM bentazone plus IAA (Figure 2.8), except when combined with 10µM IAA, when mutual antagonism occurred (Figured 2.8).

Data for the cotyledonary internode is presented in Figures 2.9 - 2.13. For this organ apical intactness had no effect on subsequent growth (Figure 2.9). Auxin stimulated elongation of this organ at all concentrations used, but this was most marked with 1µM IAA and 0.1mM IAA (Figure 2.9a). Bentazone stimulated elongation at 1mM and 0.1mM, whereas at 10µM and 1µM it was slightly inhibitory (Figure 2.9b). The greatest difference appeared to have occurred at d8, with the differences growing out of the tissue after this time.

Combinations of IAA and bentazone interacted in the cotyledonary internode as they did in the petioles. The combination of 1mM IAA with 1mM bentazone was antagonistic i.e. growth was less than that which occurred with each compound separately (Figure 2.10a). Further combinations incorporating 1mM bentazone concluded with growth being similar to that of the component which was least stimulatory when applied alone (Figure 2.10b-d). In all cases bentazone was inhibitory to the IAA response.

Similarly, 0.1mM bentazone was inhibitory to the stimulation of cotyledonary internode growth by 0.1mM and 1µM IAA (Figure 2.11b and d), yet when combined with 1mM IAA the response was virtually as that of 1mM IAA (Figure 2.11a), and when combined with 10µM IAA the response was as for 0.1mM bentazone (Figure 2.11c).

Ten millimolar bentazone reduced slightly the response to 1mM IAA when in combination with it (Figure 2.12a), and dramatically reduced the responses to 0.1mM and 1µM IAA (Figure 2.12b and d). It had little effect on the response to 10µM IAA (Figure 2.12c).



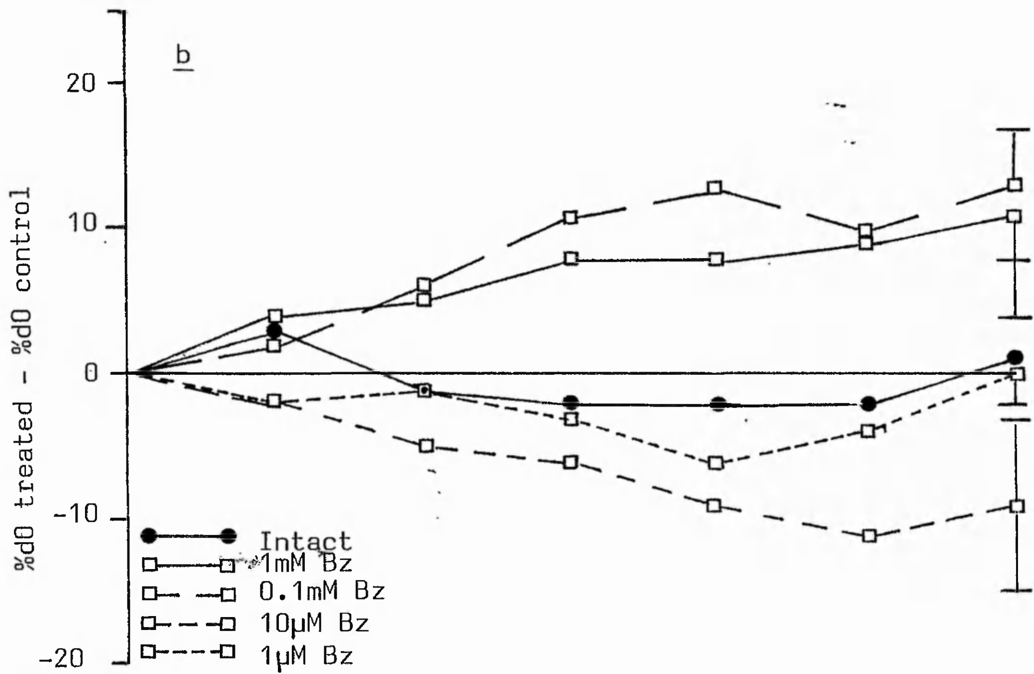


Figure 2.9 Effect of IAA (a) and bentazone (b) on growth of cotyledonary internode of *Phaseolus vulgaris* n=10, 25°C day, 15°C night, 16h photoperiod.

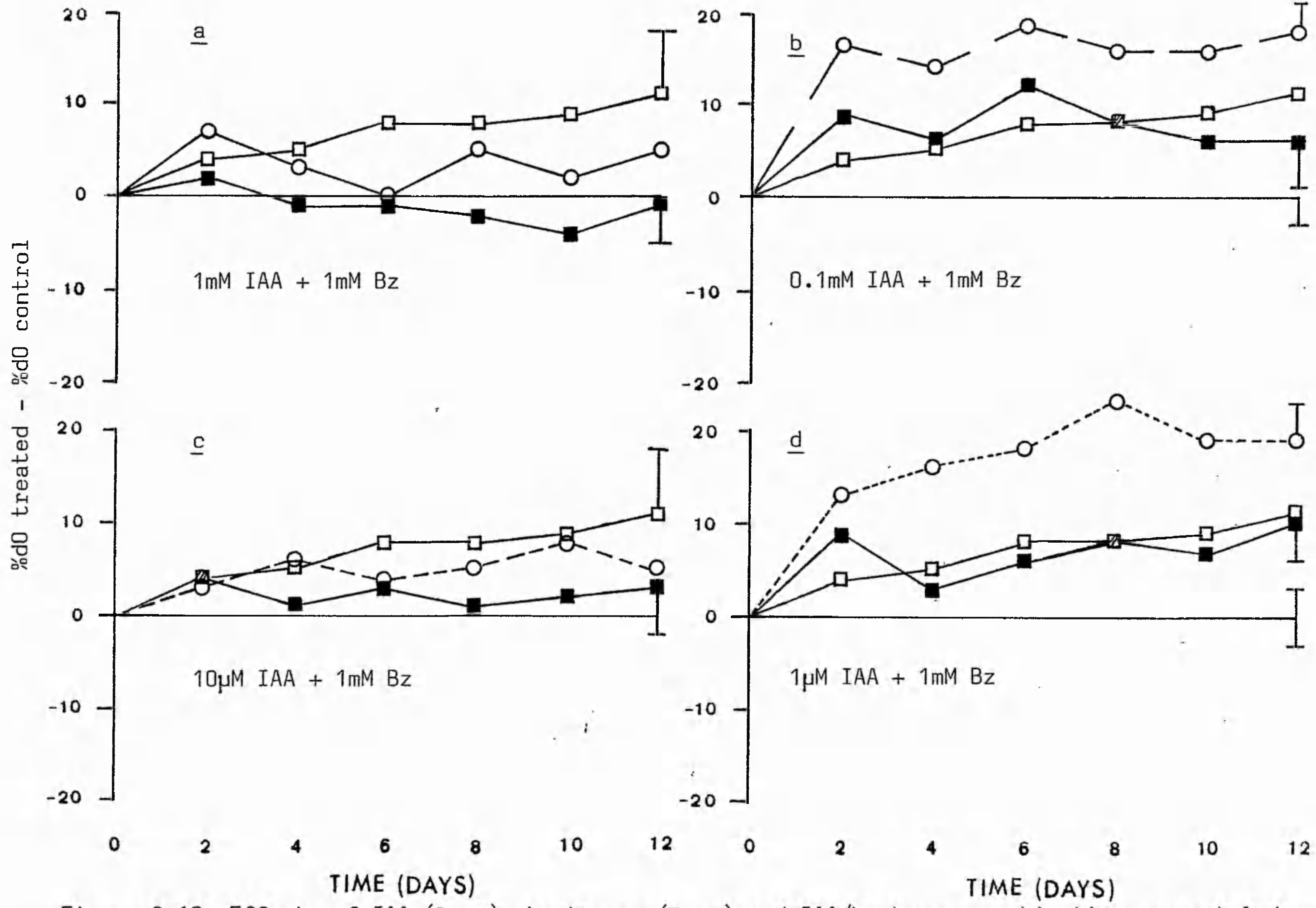


Figure 2.10 Effects of IAA (○--○), bentazone (□—□) and IAA/bentazone combinations on cotyledonary internode growth of 13d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

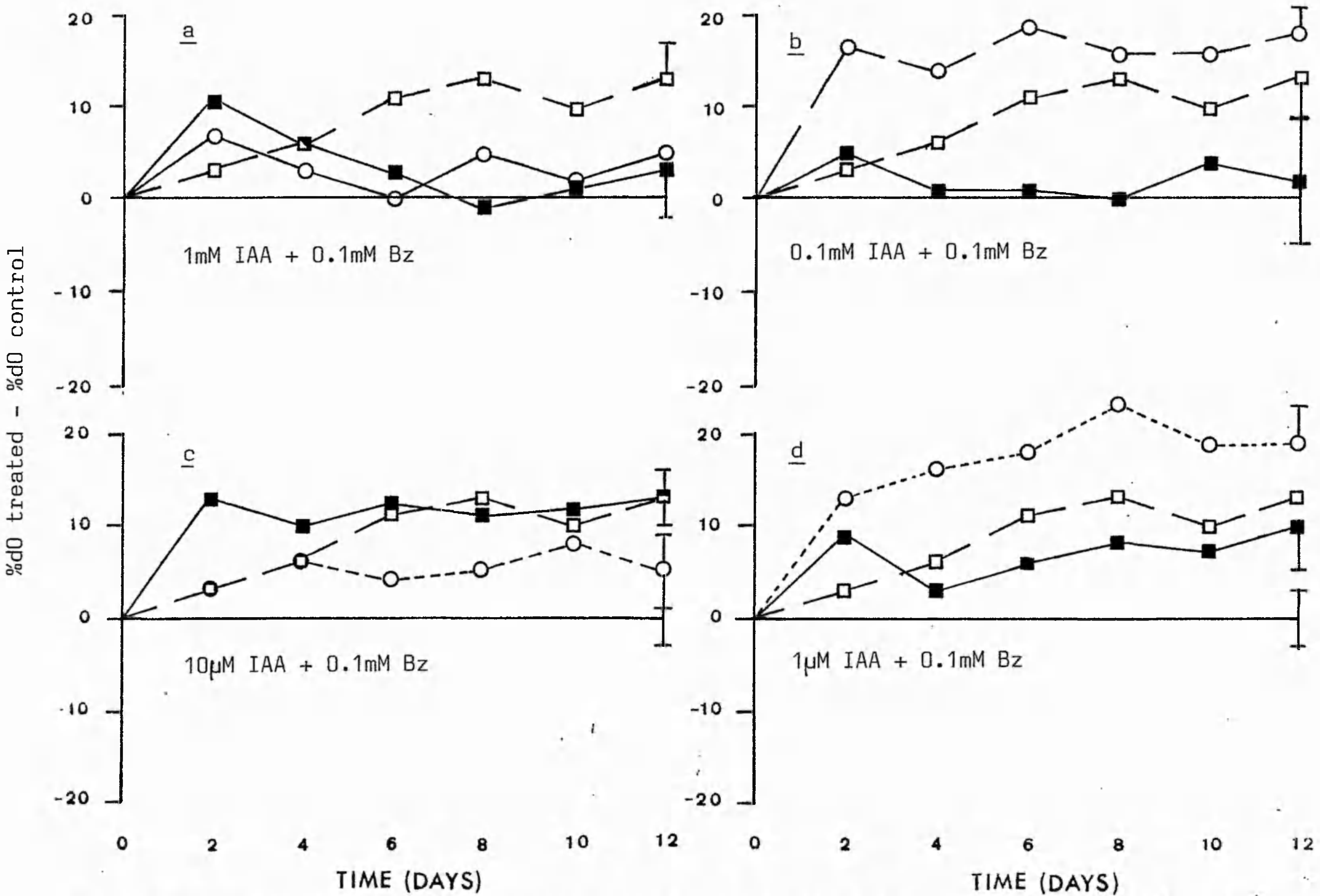
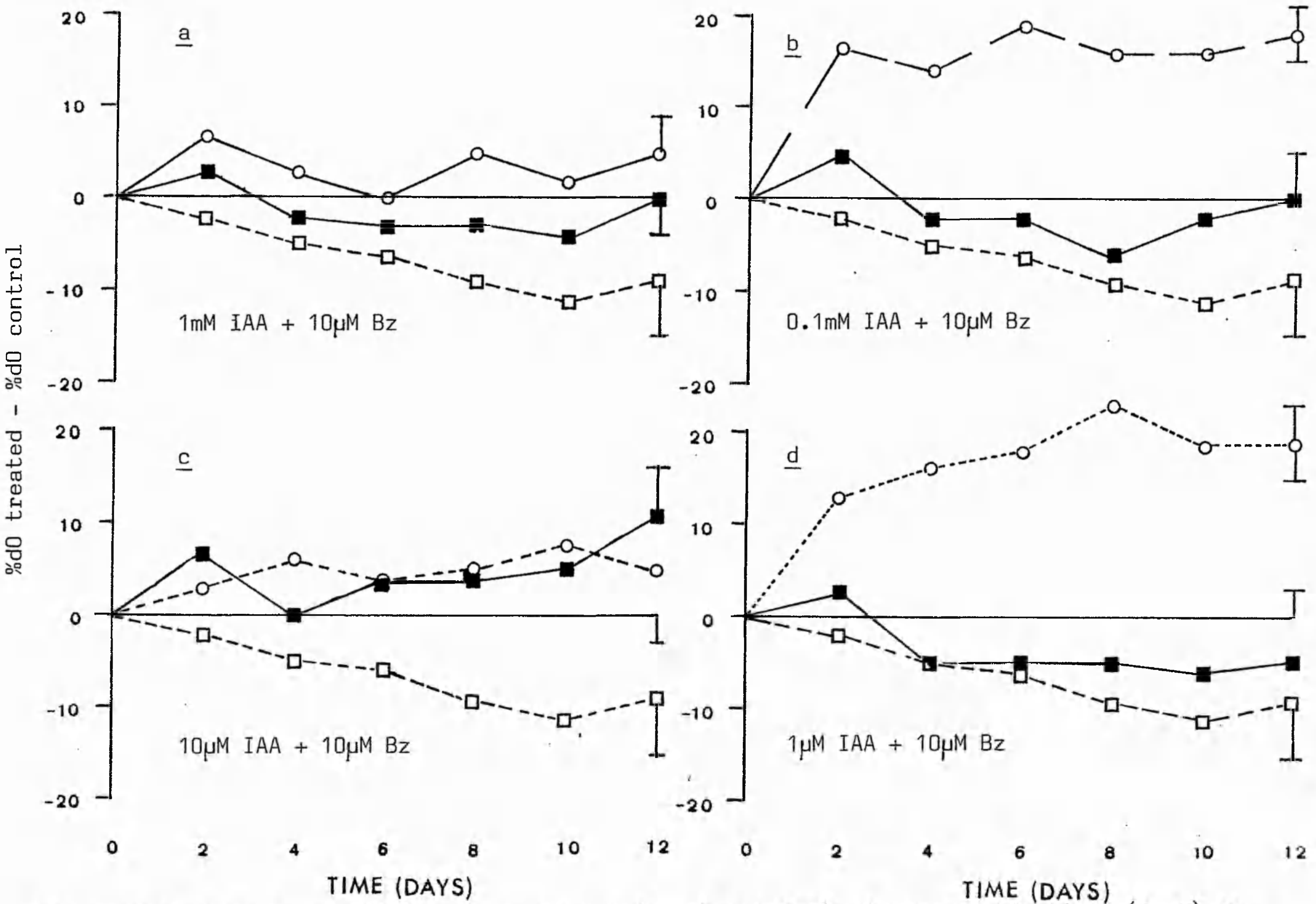


Figure 2.11 Effects of IAA (○--○), bentazone (□—□) and IAA/bentazone combinations (■—■) on cotyledonary internode growth of 13 d old *Phaseolus vulgaris*. n=10 16h photoperiod



<u>Figure 2 12</u> Effects of IAA (○--○), bentazone (□—□) and IAA/bentazone combinations (■—■) on cotyledonary internode growth of 13d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

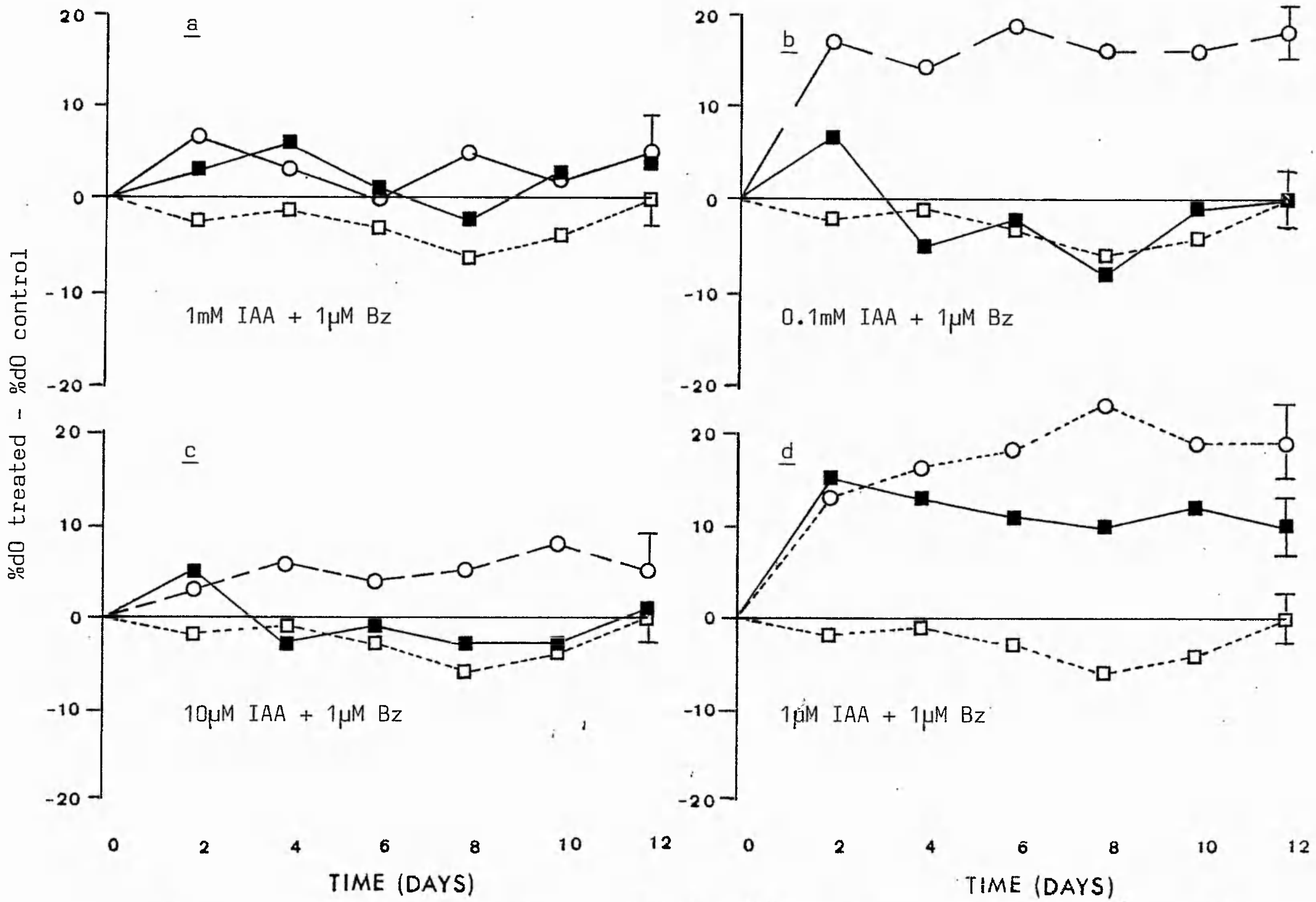


Figure 2.13 Effect of IAA (○--○), bentazone (□—□) and IAA/bentazone combinations (■—■) on cotyledonary internode growth of *Phaseolus vulgaris*. n=10, 16h photoperiod.

The effect of 1μM bentazone was to dramatically reduce the response to 0.1mM IAA (Figure 2.13b), and to reduce by a lesser degree the responses to 10μM and 1μM IAA (Figure 2.13c and d). It had little effect on the response to 1mM IAA (Figure 2.13a).

The effects of IAA and bentazone, alone and in combination, on outgrowth of the first internode of the first lateral, are presented in Figures 2.14 - 2.18.

Figure 2.14a demonstrates that apical intactness inhibited outgrowth of this internode, whereas the range of IAA concentrations stimulated the process in a concentration dependent manner. Figure 2.14b shows the similar effects induced by bentazone concentration at approximately one order of magnitude higher.

As in the other two tissues described, there were considerable interactions of IAA and bentazone in the lateral. Millimolar bentazone and 0.1mM IAA or 10μM IAA were antagonistic (Figure 2.15b and c), while 1mM bentazone and 1mM IAA combined produced a response part way between the two (Figure 2.15a). One micromolar IAA inhibited the stimulation of growth caused by 1mM bentazone (Figure 2.15d). Bentazone at 0.1mM was antagonistic with IAA at 1mM, at 0.1mM and at 10μM (Figure 2.16a-c), but with 1μM IAA the response lay part way between the two individual responses (Figure 2.16d).

Ten micromolar bentazone had little effect on the response to any concentration of IAA (Figure 2.17a-d), and the response to only one concentration of IAA, namely 10μM, was affected by 1μM bentazone (Figure 2.18).

The results clearly show that apically applied compounds can affect subsequent apical development. In summary, both IAA and bentazone stimulated petiole, cotyledonary internode and dominant lateral outgrowth, all in a concentration dependent manner (Figures 2.4, 2.9 and 2.14). The compounds were also able to interact in all three organs, and again depending on concentrations they were mutually synergistic (1mM IAA + 1mM bentazone,

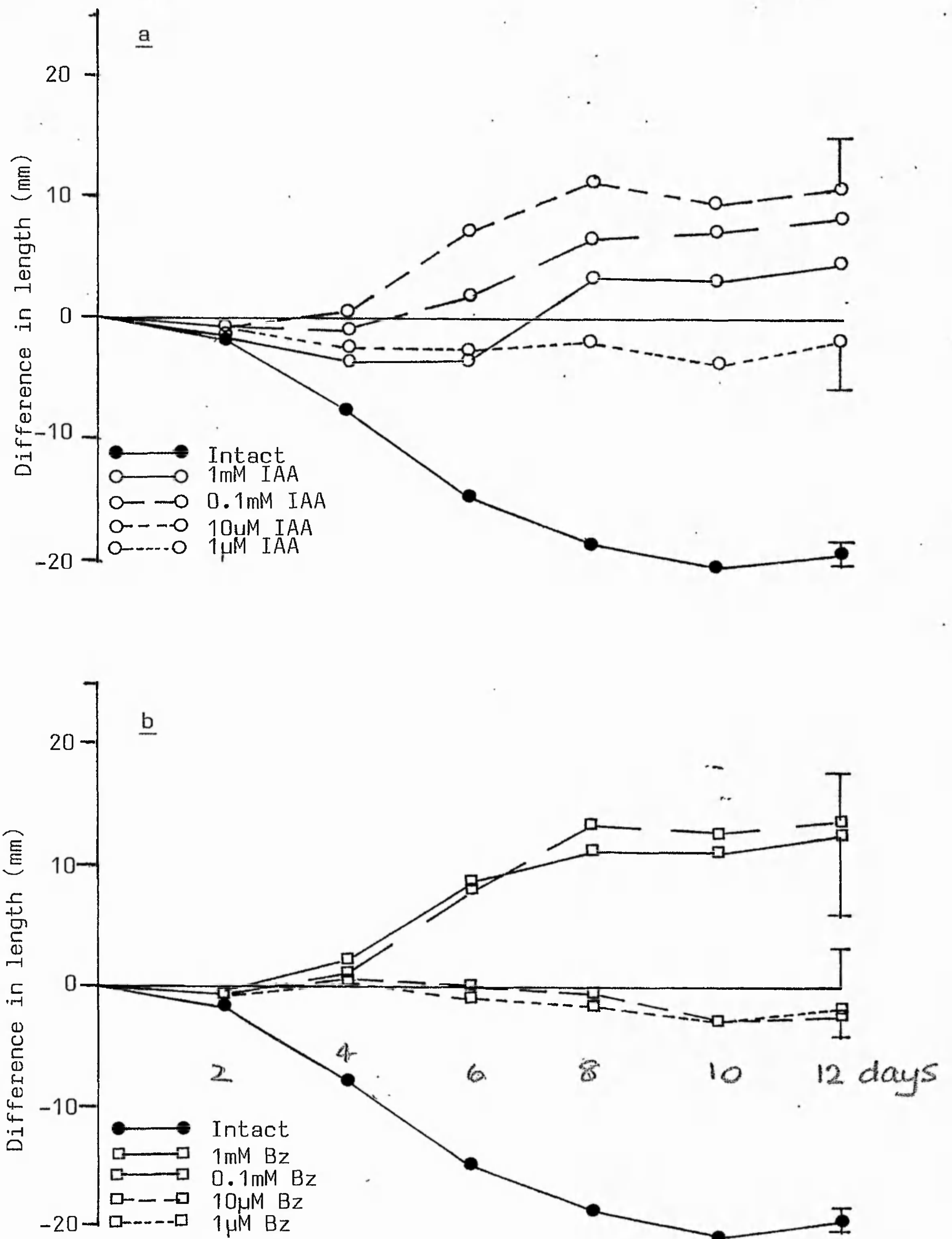


Figure 2.14 Effect of IAA (a) and bentazone (b) on lateral growth of *Phaseolus vulgaris*. n=10, 25°C day, 15°C night, 16h photoperiod.

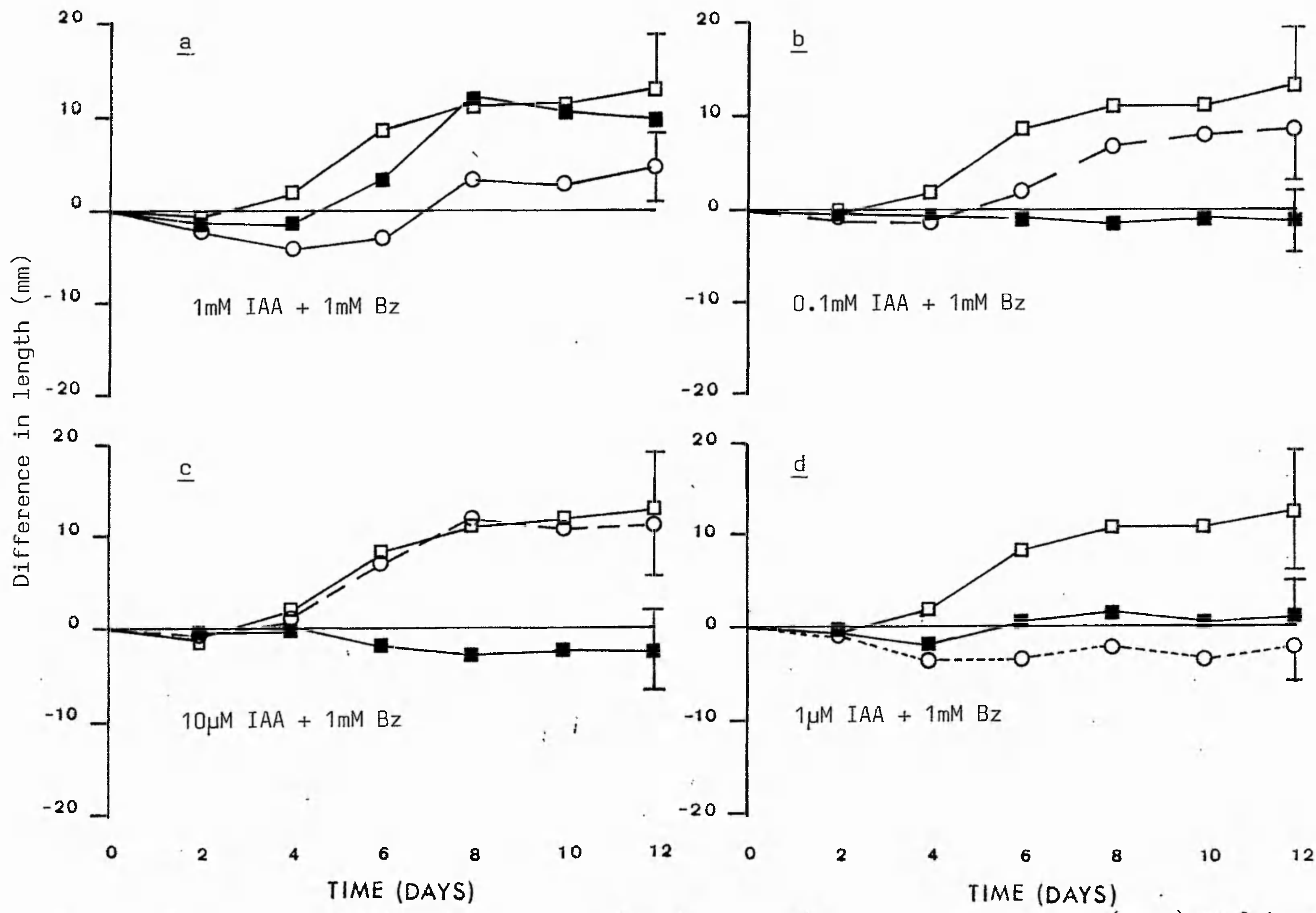


Figure 2.15 Effect of IAA (○—○), bentazone (□—□) and IAA/bentazone combinations (■—■) on lateral growth of 13d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

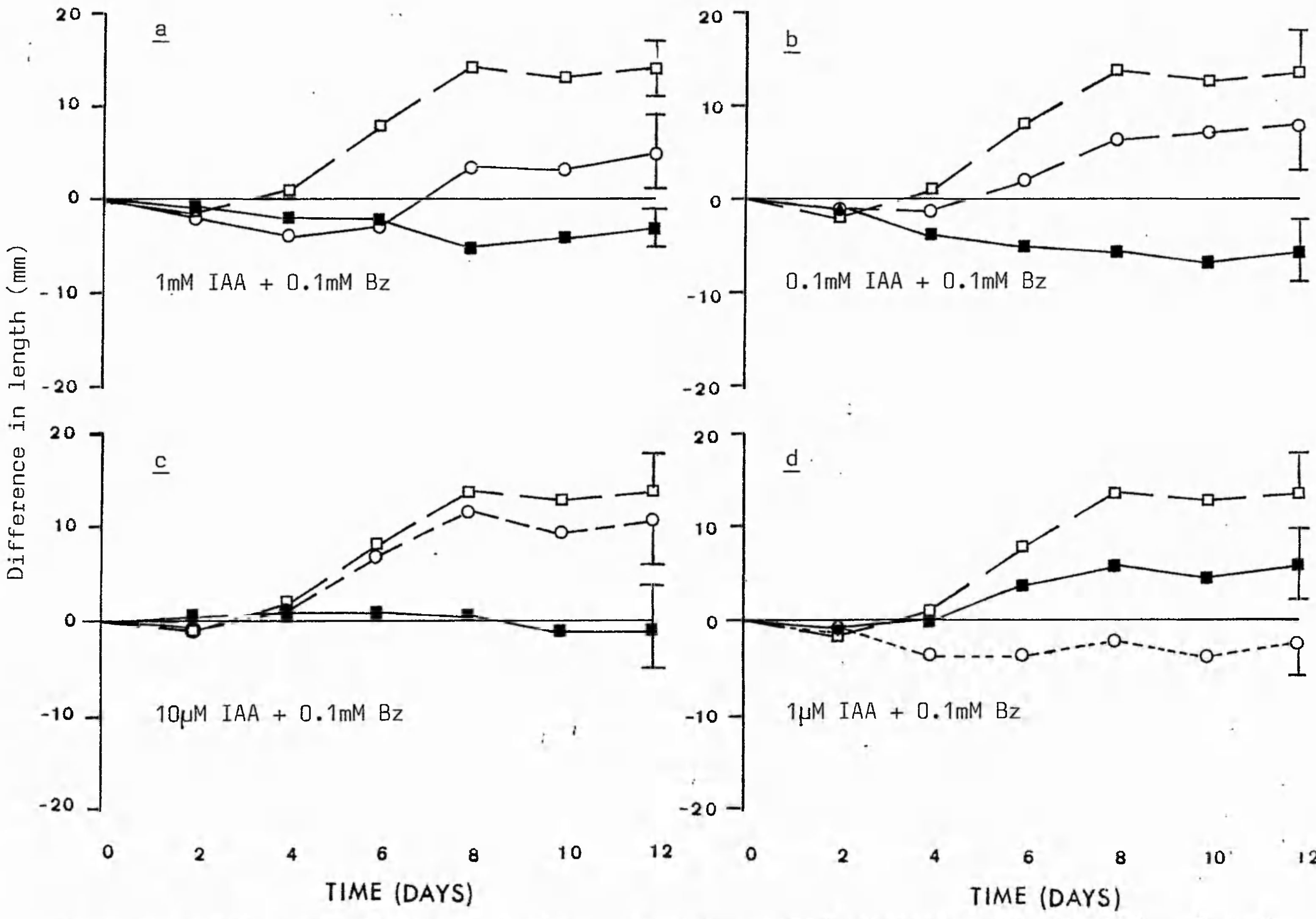


Figure 2.16 Effect of IAA (○—○), bentazone (□—□) and IAA/bentazone combinations (■—■) on lateral growth of 13d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

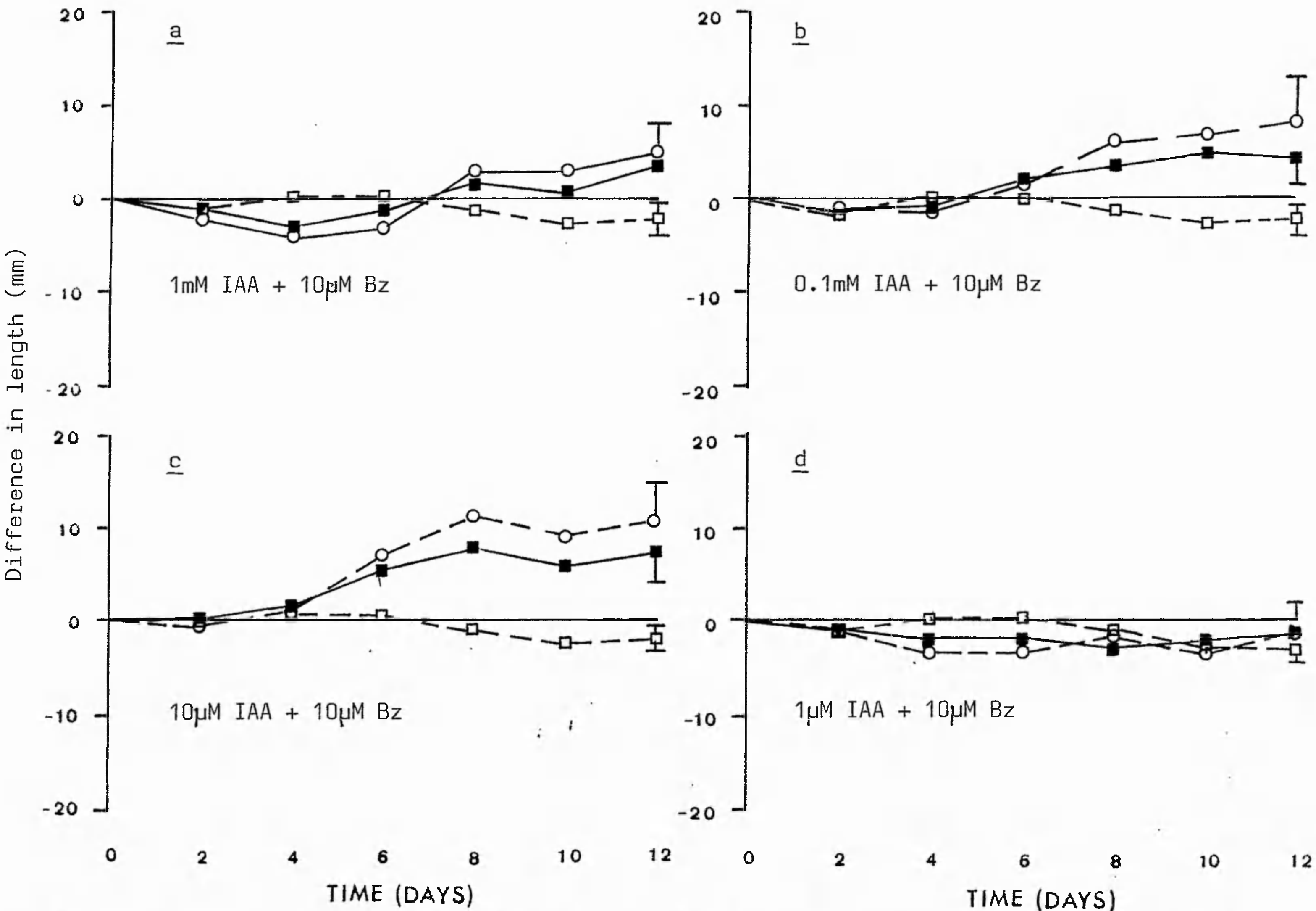


Figure 2.17 Effects of IAA (○--○), bentazone (□—□) and IAA/bentazone combinations (■—■) on lateral growth of 13d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

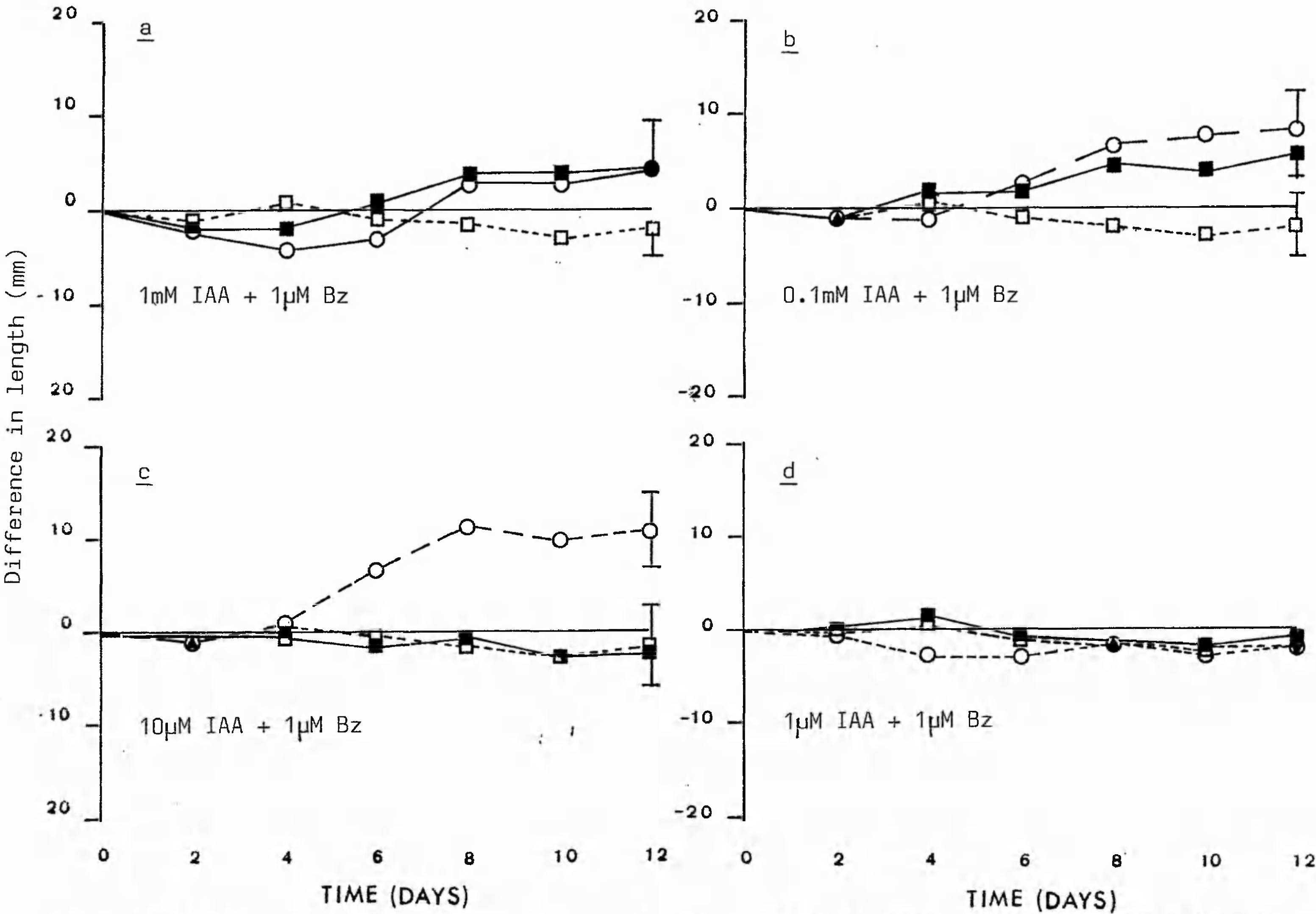


Figure 2.18 Effect of IAA (○—○), bentazone (□—□) and IAA/bentazone combinations (■—■) on lateral growth of 13d old *Phaseolus vulgaris*. n=10, 16h photoperiod.

petioles, figure 2.5a), produced a response part way between the two individual responses (1μM IAA + 0.1mM bentazone, lateral, figure 2.16d), or were mutually antagonistic (0.1mM IAA + 0.1mM bentazone, cotyledonary internode, figure 2.11b).

The data regarding lateral bud outgrowth is particularly interesting with reference to the theory of apical dominance. The theory suggests that IAA produced from the apex is responsible for preventing lateral lateral outgrowth. However, this data shows that apically applied IAA in fact stimulated lateral outgrowth, and that when the IAA activity was antagonised by bentazone there was a partial reimposition of apical dominance.

2.4.2 Cress Seed Germination

The lengths of shoots and roots of cress seedlings following 5d incubation with auxin (IAA) or bentazone, or equimolar concentrations of both, are illustrated in figure 2.19. Both IAA and bentazone at 1mM significantly inhibited both shoot and root elongation (P=0.001, all cases). Shoots and roots were significantly shorter in response to 1mM IAA (both P=0.001), but roots only with 0.1mM IAA (P=0.01). No other concentration of IAA applied alone had a significant effect in either shoots or roots. Bentazone at 0.1mM caused significantly shorter roots and shoots (P=0.001 and P=0.05 respectively), while 10μM bentazone affected neither shoot or root length. One micromolar bentazone caused significantly longer shoots than control (P=0.05), but had no effect on roots. There was no effect on shoot or root growth of 0.1μM bentazone. Bentazone at 10nM caused significantly longer shoots and roots (both P=0.001), as did 1nM bentazone (both P=0.01).

Figure 2.19 shows there to be no interaction between IAA and bentazone at equimolar concentrations in shoot tissue, but in roots there was a clear synergistic interaction between equimolar IAA and bentazone at concentrations below 1μM. The data presented is from the complete study, although the trend had been observed in preliminary studies which prompted a full investigation into IAA and bentazone interactions in this system. The data from the full study is summarised in tables 2.1 and 2.2 for roots and shoots respectively. The figures show where bentazone plus IAA treatments at a particular combination produced shoot or root length significantly different to that produced by the relevant IAA concentration applied alone. Where no significance value is given there was no significant difference in length.

No strong trend emerged from data of effect of bentazone plus IAA in shoots (Table 2.1). High bentazone concentration (0.1mM) significantly reduced shoot length in combination with both 1μM IAA and 0.1 μM IAA (both P=0.001). Ten micromolar bentazone significantly reduced shoot length with 1μM IAA (P=0.001) compared to 1μM IAA alone, but significantly enhanced the effect of

Table 2.1 Effect of IAA alone, and effect of bentazone on IAA-induced growth responses in Lepidium sativum shoots. n ⩾45, 5d, 25°C, darkness.
↑ indicates bentazone enhancement of response
↓ indicates bentazone inhibition of response
Where no probability is noted the effects were non-significant.

Conc. of Bentazone \ Concentration of IAA	0	0.1mM	10µM	1µM	0.1µM	10nm	1nM
0		↓ P=0.001					
0.1mM	↓ P=0.02			↓ P=0.001	↓ P=0.001		
10µM				↓ P=0.001			↑ P=0.01
1µM	↑ P=0.05						
0.1µM		↑ P=0.05					
10nM	↑ P=0.001	↓ P=0.01	↓ P=0.001		↑ P=0.001		
1nM	↑ P=0.01		↓ P=0.01				

Table 2.2 Effect of IAA alone, and effect of bentazone on IAA-induced growth responses in Lepidium Sativum roots. n ⩾45, 5d, 25°C, darkness.
↑ indicates bentazone enhancement of response
↓ indicates bentazone inhibition of response
Where no probability is noted the effects were non-significant.

Conc. of Bentazone \ Concentration of IAA	0	0.1mM	10µM	1µm	0.1µM	10nM	1nM
0		↓ P=0.001	↓ P=0.01				
0.1mM	↓ P=0.001			↓ P=0.05		↓ P=0.001	↓ P=0.001
10µM						↓ P=0.01	
1µM							
0.1µM		↑ P=0.05			↑ P=0.001	↑ P=0.001	
10nM	↑ P=0.001			↑ P=0.001	↑ P=0.001	↑ P=0.001	↑ P=0.01
1nM	↑ P=0.01			↑ P=0.01	↑ P=0.001	↑ P=0.001	↑ P=0.05

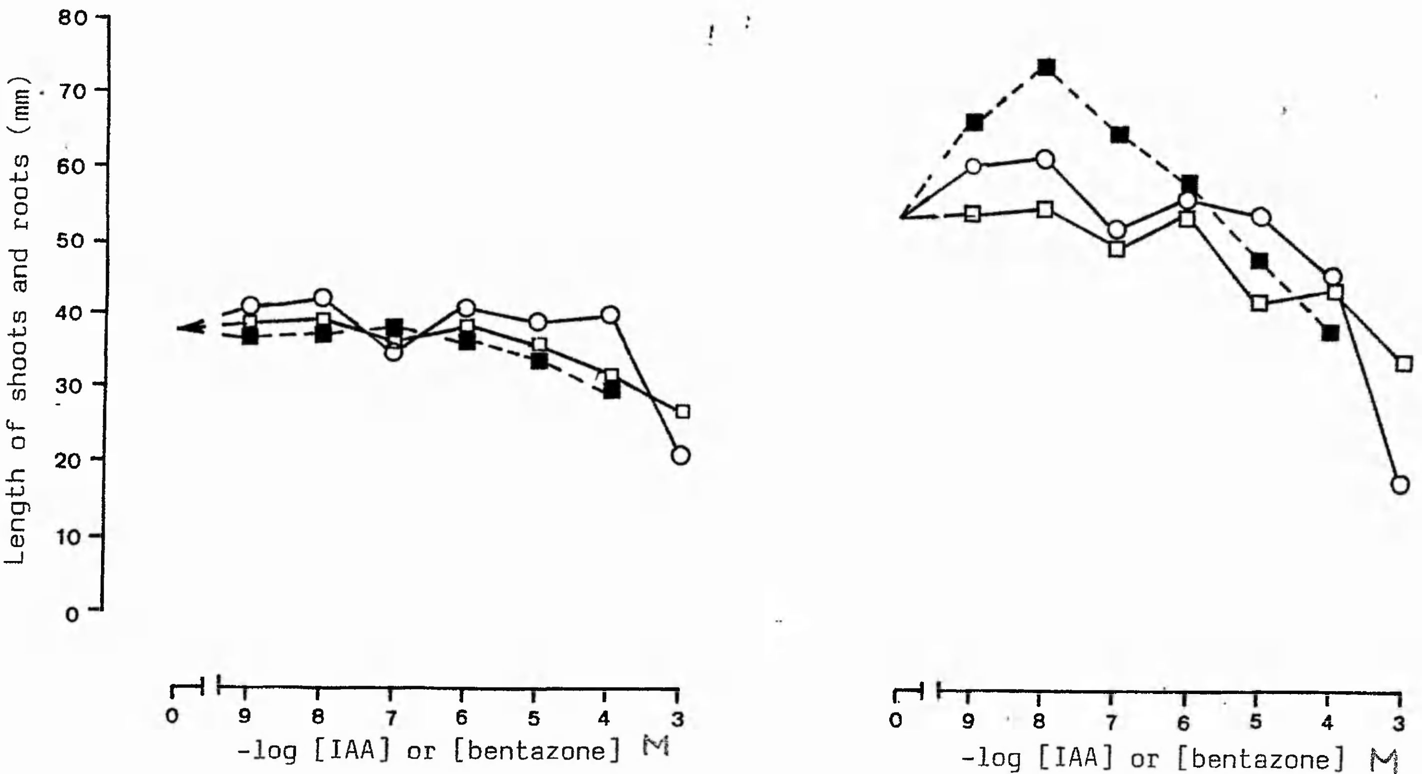


Figure 2.19 Effect of IAA (O——O) or bentazone (□——□) on growth of shoots (a) or roots (b) of *Lepidium sativum*, and effect of equimolar combinations of IAA and bentazone (■- - -■) on shoots (a) and roots (b). n=45, 25°C, 5d, darkness.

1nM IAA (P=0.01). Bentazone at 1µM did not significantly alter IAA effects at any concentration of IAA, and 0.1µM bentazone significantly enhanced only the effect of 0.1mM IAA (P=0.05). Ten nanomolar bentazone significantly reduced length of shoots in combination with 0.1mM IAA (P=0.01) and 10µM IAA (P=0.001) compared to those concentrations of IAA alone, but it significantly (P=0.001) enhanced the length of shoots with 0.1µM IAA compared to 0.1µM IAA alone. The length of shoots treated with 10µM IAA plus 1nM bentazone was significantly less than those with 10µM IAA alone (P=0.01).

In contrast to the lack of a clear trend in IAA/bentazone interaction data in shoots there was a clear pattern in the corresponding data for root, and this is illustrated in figure 2.20, with detailed statistical information provided in Table 2.2.

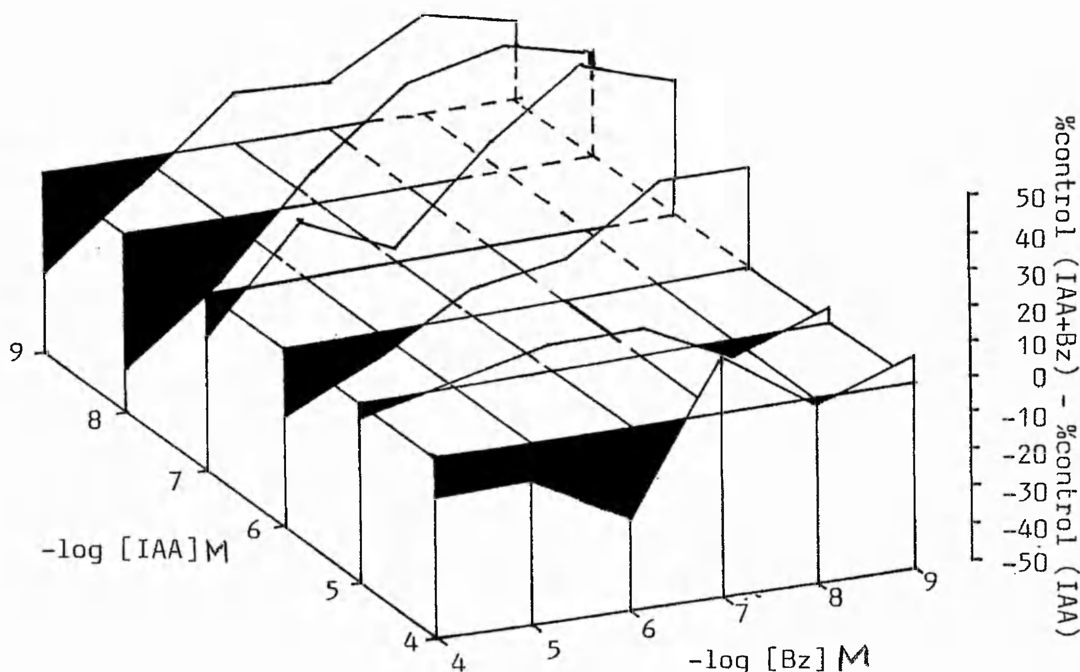


Figure 2.20 Effect of bentazone on IAA-induced root growth of cress seedlings. 5d, 25°C, darkness, n≥45.

Figure 2.20 shows that the higher concentrations of bentazone used herein (0.1mM, 10μM and 1μM bentazone) reduced the length of roots, when in combination with all concentrations of IAA, compared to IAA alone roots. For 0.1mM bentazone this was a significant reduction with 1μM IAA (P=0.05), 10nM IAA, and 1nM IAA (both P=0.001), while for 10μM bentazone this was significant only with 10nM IAA (P=0.01), compared to 10nM IAA alone. For 1μM bentazone there was no significant reduction of IAA-induced root length. In contrast to the reduction of IAA response by the higher bentazone concentrations, the lower concentrations of bentazone enhanced effects of IAA, particularly when concentrations of both compounds were sub-micromolar. This effect is illustrated in Figure 2.20. Bentazone at 0.1μM significantly enhanced root length when in combination with IAA, compared to IAA alone, at 0.1mM IAA (P=0.05) and 0.1μM and 10nM (both P=0.001, Table 2.2). Ten nanomolar bentazone significantly enhanced the effects of 1μM, 0.1μM and 10nM IAA (all P=0.001) and of 1nM IAA (P=0.01, Table 2.2). A similar effect was induced by 1nM bentazone, which significantly enhanced effects of 1μM IAA (P=0.01), 0.1μM and 10nM IAA (both P=0.001), and 1nM IAA (P=0.05, Table 2.2).

To summarise, IAA at lower concentrations had little effect alone on growth of either shoots or roots, but higher concentrations were inhibitory to both roots and shoots. Bentazone was similarly inhibitory to both shoot and root growth at high concentrations, but was stimulatory to growth of both at low concentrations. There was little significant interaction of IAA and bentazone in shoots, but in roots the stimulatory effect of low bentazone concentration acted synergistically with low IAA concentrations which had themselves done little alone.

2.4.3 *Avena sativa* Coleoptile Elongation

Using a different set of 15 coleoptile segments at each 5 min time interval, time-course studies were performed which showed that, up until 1h after start of incubation, coleoptile elongation was linear, and the rates of elongation and correlation coefficients are presented in Table 2.3.

Table 2.3 Rates of elongation (μm min^{-1} ± standard errors) and correlation coefficients (r) of *Avena sativa* coleoptile segments (n ≥ 45, 25°C, darkness).

Con'tn of Bz	Concentration of IAA 0	0.1mM	10μM	1μM
0	3.764 ± 0.3345 r = 0.9199	8.699 ± 0.8118 r = 0.8612	9.411 ± 0.9782 r = 0.8620	7.102 ± 0.5118 r = 0.9150
0.1mM	6.818 ± 0.6873 r = 0.8650	7.564 ± 0.5199 r = 0.9399	7.770 ± 0.6954 r = 0.9011	7.087 ± 0.6098 r = 0.9215
10μM	7.092 ± 0.8618 r = 0.8618	7.891 ± 0.6355 r = 0.9420	6.339 ± 0.6355 r = 0.9322	5.559 ± 0.6543 r = 0.9099
1μM	5.848 ± 0.5036 r = 0.9273	8.462 ± 0.8941 r = 0.9255	9.785 ± 0.7309 r = 0.9606	8.015 ± 0.7394 r = 0.9417

Having established that growth over this initial hour was linear it became possible to use lengths of segments at 60 min as a valid comparison, and this was subsequently used.

The preliminary data showed that the elongation responses to both IAA and bentazone were concentration dependent, both with apparent optimum concentration of 10μM, and they also show interactions between IAA and bentazone (Table 2.3). When the two optimum concentrations were applied together the rate of elongation was significantly lower than that induced by 10μM IAA alone (P=0.05), and bentazone also significantly reduced the response to 1μM IAA alone (P=0.05). However, despite the rate of elongation of combination treatments being generally lower than those of the appropriate IAA alone treatment i.e. bentazone having a partially antagonistic effect on IAA, low bentazone (1μM), when combined with 10μM IAA and 1μM IAA, caused the elongation rate to be slightly higher, i.e. there was an apparent synergism occurring. These observations dictated that

a moredetailed study of IAA and bentazone interactions be made. This study was conducted, and the results are presented in Figure 2.21 and in Table 2.4.

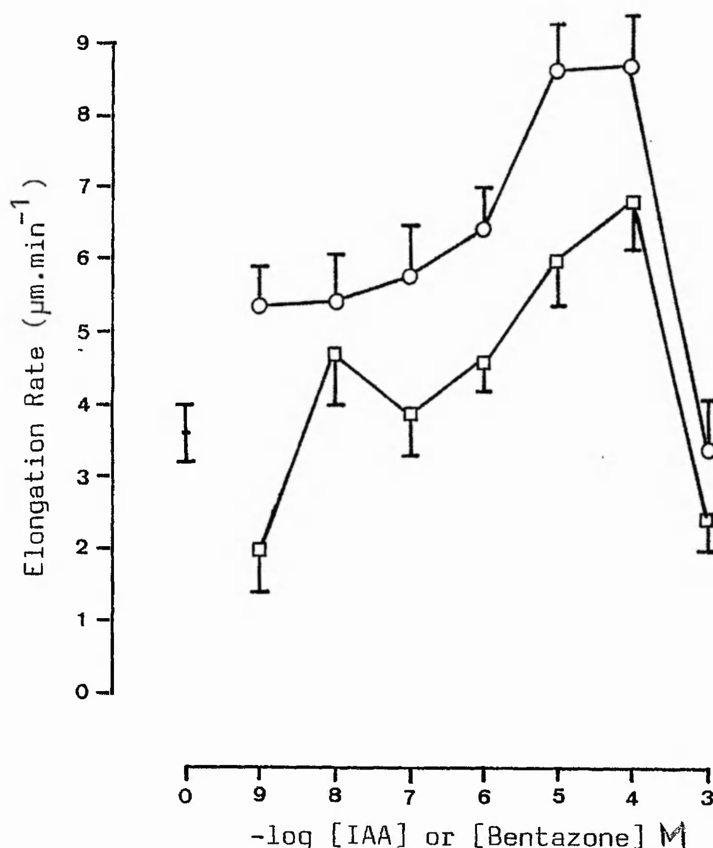


Figure 2.21 Elongation rates of Avena coleoptile segments treated with IAA (O———O) or bentazone (□———□). n ≥ 45, 25°C darkness.

Figure 2.21 confirms the concentration dependence of the responses to both IAA and bentazone applied alone. For both compounds the optimum concentration lies at around 10-100µM. Millimolar IAA and bentazone are clearly supra-optimal, and return elongation rate to near (IAA) or below (bentazone) that of control tissue. Low concentrations of IAA (1nM, 10nM) were stimulatory to coleoptile elongation, whereas lower concentrations of bentazone (1µM and lower) had little effect and proved somewhat erratic (Figure 2.21).

Table 2.4 Elongation Rates of *Avena sativa* coleoptile segments ($\mu m.min^{-1}$) incubated with IAA and/or bentazone at various concentrations. Elongation rates $\pm$ Standard Errors, and for combinations whether this is a reduction (↓) or stimulation (↑) of the IAA alone - response by bentazone, and whether this is significantly different to the IAA alone response. *P=0.05 **P=0.01 ***P=0.001 NS, Not Significant. n ≥ 45, 1h, 25°C, darkness.

Concentration of IAA

Conc'n of Bz	0	1mM	0.1mM	10µM	1µM	0.1µM	10nM	1nM
0	3.63 ± 0.385	3.45 ± 0.653	8.70 ± 0.812	8.61 ± 0.665	6.48 ± 0.528	5.80 ± 0.700	5.48 ± 0.617	5.40 ± 0.483
1mM	2.50 ± 0.533							
0.1mM	6.82 ± 0.678		7.56 ± 0.520 NS ↓	7.77 ± 0.695 NS ↓	7.09 ± 0.610 NS ↑	5.17 ± 0.633 NS ↓	4.65 ± 0.567 NS ↓	4.60 ± 0.700 NS ↓
10µM	6.04 ± 0.605		7.89 ± 0.726 NS ↓	6.34 ± 0.636 * ↓	5.56 ± 0.654 NS ↓	4.73 ± 0.533 NS ↓	5.63 ± 0.517 NS ↑	5.18 ± 0.653 NS ↓
1µM	4.60 ± 0.383		8.46 ± 0.894 NS ↓	9.79 ± 0.731 NS ↑	7.76 ± 0.661 NS ↑	6.40 ± 0.583 NS ↑	6.47 ± 0.550 NS ↑	4.35 ± 0.600 NS ↓
0.1µM	3.83 ± 0.583		7.08 ± 0.567 NS ↓	7.08 ± 0.867 NS ↓	6.93 ± 0.583 NS ↑	6.32 ± 0.567 NS ↑	5.28 ± 0.617 NS ↓	3.95 ± 0.667 NS ↓
10nM	4.68 ± 0.650		5.98 ± 0.450 ** ↓	6.93 ± 0.683 NS ↓	6.32 ± 0.583 NS ↓	7.50 ± 0.450 * ↑	3.85 ± 0.533 NS ↓	3.82 ± 0.717 NS ↓
1nM	1.95 ± 0.583		6.03 ± 0.617 * ↓	5.45 ± 0.667 ** ↓	7.98 ± 0.483 * ↑	5.95 ± 0.600 NS ↑	5.87 ± 0.733 NS ↓	5.13 ± 0.750 NS ↓

The interactions between the compounds are representd in Table 2.4. The interactive data has been assessed by the effect of bentazone on the response to the appropriate concentration of IAA applied alone. Bentazone at 0.1mM generally reduced the effect of IAA, except at 1μM IAA, but in no case was the effect of 0.1mM bentazone significant in its effect on IAA. However, 10μM bentazone significantly reduced the response to 10μM IAA (P=0.05) i.e. the combination round the two optima inhibited IAA, which confirmed the finding of the preliminary study (Table 2.3). The general effect of 10μM bentazone on IAA was to reduce the IAA effect. There was no significant effect of either 1μM or 0.1μM bentazone on any concentration of IAA, but the general effect of 1μM bentazone was to enhance the IAA response, while 0.1μM bentazone slightly reduced the effect of 0.1mM, 10μM, 10nM and 1nM IAA and enhanced 1μM and 0.1μM IAA. Ten nanomolar bentazone significantly reduced the the response to 0.1mM IAA (P=0.01) i.e. the optimum concentration of IAA, and also reduced (non-significantly) the response to every other concentration of IAA except 0.1μM IAA, which it significantly enhanced (P=0.05). The lowest concentration of bentazone (1nM) significantly enhanced the response to 1μM IAA (P=0.05), and non-significantly enhanced the response to 0.1μM and 10nM IAA. It non-significantly reduced the response to 1nM IAA (Table 2.4).

Where the interactions significantly reduced the responses to IAA, the reduction was of the response to optimum IAA concentration in all cases i.e. 10μM IAA + 10μM bentazone, 10μM IAA + 1nM bentazone, and 0.1mM IAA plus both 10nM and 1nM bentazone. Where the response to IAA was enhanced by bentazone the IAA concentration was micromolar or lower i.e. 1μM IAA + 1nM bentazone, and 0.1μM IAA + 10nM bentazone. Clearly the optimum concentration of IAA is very sensitive to inhibition by bentazone at all concentrations, with the combination of compounds (IAA and bentazone) both at sub-micromolar concentrations giving mutual enhancement of response.

This data, illustrating similar concentration dependence of response to IAA and bentazone, albeit with bentazone producing a response of smaller magnitude on an equimolar basis, and the interaction of IAA and bentazone, which could be interpreted as a competitive inhibition for binding sites, suggested that the two compounds were producing the observed responses via the same, or very closely related mechanisms. One possible approach to establish similarity of mechanisms is to assess the sensitivity of the responses to other compounds. It was this approach which was adopted in this study, and the effects of other growth regulatory compounds, the presence of divalent cations, and the effect of metabolic inhibitors on the response to near optimum IAA and bentazone concentrations (both 10µM) was subsequently assessed. The experiment was repeated on at least three separate occasions for each treatment i.e. n = 45, and length of coleoptile segments at 60 min were compared to assess the degree of inhibition occurring. The findings are presented in Table 2.5. This experiment largely supported the supposition that the two compounds were causing coleoptile elongation by the same, or closely related mechanisms.

Table 2.5 Effect of additional compounds on responses of *Avena* coleoptile segments to near-optimum concentrations of IAA and bentazone (both 10µM). *P=0.05, **P=0.01, ***P=0.001, NS Not Significant. n ≥ 45, 25°C, darkness 1h.

Added Compound +Concentration	% Inhib'n of IAA	% Inhib'n of Bz	Compound + Conc'n	% Inhib'n of IAA	% Inhib'n of Bz
1mM GA3	38**	32*	0.1mM CCCP	86***	62**
0.1mM GA3	35**	64***	10µM CCCP	42**	33*
10µM GA3	19NS	51***	1µM CCCP	25NS	12NS
0.1mM GA4/7	42**	33*	0.1mM Ou'bn	16NS	21NS
10µM GA4/7	41**	16NS	10µM Ou'bn	21NS	36*
1µM GA4/7	30*	23NS	1µM Ou'bn	33*	3NS
1mM BAP	54***	85***	0.1mM DES	46***	53***
0.1mM BAP	33**	29NS	10µM DES	39***	41***
10µM BAP	38*	31NS	1µM DES	31***	22*
0.1mM ABA	61***	57***	0.1mM NaVO4	86***	75***
10µM ABA	52***	54***	10µM NaVO4	87***	87***
1µM ABA	36*	66***	1µM NaVO4	75***	92***
1mM Eth'C'	33*	36*	10mM KNO3	74***	72***
0.1mM Eth'C'	22NS	31*	1mM KNO3	59***	55**
10µM Eth'C'	25NS	35*	0.1mM KNO3	69***	73*

For most of these additional compounds the IAA and bentazone responses were inhibited by similar magnitude, but where this was small the inhibition may be significant in IAA but not bentazone treated tissue e.g. with BAP (Table 2.5). With some of the compounds the degree of inhibition produced was dependent upon the concentration of the inhibitor, e.g. CCCP with both IAA and bentazone, and DES with bentazone. With compounds where this was not the case e.g. $NaVO_4$, KNO_3 it may be that the work was conducted within an inappropriate concentration range of inhibitor. It may also be the case that the physiological activity of these compounds is insufficiently specific to allow assessment of the sensitivity of the theoretical mechanisms of this response to them.

The acid growth theory proposes that elongation of cells occurs because of turgor pressure within a loosened plant cell wall, this loosening having been brought about by the auxin stimulated pumping of protons from the cytoplasm to the cell wall by a membrane bound, ATP requiring protein. Compounds such as DES and CCCP, which were observed herein to inhibit auxin stimulated elongation, are thought to act by inhibition of the membrane bound proton pump (Hager et al, 1971; Balke and Hodges, 1979; Cleland, 1982) i.e. by inhibiting proton excretion. It was therefore suggested that in this experiment bentazone acted as an auxin, causing elongation within this system by stimulation of the proton pump , as it was sensitive in the same way as IAA to such inhibitors as DES and CCCP. It was therefore felt appropriate to investigate the effects of IAA and bentazone in proton efflux from Avena coleoptile segments.

2.4.4 Proton Efflux from *Avena* Coleoptile Segments

The patterns of auxin induced medium acidification are illustrated in Figure 2.22a and b. The pH of bathing medium in control remained stable for 90 min, after which it began to decline (Figure 2.22). Therefore, any pH changes occurring within 90 min are attributable to treatments. IAA at all concentrations produced rapid medium acidification, commencing with a lag of 10-15 min, and continuing linearly until 60, and sometimes, 90 min (Figure 2.22). Concentrations of IAA from 0.1μM IAA to 100μM IAA produced very similar patterns of acidification, with 250μM IAA apparently supra-optimal, and 10nM and 1nM IAA having less effect i.e. there is an apparently broad optimum concentration optimum. The patterns are linear to 60 min, and therefore comparison of ΔpH (control vs treated) at this time can give an accurate representation of acidification rate. This data is presented in figure 2.24. This confirms the broad concentration optimum, and that 250μM was supra-optimal, with 10nM and 1nM IAA being sub-optimal.

The effects of bentazone on medium acidification by *Avena* coleoptile segments is illusrated in Figure 2.23. Milliomolar bentazone caused slow medium acidification and was apparently supra-optimal. Bentazone at 250μM caused the greatest degree of acidification, with other concentrations being less effective. Again, by taking ΔpH values at 60 min , all responses being essentially linear to that time, the concentration dependence of the response can be simply illustrated (Figure 2.24). Figure 2.24 shows the narrow optimum concentration of bentazone, and the minimal effect on medium acidification of low concentrations (1μM, 10μM bentazone). The maximum bentazone induced response was only 60% of that of the maximum IAA response, but perhaps more significant is the broad concentration optimum of IAA compared to bentazone (Figure 2.24).

From some of the work done previously in this study an interaction with high (optimum) concentrations of IAA and bentazone had been identified i.e. significant reduction of the response to optimum IAA was caused by optimum bentazone in the

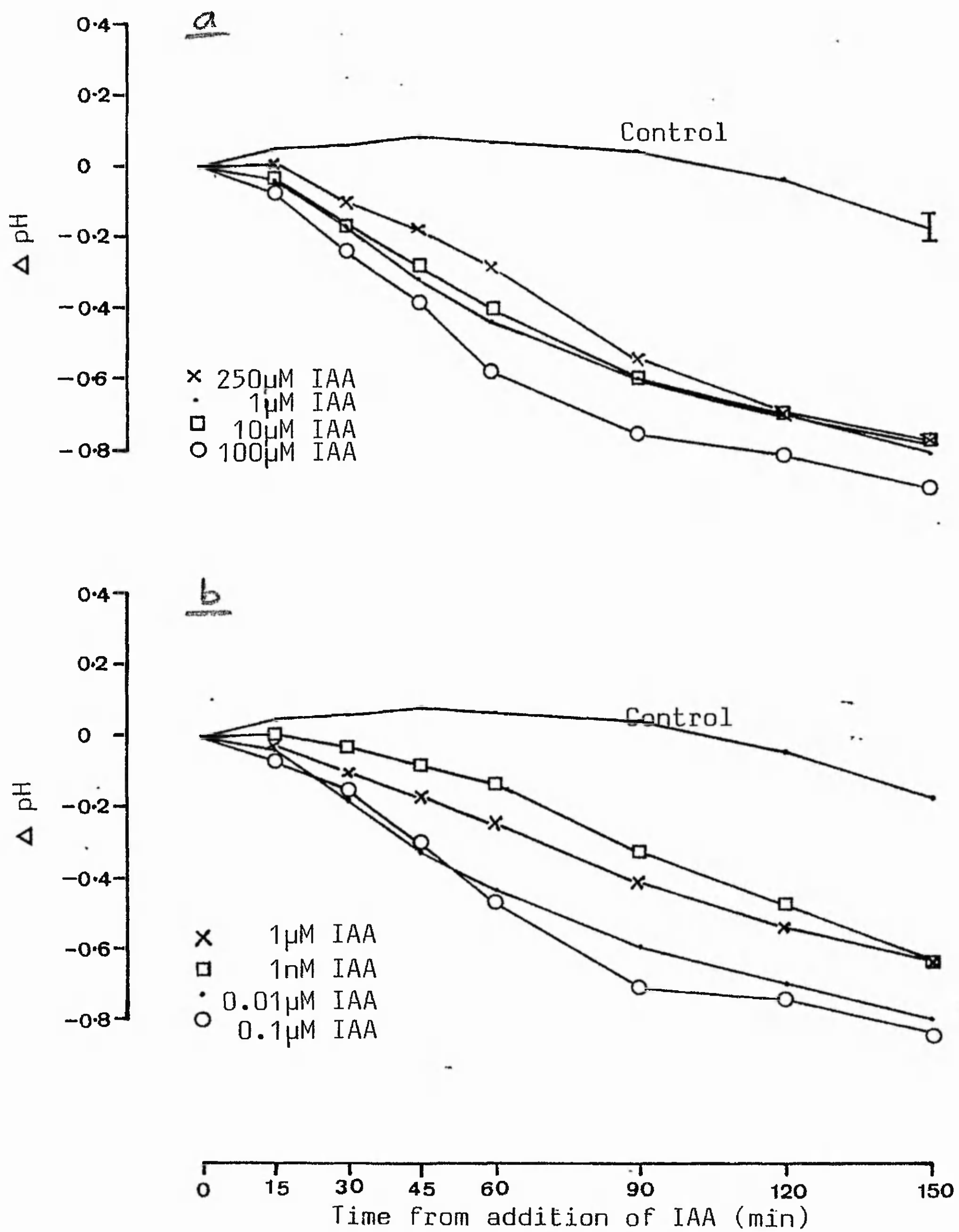


Figure 2.22 Effect of IAA on medium acidification by *Avena* coleoptile segments. n = 51, 25°C, light.

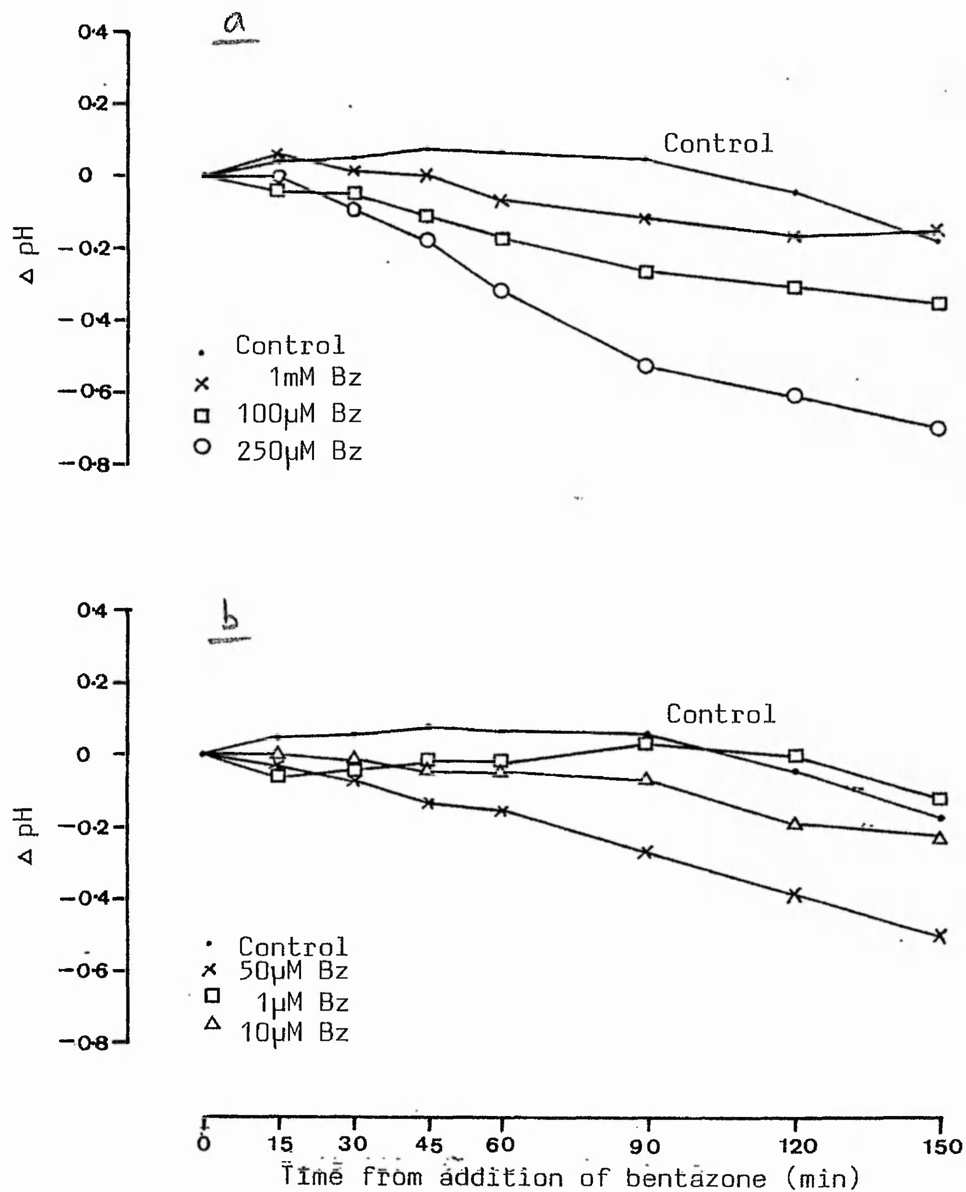


Figure 2.23 Effect of bentazone on medium acidification by *Avena* coleoptile segments. n = 51, 25°C, light.

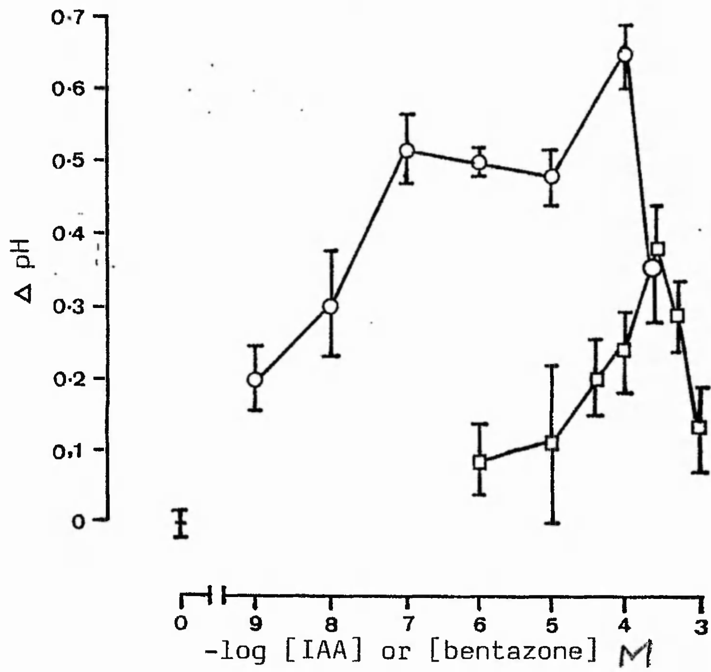


Figure 2.24 Effect of IAA (O——O) and bentazone (□——□) on medium acidification by *Avena* coleptile segments. n ≑ 51, 25°C, light, 60 min.

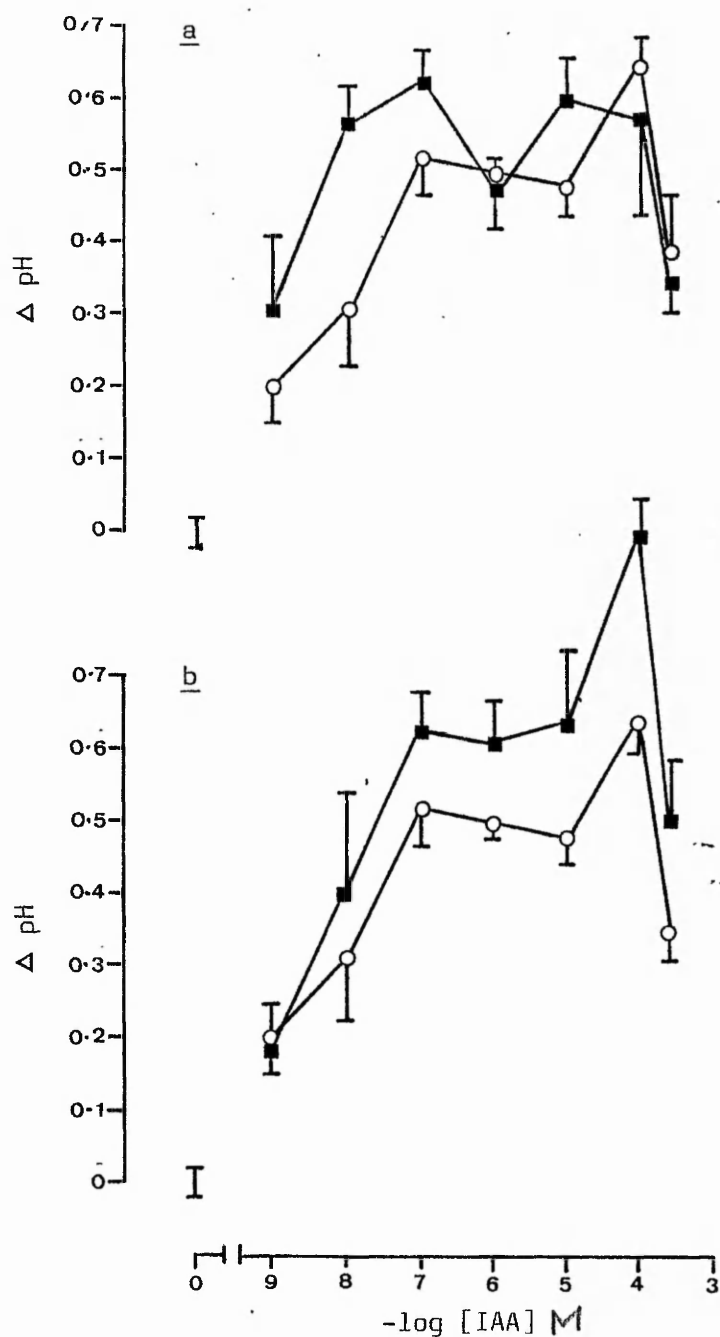


Figure 2.25 Effect of bentazone at 250μM (a), and 50μM (b), on IAA induced medium acidification by *Avena* coleoptile segments. n = 51, 25°C, light, 60 min. Closed squares, IAA + Bentazone. Open circles, IAA alone.

Avena coleoptile segment elongation bioassay. Significant interactions were also observed when the two compounds were applied at very low concentrations e.g. mutual synergism of root growth in cress germination, and enhancement of the response to 0.1mM IAA by 10nM bentazone in the coleoptile elongation experiment.

Although an investigation of the full range of IAA/bentazone interactions in medium acidification by *Avena* coleoptile segments would have been desirable, the constraints of time dictated an investigation of the effects of optimum bentazone i.e. 250μM, and sub-optimal bentazone (50μM selected) on the concentration dependence of the IAA response. The results of this investigation are presented in Figure 2.25. Bentazone at 250μM had no effect on acidification caused by IAA at concentrations greater than 1μM (Figure 2.25a). Fifty micromolar bentazone enhanced medium acidification across the full range of IAA concentrations tested with the exception of 1nM IAA (Figure 2.25b). The concentration dependence of the curve remains, but rates of acidification by 0.1μM, 1μM and 10μM IAA are raised to the level of the maximum attained by IAA alone, and the maximum ΔpH at 60 min is increased from 0.65 to 0.90, i.e. an increase of almost 40%. In all these situations where the IAA induced acidification was enhanced by bentazone the larger ΔpH was less than an additive effect, suggesting that the compounds are not acting independently.

2.5 Bentazone Activity in Auxin Bioassays - Discussion

Four areas of discussion are relevant to this chapter, these being the suitability of the methods used, the findings in relation to the literature, possible mechanisms producing the responses observed, and areas for possible future research. Each of these four spheres are now discussed in relation to the auxin like activity of bentazone.

2.5.1 A Consideration of the Techniques Used

There are many aspects of experimental procedure to be considered in studies of plant physiology. Consideration should be given to species, organ, age of material, uniformity, and conditions under which it had previously been grown e.g. in light or darkness, and water and temperature status.

Phaseolus vulgaris is the standard species which has historically been employed in studies on apical dominance (Hillman, 1970; Phillips, 1975). The method employed herein is essentially that developed by Nichols (1984), and enables the monitoring not just of lateral outgrowth, but of the development of other sub-apical organs subsequent to decapitation, thereby providing considerably more information of activity of various exogenous compounds in development of the plant apex.

Age and developmental stage of tissue may be important in this type of experiment, and the tissue used herein was at the most juvenile stage suitable for such a study. The apex above the pair of primary leaves had elongated by 10-15mm, i.e. it was at a length suitable to facilitate decapitation and subsequent application of lanolin paste to the stump. If the plants had been older the apex would have been more complex, making assessment of developmental changes more difficult.

As with other experiments in this series, the degree of replication is important. It is considered that number of plants per treatment, and their uniformity, was satisfactory in this

study, as it enabled the detection of significant differences between treatments in several tissues. The analysis of growth patterns in the apical dominance experiment was performed on a 48h cycle which, unlike the *Pisum sativum* assay for gibberellin activity (Ch. 3), was non destructive, and which permitted detection of the kinetics of the response. This was justified when it was shown that maximum differences had been reached in 6d in laterals, in 8d in cotyledonary internode and in 10d in petioles.

It was described by McIntyre (1973) that lateral bud outgrowth could occur in the presence of the apex if nutrition was exceptionally good. It could be argued, therefore, that the lateral bud outgrowth detected herein was a result of nutritive status rather than an effect of exogenous PGRs. However, this proposal can be discounted when it is recalled that intact plants retained apical dominance, i.e. nutritive conditions alone did not stimulate lateral outgrowth, decapitation was also required.

The effects of auxins on roots has been investigated (see Evans, 1985), with the conclusion at present being that, as in shoots, auxin stimulated elongation and proton efflux are closely correlated. This has been established from research largely employing roots of *Zea mays*, *Pisum sativum* and *Phaseolus vulgaris* (Lado *et al*, 1976; Evans, 1985), but the roots of *Lepidium sativum* in this assay responded as expected to a range of concentrations of IAA, and would appear therefore to be typical. It is therefore considered that tissue and methods used herein were suitable.

One aspect of procedure in this experiment which could be improved upon is the measuring of shoot and root length daily, in order to obtain information on the kinetics of the response. However, the cress tissue proved to be fragile, and non destructive analysis would have been very difficult. In order to monitor kinetics of the responses a large number of tissue samples would be required, but this would be possible.

Tissue selection for the study of IAA and bentazone in cell elongation in a well characterised system was relatively straight forward. There exists a substantial literature on the use of etiolated *Avena sativa* coleoptiles to elucidate mechanisms of auxin action in causing cell elongation (e.g. Rayle, 1973; Evans, 1985). This study, and that in measuring proton efflux from coleoptile segments, constituted a large part of this project, and as such considerable work was undertaken to ensure that the method used was reliable.

Having decided to use *Avena* coleoptiles, a suitable variety had to be found. Initially, two varieties of winter oat (Pennal and Panema) and one of spring oat (Maris Tabard) were studied. Both Pennal and Panema took 4d to reach a workable size of 3-4cm, while Maris Tabard took 3d. On visual assessment at 4d (Pennal and Panema) or 3d (Maris Tabard) the population of Maris Tabard appeared more uniform. Uniformity of tissue in short term experiments (1h) is particularly important, as Trewavas (1981) demonstrated in describing how sensitivity of wheat coleoptiles to auxin varied in time (see Chapter 1). In such a population physiological age (stage of development) also changed with time, and a non uniform population clearly contains many individual plants of differing physiological stage, and therefore probably differing ability to respond.

Preliminary investigative studies of elongation of coleoptile segments of the three varieties showed dose response of Maris Tabard to IAA to be the most uniform and reproducible, and this variety was selected to continue the study.

Having established a suitable tissue for the work several aspects of experimental procedure remained to be addressed.

1. Decapitation of coleoptiles. As described in Chapter 1 the tip has historically been considered as the site of IAA synthesis. While there is doubt about this there is usually a high IAA content in the tip, and as such it should be removed in order to examine the effects of exogenous compounds in a tissue of low endogenous IAA.

2. Storage of coleoptile segments while others are being prepared. Storage of coleoptiles in aqueous medium would have effectively lengthened pre incubation, this was found to be technically important. Tissue was therefore stored dry for approx 30 min. There was concern that water loss during this period may upset the coleoptile physiology, but figure 2.26 shows that only about 0.8% of weight was lost, presumably by dehydration, over 30min, and as such this should have little effect, particularly as the population was randomly sub divided.

3. Period of pre-incubation. A pre-incubation period to deplete endogenous auxin was adopted (see e.g. Evans and Schmitt, 1975). Figure 2.27 illustrates how preliminary studies showed growth of segments to stop at 45 min of incubation in auxin free medium, with spontaneous elongation recommencing some 30 min later. Pre-incubation period was therefore 45 min, with segments then transferred to fresh experimental medium.

4. Period of incubation. This was of 5-60 min, preliminary studies showing elongation as linear over the first hour (Chapter 2.4.3). Initially, a different sample of 15 segments was used to obtain mean length for each time period. It would have been more desirable to measure elongation of a single sample of 15 segments continuously over the first hour. This raises the point of measurement of length of tissue segments.

5. Measurement of segment lengths. While 15 segments enabled accurate determination of mean length at any particular time e.g. 60 min, the finding that elongation over the first hour was linear does not agree with documented responses, these showing a lag of 10-15 min (Vanderhoef et al, 1976). It is very difficult to explain a purely linear response in physiological terms, and as such it is suggested that a better method of measurement could be found. Had the subsequent extent of this particular study been realised at its inception it is felt that development of a technique for constant monitoring of elongation of elongation would have been a worthwhile investment. Such a technique is described by Penny et al (1972), albeit employing dicot tissue. Clearly constant measurement allows determination of kinetics, useful in determining sensitivity from initial rates of response as practiced by Weyers et al (1987).

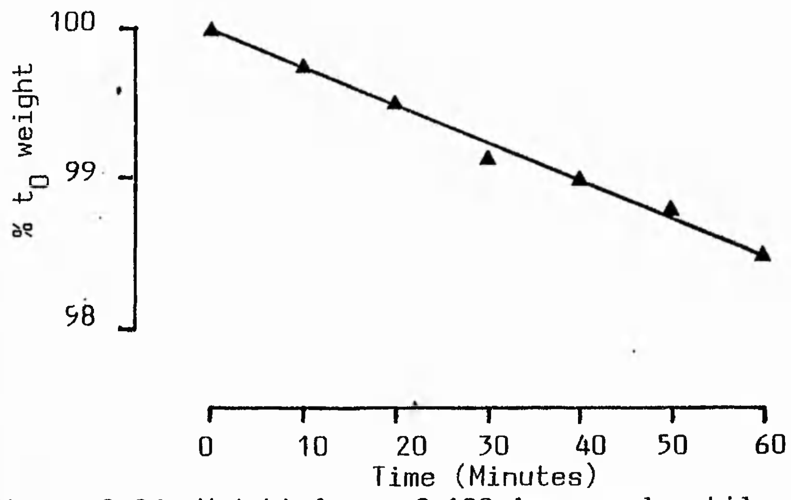


Figure 2.26 Weight loss of 100 <u>Avena</u> coleoptile segments following excision.

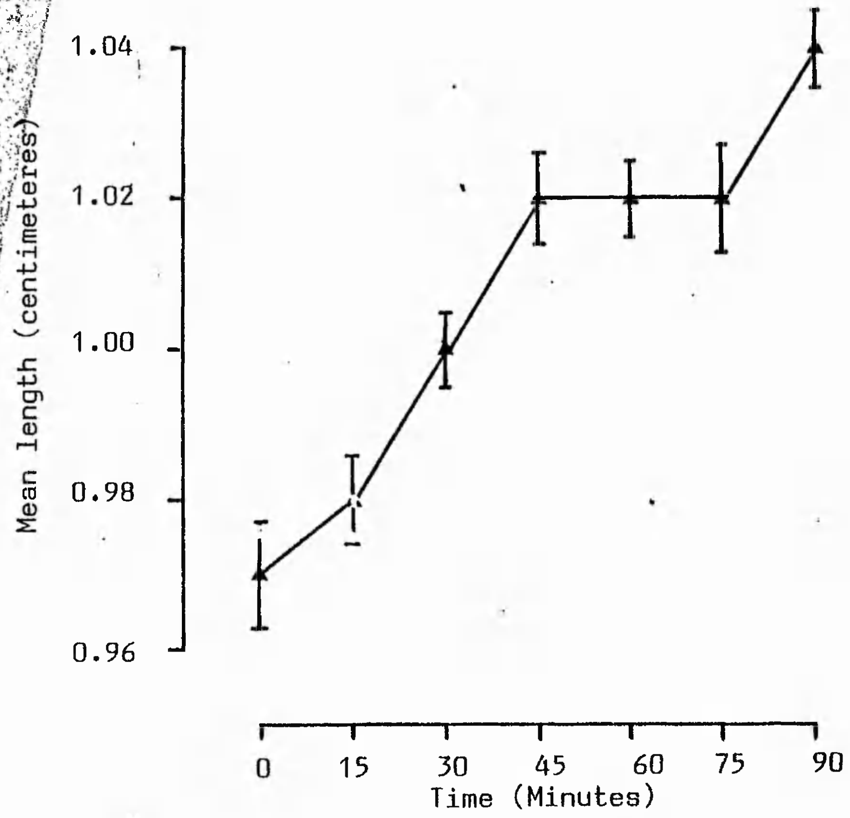


Figure 2.27 Spontaneous elongation of <u>Avena</u> coleoptile segments following excision. n=45, 25°c, darkness, pH 6.0.

6. Intactness of tissue. Intactness can present a number of problems. The cuticle of the coleoptile segment does not permit free movement of protons between the tissue and the bathing medium. As such the internal pH can only be assumed as being the same as that externally. As degree of dissociation of molecules such as IAA, and therefore their activities, are affected by pH this has serious implications. The cuticle should not however present a problem to IAA uptake, as this is an active process which can occur very rapidly through the cut ends of coleoptile segments (Evans, 1985).

One approach to ensure equilibration of pH between interior and exterior of the tissue is to remove the coleoptile epidermis, and with it the cuticle. However, peeling of tissue is undesirable according to Pope (1982), who demonstrated that the extent of peeling proportionally inhibited the extent of coleoptile elongation. There is a clear conflict here of requiring to know conditions within the tissue segment, and maintaining tissue as close as possible to its natural state. The same concern arose in the experiment to monitor reduction in bathing medium pH by *Avena* coleoptile segments. Removal of the cuticle is required to facilitate escape of excreted protons into the medium and hence to the pH electrode, yet a "normal" state of tissue is desirable. The light abrasion of tissue using a carborundum slurry, as practiced herein, may be a suitable compromise. However, care is needed in interpretation since the epidermis is the proposed location of auxin binding proteins, possibly having a receptor function, (Firn and Digby, 1977; Brummell and Hall, 1980; Lobler and Klambt, 1985b; Kutshera *et al*, 1987). Gentle abrasion of the coleoptile cuticle should permit efficient penetration of the exogenous compounds, although may not be necessary for this to occur, and the fact that bathing medium pH was seen to drop suggests that an equilibration of proton concentration is facilitated.

As with other experiments described herein (*Pisum sativum*, *Lepidium sativum*, *Avena sativa* coleoptile elongation) it is desirable to collect data on kinetics of the response. While the system employed herein enabled regular assessment of pH change

from the same tissue sample (15 min sampling interval) extrapolation to lag time could be assessed reasonably accurately. However, a continuous monitoring via a chart recorder would provide more accurate data on kinetics. This would however be considerably more time consuming.

The conditions of initial pH, buffering capacity of medium, aeration of vessels etc were developed in this laboratory by Dr P.J. Fitzsimons, and provided a reliable and reproducible system for use in this study.

2.5.2 Interpretation in Relation to the Literature

Documented responses in studies of apical dominance in species including Phaseolus vulgaris suggest that apical intactness retains apical dominance i.e. inhibits outgrowth of axillary laterals (Phillips, 1975; Tomar, 1985). This phenomenon was observed in this study. The literature would also suggest that apically applied exogenous IAA can substitute for the apex in retaining apical dominance. However, this was not observed; in fact the reverse occurred. The decapitation of the apex released the lateral from apical dominance, and IAA stimulated the subsequent outgrowth in a concentration dependent manner, with an apparent optimum at around 10-100μM (Figure 2.14). As with exogenous IAA, bentazone also stimulated lateral outgrowth in a concentration dependent manner, optimum at 0.1-1mM. It may be that inappropriate concentrations of IAA and bentazone were used in this experiment, or that insufficient IAA or bentazone was present in the applied lanolin paste to cause reimposition of apical dominance. Perhaps tissue age was wrong, or that imposed environmental conditions produced this atypical response. It is felt that the similarity of effects of IAA and bentazone in this tissue in this assay is a significant point. Also significant is the degree of interaction between the two compounds in this tissue (Figures 2.15-2.18).

As mentioned previously, the author is unaware of many published data on effect of apical decapitation on subsequent growth of sub-apical organs, and so comparisons of effects observed remain difficult. In the other organs studied, apical decapitation led to enhanced growth of primary leaf petioles which was further enhanced by IAA in a concentration dependent manner. As in the first lateral, bentazone behaved similar to IAA in this organ. Again, there were interactions between the two compounds in this tissue. Similarly, in the cotyledonary internode, apical intactness had no effect, but IAA and bentazone could independently stimulate elongation. However, depending on concentration, bentazone could also inhibit elongation of this tissue. IAA and bentazone also interacted in this tissue. Hall, Brummell and Gillespie (1985) reported that apically applied exogenous IAA

caused stimulation of growth of internodes of *Pisum sativum* in the short term (up to 4h), but that this extra growth had been made up by control plants after 24h, so the demonstration of apically applied IAA effecting growth of sub-apical organs is not without precedence.

In the bioassay examining effects of exogenous IAA and bentazone on early growth of dark grown *Lepidium sativum* (cress) it was observed that 1mM IAA was supra optimal to elongation of both shoots and roots, and this concentration is considered supra-optimal to growth in all auxin sensitive tissues (Evans, 1985). A stimulation of shoot elongation may have been expected by lower concentrations of IAA, but this was not observed, due perhaps to the intactness of the plant, or to the conditions imposed. Bentazone at lower concentrations partially stimulated shoot elongation, but not significantly (Figure 2.19). Bentazone behaved similarly to IAA in root tissue, being inhibitory at high, and slightly stimulatory at low concentrations. As in the experiment on apical dominance in *Phaseolus vulgaris* bentazone, when active, has activity similar to IAA, and in no instance is its activity radically different to that of IAA. In shoots, low concentrations of bentazone significantly reduced growth in the presence of the higher concentrations of IAA, while high concentrations of bentazone reduce responses to 1μM and 0.1μM IAA, i.e. bentazone is largely antagonistic to IAA in shoots. In roots there were very clear interactions, with high bentazone reducing effects in presence of low IAA, and a particularly clear interaction between low IAA and low bentazone, which coacted synergistically. Possible mechanisms for these interactions will shortly be addressed.

Unlike the experiment on *Lepidium sativum* growth, auxin induced elongation of *Avena sativa* coleoptile segments is a particularly well documented response to exogenous PGRs, as it has received considerable input as a method to probe the initial mechanism of action of auxin. As mentioned in section 2.3, it was found in this study that elongation was linear. As discussed

earlier in this section it is felt that this was due to the method of measurement employed, as the weight of documented evidence indicates a lag period of 10 min (Evans, 1969), or 12-15 min (Dela Fuente and Leopold, 1970) in Avena or Zea coleoptiles, or 15-20 min in Lupinus hypocotyls (Penny, Penny, Marshall and Heyes, 1972). Evans (1985) summarised lag period of response of various tissues to exogenous auxin as averaging 6-15 min. Clearly the existence of a lag period has more relevance physiologically than a linear response. It is felt that a higher definition method of measurement of elongation would have been more desirable. However, the concentration dependence of the IAA response observed herein (optimum c. 10μM, 0.1mM IAA) is similar to the that reported by Evans (1985) at c. 5mM IAA. There are no previous reports known of activity of bentazone in a coleoptile system, however on patterns of growth induced and concentration dependence it can reasonably be concluded that it acts as an auxin.

Further interesting observations were made when either IAA or bentazone were applied with various inhibitors of coleoptile elongation. This author is unaware of reports of GA3 or GA4/7 activity in a coleoptile system, but it was similarly inhibitory to IAA and bentazone responses. The bentazone and IAA responses were similarly inhibited by a range of BAP concentrations, BAP being known as an inhibitor of the first (acid) phase of auxin action (Vanderhoef, Stahl, Siegel and Zeigler, 1973). Similarly, with ABA, which inhibited IAA and bentazone responses by a similar magnitude. Studies on kinetics would have been useful here, as Philipson, Hillman and Wilkins (1973) showed that ABA inhibited IAA rapid growth responses in Avena coleoptiles inhibition commencing 30 min into the incubation. Rehm and Cline (1973) also reported an inhibition of the IAA response in Avena coleoptile segments, but they detected a lag of only 4 or 5 minutes. The opposite effect of the ethylene generating compound, Ethrel C, on IAA and bentazone effects agrees with the documented interactions of ethylene and auxin (Burg and Burg, 1966). In rice coleoptiles Ishizawa and Esashi (1983) found IAA and ethylene to interact, and suggested that elongation was regulated cooperatively by the two compounds in this tissue.

Several metabolic and plasmalemmal ATPase inhibitors were also investigated in their effects on IAA and bentazone responses.

CCCP is a compound known to be a metabolic inhibitor (Heytler and Pritchard, 1962), and Hager et al (1971) also described it as rendering membranes "leaky" to protons. If this aspect of its activity was operating here it would inhibit formation of a proton gradient and hence nullify the acid growth phase. If an uncoupler it would prevent active cell wall acidification. However it is active, it similarly inhibited both the IAA and bentazone responses. Balke and Hodges (1979) described diethylstilbestrol (DES) as an inhibitor of plasma membrane ATPases. This compound also similarly inhibited the IAA and bentazone responses. Sodium orthovanadate ($NaVO_4$) also disrupted IAA or bentazone enhanced coleoptile elongation.

From the aforementioned findings a picture emerged of IAA and bentazone enhancing elongation in coleoptiles via a similar, or the same mechanism, one which occurred rapidly in a concentration manner, and required ATP, and possibly required formation of a proton gradient across the plasmalemma. The data appeared to fit the acid growth theory of auxin action, with bentazone having auxin activity. The next logical step was to conduct an experiment monitoring the effects of IAA and bentazone on the proposed mechanism of this response i.e. proton efflux from Avena coleoptile segments. The study of this phenomenon produced proton efflux (medium acidification) with a lag of 10-15 min, which is the same as the documented response for the same tissue of approx 10-12 min (Cleland and Rayle, 1978), 15 min (Cleland, 1976), and approx 20 min (Rayle, 1973). The exact drop in pH of bathing medium is dependent on many factors including concentration of auxin, and buffering capacity of bathing medium. However, the shape of pH drop curves obtained herein compare well to those obtained by Rayle (1973) who also demonstrated that pH of control tissue medium remained constant for around 100-120 min (Figure 4 and figure 6 in Rayle, 1973). The medium acidification observed here occurred without the requirement of additional calcium, which Cohen and Nadler (1976) had proposed as being essential. Presumably there was sufficient calcium within the tissue for only 2h experiment. Of course, a full investigation could be

undertaken if deemed worthwhile. Rayle (1973), having shown that 2,4-D (a growth active auxin) could stimulate proton efflux, while 3,5-D (growth inactive) could not, proposed the causal link between auxin stimulated elongation and proton efflux. It can be proposed here, if these parameters are applied, that bentazone is an auxin.

This author is unaware of any publications from outside this laboratory on interactions of two or more active compounds in proton efflux studies, and so the interactions between IAA and bentazone observed here are difficult to place in context. These findings are however of considerable interest with regard to currently developing models of herbicide action and interaction, and these ideas are fully described and discussed in Chapter 5.

2.5.3 Mechanisms of Action and Interaction

There are several mechanisms by which IAA and bentazone could be acting to produce the responses observed. In the apical dominance (Phaseolus vulgaris) experiment effects are measured in the long term i.e. several days. It is unlikely therefore that rapid effects of IAA or bentazone could account for these effects. There is a possible effect of long term RNA and protein syntheses, developmental processes known to be sensitive to exogenous auxins in both the short term (Theologis, 1986a) and long term (Zurfluh and Guilfoyle, 1980). Other PGRs e.g. ethylene can also effect these processes (Theologis, 1986b). Indeed, bentazone has been shown to be active in inhibiting RNA and its synthesis in the short term in Glycine max (Al-Mendoufi and Ashton, 1984). Longer term studies could evaluate the effects and significance of such findings in relation to IAA and bentazone action and interaction. As in the apical dominance experiment, long term (5d) effects were recorded in the cress seed germination experiment. Similar long term studies on RNA and protein syntheses could evaluate their significance in observed IAA and bentazone actions and interactions.

A second area in which the interactions could occur is in the effects on IAA/bentazone uptake, in both the apical dominance and cress germination experiment. It is highly doubtful whether the compounds could interact in uptake from a lanolin paste into a cut stem end, or from plants in almost total contact with filter papers moistened with test solutions. However, uptake studies using radiolabelled compounds could be made if required.

A further possible area of interaction is in effect of bentazone on IAA transport, or vice versa. Jacobs and Rubery (1988) have recently suggested that endogenous flavonoids may effect transport of endogenous IAA through binding at a non IAA receptor at the plasmalemma, and thereby indirectly disrupting IAA transport. It is not inconceivable that exogenous compounds such as herbicides could affect this or similar processes.

Interactions may also occur between the two compounds (IAA and bentazone) in relation to endogenous PGRs. One compound which immediately comes to mind is ethylene. It is known that exogenous auxin can stimulate ethylene biosynthesis (Yang and Hoffman, 1984), and ethylene effects often counter those of auxin (Lieberman, 1979). Perhaps IAA and bentazone in combination stimulate ethylene production disproportionately to their individual effects combined, and that this over-production of ethylene inhibits the elongation due to both compounds. This suggestion could be investigated by monitoring of ethylene production using e.g. gas chromatography.

All the possible mechanisms of action and interaction mentioned so far describe gross, or "end product" effects. They do not describe primary binding events and molecular bases for mechanisms of action. All responses in plant physiology must commence with a molecular binding event. It is assumed that subsequent observed responses occur in proportion to degree of molecular binding, probably involving secondary messenger systems acting as a signal amplification, and gross, end product effects, such as long term stem elongation, occur some way further down the cascade of events. The acid growth phase of cell elongation in Avena coleoptiles, if confirmed through ongoing research, is a simple system in which end effect i.e. cell elongation, occurs almost as the first event following binding of auxin at the plasmalemma. Indeed, the only intermediate event in this response is the pumping of protons, and it is even proposed that the auxin binding protein is an ATPase. (This proposition is still the subject of considerable research effort and academic debate). As previously described, action and interaction of IAA and bentazone on proton efflux from Avena coleoptile segments was observed in this study i.e. at an event close to the crucial initial binding event. It is therefore proposed that the IAA and bentazone interactions observed throughout this study occur at or near the initial binding event, and that all gross effects and interactions can be explained by events here. The various possible mechanisms of action and interaction are described and discussed in Chapter 5.

Chapter 3.

3.1 Occurrence, Metabolism and Action of other PGRs

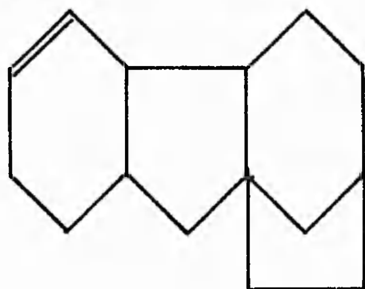
3.1.1 Gibberellins - Introduction

"Foolish Seedling" is a disease of rice, classic symptoms of which are uncontrolled stem elongation with subsequent lodging and death. The pathogen of the disease is a fungus, *Gibberella fujikuroi* (now reclassified as *Fusarium moniliforme*), and it was discovered in the 1930s that an extract of this fungus could induce characteristic stem elongation (Yabuta and Hayashi, 1939). Work over the following two decades further characterised this response (Brian, Elson, Hemming and Radley, 1954; Curtis and Cross, 1954), and the active compound was named "gibberellin", after the fungus. Since that time many related have been discovered and isolated from various sources including many algae, fungi and higher plants (MacMillan, 1985; Yamane, Yamaguchi, Takahashi, Sato, Takahashi, Iwatsuki, Phinney, Spray, Gaskin and MacMillan, 1985), with one recent count putting the total number of gibberellins at seventy two (MacMillan, 1985).

The gibberellins (GA1, GA2, ⟶GA72) now constitute a major group of endogenous compounds having dramatic and profound physiological activity, ranking second to the auxins in terms of knowledge of their structure, metabolism and physiology.

Differences in physiological activity exist between the various gibberellins, yet they all possess structural and chemical similarities. The gibberellins fall into two groups; those possessing the full diterpenoid complement of 20 carbon atoms, and those possessing only 19 carbons, the twentieth having lost by metabolism. The 19 or 20 carbon atoms form either a 4 or 5 ring system, all possessing the gibbane skeleton (figure 3.1), and having at least one carboxyl group. A number of side chains at differing carbon atoms produces the diversity. A widely commercially available gibberellin is GA3, and many studies, including the present, have been conducted using GA3 (figure 3.1 b) as an exogenous gibberellin standard.

a



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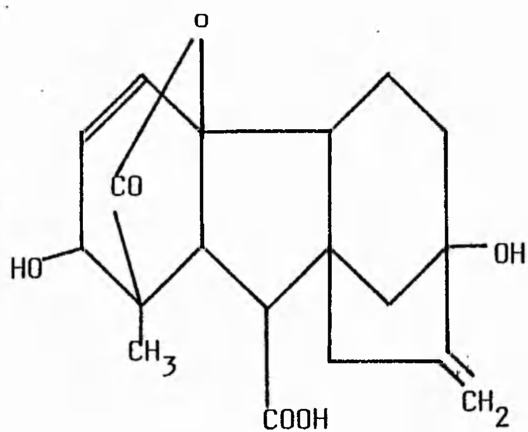


Figure 3.1 Structures of the gibbane skeleton (a) and of GA_3 (b).

Gibberellin Biosynthesis

Biosynthesis of the gibberellins occurs in very young shoot tissue i.e. in buds, and also in root tips and developing seeds. The pathway of synthesis is from acetyl CoA via mevalonate, kaurene and kaurenoic acid to GA12-aldehyde, which is the first compound in the pathway possessing a true gibberellane ring. This compound acts as the major intermediate for further molecular transformations (figure 3.2).

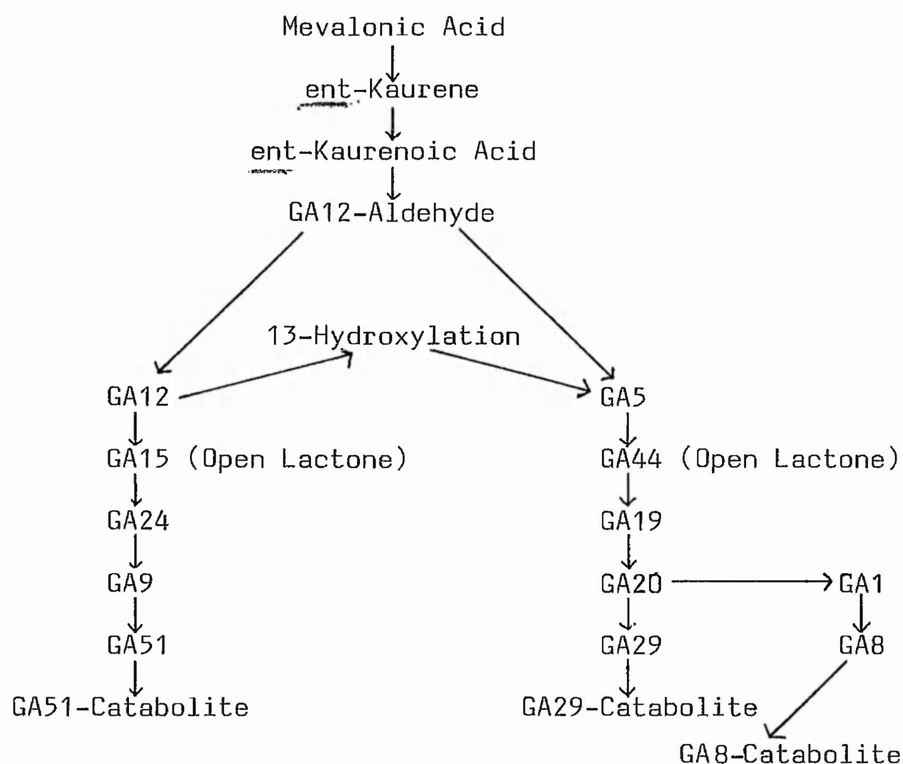


Figure 3.2 Gibberellin metabolic pathways in *Pisum sativum*. (From Sponsel, 1985)

Once synthesised, gibberellin follows a pattern of distribution consistent with transport in phloem, and exogenous gibberellin demonstrates typical phloem transported characteristics (Krishnamoorthy, 1975 Ch. 3). Gibberellins are also transported in the xylem, having been identified in xylem exudates (Krishnamoorthy, 1975 Ch. 3).

Gibberellins can exist in the plant in two main forms, as "free" gibberellin (considered the active molecule), or as

conjugates with sugars (glycosides). The glycosylation (binding of free gibberellins to sugars) of gibberellin and hydrolysis to free gibberellin is a common phenomenon in higher plants and is thought to act as a mechanism by which the concentration of active gibberellin is regulated. (For detailed discussion of gibberellin synthesis, interconversion and transport see e.g. Graebe, 1987).

Exogenous gibberellins can effect many processes of plant growth and development. They can release seeds of many species from dormancy, they can affect patterns of flowering and of fruit and seed development, and alter the processes of leaf abscission (Krishnamoorthy, 1975). However the most spectacular effect of exogenous gibberellin is the effect on shoot growth in intact plants. Gibberellins are unique in that, when applied exogenously, they can alter patterns of development in whole plants. Gibberellins can cause shoots of rosette plants to elongate and flower, and can transform dwarf varieties to resemble normal varieties of the same species. It has been suggested that the dwarf habit of such plants is due to either a total lack of endogenous gibberellins, or that those types which are present naturally are of the wrong type to cause normal growth and development (Ingram, Reid and MacMillan, 1986). One species which has been extensively studied in this respect is Pisum sativum, and the genetic factors and role of gibberellins in the response is discussed by Sponsel (1985) and by Ingram et al, (1986). It is the phenomenon of gibberellin induced internode elongation and stem growth, and the effects of IAA and bentazone on the process, which was studied herein.

Shoot growth is the visible outcome of two major processes occurring within the shoot, namely cell division and cell expansion. Cell division occurs in the apical meristem, in proximal sub-apical tissue, and in as many as 7 or 8 internodes below the apex (Brian and Hemming, 1955). In Pisum sativum cv. Meteor, exogenous GA has been shown to affect mainly cell division (Arney and Mancinelli, 1966). Cell expansion (elongation) occurs throughout actively elongating young tissues.

Gibberellins are known to stimulate cell division, particularly in the sub apex of several species (Wardlaw, 1975; Liu and Loy, 1976).

The effects of gibberellins on cell expansion are more widely documented. Gibberellins are reported to stimulate cell expansion in excised tissues including _Avena_ stem segments (Kauffman, Petering and Adams, 1969), rice coleoptile segments (Soni and Kauffman, 1972) and excised lettuce hypocotyl. Cell expansion is also enhanced by gibberellin in intact plants of various species, and in dwarf and normal varieties of the same species (Phinney and West, 1969; Kauffman _et al_, 1969). Gibberellin has been shown to enhance both cell division and elongation simultaneously in the same tissue sample (Feucht and Watson, 1958). For a review of the gibberellin enhancement of cell division and expansion the reader is referred to Evans (1985).

Although gibberellins affect both cell division and expansion the dual activities may not be independent. It has been suggested that the stimulation of cell elongation by gibberellin in turn stimulates cell division by physiological feedback, rather than by direct gibberellin effects on both processes.

Whether by stimulation of either cell division or expansion, or by a combination of both, exogenous gibberellin produces elongation of stems in intact plants. Using GA3 as an exogenous gibberellin standard the effect of auxin (IAA) and bentazone upon gibberellin mediated changes was studied herein, as were the effects of IAA and bentazone alone.

3.1.2 Cytokinins - Introduction

A further major group of low molecular weight compounds with physiological activity in plants is the cytokinins. Cytokinins are reported to have been identified in several plant tissues including immature *Zea mays* kernels, crown gall tissue of *Nicotiana tabacum*, *Raphanus sativus* seed, and seed and pod wall of *Lupinus augustifolius* by McGaw, Scott and Horgan (1984). Cytokinins have also been identified in primary leaves of *Phaseolus vulgaris*, but McGaw *et al* (1984) are forced to concede that the picture of cytokinin occurrence is still bleak and incomplete.

Cytokinins, both naturally occurring and synthetic, are generally adenine derivatives with various side chains attached at the C_6 position on the purine ring (Figure 3.3).

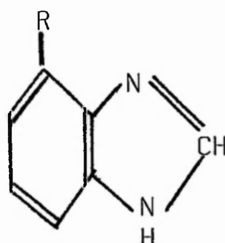


Figure 3.3 Cytokinin skeleton.

Cytokinins can exist in the plant in a number of forms. They can occur as free molecules, Letham and Palni (1983) reported that 15 free cytokinins had by then been identified with the aid of modern analytical techniques. Cytokinins can also exist as constituents of tRNA, and another 15-20 have been identified in this form (McGaw *et al*, 1984), who also reported that endogenous concentrations of cytokinin are less than 1nM g^{-1} fresh weight. Cytokinins can also exist as conjugates of sugar (glucosides) or of alanine, which are thought to act as short term storage techniques (Letham and Palni, 1983).

McGaw *et al* (1984) suggested that cytokinin biosynthesis appeared to be a minute secondary pathway of metabolism of

adenine, which therefore presents enormous experimental difficulties. However, the hydrolysis of certain tRNA species is a possible source of cytokinins. These two sources of cytokinin were considered by Maas and Klambt (1981), who concluded that cytokinin biogenesis derived 40-50% on tRNA breakdown, and 50-60% on the breakdown of isopentenylated mRNA. They doubted that a de novo synthesis was involved in cytokinin production (Maas and Klambt, 1981). The lack of sound knowledge in this area is reflected by the conclusion of Letham and Palni (1983) that studies in cytokinin metabolism, biosynthesis and metabolism are in their infancy.

Despite this lack of knowledge the site of synthesis of endogenous cytokinins appears to have been widely accepted as being the root tip (Letham and Palni, 1983), one piece of supportive evidence being that exogenous cytokinin can restore normal functioning to plants in which the roots are flooded (Reid and Railton, 1974). Cytokinins move in the xylem from the root to the shoot where they participate in the control of both development and senescence.

Cytokinins are active in a number of developmental processes, and can stimulate cell division (Letham, 1971), can delay senescence (described later), and can influence lateral bud development and chloroplast development.

As mentioned one of the effects of cytokinin is the retardation of senescence of green plant tissue. The effect of cytokinins in this respect can be measured by monitoring chlorophyll retention in treated tissues. Kuhnle, Fuller, Corse and Mackey (1977) assessed the relative activities of 20 naturally occurring and synthetic cytokinins in preventing chlorophyll loss from excised wheat leaves over a 6d, 25°C incubation in darkness. They found that the synthetic cytokinin, kinetin, with a relative activity of 1000, was most active, and 6-benzylaminopurine (BAP) had a relative activity of 369. The most active of the naturally occurring cytokinins tested had a relative activity of 336 (2-methylthioisopentenyladenine; Kuhlne et al, 1977).

Biddington and Thomas (1978) also reported that BAP and kinetin were more active in delaying senescence than the naturally occurring cytokinins, their study being conducted on oat leaves. Schistad and Nissen (1984) also found BAP and kinetin to be active in preventing chlorophyll degradation in barley leaves, but discovered a particularly complex dose response relationship. They were however unable to suggest a cause for this complexity, other than suggesting that multi-phasic uptake may be involved (Schistad and Nissen, 1984).

The mechanism of action of cytokinins in such a system remains unknown, however Sabater and Rodriguez (1978) suggested that cytokinins act through affecting activities of hydrolytic enzymes including chlorophyllase and other proteases and nucleases.

In the present study the effects of BAP and kinetin in preventing chlorophyll loss from excised cotyledons of *Raphanus sativus* was assessed, and the effect of bentazone , alone and in combination with these cytokinins in this bioassay was also assessed.

3.1.3 Abscission - Introduction

Abscission is the process by which plants shed organs including leaves, fruits and floral parts. The process of abscission is widespread in plant tissue, and this phenomenon has been of botanical interest for many years, with considerable input during the early part of this century (e.g. Sampson, 1918; Lloyd, 1927; Gane, 1934). Abscission occurs as part of the normal development of a plant, often as a process to ensure efficiency in plant survival e.g. shedding of shaded leaves as they become disadvantaged by the formation of a canopy above, the shedding of leaves of deciduous trees and shrubs in autumn, the abscission of fruits in order to facilitate seed dispersal, and the abscission of floral parts once their purpose has been served. These types of abscission are dependent upon environmental factors which determine normal patterns of plant development, including light, O_2, temperature, nutrient and water status. However, should any environmental factor become imbalanced then abscission can occur in response. Such "stress" abscission can occur in situations of low light intensity, extremes of temperature e.g. frost, extremes of water status, and mineral imbalance e.g. nitrogen deficiency.

As mentioned, study of abscission was underway early in this century, with most work involving the study of seasonally gathered material and attempts to correlate observed changes in abscission patterns with changing environmental conditions and tissue state (Sexton and Roberts, 1982). As with all such work it is likely that tissue uniformity was poor and subsequently observations poorly reproducible. However it was discovered that removal of plant organs such as the leaf accelerated abscission of the subtending petiole (Kuster, 1916). A further technique discovered to promote abscission was the use of gaseous compounds e.g. tobacco smoke (Fitting, 1911; Kendal, 1918; Hodgson, 1918; all in Sexton and Roberts, 1982). It is now known, of course, that the active gas is ethylene, which has been demonstrated to effect many physiological processes of plants (Abeles, 1973; Sexton and Roberts, 1973).

A further advance in methodology of study of abscission was the introduction of the use of explants, which gave large quantities of relatively uniform tissue, and enabled more strict control of environmental conditions. While it can be argued that explants are atypical of intact tissue and therefore misleading and unrepresentative, Sexton and Roberts (1982), in their review concluded that no major differences in the cell biology of abscission have subsequently emerged, and that it is therefore acceptable to assume that the processes of abscission, and those factors influencing them, including plant "hormones", enjoy a similar relationship in both systems. Indeed, the exogenous factors controlling abscission have largely been characterised by studying their effects on explants of several genera (Addicott, 1982, Ch.3), and an explant system was employed in the current study.

Abscission normally occurs in an anatomically distinct region at the base of the abscising organ, this region being known as the abscission zone. This zone consists of 5-50 tiers of cells, of which 1-5 layers are typically involved, these cells being characterised by being smaller than neighbouring cells, having smaller intercellular spaces, being more densely protoplasmic, having large deposits of starch and having highly branched plasmodesmata. Lignification is often weak or non existent within the abscission zone. (For a detailed description see reviews by Addicott, 1982, and Sexton and Roberts, 1982).

The abscission process typically follows senescence of the subtending organ i.e. the organ distal to the abscission zone. The process, although very widespread, is a highly coordinated sequence of biochemical events leading to wall breakdown within the 1-5 layers of cells around the ultimate fracture line. As abscission approaches there is an increase in metabolism within this region, and hydrolysis of middle lamellae and cell wall components ensues. These hydrolyses can occur in tandem with a series of cell divisions proximal to the abscission layer to form a layer of periderm cells to protect the abscission scar. (For a review see e.g. Addicott, 1982).

The abscission process has been discovered to consist of two distinct phases. While early work characterised simply the time taken to reach 100% abscission, further work identified a "lag" phase, during which the force required to break the abscission zone remains constant, this period can be indefinite depending upon auxin status of the tissue. During the second phase the breakstrength of the abscission zone rapidly declines over a period of 9-24h (Sexton and Roberts, 1982, and references therein). Exogenous factors controlling these two phases in explants, including the roles of the major plant growth regulatory compounds, have been extensively studied, mainly using Phaseolus or Coleus tissue, and the findings have been extensively described by Sexton and Roberts (1982), and by Addicott (1982, Ch.3).

The lag phase of abscission has been further subdivided into two stages by plant physiologists, the first being delimited by the period inside which exogenous auxin will continue to delay the onset of abscission. This appears to be more of an experimentally important phase than a strictly physiological one. If application of auxin is made within stage 1 then entry into stage 2 is delayed, and can be indefinitely delayed if auxin supply is maintained. This ability of auxin to delay entry into stage 2 of the lag phase is correlated directly to the ability of a particulate membrane fraction from the abscission zone to bind IAA, and to promote H^+ efflux, a good indicator of degree of auxin binding (Jaffe and Goren, 1979; Fitzsimons, Miller and Cobb, 1987). Stage 2 of the lag phase is correlated to the induction of syntheses of RNA and subsequently proteins, which will later, during the separation phase, drive the separation process.

As mentioned, exogenous auxin can indefinitely delay the entry into stage 2, if its concentration is maintained during stage 1, and this is interpreted in the whole plant situation as the declining auxin concentration from the subtending leaf, eventually falling sufficiently low to allow abscission to proceed i.e. stage 2 to be entered. Once stage 2 of the lag phase is entered exogenous auxin may, paradoxically, accelerate abscission, probably due to its effect on ethylene metabolism.

As implied in the preceding sentence, ethylene is a further growth regulatory compound strongly implicated as having a regulatory role in the abscission process (Abeles, 1973; Addicott, 1982). Ethylene is inactive in stage 1 of the lag phase i.e. that during which auxin concentration remains high, but once auxin concentration declines and stage 2 is entered, ethylene substantially accelerates the abscission process, possibly through acceleration of syntheses of hydrolytic enzymes via accelerated RNA synthesis (Sexton and Roberts, 1982, and references therein). It has been suggested, in view of the relationship of ethylene, RNA synthesis and abscission that ethylene is the inducer of abscission. However, this hypothesis has not been universally accepted. Whether ethylene is an inducer or simply an accelerator of abscission it clearly has a central role in abscission, as inhibition of its synthesis inhibits the process (Sexton and Roberts, 1982). The mechanism of ethylene action remains unknown.

Another compound having a profound effect on the lag phase of abscission is abscisic acid (ABA). When applied exogenously during stage 2 of the lag phase ABA accelerates the process of abscission, but it is unclear whether ABA acts directly or via an effect on ethylene metabolism. While stages 1 and 2 of the lag phase appear primarily dependent on auxin status of the tissue, it is unlikely to be a distinct boundary between the stages, given the nature of "hormone" interactions with regards to absolute concentrations, relative ratios of "hormones", and the nature of the tissue, and environmental sensitivities.

As with the lag phase, the separation phase of abscission is also sensitive to the plant "hormones". The linear pattern of loss of breakstrength is rapidly accelerated by exogenous ethylene, which is required for acceleration rate to be maintained, with decline in breakstrength having been demonstrated to be reversible on the withdrawal of ethylene (Abeles, Leather, Forrence and Craker, 1971). While this reversibility has been detected it is widely held that phase 2 is irreversible, and there is no reported instance of IAA either arresting or slowing down the events of the separation phase once it has commenced. Generally speaking, the effects of exogenous auxin are opposite

to those of both ethylene and abscisic acid, auxin retarding the processes of abscission, with ethylene and ABA promoting and accelerating abscission.

Of the other PGRs, exogenous gibberellins may stimulate abscission in explants, but they are thought to have very little effect in the intact plant. Cytokinins generally prevent abscission, by helping to maintain "sink" status of the organs in which their concentrations are high.

Addicott (1982) fully reviewed the subject of abscission, and concluded that the process is under "hormonal" control, with interactions occurring between the differing concentrations of various compounds in the subtended organ, the abscission zone, and other plant parts. IAA appears to be particularly important, mainly in its interactions with ethylene and ABA, but the process of abscission is complex, involving many factors, and remains incompletely understood. The possible roles of these compounds are summarised in figure 3.3a.

In this study, the effects of exogenous IAA, ethylene and ABA were investigated, along with assessment of any activity of bentazone in an explant system, using tissue obtained from Coleus blumei and Iresene herbstii.

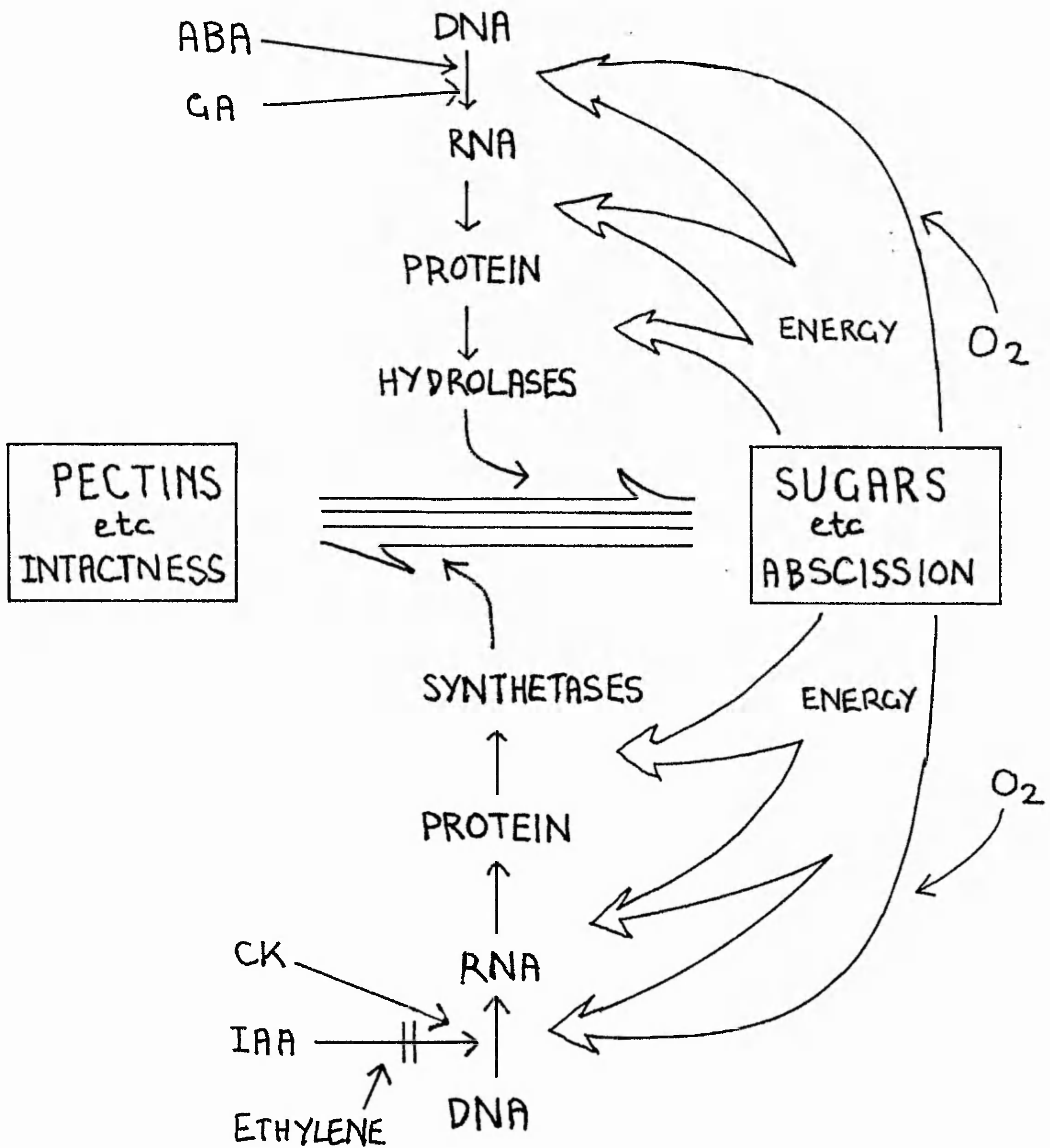


Figure 3.3a Model of the roles of the major PGRs in the abscission process. from Addicott, 1982.

3.1.4 Ethylene

Ethylene is perhaps the simplest structure of the plant "hormones", but is unusual in that it is gaseous under normal physiological conditions. It is found almost universally in plant tissues, Yang and Hoffman in their recent review (Yang and Hoffman, 1984) reported that ethlene had been found in leaves, stems, roots, flowers, fruits, tubers and seedlings of the various species' studied. The same authors summarised that discovery of the biosynthetic pathway for ethylene had been difficult as homogenates of ethylene producing tissues did not evolve ethylene.

Despite such difficulties, and following investigation of various possible precursors for ethylene biosynthesis, it has been found that methionine is the major, and perhaps the only, precursor of endogenous ethylene (Yang and Hoffman, 1984). In reviewing the literature further Yang and Hoffman (1984) suggest that the following has been identified as the pathway of ethylene biosynthesis, discovered in studies using apple.

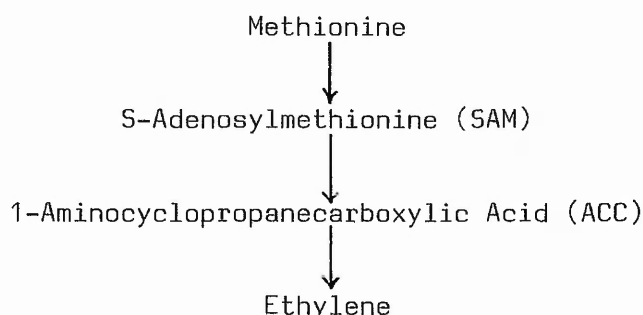


Figure 3.4 Pathway of ethylene biosynthesis in apple. (From Yang and Hoffman, 1984)

In several studies relating to ethylene effects in plant tissue aminoethoxyvinylglycine (AVG) has been employed as an inhibitor of ethylene biosynthesis (Mulkey, Kuzmanoff and Evans, 1982a and 1982b). AVG is thought to act by inhibiting the enzymic conversion of SAM to ACC (Yang and Hoffman, 1984).

As ethylene is found universally in plant tissues, so are all plant tissues capable of its synthesis (Yang and Hoffman, 1984). The control of ethylene biosynthesis is however a complex process, with many endogenous and environmental factors having an effect. Ethylene is synthesised and initiates and sustains responses as a part of the normal development of a plant e.g. in assisting the ripening of many fruits. In addition to such production in "healthy" plants ethylene production is often accelerated as a consequence of damage or stress to the plant, including physical wounding, bruising, chilling and freezing (Lieberman, 1979), which could all be encountered at some point during the normal life of almost any plant. However, enhanced ethylene production is also a response to concentrations of exogenous auxin (IAA) which are supraoptimal for e.g. cell elongation responses, and this can be detected with a lag as short as 20 minutes (Evans, 1974; Franklin and Morgan, 1978). Ethylene production can also be accelerated by application of herbicides such as 2,4-D and clopyralid (Thompson, 1989).

Ethylene, whether occurring as part of normal development, or in response to stress, can affect many physiological processes within the plant. Lieberman (1979) reviewed such effects. Ethylene can cause reduction of rate of stem elongation, thickening of stem and disruption of geotropism in etiolated _Pisum sativum_, known as the "triple response", and used as a bioassay for ethylene. Ethylene can also induce epinastic responses, and can promote flower and fruit maturation, and leaf abscission (Lieberman, 1979). Ethylene can also interact with auxin in regulating rice coleoptile elongation (Ishizawa and Esashi, 1983), and auxin inducible root elongation (Mulkey _et al_, 1982a and 1982b), which along with a promotion of abscission suggest the effects of ethylene to be essentially opposite to those of auxin in many aspects. Mulkey _et al_ (1982a) illustrated that auxin was able to induce H^+ efflux from maize root segments in the presence of the ethylene biosynthesis inhibitor AVG, but not in its absence, suggesting an auxin/ethylene interaction close to the site of action of both compounds.

Inherent in the theories about ethylene mode of action, as with those for the other "hormones", is the assumption that changing concentration of "hormone" causes changes in the response of the tissue (for a discussion of this, see Chapter 1). However, in support of the developing theories that changing sensitivity of tissue is an important factor in causing response to a "hormone" (Chapter 1), is a recent publication by Sanders, Smith and Hall (1986). These workers demonstrated that the region of pea epicotyl showing greatest response to a constant concentration of ethylene was that in which degree of ethylene/receptor binding was highest i.e. that which was most sensitive to ethylene. In their mini-review Sanders et al (1986) were able to conclude that ethylene binding sites (or receptors) appear to be membrane proteins. However, Sanders et al (1986) were forced to conclude that the basic mechanism of action of ethylene at a molecular level remained shrouded.

Despite the fact that there is no experiment herein which was conducted as a specific bioassay for ethylene, it is clear that ethylene is almost universal in plant tissues, and that it interacts with other "hormones" (Lieberman, 1979), and as such it is felt that the preceding brief description of ethylene and its effects is appropriate in this chapter.

3.2 Materials and Methods

3.2.1 Gibberellin Bioassay: Internode Elongation in Meteor Pea

Approx 100 seeds of *Pisum sativum* cv. Meteor were sown in tap watered vermiculite, and grown for 10d at 25°C under 16h photoperiod. At 10d a 10µl droplet of buffer or the appropriate PGR solution was placed in the axil of the second leaf from the base. There were at least 6 plants per treatment, and some treatments were repeated on up to 3 occasions, giving 19 or 20 plants. The plants were maintained in the same conditions for a further 5d, after which the lengths of all internodes were measured. The corresponding internodes of treated and control plants were compared using Student's t-test.

Growth regulator solutions were prepared by dissolving the required mass of GA3, IAA or bentazone in 1ml acetone. The volumes of stock solutions (1mM for all three compounds) were made to 100ml using 5mM K_2HPO_4/KH_2PO_4 buffer pH6. Adjustment to pH6, if required, was made using dilute HCl or NaOH in distilled water. Serial dilutions were prepared from these stocks using the above buffer. Final acetone concentration was never $>$ 1%, and usually $\leqslant$ 0.1%.

3.2.2 Cytokinin Bioassay: Chlorophyll Retention in Radish Cotyledons

Fodder radish (*Raphanus sativus*) seeds were densely sown at 0.5cm depth in 2.5cm vermiculite, tap watered, and propagated for 10d at 25°C, 16h photoperiod. Ten pairs of cotyledons were excised directly onto 25ml of test solution (BAP, Kinetin and bentazone in various combinations at various concentrations, see 3.3.2) in petri dishes. After 5d incubation (25°C, darkness) chlorophyll content was determined by the following method. The incubation medium was strained off the cotyledons which were blotted dry on tissue paper, this procedure being carried out under normal laboratory conditions. All subsequent steps were performed using ice cold acetone with samples kept on ice, in darkness, wherever possible. The tissue was weighed, and was ground in a chilled mortar and pestle with acid washed sand and 80% acetone. The resulting slurry was centrifuged under maximum

speed for 2 min, after which the supernatant was collected and its volume recorded. Readings were taken from the sample in a spectrophotometer at 645 and 663 nm. The chlorophyll content was determined by equation a.

$$Chl = \frac{(8.02 \times OD_{663nm}) + (20.2 \times OD_{645nm})}{100} = mg/0.5ml \text{ solvent} \quad (a)$$

Test solutions were prepared by dissolving BAP and kinetin in 2ml 0.1M NaOH, and bentazone in 2ml acetone. Required pH adjustments were made using dilute NaOH prior to final volume adjustment with 5mM K_2HPO_4/KH_2PO_4 buffer pH6.0, which was also used to prepare serial dilutions from stocks.

The experiment was repeated on three separate occasions, data was pooled, and mean values between treatments were compared using Student's t-test.

3.2.3 Abscission Bioassay: Abscission in *Coleus* and *Iresene* Explants

A population of nodal explants was obtained from mature *Coleus blumei* or *Iresene herbstii* plants which had been grown under glasshouse conditions of 14h photoperiod, 20-25°C day, 10°C night for several months. Each explant consisted of a stem section 2-3cm in length bearing a node with petioles trimmed to approximately 1.5cm. Lanolin pastes (see later for a description of their preparation) containing PGRs (ABA, IAA, bentazone) at 10µM to 1mM concentrations were smeared onto the cut ends of the petioles. Explants were then placed in petri dishes spanning a groove cut in agar, 5 *Coleus* or 6 *Iresene* per treatment. The procedure to this point was performed under normal laboratory conditions. Petri dishes containing either 2 or 3 explants were then placed in darkness at 25°C. At 24h intervals the explants were scored for number of abscissions. The experiment was repeated on 2 occasions and took 7 days in each case.

Lanolin pastes were prepared by mixing of PGR solutions previously prepared in 5mM K_2HPO_4/KH_2PO_4 buffer pH6.0 with anhydrous lanolin in the ratio 1 lanolin: 2 aqueous solution. Abscisic acid (ABA), IAA and bentazone were initially dissolved in acetone (Final concentration $\leqslant$ 0.1%).

3.3 Results

3.3.1 Gibberellin Bioassay: Internode Elongation in Meteor Pea

Figure 3.5 illustrates that exogenous GA3 caused a concentration dependent stimulation of stem growth of 10d old *Pisum sativum* cv. Meteor over the following 5d period, the highest of the concentrations used (1mM) stimulating the greatest elongation. Figure 3.5 also shows that IAA and bentazone, over a similar concentration range, caused little or no change in subsequent stem growth. Further analysis of those internodes in which the extra growth increment occurred is presented in Tables 3.1 and 3.2.

Table 3.1 Effect of a range of GA3 concentrations on growth of internodes (positions relative to node of droplet application) of 10d old *Pisum sativum* cv. Meteor (5d incubation, 25°C, light). Figures are percentage of control -100. ***P=0.001 **P=0.01 *P=0.05.

	Gibberellin Concentration				
Internode	1mM	0.1mM	10μM	1μM	0.1μM
	n=20	n=20	n=6	n=7	n=5
Below	+ 9	- 6	- 15	- 20	- 27**
1st above	- 6	- 6	- 12	- 7	- 13
2nd above	+ 72***	+ 32*	- 3	- 12	- 17
3rd above	+199***	+100***	+ 10	- 6	- 16
4th above	+ 38**	+ 11	+ 8	- 20	- 30**

One millimolar GA3 produced significantly more growth than control plants in the 2nd, 3rd and 4th internodes above the node of droplet application (n.d.a.). Additional growth in 0.1mM treated plants occurred in the internodes 2nd and 3rd above n.d.a.. Of the other concentrations tested only 0.1μM GA3 had a significant effect on internode growth, but since replication at this concentration was limited (n=5-7) more data points are considered necessary to confirm this observation.

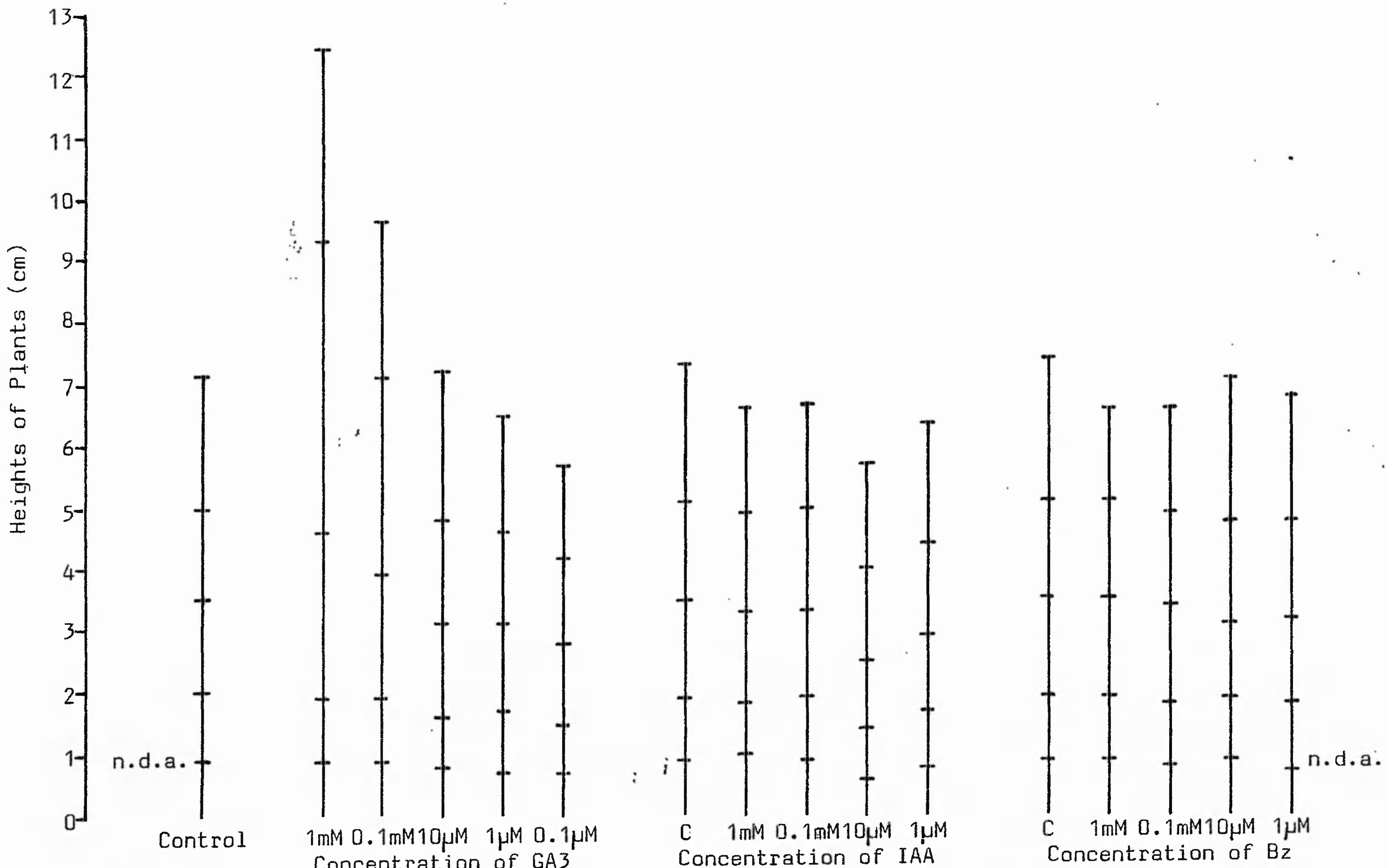


Figure 3.5 Effects of a range of concentrations of GA3, IAA and bentazone on growth of dwarf *Pisum sativum* cv. Meteor. n=20 or 6 or 7 (see Results). 25°C day, 16°C night, 5d.

Both IAA and bentazone at the highest concentrations tested (1mM and 0.1mM) had a significant effect only on the 4th internode above n.d.a. (Table 3.2) which both compounds at both concentrations significantly reduced in length by 26-35%. Lower concentrations (10μM, 1μM) of IAA apparently had a significant effect, but again replication was limited and the experiment would need to be repeated to establish the real significance of this response.

Table 3.2 Effect of a range of IAA and bentazone (Bz) concentrations on growth of internodes (positions relative to node of droplet application) of 10d old *Pisum sativum* cv. Meteor (5d incubation, 25°C, light). Figures are percentage of control -100. ***P=0.001 **P=0.01 *P=0.05.

	Concentration of IAA and Bz								
	1mM		0.1mM		10μM		1μM		
Internode	IAA	Bz	IAA	Bz	IAA	Bz	IAA	Bz	S.E.
	n=19	n=19	n=19	n=20	n=6	n=6	n=6	n=5	
Below	-15*	- 3	- 2	- 7	-35***	+ 2	-11	-16	± 6.3
1st above	-12	- 5	- 9	- 7	-20**	- 8	-15	-11	± 5.7
2nd above	- 5	- 1	- 4	+ 3	-27**	-21	-24*	- 7	± 5.8
3rd above	+ 1	+ 3	+ 4	- 5	- 6	+ 7	- 3	+ 3	± 6.1
4th above	-26**	-35**	-28**	-28**	-25*	- 2	-16	-12	±10.2

Table 3.3 presents the results of an experiment where IAA or bentazone were applied simultaneously with GA3. The results presented in Table 3.3 are relative to the GA3-only response.

Table 3.3 Effect of auxin (IAA) and bentazone (Bz) on GA3 stimulated elongation of internodes (relative to node of droplet application) of 10 d old *Pisum sativum* cv. Meteor (5d incubation, 25°C, light). Figures are percentage GA3-alone length -100. ***P=0.001 **P=0.01 *P=0.05.

	1mM GA3 plus:-				0.1mM GA3 plus:-			
Internode	1mM IAA	1mM Bz	0.1mM IAA	0.1mM Bz	1mM IAA	1mM Bz	0.1mM IAA	0.1mM Bz
	n=13	n=14	n=13	n=14	n=13	n=13	n=13	n=14
Below	-17*	+ 5	-19*	-12	- 8	+ 7	+27***	+ 4
1st above	- 5	+14	+ 8	+20	+ 1	+ 6	+12*	+ 8
2nd above	- 8	0	-14	+ 6	- 3	+21	+17	- 5
3rd above	- 9	+ 1	-34**	+ 3	-43***	-34***	-35***	-40***
4th above	- 5	- 4	-10	- 1	-39***	-16	-24	-16

IAA at 1mM significantly reduced the 1mM GA3 response in the internode below n.d.a. (P=0.05), and had a negative effect over the rest of the plant. Bentazone at 1mM had no significant effect on the response produced by 1mM GA3. IAA at 0.1mM significantly reduced the 1mM GA3 response in the internodes below (P=0.05) and 3rd above n.d.a. (P=0.01), while again 0.1mM bentazone had no effect on the 1mM GA3 response. IAA at 1mM significantly reduced the 0.1mM GA3 response in both the 3rd and 4th internodes above n.d.a. by 43% (P=0.001) and 39% (P=0.01) respectively. Bentazone at 1mM similarly reduced the GA3 response in this part of the plant but by a smaller, and only significantly in the internode 3rd above n.d.a. (34%, P=0.001). IAA at 0.1mM stimulated the GA3 response by 27% (P=0.001) in the internode below n.d.a. and by 12% (P=0.05) in the internode 1st above n.d.a.. Bentazone at 0.1mM had no effect on the GA3 response in this part of the plant. In the internode 3rd above n.d.a. both 0.1mM IAA and 0.1mM bentazone significantly (both P=0.001) reduced the GA3 response, by 35% and 40% respectively They also reduced the response in the internode 4th above n.d.a., but both non significantly.

In addition to the four internodes above n.d.a., some treatments produced an expanded internode five above n.d.a.. Table 3.4 presents data for treatments which treatments produced this response, the percentage of plants on that treatment, and the mean length of this internode 5th above n.d.a..

Table 3.4 Treatments which produced expansion of internodes distal to the 4th above n.d.a.

Treatment	n	% of plants producing 4th internode	Mean length of 4th Internode
Control	20	0	———
1mM GA3	20	60	26.9 ± 2.38
0.1mM GA3	20	40	27.5 ± 4.69
10µM GA3	6	17	13.0 (one only)
1µM GA3	6	33	15.0 ± 3.0
0.1µM GA3	6	33	17.5 ± 2.5
1mM IAA	20	35	16.0 ± 1.99
0.1mM IAA	20	58	12.8 ± 1.84
10µM IAA	6	33	22.0 ± 1.00
1µM IAA	6	33	16.5 ± 1.15
1mM Bz	20	50	13.8 ± 2.70
0.1mM Bz	20	40	8.6 ± 1.44
10µM Bz	6	17	15.0 (one only)
1µM Bz	6	17	14.0 (one only)
1mM GA3 +1mM IAA	13	38	25.6 ± 2.09
1mM GA3 +0.1mM IAA	13	62	22.6 ± 4.67
1mM GA3 +1mM Bz	14	42	25.5 ± 3.92
1mM GA3 +0.1mM Bz	14	50	26.9 ± 3.99
0.1mM GA3 +1mM IAA	13	23	20.7 ± 7.69
0.1mM GA3 +0.1mM IAA	13	62	17.9 ± 3.78
0.1mM GA3 +1mM Bz	14	28	24.2 ± 7.03
0.1mM GA3 +0.1mM Bz	14	50	20.0 ± 4.62

3.3.2 Cytokinin Bioassay: Chlorophyll Retention in Radish Cotyledons

Figure 3.6 illustrates the effects of the three compounds on chlorophyll content of radish cotyledons after 5d incubation when applied alone. Kinetin had no effect on chlorophyll content at any concentration tested. Ten micromolar BAP-treated cotyledons contained significantly more chlorophyll after 5d than did control tissue (P=0.05). No other concentration of BAP had a significant effect, but there appears to be a trend to a concentration dependent response (Figure 3.6). Bentazone did not affect chlorophyll content at any concentration studied (Figure 3.6).

When applied in combination with either BAP or kinetin bentazone did not significantly affect chlorophyll retention under the influence of either cytokinin i.e. there was no interaction (Tables 3.5a and b). A slight trend is observed in that for both cytokinins (kinetin and BAP), cytokinin plus bentazone treated tissue had higher chlorophyll contents than cytokinin alone treated tissue. However, as stated, this is not significant in any case.

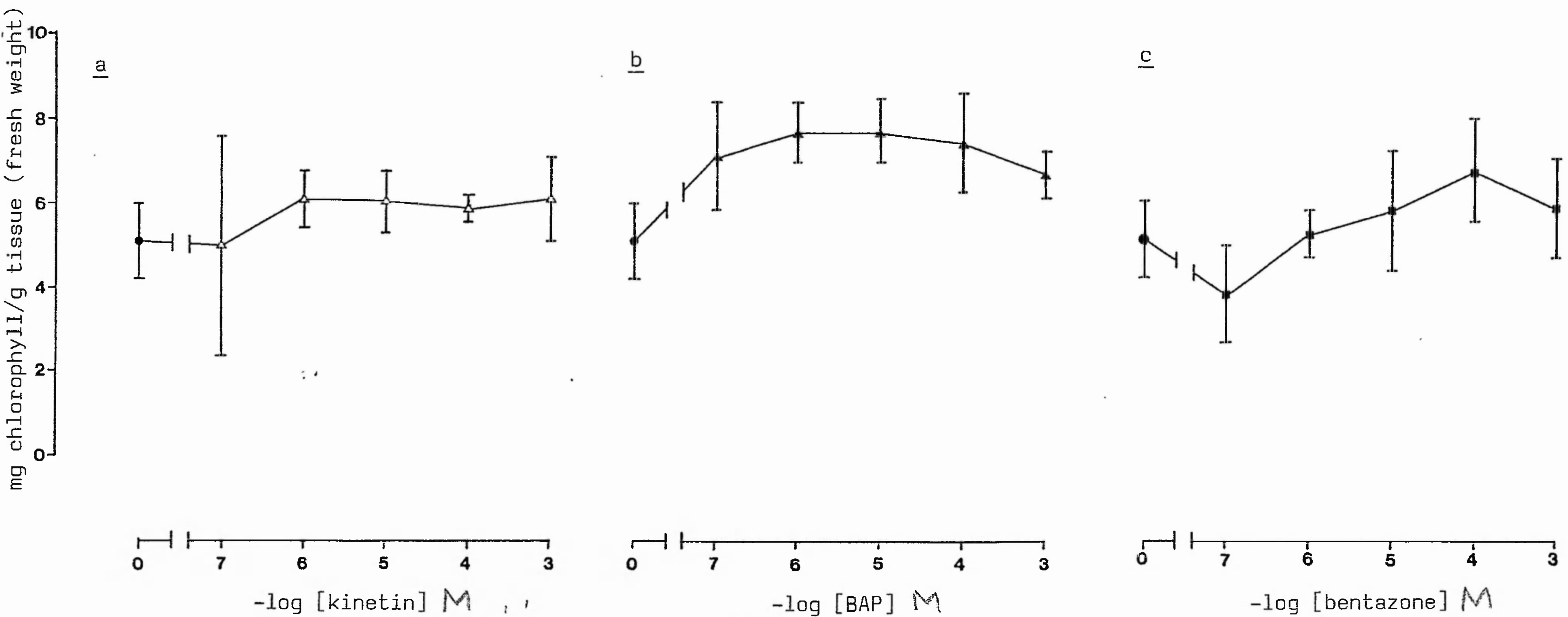


Figure 3.6 Effect of kinetin (a), BAP (b), and bentazone (c) on chlorophyll retention in excised cotyledons of *Raphanus sativus*. n=20 (10 pairs), 25°C, darkness, 5d.

Table 3.5 Chlorophyll content (mgg^{-1} fresh weight) of *Raphanus sativus* cotyledons incubated with kinetin (a) and BAP (b) alone, and each cytokinin in combination with bentazone.

a

Concentration of Bentazone	Concentration of Kinetin: 0	0.1mM	10µM	1µM
0	5.09 ± 0.84	5.85 ± 0.31	6.03 ± 0.72	6.09 ± 0.69
0.1mM	6.66 ± 1.22	6.65 ± 0.68	5.32 ± 1.01	7.95 ± 0.97
10µM	5.78 ± 1.39	8.08 ± 1.36	8.34 ± 0.64	6.02 ± 0.87
1µM	5.20 ± 0.55	7.41 ± 0.94	7.26 ± 0.70	6.57 ± 0.38

b

Concentration of Bentazone	Concentration of BAP: 0	0.1mM	10µM	1µM
0	5.09 ± 0.84	7.43 ± 1.19	7.69 ± 0.27	7.69 ± 0.69
0.1mM	6.66 ± 1.22	7.10 ± 0.53	7.74 ± 1.22	6.28 ± 1.27
10µM	5.78 ± 1.39	7.71 ± 0.84	7.54 ± 1.19	7.55 ± 0.84
1µM	5.20 ± 0.55	8.87 ± 0.80	8.05 ± 0.54	7.16 ± 0.65

3.3.3 Abscission Bioassay: Abscission in *Coleus* and *Iresene* Explants

Several treatments were common to the two experiments (one with *Coleus* explants, one with *Iresene*), and where this was the case the results are similar enough to be described as one. Where a treatment is unique to either experiment this will be indicated.

There was no abscission after 24h incubation for control tissue, but after 2d abscission proceeded rapidly and was complete within 3d (*Coleus*, Figure 3.7) or 4d (*Iresene*, Figure 3.10). The onset of abscission was delayed by IAA in a concentration dependent manner (both species, Figures 3.7a-c, *Coleus*; 3.10a-c, *Iresene*), with higher concentrations of IAA retarding abscission the most. After the onset of abscission in IAA treated explants, the process proceeded less rapidly than in control explants for *Coleus* (Figure 3.7a-c), but for *Iresene* explants the rate of abscission was similar to the maximum achieved in control explants by the end of the experimental period (Figure 3.10b and c).

Bentazone was successful in delaying the onset of abscission by one day when applied at its highest concentration (1mM, Figures 3.7d and 3.10d), but had little effect on the subsequent rate of abscission. At 0.1mM bentazone had no effect on abscission in either *Coleus* (Figure 3.7e) or *Iresene* (Figure 3.10e). Bentazone at 10µM apparently enhanced the processes of abscission (*Iresene* only, Figure 3.10f), but had no effect in *Coleus* (Figure 3.7f).

In *Coleus* explants 1mM ABA appeared to slightly retard the onset of abscission and to slow the rate of abscission once 50% abscission is reached (Figure 3.8a). However, in *Iresene* explants 1mM ABA increased the rate of abscission but had no effect on time to its onset (Figure 3.11a). In both species 0.1mM ABA increased rate of abscission without effecting its onset (Figure 3.8b, *Coleus*; Figure 3.11b, *Iresene*). In *Coleus* explants 10µM ABA had no effect on either onset or subsequent rate (Figure 3.8c), while in *Iresene*, 10µM ABA increased rate of

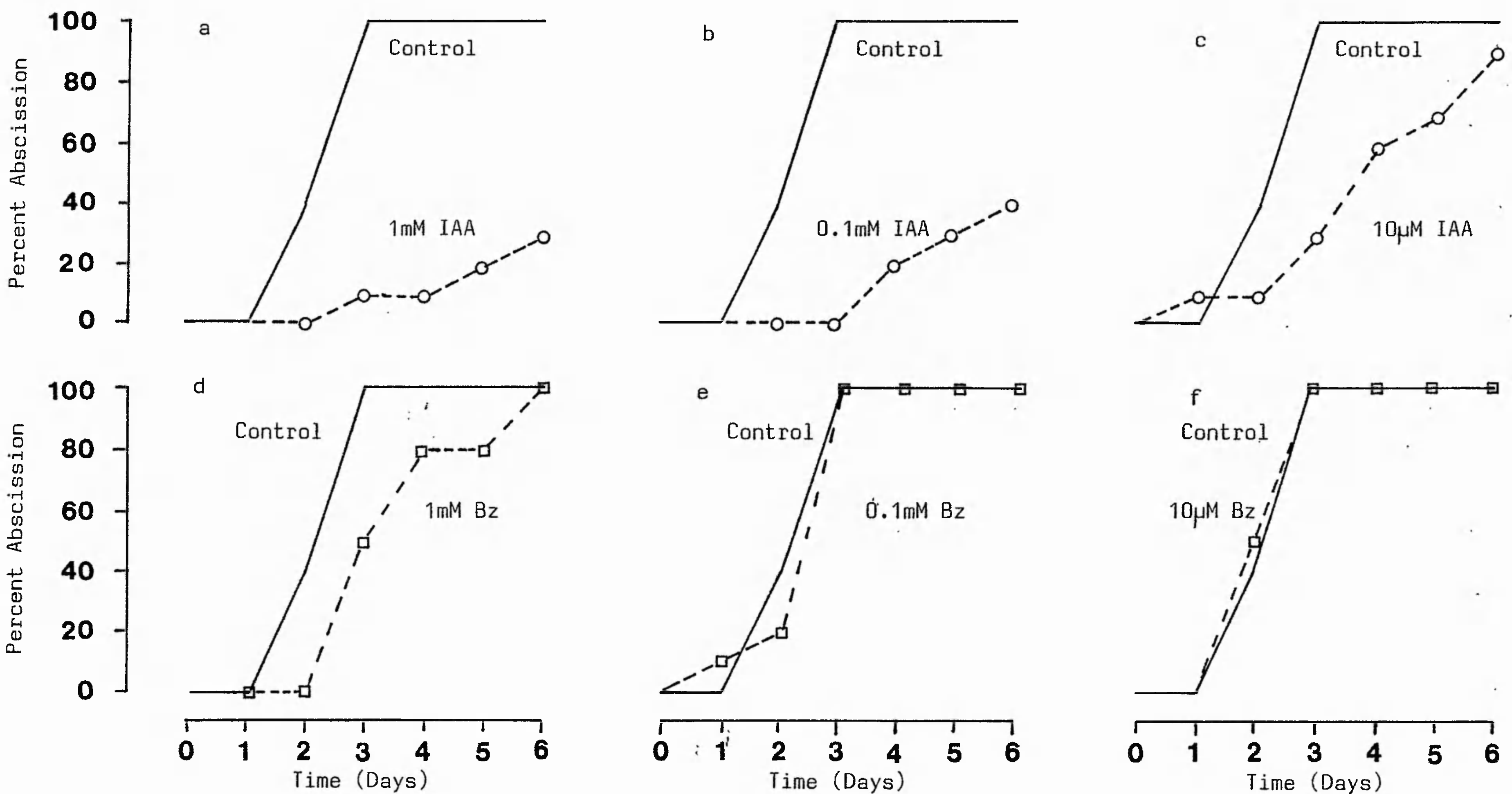
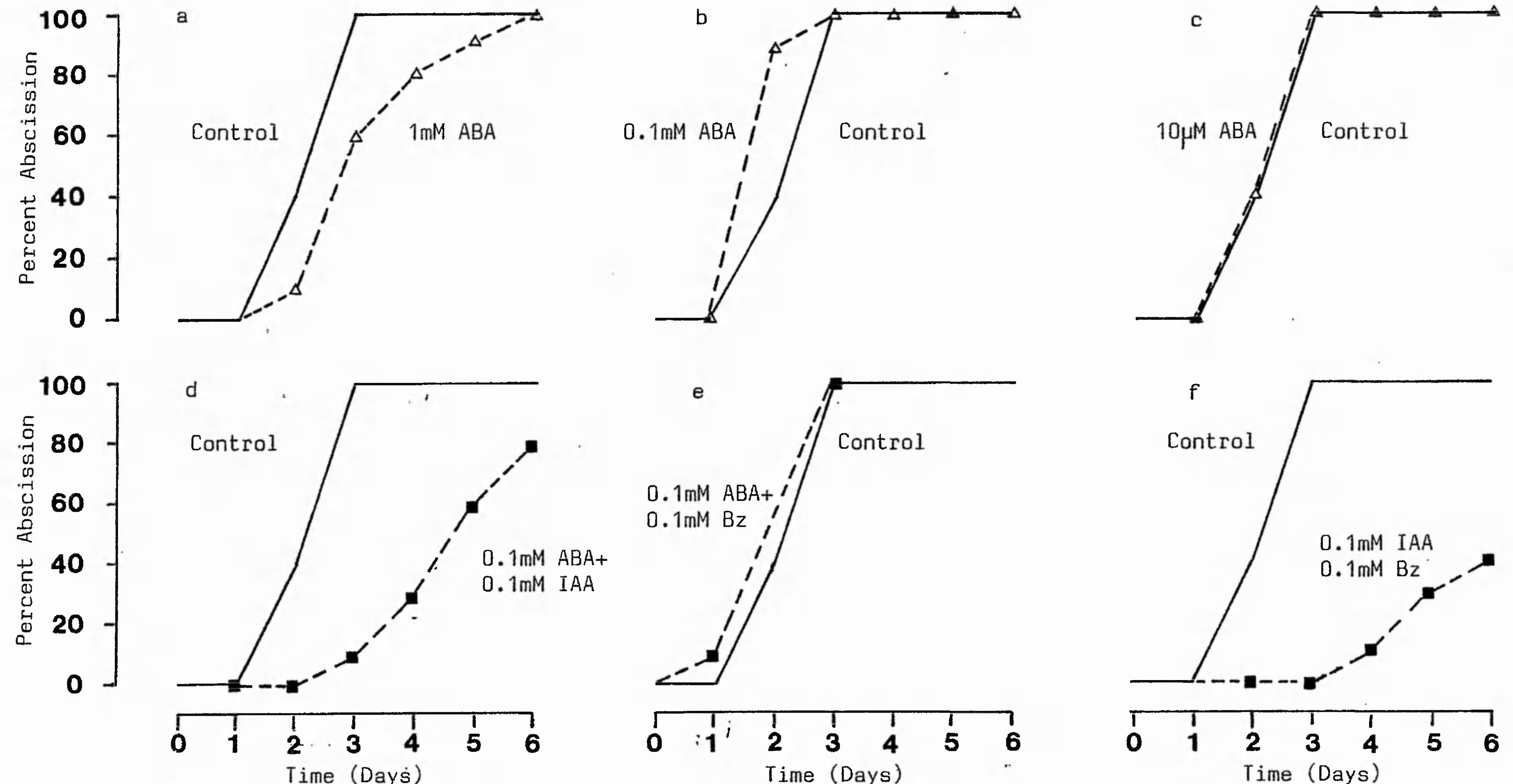


Figure 3.7 Patterns of abscission of Petioles of *Coleus blumei* explants treated with IAA and bentazone. 25°C, darkness.



<u>Figure 3.8</u> Patterns of abscission of Petioles of *Coleus blumei* explants treated with ABA and/or IAA and/or bentazone. 25°C Darkness.

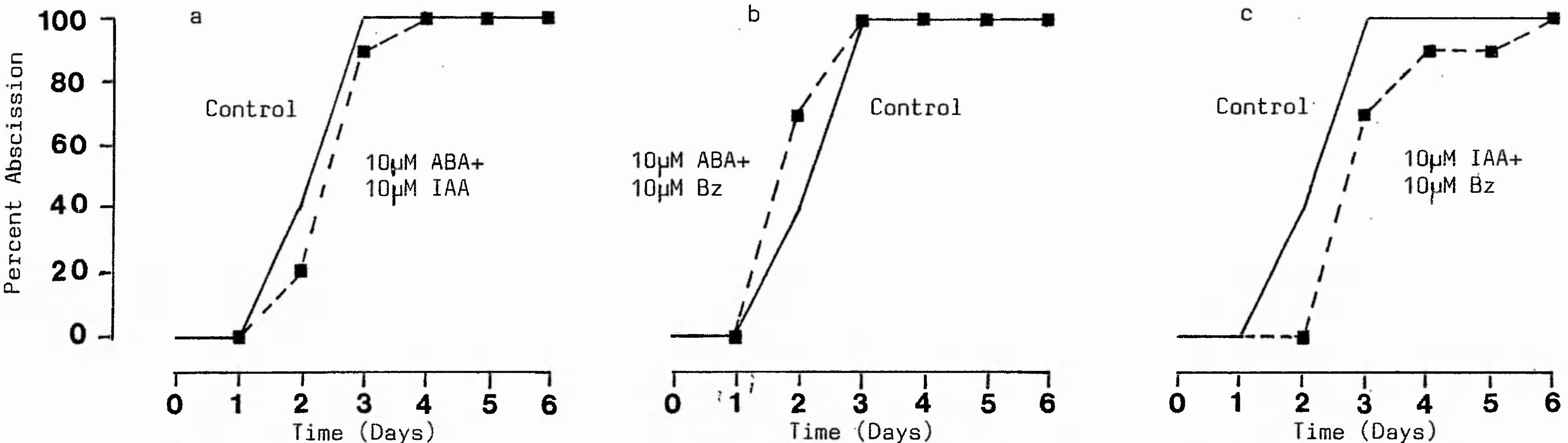


Figure 3.9 Patterns of abscission of petioles of *Coleus blumei* explants treated with ABA and/or IAA and/or bentazone. 25°C, darkness.

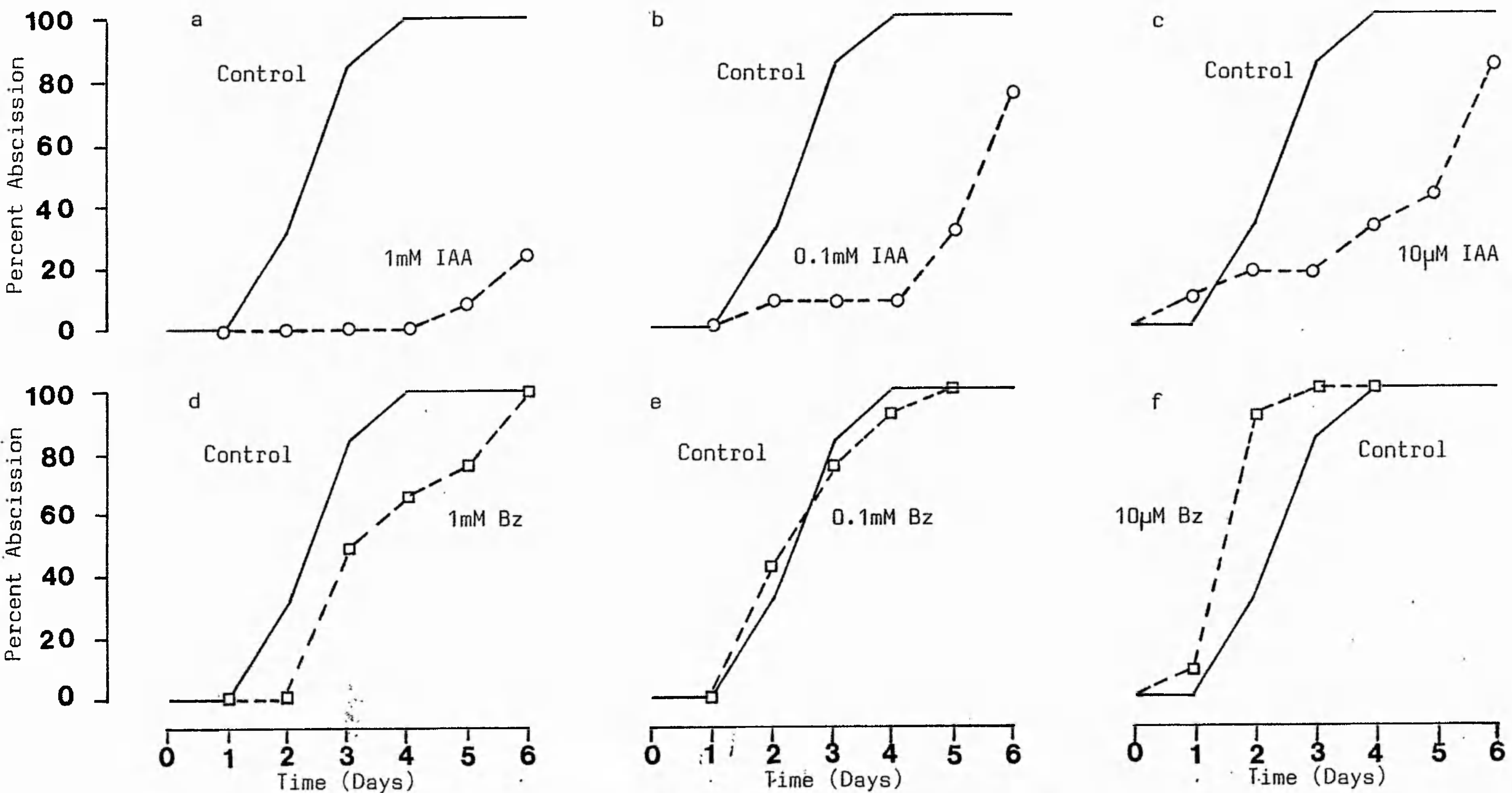


Figure 3.10 Patterns of abscission of Petioles of *Iresene herbstii* explants treated with IAA and bentazone.

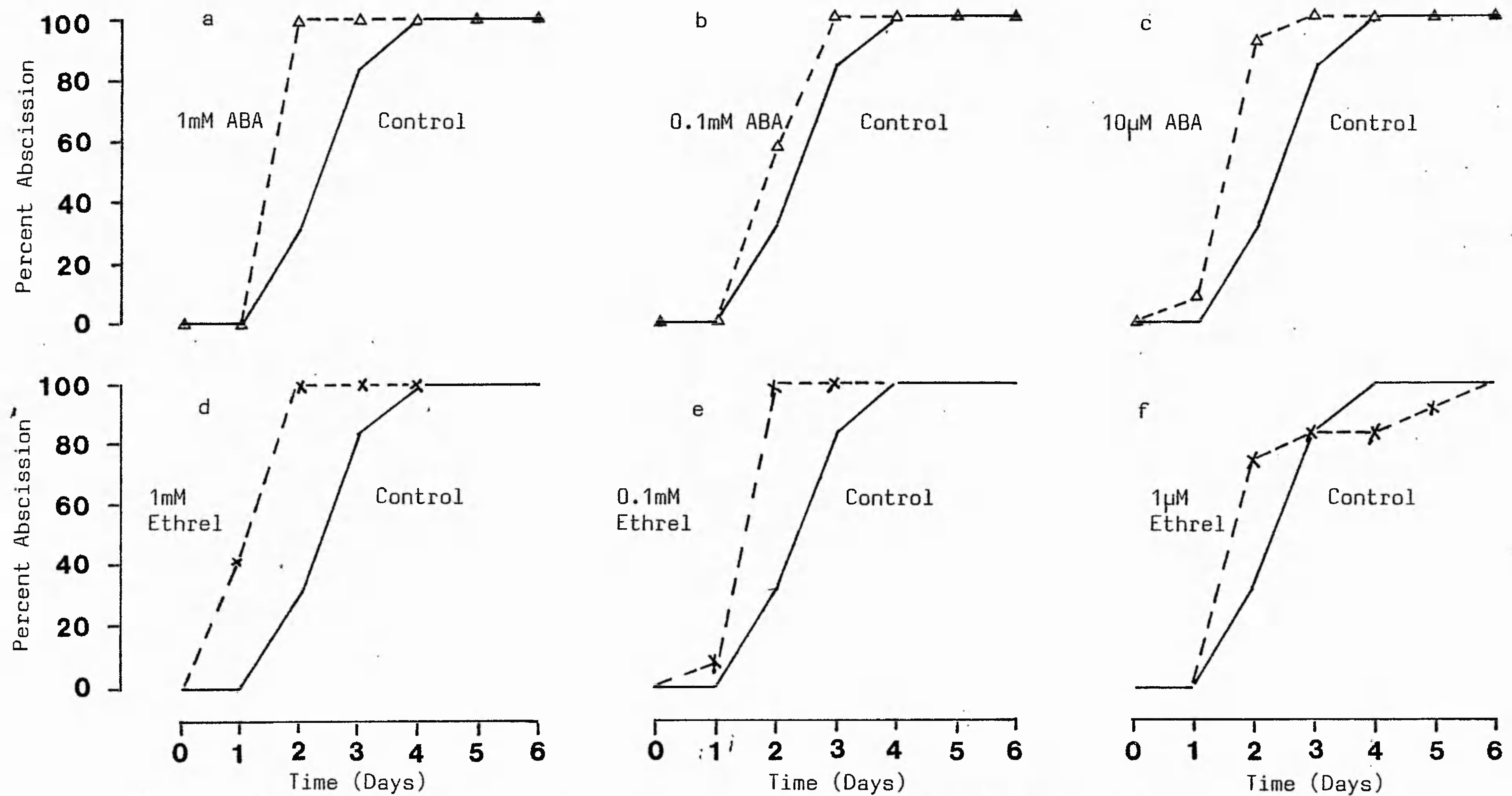


Figure 3.11 Patterns of abscission of petioles of *Iresene herbstii* treated with ABA or Ethrel C. 25° C, darkness

abscission (Figure 3.11c). The ethylene generating compound, Ethrel C, was applied to explants of Iresene only, and the results are shown in Figure 3.11d-f). At 1mM active ingredient, Ethrel C caused abscission with virtually no lag period, and 100% abscission was reached after 2d (Figure 3.11d). Ethrel C at 0.1mM had little effect on time to onset of abscission but caused the process to continue much more rapidly once initiated (Figure 3.11e). Ethrel C at 10µM caused rapid abscission to 70%, but the final 30% occurred at a much slower rate than in control explants (Figure 3.11f).

IAA at 0.1mM was successful in extending the time of onset of abscission and reducing the rate of abscission when applied in combination with 0.1mM ABA in both Coleus explants (Figure 3.8d) and in Iresene explants (Figure 3.13c) i.e IAA was able to overcome tne effects of ABA at this concentration. IAA at 10µM had very little effect in overcoming the effect of 10µM ABA in Coleus explants (Figure 3.9a, Coleus only).

Bentazone at 0.1mM had virtually no effect in reducing the effect of 0.1mM ABA (Figure 3.8e, Coleus; Figure 3.13d, Iresene). Ten micromolar bentazone similarly had no effect on pattern of abscission induced by 10µM ABA, which in itself is minimal compared to control (Figure 3.9b, Coleus only).

The effect of 0.1mM Ethrel C in promoting abscission was completely overcome by 10µM IAA (Iresene only, Figure 3.12a), and was partially overcome by 10 µM IAA (Iresene only, Figure 3.12b). Similarly, both 0.1mM IAA and 10µM IAA overcame the effects of 10µM Ethrel C in a concentration dependent manner (Iresene only, Figure 3.12c and d).

Bentazone at 0.1mM had no effect on the ability of 0.1mM IAA to delay and slow down abscission in either species (Figure 3.8f, Coleus; Figure 3.12e, Iresene), but 10µM bentazone slightly reduced the abscission delaying effect of 10µM IAA (Coleus only, Figure 3.9c).

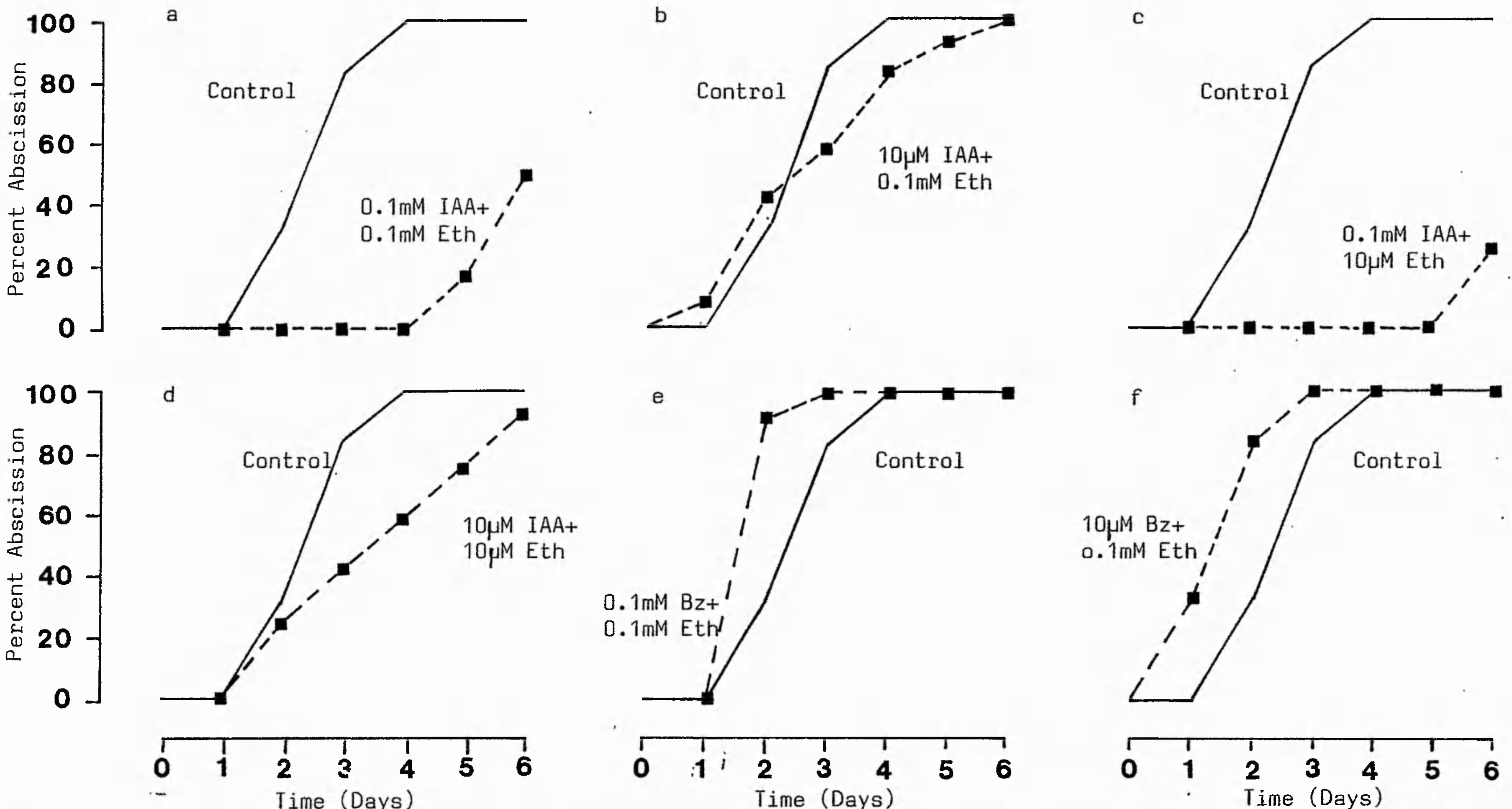


Figure 3.12 Patterns of abscission of petioles of *Iresene herbstii* treated with IAA + Ethrel C or bentazone + Ethrel C. 25° C., darkness.

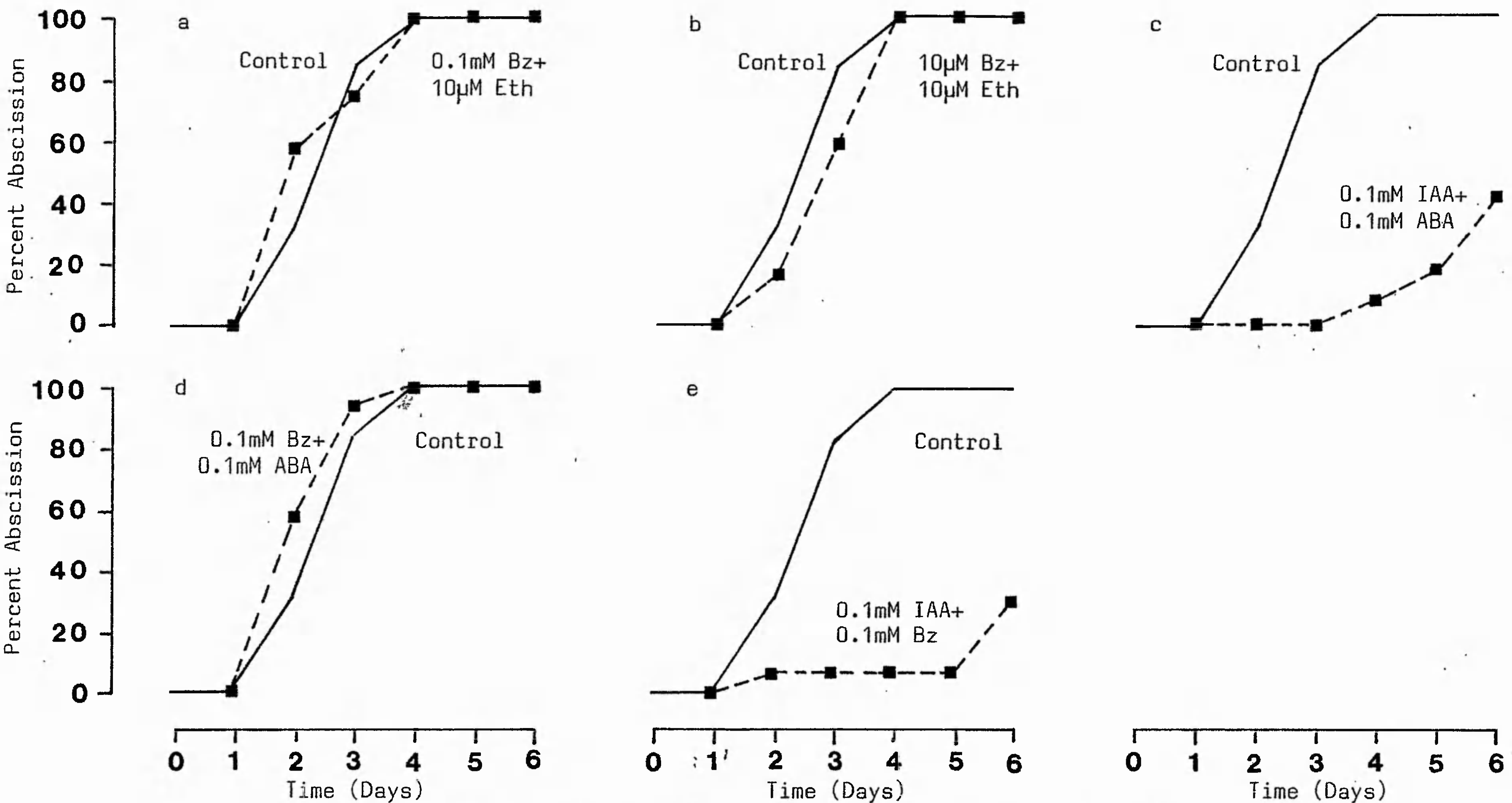


Figure 3.13 Patterns of abscission of petioles of *Iresene herbstii* explants treated with bentazone + Ethrel C or ABA, or IAA + ABA or bentazone. 25°C, darkness.

3.4 Bentazone activity in bioassays for activity of PGRs other than auxin - Discussion

3.4.1 A Consideration of the Techniques Used

There are many aspects to be considered in terms of experimental procedure in studies of plant physiology. Not least of these is the choice of plant tissue. Species, organ, plant age, uniformity and growth conditions are all important.

In this study a light grown dwarf cultivar of *Pisum sativum* was used in an investigation of bentazone activity in a gibberellin sensitive system. Dwarf *Pisum sativum* seedlings have been widely used in such work (e.g. Sponsel, 1985; Ingram *et al*, 1986), and are useful in that intact plants grown under normal conditions of light and temperature respond to exogenous gibberellin. Tissue uniformity was satisfactory and enabled statistically significant differences in internode lengths to be detected. Responses to exogenous gibberellin were entirely as expected, and as such the choice of tissue is considered to have been vindicated. However, there are possible shortcomings in the methods applied. By application of a drop of PGR solution externally on the plant the degree of uptake cannot be assessed. Uptake may be a concentration dependent process and if so may account for the concentration dependent processes observed. Extent of uptake could be monitored, if required, by studies using radiolabelled compounds. A somewhat arbitrary 5d incubation was administered to this tissue following PGR application, and no information of kinetics of the response was obtained. This could be obtained by a fuller experiment, possibly measuring plants daily.

The cytokinin bioassay employed *Raphanus sativus* cotyledons. Letham (1971) used cotyledons from this species to assay for cytokinin activity by monitoring cell division. While Letham (1971) employed etiolated cotyledons, light grown cotyledons were used herein, and failed to respond to exogenous cytokinin (measured as chlorophyll retention). Other studies of cytokinin

influence in preventing chlorophyll loss have employed monocot tissue e.g. *Triticum* or *Avena* leaves (Kuhlne *et al*, 1977; Biddington and Thomas, 1978), and possibly the choice of tissue here was wrong, as it failed to respond to either cytokinin or to bentazone. Possibly the experimental procedure was at fault. Age of tissue may have been wrong, or perhaps replication was insufficient, or imposed conditions were wrong. It is doubtful that temperature, pH or medium composition are crucial, as neither Kuhnle *et al* (1977) nor Biddington and Thomas (1978) controlled pH, incubating tissue in distilled water, at 22-23°C, similar to the 25°C imposed here.

Choice of tissue for the abscission experiment was straightforward. *Coleus* explants have been extensively used historically (Sexton and Roberts, 1982). Explants of *Iresene herbstii* had previously been shown to behave similarly to those of *Coleus blumei* in abscission experiments performed in this laboratory (Cobb, pers. comm.), and the similarity of their responses as presented in Chapter 3 justify the use of this species.

In abscission work with explants the delay before application of exogenous PGRs after cutting of explants is considered important. If the critical period is exceeded the tissue moves from the physiological lag phase into the phase during which active abscission commences (Sexton and Roberts, 1982). This period is considered to be as much as 18h (Barlow, 1950; Yamaguchi, 1954), and as all exogenous compounds were applied within 90 min in this work this delay is not considered important. The application of lanolin paste may also be a source of error. Quantity of paste is difficult to control due to the stickiness of the paste, but development of the method using possibly a syringe would be feasible. The quantity of PGR diffusing from the paste into the tissue is critical in this assay, as the explant cannot enter the active phase (phase 2) of abscission until diffusing auxin has reduced to below a critical level. The size of the "pool" of IAA and other PGRs in differing quantities of lanolin paste will clearly affect this process. Despite this, results were largely consistent with theory, and so the technique is considered satisfactory.

3.4.2 Discussion of Findings

Gibberellin (GA3) enhanced growth of intact *Pisum sativum* cv. Meteor in a concentration-dependent manner, this being expressed in longer internodes and increased internode number, documented responses to exogenous gibberellin (Brian and Hemming, 1955; Sponsel, 1985; Ingram *et al*, 1986). Arney and Mancinelli (1966) reported increased cell division in response to exogenous GA3, and this could account for accelerated apical development i.e a reduced plastochron, with subsequent enhanced outgrowth. The higher concentrations of GA3 enhanced growth lower down the plant, where possibly endogenous concentrations are growth limiting. However, considering current ideas on plant sensitivity (Chapter 1), it may be that receptor / gibberellin affinity is altered in lower internodes as the tissue ages. Gibberellins also enhance cell elongation (Fisher, 1970), but without studying the histology of the observed responses the contributions of cell division or elongation cannot be assessed.

Both exogenous IAA and bentazone at high concentrations produced very similar responses when applied alone, and when applied in combination with exogenous GA3. The lack of significant responses to IAA or bentazone in internodes to 3rd above n.d.a. are not unexpected, as there are few recorded instances of exogenous auxin affecting growth of intact plants. However, both IAA and bentazone significantly inhibited growth of tissue most juvenile at time of droplet application i.e. the internode 4th above n.d.a.. These compounds could be acting via a direct effect on developmental processes of cell division and elongation subject to stimulation by exogenous and endogenous gibberellin. Support for this proposal is provided by the observation of IAA and bentazone similarly affecting exogenous GA3 stimulated outgrowth in a manner dependent on GA3 concentration. IAA and bentazone inhibited the GA3 response in the internodes in which GA3 was most active. Gibberellin and auxin are often held to coact in stimulating cell elongation (Brian and Hemming, 1958) so their antagonism as seen here is surprising, but may be due to concentrations or ratios of the two compounds, or due to the tissue or experimental conditions imposed.

Cotyledons treated with 10μM BAP contained significantly more chlorophyll than controls, this being a known effect of cytokinins (Biddington and Thomas, 1978). However, they reported an effect at the lower concentration of 10nM, albeit using monocot tissue. No other concentration of BAP, or any concentration of kinetin or bentazone had an effect in this system. Perhaps the choice of tissue was mistaken, but this species is known to be sensitive to cytokinin in other bioassays e.g. cotyledon enlargement (Letham, 1971). There was no interaction between either cytokinin and bentazone in this system. The previously reported ability of bentazone to cause modification of chloroplasts to "shade types", and its effect on the photosynthetic apparatus (Lichtenthaler *et al*, 1980) does not appear to be related to any cytokinin properties. This is not surprising, as bentazone does not fulfil the structural criteria of a cytokinin, i.e. it is not an N^6 substituted adenine derivative.

In the study of abscission in explants of *Coleus* and *Iresene* ABA enhanced abscission, a finding consistent with the documented response (e.g. Addicott *et al*, 1964). The observation that 1mM ABA slowed progression of abscission in *Coleus* has been noted previously, and was attributed to the juvenility of tissue (Osborne, 1964, cited in Addicott, 1970). In the present study the uniformity of tissue between treatments was stringently controlled, and so it is difficult to assess the reason for this response. IAA retarded the process in a concentration dependent manner, a documented response to IAA applied distal to the abscission zone representing the presence of a green, non senescing leaf (Addicott, 1982). The effect of IAA was to prolong the lag phase i.e. the phase representing depletion of endogenous auxin, and also slowed the subsequent rate of progression of the process. This can be explained by considering the lanolin paste as a reservoir which continues to supply IAA during stage 2 of abscission, although Sexton and Roberts (1982) considered that this aspect of abscission cannot be slowed or reversed.

Ethylene (as "Ethrel C") also behaved as documented responses would lead one to expect, this being an almost invariable

enhancement of abscission. This was observed herein, but at concentrations of less than 0.1mM active ingredient the rate of abscission fell dramatically once 75% abscission had been achieved. This effect may be artefactual due to tissue age, and may be due to insufficient replication. However, it is not unreasonable to expect endogenous ethylene to be active by this time in abscising tissue, and so the reason for this observation remains obscure. Ethylene frequently accompanies abscission, and is commonly seen as a requirement for abscission to occur (Sexton and Roberts, 1982), so possibly the reservoir of ethylene in the lanolin paste was insufficient to drive the process fully to its conclusion. However, it is difficult to understand how the process could be suddenly arrested. This may be a reminder that the control of developmental processes is a complex phenomenon that cannot be explained in a simplistic fashion. More studies towards a fuller understanding of abscission will provide information by which such observations may be accounted for in future.

The effects of bentazone in this assay were minimal, except at high concentration (1mM), when it showed marginal auxin type retardation of abscission. Bentazone may be active in this system, but it has been shown to have restricted basipetal movement (e.g. in Helianthus, Irons and Burnside, 1982), and if this occurs in the species' studied herein bentazone would not be transported to the abscission zone where it could affect the abscission processes, particularly at the lower concentrations of bentazone applied. Responses when IAA and bentazone were applied together were essentially as for IAA alone i.e there was no interaction between the compounds in this assay.

As for the responses observed in Chapter 2 there are several possible explanations to account for the actions and interactions of the various PGRs in causing the responses observed in this chapter. In the gibberellin bioassay (internode elongation in Pisum sativum) it is feasible that IAA or bentazone inhibit GA3 stimulated cell division or elongation, and thereby inhibit apical development. Perhaps the effect was on ethylene bio-

synthesis which in turn inhibited GA3 enhanced responses. These events could be independent, or may involve opposite actions on the same biochemical and physiological processes. Again, there may be an effect on GA3 uptake or transport, which could be investigated using radiolabelled compounds, if deemed a worthwhile study. In the study of abscission there are similar possible mechanisms of action and interaction. These are possible effects on uptake, transport, and through ethylene and other PGR metabolisms. As described in Chapter 2, such responses are dependent upon initial binding events, second messenger systems, and amplification of signals to produce final events. It is felt that many aspects of action and interaction of PGRs can be explained through consideration of binding and early post-binding events. These aspects are addressed in Chapter 5.

Chapter 4. USE OF PROTOPLAST SYSTEMS TO STUDY BENTAZONE ACTIVITY

4.1 Protoplasts and Fluorescent Amines

As described in Chapter 2, bentazone was found to have measureable and significant effects in a number of auxin sensitive bioassays, in both the short term (< 1h) and long term (12d) experimental systems. Of particular interest with regard to the effects of bentazone on stomatal movement, and possible implications for its herbicidal activity and interactions (Nichols, 1988), are the *Avena* coleoptile elongation bioassay, and the proton efflux (measured as reduction in bathing medium pH) from abraded *Avena* coleoptile segments. Bentazone has been found to be active in both these systems in this study (Chapter 2), and has also shown significant interaction with exogenous IAA. There is clear implication here with regard to the acid growth theory (Hager, Menzel and Krauss, 1971), and proposed action at the plasmalemma (Cobb, Rees, Nichols, Miller and Pallett, 1985). As discussed in Chapter 2, there still exists doubt that there is a causal relationship between proton efflux and cell and tissue elongation (Brummel and Hall, 1987), but there are clear correlations between ability of an auxin-like compound to cause H^+ efflux, and its growth promoting activity (Evans, 1985). It has also been established that growth active auxins bind at the plasmalemma of receptive cells (Batt and Venis, 1976; Lobler and Klambt, 1985b). It would therefore be of interest to examine the effects of such compounds, in particular IAA and bentazone, at the plasmalemma, in the absence of the cell wall. The plasmalemma of the plant cell can be exposed by the preparation of protoplasts, and the effects of exogenous compounds on certain aspects of protoplast physiology including swelling and / or contraction of protoplasts (Rees, 1986; Cobb *et al*, 1985) and changing differences between internal and external pH, can be assessed, and theoretically correlated to effects of the exogenous compound at the plasmalemma. For example, Cobb *et al* (1985) reported a rapid (30 min), concentration-dependent, bentazone-induced swelling of protoplasts from *Glycine max* mesophyll, apparently dependent on mitochondrial ATP, and proposed that herbicide binding to active site(s) at the plasmalemma caused this by altering cation and water flux.

The main findings about bentazone activity in the *Avena* coleoptile experimental systems as described are that it is active in causing elongation and proton efflux, and these, along with those of Cobb *et al* (1985) relating to protoplast swelling, suggest that a study of effects of IAA and bentazone on proton pumping from protplasts would be worthwhile. Such a study could potentially give data relating to activities at the plasmalemma, without interference from the cell wall. The pH at the plasmalemma would be known rather than assumed (as is the case with cell wall present), and greater accuracy in determining kinetics of the rapid, initial response would be possible, along with greater accuracy in determination of kinetics of any potential interactions.

Rottenburg (1979) described methods to measure membrane potential and ΔpH in cells, organelles and vesicles from various sources including mammalian liver mitochondria, chloroplast vesicles and bacterial vesicles. Among the techniques for determination of pH across the membranes of cells, organelles and vesicles are the ion distribution techniques.

"The basis of these methods is the use of acids or amines, the neutral species of which diffuses across the membrane while the ion is impermeable. When a permeable acid has reached an equilibrium distribution, $AH_{in} = AH_{out}$. Since the acid dissociates on both sides (and assuming the dissociation constant is not changed),

$$K_a = \frac{H^+_{in} \, A^-_{in}}{AH_{in}} = \frac{H^+_{out} \, A^-_{out}}{AH_{out}} \qquad (1)$$

it follows that in equilibrium

$$pH_{in} - pH_{out} = pH = \log \frac{A^-_{in}}{A^-_{out}} \qquad (2)$$

If pH $>$ pK+1, most of the acid is ionised on both sides of the membrane, and measurement of the distribution ratio of the acid allows the calculation of ΔpH according to Eq. 2.

Otherwise, a significant fraction of the acid would be un-ionised and, since the total acid concentration $A^{+} = A^{-} + AH$, at equilibrium

$$\frac{A^{+}_{in}}{A^{+}_{out}} = \frac{1/Ka + 1/H^{+}_{in}}{1/Ka + 1/H^{+}_{out}} \qquad (3)$$

solving for internal pH,

$$pH_{in} = \log \frac{A^{t}_{in}}{A^{t}_{out}} (10^{pK} + 10^{pH_{out}}) - 10^{pK} \qquad (4)$$

When pH $<$ pK the acid ratio becomes less sensitive to ΔpH. Since the ionised acid is concentrated on the more basic side of the membrane, this method is useful only when $pH_{in} > pH_{out}$, (i.e. positive ΔpH). When $pH_{in} < pH_{out}$ the equilibrium distribution of amines can be used. For amines in which only the neutral species is permeable, the same considerations as above yield

$$-pH = \log \frac{AH^{+}_{in}}{AH^{+}_{out}} \qquad (5)$$

When pH $>$ pK-1 we must again consider the neutral form ($A_t = A + AH^{+}$),

$$\frac{A^{t}_{in}}{A^{t}_{out}} = \frac{Ka + H_{in}}{Ka + H_{out}} \qquad (6)$$

and solving for internal pH

$$pH_{in} = -\log \frac{A^{t}_{in}}{A^{t}_{out}} (10^{-pK} + 10^{-pH_{out}}) - 10^{-pK} \qquad (7)$$

from: Rottenburg, 1979

Methods in Enzymology LV, 547

An extension of this principle is the measurement of Δ pH by change in external concentration, with fluorescent amines proving particularly useful. Again described by Rottenburg, 1979.

> "A very useful method for the estimation of Δ pH from the extent of amine uptake involves the use of fluorescent amines. Various fluorescent amines are quenched when taken up by organelles, cells and vesicles. The extent of quench - ing is used to calculate the extent of uptake and hence the internal concentration. The residual fluorescence is propor- tional to the external concentration. The most suitable amines for this purpose are 9-aminoacridine (pK=10.0) and N-(1-naphthyl)ethylene diamine (pK=9.5). Assuming that all amine is free but completely quenched we obtain from equation 5.
>
> $$pH = \log \frac{Q}{1-Q} \frac{V_{in}}{V_{out}}$$
>
> where Q is the fraction of the total amine that was quenched, and V_{in}/V_{out} is the ratio of internal volume to the suspen- sion volume.

from Rottenburg, 1979

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This method was introduced in a paper by Schuldiner and Avron (1971) who demonstrated that in light-induced quenching of atebrin (an uncoupler and fluorescent amine) fluorescence clearly behaved parallel to the proton uptake in isolated lettuce chloroplasts. They concluded that the fluorescence quenching of atebrin (and by implication other fluorescent amines) may serve as a powerful tool for following proton gradients generated across the chloroplast membrane. They also concluded that the quenching was due to a screening of the fluorescent molecule by chlorophyll (Schuldiner and Avron, 1971).

The same group expanded on these findings in a subsequent publication, Schuldiner, Rottenburg and Avron (1972), in which they assessed the value of several different amines for use with

this technique. They calculated that an amine of high pK will be distributed across the membrane in accordance with the proton concentration gradient, and that it is therefore possible to calculate the proton distribution ratio, or ΔpH, from measuremennts of residual fluorescence (Schuldiner et al, 1972).

This technique was employed, apparently successfully, by Weigel and Weiss (1984) in the determination of ΔpH across the tonoplast membrane of isolated leaf mesophyll vacuoles of Valerianella locusta (L.) using 9-aminoacridine (9aa). Weigel and Weiss (1984) discovered the quenching of amine fluorescence to be dependent upon amine concentration, due to the ability of these compounds to act as uncoupling agents. This supports the earlier work of Schuldiner and Avron (1971) who demonstrated that the process was energy dependent. The observation by Schuldiner et al (1972) that chlorophyll screens internally held amine from exciting wavelengths of light in chloroplast systems cannot be sustained in chlorophyll free vacuoles, and Weigel and Weiss (1984) attribute fluorescence quenching to internal dimerisation of the amine, a finding of Bucholz (1977) quoted therein. In his review of the technique Rottenburg (1979) made no reference to the mechanism by which quenching occurs.

There is a report of the use of this technique in investigation of herbicidal disruption of proton gradients in tonoplast and plasma membrane vesicles from oat roots (Ratterman and Balke, 1987). It was found by these authors that the herbicides oryzalin and oxyfluorfen produced concentration dependent effects on proton gradients across tonoplast enriched vesicles as monitored via quinacrine fluorescence, and oryzalin similarly affected plasma membrane enriched vesicles (Ratterman and Balke, 1987). It was concluded that some herbicides can affect directly the functioning of plant cellular membranes (Ratterman and Balke, 1987).

It was therefore felt appropriate to employ such a method in the determination of ΔpH between Avena coleoptile protoplast cytoplasm and bathing medium, and whether IAA and/or bentazone are able to alter this relationship.

4.2 Materials and Methods

Growth Conditions

Spring oats (*Avena sativa*) cv. Maris Tabard seeds were germinated and grown in tap watered vermiculite in darkness, at 24 ± 1°C in high humidity, for 88-96h.

Protoplast Isolation

Forty coleoptiles were harvested and lightly abraded between thumb and forefinger with a slurry of 800 grit silicon carbide powder in distilled water, and were stored in distilled water until all were prepared. Segments of 15mm were cut from each coleoptile, commencing 5mm from the tip, and were placed in distilled water and washed for 10min using vigorous aeration. Washed segments were halved longitudinally, deleafed, and placed in a preplasmolysis medium consisting of 500mM mannitol, 10mM MES, 0.1mM $CaCl_2$ and 0.25% (w/v) bovine serum albumin, adjusted to pH 5.8 using KOH, and were preplasmolysed for 30 min at 25 ± 0.5°C. Tissue was transferred into digestion medium consisting of preplasmolysis medium with the addition of 0.2% (w/v) cellulysin and 0.013% (w/v) pectolyase, at 25 ± 0.5° C, and digested for 2h. Tissue was agitated to facilitate release of protoplasts, and was strained through 50 micron mesh nylon muslin. The resulting fluid was spun at 100g for 5 min, and the digestive supernatant was decanted off. The pellet was suspended in 5mls 20% Percoll in distilled water. Harvest of protoplasts was assessed by counting number and size of protoplasts in a known volume haemocytometer and graduated eyepiece on the microscope.

Fluorescence Measurements

The constraints of time prevented any measurements of amine fluorescence from the protoplast suspension obtained from the above method.

4.3 Preliminary Results

Protoplasts were successfully isolated by the method described, and their number and sizes in 20µl of resuspension medium was assessed using a haemocytometer. The findings from the five separate occasions are presented in table 4.1.

Table 4.1 Numbers and diameters of protoplasts in 20µl resuspension medium, obtained from the method described in the previous section (4.2)

Experiment	Protoplast Diameter (µm)								Mean Volume	Total Number	Total Volume (nl)
	2	3	4	5	6	7	8	9			
a	123	159	70	63	18	3	0	0	0.027	436	11.8
b	71	72	61	32	20	8	6	1	0.041	271	11.1
c	108	135	105	78	22	8	0	0	0.033	456	14.9
d	69	53	59	32	16	7	2	0	0.037	238	8.7
e	51	88	53	75	39	24	2	0	0.052	332	17.4

Over the five isolations numbers of protoplasts obtained varied by a factor of two, as did mean protoplast volume and total internal volume. Lowest total internal volume coincided with reduced number of protoplasts obtained, whereas large total internal volume was contributed to by a greater number of protoplasts 6um diameter and greater. The distribution of sizes for each isolation is presented in figure 4.1a-e, with an overall size distribution for all isolations combined presented in figure 4.2.

The viability of such protoplasts was assessed by their ability to absorb fluorescein diacetate (FDA) and fluoresce when irradiated with ultraviolet (UV) light. Protoplast density in each preparation was low, but viability appeared to be almost complete. The fluorescence from such protoplasts is illustrated in plate 4.1.

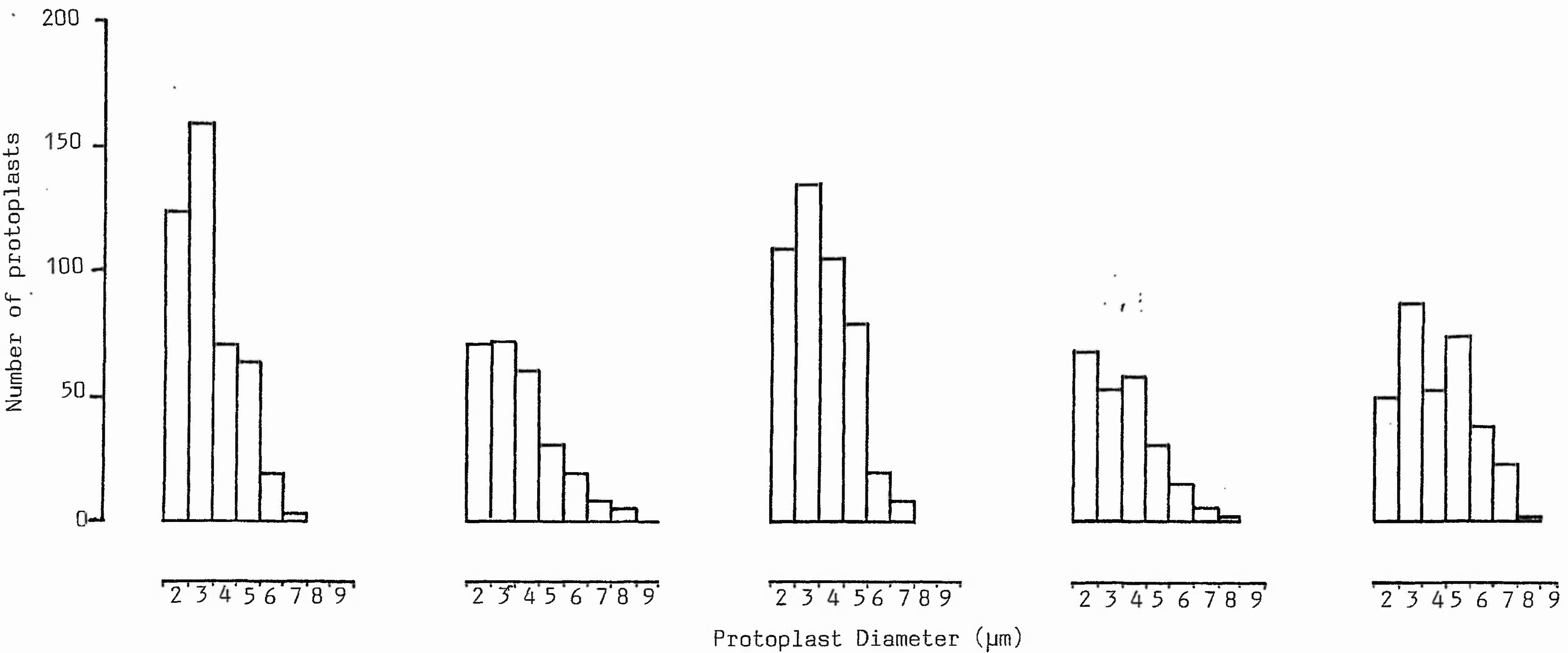


Figure 4.1 Protoplast diameter and number for five separate isolations as assessed by the method described.

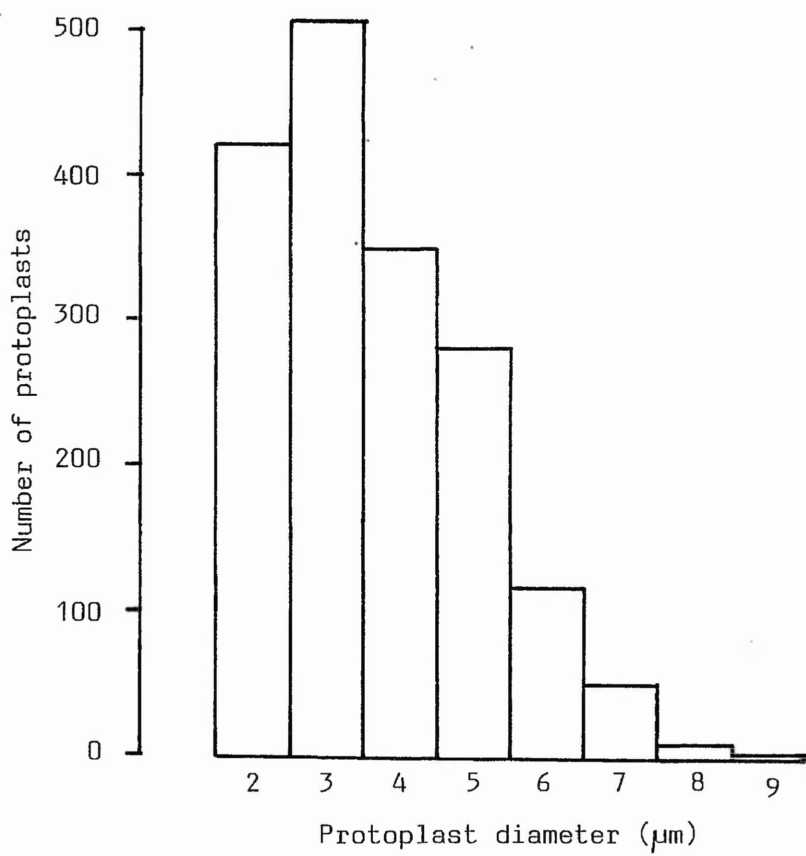


Figure 4.2 Total number of protoplasts obtained and their diameters, aggregate of five isolations.

4.4 Protoplasts and Fluorescent Amines - Discussion

As described in the introduction to this chapter, the monitoring of fluorescence from fluorescent amines provides considerable potential, as it permits continuous measurement of ΔpH without separation of the membrane bound particles from the medium. Weigel and Weiss (1984) employed the technique using 9 amino acridine as the fluorescent species in measuring ΔpH across the tonoplast of isolated mesophyll vacuoles. The use of single compartment systems such as vacuoles appears relatively straightforward, but the use of the technique in multicompartmental systems raises several points which remain to be resolved in this author's mind.

If the fluorescent amine equilibrates freely across the plasmalemma in a protoplast system, presumably internal amine equilibrates across internal cellular membranes including the tonoplast, mitochondria and vesicles. Any change in equilibria between internal organelles and the cytoplasm, depending upon relative pH, will effect the equilibrium between the cytoplasm and external medium. The degree of fluorescence quenching may then be dependent upon any activity at internal membranes, but could be mistakenly interpreted as an effect at the plasmalemma.

A second aspect of the technique which remains to be resolved is the cause of quenching of fluorescence. Schuldiner and Avron (1971) attribute quenching to simple screening of amine by membranes, and/or by cellular pigments, while Weigel and Weiss (1984) suggest that dimerisation is the cause. If dimerisation, the equilibrium relationship between the internal monomer and dimer, and the external monomer and dimer, and the pH dependence of this, if any, must be established.

These potential difficulties must be investigated and understood if this technique can be employed with purpose. However it is suggested that the monitoring of fluorescence from isolated Avena coleoptile protoplasts could provide a useful technique in studying the membrane activity of IAA, bentazone and other herbicides, towards a full understanding of their mechanisms of action and interaction.

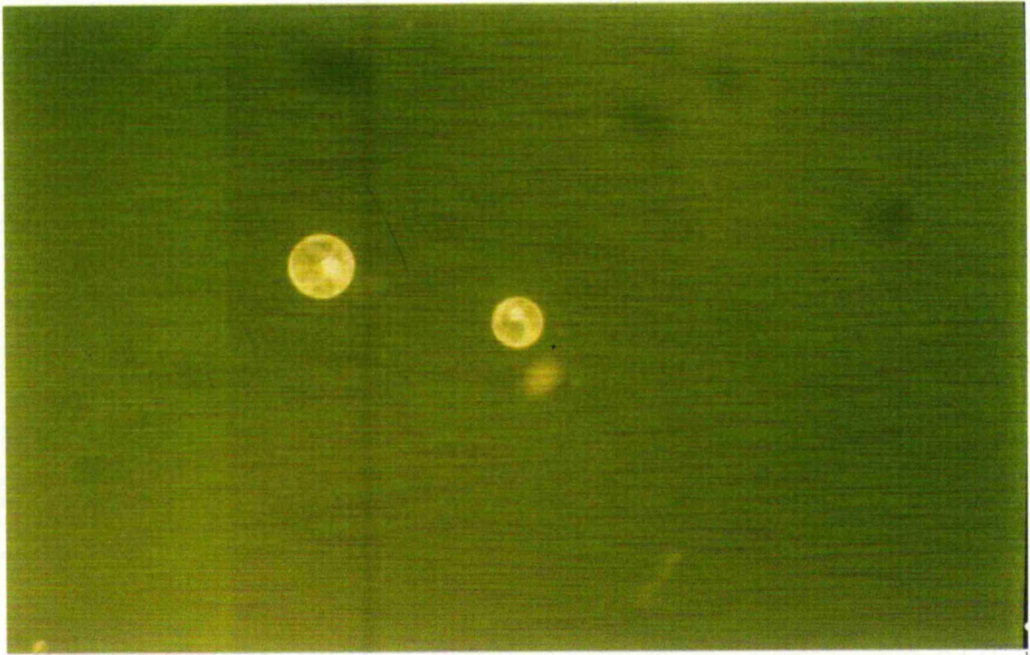


Plate 1. FDA stained protoplasts from *Avena* coleoptiles.

Chapter 5. GENERAL DISCUSSION

Studies in this thesis have clearly demonstrated that there are several mechanisms by which IAA and bentazone may act and interact in a non-photosynthetic manner. These observations describe complex physiological responses, and it is suggested in sections 2.5.3. and 3.4.3 that an initial binding event (or events) between the PGR and its receptor underpins these physiological responses i.e.

1. RNA and protein syntheses
2. IAA/bentazone uptake
3. IAA/bentazone transports
4. Relations with endogenous PGRs (metabolism and/or action).

Each response results from a cascade of events following binding.

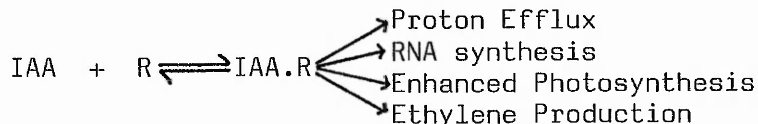
Trewavas (1981), in arguing that plant sensitivity is an important factor in controlling responses mediated by endogenous and exogenous PGRs, simply described PGR/receptor interaction thus:-

Growth Substance + Receptor $\rightleftharpoons$ Growth Substance/Receptor Complex

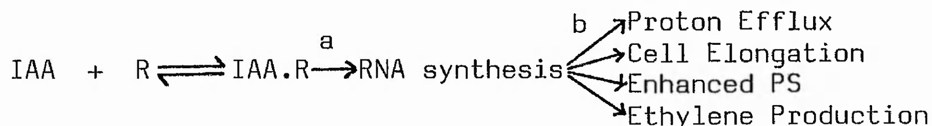
If it is assumed that the degree of GSR formation is directly related to the magnitude of the measured response, we then obtain:-

where 'a' implicates secondary messenger amplification systems (see 1.4).

If we restrict the discussion at present to auxin responses, it can be asked whether there is only one auxin receptor within a particular species, i.e. does the following apply?



However, the model can be increased in complexity. For example, Davies (1973) assessed that de novo RNA synthesis in response to IAA would not be sufficiently rapid to have a role in IAA-induced proton efflux, but more recent work has suggested that de novo RNA synthesis is sufficiently rapid (Theologis and Ray, 1982). There is also the debate that proton efflux is not causal to cell elongation (Brummell and Hall, 1987). Hence, the following could also apply:-



where both a and b implicate secondary messengers

The nature of the receptor, and of subsequent secondary messenger / amplification mechanisms is currently the subject of intense research interest (Venis, 1985). Despite this, subsequent responses are still thought to be dependent upon the fundamental event of GSR formation (Figure 5.1).

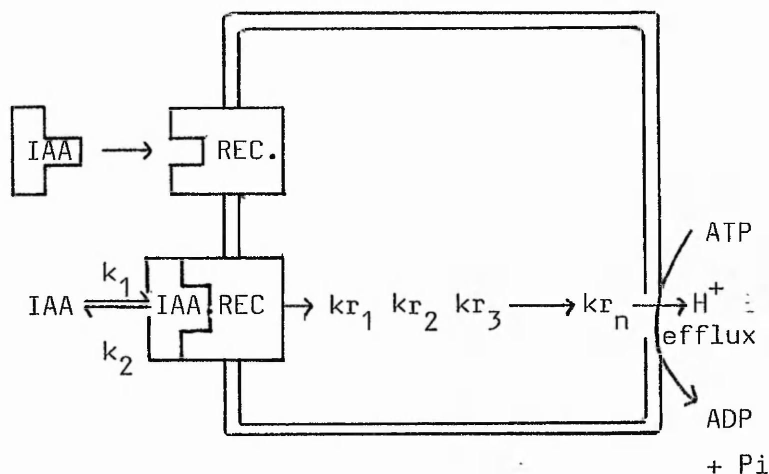


Figure 5.1 H^+ Efflux Dependence upon GSR (IAA.REC) Formation.

The degree of GSR formation is dependent upon the relationship between k1 and k2 (association and dissociation constants respectively). This relationship can be altered depending upon three major factors:-

1. Concentration of GS
2. Concentration (density) of R
3. Affinity of GS for R (Weyers *et al*, 1987)

Trewavas (1981) and Nissen (1985 and 1988) have presented evidence that factors 2 and 3 are more significant in regulating responses than factor 1, which has been traditionally been considered as most important (section 1.3). The magnitude of response (H^+-efflux) is subsequently dependent upon k_r1, $k_r2 \longrightarrow k_rn$ (Figure 5.1) as well as efficiency of ATPase, i.e. phenomena occurring "downstream" to the initial binding event.

Data accumulated in this study shows bentazone to have auxin activity in a number of bioassays (Chapter 2) and to behave as an auxin in other PGR bioassays (Chapter 3). The principal pieces of evidence are that:-

1. IAA and bentazone both stimulate growth of primary leaf petioles, cotyledonary internode and first lateral over a 12d period, of light grown, 13 or 14d old, decapitated *Phaseolus vulgaris*, depending on concentration.

2. IAA and bentazone both stimulate elongation, within 1h, of etiolated *Avena* coleoptile segments, with similar concentration dependence.

3. IAA and bentazone stimulation of *Avena* coleoptile segment elongation is similarly sensitive to a range of inhibitors.

4. Both IAA and bentazone stimulate H^+- efflux from *Avena* coleoptile segments in a concentration dependent manner.

5. Both IAA and bentazone similarly inhibited internode elongation of Pisum sativum, and similarly inhibited its response to exogenous GA3.

6. Bentazone at high concentrations delayed abscission in a manner similar to low concentrations of IAA.

The data for experiments using Avena sativa coleoptile tissue was collected for responses within 1h, and as such represents responses closer to the initial binding event than that for the other systems, where responses were observed over periods of days. Avena sativa coleoptile elongation and proton efflux have been the subject of considerable experimental input over the last two decades, as attempts have been directed at elucidating the primary mechanism of auxin action. These responses are thought to be mediated by a plasmalemma bound, proton pumping ATPase, which may not be the IAA receptor protein (Hager et al, 1971). There is doubt that there is a causal relationship between proton efflux and cell elongation (Kutschera and Schopfer, 1984; Brummell and Hall, 1987), but the basic model (Figure 5.1) remains valid, with at least one IAA receptor having been identified as plasmalemmal (e.g. Lobler and Klambt, 1985b). Considering this hypothesis, and the evidence that bentazone is similarly and additionally active to IAA in both protoplast (Rees, 1986) and stomatal systems (Nichols, 1988), it is speculated that bentazone is active at the same (or closely related), binding site as IAA, at the plasmalemma. The development of longer term responses are also dependent degree of GSR formation i.e.

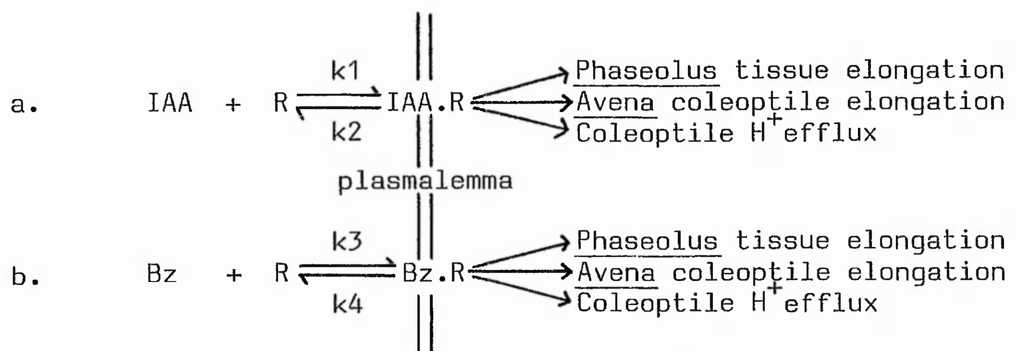


Figure 5.2 Proposed shared mechanism for IAA and bentazone (Bz) responses.

Despite the similarities of response to IAA and bentazone, the optimum bentazone-induced response is consistently smaller in magnitude that the optimum IAA response. To accommodate this in the above model the factors affecting IAA.R formation and Bz.R formation must be considered. It can reasonably be assumed that receptor density is the same for both IAA and bentazone treated, as uniform material was randomly sub-divided between treatments in all experiments. The concentration of GS, and the affinity between GS and R, must therefore be considered. Again, for <u>Avena</u> coleoptile elongation and Proton efflux, being rapid responses following a binding event, it is recalled that optimum elongation in response to c.0.1mM IAA of approximately $9\mu m.min^{-1}$ compares to optimum response to 0.1mM bentazone of approximately $6.5\mu m.min^{-1}$. For proton efflux, optimum ΔpH at 1h (response to 0.1mM IAA) was approx. 0.65, for response to 0.5mM bentazone this was approx. 0.38. Clearly then, magnitude of response is not solely dependent upon concentration of GS. The affinity between receptor and GS is probably therefore important. For IAA, this is the relationship between k1 and k2 (Figure 5.2), and for bentazone, the relationship between k3 and k4 (Figure 5.2). It is proposed that $k1/k2 \succ k3/k4$, giving a higher IAA.R than Bz.R, leading to larger responses.

In addition to the responses of the various tissues to IAA and bentazone alone, clear interactions between the compounds were observed in a number of cases including:-

1. Bentazone consistently reduced the responses to range of IAA concentrations of primary leaf petioles (Figures 2.5-2.8) and cotyledonary internode (Figures 2.10-2.13), with effects in some cases in laterals (Figures 2.15-2.18) of <u>Phaseolus vulgaris</u>.

2. Ten micromolar and 0.1mM bentazone reduced the responses to a range of auxin concentrations of <u>Lepidium sativum</u> roots (Figure 2.20), while:-

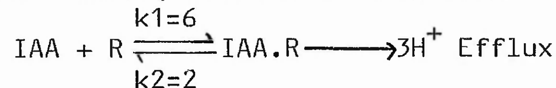
3. Sub micromolar concentrations of bentazone enhanced the responses to micromolar to nanomolar IAA in <u>Lepidium sativum</u> roots (Figure 2.20).

4. The elongation response of *Avena sativa* coleoptiles to optimum IAA (10μM) was significantly reduced by optimum (10μM) bentazone (Table 2.4), and 10nM and 1nM bentazone significantly reduced the elongation response of *Avena* coleoptile segments to circa optimum IAA (0.1mM, 10μM; Table 2.4). However, the response to sub optimal IAA (0.1μM) was significantly enhanced by 10mM bentazone (Table 2.4).

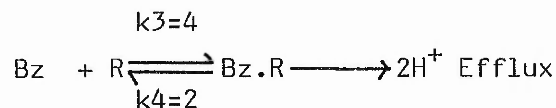
5. Proton efflux from *Avena sativa* coleoptile segments in response to sub micromolar IAA was enhanced by 250μM bentazone (Figure 2.25a) and proton efflux to all concentrations of IAA was enhanced by 50μM bentazone (Figure 2.25b).

It is proposed therefore that when IAA and bentazone are applied simultaneously at high (optimum) concentrations, the degree of GSR formation is reduced. Bentazone could reduce degree of IAA.R formation by either decreasing rate of association of IAA and R i.e. decreasing k1, or by increasing rate of of dissociation i.e increasing k2. Similarly, IAA could either decrease k3 or increase k4 (Figure 5.2). This speculative model is presented in Figure 5.3.

a. Optimum IAA alone, efficient IAA.R formation, k1/k2=3=3H .



b. Optimum Bz alone, less efficient GS.R formation, k3/k4=2.



c. Optimum IAA + optimum Bz, efficiency of GSR formation reduced
k1/k2 reduced, k3/k4 reduced.

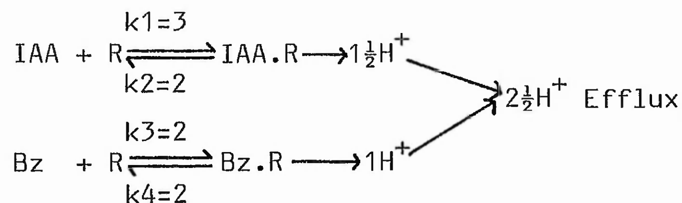
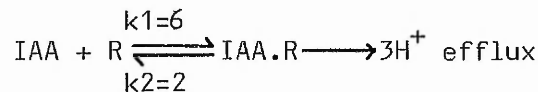


Figure 5.3 A model of interaction of optimum IAA and bentazone in proton efflux.

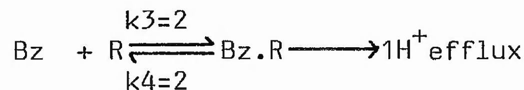
In figure 5.3 proton efflux is proportional to the magnitude of the other responses e.g. in Phaseolus vulgaris tissue, in Lepidium sativum roots, and in Avena sativa coleoptile elongation, the presence of optimum bentazone consistently reduced the magnitude of the response when in combination with IAA alone.

Again, considering proton efflux as the most rapid response following binding, when IAA at all concentrations was combined with low bentazone concentration, an enhancement of IAA response was observed. The model for high IAA and low bentazone is presented in Figure 5.4.

a. Optimum IAA alone, efficient IAA.R formation, k1/k2=3.



b. Low bentazone, low Bz.R formation, k3/k4 = 1.



c. Optimum IAA + low bentazone, minor effects on GSR formation, Response greater than IAA alone.

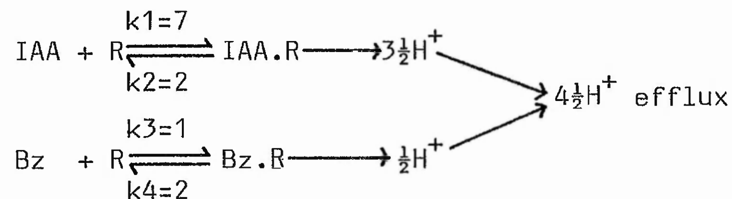
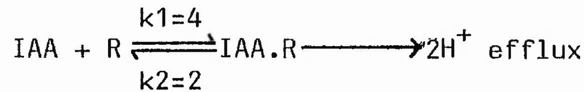


Figure 5.4 A model of optimum IAA combined with low bentazone.

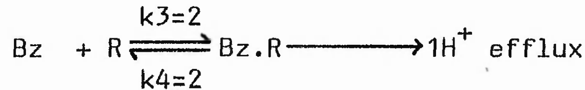
The model for low IAA plus low bentazone for proton efflux is presented in figure 5.5.

In Figures 5.3-5.5 high concentration of bentazone reduces efficiency of IAA.R formation, with high IAA reducing efficiency of Bz.R formation. Low concentration enhances IAA.R formation while low IAA enhances Bz.R formation.

a. Low IAA, lower IAA.R formation, k1/k2=2.



b. Low Bz, low Bz.R formation, k3/k4=1



c. Low IAA plus low Bz, higher overall GSR formation, larger response than for IAA alone.

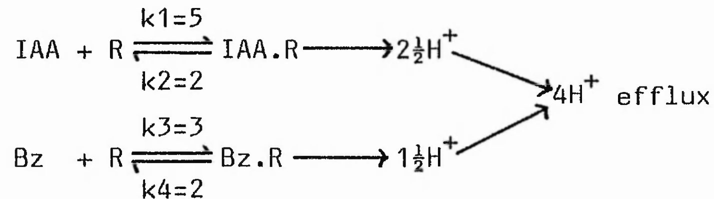


Figure 5.5 A model of interaction of low IAA plus low bentazone in proton efflux.

The consequence of this is observed in high concentration combinations causing antagonism of response in *Phaseolus* cotyledonary internode and lateral, in *Lepidium* roots, and in *Avena* coleoptile elongation. These responses are all proportional to rate of overall GSR (IAA.R + Bz.R) formation represented in Figure 5.3. The antagonistic responses of high IAA plus low bentazone observed in *Phaseolus* petioles and *Avena* coleoptile elongation are proportional to total GSR formation represented in Figure 5.4, and low concentration combination mutual synergisms, observed in *Lepidium* root growth and *Avena* coleoptile proton efflux are represented by enhanced GSR formation in Figure 5.5.

In Chapter 3 there is the example of IAA and bentazone independently reducing GA3 responses in *Pisum sativum* internodes, and it is proposed that, in the absence of exogenous GA3 both IAA and bentazone reduce endogenous GA3.R formation in juvenile internodes where sensitivity is high. The same effect is observed for exogenous GA3, and it is proposed that IAA and bentazone are inhibiting exogenous GA3.R formation in internodes 3rd and 4th above node of droplet application, thereby reducing GA3-enhanced responses.

The precise nature of the optimum IAA/optimum Bz interaction at the binding site remains to be elucidated. However, there are features that suggest direct competition for the site. If bentazone is considered to be the inhibitor of IAA action, despite having activity itself, formation of Bz.R reduces IAA.R formation. IAA.R formation causes greater response than Bz.R formation, so Bz.R formation reduces the overall, measured response. Competitive inhibition can be overcome by increasing the concentration of substrate (IAA), but the same effect can be achieved by reducing the concentration of inhibitor (Bz) i.e. increasing the substrate:inhibitor (IAA:Bz) ratio. When this was done an enhancement of response was observed (proton efflux, Figure 5.4).

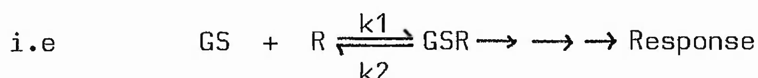
Explanation of low concentration synergisms is less straight forward. It may be that the presence of bentazone at low concentrations increases affinity between IAA and the receptor, possibly by effecting a conformational change to the receptor.

The models of IAA/bentazone interactions in Figures 5.3-5.5 can account for interactions close to the binding event i.e. proton efflux within 1h, and can help explain some of the interactions observed in the longer term experiments. There are similarities of interactions in all these systems, and it is therefore reasoned that a common and fundamental feature of all responses is affected by the interaction. This is proposed to be at a plasmalemma bound receptor protein, activation of which initiates the cascade of events resulting in short and longer term measured responses. Deviations from interactions described can be accommodated by implicating negative feedback mechanisms and subsequent effects of responses having a common initiation. For example, ethylene production is enhanced by auxins, yet ethylene is known to interact with auxin in regulating e.g. coleoptile elongation (Ishizawa and Esashi, 1983). Theologis and Ray (1982) have reported enhanced mRNA synthesis in pea epicotyls, within 20min of application of exogenous IAA. Possibly proteins resulting from this RNA synthesis have a role to play in e.g. cell wall synthesis, and as such affect progression of longer term tissue growth.

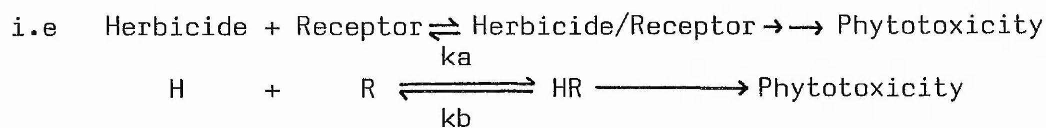
It is clear that consideration of models of GSR formation and the factors affecting it can help explain plant tissue responses to PGRs. Can similar models assist in explanation of herbicide action and interaction?

The selective action of some herbicides can be accounted for by differences in uptake, translocation and metabolism in susceptible and resistant species. Bentazone is one such herbicide (Chapter 1.2). However, there are other compounds for which this is not the case e.g. clopyralid (Thompson, 1989). The selectivity of such herbicides is dependent upon some as yet unidentified inherent feature of the plant i.e. on its ability to respond to the herbicide. Trewavas (1981) considered tissue sensitivity as a factor in being able to respond to endogenous and exogenous PGRs, and described that response is dependent upon the interaction between PGR and its receptor.

i.e



In sensitive tissues GSR is high i.e. k1 > k2, while in non-sensitive tissue it is low. GSR formation is dependent upon concentration of GS, density of R, and affinity of GS for R. Response is also subject to change depending on limitation of further biochemical and physiological pathways. An analogy of this model can be proposed for herbicide action.



Again analagous to the PGR situation, the formation of HR is considered to be dependent upon three factors:-

1. Concentration of herbicide
2. Concentration (density) of receptor
3. Affinity of herbicide for receptor (relationship of ka and kb).

Concentration of applied herbicide has an effect on phytotoxicity e.g. clopyralid in Matricaria (Thompson, 1989), however of more relevance is concentration of herbicide penetrating to active site e.g. at the plasmalemma. It is proposed that density and/or affinity of receptor also exerts some control in formation of HR. Examples in which nature of the receptor could be important are:-

1. In differences between resistant and susceptible species. Perhaps resistant species in which there is a lack of a role of uptake, transport and metabolism have a lower receptor density, or a different affinity than susceptible species exhibiting similar uptake capabilities.

2. Herbicide efficacy as affected by environment. Trewavas (1981) reasons that nutritional status influences the ability of a plant to respond to endogenous PGR through an effect on density and affinity of receptor proteins. High nutrition facilitates high receptor density and affinity leading to high responses. Perhaps the poor efficacy of bentazone during the hot, dry summers of 1975 and 1976 (Chapter 1) was a function of receptor status rather than differential uptake and translocation involving stomata.

3. Antagonism between simultaneously applied herbicides in the field. This is perhaps the most commercially relevant of these aspects, as simultaneous applications of two or more herbicides are desirable in that they facilitate control of a broader weed spectrum for the same cost in energy and time.

Cobb (1988) has described various field antagonisms of herbicides, including bentazone antagonism with diclofop-methyl, haloxyfop-methyl and sethoxydim in soybean. Various contributory factors have been proposed, including environmental stress, effects on uptake, translocation and metabolism, and competition for regulatory binding sites (Cobb, 1988). Furthermore, it was proposed that interaction between these compounds occurs at the plasmalemma, possibly involving auxin receptors and ATPase.

Cobb (1988) presented the following supporting evidence:-

1. Herbicides which antagonise diclofop methyl in the field have auxin activity, compatible herbicides generally do not.

2. Bentazone, having auxin activity (this thesis), antagonises phenoxypropanoates and cyclohexenones.

3. Diclofop methyl and sethoxydim are active at the plasmalemma.

4. Bentazone antagonises auxin activity (Nichols, 1988; this thesis).

Further work in this laboratory has further characterised the speculative proposed model of Cobb (1988), by assessing effects of auxin type herbicides on the well characterised, plasmamembrane mediated, auxin sensitive proton efflux response from etiolated *Avena* coleoptile segments (Fitzsimons, Barnwell and Cobb, 1988). The data therein illustrate that the maximum response is equal for most auxin type compounds, but the concentration of "auxin" required to produce the response varies from a relative value of 1 for IAA, to over 6000 for clopyralid. This indicates a higher receptor affinity for IAA than for the other herbicides, with receptor/clopyralid affinity being the lowest (Fitzsimons *et al*, 1988). Having established relative affinities Fitzsimons *et al* (1988) also investigated the effect of diclofop methyl on receptor affinity to MCPA (two compounds representative of a field antagonistic combination). It was found that diclofop methyl reduced affinity of receptor for MCPA depending on concentration. Diclofop methyl also reduced the maximum response obtained by MCPA. It was concluded that antagonism of MCPA action was due to both competitive and non competitive inhibition of the H^+ efflux mechanism (Fitzsimons *et al*, 1988).

It would appear therefore, that by investigation of responses close to the point of PGR or herbicide binding with receptor, important and useful information can be obtained on the nature of any interaction. The measurement of proton efflux is a useful technique for this, and a full characterisation of the IAA/bentazone interaction by the method of Fitzsimons _et al_ (1988) would provide further useful information on the nature of bentazone action. The technique could also enable assessment of bentazone interaction with those herbicides which it antagonises in the field. However, it is considered that this technique does have limitations including:-

1. Herbicide concentration _at the plasmalemma_ can only be assumed.

2. It can only be assumed that protons escape the tissue to the pH electrode linearly to their rate of efflux.

3. The lag times of herbicide penetration, H^+ pumping apparatus activation and proton escape cannot be assessed independently, only a composite picture is obtained. Further characterisation of these elements could be made through employment of binding studies using radiolabelled herbicides and auxins, and through use of calcium agonists and antagonists in order to evaluate the role of proposed secondary messengers in mediating this response (Brummell and Hall, 1987).

For the reasons listed above it is suggested that development of the technique of fluorescence quenching in _Avena_ protoplasts (Chapter 4) be developed, as it permits:-

a. direct contact of herbicide/PGR solution and plasmalemma.

b. assessment of Δ pH without separating tissue from medium.

c. precise and accurate measurements of initial rates of response.

This would permit rapid and accurate assessment of PGR and/or herbicide action and interaction at the plasmalemma, in isolation from influence from other plant parts e.g. the cell wall.

It is clear that PGR and herbicide actions and interactions are complex involving many factors including poorly understood aspects of plant physiology. Due to the poor understanding of the molecular bases of PGR action the reasons for bentazone interactions with IAA and GA3 observed in this work remain obscure. This study provides a speculative attempt to explain some of the effects observed, but only more work on plant physiology and biochemistry will provide the basic information required before such observations can be satisfactorily explained.

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Addendum

Figure 2.2 is absent in this thesis.

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

Rapid and long-term non-photosynthetic interactions of bentazone with plant growth and development

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Summary: The non-photosynthetic interactions of bentazone with plant growth and development were investigated in two experimental systems sensitive to applied auxin. Bentazone stimulated rapid coleoptile elongation in the *Avena sativa* assay in darkness, and altered longer-term sub-apical growth in light grown *Phaseolus vulgaris*. In both systems bentazone behaviour was similar to auxin, and significant interactions were observed when both substances were applied together. These findings are discussed in relation to the mode of action of bentazone and the use of these assays in herbicide studies.

INTRODUCTION

Bentazone is a selective postemergent herbicide for the control of broad-leaved weeds in several major crops including soybean, maize, rice and peanuts. Studies conducted during the 1970's on the mode of action of bentazone clearly demonstrated an inhibition of photosynthetic electron flow at photosystem II in isolated thylakoids (Pfister *et al*., 1975), chloroplasts (Mine and Matsunaka, 1975) and intact leaves (Retzlaff and Hamm, 1976; Potter, 1977). However, subsequent studies in this laboratory clearly demonstrated that; (1) Bentazone efficacy was particularly sensitive to environmental conditions both before and after treatment (Taylor *et al*., 1981). (2) Bentazone can alter stomatal movement in *Chenopodium album* epidermes (Dunleavy and Cobb, 1984 a and b), intact leaves (Dunleavy *et al*., 1982; Cobb *et al*., 1983) and in the epidermes of several other species (Nichols, unpublished). (3) Bentazone can induce rapid concentration - dependent turgor changes in both light and darkness in isolated protoplasts, epidermes and coleoptiles (Cobb *et al*., 1985). These observations imply a rapid action of bentazone at the plant cell membrane that is independent of any inhibition of photosynthesis, and an interaction with a membrane-bound ATPase has been recently proposed to accommodate these data obtained from several experimental systems (Cobb *et al*., 1985).

A well-documented and rapid turgor response is the elongation of etiolated *Avena sativa* coleoptiles. This process is widely believed to result from the active secretion of protons from the cell causing the loosening of acid-labile hydrogen bonds in the cell wall polysaccharide matrix, and the resultant influx of water causes an increase in cell length (Cleland, 1980). Proton efflux is particularly sensitive to cations (Ca^{2+} and Mg^{2+}), ATPase inhibitors (e.g. CCCP) and auxin concentration (Bandurski and Nonhebel, 1984). Furthermore, cell elongation proceeds as a biphasic response. The first phase lasts approximately 60 minutes and is directly proportional to proton efflux i.e. acid growth (Rayle, 1973), whilst the later, second phase of elongation involves the participation of protein synthesis required for the formation of new cell wall material (Vanderhoef *et al*., 1976 a and b). This study has investigated the action and interaction of bentazone with

auxin in the first phase of cell elongation, to establish a rapid action of this major herbicide in a non-photosynthesising tissue. Preliminary observations have recently been reported (Cobb et al., 1985).

A further series of experiments was performed using young, intact Phaseolus vulgaris grown in the light to establish any long-term consequences of this cell membrane action of bentazone. P. vulgaris was chosen because of a widespread literature on apical dominance in this species, in which outgrowth of lateral buds is inhibited by the presence of the shoot apex (Hillman, 1984). Decapitation permits lateral growth which may then be examined in the presence of natural or synthetic growth regulators applied in lanolin to the apical stump. Thus, the reimposition of apical dominance and other growth parameters may be examined in rapidly growing tissues. Apical dominance, however, is a complex growth process involving not only natural auxin but other growth regulators and environmental factors (Hillman, 1984).

This paper presents data for both short and long-term growth responses of bentazone and serves to characterise the auxin-activity of this herbicide as an additional, albeit secondary, mode of action. The potential use of these physiological assays in agrochemical screening is also discussed.

MATERIALS AND METHODS

Rapid Elongation of A. sativa Coleoptiles: Avena sativa L. cv. Maris Tabard (spring oat) was grown in moist vermiculite for 3d in total darkness at 25°C. Coleoptiles (total length 3-4 cm) were removed in dim light, the apical 0.5 cm discarded (to remove the endogenous source of IAA) and 1 cm segments preincubated on 25 ml 5mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) for 45 min at 25°C in total darkness, to allow for the outward diffusion of endogenous auxin. For each assay 105 segments were prepared and subdivided into seven populations of 15 for preincubation and incubation. Segments were carefully transferred to 25 ml of incubation medium (buffer plus bentazone and/or IAA) and incubated in total darkness at 25°C for 0,10,20,30,40,50 and 60 min periods. Mean segment length following incubation was precisely determined using an overhead projector with a magnification factor of 5. Each experimental treatment was replicated up to five times and scatter graphs prepared. Generalised Linear Interactive Modelling (GLIM) was then used to establish a precise linear relationship of elongation versus time of incubation, and correlation coefficients of approx. 0.9 were obtained. Technical grade bentazone (c.99.8% pure) and IAA (Sigma) were initially dissolved in a minimum quantity of acetone, and stock solutions of 10^{-3}M were prepared, from which dilutions in buffer were made.

Sub-Apical Growth of Phaseolus vulgaris: Phaseolus vulgaris L. cv. Masterpiece (Dwarf French Bean) was grown in J Arthur Bowers potting compost in 12.5 cm diameter pots (16h photoperiod, 27.5 $\pm$ 2.5°C day, 16.5 $\pm$ 1.5°C night) for 13-14 days, by which time the first internode above the primary leaves was approx. 0.5 cm long. Shoot apices were decapitated with a sharp blade and chemical treatments in lanolin paste immediately applied to the cut stump. Known weights of auxin (IAA) and bentazone were dissolved in a minimum volume of acetone and distilled water, and thoroughly mixed with pure lanolin (BDH) in the ratio 2 parts IAA or bentazone to one part lanolin, to give final concentrations of 10^{-3} to 10^{-6} M. 10 plants were used for each treatment and measurements made at two day intervals of cotyledonary internode length, the length of primary leaf petioles, and the length of the first internode of the dominant lateral (Fig. 1). Measurements were recorded for up to 12 days after treatment and cotyledonary internode and petioles' lengths calculated as percentage change in length over the growth period, i.e. -

$$\Delta\,\% = \left(\frac{\text{length, day }X}{\text{length, day }0}\cdot 100\right)_{\text{treated}} - \left(\frac{\text{length, day }X}{\text{length, day }0}\cdot 100\right)_{\text{control}}$$

Where X = 6 for colyledonary internode, and
X = 10 for petioles.

Measurements of growth of the dominant lateral shoot are presented as the differences in length of treated and control (lanolin + distilled water only) plants at day 8. Measurement of leaf length and width (Fig. 1) were also made, but no differences were observed between control and treated plants. A Student's t-test was employed for the statistical comparison of selected mean data values.

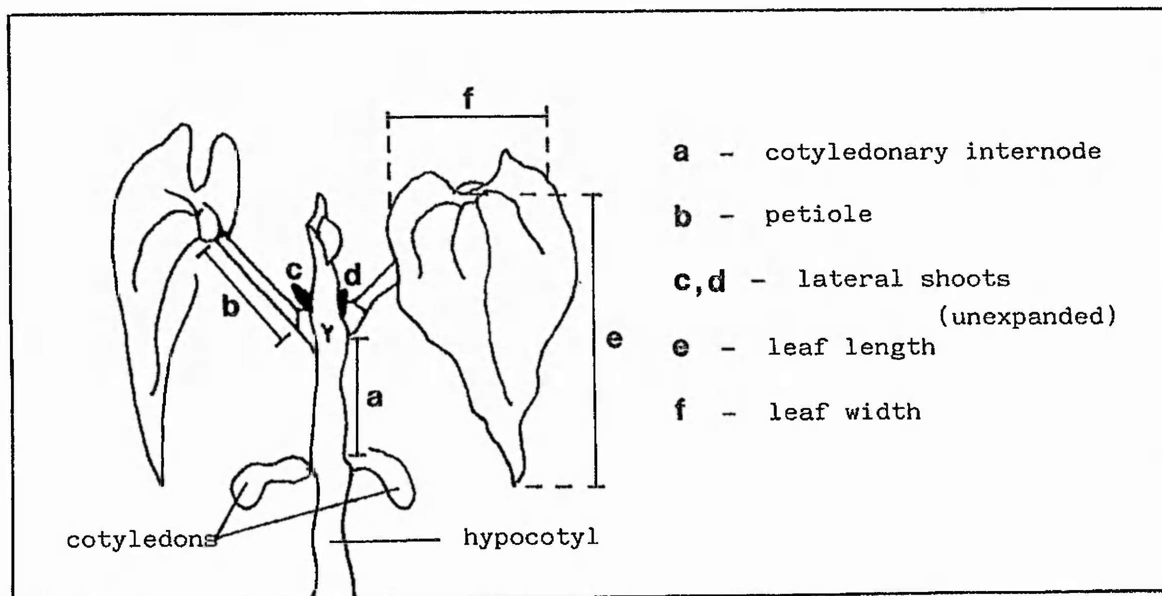

Fig. 1. Shoot of 13/14 day old *Phaseolus vulgaris* at time of treatment. a - f, parameters measured.

RESULTS

The short-term assay of etiolated *A. sativa* coleoptile length clearly demonstrates that bentazone is capable of inducing rapid cell elongation in a concentration-dependent manner similar to IAA(Fig. 2A). However, the magnitude of the response is not as great as that observed with IAA. When coleoptiles were incubated with both IAA and bentazone a marked interaction was observed i.e. auxin-induced elongation was significantly reduced ($p = 0.02$) in the presence of bentazone (Fig. 2B). This interaction was observed over a wide range of both auxin and bentazone concentrations (10^{-4} to 10^{-6}M; Miller, unpublished).

The long-term study of *P. vulgaris* growth clearly indicates that 10^{-4}M bentazone, when applied in lanolin to decapitated apical stumps, promotes the elongation of cotyledonary internodes, petioles, and the first internodes of the lateral shoots (Fig. 3). For the comparison of experimental treatments, the time to maximum observed growth was used, i.e. petioles from bentazone treated plants had increased in length by a maximum of 25% more than the control 10 days after treatment, similarly 8 days and 6 days for 1st internode of dominant lateral and cotyledonary internode respectively. These

responses proved similar to those observed with auxin, although bentazone was less active at lower concentrations (10^{-5} and 10^{-6}M; Fig. 4,A,C,E). However, when elongation was measured in the presence of both 10^{-4}M auxin and bentazone, interactions were again observed (Fig. 4,B and D) and a significant inhibition ($P = 0.01$) was noted with the elongation of the first internode of the lateral shoot (Fig. 4F).

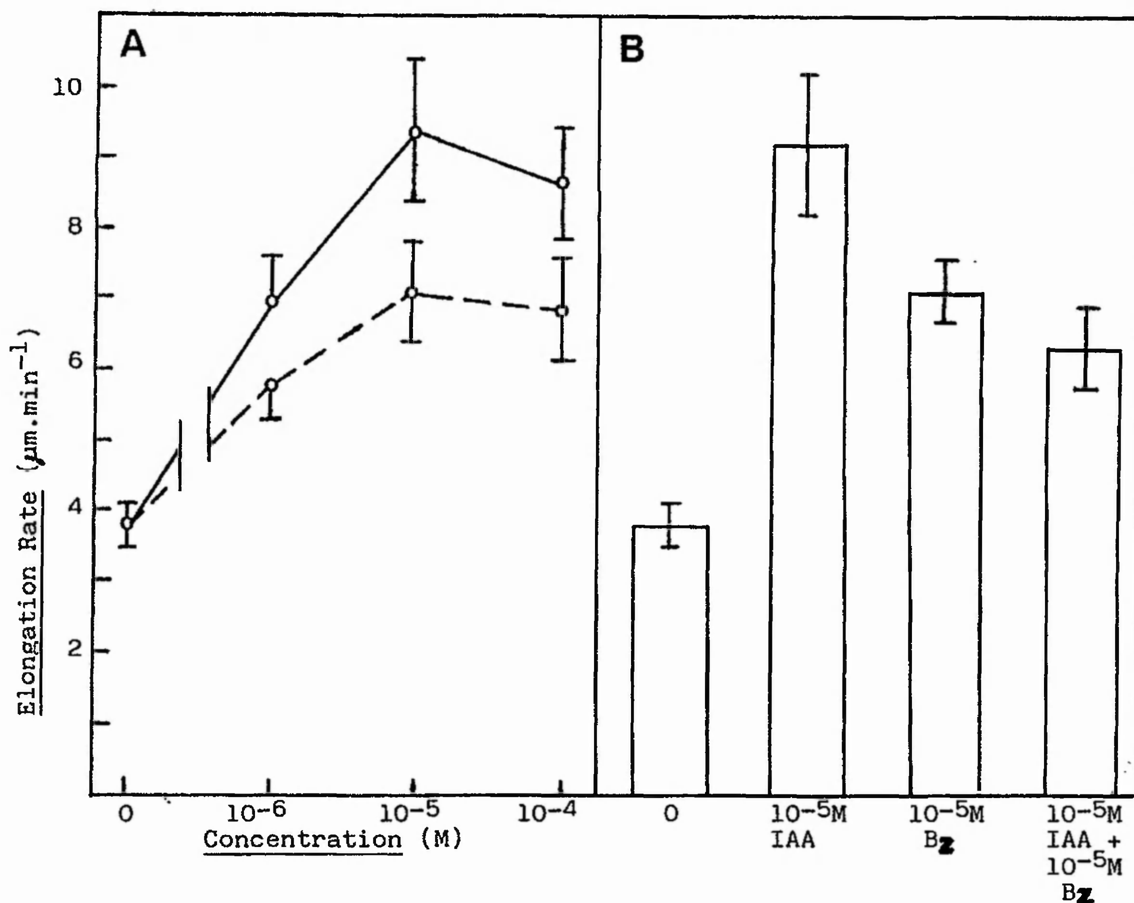

Fig 2. (A) Dose-response of A.sativa coleoptiles to auxin (•——•) and bentazone (•– –•). (B) Interaction of auxin and bentazone. (Elongation rates measured over a 60 min period).

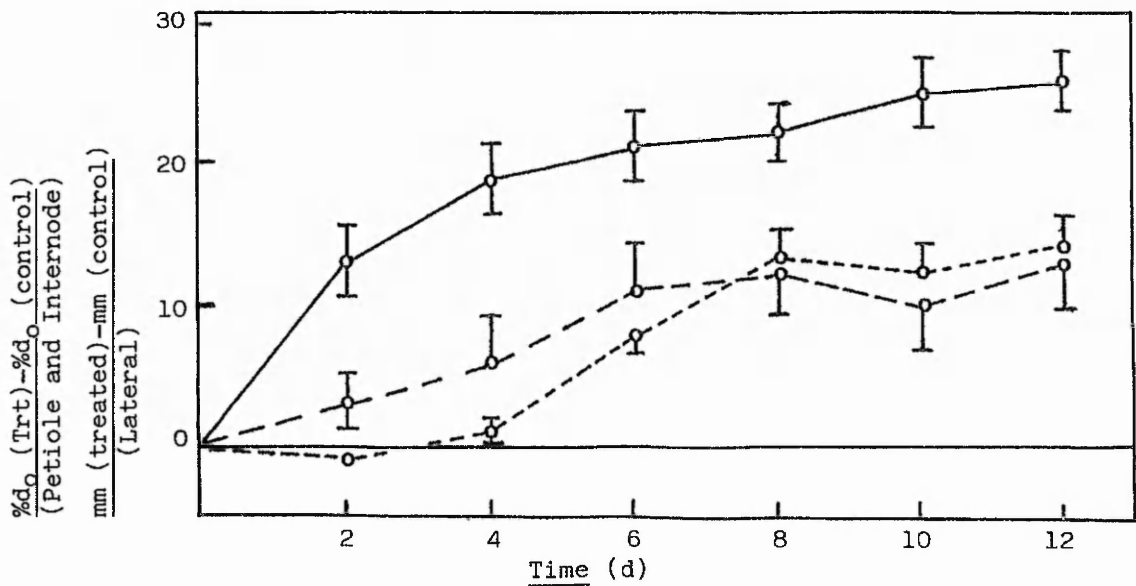

Fig 3. Growth characteristics of P.vulgaris following treatment with 10^{-4}M bentazone. 1st internode (•— — —•, n=10), petioles (•———•, n=20), laterals (•- - - - -•, n=10).

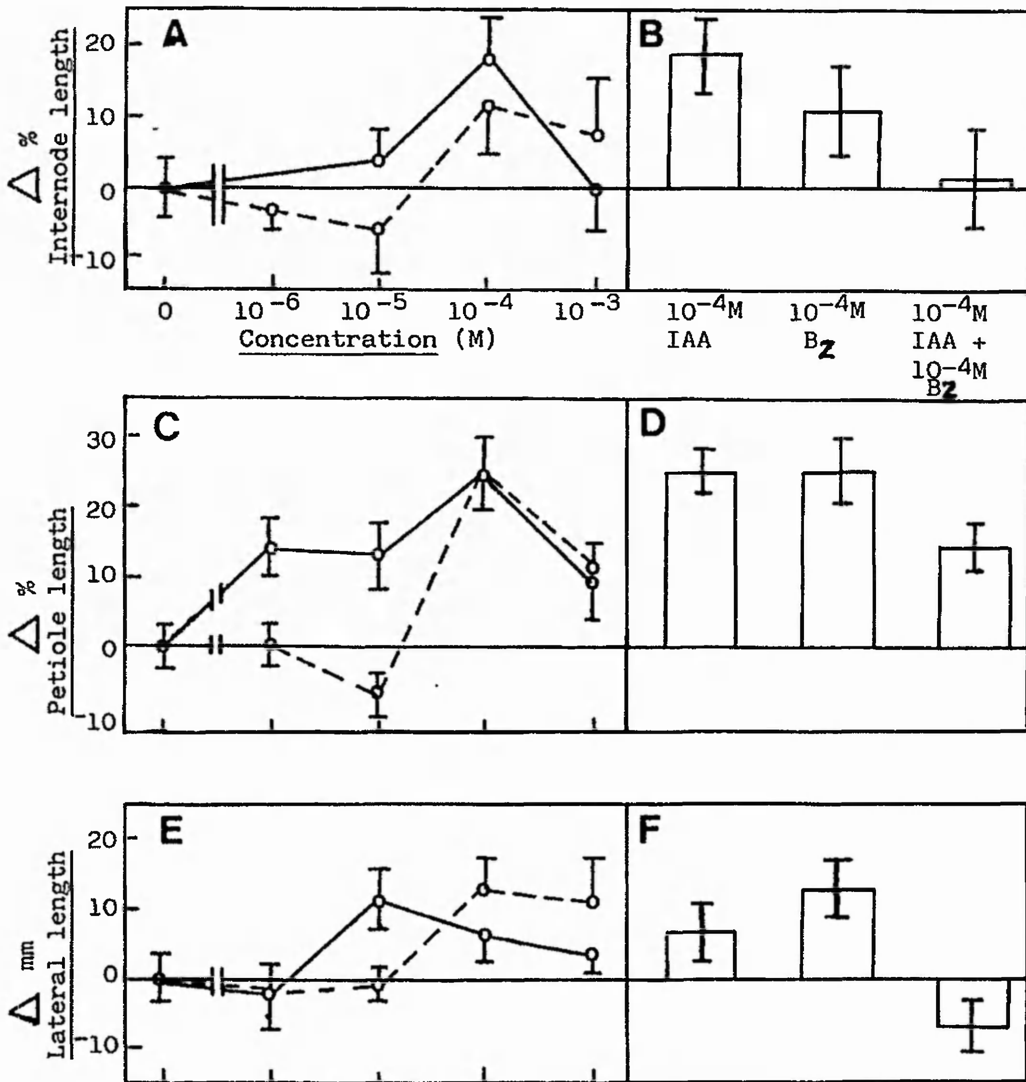

Fig 4. Dose-response of P. vulgaris lst internode (A), Petioles (C) and lst Internode of Lateral shoot (E), to auxin (•——•) and bentazone (•– –•). Interaction of auxin and bentazone in lst Internode (B), Petioles (D) and lst Internode of lateral shoot (F). (Values are means + SE, n = 10 or 20).

DISCUSSION

Although the literature describes bentazone as an inhibitor of photosynthetic electron flow at photosystem II (Moreland, 1980), recent findings have clearly indicated an additional site of action at the plant cell membrane (Cobb et al., 1985). The present study has also shown bentazone to rapidly induce cell elongation in etiolated A. sativa coleoptiles and has demonstrated a significant interaction of auxin and bentazone in this process. Bentazone may behave as an auxin since its molecular dimensions are in accord with the hypothesis that a strong negative charge is separated from a weak positive charge by 0.55 nm (Thimann, 1963). In this way, bentazone may stimulate proton efflux in a similar manner to auxin (Evans, 1985). It is therefore not surprising that inhibitory trends are observed when both compounds are present in the assay, since competition for a common active site may take place. Bentazone was also shown to have auxin-type activity in the long-term, sub-apical elongation growth of P. vulgaris, and interacted with auxin in a similarly inhibitory fashion. Indeed, the presence of both 10^{-4}M bentazone and 10^{-4}M IAA caused a significant reduction in the length of the first internode of the dominant lateral shoot compared to individual treatments (Fig. 4F), and indicates a possible reimposition of apical dominance when the two compounds interact. However, possible mechanisms underlying these interactions in the P. vulgaris study are many and complex. Whereas the A. sativa system, employing dark-grown monocotyledonous tissue, is essentially rapid and confined to the cell membrane over a 60 min period, the P. vulgaris system studies growth of a light-grown dicot over a 12 day period, and clearly involves many endogenous and exogenous determinants. Therefore, extrapolation from the former to the latter experimental system should be avoided until the contribution of the following factors is understood. (1) Transport mechanisms: To achieve the growth responses observed, exogenously applied IAA and bentazone must be basipetally transported to the rapidly elongating tissues. A study of the movement of radiolabelled compounds would be needed to identify any interaction during transport. (2) Competition at active sites: Kinetic studies are needed, again with radiolabelled bentazone and IAA, to establish an interaction of these compounds at the cell membrane. Furthermore, an interaction may occur at the genome level, since convincing evidence exists in the literature for the promotion of protein synthesis by auxin and the phenoxy acid herbicides (Fedtke, 1982). (3) Auxin metabolism: Auxin activity in vivo is closely dependent on concentration and various mechanisms of synthesis and degradation are thought to operate to ensure an optimum IAA concentration in a given tissue. Benzatone may interfere with auxin synthesis and conjugation, or conceivably interact with IAA oxidase activity. (4) Other growth regulators: Natural plant growth regulators seldom act alone but interact with each other to produce complex growth responses. For example, auxin is known to stimulate ethylene biosynthesis in some tissues which may then be inhibitory to cell elongation (Beyer, Morgan and Yang, 1984). (5) Metabolites: Detailed analytical procedures are necessary to establish whether, after a 12 day period of growth, an interaction of IAA and bentazone is evident, or whether their metabolites are producing the observed responses. For example, bentazone may become hydroxylated in the 6- or 8- position (Mine, Miyakado, and Matsunaka, 1975) and these derivatives have different physiological activity to the parent molecule (Cobb, Miller and Nichols, unpublished).

Hence, further studies are required on the action and interaction of bentazone with auxin. Such information would clearly be of value in establishing a complete mode of action of this herbicide both in the laboratory and in the field, and may help to explain the many recorded instances of the interactions of other herbicides, particularly with the phenoxy acids and pyridine derivatives, with auxin-type activity.

The methods used in this study are of general value in establishing the auxin-type activity of a herbicide. The 60 minute A. sativa assay gives highly reproducable data, is simple and rapid to perform, is supported by a considerable literature, and is specific to cell elongation. A study of P. vulgaris growth is also simple to perform, although time-consuming, and has the potential for a more widespread screen for growth regulator activity and interaction.

ACKNOWLEDGEMENT

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AUXIN-INDUCED H^+-EFFLUX: HERBICIDE ACTIVITY AND ANTAGONISM

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ABSTRACT

A method is described for the accurate measurement of proton-efflux from *Avena* coleoptile segments. Auxin induces rapid, linear rates of H^+-efflux after a short lag-period and the ability of various herbicides to mimic this process is reviewed. The 'dose-response' characteristics of 2,4-D have been determined in the presence and absence of fluazifop-butyl, a phenoxypropanoate graminicide. The antagonism observed between these two herbicides in the field is reflected in H^+-efflux assays in a manner indicating fluazifop-butyl has anti-auxin activity. The general importance of the anti-auxin behaviour of phenoxypropanoate graminicides is discussed.

INTRODUCTION

Despite forty years of intensive study devoted to synthetic and natural auxins there are still many features of their herbicidal activity which are poorly understood and merit further investigation (Pillmoor and Gaunt, 1981). Perhaps one reason for this has been the choice of experimental systems used to study 'auxin-type' responses. A common method used to detect auxin activity is the *Avena* coleoptile straight-growth bio-assay. However,auxins do not induce rapid growth directly but act *via* the induction of H^+-efflux leading to the acidification of the cell-wall matrix (Rayle, 1973). Proton efflux, therefore, is the first detectable effect of an auxin on sensitive (i.e. receptive) plant cells and as such precedes all other auxin-induced phenomena (altered genome expression etc.). Indeed, the ability of a compound to induce H^+-efflux can be viewed as a measure of its potential toxicity as an auxin-type herbicide (assuming it is not metabolised significantly by the target species), even though H^+-efflux itself may not be the cause of plant death.

An important advantage of studying H^+-efflux (instead of growth) is that the resulting data may be interpreted in terms of a simple molecular model. H^+-efflux is brought about through the activation of a plasmalemma-bound H^+-pumping ATPase (Evans, 1985). Auxin (IAA) does not appear to directly affect this enzyme *in vitro* but *in vivo* stimulates its activity *via* a putative secondary-messenger system, itself initiated by auxin binding to receptors in the plasmalemma and possibly elsewhere (Libbenga *et al*., 1986). It should be possible to determine the relative affinity of various auxins for the receptor protein binding site. In order to do this we require detailed information about rates of H^+-efflux over a wide range of 'auxin' concentrations (Weyers *et al*., 1987).

In this paper we describe a method for accurately measuring rapid, linear H^+-efflux from *Avena* coleoptiles and present comparative data for a number of well known synthetic auxins and auxin-type herbicides. We have analysed the activity of 2,4-D in detail and present data comparing the effect of this compound in the presence and absence of fluazifop-butyl, a phenoxypropanoate (ester) graminicide. These herbicides were chosen as an example of a mixture in which a broad-leaf weed-killer antagonises graminicide action in the field (Cobb, 1987). It is suggested that the

anti-auxin activity of the phenoxypropanoates may be a more important facet of their herbicidal activity than previously considered.

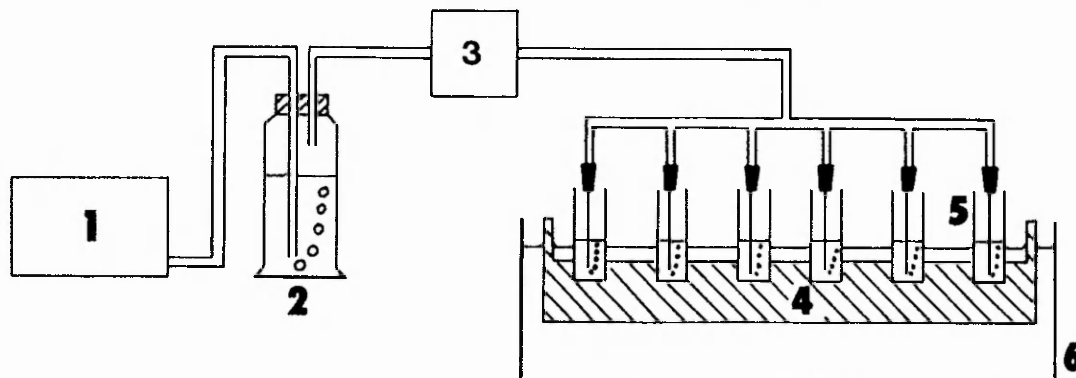

Fig. 1. The apparatus. 1) Air pump 2) Humidifier 3) Flow meter 4) Cuvette holder 5) Aeration chambers 6) Water bath.

MATERIALS AND METHODS

Avena sativa var. Maris Tabard seeds were sown in moist vermiculite (tap-water) and seedlings harvested after 88-94 h growth at 24 ^{o}C (darkness, high humidity). For each experiment c. 100 coleoptiles (35-60 mm) were gently abraded with a carborundum paste (800-grit silicon carbide powder in water) and stored/washed in distilled water. Segments were cut 3-18 mm behind the apex and stored (in darkness) in 50 cm^3 of 0.15 mM MES-Tris buffer, pH 6.5. The segments were washed and kept stirred by vigorous aeration (600 cm^3/min) for 15 min, rinsed and transferred (10 x 10) into plastic vials (7 cm^3 total volume) containing 1.8 cm^3 of MES-Tris buffer plus 1.0 mM KCl (dim lab-light conditions, c. 10 µmol photons/m^2/s, PPFD). The vials were maintained at a constant temperature of 25 ^{o}C and individually aerated (50 cm^3/min) with humidified lab-air (Fig. 1). After 40-50 min continuous aeration various chemicals were added as required and pH readings commenced (110-120 min from starting tissue preparation). Approximately 1.5 cm^3 of each bathing medium was transferred in sequence to a constant-temperature stirring vessel (Clark-type O_2-electrode cuvette) and pH measured to the nearest 0.01 unit (Russell semi-micro pH electrode, Corning 140 pH meter). 'Auxins' were dissolved in acetone (10-100 mM), diluted with Tris buffer to neutralize, then further diluted with the K^+-MES-Tris buffer to give stock solutions 100-times the final concentration required in the assay. Fluazifop-butyl (PP009, 90%) was dissolved in acetone (10 mM) before use. Over the pH range 6.3 - 5.5, the bathing medium buffering capacity was linear thereby allowing the rates of change of pH to be used as a direct measure of increase in [H^+]. For each treatment the rate of H^+-efflux was determined from at least two-to-four consecutive estimates of pH. Dose-response curves were analysed using a computer program developed by Weyers *et al.* (1987), based on a model of hormone-receptor complex formation at specific binding sites in plant cells.

RESULTS AND DISCUSSION

The effect of IAA on the pH of the coleoptile segment incubation medium is shown in Fig. 2. In the experiment shown 10 µM auxin induced rapid H^+-efflux after a lag period of 12 min and the rate of efflux was linear with respect to time for at least 30 min thereafter. Eventually, the rate of change of pH slowed down so that after 60 min no further substantial change in the pH difference relative to control was seen. At the lower

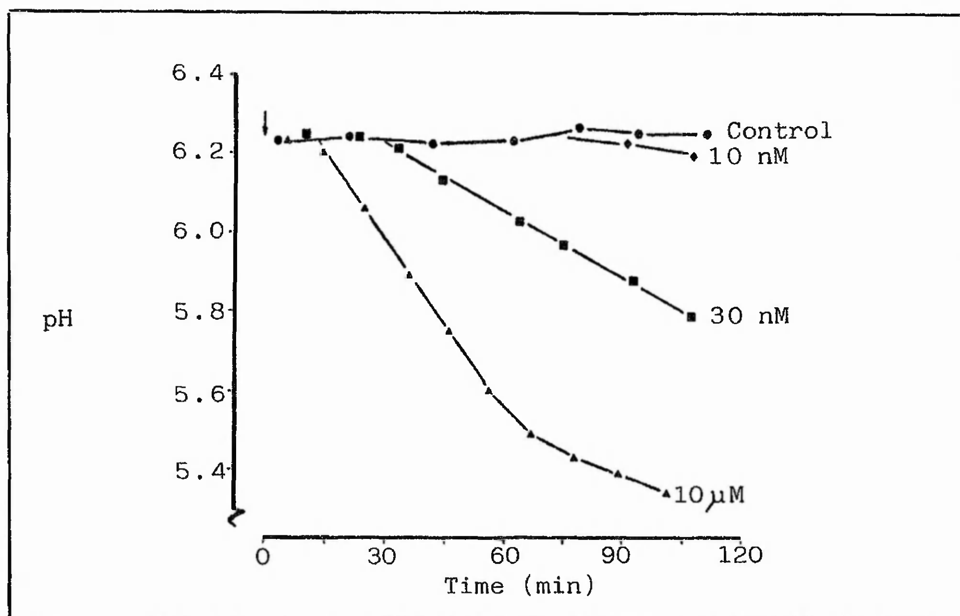

Fig. 2. The effect of various concentrations of IAA on bathing medium pH.

concentration of 30 nM, auxin was still able to promote appreciable rates of H^+-efflux in a linear fashion, with only a slightly extended lag period. At concentrations of 10 nM or less virtually no change in bathing medium pH was observed at all. Each curve of pH change against time has two important features: a) Lag period (L) ; b) Initial rate of response (R)

The lag-period for auxin-induced H^+-efflux varied as a function of external concentration (12-40 min, 10^{-4} - 10^{-8} M), however, irrespective of the length of the lag-period the rate of H^+-efflux was always linear with respect to time at least until the external pH had fallen to c. 5.5.

The relationship between the initial rate of H^+-efflux and external auxin concentration shows features common to a 'classical' hormone-receptor interaction (Fig. 3, O'Brien, 1979). The transition from 10 to 90% effect takes place over a narrow (physiologically relevant) concentration range and there is an even level of near-maximum response over a wide range of higher concentrations (this contrasts with low specificity and distinct optima often seen in growth curves; Nissen, 1985). These observations show that the rapid measurement of H^+-efflux is the most appropriate means of describing the response of Avena coleoptiles to various 'doses' of auxin. Data of this type (Fig. 3) can be analysed with respect to a simple model (based on Michaelis-Menten principles) which compares the rate of reaction (R) with the rate of 'hormone'(H)-receptor (Rec) complex formation.

$$\text{i.e.} \quad H + Rec \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HRec \xrightarrow{k_r} \text{Response } (H^+\text{-efflux})$$

where k_1, k_2 are rate-constants for HRec association/ dissociation and k_r is the rate constant for the series of events leading from the formation of HRec to the induction of H^+-efflux. It has been shown that such responses can be described in the following terms:

$$R = \frac{R_{max}\ [H]^p}{[H]^p + K_D}$$

where R_{max} = maximum initial rate of response
K_D = dissociation constant for HRec

$[H]_{50}$ = $K_D{}^{1}/p$, the 'hormone' concentration giving 50% response

p = interaction coefficient, allows deviations from Michaelis-Menten kinetics to be accommodated into the model.

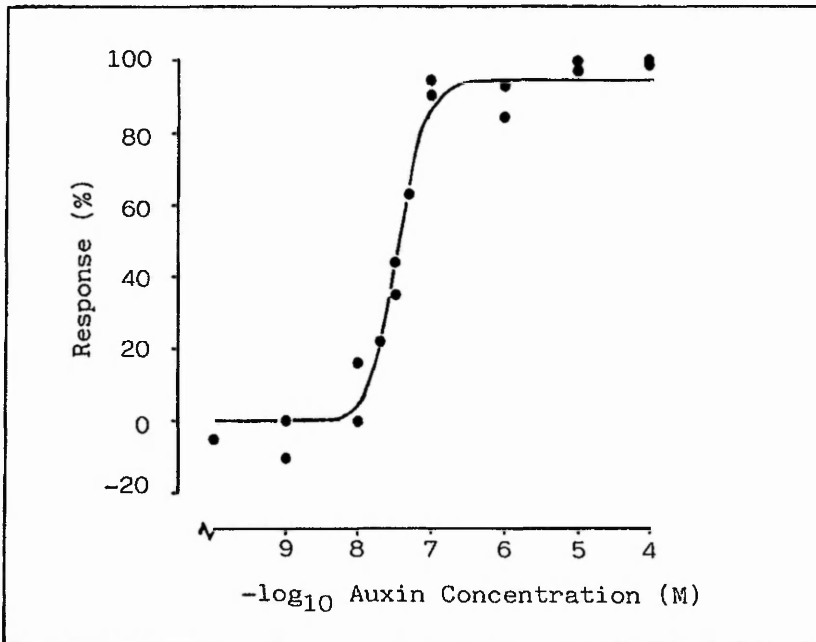

Fig. 3. IAA dose-response curve : initial rate of H^+-efflux. The data plotted were obtained in two separate experiments. The rate of response has been standardised so that the maximum observed rate (at 10^{-4}M) = 100. Maximum rates for each experiment were 1) 0.62 pH units/h 2) 0.87 pH units/h.

The above three parameters may be used to describe any dose-response curve and are termed 'sensitivity' parameters (Weyers et al., 1987). Using a computer program we have been able to make objective estimates of these parameters for auxin-induced H^+-efflux.

R_{max}	K_D	$[H]_{50}$	p
94.4	4.05×10^{-10}M	3.41×10^{-8}M	2.31

The relative activity of a number of different synthetic auxins (plus fusicoccin) is shown in Table 1. Fusicoccin is a phytotoxin known to promote high rates of H^+-efflux in a wide range of plant tissues, irrespective of their sensitivity to auxin (Marrè, 1979). The chemicals are grouped into activity classes as a crude means of defining their ability to mimic IAA in H^+-efflux assays. Of the herbicides tested, only benazolin was

able to promote H^+-efflux at appreciably faster rates than auxin (100-500 µM, 109-125%) although 2,4-D was able to induce final effects greater than auxin (data not shown). Clearly, fusicoccin and the auxin-herbicides may be less potent than auxin in terms of concentration but are characterised by causing more sustained levels of H^+-efflux. The resulting apoplast pH values of less than 5.0 are likely to cause toxic effects in target tissues.

Table 1. The H^+-efflux activity of Fusicoccin, synthetic auxins and auxin herbicides.

Chemical	Concentration (µM)	R (%)	L (min)	ACTIVITY
Fusicoccin	0.1	77	5	
"	1.0	179	3	HIGH
NAA	1.0	96	14	
Fluroxypyr	1.0	75	25	
"	10	101	17	
IPA	10	98	21	MEDIUM
2,4,5-T	10	85	16	
BAS 518	10	91	19	
2,4-D	10	83	20	
Benazolin	100	109	13	
BAS 514	100	75	20	
Fenoprop	100	70	16	LOW
Clopyralid	100	50	35	
"	1000	80	20	
2,3-D	10			
Ioxynil	100			NONE
Acifluorfen	100			
Fluazifop-H^+	100			

The data shown are those for the minimum concentration which gave rates of H^+-efflux close to those induced by an auxin standard (1-10µM; R = % relative effect).

Table 2. 2,4-D Sensitivity parameters.

Sensitivity parameter	2,4-D (A)	2,4-D + 100 µM Fluazifop-butyl (B)	B/A
R_{max}	117	119	1.0
K_D (µM)	2.1	14.8	7.2
$[H]_{50}$ (µM)	2.3	20.5	8.9
p	0.86	0.89	1.0

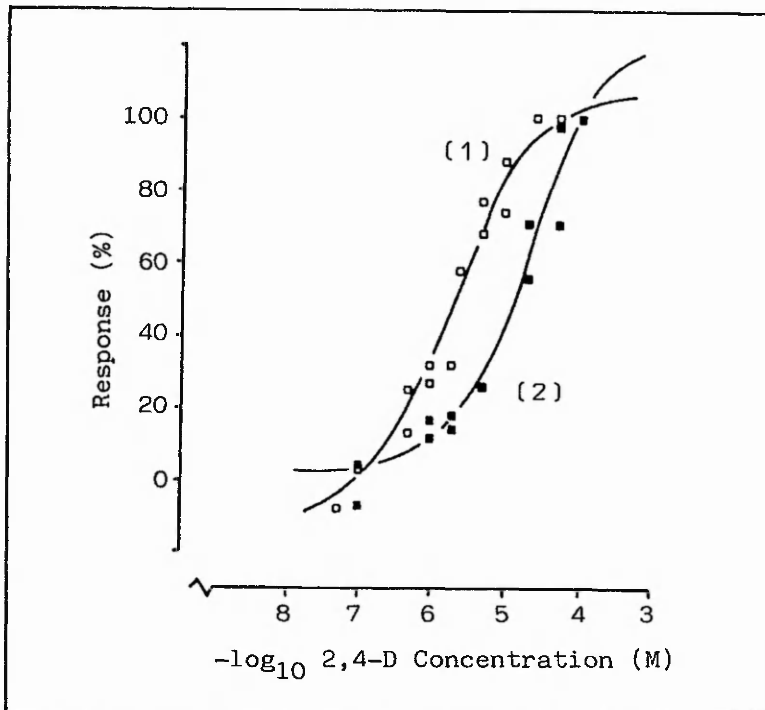

Fig. 4. 2,4-D dose-response curve ± 100 µM Fluazifop-butyl. Curve 1; 2,4-D only; Curve 2; plus fluazifop-butyl. Each curve represents the pooled data from two separate experiments. 2,4-D only : 100 = 0.65, 0.69 pH units/h. Plus fluazifop-butyl : 100 = 0.70, 0.82 units/h.

Fig. 4 (curve 1) shows the dose-response characteristics for initial rates of 2,4-D-induced H^+-efflux. 2,4-D (20-100 µM) can induce H^+-efflux at the same rate as auxin but is almost completely ineffective below 0.5 µM ($[H]_{50}$ = 2.3 µM, c. 70 x auxin $[H]_{50}$). Fluazifop-butyl alone was inactive in promoting H^+-efflux but was able to modify the activity of 2,4-D when the two herbicides were added simultaneously. Fig. 4 (curve 2) shows the dose-response curve for 2,4-D-induced H^+-efflux in the presence of 100 µM fluazifop-butyl. At high 2,4-D concentrations (100 µM) the graminicide did not affect rates of H^+-efflux but as the 2,4-D concentration was lowered an increasing level of inhibition was observed. Analysis of the two dose-response curves (Table 2) reveals that the affinity of the 'auxin' receptor for 2,4-D is reduced (K_D increased) by fluazifop-butyl although R_{max} remains unaffected. Such a pattern of effect is indicative of a simple competitive inhibition between these two herbicides for binding at an auxin-receptor site.

CONCLUSIONS

Whilst the elongation of Avena coleoptiles is a convenient bioassay for auxin activity, the rapid promotion of growth is not a direct response to auxin but an indirect consequence of apoplast acidification (Evans, 1985). The method described here for studying H^+-efflux allows the accurate determination of a rapidly induced auxin-mediated effect. Using this method we have obtained data which is suitable for analysis with respect to a molecular model of plant 'hormone' responses (Weyers et al., 1987).

As expected, several synthetic auxins and auxin-herbicides were active in a qualitatively similar fashion to IAA. The relative activity of each compound was determined by comparison with 1 to 10 μM auxin-induced rates of H^+-efflux (Table 1). The ability of each compound to induce H^+-efflux can be seen as a reflection of its ability to fit the auxin-binding receptor protein. Interesting information about structure-activity relationships can be obtained. For instance, the closely related herbicides fluroxypyr and clopyralid or BAS 514 and BAS 518 occupy different activity groups in Table 1. Even more acute dependency on specific molecular structure can be seen when comparing the activity of 2,4-D with that of 2,3-D.

We have applied the principles of hormone-receptor binding kinetics (cf. Michaelis-Menten analysis) to describe the auxin activity of 2,4-D in detail. Fluazifop-butyl, a graminicide susceptible to antagonism by 2,4-D in field applications, alters the dose-response characteristics for 2,4-D-induced H^+-efflux in a manner consistent with a simple competitive inhibition of 2,4-D/auxin-receptor complex formation. Fluazifop-butyl has no auxin activity alone but in combination with 2,4-D behaves as an anti-auxin. It cannot be determined from these studies whether fluazifop-butyl binds at the same or a different site as 2,4-D on the auxin receptor. The observed kinetics could be explained by fluazifop-butyl actually occupying the auxin binding site itself (scheme 1, Fig. 5) or by binding to a separate site on the receptor (scheme 2, Fig. 5) giving the same overall effect.

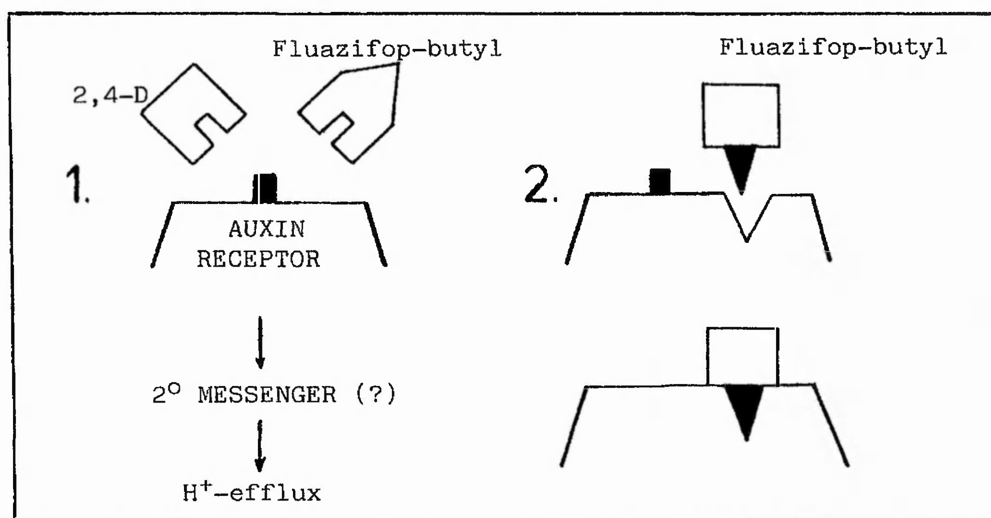

Fig. 5. Schematic representation of two models of fluazifop-butyl/2,4-D antagonism. Scheme 1: competition for common binding site. Scheme 2: separate binding sites; occupation of fluazifop-butyl site results in loss of 'auxin'-binding ability.

Can our experimental observations explain graminicide/broad-leaf weedkiller antagonisms observed in the field? The anti-auxin behaviour of fluazifop-butyl can be seen in target grass weeds which exhibit reduced internode elongation (eg. in *Setaria viridis*; Carr, 1986). In the presence of 2,4-D these sub-lethal symptoms may not develop, consequently resulting in increased vigour in the target weeds. The ultimate phytotoxicity of the phenoxypropanoates seems to be dependent on the accumulation of the acidic moiety (fluazifop, diclofop) in apical tissues where it inhibits lipid synthesis leading to necrosis and plant death (e.g. Carr *et al*., 1985, 1986; Shimabukuro *et al*., 1979). More information about the uptake,

hydrolysis and movement of graminicides in treated plants may reveal whether the short-term or long-term effects of 2,4-D on grass weeds can best explain the observed antagonism. We are currently extending our studies of H^+-efflux to include other graminicide/broad-leaf weedkiller mixtures. It should be possible in future to demonstrate whether anti-auxin properties are a general feature of the phenoxypropanoates and the extent to which such properties are the basis of herbicide antagonisms observed in the field.

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THE CELL MEMBRANE AS A SITE FOR BENTAZONE ACTION

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ABSTRACT

Bentazone can induce rapid, concentration-dependent turgor changes in both light and darkness in isolated protoplasts (from *Glycine max* mesophyll cells and *Commelina communis* guard cells), abaxial epidermes (from *C.communis*) and etiolated coleoptiles (from *Avena sativa*). Hence, bentazone can alter protoplast size, stomatal apertures and coleoptile elongation, and each experimental system is sensitive to specific cations and may be inactivated by inhibitors of cytoplasmic ATP synthesis. A unifying scheme is proposed that interprets this data in terms of the cell membrane as a site of bentazone action, that is independent of any role in photosynthesis.

INTRODUCTION

Bentazone (3-isopropyl-1H-2,1,3-benzothiadiazine-4(3H)-one 2,2-dioxide) is a selective herbicide for the postemergence control of broadleaved weeds in several important crops including soybean, rice, peanut, pea, maize and beans. Studies conducted during the 1970s by several workers showed that this major herbicide inhibited electron flow at photosystem II, using isolated thylakoids (Pfister *et al*. 1975), chloroplasts (Mine & Matsunaka 1975, Böger *et al*. 1977) and cells (Retzlaff *et al*. 1979), and also inhibited photosynthesis in intact leaves (Penner 1975, Retzlaff & Hamm 1976, Potter 1977). However, subsequent studies in this laboratory clearly demonstrated that bentazone efficacy was particularly sensitive to environmental conditions both before and after application (Taylor *et al*. 1981), and this led to the hypothesis that bentazone could alter stomatal movement in *Chenopodium album* leaves (Dunleavy *et al*. 1982, Cobb *et al*. 1983) and isolated abaxial epidermes (Dunleavy & Cobb 1984 a and b). More recent observations (Nichols unpublished) have demonstrated that this response is not confined to *C.album* but is more widespread, suggesting that a feature of the bentazone molecule is an ability to alter stomatal movement, presumably by changing the permeability of the stomatal guard cell membranes.

The present study was conducted to examine further this membrane response in isolated protoplasts, epidermes and coleoptiles, in both light and darkness and to gain further insight into the role of the cell membrane as a site of bentazone action.

MATERIALS AND METHODS

Glycine max mesophyll cell protoplasts

Protoplasts were enzymatically prepared from mesophyll cells of first trifoliate leaves of *Glycine max* L. Merrill cv Fiskeby *V* using the method of Rees *et al*. (1985). Protoplasts were highly intact, *c*. 90% pure and capable of photosynthetic rates of up to 90 µmoles O_2 evolved.(mg.chlorophyll)$^{-1}$.hr^{-1}. For volume determinations protoplasts (2 x 10^6 ml^{-1}) were first incubated for 10 min at 25°C at a photon flux density (PFD) of 100 µmol.m^{-2}.s^{-1} (photosynthetically active radiation, PAR). An appropriate concentration of bentazone was then added to the reaction mixture (final concentration 0, 1, 10, 100 and 1000 µM) which was stirred (80 rpm), illuminated at 500 µmol.m^{-2}.s^{-1} and maintained at 25°C pH 7.6 for up to

90 min. Aliquots were withdrawn at regular intervals and protoplast diameters determined microscopically and, assuming the protoplasts to be spherical, converted to volumes using the formula $4/3\ \pi r^3$. Each bentazone treatment was replicated 4 times and mean protoplast volumes calculated ± S.E. (n = 40).

Commelina communis epidermes and guard cell protoplasts

Fully expanded leaves of Commelina communis L. were pretreated for 70 min on 25 ml 0.1 mM $CaCl_2$ and exposed to a photon flux density of 50 µmol.m^{-2}.s^{-1} (PAR). Abaxial epidermes were carefully prepared, all visible contamination removed and the peels floated on pretreatment solutions for 60 min to induce either stomatal opening or closure. Opening was induced by the presence of 50 µmol.m^{-2}.s^{-1} (PAR), 25°C, 100 mM KCl and < 50 µl/l CO_2, whereas closure was obtained by darkness, 25°C, > 500 µl/l CO_2 and the absence of KCl. Epidermal peels were then transferred to treatment solutions of varying KCl concentration ± 100 µM bentazone for 2 h and the environmental conditions maintained as described above. All solutions were maintained at pH 6.0 by the addition of KOH and pH remained constant throughout the period of study. Stomatal apertures were directly measured microscopically, each treatment was replicated 3 times and mean apertures calculated ± S.E. (n = 60).

Guard cell protoplasts were enzymatically isolated from abaxial epidermes according to the method of Fitzsimons and Weyers (1983). Protoplasts were incubated in buffer (pH 5.5) containing 10 mM KCl and 100 µM bentazone for up to 60 min at 25°C in darkness. Protoplast diameters were measured microscopically and mean volumes calculated ± S.E. (n = 30).

Coleoptile elongation in etiolated Avena sativa

Avena sativa L. cv Maris Tabard was grown in moist vermiculite for 3 d in total darkness at 25°C. Coleoptiles were removed in dim light, the apical 0.5 cm discarded and 1 cm segments preincubated on 25 ml 5 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) for 45 min at 25°C in darkness. Segments were then transferred to 25 ml of incubation medium (buffer plus bentazone or IAA) and maintained in darkness at 25°C for up to 60 min. Fifteen segments were carefully removed at predetermined intervals and their lengths precisely determined using an overhead projector. Each treatment was replicated at least 3 times, mean lengths calculated ± S.E. (n = 45) and regression values determined where appropriate. In Fig. 3A, r = 0.69, control; r = 0.71, 1 µM; r = 0.71, 10 µM and r = 0.72, 100 µM bentazone.

RESULTS

The data presented in Figures 1, 2 and 3 clearly indicate an action of bentazone at the cell membrane of mesophyll and guard cell protoplasts, abaxial epidermes and etiolated coleoptiles. In each case the bentazone-induced responses were rapid and suggested large turgor changes within the treated cells.

Physiologically competent protoplasts isolated from G.max mesophyll cells showed a rapid increase in volume in the presence of bentazone (Fig. 1A). This response was concentration-dependent, in that 10 µM bentazone induced a near doubling in protoplast volume (197% of control values) after 30 min of incubation in the light (Fig. 1B). No strict light-dependency was evident in this response, since 10 µM bentazone was also found to cause protoplast swelling in total darkness (161% of control values after 30 min incubation). 100 µM bentazone induced a similar volume increase in protoplasts isolated from C.communis abaxial epidermes (Fig. 2A). This protoplast swelling in darkness was equally rapid but more transient, since

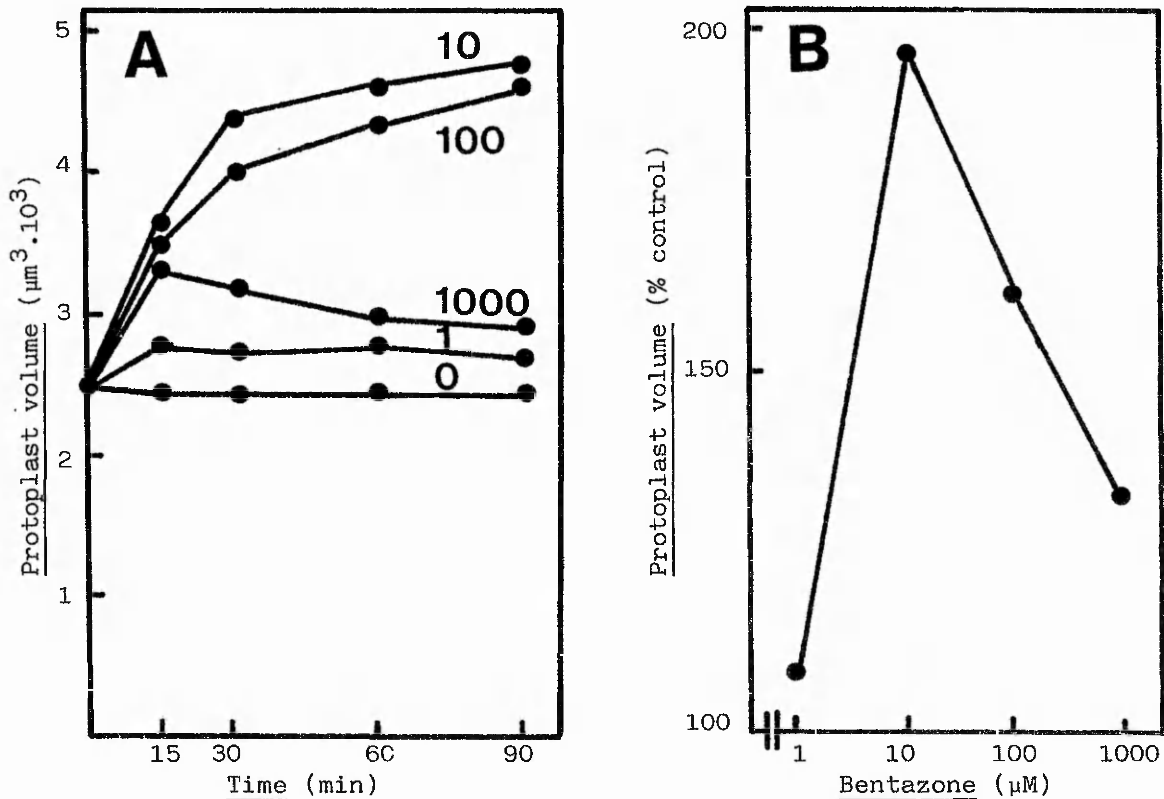

Fig. 1. Bentazone-induced protoplast swelling in isolated mesophyll cells from *Glycine max* incubated in the light, 25°C, pH 7.6 and in the presence of 0-1000 µM bentazone. Fig. 1A illustrates the time-course of protoplast swelling over a 90 min period, whereas Fig. 1B indicates a concentration-dependent response, expressed as % of control values after 30 min incubation.

volumes were observed to return to control values after 40 min of incubation, possibly implying a reversible response in this cell type. In both experimental systems little or no protoplast rupture was observed as a result of bentazone treatment. Furthermore, staining with neutral red, Evan's blue or fluorescein diacetate indicated a high degree of plasmalemma integrity suggesting fully functional cell membranes.

Guard cells function *in vivo* to control stomatal apertures by changes in their turgor. Hence, increased guard cell turgor, caused by potassium ion and water influx, induces stomatal opening, whilst decreased turgor, potassium ion and water efflux, results in stomatal closure. 100 µM bentazone was therefore added to functional *C.communis* abaxial epidermes for 2 h to examine the effect of turgor changes induced by bentazone in the incubation medium (Fig. 2B). In the light, where experimental conditions favoured stomatal opening, 100 µM bentazone induced a potassium concentration-dependent response. Below *c*. 80 mM potassium, bentazone caused stomatal opening relative to the control, whereas above *c*. 80 mM potassium, apertures were less than control values. On the other hand, 100 µM bentazone produced wider stomatal apertures than the control throughout the potassium concentration range when incubated in darkness. Thus, the

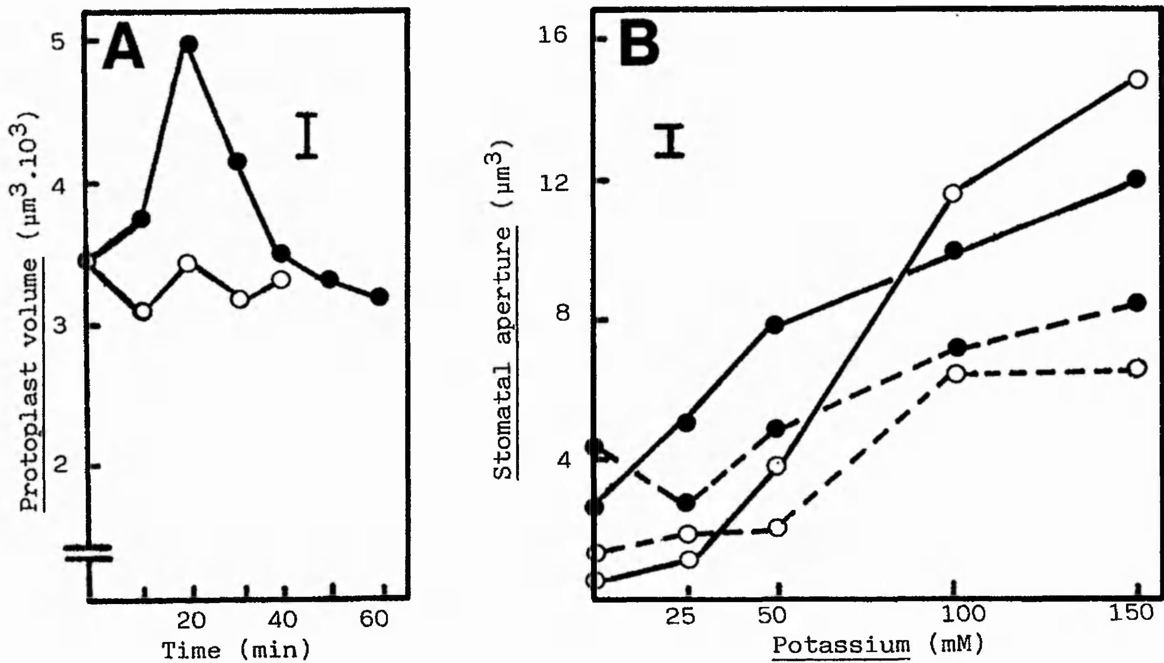

Fig. 2A. Bentazone-induced protoplast swelling in isolated guard cell protoplasts from Commelina communis incubated in darkness, 25°C and pH 5.5 for up to 60 min in the presence (●-●) or absence (O-O) of 100 µM bentazone. Fig. 2B. Effect of 100 µM bentazone on stomatal movement in abaxial epidermes of C.communis following 2 h incubation in either opening or closing conditions, at 0-150 mM KCl. Solid lines, opening conditions; broken lines, closing conditions; open circles, bentazone absent; closed circles, bentazone present.

bentazone-induced turgor changes observed with guard cell protoplasts in darkness (Fig. 2A) are reflected in wider stomatal apertures in dark-incubated epiderms (Fig. 2B).

Another well documented, rapid turgor response of plant cells is the elongation of etiolated Avena coleoptiles, particularly in the presence of indole acetic acid (IAA). Proton excretion from the coleoptile cells is thought to loosen the cell wall polysaccharides and the resultant influx of water causes an increase in cell length. Figure 3A clearly demonstrates that bentazone is able to induce coleoptile elongation in darkness in a concentration-dependent fashion. Indeed, 10 µM bentazone induced a three -fold increase in the rate of coleoptile elongation when compared to the control values, although it was not as active as IAA (Fig. 3B).

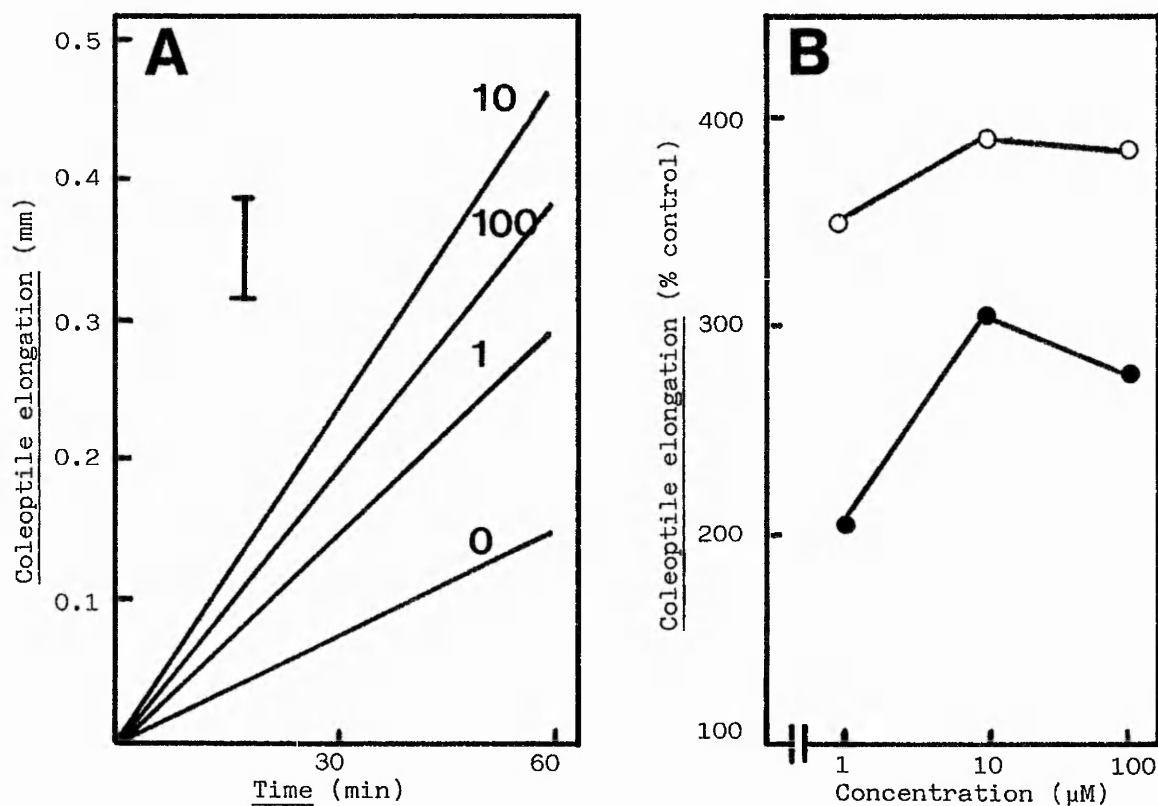

Fig. 3. Bentazone-induced coleoptile elongation in etiolated Avena sativa. Fig. 3A. Time-course of the response in the presence of 0-100 µM bentazone. Fig. 3B. Concentration-dependence of the response in the presence of bentazone (●-●) and auxin (O-O), expressed as % of control values after 60 min incubation.

DISCUSSION

Although there is abundant evidence in the literature designating bentazone as an inhibitor of photosynthetic electron flow at photosystem II (Moreland 1980), these findings clearly indicate a further action of bentazone at the plant cell membrane. Figures 1, 2 and 3 suggest that bentazone can induce rapid changes in the turgor of protoplasts, epidermes and coleoptiles in both light and darkness, and may therefore be considered to be independent of photosynthesis. Indeed, bentazone-induced swelling of G.max mesophyll cell protoplasts in darkness is particularly sensitive to inhibition by 10 µM rotenone and antimycin A (Rees, unpublished), from which it may be inferred that protoplast swelling has a requirement for ATP derived from mitochondria.

Protoplasts, epidermes and coleoptiles are all capable of rapid changes in cell turgor, mediated by the influx of cations and water into the cell in exchange for proton efflux, each possibly involving the action of membrane bound ATPases. Rees (unpublished) has demonstrated that G.max protoplast swelling is particularly sensitive to the magnesium ion concentration of the bathing medium and may be inhibited by compounds that prevent electron flow in mitochondria. Similarly, in stomatal movement potassium is the osmotically active cation (Fischer 1968) whose transport

into the guard cell may be prevented by several metabolic inhibitors (Outlaw 1983). Furthermore, coleoptile elongation in darkness has a requirement for calcium ions (Cohen & Nadler 1976) and is inactivated by inhibitors (see Davies 1973). Thus, there is good evidence in the literature for active cation transport at the cell membrane in each of the three experimental systems used in the study, although how bentazone may interact in each system remains to be established.

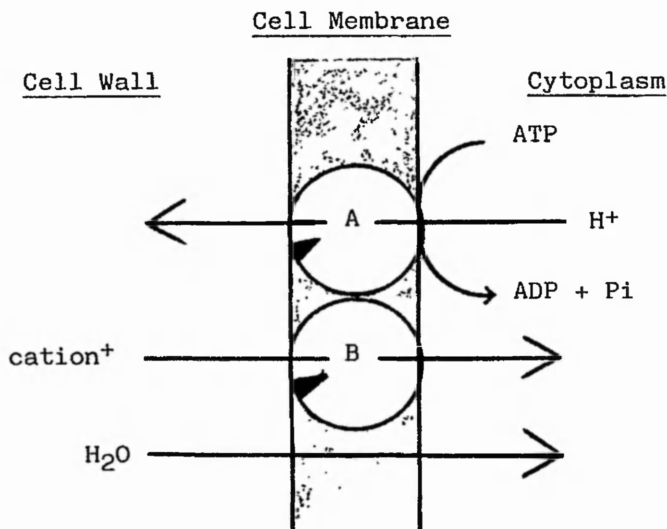

Fig. 4. A hypothetical scheme for bentazone action at the plant cell membrane. An ATPase proton pump (A) is envisaged to drive the efflux of protons in exchange for cations at a cation-specific transport site (B). One or both transport molecules may contain regulatory sites (▼) that are sensitive to bentazone and/or other regulatory molecules, such as IAA.

Since each bentazone-induced response is both rapid and concentration-dependent it is proposed that this herbicide may bind to active site(s) on the cell membrane altering cation and water flux, as represented in Fig. 4. In this scheme an ATPase proton pump is envisaged to drive the efflux of protons in exchange for cations at a cation-specific transport molecule. Bentazone could conceivably bind at one, both or at other sites on the membrane to alter cation influx and hence cell turgor. Evidence does exist, particularly with regard to coleoptile elongation, that the endogenous plant growth regulator IAA may induce increased cell turgor by binding to the cell membrane ATPase (eg Davies 1973). Furthermore, since bentazone can interact with auxin in this experimental system (Miller unpublished), an action of bentazone at a regulatory site on the cell membrane is conceivable. Indeed, the stoichiometry of cation transport in the presence or absence of bentazone, and other membrane active herbicides and regulators, is an active area of study in this laboratory, which may yield a greater understanding of bentazone and other herbicide actions at the cell membrane.

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