THE PHYSIOLOGY OF FLOWERING OF *BORONIA MEGASTIGMA* (NEES.) AND *HYPOCALYMMA ANGUSTIFOLIUM* (ENDL.).

A thesis submitted for the degree of Doctor of Philosophy
in the department of Horticulture, Viticulture and Oenology
at the University of Adelaide.

by

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DECLARATION

This thesis embodies my independent research conducted in the CSIRO Division of Horticulture, Adelaide and the Department of Horticulture, Viticulture and Oenology, The University of Adelaide, and does not incorporate material previously submitted for another degree in any university. To my knowledge this thesis contains no material previously published or written by another person except where due reference is made in the text. I consent to this thesis being made available for loan or photocopying if it is accepted for the award of the degree.

Jamie S. Day

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ABSTRACT

The variety and beauty of Australian native plants has encouraged growers and researchers to view these plants with increasing interest, however the paucity of knowledge about Australian plants has restricted their commercial exploitation. Current research is focussed on selection, breeding and improved cultivation practises for these plants. Areas that require further research include propagation and the control of growth and flowering using environmental and chemical means. This was the major focus of the following study.

The floral development of *B. megastigma* and *H. angustifolium* was examined using scanning electron and light microscope techniques. Distinct stages of flower development were defined to facilitate the correlation of these morphological changes to measured physiological changes.

Boronia megastigma and *Hypocalymma angustifolium* were investigated with a view to gaining control over floral development through the use of environmental and chemical treatments. Flowers developed from the leaf axils of both species when placed in a cool temperature (17°C day / 9°C night) but photoperiod was without effect. Ten weeks in the above temperature regime was required to commit *H. angustifolium* plants to flower and fifteen weeks was required by *B. megastigma*. Thus an extension of the flowering season could be obtained by moving plants outside from a heated glasshouse throughout the winter as long as the requires number of weeks of cool temperature remained in the season. Flowering in both species was inhibited by a large difference between day and night temperature (21°C day / 5°C night) and enhanced if day temperature was lower than night temperature (9°C day / 17°C night). The temperature of the aerial parts of the plant controlled flowering, whereas vegetative growth was controlled by root temperature. Consequently, while less vegetative growth occurred during the production of flowers of both species than during vegetative phases of growth, flowering can occur while vegetative growth continues.

Plant growth regulators had a variety of effects on vegetative and floral development of *Boronia megastigma* and *Hypocalymma angustifolium*. Because flowers of both species develop in the axils of leaves, the balance between apical and axillary growth is an important control of flowering. Auxin transport inhibitors and benzyladenine stimulated lateral vegetative growth in warm conditions. Benzyladenine treatment of plants in cool conditions (17°C day /

9°C night) increased the rate of flower development and in *B. megastigma* reduced the number of weeks required to reach anthesis from 22 to 14 weeks. Treatment of plants with paclobutrazol also increased the rate of floral development in *B. megastigma* and increased the percentage of axils with flowers. *H. angustifolium* plants flowered in non-inductive conditions after treatment with paclobutrazol. Conversely many treatments that encouraged vigorous vegetative growth inhibited flowering in both species.

Because benzyladenine greatly enhanced the rate of floral development, it is likely that cytokinins play some role in the development of flowers in *B. megastigma.* This hypothesis was strengthened by measurement of changes in the endogenous cytokinins when *B. megastigma* plants were transferred from a non-inductive to a flower-inductive environment. Cytokinins were separated using High Pressure Liquid Chromatography, quantitatively measured using Radio-immuno assays and qualitatively measured using Gas Chromatography-Mass Spectrometry. Soluble sugars and starch were also measured. There were changes in the concentration of cytokinins and carbohydrates in the first few days after transfer to cool conditions. While the concentrations of cytokinins returned to the original level within four days of transfer, the carbohydrate concentrations remained high until flower buds reached the stage of commitment to flower. At this stage the carbohydrate concentrations decreased dramatically and the cytokinin concentrations became variable, increasing and decreasing in concentration through to anthesis.

B. megastigma and *H. angustifolium* cuttings root easily, however there are many treatments that can enhance the number of cuttings that form roots. A maximum percentage of *B. megastigma* cuttings formed roots when cuttings were placed in a peat-based propagation medium and a fog propagation house. *H. angustifolium* cuttings required treatment with auxins and the use of stem rather than tip cuttings to maximise the percentage of cuttings that formed roots. The number and length of roots and the number of cuttings with new top growth was generally enhanced by those treatments which reduce water loss from the cuttings such as fog rather than mist, peat rather than pine-bark propagation medium and stem rather than tip cuttings. Cuttings of both species can be taken at most times of year from stock plants maintained outside and yield consistently good plants. Only when *B. megastigma* flowers were in a late stage of development and when *H. angustifolium* plants were growing vigorously during summer was there a decrease in the percentage of cuttings with roots.

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CONFERENCE SEMINARS

Sections of this thesis have been presented as seminars at the following conferences.

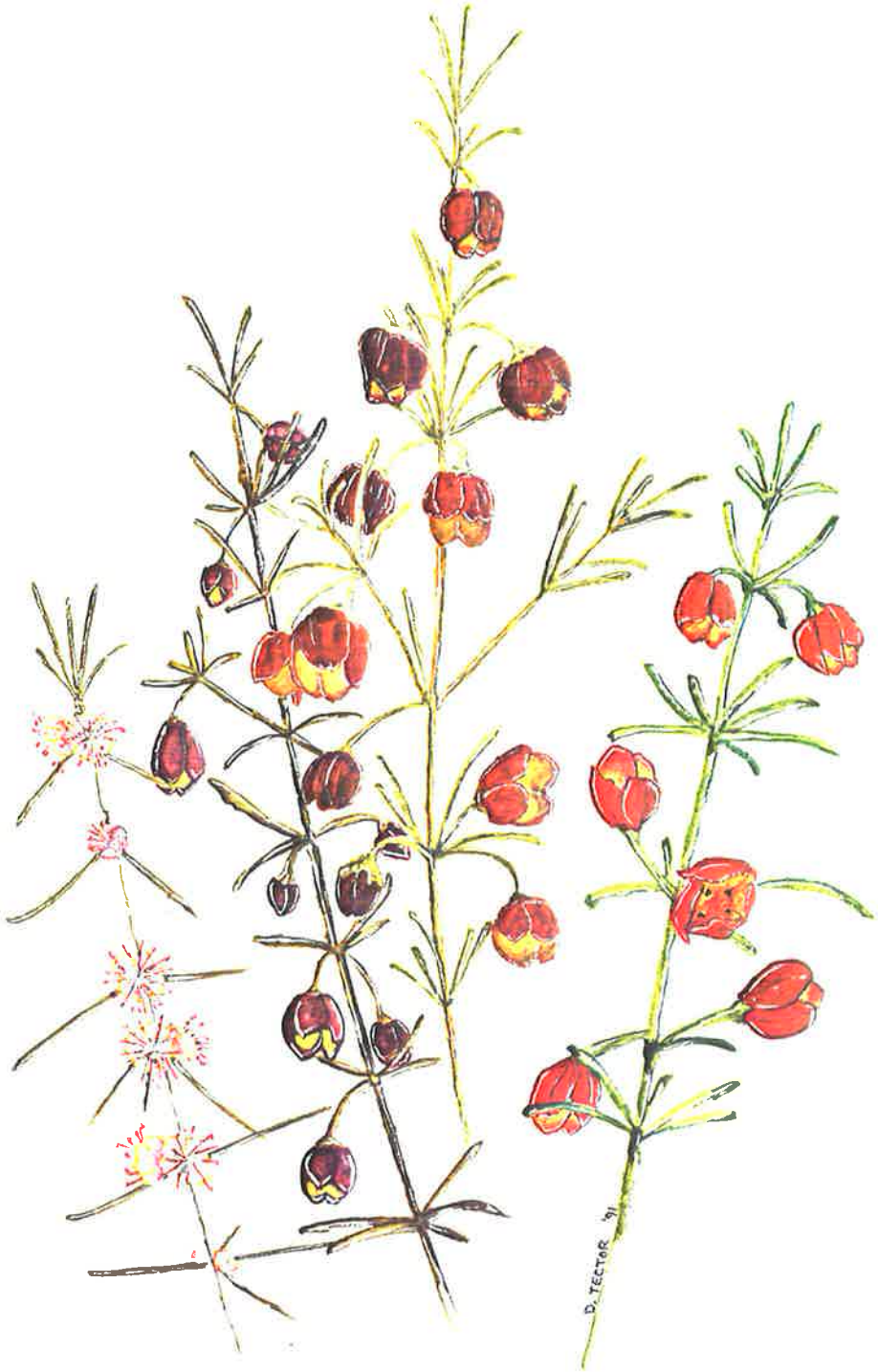
Day, J.S., B.R. Loveys & D. Aspinall **(1990)** Physiology of floral development in *Boronia megastigma* Nees. (Brown Boronia), Rutaceae. The Australian Society of Plant Physiologists Thirtieth Annual General Meeting, September 1990, The University of New South Wales. Programme and Abstracts.

Day, J.S., B.R. Loveys & D. Aspinall **(1991)** The role of cytokinins in the development of flowers of *Boronia megastigma* Nees. (Brown Boronia). Abstracts of the Australian Society of Plant Physiologists, 31st Annual General Meeting, October 1991, Australian National University.

ABBREVIATIONS

ABA	abscisic acid
AMG	amylo-glucosidase
AMP	adenosine monophosphate
AOA	amino-oxy acetic acid
ATP	adenosine triphosphate
AVG	amino-ethoxy vinyl glycine
BA	benzyladenine, N-(phenylmethyl)-1H-purine-6-amine
CCC	chloromequat chloride, 2-chloro-N,N,N-trimethylethanaminium chloride
Cyanamide	carbo di-imide
daminozide	Alar, butanedioic acid mono-(2,2-dimethylhydrazide)
DMSO	dimethyl-sulfoxide
DN	day neutral
DNA	dioxyribo-nucleic acid
DW	dry weight
ELISA	enzyme-linked immuno-sorbent assay
Ethrel	ethephon, 2-chloroethylphosphonic acid
FW	fresh weight
G-6-PDH	glucose-6-phosphate dehydrogenase
GA	gibberellin
GA_3	gibberellic acid
GC-MS	gas chromatography-mass spectrometry
HK	hexokinase
HPLC	high pressure liquid chromatography
IAA	indole-3-acetic acid
iP	isopentyl adenine, N_6-(Δ^2-isopentyl)adenine
[9R]iP	isopentyl adenosine, 9-ß-D-ribofuranosyl-iP
[9R-5'-P]iP	iPMP, 5'-monophosphate of [9R]iP
LD	long day
LSD	long-short day
Me -	permethyl derivative of
Morphactin	methyl-2-chloro-9-hydroxy-fluorene-9-carboxylate
mRNA	messenger RNA
NADP	nicotinamide adenine dinucleotide phosphate
Paclobutrazol	(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol
PFK	phosphofructokinase
P_{fr}	form of phytochrome sensitive to far-red light
PGI	phospho-glucose isomerase
P_r	form of phytochrome sensitive to red light

RIA	radio-immuno assay
RNA	ribo-nucleic acid
SD	short day
SEM	scanning electron microscope
SIM	selected ion monitoring
SLD	short-long day
STS	silver thiosulphate
TBS	tris-buffered saline
TEA	triethyl amine
TEAA	TEA adjusted to appropriate pH with acetic acid
TIBA	2,3,5 tri-iodo-benzoic acid
tRNA	transfer RNA
TW	tissue water
Z	zeatin
[9R]Z	zeatin riboside, 9-ß-D-ribofuranosyl zeatin
[9G]Z	zeatin-9-glucoside, 9-ß-D-glucopyranosyl zeatin
(diH)Z	dihydro zeatin
(diH)[9R]Z	dihydro zeatin riboside, 9-ß-D-ribofuranosyl-(diH)Z
(diH)[9G]Z	dihydro zeatin-9-glucoside, 9-ß-D-glucopyranosyl (diH)zeatin
(OG)Z	zeatin-O-glucoside, O-ß-D-glucopyranosyl zeatin
(OG)[9R]Z	zeatin riboside-O-glucoside, 9-ß-D-ribofuranosyl-(OG)Z
[9R-5'-P]Z	ZMP, 5'-monophosphate of [9R]Z
(diH)[9R-5'-P]Z	(diH)ZMP, 5'-monophosphate of (diH)[9R]Z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CHAPTER ONE

GENERAL INTRODUCTION

Introduction

The potential for export earnings has encouraged a surge in interest in floriculture in Australia during the last decade. Australia's southern hemisphere location and range of climates affords it a unique advantage in supplying floriculture products to markets in the northern hemisphere. The value of the ornamental horticultural industry in Australia, including both exotic and native plants, has been steadily increasing for many years and in 1989 the Nurserymen's Association estimated the retail industry to be worth $1 billion, which included flowers, plants and associated garden products (von Hentig, 1990). In 1987 the wholesale cut-flower industry alone was valued at $50.7 million and 758 companies were involved (ASRRC, 1989). Total export of cut-flowers from Australia increased from $2.8 million in 1982 to around $11 million in 1989 (Pegrum, 1990). Although increasing in value, this still represents less than 1% of world trade in cut-flowers.

While nurseries within Australia have concentrated on the production of plants which dominate the local and world market (roses, carnations and chrysanthemums), more and more people in Australia and overseas are looking for something different. With around 25 000 species endemic to Australia there are many plants from which to select and develop new cultivars. Commercial nurseries are currently growing between 4000 and 5000 native Australian species (Peate, 1990) but of these only a handful are cultivated on a large scale and may be considered of significance. However the areas under cultivation are expanding. In Western Australia, the number of native plants in cultivation increased from 100 000 in 1983 to 1.2 million in 1989, and by 1992 over 92 million stems are expected to be harvested from these plants (Pegrum and Webb, 1989). Most of these stems will be exported. The export of Australian native cut-flowers from Australia increased by 75% between 1982/83 and 1985/86 (Joyce and Burton, 1989) and is likely to continue to expand.

Many of the plants that have been introduced to cultivation have been chosen as a result of the export success of bush picked flowers. These plants have generally undergone little or no selection, and research into cultural practices has been minimal. A lack of understanding of the water and fertilizer requirements, unfamiliar diseases, many well adapted native insects (Pegrum, 1990), and poor post-harvest knowledge (Loveys, 1990) has led to failures at many stages of flower production. A great deal more market and cultivation research needs to be done to choose the most appropriate Australian native plants and to improve existing genotypes for the nursery and cut-flower industries.

There have been notable advances in the selection and development of some Australian plants within Australia. Progress has been made in the selection and breeding of Geraldton wax *Chamelaucium uncinatum* (Moody, 1989) and Kangaroo paws *Anigozanthos* and *Macropidia* (see Lawson and Goodwin (1985)), and the floral biology of *Helipterum sp.* (Sharman and Sedgley, 1988) and Kangaroo paws (Motum and Goodwin, 1987a). Cultivation techniques have been introduced to enhance propagation (Williams and Taji, 1987; Williams *et al.*, 1984a; 1984b; 1985), improve potting mixes (Handreck, 1989; 1991), control plant shape (Lamont, 1986; Lewis and Warrington, 1988), control flowering (Motum and Goodwin, 1987b; Sharman *et al.*, 1989) and improve post-harvest life of flowers (Joyce, 1988; 1989). However much of the research on Australian plants continues to be conducted in countries such as Israel, USA, Germany and the Netherlands (Frith, 1990; von Hentig and Ehlers, 1990). Many of the new cultivars of Australian native potted-plants and cut-flowers have been developed outside of Australia (Elliot and Jones, 1989). Research on native plants must be undertaken within Australia if this country wishes to maintain some control over a valuable asset.

Research is required in all aspects of plant and flower production (Frith, 1990; Pegrum, 1990). Initially there is a need for more intensive selection so that breeding and cultivation practises can be developed on superior material (Elliot, 1990). The development of effective propagation protocols must also precede large scale production of plants for both cut-flowers and potted plants (Frith, 1990). Most of the propagation protocols devised for Australian plants have been adaptations of traditional methods by commercial growers (Peate, 1990).

Cultural practises that have been used widely with exotic plants include the manipulation of plant shape and flowering by environmental or chemical treatments (Larson, 1985). Research with plants such as carnations, roses and poinsettias has reached the stage where flower production can occur continuously throughout the year (Goodwin, 1985) despite the constraints normally imposed on flowering by environmental conditions. Control of flowering has been usefully employed to increase the number of flowers, produce flowers out of season, and time flowering for particular days of the year. For example, short days are used to induce flowering and control the rate of development of chrysanthemum flowers such that flowers are ready to cut during the week before Mother's Day. This ability to control flowering so closely has only come about by the extensive study of the photoperiodic response of chrysanthemums.

Many of the techniques used to control flowering have been discovered by trial and error with little or no understanding of the physiology of flowering of the plant in question. If environmental and chemical treatments are based on a greater understanding of the floral process then the most appropriate treatment for that particular plant can be employed. Few attempts have been made to understand even the basic physiology of flowering in Australian native plants.

During research for this project the physiology and production of Australian native plants was examined. More specifically, aspects of the propagation and control of plant shape and flowering of *B. megastigma* and *H. angustifolium* were explored. A central part of the work was directed toward understanding the effects of various environmental and plant growth regulator treatments on flowering, and the relationship between the timing of the treatment and flower evocation and development.

The understanding of the physiology of flowering gained from this research was further advanced by the measurement of endogenous carbohydrates and cytokinins. Correlating changes in concentration of endogenous substances with stages of flower development allowed a more complete picture of the control of flower development in *B. megastigma*. Finally similarities in the floral processes of the two plant species from different families were assessed in an attempt to apply the findings to a wide range of Australian species.

The process of flowering

Flowering is an extremely complex process. Plants detect a variety of stimuli and the stimuli can cause many changes within the plant (Esau, 1977; Lyndon, 1990). Each species has a requirement for specific conditions which start the plant along a pathway which finally leads to flowering, and each species responds to these conditions in a unique way suggesting that it is the genetic make up of the plant that defines the development of flowers (Lyndon, 1990). Since each plant has a unique pathway leading to flowering it is difficult to present a general model of the changes that occur in plants. An overview has been presented here of the changes that have been observed and measured, the substances that are thought to be involved and the models that have been proposed for the control of flowering. Recent reviews examining aspects of the flowering process have been written by Bernier (1988), Davenport (1990) and Drews *et al.* (1991).

The process of flowering can be defined by particular stages, however the terminology used to define these stages is confusing. The definitions used here follow those published by the international working group on flowering (Salisbury, 1987). Induction of flowering occurs when the floral stimulus has been detected (McDaniel *et al.*, 1991). The induction of flowering results in the transmission of a floral signal from the site of detection to the site of floral initiation. Evocation then occurs at the meristem from which flowers are to develop. Evocation is the molecular aspect of change (Sedgley and Griffin, 1989), the changes in nucleic acids, protein and other substances necessary to trigger and supply materials for change at a higher level of organisation (Bernier, 1988). Initiation occurs when the first morphological changes are detected (Sedgley and Griffin, 1989). Usually this is when there is a change in the shape of the bud apex. As defined by Battey and Lyndon (1990), evocation can continue after the initiation of flowers because the culmination of evocation is a commitment to flower, when the control of flowering is transferred from the site of induction to the site of flower development. However, as defined by Salisbury (1987), evocation terminates with the initiation of flowers and this definition has been used. Floral organs or flowers are said to be determined when the genetic information necessary for their development has been expressed (McDaniel *et al.*,

1991). This does not imply that the flowers or floral organs are committed because their development can be interrupted by environmental or other changes. Commitment to flower is an irreversible commitment to continue development through to anthesis. After flowers are initiated, they undergo differentiation which describes the morphological changes associated with the initiation and development of the individual floral organs. Development of the flower is the on going biochemical and morphological process from initiation until flower death and thus encompasses determination, differentiation, commitment to flower and senescence of flowers.

Induction

Photoperiodism

Some plants have the ability to time physiological and morphological events using a mechanism that detects light. One of the most important roles for this system is the detection of the daylength (photoperiod) for the control of flower induction. This is one of the mechanisms which synchronises flowering of plants with the seasons and thus ensures the production of flowers and seed when conditions are favourable.

Some plants, for example spinach (Auderset *et al.*, 1980), are long-day (LD) plants. These plants only flower when the length of day is increased beyond a threshold length. Others, such as *Xanthium*, are short-day (SD) plants, requiring a daylength shorter than some threshold. This plant was used in the early work which found that it is night length rather than daylength that elicits the photoperiodic response (see Bidwell, 1977). Between these two extremes there is a range of daylength requirements. Some plants are long-short-day (LSD) plants requiring long days followed by short days, others are short-long-day (SLD) plants and still others are day neutral (DN) plants. Many forest and fruit trees are day neutral. For these plants photoperiod has no role in floral induction (Jackson and Sweet, 1972).

The requirement for a specific photoperiod may not be absolute. In a non-inductive photoperiod six different treatments can by-pass the induction mechanism and initiate flowers in *Pharbitis nil*, including poor nutrition, high irradiance, low temperature, root removal and the

application of a cytokinin or chlormequat chloride (Bernier, 1988). Other plants, such as *Helipterum roseum*, respond to photoperiod but the response is 'facultative', a particular photoperiod facilitates flower induction but is not an absolute requirement. *Helipterum roseum* will flower in any photoperiod, however flower initiation occurs sooner in long days (Sharman *et al.*, 1989). For still other plants the photoperiodic requirement may be absolute but may be altered by other environmental conditions. The fact that plants in nature do not flower at exactly the same time each year is evidence of this. *Leucospermum patersonii* is a LSD plant (Wallerstein, 1989), however if plants received only 20% full sun during the long day period then some shoots fail to flower (Napier and Jacobs, 1989). In this case, the decrease in light intensity during long days affected the ability of shoots to respond to the subsequent short days. Although a system for the detection of photoperiod may be present, the photoperiodic response can be modified by other factors.

The detection of light probably occurs in the leaves and the subsequent response by the plant is most likely under the control of two main systems, circadian rhythms and phytochrome (Moore, 1989). The circadian rhythm is an intrinsic rhythm of around 21-27 hours that seems to play a fundamental role in the timing of processes that occur in light and dark periods (Kinet *et al.*, 1973). Phytochrome is a pigment with many forms (Smith and Whitelam, 1990). All forms are converted between two stable states, P_r which absorbs red light and P_{fr} which absorbs far-red light. The balance between red and far-red light controls the interconversion between P_r and P_{fr} which induces a physiological response. The phytochrome system appears to modify the basic timing mechanism of the circadian rhythm and it is likely to be this interaction that results in the photoperiodic response (Moore, 1989).

The result of the detection of daylength by phytochrome is probably a change in the synthesis or release of proteins within the leaf, which then regulate other genes (Lyndon, 1990). The ultimate result of photoperiodic induction is the transmission of a signal from the leaves to the site of flower formation.

Vernalization

Many plants require a period of cold before they will flower. *Digitalis lanata* requires reduced temperatures for several weeks to allow the initiation of flower buds however, after initiation, the buds continue to develop in a warmer environment (Mertinat *et al.*, 1989). This species has an absolute requirement for vernalization. As for photoperiodic induction, some plants have a facultative response to low temperature. Other plants have an absolute requirement for low temperature and a facultative response to other environmental conditions. Thus for induction and subsequent development of flowers of many plants there is continuous interaction between temperature, photoperiod and other environmental conditions. For flowering of *Helipterum roseum* photoperiod and temperature interact to control the number and size of flowers, the rate of flower development and flower stem length (Sharman *et al.*, 1989).

Unlike photoperiodism the detection of cold may occur at a variety of sites, such as the roots, leaves or shoot apex (Bidwell, 1977), depending on the species. Bernier *et al.*. (1981a) report that generally the shoot apex is the site of cold detection. In the sweet orange (*Citrus sinensis*) high root temperature had little effect on floral initiation, whereas high shoot temperature inhibited flowering (Moss, 1976). A low night temperature was all that was needed to produce profuse flowering, suggesting that day temperature also may have no role in floral induction of this species (Moss, 1976). There is no known detection system for low temperature equivalent to the phytochrome system for light detection.

One reason why vernalization may be required for flower induction in some plants is to halt vegetative growth (Jackson and Sweet, 1972). Those factors which encourage vegetative growth, such as warm temperature and nitrogen fertilizers, tend to decrease floral initiation in many plant species. Richards (1985) found that warm temperature inhibited flower initiation and encouraged vegetative growth in *Boronia heterophylla.* Reddy and Menary (1989b) found that high nitrogen concentration in a nutrient solution applied to *Boronia megastigma* plants while the flowers were beginning differentiation resulted in a decrease in the number of flowers and suggest this was due to the continuation of vegetative growth throughout floral initiation.

Cold temperature can have far reaching consequences within the plant. Mohamed-Yasseen and Splittstoesser (1990) cite reports showing that plants grown in cold temperatures

have altered levels of endogenous cytokinins, chlorogenic acid, proteins, lipids and phospholipids, and a changed membrane fluidity and enzyme activity. These authors suggest the changes in enzyme activity may be due either to changes in membrane fluidity or some modification of genetic transcription. One of the enzymes affected by cold temperature in potatoes is phosphofructokinase (PFK) (Hammond *et al.*, 1990), and the inactivation of this enzyme causes an accumulation of sugars. Bodson and Bernier (1985) suggest that changes in carbohydrate distribution and metabolism may play a part in the evocation of flowers. There is also much evidence that plant growth regulators are involved in the vernalization response (Bonnet - Masimbert and Zaerr, 1987; Pharis and King, 1985). While cold temperature obviously results in many endogenous changes, it is not clear which of these changes are involved in the transition from vegetative to floral development and which are merely an unrelated response to cold temperature.

Other systems

Bernier (1990) suggests that by concentrating on photoperiodic and vernalization responses of plants there are a number of other environmental conditions that induce flowering which have been overlooked. This may be because many of the plants studied are from temperate climates where most plants can readily detect changes in daylength and temperature. In the tropics, seasonal changes in daylength and temperature are less likely to be detected by plants and thus other strategies may be used to control flowering. Bernier (1990) mentions light intensity and water stress as examples of factors that can control flower induction and lists several plants which use these strategies. In addition there is what Bernier (1988) describes as "autonomous" induction, where plants will eventually flower in any environment as long as conditions are adequate for growth. These plants may have a facultative requirement for a certain environmental condition but will eventually flower in condition considered non-favourable for flowering. Examples of this are *Xanthium*, which has a facultative requirement for SD, and *Lolium*, which has a facultive requirement for LD, both of which will flower eventually in conditions considered non-inductive (Bernier, 1988).

Evocation

Below is a description of the changes that occur during evocation in some annual plants. It is likely that similar changes also occur in perennial plants but confirmation of this is difficult because of the complexity of flowering in these plants (Jackson and Sweet, 1972). Whereas evocation may occur in annual plants after one day of specific environmental conditions (eg: one 18 hour photoperiod results in evocation in *Lolium temulentum* (McDaniel *et al.*, 1991)), perennial plants may require extended periods of exposure to particular environmental conditions before evocation occurs.

There are a number of reports of the first detectable changes that occur within the plant when flowering is induced (eg: *Sinapis alba*, Le Jeune *et al.* (1988); *Spinacia oleracea*, Auderset *et al.* (1980)). One hour after the critical long daylength there are specific measurable changes in the peroxidase activity in the leaves of spinach plants. One long day also results in an increase in the ABA level in spinach leaves (Zeevaart, 1971). Three hours after the critical photoperiod the glucose-6-phosphate dehydrogenase (G-6-PDH) activity in the shoot apex has doubled, which Auderset *et al.* (1980) suggest is a response to the need for NADPH energy for RNA synthesis. Also by this time, mitotic activity and the amount of assimilates (carbohydrates) in the central zone of the shoot apex have increased. Three to four hours after this new proteins are found in the leaves and the shape of the apex begins to change (Auderset *et al.*, 1980). Thus after induction there are changes occurring throughout the plant. Evocation occurs in the shoot apex soon after induction. In spinach, evocation begins three hours after the critical photoperiod (Auderset *et al.*, 1980) and in *Lolium temulentum* evocation is under way six hours after the completion of a critical photoperiod (McDaniel *et al.*, 1991).

In the central zone of the apex where floral development occurs, quite dramatic changes occur during evocation (Bernier, 1988). There is an increase in the number of mitochondria, an increase in the rate of respiration and the synthesis of new RNA and proteins (Lyndon, 1990). Bernier (1990) reports a 10% change in the polypeptides of the meristem of *Sinapis alba* (detectable in 2-D gel electrophoresis) during evocation and before the initiation of flowers. In *Lolium temulentum* changes in gene expression were only detected after morphological change

had begun, although it was suggested that technical difficulties may be the reason why changes in gene expression were not detected earlier (McDaniel, 1991).

The induction of flowering does not ensure that evocation will occur. Induction of flowering may result in the transmission of a signal to the site of flower formation, however if the target tissue at the floral meristem is not competent to receive and respond to the signal, evocation will not occur (Lyndon, 1990). Tissues are said to be 'competent' if they will follow a certain genetically determined developmental pathway given appropriate signals. The most obvious example of plants that are not competent to flower are those that have not undergone the phase change from juvenile to mature form (Hackett, 1985). Growth in conditions known to result in flowering in mature plants may not result in flowering in juvenile plants because juvenile tissue is not competent to respond to the inductive signals. Even after plants have attained a mature form, there are a number of situations where apices of mature plants can lose their competence to flower (Bernier, 1988).

Lyndon (1990) suggests that in plants that require vernalization, cool temperatures may be required to make the apex competent to respond to signals which may already be present. Authors have suggested different ways in which these plants may gain competence. Seeley (1990) suggests that the 'machinery' for the production of growth substances may develop during vernalization, so that when another stimulus is detected the plant can respond by rapidly modifying the endogenous concentration of plant growth regulators. Competence in this example equates with the development of the plant growth regulator 'machinery'. Alternately, the acquisition of competence may result from a reduction in DNA methylation of promoter genes important for flowering. This would result in a subsequent increase in transcription of the affected DNA. It is possible that the vernalization mediated decrease in methylation may occur specifically at site(s) for the binding of regulatory proteins (King, 1991). Evidence for this role for vernalization is provided by Bagnall, Burn and Dennis (unpublished) who observed an acceleration of flowering in vernalization-requiring *Arabidopsis* plants resulting from treatment of the plants with 8-azacytidine which reduces the methylation of DNA.

Commitment to flower

The stage of irreversible commitment to flowering is the stage when all final biochemical and morphological modifications required for flowering have taken place (Battey and Lyndon, 1990). Commitment to flower may occur soon after the transmission of a signal to the shoot apex. In *Lolium temulentum*, some apices excised from plants six hours after the end of an inductive long day and allowed to develop *in vitro* began inflorescence differentiation (McDaniel *et al.*, 1991). Fourteen hours after this all apices were florally committed and commitment occurred prior to detectable morphological changes to the apex, that is prior to initiation of flowers.

Many plants require much longer periods to commit them to flower, commitment may not occur until they pass a certain stage of development. *Leptospermum scoparium* requires short days for floral induction. It also requires short days for the first eight weeks of floral development to prevent flower abscission. After eight weeks, flowers are committed and remain on the plant in any photoperiod (Zieslin, 1985). An extreme example of the conditions required to commit plants to flower occurs in the Snowy Mountains plant *Caltha introloba* (Wardlaw *et al.*, 1989). This plant initiates flowers in the long, high light intensity days of summer, however plants kept in these conditions abort their flowers. Only after the plant is kept at 0°C for one month can the plant be returned to warmer temperatures to flower. Thus evocation and initiation of flower buds occurs prior to winter, however the buds are not committed to flower until the end of winter.

Flowers not fully committed may revert to vegetative growth. In *Pharbitis nil*, axillary meristems revert to vegetative growth if placed in a warm environment after an inductive short day (Battey and Lyndon, 1990). Gibberellic Acid (GA_3) causes the reversion of flowers in *Citrus sinensis* (Lord and Eckard, 1987) and sorghum (Bhaskaran *et al.*, 1990) if treatment occurs before flowers are fully committed. In general, reversion of flowers occurs when the environmental or hormonal conditions that induce flowering are reversed (eg: return to long-days after an inductive short-day), and may result in stems that look like peduncles or pedicels and leaves that look like bracts or sepals. The presence of bract-like leaves implies that the cells

forming the bracts are genetically determined even though the whole flower is not committed (Lyndon, 1990). Thus a commitment to flower in some species may only occur when each of the whorls of a flower are committed. This is the case in *Anagallis* where reversion to vegetative growth can occur at any time, it is even possible to get vegetative growth from the floral meristem in place of carpel development (Lyndon, 1990).

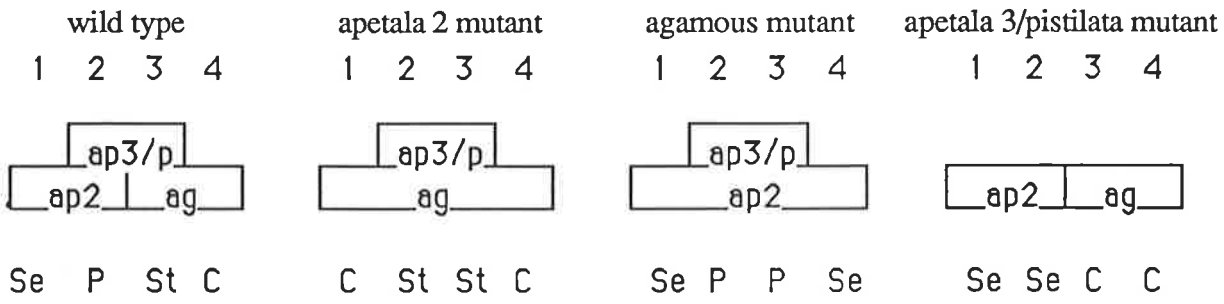
Initiation, differentiation and development

The first detectable sign of floral initiation is usually a swelling of the apical dome (Esau, 1977). Associated with this change in *Anagallis*, Hernandez *et al.* (1991) describe pre-patterning of the epidermal cells of the apical meristem. The epidermal cells form distinct patterns which can be followed through to subsequent development of floral organ primordia. These changes precede the production of much of the genetic information required for the development of floral organs and thus precede flower determination in these plants.

It is necessary to make a distinction between initiation and differentiation to explain flowering in plants such as *Citrus* (Davenport, 1990) and grape vines (Buttrose, 1974) where buds are produced that can differentiate into vegetative or floral growth. *Citrus* and *Vitis* plants initiate undifferentiated buds prior to winter, however the buds enter a rest period during the cold months. While some bud differentiation may occur in grape vines prior to winter (Buttrose, 1974), differentiation and bud swell of floral buds of *Citrus* and *Vitis* predominantly occurs in the warmer months following winter. Thus initiation and differentiation are separated in time, unlike the situation in most other plants.

Throughout floral evocation and development there are changes in the activity and production of RNA and proteins (Lyndon, 1990). This results in a range of RNA's being present in flowers, and even in specific floral organs, most of which were found nowhere else in the plant (Herdenberger *et al.*, 1990). More recently homeotic genes have been described using mutant plants of *Antirrhinum* and *Arabidopsis* (Coen, 1991). These genes define whorl identity and are similar in both of the species studied, suggesting that the genetic mechanism controlling whorl identity is highly conserved between species. In *Arabidopsis* there are three

groups of genes that interact; *apetala 2*, *agamous* and an *apetala 3/pistilata* complex (Drews *et al.*, 1991). The identity of the floral whorls found in the wild type and some of the *Arabidopsis* mutants are shown below.



(1-4 floral whorl number, Se - sepals, P - petals, St - stamens, C - carpels = actual whorls observed ; from Drews *et al.*, 1991)

In-vitro localisation has shown that the homeotic genes are expressed in concentric circles around the meristem with the expression of genes only found in the expected whorls illustrated above (Drews *et al.*, 1991). Thus there are specific genes for specific floral organs that presumably control other genes that organise pigment and the type of cells that develop (Coen, 1991). There are also regulator genes that control the expression of the homeotic genes.

Coen (1991) suggests that although homeotic genes are found associated with whorls in discrete areas of the meristem, they are patterned not by position but by time. Thus regulator genes determine when expression of the genes is turned on and off rather than where they will be expressed. This suggestion fits well with Lyndon's (1990) suggestion that the floral meristem is a relay system, where the determination of one set of organs depends on the metabolic state of the meristem. As successive whorls of organs are formed the metabolic state of the meristem changes, thus influencing the identity of the next whorl to develop. In addition, each whorl of organs may produce its own set of growth regulators which may influence the following whorl (Lyndon, 1990) . It is suggested that genes interact to control the metabolic state of the meristem and the growth regulator production of each whorl of organs and thus control the subsequent whorls. Reversion of flowering or other modifications of

flower development may occur when there is interruption of either gene action, growth regulator production or metabolic state.

Floral development can be manipulated in a range of ways. Kinet *et al.* (1985) show evidence that floral development can be affected by nutrition, light, temperature and water stress as well as plant growth regulators. These exogenous factors can affect the rate of development, abortion, number and size of flowers as well as sex expression, fertility, dormancy and flower abnormality. Long days and high temperature hastened floral development in a range of *Anigozanthos spp.* (kangaroo paws), although high temperature caused some floral abortion and weakened the colour of flowers in some species (Motum and Goodwin, 1987b).

There is ample evidence that flower development is controlled by endogenous carbohydrates (Bodson and Bernier, 1985) and growth regulators (Larson, 1985; Menhenett, 1982). Environmental conditions and exogenous applications of plant growth regulators control the development of flowers by altering the endogenous concentrations of a range of substances. The genes controlling flower development may either be directly affected by the exogenous changes or indirectly by the changes in endogenous substances mediated by exogenous factors.

The role of endogenous substances

Once flowering has been induced, a signal transmits the information to the site of flower formation. The floral signal has been defined by Lyndon (1990) as the sequence of substances, or changes in concentration of one or more substances, that cause changes in competence and determination at the shoot apex. In plants that are induced to flower by cold temperature there may be no need for a floral signal because temperature may influence the site of flower formation directly (Bernier *et al.*, 1981a; Moss, 1976). However, endogenous substances are affected by cold temperature, and the concentration changes of these substances must have some effect on floral evocation and development. The following examines the role of various substances, including plant growth regulators, in the evocation and development of flowers.

Plant growth regulators

One of the great problems with the study of plant growth regulators is the huge range of effects they can have. The same growth regulator may have different effects in different tissues, for example in a gibberellin deficient *Pisum* mutant the application of gibberellic acid (GA_3) to the stem epidermis results in an increase in cell number but not cell length whereas application to the outer layers of the cortex results in an increase in cell length but not cell number (Klee and Estelle, 1991). Also there are interactions between the various growth regulators and between growth regulators and other compounds thus ensuring that any change within a plant can not usually be attributed to only one substance. For example, flower initiation in subterranean clover is controlled by both photoperiodism and vernalization. There are probably a different set of genes that control the response to each of these environmental factors, however it is possible that some interaction occurs between the genes or between the substances produced by these genes because the environmental changes ultimately affect the same target site (Salisbury *et al.*, 1987). It is possible for all growth regulators to have some effect on the process under study (Leopold, 1987). Klee and Estelle (1991) suggest that growth regulators interact either by a particular growth regulator altering the sensitivity of a tissue to other growth regulators, or by different growth regulators utilizing the same secondary messenger system for the transmission of signals into the cell (such as the change in Ca^{2+} concentration utilized by animal systems).

Further, growth regulators and growth regulator receptors are likely to be compartmentalized within cells or tissue. The tissue may change in sensitivity to the growth regulator (either gain or lose competence) either by changing the number of growth regulator receptors or altering the affinity between the growth regulator and receptors (Davies, 1987). All this suggests that a unifying principal of growth regulator action is extremely unlikely. With that said there are many processes within plants that tend to be modified by a particular growth regulator, for example in some species flowering seems to be primarily controlled by one growth regulator.

History of growth regulators and flowering

Initially a hypothetical growth regulator 'florigen' was suggested to be the floral signal. A simple hypothesis of flowering, where the presence of florigen resulted in the initiation of flower, was proposed based on work on a limited number of plants. An inability to isolate florigen has hampered proponents of this original theory. Gibberellins, a group of naturally occurring growth regulators, were at one time thought to be florigen because of their ability to replace the long day or low temperature requirement for flowering in some plants (Pharis and King, 1985). As more plants were studied more alterations to the simple model became necessary (Moore, 1989). Because gibberellins do not promote flowering in SD plants it was suggested that anthesin (a hypothetical antagonist to gibberellin) must be present in addition to gibberellins for flowering to occur (Bidwell, 1977). When a certain balance between the concentrations of gibberellin and anthesin was reached the plant would flower. Abscisic Acid (ABA) initially seemed to be anthesin because of its role in flowering in some short day plants (Moore, 1989). To account for plants which require cold temperature to flower, vernalin was hypothesized, becoming the third hypothetical growth regulator that has never been successfully isolated. It is now widely accepted that these hypothetical growth regulators do not control flowering and that the floral signal is most likely to involve growth regulators and other substances that have already been isolated, most of which display some florigenic properties.

Auxins

The primary auxin in plants is indole-acetic acid (IAA). It is produced mainly in growing shoot and root tips and undergoes slow polar movement to the rest of the plant. Production of IAA is under feedback control, the end product of the reaction controls the rate of one or more of the synthesis enzymes. IAA tends to have two response mechanisms (Theologis, 1986). The rapid response results in cell elongation and cell wall relaxation which is connected in some way with proton secretion. The longer term response involves protein and RNA synthesis and control of nutrient movement which can then stimulate cell division, enable organ formation and tissue organization, prevent abscission of tissue and prevent lateral

bud development (ie: ensure apical dominance). IAA also stimulates ethylene production, thus ethylene may be responsible for some of the responses attributed to auxins (Bidwell, 1977).

IAA generally inhibits flowering in SD plants (Odén and Heide, 1989; Tucker, 1977), and has no effect or promotes flowering in LD plants (Simmonds, 1987). Conversely, *in vitro* flowering of the SD plant *Streptocarpus nobilis* can be stimulated by low concentrations of IAA (Simmonds, 1987).

Much interest has been directed towards the auxin/cytokinin ratio which is of great importance both in the control of apical dominance (Tucker, 1977) and in tissue culture of plants. In many perennial plants that require vernalization for floral induction, apical dominance plays a major role in the control of flowering (Bernier *et al.*, 1981a). These authors suggest that the apical meristems are insensitive to cold and thus continue vegetative growth albeit at a decreased rate, while the axillary buds respond to cold and form floral buds. Cold temperature then is a way of reducing apical activity and apical dominance which then allows the axillary buds to develop. The use of mutant plants and the insertion of genes that control cytokinin concentration have shown that it is the ratio rather than absolute levels of IAA and cytokinins that controls apical dominance (Klee and Estelle, 1991).

In *in vitro* grown tobacco, a high auxin/cytokinin ratio in the tissue culture medium results in root growth whereas a low ratio results in shoot growth (Elliot, 1982). Tissue culture of *Streptocarpus nobilis* with the same ratios as above, results in vegetative and floral growth respectively (Simmonds, 1987). Thus changes in the auxin/cytokinin ratio can result in different growth patterns of *in vitro* grown plants depending on the plant species being studied. Many *in vitro* results can not be extrapolated to *in vivo* conditions, however the results from *in vitro* grown *Streptocarpus nobilis* are what would be expected if flowering *in vivo* was controlled by apical dominance.

Gibberellins

By 1985, 72 different gibberellins had been discovered (Graebe, 1987), however any one plant species will only contain some of these. Gibberellins are produced in actively growing areas under feedback control and they move rapidly throughout the plant in both the

phloem and xylem (Bidwell, 1977). Gibberellins encourage cell elongation, protein and RNA synthesis, and generally inhibit organ formation. Probably the most obvious effects of gibberellins are stem elongation and release from dormancy (Bidwell, 1977).

Vince-Prue (1985) states "gibberellins are the only group of chemicals which evoke flowering when applied to a wide range of plant species", however they inhibit flowering in some plants and have no effect on flowering in others. In some conifers and annual ornamental plants that require cold temperatures or long days for floral initiation, gibberellins stimulate flowering (Pharis and King, 1985). In a range of fruit trees (Jackson and Sweet, 1972) and woody angiosperms in general (Pharis and King, 1985) gibberellins inhibit flowering. It is suggested that this inhibition of flowering is related to the biennial or perennial habit of these plants (Pharis and King, 1985). In *Chamelaucium uncinatum*, Shillo *et al.* (1985) found that treatment of plants with gibberellic acid or growth retardants inhibited and promoted flowering respectively and from this data suggest that a reduction in endogenous gibberellin concentration was necessary but not sufficient for flowering.

For woody angiosperms, it is unlikely that an increase in gibberellin concentration plays any role in the evocation of flowering. Gibberellins may have more involvement with floral development since many authors suggest that gibberellins increase the rate of flower development only after flowers have been initiated (Kinet and Sachs, 1984; Metzger, 1988). In a range of plants, gibberellin treatment results in the reversion of floral buds to vegetative growth (Battey and Lyndon, 1990), thus plants may need to be committed to flower before gibberellin treatment is used to increase the rate of flower development.

As previously mentioned, there are many different gibberellins and there is some evidence that different gibberellins regulate different physiological processes. In *Lolium temulentum* (Pharis *et al.*, 1987) and Sitka spruce (Moritz *et al.*, 1990) non-polar gibberellins (eg; GA_4 & GA_7) stimulate reproductive growth whereas polar gibberellins (eg; GA_1 & GA_3) have an opposite effect, causing stem elongation and inhibition of flowering. King *et al.* (1990) suggest there may be different receptors for the different types of gibberellins. This is one area that deserves further work.

Plant growth retardants are predominantly gibberellin synthesis inhibitors and reduce stem length, root length and leaf size without affecting leaf number (Grossmann, 1990). Some of the common growth retardants are chlormequat chloride (CCC), daminozide (Alar) and paclobutrazol. There is little direct evidence that CCC or daminozide decrease the concentration of endogenous gibberellins however they both are known to affect the early stages of gibberellin biosynthesis (Dicks, 1980). Triazole growth retardants, of which paclobutrazol is one, inhibit cytochrome P-450 dependent oxygenases and thus may influence many reactions within plants (Lenton, 1987). The major action of paclobutrazol in plants is an inhibition of *ent*-kaurene oxidase, an early step in the biosynthesis of gibberellins. Paclobutrazol has also been reported to inhibit sterol synthesis (Lenton, 1987).

Growth retardants generally prevent the stimulation by gibberellins of flowering in annual plants and promote flowering in woody angiosperms (Pharis and King, 1985). The growth retardant daminozide delays flowering in *Trachelium caeruleum* (Armitage, 1988) and paclobutrazol delays flowering in some other annual plants which Davies *et al.* (1989) suggests may result from a decrease in the rate of photosynthesis. However, Loveys (pers. comm., 1991) found that bayloton, one of the triazole compounds closely related to paclobutrazol, had no effect on the rate of photosynthesis of sunflower and bean plants. Further, von Hentig and Ehlers (1990) report that growth retardants advance flowering on *Pimelea ferruginea*, a perennial plant. Growth retardants are also known to delay the onset of senescence by decreasing the production of ethylene and increasing the concentration of chlorophyll, protein and mineral elements in plant tissue (Grossmann *et al.*, 1989).

Cytokinins

Cytokinins are predominantly produced in the roots and move slowly to other parts of the plant. Cytokinin production is possibly controlled both by feedback control and by abscisic acid (ABA) (Letham and Palni, 1983). Cytokinins play a role in the release from dormancy and the prevention of senescence, and they may be involved with the translation of genetic information since most tRNA has a cytokinin molecule bound to it (Letham and Palni, 1983). Cytokinins interact with auxins to induce and promote cell division, cell enlargement, organ

formation and the mobilization of nutrients. Conversely cytokinins both stimulate auxin production and overcome apical dominance, which is controlled by auxins. It is the ability of cytokinins to overcome apical dominance that has been exploited commercially most widely. Keever and Foster (1990) list a range of plants that become bushier and more commercially acceptable as potted-plants after exogenous applications of benzyladenine (a synthetic cytokinin).

Cytokinins induce flowering when applied to *Pharbitis nil* (Ogawa and King, 1980) and tomatoes (Kinet and Sachs, 1984) in an unfavourable environment. In *Sinapis alba*, cytokinins produce a mitotic wave in the shoot apex similar to that caused by photoperiodic induction although no floral primordia are produced (Le Jeune *et al.*, 1988). This suggests that cytokinins may be part of the floral signal in *Sinapis alba*. In roses (Richards and Wilkinson, 1984) and *Rhipsalidopis gaertneri* (Boyle *et al.*, 1988) cytokinin applications increase the number of flowers that develop, however this is due to an increase in lateral shoot growth in both cases, which provided more sites for flowers to develop, rather than to an increase in floral initiation *per se*.

Endogenous cytokinin concentration increased in *Begonia* plants when they were placed in environmental conditions that induce flowering (Hansen *et al.*, 1988), however these authors found no correlation between the number of flowers and the cytokinin concentration. Reddy and Menary (1989a) found that increasing the nitrogen fertilizer applied to plants prior to the differentiation of flowers increased the yield of *Boronia megastigma* flowers. They suggest that there may be some relationship between cytokinins and flowering in *B. megastigma* using work on sunflowers (Salama and Wareing, 1979) which showed that the application of nitrogen to plants resulted in an increase in endogenous cytokinin concentration.

Medford *et al.* (1989) inserted a chimeric isopentyl transferase gene into *Arabidopsis*, which dramatically increased the endogenous concentration of zeatin-type cytokinins. The morphological changes resulting from the cytokinin increase were confined to vegetative growth rather than floral development. Exogenous application of cytokinins to the shoot apex of *Arabidopsis* resulted in flower initiation. Thus these authors conclude that results from the exogenous application of cytokinins can not be used to interpret the effect of changes in

endogenous cytokinins. However, Bernier (1990) reminds us that in the above experiment the changes in endogenous cytokinins occurred throughout the plant and at all times, whereas if cytokinins were a signal for the flower initiation they would only change at a specific time and in a specific area in the plant.

It has been suggested that cytokinins have no direct effect on flowering but may have some indirect effects (Vince-Prue, 1985). Cytokinins are reported to increase the flow of substances in the phloem (Vince-Prue, 1985) and overcome ABA inhibition of lateral bud growth (Tucker, 1977) both of which could encourage flowering. Zeislin and Khayat (1990) suggest that cytokinins increase the sink strength of lateral flower buds which enhances the flowering of these buds. Thus part of the role of cytokinins in overcoming apical dominance may be to enhance the distribution of carbohydrates to lateral shoots in preference to apical shoots.

Cytokinins are able to retard senescence of flowers. Cytokinins either block ethylene biosynthesis or action (Bosse and Van Staden, 1989), or lower the responsiveness of the tissue to ethylene and thus delay the rapid increase in ethylene production which results in senescence (Veen, 1987). When dihydro-zeatin ((diH)Z) was placed in the holding solution for carnation flowers, longevity was increased (Bosse and Van Staden, 1989). Conversely, cytokinin application was found to increase respiration and ethylene production in broccoli florets. However, this treatment still increased the shelf life of the broccoli by 90% (Rushing, 1990).

All this evidence suggests that cytokinins may be involved in flowering in some plants and may be part of the floral signal resulting in floral evocation. However in most plants where cytokinins have an effect on flowering, they promote rather than induce flowering.

Abscisic acid

Abscisic acid promotes dormancy of plants and abscission of leaves and flowers (Tucker, 1977), and is closely involved with photosynthesis through its control of stomatal closure (Bidwell, 1977). ABA also may act as a gibberellin antagonist. In *Lolium temulentum*, GA_3 was required for the development of flowers in tissue culture and ABA inhibited this development (McDaniel *et al.*, 1991). ABA is thought to be involved with dormancy of buds

of many trees during winter months and it has been reported that the concentration of ABA declines during cooler weather (Powell, 1987). However, endogenous concentrations of ABA have been found to increase when plants are returned to warmer temperatures (Pilate *et al.*, 1989) suggesting there is no direct relationship between dormancy and ABA concentration.

Ethylene

Ethylene production is stimulated by auxins or ethylene itself, and probably is produced in response to an increase in the permeability of cell walls. Germination of seeds, abscission of leaves, senescence of flowers and the ripening of fruit are some of the processes that ethylene affects (Yang and Hoffman, 1984). Ethylene causes quite dramatic floral initiation in many bromeliad (Abeles, 1987) and *Xanthorrhoea* species. However it inhibits flowering in many short day plants (Abeles, 1987). Elliot (1982) suggests that some auxin/ethylene combinations may act in a similar way to cold temperature and initiate flowers by turning off vegetative growth.

Ethylene level is intimately involved in the senescence of flowers (Woltering and Harren, 1989). After anthesis, ethylene is produced at a slowly increasing low level before senescence is visually obvious. Senescence is associated with a second phase during which there is a dramatic increase in ethylene caused by a feedback mechanism which stimulates the production of more ethylene (Woltering and Harren, 1989). Most attempts to extend flower longevity have involved the application of chemicals to modify the response of flowers to high concentrations of ethylene. Three of the most commonly used compounds are silver thiosulphate (STS), amino-oxy acetic acid (AOA) and amino-ethoxy vinyl glycine (AVG) (Spikeman, 1989). AOA and AVG inhibit ethylene production by inhibiting the enzyme ACC-synthase which is the major control site for ethylene production (Spikeman, 1989). STS is probably more widely used than other compounds and is extremely effective in decreasing the response to ethylene in a range of cut-flower and potted plants. Silver ions (Ag^+) are the active part of STS and are thought to inhibit binding by ethylene to it's receptor thus preventing a response by the plant to available ethylene (Veen, 1987).

Carbohydrates

It is generally accepted that the movement and concentration of carbohydrates (assimilates) play some role in flowering. Kinet and Sachs (1984) suggest that any factors which increase the sink strength (the accumulation of assimilates) of floral apices relative to vegetative apices will tend to promote flowering. This is part of their 'nutrient diversion' hypothesis, where conditions or treatments induce flowering by increasing the assimilate level in floral apices relative to the level in non-inductive conditions. This is supported by measurement of endogenous carbohydrate concentrations which have shown that more assimilates are directed to the flower than to vegetative shoot tips during flower development in a number of plants (Bodson and Bernier, 1985). Salisbury and Marinos (1985) agree with the nutrient diversion hypothesis, but suggest that it is not purely the partition of assimilates but the carbohydrate/nitrogen ratio in the apices which determines whether floral or vegetative development will occur. A high ratio induces flowering.

There is controversy over whether the diversion of nutrients is associated with flower initiation or with flower development. An accumulation of assimilates occurs in the central zone of the shoot apex during evocation in a number of plants, all of which differ in the conditions required for flower induction (Bodson and Bernier, 1985). In *Sinapis alba* (Bodson and Bernier, 1985) and red clover (Jones, 1990), an increase in the assimilates in the shoot apex can be induced without subsequent flower initiation. This suggests that the increase in assimilates is not the cause of flower evocation and thus the 'nutrient diversion' hypothesis does not hold for these plants. Bodson and Bernier (1985) suggest that the increase in assimilates is a response to some floral signal and that while carbohydrate changes are essential, they are only a part of the system controlling floral initiation. Since growth regulators can be involved in the partitioning of assimilates, Hackett (1985) speculates that this may be how they control flowering.

There is more evidence linking changes in carbohydrate concentration with flower development. High light intensity, CO_2 enrichment and cold temperature have been found to enhance the rate of floral development in many plants (Bodson and Bernier, 1985). The first

two treatments can increase the carbohydrate concentration within the plant by stimulating photosynthetic assimilation. Cold temperature results in an accumulation of carbohydrates by causing a decrease in the use of photosynthates (Paul *et al.*, 1990). While an accumulation of carbohydrates can be associated with floral development, there is difficulty determining whether the changes in carbohydrate concentration are an essential part or only a consequence of floral development.

Other substances

There are many compounds which, although not plant growth regulators or carbohydrates, may be involved in flowering in some way and Bernier (1988) lists a number of them. Spermidine is one example. Kaul-Sawhney *et al.* (1990) have shown that spermidine added to vegetative tobacco in tissue culture causes a significant production of floral buds. An inhibitor of spermidine synthase inhibits flower buds in cultures programmed to flower. Another compound, salicyclic acid can cause the long day plant *Lemna gibba* to flower in short days (Moore, 1989).

There is a suggestion that the signal required for flowering may not be chemical because the speed of transmission excludes a role for substances which move in the symplast or phloem (Bernier, 1988). It is possible that an electrical wave of depolarization utilizing ion fluxes may transmit the signal although the detection of such a signal has not been achieved (Bernier, 1988). Ion fluxes (eg: Ca^{2+}) may be used on a cellular rather than a whole plant level to transmit a growth regulator signal from the membrane bound receptor protein into the cell (Elliot, 1986). If Ca^{2+} or some other ion was used to relay information into the cell, then plant growth regulators could interact because their respective receptor proteins may compete for the same ion channel to transmit the signal (Klee and Estelle, 1991). Evidence for a role for Ca^{2+} in flowering was reported by Friedman *et al.* (1989) who found that EGTA, a specific Ca^{2+} chelator, could inhibit flowering in the SD plant *Pharbitis nil* when applied before the flower inductive photoperiod. However these authors suggest that EGTA acts by affecting the action of phytochrome rather than by influencing growth regulator action.

Multi-factorial approach

It is apparent from the previous discussion that the floral signal consists not of a single compound but of a combination of changes in movement and level of many substances, be they growth regulators, assimilates or other compounds. There may be a range of different control mechanisms at any stage of flower evocation and development (Jackson and Sweet, 1972). This is consistent with the findings of Le Jeune *et al.* (1988) that many of the features of floral evocation in *Sinapis alba* could be produced individually by a range of treatments. However only when they all occurred together were flower primordia produced. It may not be that changes must occur all at once. Jackson and Lyndon (1990) suggest that one substance may cause changes which allow the tissue the competence to respond to another substance. Thus the timing of the changes may be as important as the quantitative changes of substances.

In some plants one compound may be primarily responsible for floral evocation, or indeed inhibition of floral evocation, and thus applications of plant growth regulators have the ability to control this compound and induce flowering. Jackson and Sweet (1972) suggest that this situation is more likely to occur in annual plants (such as spinach, *Sinapis alba*, *Pharbitis nil* and *Arabidopsis*). Perennials tend to have more varied mechanisms and a greater number of steps involved in floral evocation and development. To control flowering in these plants, a combination of plant growth regulators which more closely resemble the growth regulator action within the plant may be required (Elliot, 1982).

Specific conditions may not only be required at the beginning of flower development, but once flower buds have reached a certain stage of development. Flower buds in many fruit trees reach an intermediate stage of development and then become dormant. A change in the environment is required for the buds to `break dormancy' and develop through to anthesis. This strategy is common in plants growing in areas where the winter is extreme. If we consider again the multi-factorial approach to floral evocation and development it is easy to understand how flowering can be controlled at a number of stages. Since many processes are involved in flowering a block in any one of these can halt development and result in dormancy, cause the abortion of floral development or result in a redirection of floral to vegetative development.

The expression of genes, which ultimately control flowering, can be influenced by growth regulators or other compounds. Numerous authors have shown how growth regulators can influence RNA production (Houssa *et al.*, 1990; Perennes *et al.*, 1990; Soeda *et al.*, 1990). Lyndon (1990) suggests that growth regulators are most likely to affect regulator genes rather than directly affecting specific genes, and cites the GA_3 stimulation of mRNA which results in the production in α-amylase in barley aleurone tissue as an example. This author states there is no evidence that growth regulators have a direct effect on transcription. It is interesting that growth regulators can control some genes and conversely that other genes can control growth regulators. In the suggested multi-factorial approach to the control of flowering, the gene-growth regulator interactions are the lowest level of the complex web of signals and actions which finally result in a change in growth pattern.

Species information

Boronia megastigma

The Boronia genus is almost endemic to Australia with only one of the 95 species being found outside Australia in New Caledonia. The genus is present throughout most of Australia from hot and dry areas, to the tropics, to altitudes above 1000 metres. The genus contains many species of horticultural merit both for their attractive flowers and fragrance.

B. megastigma is endemic to the higher rainfall areas of south-west Western Australia (Anon, 1971a). It occurs in moist, seasonally wet sites on various soil types of acid to neutral pH. Generally *B. megastigma* plants are found in shady areas on the edge of Paper-Bark flats or close to creeks (Christensen and Skinner, 1978). This species has a life span of 5-7 years (Anon, 1971a) and regenerates from soil seed banks after fire or soil disturbance. Because seeds are poorly dispersed *B. megastigma* plants are generally found in clumps (Christensen and Skinner, 1978).

B. megastigma is an erect shrub with many branches. The compound leaves are opposite and the three leaflets are 1-1.5 cm long, narrow and thick. Flowers are solitary in the axils of leaves, bell shaped and about 1 cm in diameter and length. The fragrant flowers are generally red-brown outside and yellow inside (Elliot and Jones, 1982). The flower characteristics have given this boronia the common names Brown Boronia or Scented Boronia. Flowering occurs during late winter or early spring. Since cultivation, many forms have been selected including 'Lutea' with yellow-green flowers, 'Harlequin' with yellow and red-brown striped flowers and 'Heaven Scent' which has a more compact form (Elliot and Jones, 1982). The cultivar 'Chandleri' has burgundy flowers and the normal open habit.

B. megastigma was introduced into cultivation in Britain during the 1840's by James Drummond who made his living solely by the sale of seed and plants to Britain (Elliot and Jones, 1989). Commercial exploitation in Australia began around 1900 with the sale of field harvested blossom in Western Australia. Seeds began selling in Victoria around 1909. Distillation of the flowers for essential oils began in 1925. In 1969 all flowers and seeds were still harvested from wild populations. Most seed was sent to Victoria and New Zealand while most cut-flowers remained in Western Australia (Anon, 1971a). Since that time *B. megastigma* has gained in importance as a garden and pot plant, as a cut flower with a vase life greater than two weeks (Motum and Goodwin, 1982) and in plantations where essential oils are extracted from the flowers (Davies and Menary, 1983). The suburb of Boronia in Melbourne was named after the many plantations of this species growing in the area (Elliot and Jones, 1989).

Davies and Menary (1983) have isolated 150 volatile components from *B. megastigma* flowers and have identified 50% of these. The groups of compound that contribute most to the aroma are the ionones, related epoxides and dihydro compounds, various esters and sesquincineole. Some long chain acetates have been found, and may attract pollinators to these flowers (Davies and Menary, 1983).

Interest in *B. megastigma* shown by the nursery industry has led to some informal experimentation by horticulturalists (Raid, 1971). It is generally accepted that *B. megastigma* grows well in a wide range of soils but prefers a moist, well drained site in dappled or partial sun. The root system should be protected from drying out or death may result (Elliot and

Jones, 1982). Abundant leaf mulch is recommended for this reason (Cane, 1989). *B. megastigma* has been successfully grafted on to the more tolerant root stock *Boronia clavata.*

B. megastigma is sensitive to phosphorus (Thomas, 1981) and to high concentrations of nitrogen (Reddy and Menary, 1990). Reddy and Menary (1989a; 1989b; 1990) have conducted extensive studies on the nitrogen requirements of this species in both glasshouse and field conditions. Generally the addition of nitrogen caused an increase in vegetative growth which produced more leaf axils from which flowers could form. While nitrogen increases the number of flowers, it decreased their size and the percentage of flowers that fully developed (Reddy and Menary, 1989b). In the field, flower yield also increased if a slow release nitrogen source was applied early in the season. However nitrogen applied while the flower buds were developing caused a decrease in flower yield.

Roberts and Menary (1989a; 1989b; 1990) and Roberts *et al.* (1991) have investigated some aspects of flower initiation and development in *B. megastigma.* Flowers were initiated in all the environments they tried, however subsequent development was affected by both light and temperature. Extended daylength, high temperature and high light intensity caused a decrease in the number of flowers that reached maturity (Roberts and Menary, 1989b). Some of the flower buds developing in a sub-optimal environment became dormant, others formed transitional buds with both vegetative and floral characteristics and others abscised with vegetative buds developing from the axils of the bracts (Roberts and Menary, 1989a). Lewis and Warrington (pers. comm., 1988) suggest that flowering was best when plants were grown in temperatures between 10-20°C for at least 12 weeks.

Attempts have been made to alter the vegetative and floral characteristics of different Boronias using plant growth regulators. Lewis and Warrington (1988) found that promalin (a mixture of gibberellins GA_4 & GA_7 with benzyladenine) restricted plant height by 40% and doubled the number of branches of *B. megastigma* 'Lutea' plants. Daminozide decreased plant height and increased branch number, branch length and shoot dry weight. Two growth retardants, paclobutrazol and chlormequat chloride, had no effect when applied as a soil drench.

Boronia heterophylla is a species closely related to *B. megastigma* (Bentham and von Mueller, 1967a). Manual pinching of vegetative shoots of this species in early winter caused a

decrease in flowering due mainly to the abortion of flower buds (Richards, 1985). Benzyladenine application during late summer increased the branch number and thus provided more site at which flowers could form. Treatment of plants with Atrinal, which suppresses apical dominance, also caused a 250% increase in the number of branches and a subsequent increase in flower number due to an increase in the availability of sites (Wilkinson, 1981).

Hypocalymma angustifolium

The *Hypocalymma* genus is less well-known than *Boronia*. There are only 13 species, although a revision of the genus is under way (Elliot and Jones, 1990). These species are confined to the south-west of Western Australia. The genus is in the *Chamelaucium* alliance of the Myrtaceae and closely related to *Chamelaucium*, *Baeckia* and *Leptospermum* (Bentham and von Mueller, 1967b).

H. angustifolium is an erect shrub with many stems. The stems are white due to the presence of cork or phellem surrounding the stem. The phellem develops just back from the shoot tip and, in the second year of development, splits to reveal rough bark. Leaves are opposite, 2-3 cm long, very narrow and thick with pointed ends. Flowers are found two per axil and are sessile or borne on a very short pedicel. They are up to 1 cm across, five or more petalled and cup-shaped to flat with prominent stamens. Flowering occurs during late winter or early spring and a flush of vegetative growth follows anthesis in late spring (Elliot and Jones, 1990). Because of the conspicuous white flowers *H. angustifolium* has the common name White Myrtle. The colour of the flowers changes from white to deep pink during development which Lamont (1985) has correlated with loss of stigma receptivity and onset of ovule swelling. Pollinators only visit white flowers thus the colour change is a way of maximising pollination and foraging efficiency.

Both *H. angustifolium* and *Hypocalymma robustum* were introduced to England in 1843 because they are profusely flowering plants (Elliot and Jones, 1990). *H. angustifolium* is cultivated mainly as a garden plant and only on a limited scale for cut-flowers. These flowers

have a vase life greater than two weeks (Motum and Goodwin, 1982). Little work has been done to define the conditions which give the best growth and flowering of this species.

H. angustifolium plants are found naturally on free-draining, acid to neutral, sand or gravel soils commonly with a clay sub-soil which may allow some water-logging in winter (Elliot and Jones, 1990). Growth is best in these soil conditions (Anon, 1971b). Mulching to keep the roots cool and moist is useful although this plant is hardy to dry periods. Plants prefer shade during part of the day and should be sheltered from strong winds. Hard pruning is useful to prevent a sparse woody appearance (Elliot and Jones, 1990). There is one cultivar 'Densiflorum' which can be distinguished from the normal form by compact habit and smaller leaves.

There has been no investigation of flowering in *H. angustifolium* but work on *Leptospermum scoparium* (Zieslin, 1985) and *Chamelaucium uncinatum* (Shillo *et al.*, 1984; 1985), two species from similar habitats and closely related to *H. angustifolium*, may give some indication of the conditions that may control flowering in *H. angustifolium*. *Leptospermum scoparium* flowers are induced by short days in mild temperature. Long days or low temperature inhibit flowering. Plants required eight weeks of the inductive conditions before plants were committed to flower (Zieslin, 1985), thus inductive conditions were required for both induction and early flower development.

In *Chamelaucium uncinatum*, flowering was induced in short days and four weeks of these conditions were required to commit plants to flower. If short days were combined with cool temperature, vegetative axils ceased growth and flowering resembled a terminal cyme (Shillo *et al.*, 1984). At slightly higher temperatures vegetative growth continued resulting in an increase in the number of flowers. Long days inhibited flowering but the application of chlormequat chloride was able to relieve this inhibition. Gibberellin application partially inhibited flowering in short days. These results suggest that a reduction in endogenous gibberellin content is necessary but not sufficient to induce flowering in *Chamelaucium uncinatum* (Shillo *et al.*, 1985).

CHAPTER TWO

FLORAL DEVELOPMENT IN *BORONIA MEGASTIGMA* AND *HYPOCALYMMA ANGUSTIFOLIUM*

Introduction

In recent years a number of researchers working with Australian native plants have conducted microscopic studies to better appreciate the physiology of flowering (Motum and Goodwin, 1987a; Roberts and Menary, 1989a; Sharman and Sedgley, 1988). There are a number of reasons for studying floral physiology, one being to gain some control of flowering for commercial purposes. However, as Lord and Eckard(1985) state, "the complexity of the flowering process in trees (or any woody perennials) requires careful developmental documentation prior to any experimental manipulation of flowering".

Authors have described the taxonomic characteristics of *B. megastigma* and *H. angustifolium* flowers (Bentham and von Mueller, 1967a; 1967b; Elliot and Jones, 1982; 1990), however there is a paucity of information describing floral development of these species. Roberts and Menary (1989a) have described the external morphological changes that occur during *B. megastigma* flower development and describe a range of transitional buds, which are floral buds that revert to vegetative growth. These authors report that uncommitted buds are produced throughout the year in the leaf axils of *B. megastigma* plants, three to four axils basal to the apical shoot. Buds can remain dormant, or develop vegetatively or florally depending on the environmental conditions of the plant. Cool temperatures (17°C day / 9°C night) have been found to induce flowering in *B. megastigma* and *H. angustifolium* (Chapter 4).

No similar work has described the development of flowers of *H. angustifolium*, in fact the Australian Myrtaceae, excepting the *Eucalyptus* genus, have received little attention in this regard. Beardsell *et al.* (1989) described the floral nectaries in *Thryptomene calycina*. A number of authors have investigated the post anthesis development of *Eucalyptus* species to aid

their work on the breeding systems of these plants (Moncur and Boland, 1989; Sedgley and Smith, 1989). Moncur and Boland (1989) briefly examined the floral development of *Eucalyptus melliodora* from initiation to senescence and refer to a few earlier works on other *Eucalyptus* species.

In this study, the development of *B. megastigma* and *H. angustifolium* flowers have been followed from initiation through to anthesis using scanning electron and light microscope techniques. Stages of development have been assigned to distinctive phases of flowering in both species. These stages of development will be used in later chapters to relate physiological events to the morphological development of flowers.

Methods

Plant material and growth conditions

Before the commencement of this study, a range of propagation conditions were tested to determine those conditions required to obtain good quality plants (Appendix 1). The following method of propagation was then adopted.

All plants were propagated from cuttings obtained from a limited number of stock plants, these stock plants being derived from a single plant which was originally purchased from Woods and Forests, Murray Bridge, South Australia. Stock plants were maintained in a heated glasshouse. Cuttings were placed in trays of pine-bark based propagation medium in mist. After rooting, *B. megastigma* 'Heaven Scent', a compact form of Brown Boronia, and *H. angustifolium* cuttings were grown in a heated glasshouse in one litre pots in a mix of 3 parts composted pine bark, 1 part fine sand, 1 part coarse sand with 2g/litre osmocote (N:P:K, 17 :1.6 :8.7), 1g/litre osmocote coated iron sulphate and 1g/litre of a dolomite: gypsum mix (7:2). Five to six months after potting-on from cuttings, plants uniform in height and breadth were transferred to growth cabinets with a twelve hour daylength and temperature period. The cabinets were equipped with four 400W high pressure sodium lamps, five 140W fluorescent tubes and six 100W tungsten globes. Irradiance at plant height was 350 $\mu mol.m^{-2}.s^{-1}$ (400nm-

700nm). The spectral distribution of the cabinets is shown in figure 3.1. Warm conditions were provided at a day/night temperature of 25°C/17°C and cool conditions provided at 17°C/9°C. Ten leaf axils with floral buds were selected and examined each week from plants in cool conditions, beginning at the commencement of cool conditions through to anthesis.

Scanning electron microscope sample preparation

Material was fixed for two hours at room temperature in 4% glutaraldehyde in phosphate buffer (0.025M KH_2PO_4, pH 7.0). Large organs were dissected prior to fixation to allow entry of the fixatives to the inner tissues. Tissue was further fixed overnight at 0°C in 1% osmium tetroxide in phosphate buffer. The material was rinsed in RO water (H_2O passed through reverse osmosis and ion exchange, with a conductivity of less than 12mΩ.cm^3) and dehydrated in a graded acetone series (15%, 30%, 45%, 60%, 70%, 95%, 3 x 100%) before critical point drying. After mounting material on stubs, further dissection was carried out if required. The samples were sputter coated (Emscope SC 500) with 20nm of gold and viewed with a scanning electron microscope (SEM) (Cambridge Instruments Stereo scan 250 Mk 3) with an accelerating voltage of 20 kV in secondary electron mode. Electron micrographs were recorded using Ilford Pan F film.

Light microscope sample preparation

Tissue used for light microscope sections was fixed overnight in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 - 7.4. The material was rinsed in this buffer and RO water before being dehydrated in the acetone series as above. The tissue was transferred from 100% acetone to a Spurr's resin:100% acetone mix (1:1) and left for five hours at room temperature. The samples were then transferred to 100% Spurr's resin overnight (Spurr, 1969). Following this the material was transferred to fresh 100% Spurr's resin and polymerized overnight at 70°C. 1µm sections (2µm for large organs) were cut using an ultramicrotome (Reichert-Jung) and stained with 0.25% (w/v) Toluidine Blue-O in 0.1% (w/v) sodium carbonate ($NaCO_3$). A Zeiss Axioplan light microscope fitted with a Zeiss MC100

camera was used to observe and photograph the sections. Kodak Tech Pan 2415 film was used for photographs and this was developed with Kodak HC - 110, Dilution B (1:7).

Colour photographs were taken either with a Zeiss dissecting microscope fitted with a MC63 camera and controller, or without a microscope with a Canon A-1 camera. Kodak Ectachrome 100 daylight film was used in both cameras.

Results

Boronia megastigma

Figures 1 & 2 show an undifferentiated *B. megastigma* bud. These buds can be found in most leaf axils and can develop vegetatively or florally (Roberts and Menary, 1989a). In warm conditions (25°C day/ 17°C night) these buds remained dormant or developed vegetatively. Vegetative differentiation is characterised by the maintenance of decussate phyllotaxis and the tri-lobed appearance of the leaf primordia. The leaf primordia expand at a faster rate than the bract primordia of a developing flower, and the apical meristem of vegetative shoots remained rounded. The three leaflets of the compound leaf became quite distinct from one another and were attached to the leaf base (Figure 3).

In cool conditions (17°C day/ 9°C night), the uncommitted buds began floral differentiation. The formation of a flat broad meristem (Figure 4) and the initiation of the four sepals resulted in a broadening of the developing floral bud. Sepals developed in a decussate fashion but with a reduced plastochron (Roberts and Menary, 1989a). Swelling of the bracts (Figure 7) was also commonly associated with floral differentiation. At this stage it was possible to macroscopically differentiate between vegetative and floral buds. After three to four weeks in cool conditions the sepals overlapped each other and covered the meristem. Approximately a week later the decussate phyllotaxis was lost when the simultaneous initiation of the petals and stamens occurred (Figure 6). At this stage the flower stalk below the bracts began to elongate (Figure 5). The petals grew faster than the stamens and eventually covered the stamens, the meristem and the four carpels which had begun to develop (Figure 8). The

growth of the petals and general enlargement of the bud forced the sepals to emerge through the bracts. After ten weeks in cool conditions the flower stalk above the bracts began to elongate (Figure 10) and the whole flower stalk began to bend. At this stage the sepals, despite their decussate development, were all equal in size and were longer than the bracts. The lobes of the anthers could be distinguished and the four carpels that had developed independently up to this stage (Figures 9 & 11), approached each other prior to fusion to form the sessile stigma.

The development of the stigma, ovaries and the anthers commenced before much bud expansion occurred. The beginning of the phase of rapid bud expansion coincided with the development of the ovules and pollen which occurs after twelve to thirteen weeks in cool conditions (Figures 12 & 13), however the ovules and pollen were well developed before the majority of bud expansion occurred. The growth of the large stigma accompanied bud expansion and the emergence of the petals through the sepals (Figure 14). *B. megastigma* flowers have two vertically arranged ovules in each of the four carpels (Figure 13). Of the eight anthers only the four adjacent to the petals had pollen (Figure 12). These anthers were smaller than the non-fertile anthers (Figure 17) and were situated below the stigma and adjacent to the carpels (Figure 15). The pollen was produced in four locules, two locules per anther lobe (Figure 12), and the filament was attached dorsally (Figure 17). The sepaline anthers produced no pollen and show the appearance of being squashed by the lobes of the stigma (Figure 12). Rudimentary locule formation was observed in these anthers (Figure 12) and the filament was attached ventrally (Figure 17). At anthesis, the petals were red to brown on the outside and yellow inside (Figure 16). Senescence of flowers occurred in cool conditions after the flowers had been open for three to four weeks. The petals shrivelled and abscised, and the flower stalk generally abscised above the bracts (Figure 18).

Reversion of floral buds was able to occur if plants with floral buds in which the stigma, ovules and pollen had not developed, were placed in conditions that were unfavourable for the induction of flowering. There are many forms that the reversion can take, one is shown in figure 19 and Roberts and Menary (1989a) show many more. Reversion of flower buds was characterised by aberrations in the development of floral organs, the development of vegetative shoots from the axils of the bracts and eventual abscission of the flower stalk above the bracts.

Figure 1: —— 200µm

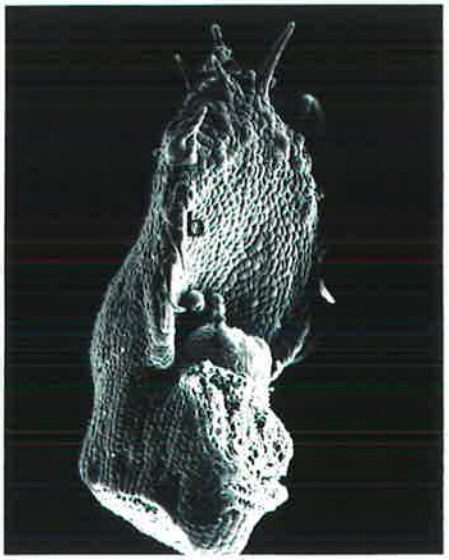


Figure 2: —— 100µm

Figure 3: —— 200µm

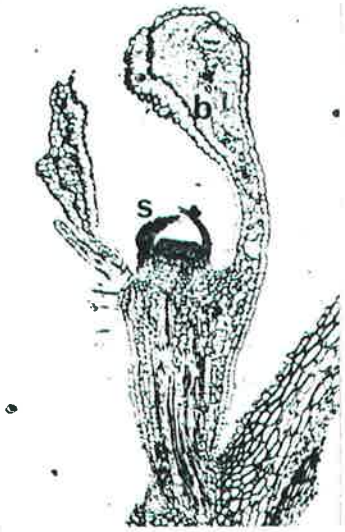


Figure 4: —— 200µm

Figure 5: —— 2mm

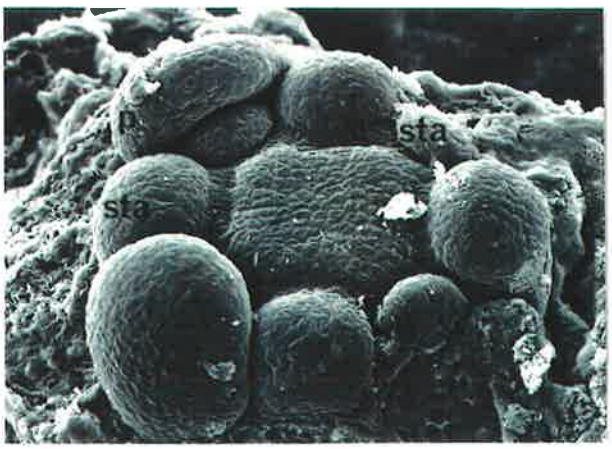


Figure 6: —— 50µm

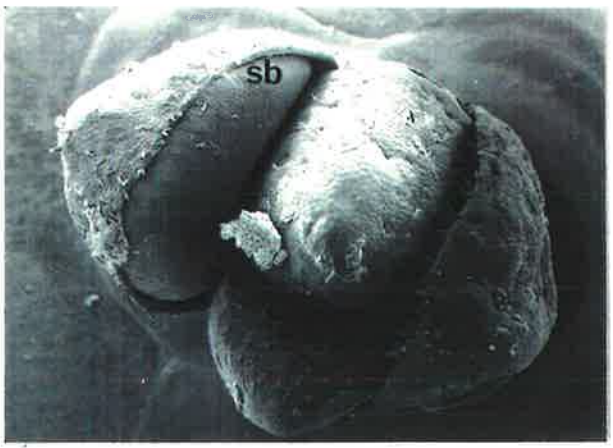


Figure 7: —— 400µm

Boronia megastigma

Figure 1 - stage 0, undifferentiated buds in the leaf axils of plants in warm conditions.
Figure 2 - stage 0, undifferentiated bud with leaf or bract removed.
Figure 3 - vegetative bud with three leaflets attached to the leaf base.
Figure 4 - stage 1, initiation of sepals and flattening of the meristem.
Figure 5 - stage 2.
Figure 6 - stage 2, bracts and sepals removed, simultaneous initiation of petals and stamens.
Figure 7 - stage 3, swollen bracts.
ub - uncommitted bud, ls - leaf scar, b - bract, i - leaf or bract initial, lt - leaflet, lb - leaf base, s - sepal, p - petal, sta - stamen, sb - swollen bract

Figure 8: —— 100µm

Figure 9: —— 100µm

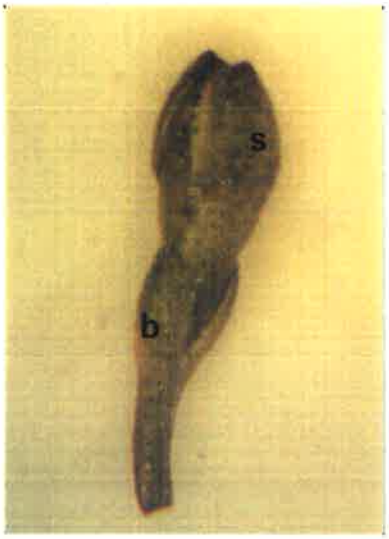


Figure 10: —— 2 m m

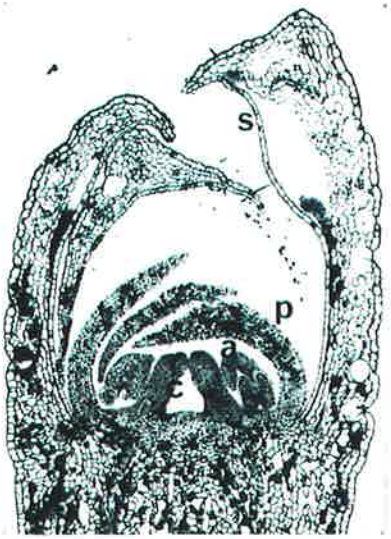


Figure 11: —— 200µm

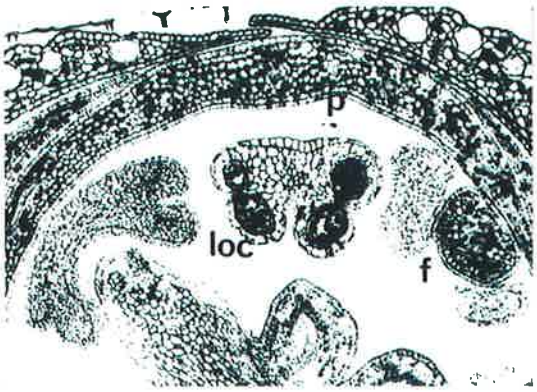


Figure 12: —— 200µm

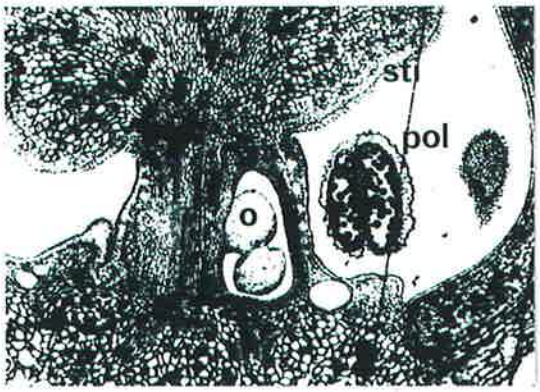


Figure 13: —— 200µm

Boronia megastigma

Figure 8 - stage 4, sepals removed, petals cover meristem.
Figure 9 - stage 4, sepals and petals removed, carpels prior to fusion protrude above developing anthers.
Figure 10 - stage 4, elongation of flower stalk above bracts.
Figure 11 - stage 4, carpels arise separately, endothecium of anther formed but no pollen.
Figure 12 - stage 5, ovaries and fertile anthers petaline, non-fertile anther squashed with possible rudimentary locule formation.
Figure 13 - stage 5, two ovules per ovary vertically arranged, pollen sac elongate.
p - petal, a - anther, c - carpel, s - sepal, b - bract, loc - locule, f - filament, o - ovule, sta - stamen, pol - pollen

Figure 14: ——— 5mm

Figure 15: ——— 400µm

Figure 16: ——— 10mm

Figure 17: ——— 500µm

Figure 18: ——— 10mm

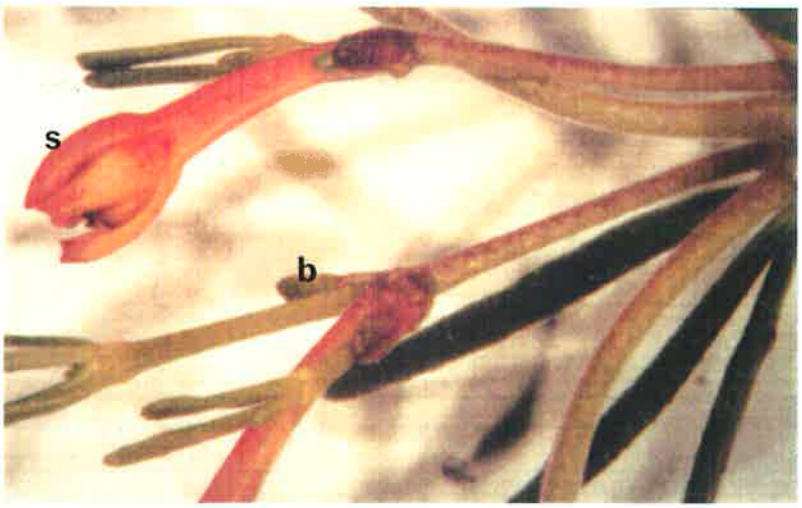


Figure 19: ——— 2mm

Boronia megastigma

Figure 14 - late stage 5, petals show through sepals.
Figure 15 - late stage 5, sepals, petals and stigma removed, carpels remain separate at base, non-fertile anthers taller than fertile anthers.
Figure 16 - stage 6, anthesis, red outer petal, stigma and non-fertile anthers, yellow inner petal and fertile anthers.
Figure 17 - stage 6, anthesis, petals removed, ventral attachment of filament to fertile anther, dorsal attachment of filament to non-fertile anther.
Figure 18 - stage 7, senescence, abscission of petals, abscission of flower stalk above bracts.
Figure 19 - reversion to vegetative growth, elongate flower stalk and sepals, vegetative growth from axils of bracts.
c - carpel, a(f)- fertile anther, a(nf) - non-fertile anther, sti - stigma, s - sepal, b - bract

Stages of *Boronia megastigma* floral development

The stages of flower development have been defined based on the distinctive morphological changes listed immediately after the stage number. Further descriptions that help identify the stage of development are listed after the defining characteristic.

Stage 0 - Undifferentiated dormant bud, less than 300µm diameter. Bract or leaf initials present.

Stage 1 - Sepals initiated in decussate fashion. Macroscopic appearance of flower buds. Swelling of bracts, sepals cover the flat meristem. Two weeks in cool temperature (17°C day / 9°C night). Flower bud less than 500µm diameter.

Stage 2 - Interruption of decussate phyllotaxis with the simultaneous initiation of petals and stamens. Beginning of elongation of flower stalk below bracts. Four to five weeks in cool temperature. Flower bud between 600µm - 700µm diameter.

Stage 3 - Initiation of carpels. Sepals visible through bracts. Petals cover meristem. Seven to eight weeks in cool temperature. Flower bud less than 1mm diameter.

Stage 4 - Carpels protrude above anthers prior to fusion to form the sessile stigma. Elongation of flower stalk above bracts and bending of flower stalk. Sepals larger than bracts. Development of anthers. Ten to twelve weeks in cool temperature. Flower bud approximately 1 mm diameter.

Stage 5 - Development of stigma, ovules and pollen. Macroscopic appearance of petals through sepals. Thirteen to fourteen weeks in cool temperature. Flower bud 1-2 mm diameter. This stage is followed by a period of bud expansion.

Stage 6 - Anthesis. Nineteen to twenty one weeks in cool temperature. Flower bud 9-12 mm diameter.

Stage 7 - Senescence. Shrivelling of petals. Abscission of petals and of flower stalk above bracts. Twenty two to twenty four weeks in cool temperature.

Hypocalymma angustifolium

As the stem of *H. angustifolium* developed, a periderm consisting primarily of a layer of large-celled phellem or cork was laid down surrounding the stem (Figure 25). This cork covered any buds that were developing in the leaf axils thus obscuring the external morphology of the buds until a late stage of development was reached. After approximately six months, the phellem began to split longitudinally revealing a woody bark underneath. Few buds developed from stems greater than one year old.

In warm conditions (25°C day/ 17°C night) buds similar to that shown in figure 20 were present in a few leaf axils, however floral meristems were not observed in warm conditions. Initially a pair of bracts developed to cover the whole meristematic tissue (Figure 20). Within the bracts the vegetative shoot developed. The leaf axils with buds were generally found in four to five nodes grouped along a stem, no buds were observed in the eight to fourteen nodes between groups. Vegetative shoots developed from these buds at much the same time resulting in a flush of lateral vegetative growth. Vegetative shoots had small rounded meristems and leaves that developed in a decussate fashion (Figure 21). The large difference in the size of sequential pairs of leaves suggest that vegetative shoots have a relatively large plastochron. The developing vegetative shoot emerged from the bracts and phellem with little splitting of the periderm (Figure 22). Most of these shoots halted growth before five nodes had developed.

When plants were transferred to cool conditions (17°C day/ 9°C night), buds developed in leaf axils excluding those five to ten nodes basal to the shoot apex and where thick woody bark was present. In the leaf axils that already had bracts and a vegetative shoot, the vegetative bud generally halted development and floral buds were initiated in the meristematic tissue flanking the vegetative shoot (Figure 20). In the other axils, bracts developed to enclose the two floral buds and the central vegetative bud. Floral buds could be easily distinguished from vegetative buds from their position within the bract (Figure 23). Each floral bud produced a pair of bracteoles in a decussate fashion (Figure 23). The sepals interrupted the decussate phyllotaxis and grew to cover the meristem (Figure 24). Eventually the sepals fused to form a flat calyx tube with five lobes which enclosed the base of the receptacle. The initiation of petals in a spiral phyllotaxis closely followed the initiation of sepals. There was some overlap of the early stages of development of the *H. angustifolium* flowers examined, caused by the ability of flowers to develop at different rates on the same plant. However, in conditions suitable for flowering, synchronous anthesis generally occurred.

As the petals and bracts (Figure 26) enlarged, the periderm covering the leaf axils began to bulge (Figure 25). The bud expansion forced the buds to lean away from each other and forced the sepals, bracteoles (Figure 27) and bracts apart. The petals emerged from the sepals at much the same time as stamens and the style began development (Figure 29), thus while the

initiation of petals closely followed that of sepals, there was a period of petal and bud expansion before stamen and style initiation. The stamens are numerous and may be joined in a single ring (Bentham and von Mueller, 1967b). Bud expansion accompanied the rapidly elongation of the filaments and style and resulted in the emergence of the buds through the periderm (Figure 28). Bud expansion preceded anther and stigma development and certainly preceded ovule and pollen development. The filaments of *H. angustifolium* flowers bent in many directions, however most bent to force the developing anthers to the base of the receptacle (Figure 30).

There was continuous filament and style elongation while the anthers and stigma developed (Figure 32). The anthers had four locules (Figure 33). The stigma was small (Figure 32). The ovary was three celled and had three prominent ridges running from the base of the style to the receptacle. There was one ovule per ovary cell and the style was inserted in a central depression in the ovary (Figure 34). Just prior to anthesis petals were green with white edges (Figure 31). At anthesis all traces of green had vanished (Figure 35) and the smaller petals nearest the calyx tube had tinges of pink. As senescence progressed, during the three to four weeks before the flowers abscised, the larger petals became pink. Just prior to abscission all petals were a deep pink (Figure 36). Senescence was characterised by the shrivelling of petals and eventual abscission of the whole flower. The petals remained attached to the receptacle.

There was no reversion of flowering in *H. angustifolium*. Placing *H. angustifolium* plants in conditions unfavourable for flowering prior to the development of the anthers and stigma generally resulted in a halt to the development of the bud. No organs transitional between floral and foliar developed and no leaves grew from the axils of the bracts or bracteoles, indicating that buds were determined to be either floral or vegetative early in development. The vegetative bud between the two floral buds generally commenced development when plants were transferred to conditions not favourable for floral induction or after the senescence of flowers.

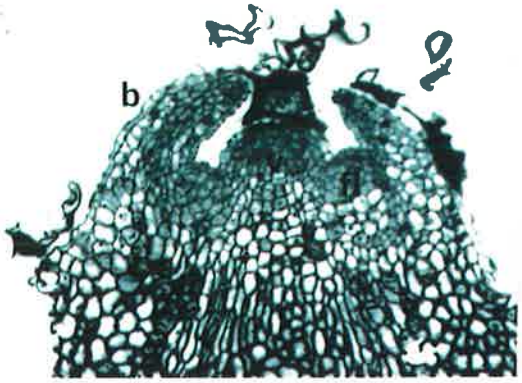


Figure 20: ——— 100µm

Figure 21: ——— 300µm

Figure 22: ——— 1mm

Figure 23: ——— 100µm

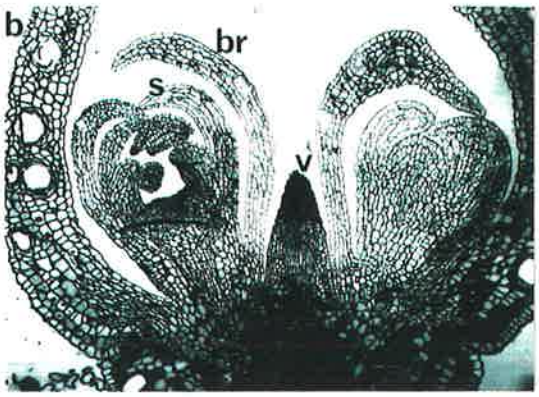


Figure 24: ——— 100µm

Hypocalymma angustifolium

Figure 20 - stage 1, initiation of bracts and development of three buds.
Figure 21 - vegetative shoot, decussate phyllotaxis and large plastochron.
Figure 22 - vegetative shoot emerging through phellem from leaf axil.
Figure 23 - early stage 2, bracts cover three buds, central bud vegetative flanked by two floral buds, initiation of bracteoles.
Figure 24 - late stage 2, initiation of sepals and petals, flattening of meristem.
b - bract, v - vegetative, fl - floral, l - leaf, s - sepal, br - bracteole

Figure 25: —— 200µm

Figure 26: —— 200µm

Figure 27: —— 200µm

Figure 28: —— 1mm

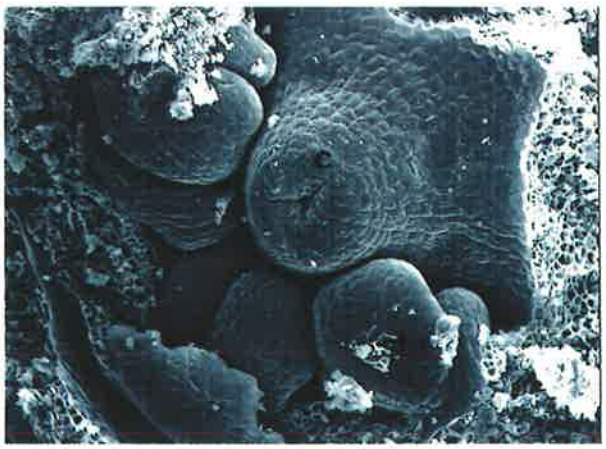


Figure 29: —— 50µm

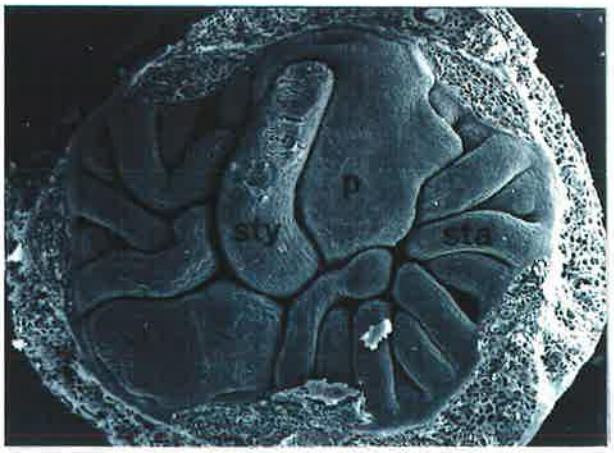


Figure 30: —— 200µm

Hypocalymma angustifolium

Figure 25 - stage 3, bulging of large celled phellem.
Figure 26 - stage 3, phellem removed, bracts covering buds.
Figure 27 - stage 3, bracts removed, two floral buds showing bracteoles and imbricate sepals.
Figure 28 - stage 4, emergence of floral buds through phellem.
Figure 29 - early stage 4, petals removed, development of stamens and carpel.
Figure 30 - late stage 4, petals removed, bending of filaments and style.
ph - phellem, ls - leaf scar, b - bract, br - bracteole, s - sepal, c - carpel, sta - stamen, sty - style, p - petal

Figure 31: ——— 2mm

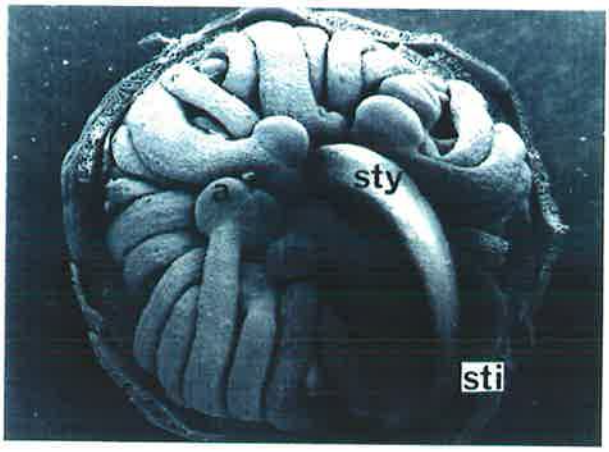


Figure 32: ——— 500µm

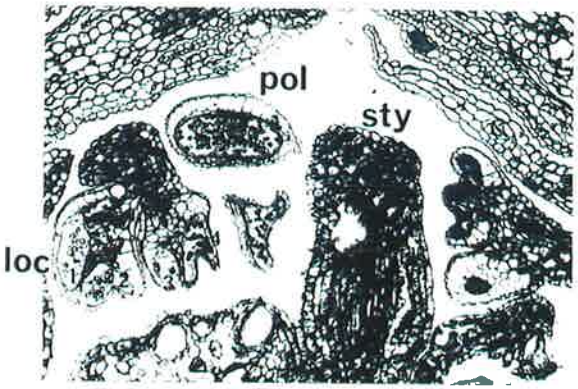


Figure 33: ——— 200µm

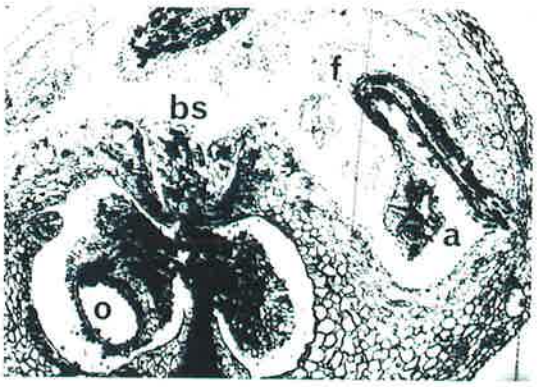


Figure 34: ——— 400µm

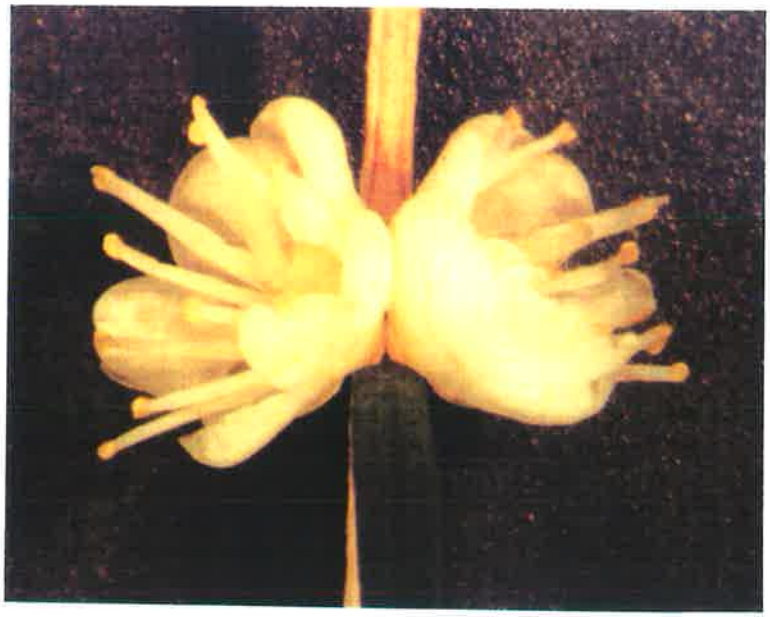


Figure 35: ——— 3mm

Figure 36: ——— 3mm

`Hypocalymma angustifolium`

Figure 31 - stage 5, flower buds in leaf axils, leaf forced perpendicular to stem.
Figure 32 - stage 5, development of anthers and stigma.
Figure 33 - stage 5, four locules per anther, pollen well developed, style inserted in top of ovary.
Figure 34 - stage 5, filament attached to edge of receptacle, one ovule per ovary.
Figure 35 - stage 6, anthesis, flower white except for yellow anthers, numerous petals.
Figure 36 - stage 7, senescence, all petals pink, flower remains intact.
a - anther, sty - style, sti - stigma, loc - locule, pol - pollen, o - ovule, bs - base of style, f - filament

Stages of *Hypocalymma angustifolium* flower development

The stages of flower development have been defined based on the distinctive morphological changes listed immediately after the stage number. Further descriptions that help identify the stage of development are listed after the defining characteristic.

Stage 1 - Initiation of floral buds. Two bracts enclosing a central vegetative bud and the two floral buds (total diameter 300 - 400 μm). Zero to two weeks in cool temperatures (17°C day / 9°C night).

Stage 2 - Initiation of bracteoles, sepals and petals. Flattening of meristem. Bulging of the periderm covering the leaf axils. Two to four weeks in cool temperature. Flower bud diameter 100 - 500μm.

Stage 3 - Initiation of stamens from the edge of the receptacle and of the style from the centre of the meristem. Flower buds emerge from bracts and bracteoles. Green-white petals emerge from sepals. Four to six weeks in cool temperature. Flower bud diameter less than 1 mm.

Stage 4 - Elongation of style and filaments. Macroscopic appearance of flower buds through periderm as periderm splits. Six to eight weeks in cool temperature. Flower bud diameter 1 - 1.5mm.

Stage 5 - Development of ovules, stigma, anthers and pollen. Continued bud expansion. Eight to eleven weeks in cool temperature. Flower bud diameter approximately 2 mm.

Stage 6 - Anthesis. Petals turn from white to pink. Seventeen to twenty weeks in cool temperature. Flower diameter 8-11 mm.

Stage 7 - Senescence. Shrivelling of petals. Abscission of whole flower. Commencement of vegetative growth from leaf axils. Twenty to twenty three weeks in cool temperature.

Discussion

Boronia megastigma

Flower development in *B. megastigma* is similar to that in other members of the Rutaceae such as *Citrus sinensis* (Lord and Eckard, 1985; 1987). Both species have undifferentiated buds that can enter a stage of rest and can develop either vegetatively or florally. In both species, once floral development has begun after the rest period, there is no interruption in development through to anthesis although the rate of development may change

with changing environmental conditions. The bracts and sepals of both species initiate with the same phyllotaxis as the leaves thus making conclusive identification of floral buds difficult until petals have been initiated. Finally, both species are able to revert to vegetative growth when transferred from flower-inducing to non flower-inducing conditions if the transfer occurs before flowers have reached a critical stage of development (Lord and Eckard, 1987; Roberts and Menary, 1989a). It is common for conditions that do not induce flowering to result in a reversion of flowering (Battey and Lyndon, 1990). In *Citrus sinensis*, flowers will not revert to vegetative growth after sepals have been formed on the terminal flower of the inflorescence, whereas in *B. megastigma*, stage five (where the stigma, ovules and pollen have begun development) must be reached before the flower is committed.

The ability of *B. megastigma* flowers to revert to vegetative growth may be related to the ability of uncommitted buds to initially develop vegetatively or florally. In species of plant that do not have this ability, for example *H. angustifolium*, flowers may not revert to vegetative growth when returned to conditions that are not favourable for flower induction. Plants that can undergo floral reversion may only be of two types, those with uncommitted buds like *Citrus sinensis* (Lord and Eckard, 1985) and *B. megastigma* (Roberts and Menary, 1989a), or those annual plants that have terminal shoots that develop into flowers (see Battey and Lyndon, 1990 for a compiled list). It is interesting to note that flower organs that resemble leaves can be found in mutant plants (Coen, 1991) and plants displaying phyllody as well as plants exhibiting floral reversion (Battey and Lyndon, 1990). Specific genes determine whether the true floral organ develops or a leaf-like floral organ (Drews *et al.*, 1991). Thus mutant plants that lack certain genes show symptoms similar to flower buds that have reverted to vegetative growth. This may suggest that the genetic control of flowering in plants that are able to show reversion is different to the genetic control in plants that do not show reversion, and that the genetic make up of buds that can be either vegetative or floral are different from those that are determined to be floral or vegetative at an early stage of development. The genetic difference may be the presence or absence of regulator genes which, it is understood, direct the determination of flowers (Coen, 1991).

Hypocalymma angustifolium

Eucalyptus melliodora and *H. angustifolium* are both members of the Myrtaceae. While there are many similarities in the morphological development of flower of these two species there are also many differences. *E. melliodora* has two deciduous bracts that enclose an inflorescence of seven flowers (Moncur and Boland, 1989), thus only floral tissue is present. In *H. angustifolium* plants in cool conditions, the bracts enclosed two floral buds and a vegetative shoot. In warm conditions only the two bracts and the vegetative shoot could be distinguished raising the possibility that bract initiation is not necessarily associated with flower initiation. Flower initiation in *H. angustifolium* was associated with the commencement of cool conditions. Initiation of lateral vegetative buds occurred in all conditions tested but were only found in a few leaf axils grouped along the stem. It is not known what signal results in the initiation of vegetative shoots, however the fact that they are initiated in groups along the stem suggests that a specific signal is required.

Bentham and von Mueller (1967b) refer to three bracts per *H. angustifolium* flower. These bracts are found "one at the top of the common peduncle and two under the calyx". This corresponds to one bract per flower of the bracts that cover the three buds in the leaf axil, and the two bracteoles under the calyx of each flower. The situation in *E. melliodora* is similar. In this species each flower of the inflorescence is subtended by two leafy bracts (bracteoles) and the inflorescence is initially enclosed in two bracts (Moncur and Boland, 1989). Bentham and von Mueller (1967b) also state that *Hypocalymma* species have five petals. The *H. angustifolium* plants used in this experiment had more than five petals, commonly up to ten or twelve were present. The first petals to be initiated were small, were pink at the time of anthesis and abscised easily when the flower was removed from the plant. Five to seven petals were visible when looking at the flower face. These were the largest petals, the last to be initiated and were white at anthesis. Possibly plants with more petals have been selected for horticultural purposes.

Conclusions

The floral development of *B. megastigma* and *H. angustifoium* are quite different, as would be expected in species from different families, however both species show similarity to the floral development of other members of their respective families. The major differences between these two plants relates to the initial buds from which flowers develop. *B. megastigma* plants have buds that initiate autonomously as the plant grows, can develop vegetatively or florally and can revert to vegetative growth if plants are placed in non-inductive conditions prior to stage five of development. *H. angustifolium* flower buds are only initiated when plants are transferred to cool conditions, can only form flowers and halt development if placed in non-inductive conditions. The differences must relate to differences in the genetic code of the plants, however there is a possibility that there is a more fundamental difference in the regulator genes controlling flower determination between flowers that are able to revert to vegetative growth and those that halt development when plants are placed in non-inductive conditions.

CHAPTER THREE

ENVIRONMENTAL CONTROL OF FLOWERING OF *BORONIA MEGASTIGMA* AND *HYPOCALYMMA ANGUSTIFOLIUM*

Introduction

Since, in natural conditions, both *B. megastigma* and *H. angustifolium* flower for a brief period in late winter and early spring it is likely that a specific environmental cue such as temperature or photoperiod is responsible for the induction of flowering. No previous research has investigated the effects of environment on the flowering response of *H. angustifolium*, but *Chamelaucium uncinatum* (Shillo *et al.*, 1984; 1985) and *Leptospermum scoparium* (Zieslin, 1985), other members of the Myrtaceae, are induced to flower by exposure to short photoperiod.

There are a number of reports on the effects of environmental conditions on flowering of *B. megastigma*. Lewis and Warrington (pers. comm., 1989) found that flowering in *B. megastigma* 'Lutea' was not affected by photoperiod but occurred in plants following 12 weeks of 10°C to 20°C temperatures. Roberts *et al.* (1989b) found that short days and low night temperature promoted flowering in this species although flowering occurred in all environmental conditions they tested.

The following research aimed to examine the role of environmental conditions in the control of flowering of *B. megastigma* and *H. angustifolium*.

Methods

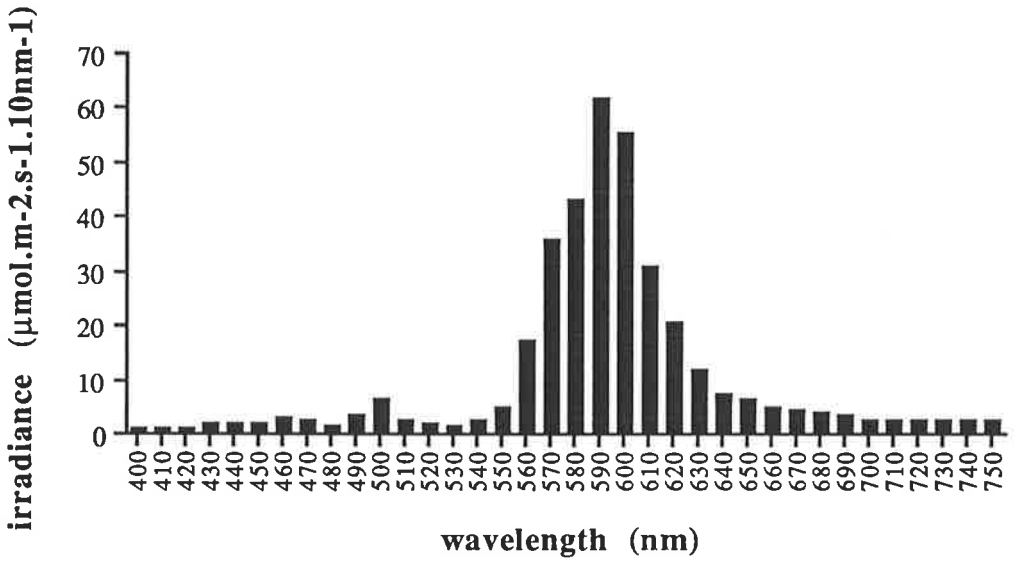
Plant material

Plants were propagated and grown as described in chapter 2. Plants uniform in height and breadth were selected for experiments from a heated glasshouse, between five and six months after potting-on from cuttings.

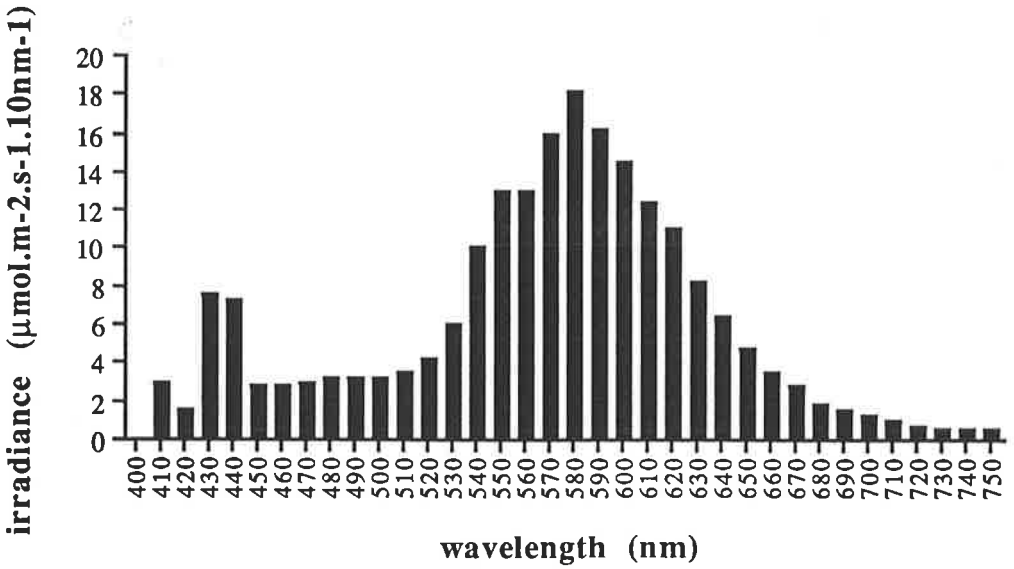
Figure 3.1 - The spectral distribution of light in the growth cabinets.

Light intensity was measured at the stated wavelengths with a spectro-radiometer (Isco SR).

a) Spectral distribution in the growth cabinets used for tables 3.1,3.2 & 3.3, predominantly lit with sodium lamps. Total irradiance = 358 $\mu mol.m^{-2}.s^{-1}$ (400-700nm).



b) Spectral distribution in the growth cabinets used for figure 3.3 and table 3.4, predominantly lit with fluorescent lamps. Total irradiance = 209 $\mu mol.m^{-2}.s^{-1}$ (400-700nm).



Photoperiod and temperature

The role of temperature and photoperiod on flowering of the two species and was conducted in the open and in growth cabinets with a twelve hour temperature period. The

plants would experience in their natural habitat. Plants were grown in cabinets equipped with four 400W high pressure sodium lamps, five 140W fluorescent tubes and six 100W tungsten globes (spectral distribution, figure 3.1a). Irradiance at plant height was 350 $\mu mol.m^{-2}.s^{-1}$ (400-700nm). Day light extension for photoperiodic treatments was provided for six hours at 25 $\mu mol.m^{-2}.s^{-1}$ by two 140W fluorescent tubes and two 100W tungsten globes.

Plants grown in the open or in glasshouses (Table 3.1) grew under natural light from May to September, late autumn to early spring. The daily minimum and maximum temperatures were recorded for the year 1990 in both glasshouses and outside. Mean monthly minimum and maximum temperatures and their associated standard errors were calculated and are presented in Figure 3.2.

Because photoperiod was found to have no effect on flowering of *B. megastigma* and *H. angustifolium*, all the following experiments were conducted with a daylength and temperature period of twelve hours.

Commitment to flower

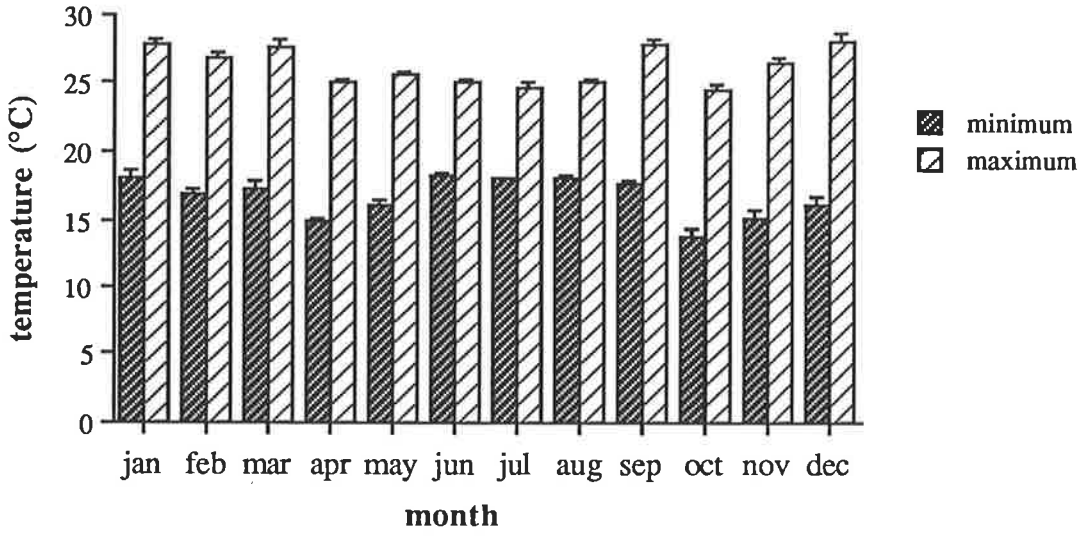
The number of weeks of cool temperature (17°C day / 9°C night) required to obtain maximum flowering was determined using plants grown in growth cabinets lit by twenty eight 140W fluorescent tubes, which provided an irradiance of 200 $\mu mol.m^{-2}.s^{-1}$ (400-700nm) at plant height (spectral distribution, figure 3.1b). Plants were grown in cool conditions and transferred to a warm growth cabinet (25°C day / 17°C day) at either one or two week intervals. The percentage of leaf axils with flowers was recorded if or when anthesis occurred.

Intermediate temperature

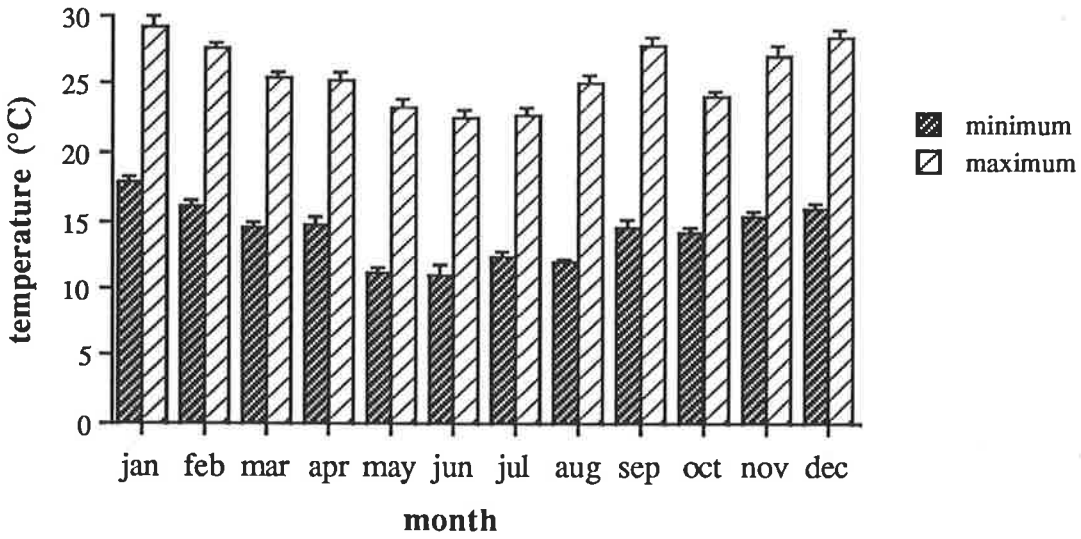
This experiment examined the effect of growth regulators on flowering of plants grown in a temperature regime of 19°C day / 11°C night. Light quality and quantity in the growth cabinet was as in the 'photoperiod and temperature' experiment above. Benzyladenine was donated by Abbots Laboratories, Chicago and paclobutrazol was a gift from ICI, Australia. Plants that were treated with benzyladenine received three applications of $50mg.l^{-1}$, one week apart, sprayed until run off (approximately 25ml per plant). Paclobutrazol was applied once as

Figure 3.2 - The monthly minimum and maximum temperatures in the glasshouses and outside.

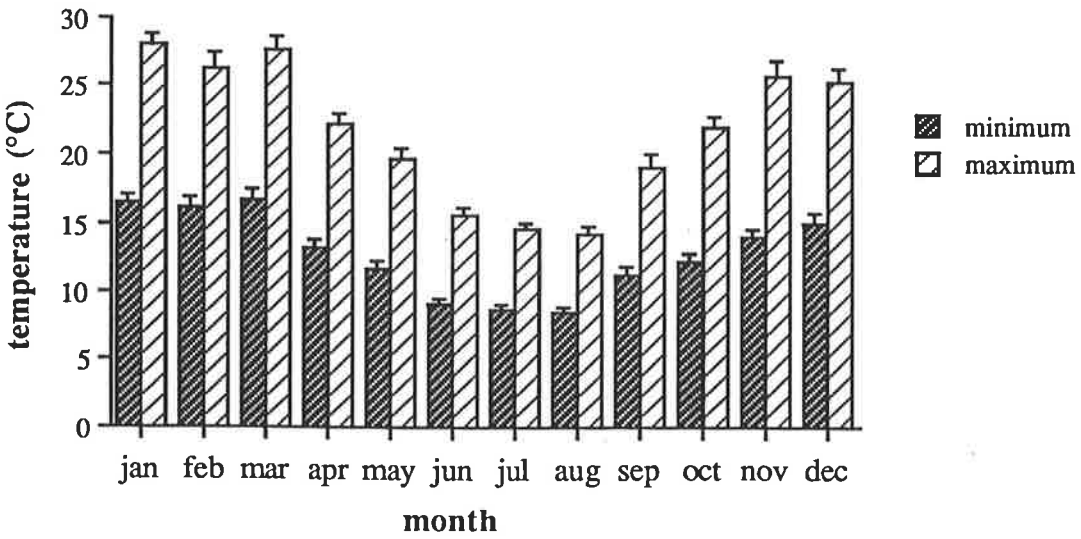
a) Heated glasshouse. Values are mean ± standard error.



b) Unheated glasshouse. Values are mean ± standard error.



c) Outside. Values are mean ± standard error.



a soil drench, 50ml per one litre pot. The commencement of growth regulator treatment coincided with the transfer from a heated glasshouse to the growth cabinet.

Day and night temperature

Plants were grown in growth cabinets with various day and night temperatures to observe any effect on flowering of the two species. Light quality and quantity in the growth cabinets was as for the 'photoperiod and temperature' experiment above. All temperature combinations exposed the plants to a mean temperature of 13°C over the 24 hours period, with twelve hours exposure to each of the temperatures stated in table 3.3.

Root and shoot temperature

This experiment aimed to determine whether the roots or aerial parts of the plants were the site for the detection of cool temperature. Plants were grown in either cool (17°C day / 9°C night) or warm (25°C day / 17°C night) growth cabinets with light conditions as in the 'commitment to flower' experiment above. The temperature of the roots were controlled in half the plants using thermostatically controlled water which was circulated through copper coils in close contact with the pots. The pots and copper coils were externally insulated. This arrangement proved effective for maintaining the roots of plants at a relatively constant temperature while not affecting the temperature of the aerial parts of the plants. The temperature of the soil in the pots was continuously monitored using thermocouples placed in the soil and attached to a chart recorder. The cooled roots of plants in the warm growth cabinet were maintained at 13.5°C day / 11°C night and the warmed roots in the cool growth cabinet at 23.5°C day / 20.5°C night. Plant height and percentage of leaf axils with flowers was recorded at anthesis.

Extension of natural flowering

An attempt was made to extend the natural season of flowering by moving plants outside from a heated glasshouse throughout the winter and early spring. At anthesis the date and the percentage of axils with flowers was recorded. Some *H. angustifolium* plants were treated with 10mg paclobutrazol per pot at the time of transfer.

Measurements

In all experiments four plants were used for each treatment. The percentage of axils with flowers were counted on a branch which represented between 20 - 40 % of the plant. The percentage of axils with flowers and plant height were measured at anthesis. Where appropriate, the results were compared using one way Analysis of Variance (ANOVA). For means that were significantly different (p = 0.05), LSD values were calculated using Fisher's Least Significant Difference (LSD) test (Sokal and Rohlf, 1981).

Results

Photoperiod and temperature

Under natural conditions, it is likely to be temperature and not photoperiod which

Table 3.1 - The effect of temperature and photoperiod on the flowering of *Boronia megastigma* and *Hypocalymma angustifolium*

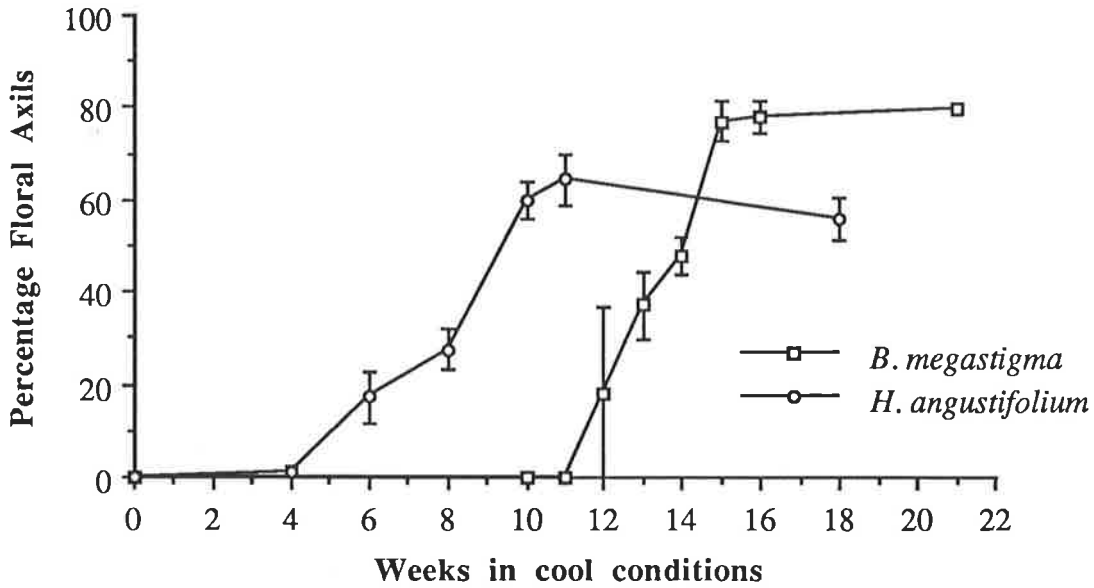
Values are means ± standard error. p = 0.05. [1] - daylight extension = 25µmol.m^{-2}.s^{-1}.

treatment		percent floral axils	
		B. megastigma	*H. angustifolium*
heated glasshouse		0	0
unheated glasshouse		70.53 ± 2.77	56.15 ± 8.17
outside		55.50 ± 4.94	48.75 ± 7.18
	LSD	*10.46*	*19.88*
Daylength (hours)	Day/Night temperature (°C)		
9	25 / 17	0	0
9	17 / 9	72.53 ± 2.67	54.80 ± 3.07
9 + 6[1]	25 / 17	0	0
9 + 6	17 / 9	76.59 ± 3.47	49.20 ± 3.88
	LSD	*6.94*	*7.24*

controls flowering in *B. megastigma* and *H. angustifolium*. No flowering occurred in warm conditions whether in a glasshouse with natural daylength variation, or in growth cabinets with controlled daylength (Table 3.1). Plants of both species grown in a heated glasshouse for two years have remained vegetative. Daylength had no influence on the number of flowers that developed in cool conditions. Less vegetative growth occurred in plants grown in the unheated glasshouse and in cool growth cabinets than in warm conditions. Even less vegetative growth occurred in those plants grown outside, resulting in a more compact habit. These plants also had fewer flowers and their flowering was slightly delayed in comparison to plants in the unheated glasshouse.

Figure 3.3 - Commitment of *B. megastigma* and *H angustifolium* flowers.

Plants were moved from cool (17°C day / 9°C night) to warm (25°C day / 17°C night) conditions after the number of weeks shown. Percent floral axils were recorded at anthesis. Anthesis occurred in cool conditions after eighteen weeks for *H. angustifolium* and twenty-one weeks for *B. megastigma*. Values shown are means ± standard error.



Commitment to flower

Figure 3.3 shows the number of weeks of cool temperature (17°C day / 9°C night) required before plants were committed to flower, that is before a maximum number of flowers continued to develop through to anthesis when the plants were transferred to a warm

environment (25°C day / 17°C night). The number of weeks required to obtain maximum flowering of *H. angustifolium* plants was shorter than that for *B. megastigma.* Some *H. angustifolium* buds required only four weeks of cold temperature to commit them to flower. If we consider the period between when no flowers were committed and maximum flowering, seven weeks was required for *H. angustifolium* (weeks 3-10) and four weeks for *B. megastigma* (weeks 11-15). *H. angustifolium* required eight weeks (weeks 10-18) in cool conditions to reach anthesis after commitment to flower whereas *B. megastigma* required six weeks (weeks 15-21).

Intermediate temperature

Flower initiation and development in *B. megastigma* was affected by an intermediate temperature regime (19°C day / 11°C night) in a different way than flowering of *H. angustifolium* (Table 3.2). This temperature proved to be cool enough to allow the production of flowers in both species. Flower initiation of *B. megastigma* was not affected by an intermediate temperature, however the rate of flower development was slow, flowers taking ten

Table 3.2 - Flowering of *B. megastigma* and *H. angustifolium* in an intermediate temperature (19°C day / 11°C night).

Values are mean ± standard error. * significantly different from control (p = 0.05).

	treatment	percent floral axils	time to anthesis (weeks)
B. megastigma	control	67.00 ± 2.77	31
	50mg.pot^{-1} paclobutrazol	73.75 ± 5.17	25
	50mg.l^{-1} benzyladenine	1.25 ± 1.25*	13
	LSD	*11.41*	
H. angustifolium	control	28.37 ± 13.95	22
	2.5mg.pot^{-1} paclobutrazol	50.94 ± 6.69	21
	10mg.pot^{-1} paclobutrazol	65.41 ± 4.63*	21
	50mg.pot^{-1} paclobutrazol	72.50 ± 5.41*	21
	LSD	*26.61*	

weeks longer to develop than in a more favourable temperature (compare with Figure 3.3). Paclobutrazol and benzyladenine increased the rate of flower development of *B. megastigma*, however benzyladenine decreased the percentage of axils with flowers to near zero and many vegetative shoots were produced. Initiation or early development of *H. angustifolium* flowers was affected by an intermediate temperature regime resulting in a decrease in the percentage of axils with flowers. In contrast to *B. megastigma*, paclobutrazol treatment of *H. angustifolium* plants results in an increase in number of flowers rather than a decrease in time to flowering.

Table 3.3 - The effect of different day/night temperatures on flowering of *B. megastigma* and *H. angustifolium*.

Values are means ± standard error. * significantly different from other values (p = 0.05).

	B. megastigma		***H. angustifolium***	
day/night temperature (°C)	% floral axils	time to anthesis (weeks)	% floral axils	time to anthesis (weeks)
21 / 5	0*	---	21.25* ± 8.26	18
17 / 9	70.5 ± 2.10	22	67.50 ± 3.23	17
13 / 13	69.00 ± 4.38	20	66.75 ± 5.12	17
9 / 17	76.25 ± 2.31	15	63.94 ± 2.32	17
LSD	*8.51*		*18.21*	

Day and night temperature

Variation in day/night temperature affected flowering in both *B. megastigma* and *H. angustifolium* (Table 3.3). *H. angustifolium* plants all flowered after the same number of weeks regardless of day/night temperature, however significantly fewer flowers formed on those plants grown in the warmest day coupled with the coolest night (21°C/5°C). In the three temperature regimes with the lower day and higher night temperatures, *B. megastigma* plants had a consistently high percentage of axils with flowers, however the cooler the daytime temperature (and higher the night temperature) the less time those flowers took to develop. *B. megastigma* plants in the highest daytime temperature had no flowers that reached anthesis although microscopic examination showed that floral differentiation had begun. Petals and

Table 3.4 - The effect of root and shoot temperature on flowering in *B. megastigma* and *H. angustifolium*.

Values are means ± standard error. p = 0.05.

[1] - Root and shoot temperature = 25°C day /17°C night.

[2] - Root temperature = 13.5°C day /11.0°C night, shoot temperature = 25°C day /17°C night.

[3] - Root temperature = 23.5°C day /20.5°C night, shoot temperature = 17°C day /9°C night.

[4] - Root and shoot temperature = 17°C day /9°C night.

root/shoot temperature	*B. megastigma*		*H. angustifolium*	
	% floral axils	plant height (cm)	% floral axils	plant height (cm)
warm/warm[1]	0	30.25 ± 1.11	0	42.48 ± 3.57
cool/warm[2]	0	19.75 ± 2.06	0	22.29 ± 1.80
warm/cool[3]	79.64 ± 0.72	28.00 ± 2.12	65.82 ± 4.80	42.10 ± 4.32
cool/cool[4]	77.45 ± 2.70	19.25 ± 0.85	63.91 ± 7.93	19.53 ± 2.33
LSD	*4.30*	*5.04*	*14.28*	*9.75*

stamens had been initiated in flower buds but no further development occurred after fifteen weeks at this temperature.

Root and shoot temperature

Temperature was perceived by the aerial parts of *B. megastigma* and *H. angustifolium* plants (Table 3.4). Only when the shoot temperature was low did flowers develop and root temperature had no effect on flowering. Plant height was retarded by cool root temperature regardless of the temperature of the top of the plant, which had no influence on vegetative growth. The decrease in height observed in both *B. megastigma* and *H. angustifolium* in response to cool root temperature was due to a decrease in both number and length of internodes. Thus flowering was controlled by the temperature of the top of the plant and was not affected by root temperature, whereas control over vegetative growth was exerted by root temperature.

Table 3.5 - Flowering of *B. megastigma* plants transferred outside from a heated glasshouse at intervals through autumn and winter.

Values are mean ± standard error. * significant decrease from plants transferred prior to 9 July (p = 0.05).

date moved	% floral axils	time of anthesis
19 March	61.75 ± 2.84	10 September
16 April	56.25 ± 5.91	10 September
14 May	55.50 ± 4.94	25 September
11 June	56.25 ± 6.58	3 October
25 June	55.42 ± 7.36	15 October
9 July	53.75 ± 3.15	25 October
23 July	33.75 ± 8.98*	25 October
6 August	0*	---
LSD	*13.61*	

Extension of natural flowering

Because a longer period of cold was required by *B. megastigma* than by *H. angustifolium* to commit plants to flower (Figure 3.3), less extension of the flowering season was gained by moving *B. megastigma* plants outside from a heated glasshouse throughout the winter and spring (Tables 3.5 & 3.6). Anthesis of the population of *B. megastigma* plants could be spread over a period between the 10th of September and the 25th of October ($6^1/_2$ weeks) whereas anthesis of *H. angustifolium* plants covered the period between the 20th of September to the 21st of November (9 weeks). Paclobutrazol treatment of *H. angustifolium* plants extended the period during which anthesis occurred by at least a further three weeks to the 11th of December. Towards the end of this extended period, the percentage of axils with flowers was significantly lower and the length of time flowers on a particular bush remained open was shorter for both *B. megastigma* and *H. angustifolium*.

Table 3.6 - Flowering of *H. angustifolium* plants transferred outside from a heated glasshouse at intervals through autumn, winter and early spring.

Paclobutrazol application occurred at the time of transfer. Values are mean ± standard error. * significant decrease from plants transferred prior to 11 June (p = 0.05).

	no paclobutrazol		10mg.pot^{-1} paclobutrazol	
date moved	% floral axils	time of anthesis	% floral axils	time of anthesis
19 March	55.36 ± 6.20	20 September	62.75 ± 4.57	20 September
16 April	52.70 ± 7.26	3 October	63.00 ± 7.29	3 October
14 May	53.68 ± 4.35	10 October	60.75 ± 5.45	10 October
11 June	50.11 ± 3.54	20 October	67.50 ± 7.71	25 October
25 June	48.75 ± 14.20	25 October	65.63 ± 2.04	25 October
9 July	35.00 ± 16.21	30 October	28.75 ± 12.97*	30 October
23 July	18.85 ± 17.12*	9 November	55.32 ± 8.66	9 November
6 August	12.00 ± 9.39*	21 November	36.25 ± 15.19*	21 November
20 August	0*	---	12.50 ± 12.50*	3 December
3 September	0*	---	20.50 ± 7.60*	11 December
LSD	*27.79*		*26.74*	

Discussion

The cabinet lit with predominantly sodium lamps provided a relatively narrow distribution of light intensity (Figure 3.1). The light spectrum provided by these lamps was not optimal for growth. After extended periods (greater than 20 weeks) in these cabinets it was possible to detect a yellowing of the leaves of *B. megastigma* and a general reduction in vegetative growth of both species grown in warm conditions. Plants in cool conditions showed no or less effect. In the cabinet with fluorescent lights and in the glasshouses the plants appeared healthier. These poorer growth conditions did not interfere with the temperature or photoperiod effect which was tested both in growth cabinets and in glasshouses (Table 3.1).

Roberts (1989a) showed that undifferentiated buds are found in the axils of leaves of *B. megastigma* plants and their production coincides with the cessation of leaf expansion, 2-3

nodes back from the vegetative growth tip. Flower buds were found in a similar position in the axils of *H. angustifolium* leaves upon transfer to cool conditions (Chapter 2). In *B. megastigma*, undifferentiated buds either form flowers or vegetative shoots or remain dormant depending on the environment of the plant. In warm conditions (25°C day / 17°C night), *B. megastigma* and *H. angustifolium* did not naturally form flowers (Table 3.1). Flowering in both species occurred in cool conditions (17°C day / 9°C night) irrespective of photoperiod (Table 3.1).

For *B. megastigma*, it has been suggested that both low night temperature and short days are conditions that promote flowering (Roberts and Menary, 1989b). However, these authors provided daylight extension to plants at 150μmol.m^{-2}.s^{-1}, presumably well above the light compensation point for this species. Providing light of this intensity would allow a significant increase in the amount of photosynthetic assimilation occurring in long-day plants relative to short-day plants. A difference in the rate of assimilation would interfere with the detection of a true photoperiodic response. Lewis and Warrington (pers. comm., 1989) found photoperiod to have no effect on flowering in *B. megastigma* 'Lutea', a result which corresponds to the results presented in table 3.1.

To obtain a maximum percentage of flowering axils, *H. angustifolium* required 10 weeks of 17°C day / 9°C night temperatures and *B. megastigma* required 15 weeks (Figure 3.3). This number of weeks corresponded to the number of weeks required for flowers to reached stage five of flower development (Chapter 2). Plants transferred to warm conditions before the required number of weeks showed either no or limited flowering. Because *H. angustifolium* requires fewer weeks of cool temperatures to commit plants to flower than *B. megastigma*, flowering can be forced to occur later in the season (Tables 3.5 & 3.6). Many species of plant require a number of weeks of appropriate environmental conditions to fully evoke flowering. *Chamelaucium uncinatum* required four weeks of short days (Shillo *et al.*, 1985) and *Leptospermum scoparium* required short days for eight weeks (Zieslin, 1985). Olives required ten weeks of cold (Badr and Hartmann, 1971) and Lewis and Warrington (pers. comm., 1989) found that twelve weeks were required to commit *B. megastigma* 'Lutea' plants to flower.

H. angustifolium plants returned to warm conditions from cool temperatures before they were committed to flower halted floral development. After a number of weeks in warm temperatures, flowers lost the ability to continue floral development when returned to flower inductive conditions. *B. megastigma* plants transferred to warm conditions showed signs of floral reversion. Floral buds that were not committed to flower halted development or developed leaf-like sepals and eventually abscised. Vegetative growth commonly commenced from the axils of floral bracts (Figure 19, Chapter 2). Similar observations were made by Roberts (1989a). It is common in perennial temperate plants for exposure to non-inductive conditions to result in the reversion from floral to vegetative growth (Battey and Lyndon, 1990; Jackson and Sweet, 1972).

The transition from no flowering to maximum flowering in *B. megastigma* plants occurred over four weeks, between the 11th and 15th weeks of low temperature. *H. angustifolium* required seven weeks to make this transition (Figure 3.3). This suggests that individual flowers on *B. megastigma* plants developed at much the same rate and reached the state of commitment to flower at much the same time. Evocation and early development of *H. angustifolium* flowers was more uneven, with flowers on different parts of the plant being initiated and developing earlier or faster than others. This corresponds with the observation that in an environment marginally favourable for flower induction (Tables 3.2 & 3.3), *H. angustifolium* had the ability to flower on some stems and not others. It is not individual flowers that develop at different rates, but flower groups on adjacent axils that develop at different rates to other groups of flowers on different stems. Thus *H. angustifolium* flower evocation and development relies on changes specific to individual stems of the plant as well as changes that occur throughout the plant.

Data in table 3.2 suggest that 19°C day / 11°C night is a marginal environment for flowering of *B. megastigma* and *H. angustifolium* . Other researchers have investigated the intermediate or critical temperature, the highest temperature that still allows flowering to occur in plants that require cold temperature (Badr and Hartmann, 1971; Roelofse and Hand, 1990). A constant temperature of 12.5°C for olive (Badr and Hartmann, 1971) and 14°C for celery (Roelofse and Hand, 1990) was found to be critical for flowering of these species. These constant temperatures are closer to the cool temperatures used in the experiments (13°C, Table

3.3), suggesting that the temperature required to inhibit flowering in *B. megastigma* and *H. angustifolium* is higher than that for olive and celery. A temperature regime of twelve hours each of 19°C day / 11°C night equates to a mean temperature of 15°C over 24 hours, however as we see in table 3.3 a mean temperature may not adequately describe the optimal temperature for flowering.

Flowering of *H. angustifolium* in a marginally inductive environment was enhanced if plants were treated with paclobutrazol (Table 3.2), suggesting that endogenous gibberellins play some role in the evocation and early development of flowers. The enhancement of flowering resulting from application of paclobutrazol to plants in a marginal environment is most obvious in the extension of flowering season observed in table 3.6. In contrast to this, the evocation of *B. megastigma* flowers in a marginal environment was not affected by treatment with paclobutrazol but paclobutrazol increased the rate of development of flowers (Table 3.2). For this species endogenous gibberellins may be involved with the development of flowers rather than with evocation. Benzyladenine treatment of *B. megastigma* plants in intermediate temperatures greatly inhibited flowering and the axillary buds developed into vegetative shoots rather than floral buds. More vegetative growth occurred on plants of both species in this marginal temperature than in cooler temperatures. Plants grown outside late in the flowering season (Tables 3.5 & 3.6) also had a greater proportion of stems with actively growing tips than earlier in the flowering season and the amount of flowering that occurred was reduced. Paclobutrazol treatment of plants in these marginal environments decreased the vigour of many of the shoot tips and flowering was enhanced.

Plants would experience a range of conditions throughout the season when they are transferred outside from a heated glasshouse. Plants transferred in March required longer to reach anthesis than plants transferred later in the year (Tables 3.5 & 3.6). There are two reasons for this. In March, plants did not begin floral initiation or differentiation immediately probably because the temperature was still too warm (mean maximum 27.7°C / minimum 16.6°C, figure 3.2c). In addition, after commitment to flower the rate of development of flowers was dependent on temperature (Table 4.7), thus plants reaching this stage of flower development earlier in the season when the temperature was still cool would develop slower than those flowers that reach commitment to flower in warmer weather. Therefore, *B.*

megastigma plants took 21 weeks to reach anthesis if transferred outside on the 16th of April and only $13^1/_2$ weeks if transferred on the 23rd of July. *H. angustifolium* plants reached anthesis after 24 weeks if transferred on the 16th of April and after 15 weeks if transferred on the 6th of August. *B. megastigma* plants grown outside in Adelaide reached stage five of flower development (commitment to flower, Chapter 2) around the beginning of August, and *H. angustifolium* plants were committed to flower during the last week of July.

Table 3.5 shows that *B. megastigma* plants did not begin flower differentiation until after the 16th April, those plants transferred to the open on the 19th March reached anthesis on the same date as those transferred on the 16th April. This is not true for *H. angustifolium* plants (Table 3.6), suggesting that the threshold above which no floral differentiation will occur is higher for *H. angustifolium* than for *B. megastigma*. Further support for this hypothesis is presented in table 3.3 where, in a temperature regime of 21°C day / 5°C night, 21% of total leaf axil of *H. angustifolium* plants had flowers whereas no flowers developed on *B. megastigma* plants.

Roberts (1989b) suggested cold night temperature was required for flowering of *B. megastigma*. Moss (1976) also found this to be so with *Citrus sinensis*. In olives an inhibition of flowering was caused by high day temperature (Badr and Hartmann, 1971). These authors grew olive plants in 18°C day / 7°C night and at a constant temperature of 12.5°C and only observed flowering in the second treatment. Even plants grown in 16°C day / 1.5°C night did not flower, suggesting that a mean temperature was not adequate to describe conditions necessary for flowering of olive. Both *B. megastigma* and *H. angustifolium* respond in the same way as olive (Table 3.3). A mean temperature does not adequately describe the optimal conditions for flowering because, even with the same mean temperature, flowering was inhibited in plants grown in a high day temperature. This suggests that it may be day temperature rather than night temperature that controls flowering in *B. megastigma* and *H. angustifolium*. However it is also possible that 21°C, during the day or night, is above the maximum temperature threshold for these plants, and above this threshold only limited flowering will occur.

A large difference in day and night temperature caused stem elongation similar to that seen when plants were treated with Gibberellic Acid (GA_3) in *Campanula isophylla* (Moe,

1990). As the temperature difference decreased, stems elongated less. Finally, when night temperature was greater than day temperature, stem elongation was retarded in a similar way to plants treated with a growth retardant. In *B. megastigma* and *H. angustifolium*, a similar situation may exist since GA_3 treatment inhibited flowering (Chapter 4) as did a temperature regime of 21°C day / 5°C night (Table 3.3), and paclobutrazol increased the rate of flowering of *B. megastigma* (Table 3.2 & Chapter 4) as did a day/night temperature of 9°C/17°C.

If the day/night diurnal change in temperature was gradual rather than dramatic, then the critical temperature for flowering of olive trees was higher (Badr and Hartmann, 1971). Comparing figure 3.2 and table 3.1 shows that *B. megastigma* and *H.angustifolium* plants flowered at a higher average temperature in the unheated glasshouse than plants in cool growth cabinets. The mean maximum and minimum temperatures in the unheated glasshouse over the period flowers developed (May to September) was 24.34°C ± 0.53 / 12.21°C ± 0.46, much greater than 17°C / 9°C, yet percent of axils with flowers remained high and time to anthesis was not affected. Possibly the gradual diurnal changes in temperature occurring in the unheated glasshouse allowed this to occur.

The detection of photoperiod occurs in the leaves of plants through the phytochrome system (Smith and Whitelam, 1990). There is no similar system known for the detection of cold. Cold temperature causes many changes within plants including an increase in the cytokinin concentration (Hansen *et al.*, 1988), a decrease in the gibberellin concentration (Pinthus *et al.*, 1989), an accumulation and redistribution of sugars (Paul *et al.*, 1990; Wolf *et al.*, 1990) and a reduction of vegetative growth (Roelofse and Hand, 1990) amongst others. In Satsuma mandarin (*Citrus unshiu*) cool root temperature was found to increase starch in leaves and decrease the nitrogen content of the plant (Poerwanto and Inoue, 1990b). Low air temperature caused a reduction in the measured gibberellin-like activity in Satsuma mandarin and allowed floral development to proceed. This coincides with the results presented in table 3.4 and with those in *Citrus sinensis* (Moss, 1976), where flowering was controlled by the temperature of the aerial parts of the plant. In *B. megastigma* and *H. angustifolium* root temperature had no effect on flowering, however low root temperature decreased the amount of vegetative growth that occurred. Poerwanto (1990a) suggested that a similar result observed in Satsuma mandarin was due to a reduction in root growth which resulted in a subsequent

reduction in cytokinin transport from the root to the shoot finally resulting in a decrease in vegetative growth.

A reduction in vegetative growth coincides with the production of flowers in many woody plants (Jackson and Sweet, 1972; Richards, 1985; Roberts and Menary, 1989b). As we have discussed above, a reduction in vegetative growth resulting from treatment with a growth retardant (Table 3.2) or a higher night temperature than day temperature (Table 3.3) was associated with an enhancement of flowering. These treatments probably allowed the redistribution of nutrients and carbohydrates from vegetative shoots to floral buds (Weaver and Johnson, 1985). However, table 3.4 shows that a reduction in vegetative growth is not necessary for flowering to occur in *B. megastigma* and *H. angustifolium*, and flowering and vegetative growth were controlled by distinct parts of the plant. Thus in these plants, a reduction in vegetative growth may enhance flowering but is not part of the mechanism controlling flower induction.

B. megastigma and *H. angustifolium* are from different families, but have a similar mechanism for the control of flowering. Their responses to photoperiod, changes in day/night temperature and root/shoot temperature are similar. This similarity may be related to the 'Mediterranean' climate in which these species naturally grow and have evolved. The control of flowering of olive, which also grows naturally in a Mediterranean climate, shows many similarities with flowering in *B. megastigma* and *H. angustifolium* (Badr and Hartmann, 1971). Conversely, *Chamelaucium uncinatum* (Shillo *et al.*, 1985) and *Leptospermum scoparium* (Zieslin, 1985) require short days for floral initiation and warm or mild temperatures for floral development. Cold temperature either prevents or decreases the amount of flowering. These two species grow naturally in the same temperate climate as *B. megastigma* and *H. angustifolium* and are from the same family as *H. angustifolium*. Thus closely related species that grow in the same natural environment can require different environmental cues for the evocation and development of flowers.

CHAPTER FOUR

CONTROL OF FLOWERING OF *BORONIA MEGASTIGMA* AND *HYPOCALYMMA ANGUSTIFOLIUM* USING PLANT GROWTH REGULATORS

Introduction

In many woody plants, flowering requires the cessation of apical vegetative growth (Jackson and Sweet, 1972). Since both *B. megastigma* and *H. angustifolium* flower from leaf axils the control of flowering in these species probably depends of the balance between apical and axillary development. Tucker (1977) suggests that lateral or axillary growth is primarily controlled by indole-acetic acid (IAA), cytokinins and abscisic acid. High IAA levels encourage apical dominance and abscisic acid prevents lateral bud outgrowth. Cytokinins overcome both the IAA mediated apical dominance and the abscisic acid inhibition of lateral bud outgrowth and allow axillary growth to commence. Consequently axillary development may be encouraged by treating plants with auxin transport inhibitors or cytokinins. Keever and Foster (1990) show that benzyladenine, a synthetic cytokinin, can encourage lateral bud break in a range of ornamental plant species.

Apical vegetative growth is encouraged by high endogenous gibberellin levels, and treatment with gibberellins inhibits flowering in most woody plants (Jackson and Sweet, 1972). Different types of gibberellins may affect growth and flowering of plants in different ways (Metzger, 1988; Pharis and King, 1985). Pharis *et al.* (1987), working with *Lolium*, suggests that the more polar gibberellins (eg: GA_1 & GA_3) tend to encourage vegetative growth whereas the less polar gibberellins (eg: GA_4 & GA_7) encourage flowering. The plant growth retardant paclobutrazol inhibits gibberellin and sterol synthesis (Grossmann, 1990), and treatment of plants with paclobutrazol results in a reduction in internode length and an

enhancement of flowering in many woody plants (Lamont, 1986; Wilkinson and Richards, 1991).

Plant growth regulators are used widely in the agronomic and horticultural industries (Lever, 1982). In the horticultural industry, growth retardants are used to dwarf pot-plants (Menhenett, 1982) and may also enhance flowering (Goodwin, 1985). Flowering is induced commercially by treating bromeliads with ethylene, and gibberellic acid is used to induce flowering in a number of plants including *Spathiphyllum* (Goodwin, 1985). The effects of a number of plant growth regulators on vegetative and floral development of *B. megastigma* and *H. angustifolium* have been tested.

Methods

Plant material and growth conditions

All plants were propagated and grown in a heated glasshouse as described in chapter 2. Plants uniform in height and breadth were chosen for experiments between five and six months after potting-on from cuttings. Four plants were used for each treatment.

Growth regulators

Gibberellic acid (GA_3), benzyladenine (BA), GA_{4+7} + BA and the wetting agent used for all sprays were donated by Abbots Laboratories, Chicago and paclobutrazol was a gift from ICI, Australia. Morphactin was purchased from Merck and TIBA from Fluka. All treatments other than paclobutrazol and morphactin were sprayed to run off (approximately 25 ml per plant). Those plants sprayed more than once were sprayed weekly until the required number of sprays was reached. Paclobutrazol and morphactin were used as a soil drench, 50 ml per one litre pot.

For each growth regulator, groups of plants were treated with a range of concentrations covering two orders of magnitude. Due to difficulties in presentation only the results of a few of these concentrations are presented.

Warm conditions

Warm conditions were provided by a heated glasshouse under natural light, temperature conditions shown in figure 3.2a. All measurement of plants in warm conditions was carried out after three months except for the percentage of axils with flowers which was measured at anthesis.

Cool conditions

Cool conditions were provided by growth cabinets with a day/night temperature of 17°C/9°C, light conditions as for sodium lamp lit growth cabinets (Figure 3.1a). All measurements were conducted at anthesis except the % vegetative axils which was measured after three months (before anthesis in all cases). Flower diameter and petal length was measured with vernier callipers. Ten flowers per plant were selected for this measurement, thus values represent the mean of forty flowers per treatment.

All plants received water daily. Plant with a high nutrient status were equivalent to control plants except they were watered with a solution purchased from Top Australia Ltd. which was prepared such that the nutrient concentration was similar to half strength Hoaglands solution.

The '5cm cutting' was a cutting with roots and 5cm of new vegetative growth. Young plants had this form approximately one month after being removed from propagation conditions.

Plants were girdled by removing a three millimetre strip of bark from the stem approximately three centimetres above soil level. The phloem did not reconnect during the experiment. The stem bulged above the girdle.

Treatment of plants two months prior to transfer to cool conditions

In this experiment, plants were treated in warm conditions and remained in this environment for two months before being transferred to cool conditions. Prior to transfer to cool conditions, the starch concentration from a section of stem with two nodes and attached leaves was measured. Starch from two sections from each plant was extracted and measured as

described for benzyladenine treated plants in chapter 5. Other measurements were made at anthesis.

Treatment of plants after commitment to flower

H. angustifolium and *B. megastigma* plants used for this experiment grew in cool conditions (17°C day / 9°C night) for eleven weeks and fifteen weeks respectively. After this time four plants of each species were transferred to warm conditions (25°C day / 17°C night). All other plants remained in cool conditions. Ten flowers per plants were tagged and used to measure the days to anthesis and the number of days that flowers stayed open. The flower and flower stalk parameters of these ten tagged flowers per plant were measured with vernier callipers, thus all flower measurements represents the mean of forty replicates per treatment.

Measurement

At the beginning of the experiments, nodes approximately one centimetre basal to the apical shoot were tagged on two lateral shoots per plant. The internode length was calculated from the growth distal to the tagged nodes. Percent vegetative axils represents the percentage of total axils from which vegetative shoots were growing and thus is a measure of the number of lateral vegetative shoots. Percent floral axils and percent vegetative axils were calculated using a tagged representative branch of between 20 - 40% of the plant. Where flowering occurred, the percent floral axils were calculated from the number of the total axils with flowers at anthesis.

The data was initially compared using Analysis of Variance (ANOVA). Where differences were significant ($p = 0.05$), LSD values were calculated using Fisher's Least Significant Difference test (Sokal and Rohlf, 1981). In the tables, treatments that significantly differ from the control are marked with an asterisk.

Results

Undifferentiated buds can be found in most leaf axils of *B. megastigma* plants at all times of year. In warm conditions these buds remained undifferentiated and dormant. Treatments in warm conditions that initiated floral differentiation in *B. megastigma*, include benzyladenine, paclobutrazol, morphactin, TIBA, cyanamide, chloromequat chloride, ethrel and tip-pruning. Microscopic examination of the developing flowers from the above treatments showed that development reached the stage where petals and stamens had been initiated. When this stage was reached development stopped and either the developing flowers abscised or vegetative shoots developed in the axils of the bracts. Continuation of the treatment in warm conditions, for example soil drench with paclobutrazol or eight weeks of benzyladenine application at low concentration, did not advance floral development past this early stage.

Table 4.1 - Effect of various treatments on growth and flowering of *H. angustifolium* in warm conditions (25°C day / 17°C night).

Values are mean ± standard error. % vegetative and % floral axils represent the number of total axils with vegetative shoots and flowers respectively. [1] - Second tip-pruning occurred one month after the first prune. * - significantly different from control (p = 0.05).

	internode length (mm)	plant height (cm)	% vegetative axils	% floral axils
control	11.21± 0.64	42.25 ± 2.53	5.80 ± 0.77	0
twice tip-pruned[1]	10.80 ± 1.39	30.25* ± 2.02	12.78* ± 1.47	0
2 X 200 mg.l^{-1} BA	8.34* ± 0.81	33.75 ± 2.29	12.81* ± 1.36	0
2.5 mg.pot^{-1} Morphactin	3.65* ± 0.34	18.67* ± 1.20	91.07* ± 4.93	8.13* ± 5.32
3 X 600 mg.l^{-1} TIBA	8.59* ± 0.87	34.00 ± 5.29	65.69* ± 7.27	0
LSD	*2.58*	*9.95*	*6.72*	*4.67*

Warm conditions

Tables 4.1, 4.2 and 4.3 report attempts to induce flowering in *B. megastigma* and *H. angustifolium* in warm conditions. Morphactin and TIBA are auxin transport inhibitors and

thus may be expected to overcome auxin control of apical dominance. This occurred with *H. angustifolium* plants (Table 4.1) and *B. megastigma* plants (Table 4.3). These growth regulators decreased internode length and plant height, and encourage lateral development. Growth from leaf axils was mostly vegetative rather than floral, only *H. angustifolium* plants in warm conditions treated with morphactin flower to some extent (Table 4.1).

Table 4.2 - Effect of paclobutrazol on growth and flowering of *H. angustifolium* in warm conditions (25°C day / 17°C night).

Values are mean ± standard error. * - significantly different from control (p = 0.05). N.S. - not significantly different.

paclobutrazol treatment.pot^{-1}	internode length (mm)	plant height (cm)	% vegetative axils	% floral axils	time to anthesis
0	9.87 ± 0.66	42.50 ± 3.57	7.57 ± 2.04	0	---
1 mg	8.05* ± 0.53	37.5 ± 1.32	6.27 ± 0.94	0.36 ± 0.36	22 weeks
2.5 mg	4.60* ± 0.35	37.67 ± 5.24	5.87 ± 0.98	12.25 ± 12.25	24 weeks
7.5 mg	3.67* ± 0.51	31.33* ± 2.60	7.86 ± 1.58	32.27* ± 9.11	23 weeks
20 mg	3.48* ± 0.27	30.00* ± 0.58	9.26 ± 2.39	49.03* ± 7.86	23 weeks
50 mg	2.97* ± 0.08	29.00* ± 2.08	8.68 ± 1.67	41.59* ± 3.46	22 weeks
LSD	*1.43*	*8.84*	*N.S.*	*21.24*	---

Benzyladenine (BA) treatment in warm conditions encouraged vegetative lateral development in *B. megastigma* (Table 4.3) and in *H. angustifolium* (Table 4.1). No flowering resulted from benzyladenine treatment in warm conditions. Tip-pruning had a similar effect to benzyladenine treatment on *H. angustifolium* plants, an increase in vegetative shoots but no effect on flowering. Tip-pruning of *B. megastigma* in warm conditions had no effect on flowering or vegetative growth (Table 4.3) possibly because growth parameters were measured three months after treatment thus allowing time for regrowth after the removal of tips.

In warm conditions, treatment of *B. megastigma* plants with both GA_3 and GA_{4+7} + BA resulted in an increase in internode length. GA_3 also resulted in an increase in plant height whereas GA_{4+7} + BA increased the number of lateral shoots that developed. Paclobutrazol

treatment of *B. megastigma* plants resulted in a reduction of internode length and plant height but had no effect on flowering of this species in warm conditions (Table 4.3). *H. angustifolium* plants in warm conditions were induced to flower by paclobutrazol treatment (Table 4.2). Even at low rates of application some flowering occurred but rates greater than 7.5 $mg.pot^{-1}$ were required for maximum flowering. In addition, paclobutrazol treatment reduced internode length and plant height in both species.

Table 4.3 - Effect of various treatments on the growth and flowering of *B. megastigma* in warm conditions (25°C day / 17°C night).

Values are mean ± standard error. [1] - $100mg.l^{-1}$ of each plant growth regulator. * - significantly different from control (p = 0.05).

	internode length (mm)	plant height (cm)	% vegetative axils	% floral axils
control	9.74 ± 0.59	30.25 ± 1.11	20.73 ± 1.74	0
tip-pruned	10.59 ± 0.27	30.25 ± 0.75	23.33 ± 3.37	0
4 X 50 $mg.l^{-1}$ BA	9.21 ± 0.79	28.33 ± 0.67	65.38* ± 6.71	0
3 X GA_{4+7} + BA[1]	15.60* ± 0.62	29.75 ± 0.25	86.88* ± 3.01	0
3 X 100 $mg.l^{-1}$ GA_3	12.63* ± 0.61	35.53* ± 0.96	12.28 ± 0.50	0
50 $mg.pot^{-1}$ paclobutrazol	5.44* ± 0.31	23.50* ± 0.29	32.56 ± 6.74	0
1.5 $mg.pot^{-1}$ morphactin	3.75* ± 0.63	24.00* ± 1.53	64.40* ± 3.63	0
LSD	*1.85*	*2.86*	*14.82*	---

Cool conditions

In cool conditions, paclobutrazol treatment of *H. angustifolium* plants had no significant effect on flowering (Table 4.4), however gibberellic acid totally inhibited flowering, suggesting that gibberellins may play a role in flowering of *H. angustifolium*. Rooted cuttings with 5cm vegetative growth, benzyladenine treated plants, plants with a high nutrient status and tip-pruned plants all had vigorous vegetative growth and a reduced percent floral axils in cool conditions. In these treatments, flowers only developed on the older stems of the plant. Those

flowers that did develop on benzyladenine treated *H. angustifolium* plants were larger than those on control plants. No other treatments significantly changed the size of flowers of *H. angustifolium* or *B. megastigma* plants when treatment coincided with transfer to cool conditions (Tables 4.4 & 4.5).

Table 4.4 - Effect of various treatments on flowering of *H. angustifolium* in cool conditions (17°C day / 9°C night).

Values are mean ± standard error. * - significantly different from control (p = 0.05).

	% vegetative axils	% floral axils	time to anthesis	flower diameter (mm)
control	6.72 ± 0.40	55.10 ± 4.62	17 weeks	7.88 ± 0.17
tip-pruned	13.42* ± 1.47	27.77* ± 6.69	16 weeks	8.00 ± 0.13
2 X 200mg.l^{-1} BA	26.14* ± 2.67	1.5* ± 1.5	13 weeks	8.49* ± 0.19
3 X 100mg.l^{-1} GA_3	2.77* ± 0.45	0*	---	---
5mg.pot^{-1} paclobutrazol	6.60 ± 0.57	66.48 ± 4.00	17 weeks	8.09 ± 0.12
high nutrient status	4.54 ± 0.78	16.25* ± 9.87	18 weeks	8.07 ± 0.14
5cm cutting	5.81 ± 0.63	0*	---	---
LSD	*3.44*	*14.99*		*0.42*

Benzyladenine treatment of *B. megastigma* plants concurrent with commencement of cool conditions had no effect on the number of flowers but greatly increased the rate of flower development (Table 4.5). Girdling *B. megastigma* plants and placing them in cool conditions had no effect on flower number but the rate of floral development was slower than in the control. *H. angustifolium* and *B. megastigma* rooted cuttings with 5cm vegetative growth differed in their flowering response. Whereas *H. angustifolium* rooted cuttings did not flower (Table 4.4), flowering of similar *B. megastigma* cuttings was comparable to that of older plants (Table 4.5).

Gibberellic Acid and GA_{4+7} + BA treatment of *B. megastigma* plants reduced flowering as did tip-pruning and treatment with the auxin transport inhibitors TIBA and morphactin (Table

4.5). Plants with a high nutrient status also flowered less than control plants. Paclobutrazol increased the percent of axils with flowers (Tables 4.5), however high rates of application were required. These rates of application also inhibited vegetative growth. Rates of application similar to those that affected flowering in *H. angustifolium* had no effect on growth and flowering of *B. megastigma* (results not shown).

Table 4.5 - Effect of various treatments on flowering of *B. megastigma* in cool conditions (17°C day / 9°C night).

Values are mean ± standard error. [1] - 100mg.l^{-1} of each plant growth regulator. * - significantly different from control (p = 0.05). N.S. - not significantly different from control.

	% vegetative axils	% floral axils	time to anthesis	petal length (mm)
control	19.39 ± 2.95	63.58 ± 1.62	22 weeks	6.48 ± .061
tip-pruned	25.61 ± 1.86	35.00* ± 8.66	18 weeks	6.64 ± .086
girdled	17.60 ± 2.19	58.75 ± 2.39	27 weeks	
3 X 50 mg.l^{-1} BA	33.67* ± 4.63	63.40 ± 2.00	14 weeks	6.67 ± .087
2 X GA_{4+7} + BA[1]	46.55* ± 7.21	14.72* ± 9.72	13 weeks	
2 X 100 mg.l^{-1} GA_3	8.21 ± 2.48	25.09* ± 7.31	25 weeks	6.21 ± .054
50 mg.pot^{-1} paclobutrazol	19.33 ± 1.45	78.33* ± 1.31	20 weeks	6.34 ± .140
0.75 mg.pot^{-1} morphactin	64.12* ± 10.23	0*	---	
200 mg.l^{-1} TIBA	29.12 ± 1.99	47.71* ± 6.47	22 weeks	
high nutrient status	17.78 ± 2.29	51.45* ± 4.23	24 weeks	
5cm cutting	31.00 ± 9.63	56.56 ± 1.83	25 weeks	
LSD	*13.28*	*11.33*		*N.S.*

Treatment of plants two months prior to transfer to cool conditions

Two months after treatment of *B. megastigma* plants in warm conditions with benzyladenine, GA_{4+7} + BA or GA_3, a decrease was measured in their endogenous starch concentration (Table 4.6). When these plants were subsequently transferred to cool conditions less flowers developed than on control plants. Treatments such as paclobutrazol and tip-

pruning of *B. megastigma* plants, which did not result in an increase in plants height or an increase in the number of vegetative shoots in warm conditions (Table 4.3), did not decrease the endogenous starch concentration and did not decrease flowering when plants were transferred to cool conditions (Table 4.6).

Table 4.6 - Effect of various treatments on starch concentration and flowering of *B. megastigma* plants treated in warm conditions (25°C day / 17°C night) two months prior to transfer to cool conditions (17°C day / 9°C night).

Starch concentration was measured at the time of transfer. Values are mean ± standard error. [1] - 100 $mg.l^{-1}$ of each plant growth regulator. * - significantly different from control (p = 0.05).

	starch concentration (mg starch.g^{-1} DW)	% floral axils	time to anthesis
control	55.54 ± 2.07	60.51 ± 7.24	20 weeks
tip-pruned	52.18 ± 3.73	66.41 ± 4.11	21 weeks
3 X 50 $mg.l^{-1}$ BA	27.64* ± 2.69	42.76* ± 7.46	22 weeks
3 X GA_{4+7} + BA[1]	19.36* ± 4.03	0*	---
3 X 100 $mg.l^{-1}$ GA_3	31.80* ± 2.63	21.81* ± 5.15	23 weeks
50 $mg.pot^{-1}$ paclobutrazol	60.57 ± 1.96	89.02* ± 2.39	17 weeks
LSD	*11.35*	*15.33*	

Treatment of plants after commitment to flower

After plants were committed to flower the control of development changed dramatically from that before commitment. The rate of development of both *B. megastigma* and *H. angustifolium* flowers was greatly increased by a warm environment (Table 4.7), whereas before commitment to flower warm temperature inhibited or halted flower development. Warm temperature also significantly decreased the size of the flowers and the number of days the flowers remained open but increased the number of vegetative shoots present at anthesis. Gibberellic Acid and benzyladenine treatment of *B. megastigma* plants committed to flower had little effect on the rate of flower development or days that flowers remained open, however both

Table 4.7 - Flowering of *B. megastigma* and *H. angustifolium* when treated after plants are committed to flower.

B. megastigma plants were treated after 15 weeks in cool conditions (17°C day / 9°C night) and *H. angustifolium* plants were treated after 11 weeks in these conditions. Control plants remained in cool conditions. 'Warm' plants were transferred to 25°C day / 17°C night. All growth regulator treatments in cool conditions. Values are mean ± standard error. * - significantly different from control (p = 0.05). N.S. - not significantly different.

				length (mm)			
	% floral axils	days to anthesis	days flowers open	below bracts	above bracts	petal	% vegetative axils at anthesis
B. megastigma							
control	74.39 ± 2.62	44.0 ± 2.0	43.0 ± 1.0	4.05 ± 0.22	5.06 ± 0.26	6.56 ± 0.14	15.18 ± 2.74
warm	70.60 ± 3.71	17.3* ± 3.3	17.3* ± 2.03	3.96 ± 0.13	4.88 ± 0.16	6.23* ± 0.05	40.31* ± 3.09
3 X 100 GA_3	72.34 ± 4.11	38.0 ± 0.0	44.0 ± 1.0	7.29* ± 0.53	8.33* ± 0.42	7.17* ± 0.11	21.83 ± 3.37
3 X 50 BA	75.76 ± 2.97	42.0 ± 2.0	43.3 ± 1.3	4.35 ± 0.18	5.35 ± 0.24	7.01* ± 0.11	22.07 ± 1.51
LSD	*N.S.*	*7.13*	*4.58*	*0.97*	*0.88*	*0.31*	*9.03*
H. angustifolium				**flower diameter (mm)**			
control	62.14 ± 4.82	64.2 ± 3.5	41.9 ± 2.7	8.11 ± 0.09			4.64 ± 1.88
warm	57.93 ± 7.05	27.2* ± 2.3	18.9* ± 1.8	7.74* ± 0.17			46.23* ± 8.77
LSD	*N.S.*	*8.89*	*5.97*	*0.35*			*16.34*

treatments resulted in larger flowers and GA_3 increased the length of the flower stalk both above and below the bracts.

Summary of results

B. megastigma and *H. angustifolium*.plants had more axillary vegetative growth than control plants when treated in warm conditions with auxin transport inhibitors or benzyladenine. However, the species differed in their flowering response when treated with various other plant growth regulators. *H. angustifolium*.plants did not flower when treated with gibberellic acid in cool conditions, and paclobutrazol treatment was able to substitute to some extent for the cold required for flowering in this species. *B. megastigma* plants responded less than *H. angustifolium*.to treatment with gibberellic acid but the rate of floral development was increased when plants were treated with benzyladenine. Many treatments, such as GA_3, GA_{4+7} + BA, high nutrient status and tip-pruning, that encouraged vegetative growth in cool conditions resulted in a reduction in flowering in both species. After *B. megastigma* plants were committed to flower, the rate of flower development was enhanced by warm conditions whereas the size of flowers was increased by treatment with benzyladenine and GA_3.

Discussion

Many authors have questioned the use of results from exogenous applications of plant growth regulators to discuss changes in plant development (Bernier *et al.*, 1981b; Medford *et al.*, 1989). Endogenous plant growth regulators are compartmentalized within cells and organs and subtle, continuous changes in concentration of endogenous plant growth regulators are probably all that is required for plant development changes (Klee and Estelle, 1991). Exogenously applied plant growth regulators are usually applied at high rates and at discrete times, and are unlikely to mimic the compartmentalisation of their endogenous counterparts. The changes in plant development that result from the application of exogenous plant growth

regulators will not exactly replicate the changes resulting from variation in endogenous plant growth regulators but the physiology of the plant is affected and thus results such as those presented here are worth discussing. This is especially true for plants used for horticultural purposes since growth regulator treatments are an accepted part of horticultural practice (Lever, 1982).

Control of apical dominance is important for flowering in many woody plants especially when flowers develop from the leaf axils. These plants generally require a reduction in apical vegetative growth before axillary floral initiation will occur (Jackson and Sweet, 1972). It is generally accepted that auxins play a major role in the control of apical dominance (Tucker, 1977). Thus treatment of plants with TIBA and morphactin, which prevent auxin transport, encouraged lateral growth in *B. megastigma* and *H. angustifolium* (Tables 4.1 & 4.3). In warm conditions, treatment with these auxin transport inhibitors primarily resulted in vegetative lateral growth, although some flowers did develop in *H. angustifolium* plants treated with morphactin (Table 4.1). The flowering that occurred in *H. angustifolium* plants may be a result of the growth retardant effect of morphactin (Schneider, 1970) rather than of an effect on auxin transport. In cool conditions, TIBA and morphactin inhibited flowering of *B. megastigma.* Auxin transport may have a limited role in the control of flower induction of *B. megastigma* and *H. angustifolium.*

Cytokinin application and tip-pruning of plants caused an increase in the rate of flower development of *B. megastigma* and *H. angustifolium* (Tables 4.4 & 4.5). Cytokinins are known to alter the transport and metabolism of assimilates in favour of axillary buds (Abou-Haidar *et al.*, 1985; Ogawa and King, 1979). Tip-pruning plants would also initially ensure that lateral buds received more assimilates due to the absence of apical shoots. Further, gibberellic acid application resulted in a decrease in the rate of floral development of *B. megastigma* (Table 4.5), and Weaver (1985) suggests that gibberellins direct assimilates to apical shoots and deprive the axillary buds of nutrition. Thus there are a number of treatments which increase or decrease the rate of floral development in *B. megastigma* and *H. angustifolium*, probably through the direction of assimilates toward or away from axillary floral buds.

Treatment of *B. megastigma* plants with benzyladenine, GA_{4+7} + BA or GA_3 in warm conditions resulted in a decrease in the endogenous starch concentration (Table 4.6) and an increase in vegetative growth (Table 4.3). When these plants were transferred to cool conditions, less flowering occurred than in control plants (Table 4.6). These results indicate that growth regulators can affect the development of plants long after treatment is completed. The reduction of endogenous starch concentration did not decrease the rate of flower development as might have been expected. The carbohydrate status of plants when they are transferred to cool conditions does not affect subsequent flowering since *B. megastigma* and *H. angustifolium* plants flowered normally after being grown in dark conditions for two weeks before transfer to cool conditions. Growth in the dark for two weeks depleted the starch concentration (*B. megastigma*, 24.9 ± 0.8 to 0.37 ± 0.09 mg(starch).g^{-1}DW; *H. angustifolium*, 12.3 ± 1.36 to 0.35 ± 0.14 mg(starch).g^{-1}DW) and the soluble sugar concentration (*B. megastigma*, 79.9 ± 3.8 to 1.25 ± 0.17 µmol(sucrose).g^{-1}TW; *H. angustifolium*, 61.7 ± 7.4 to 0.62 ± 0.10 µmol(sucrose).g^{-1}TW). Thus rate of floral development may not be controlled by carbohydrate level per se, but by the distribution of carbohydrates to floral buds rather than vegetative shoots. The reduction in flowering resulting from benzyladenine, GA_{4+7} + BA or GA_3 treatment two months prior to transfer to cool conditions is likely to be associated with the increased vegetative growth occurring in these plants rather than the measured reduction in starch concentration.

Girdled *B. megastigma* plants had a slower rate of floral development that control plants (Table 4.5). Girdling has been used to increase flowering and fruiting of Satsuma mandarin (Iwahori *et al.*, 1990). These authors found that girdling increased the rate of flower development and allowed more flower buds to reach a certain stage of flower development before the winter rest period. Starch accumulated above the girdle in *B. megastigma* plants (24.9 ± 0.8 to 33.8 ± 2.7 mg(starch).g^{-1}DW), which may suggest that the rate of flower development should increase. However, in addition to the accumulation of carbohydrates, girdling can also affect the phenol and auxin content (Rao *et al.*, 1989), the protein content (Iwahori *et al.*, 1990) and the ABA concentration (Loveys and Kriedemann, 1974) of plants above the girdle. In *B. megastigma*, the rate of floral development may not have increased in

girdled plants because of the endogenous changes other than carbohydrates that result from girdling. For example, it is possible that girdling plants resulted in a decrease in root growth which caused a decrease in cytokinin production and transport to the shoot. Floral development may have been slower in girdled plants because of a lower concentration of cytokinins in the top of the plant.

In cool conditions there are many treatments that resulted in a reduction in flowering in *B. megastigma* and *H. angustifolium* including tip-pruning, high nutrient status and gibberellic acid treatment of plants (Tables 4.4 & 4.5). Reddy and Menary (1989a) also found the application of nitrogen fertilizer during floral development caused a decrease in flowering in *B. megastigma* plants. Rooted cuttings of *H. angustifolium* and plants treated with benzyladenine also had fewer flowering axils than controls as did *B. megastigma* plants treated with GA_{4+7} + BA. The vegetative growth of plants in all these treatments was more profuse than in the control, reinforcing the apparent negative correlation between vegetative and floral growth reported by others (Reddy and Menary, 1989a; Richards, 1985; Roberts and Menary, 1989b). However in chapter 3 it was found that while flowering was normally associated with a reduction in vegetative growth in *B. megastigma* and *H. angustifolium*, vegetative growth could continue throughout the development of flowers. The possibility still remains that whatever is encouraging vegetative growth may also result in a reduction of flowering.

The reduction of flowering and increase in vegetative growth observed in response to the above treatments may have resulted from an increase in endogenous gibberellin concentration. Roberts *et al.* (1991) reported a decrease in gibberellin concentration of *B. megastigma* leaf tissue that may be associated with the initiation of flowers. Gibberellins are known to inhibit organ formation and force the reversion of floral buds to vegetative shoots (Bhaskaran *et al.*, 1990, Odén, 1989 #130). In *B. megastigma* and *H. angustifolium*, gibberellic acid treatment inhibited flower formation and paclobutrazol enhanced flowering particularly in *H. angustifolium* (Tables 4.2, 4.4 & 4.5). The major effect of paclobutrazol is an inhibition of gibberellin synthesis (Graebe, 1987). The fact that paclobutrazol can partially substitute for the cold required for flowering of *H. angustifolium* plants suggests that the

inhibition of gibberellin synthesis may play a major role in the control of flowering in this species.

H. angustifolium and *Chamelaucium uncinatum* belong to the *Chamelaucium* alliance in the Myrtaceae family (Johnson and Briggs, 1983). *Chamelaucium uncinatum* requires four weeks of short days for the initiation and/or early development of flower buds (Shillo *et al.*, 1985). Treatment of *C. uncinatum* with chlormequat chloride promoted flowering in long days, and gibberellins partially inhibited flowering in a favourable photoperiod. These authors suggest that a reduction in endogenous gibberellins is necessary but not sufficient to induce flowering in *C. uncinatum*. Thus the role of gibberellins in flowering of two closely related species of plant is similar even though flowering in one is controlled by temperature and the other by photoperiod. This may suggest that the environmental cues for flowering in these two species produce similar endogenous hormonal changes. Bagnall (1992) has observed a similar relationship between vernalization and light quality (red:far red ratio) in the flowering of a number of late flowering mutants of *Arabidopsis thaliana*. Those mutants that are most responsive to vernalization treatment are also more responsive to changes in light quality, suggesting that there is some interaction between systems responding to vernalization and the systems affected by phytochrome light detection.

Gibberellic acid treatment of *B. megastigma* plants resulted in a section of the plant with long internodes from which flowers did not develop. Microscopic investigation showed that undifferentiated buds were present in the leaf axils of this section of stem but differentiation and development did not proceed in either the vegetative or floral direction. Limited flowering did occur on the new vegetative growth above these regions. The undifferentiated buds that were initiated on those sections of stem that developed under the influence of high gibberellic acid may have lost their competence to respond to later endogenous changes or external environment changes (Davies, 1987; Jackson and Lyndon, 1990).

High rates of paclobutrazol application (50 $mg.pot^{-1}$) were required to produce any observable effects on vegetative or floral growth in *B. megastigma*. Lewis and Warrington (1988) also found that soil drench application of paclobutrazol to *B. megastigma* 'Lutea' plants at rates below 10 mg per pot had no effect. This lack of response may be a feature of many

Rutaceae since lemon plants (*Citrus limon*) required rates of 100 mg paclobutrazol in a 2.5 litre pot to reduce growth (Bausher and Yelenosky, 1986). The increase in percent of axils with flowers in *B. megastigma* plants treated with paclobutrazol resulted not from an increase in the number of flowers but an inhibition of vegetative growth (a reduction in the number of nodes formed) which allowed flowers to develop closer to the stem tips. It is possible that at high concentrations of paclobutrazol, sterol synthesis is being inhibited (Lenton, 1987). Sterols are required for new membrane production associated with dividing cells (Haughan *et al.*, 1988) and therefore are required for both vegetative and floral growth. An inhibition of sterol synthesis may have been the reason for the (non-significant) decrease in petal length observed in *B. megastigma* plants treated with paclobutrazol (Table 4.5).

After *B. megastigma* plants were commitment to flower, transfer to warm conditions or gibberellic acid treatment did not inhibit flowering (Table 4.7). Prior to commitment to flower these treatments resulted in an inhibition of flowering (Figure 3.3 & Table 4.5). Benzyladenine and gibberellic acid treatment increased *B. megastigma* flower size (Table 4.7). As mentioned above gibberellins and cytokinins attract nutrients toward the tissue in which they reside (Weaver and Johnson, 1985) and encourage cell elongation and cell division respectively, both of which could be reasons for the observed increase in size of flowers of *B. megastigma.*

In addition to decreasing the time to anthesis, decreasing the number of days flowers remain open and decreasing flower size, the transfer of plants to warm conditions after commitment to flower results in a stimulation of vegetative growth. The stimulation of vegetative growth probably deprives the flowers of nutrients and results in a reduction in the size of flowers and possibly a reduction in time for which the flowers remain open.

CHAPTER FIVE

CHANGES IN CYTOKININ AND CARBOHYDRATE CONCENTRATION IN *BORONIA MEGASTIGMA* DURING FLOWERING

Introduction

There is ample evidence that cytokinins are involved in flowering in many plants. Le Jeune *et al.* (1988) report that application of cytokinins to the shoot apex of *Sinapis alba* plants in non-inductive conditions results in a mitotic wave. This wave of cell divisions is thought to be one of the evocation events occurring in all plants, and normally occurs in *Sinapis alba* after a single inductive long-day. Le Jeune *et al.* (1988) also found that the endogenous cytokinin concentration increased dramatically throughout the plant at the end of an inductive long-day. Roberts *et al.* (1991) measured an increase in concentration of two fractions (Z + [9R]Z and (diH)Z + (diH)[9R]Z) extracted from *B. megastigma* during the first half of flower development, and in chapter 4 it was shown that treatment of *B. megastigma* plants with benzyladenine shortened the time required for flowering in cool conditions from twenty weeks to fourteen. This evidence suggests that cytokinins play some role in the development of flowers in *B. megastigma.*

Conversely, there have been many reports suggesting that cytokinins play no role in flowering. Treatment of *Boronia heterophylla* plants with benzyladenine increased the number of flowers but only by increasing the number of sites at which flowers could form (Richards, 1985). Flowering was delayed in both *Boronia heterophylla* (Richards, 1985) and *Rhapsaldopis gaertneri* (Boyle *et al.*, 1988) plants when treated with benzyladenine. Hansen *et al.* (1988) found no relationship between flower number and cytokinin concentration in *Begonia* plants. Finally Roberts *et al.* (1991) report that flowering of *B. megastigma* can be induced *in vitro* without the presence of cytokinin in the tissue culture medium.

Many authors have presented results suggesting that carbohydrates are involved in both the evocation and development of flowers. An increase in the soluble sugars in the shoot apex immediately prior to flower initiation has been found in both *Pharbitis nil* (Abou-Haidar *et al.*, 1985) and red clover (Jones, 1990). However, Jones (1990) found that in red clover an increase in soluble sugars was not sufficient to cause flowering, thus an increase in the supply of carbohydrates is only part of the system controlling flowering in this species. Bodson and Bernier (1985) suggest that this conclusion can be extended to many plants.

A high endogenous carbohydrate concentration may aid the development of flowers more than flower initiation (Bernier *et al.*, 1981b). Bodson and Bernier (1985) cite results to show that a higher concentration of carbohydrates is required for flowering than for vegetative growth, and that higher irradiance generally results in an enhancement of flowering. In *B. megastigma*, Roberts and Menary (1990) fed $^{14}CO_2$ to lateral shoots and subsequently extracted various fractions from this tissue. They report an increase in the percentage of the total ^{14}C partitioned into a fraction containing soluble sugars during the initiation of flowers relative to other periods of early flower development, suggesting that the demand for photosynthetic assimilates is high during the initiation of flowers.

Many plant growth regulators can affect the metabolism and distribution of endogenous carbohydrates (Weaver and Johnson, 1985). In particular, the presence of cytokinins in a tissue will increase the sink strength of that tissue, cytokinins favour the distribution of carbohydrates to tissues in which they reside. Zeatin and zeatin riboside are implicated in the mobilization of carbohydrates in chick pea embryos (Muñoz *et al.*, 1990) and cytokinins can affect sucrose metabolism enzymes in rose flowers (Zieslin and Khayat, 1990). Thus there is support for a role for cytokinins in the metabolism and distribution of carbohydrates. More specifically, benzyladenine application to *Pharbitis nil* results in an increase in the assimilates at the shoot apex and an increase in the number of flowers (Abou-Haidar *et al.*, 1985; Ogawa and King, 1979).

In *Pharbitis nil*, both cold temperature and benzyladenine treatment of plants have a similar influence on the movement of assimilates and flowering (Abou-Haidar *et al.*, 1985). Both these treatments also affect sucrose metabolism enzymes (Zieslin and Khayat, 1990) and

thus exert some feedback control on photosynthesis. It is possible that some of the consequences observed when plants are transferred to cold conditions are the result of changes in the endogenous cytokinin concentration. Cytokinins increase in *Begonia* plants (Hansen *et al.*, 1988) and potato tubers (Turnbull and Hanke, 1985) when they are transferred to cold. However in plants such as *Pseudotsuga menziesii* (Pilate *et al.*, 1989) and apple (Tromp and Ovaa, 1990; Young, 1989) that have a dormancy period, the concentration of zeatin type cytokinins was found to decline while plants were in cold conditions and increase during bud swell when plants are returned to warm conditions.

Cold temperatures can affect much more than the cytokinin concentration of plants. In endive, cold temperature affects many enzymes and substrates within the plant (Mohamed-Yasseen and Splittstoesser, 1990). These authors suggest the changes are a result of an alteration of membrane fluidity or of changes to the transcription of genes. One of the major results of cold temperature is an accumulation of carbohydrates. Cold temperatures can control carbohydrate concentration by directly affecting photosynthesis (Martindale *et al.*, 1991) and sucrose metabolism enzymes (Zieslin and Khayat, 1990), by an inhibition of carbohydrate translocation and by restriction of the use of carbohydrates (Paul *et al.*, 1990).

The above examples suggest that there is much interaction between cytokinins, carbohydrates, cold temperature and flowering in a number of plants. The following experiments examined the effect of cold temperature on cytokinin and carbohydrate concentration in *B. megastigma*. The observed changes were then related to the initiation and development of flowers.

Methods

Three separate experiments were conducted. A short term study examined the first three weeks in cool conditions during which floral differentiation began. This study involved RIA measurement of Z, (diH)Z, iP, and their respective ribosides, soluble sugar and starch concentration, and the rate of photosynthesis in plants in both warm and cool conditions.

Cytokinins were measured in leaf, stem and root tissue to give some indication of the distribution of cytokinins during this period.

The second experiment was a long term study which covered the time from transfer to cool conditions through to anthesis. Z and [9R]Z were measured using RIA's in a combined leaf and stem extract at two week intervals from both warm and cool plants. Soluble sugar and starch concentrations were measured for the same tissue. After twelve weeks in cool conditions when plants were committed to flower, a number of plants were transferred to warm conditions to allow comparison with plants that remained in cool conditions through to anthesis. It was hoped that measurement of cytokinin and carbohydrate concentrations could be related to stage of flower development rather than environmental conditions after commitment to flower. After fourteen weeks in cool conditions flower buds were large enough to allow measurement of carbohydrate and cytokinin concentration separately from leaf and stem tissue.

In addition to the measurement of cytokinins and carbohydrates in plants in cool conditions, measurement of carbohydrates in benzyladenine treated plants was conducted in both in the short term and long term experiments. This enabled a further examination of the relationship between carbohydrates and the rate of flower development since benzyladenine treatment of plants greatly enhanced the rate of flower development (Chapter 4). Plants were sprayed three times, one week apart, with 50mg.l^{-1} benzyladenine.

Finally, a gas chromatograph - mass spectrometry (GC-MS) study was undertaken to confirm and extend the qualitative assessment of cytokinins.

The nomenclature for the various cytokinins listed in Horgan and Scott (1991) has been used throughout (see abbreviations at the front of the thesis).

Radio-immuno assay (RIA)

Plant material

Plants were propagated and grown as described in chapter 2. At an age of five to six month, plants of similar height and breadth were transferred from a heated glasshouse to a warm growth cabinet (25°C day/17°C night) and allowed to adjust to the cabinet conditions for

two weeks. After this time, half the plants were transferred to a cool growth cabinet (17°C day/ 9°C night) to allow comparison with the plants in warm conditions. Growth cabinet conditions were as for fluorescent lamp lit cabinets (Figure 3.1b).

The four plants used as replicates for each treatment were removed from growth cabinets at 10am, two hours after the beginning of the light period, on the days shown in the figures. The aerial parts of the plant were cut from the roots at soil level, weighed, sealed in plastic and stored at -30°C until required for analysis. If roots were required they were shaken free of soil, washed, surface dried, weighed, sealed in plastic and stored as for the other tissue.

Extraction

Twelve extracts were conducted in parallel. Only two of the four replicates for each treatment and time period were processed at any one time. Tissue was removed from -30°C and separated if required (eg: leaf from stem tissue, flower buds from leaf and stem tissue). One gram fresh weight of tissue (less of young flower buds) was extracted in 10ml 80% ethanol (0°C) to which ^{3}H-(diH)Z (approximately 20000-30000 Bq) was added as an internal standard (Appendix 2 - loss of internal standard & interference of internal standard in RIA's). Two 200µl aliquots of the extracting solvent were measured to accurately determine the initial radioactivity. The tissue was homogenized and left overnight at -20°C (Horgan, 1978). The extract was centrifuged for ten minutes at 2000g and the supernatant retained. The residue was again suspended in 10ml 80% ethanol and then filtered through Whatman No. 1 filter paper. The solid material was retained on the filter paper and oven dried to allow the calculation of dry weight. Starch concentration was measured using this tissue.

The combined supernatants were evaporated to aqueous with a vacuum rotary evaporator (35°C), made up to 5ml with water and frozen overnight. The solution was thawed and centrifuged for fifteen minutes at 40000g. 25µl were taken for measurement of soluble sugars. The supernatant was adjusted to a pH of 8.2-8.4 with NaOH and partitioned three times with water saturated butanol (Horgan, 1978). The butanol fractions were combined, 10ml of TEAA buffer added (20mM Triethyl-amine adjusted to pH 3.35 with glacial acetic acid) and rotary evaporated to approximately 500µl. The sample was made up to 1.2ml with 10%

Acetonitrile (CH_3CN) in TEAA buffer ready for High Pressure Liquid Chromatography (HPLC).

High pressure liquid chromatography

A range of HPLC methods were tested (Appendix 2) before the following method was adopted.

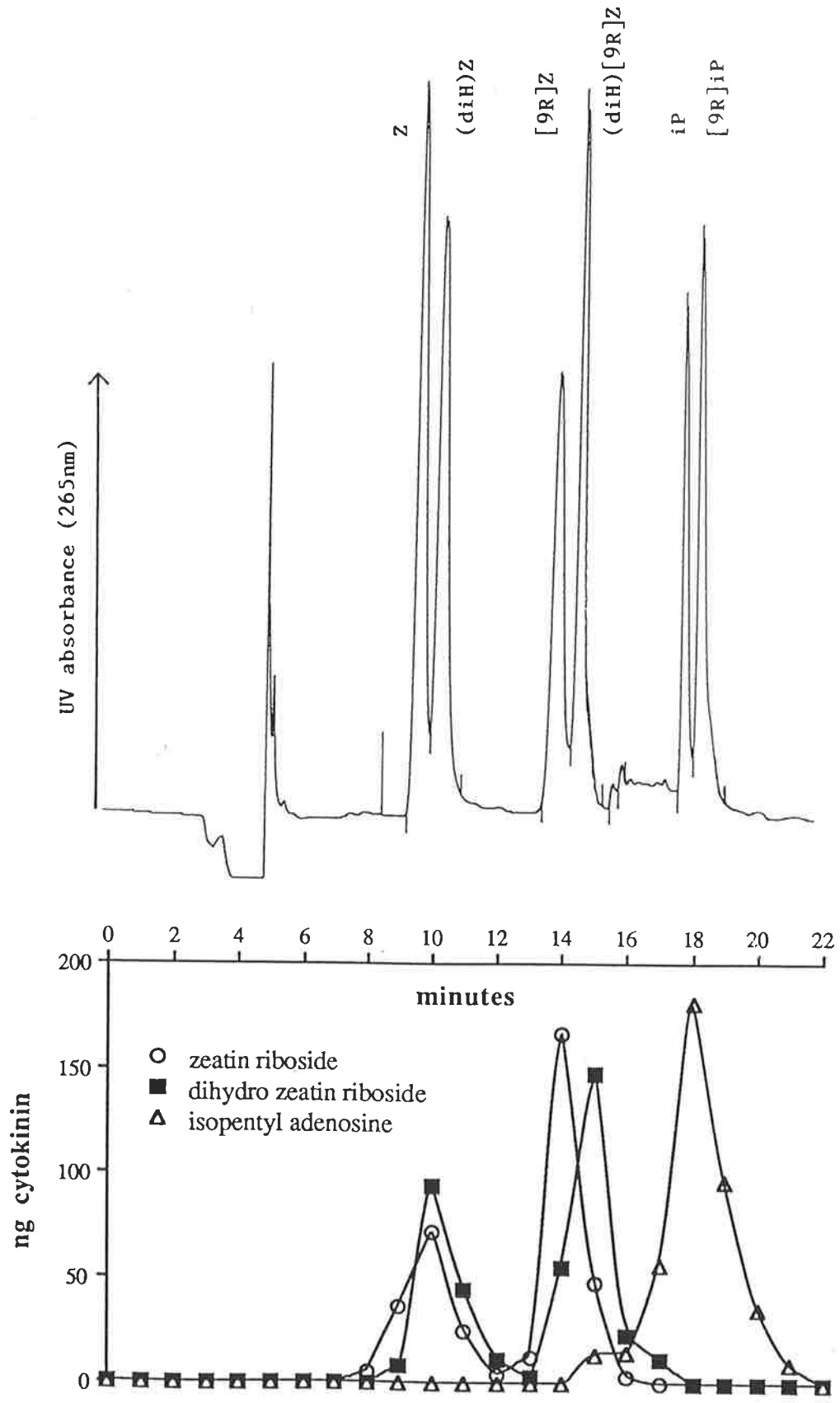
time (minutes)	percent CH_3CN	percent TEAA buffer
0	12	88
10	12	88
10.5	30	70
20	30	70
21	100	0

A Brownlee Labs 250 x 4.6mm, 10µm spherisorb C_{18} column was used with a Varian Vista 5500 HPLC. The column temperature was maintained at 30°C and the pressure ranged from 22 - 48 atmospheres at 1ml per minute. The UV detector measured absorbance at 265nm. 1ml injections were used and an automatic relay allowed flow through the injection loop between 0.10 and 2.00 minutes. Standards were injected at a concentration of 250ng/ml. A combined injection of zeatin (Z), zeatin riboside ([9R]Z), di-hydro zeatin ((diH)Z), di-hydro zeatin riboside ((diH)[9R]Z), iso-pentyl adenine (iP) and iso-pentyl adenosine ([9R]iP) was tested before and after a group of twelve extracts (Figure 5.1a). The variation in retention time of the standards was never more than 0.15 minute. All standards were made up in 10% CH_3CN in TEAA buffer.

One minute fractions were collected from the HPLC between seven and twenty-one minutes and evaporated to dryness in a vacuum centrifuge (Savant). Fractions were redissolved in 250µl of 10% methanol. Fractions two to ten were measured for zeatin and zeatin riboside, fractions three to eleven for di-hydro zeatin and di-hydro zeatin riboside and fractions nine to fifteen for iso-pentyl adenine plus iso-pentyl adenosine (Figure 5.1b).

Figure 5.1 - Cytokinin standards separated by HPLC and fractions subsequently measured by RIA.

a) UV absorbance (265nm) trace of cytokinin standards after separation by HPLC.
b) Measurement of cytokinins by RIA. One minute fraction of the standards separated by HPLC above were assayed with the three different poly-clonal antibodies.



Radio-immuno assay

This radio-immuno assay method was adapted by C. Turnbull from Turnbull and Hanke (1985). The ribosyl-[3H]-dialcohol tracer compounds and the cytokinin antibodies were purified and donated by C. Turnbull. The basis of the RIA technique that the antibody will bind non-selectively to labelled and unlabelled cytokinins, thus an increase in concentration of unlabelled cytokinin in the assay will result in a decrease in binding of labelled cytokinin. Therefore, an increase in amount of unlabelled cytokinin is detected as a decrease in the final radioactivity in the assay.

Duplicate micro centrifuge tubes were used for each fraction. In each tube was placed 90µl Tris buffered saline (TBS; 0.1 M Tris base, 0.1M NaCl adjusted to pH 7.2 with HCl), 10µl γ-globulin (2% w/v in TBS) and 10000 Bq [^{3}H] of either [9R]Z, (diH)[9R]Z or [9R]iP tracer. The cytokinin antibodies could not distinguish between these tracers and native ribosides. To the tubes was added 50µl of standard or sample and 50µl of antibody solution. The antibody powder was tested at various concentration to obtain a B_0 = 35% (Appendix 2), where B_0 is the radioactivity bound in the absence of standard or sample as a percentage of the total radioactivity.

The tubes were mixed and left at room temperature for one hour before the addition of 200µl of saturated ammonium sulphate ($(NH_4)_2SO_4$) which precipitates the antibody-γ globulin complex. The mixture was left for twenty minutes then centrifuged for three minutes at 14000g after which the supernatant was decanted and the tubes allowed to drain. 150µl of 50% saturated ammonium sulphate was added to each tube. The tubes were mixed, centrifuged and decanted as before. The pellet was dissolved in 200µl distilled water and left for fifteen minutes before the addition of 1.2ml Beckman Ready-Solv EP scintillant. The tubes were capped, shaken and counted for two minutes in a Beckman LS 3801 scintillation counter.

The scintillation counter calculated a standard curve from tubes using unlabelled standard concentrations of cytokinin. The concentrations of cytokinin used were [9R]Z - 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0ng per tube, (diH)[9R]Z - 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0ng per tube and [9R]iP - 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0,

20.0ng per tube. A new standard curve was constructed for each set of assays. The curve was linearised using a logit transformation

$$x = \log [\text{cytokinin concentration}] \text{ (ng per tube)}, \quad y = \ln\left[\frac{\%(B/B_0)}{100-\%(B/B_0)}\right]$$

where B_0 is the radioactivity bound in the absence of standard or sample as a percentage of the total radioactivity and B is the radioactivity bound in the presence of sample or standard as a percentage of the total radioactivity. All radioactivity values were corrected for non specific binding which represented the amount of radioactivity bound in the absence of antibody.

Amount of cytokinins were calculated by pooling the ng of cytokinin present in those fraction corresponding to the HPLC elution time of standards (Figure 5.1) and subtracting a background (ng-cytokinin measured in a fraction that did not co-elute with a standard). Final cytokinin concentrations took into account the percent loss of internal ^{3}H-(diH)Z during extraction and the cross reactivity of the cytokinin with the antibody (Appendix 2). In those tissues where a measurement of (diH)Z was required, ^{3}H-(diH)Z was added to only two of the four extracts of that tissue, the resulting losses were averaged and this average was used in the calculation of cytokinins in non-spiked extracts. The loss of all measured cytokinins was assumed to be equivalent to the loss of ^{3}H-(diH)Z. Individual t-tests were used to compare values at any one time period.

Gas Chromatography - Mass Spectrometry

The following gas chromatograph - mass spectrometry method was adapted by R. Horgan from Scott and Horgan (1984) and McGaw *et al.* (1985). The ^{2}H and ^{15}N tracer cytokinins were synthesized by R. Horgan (Scott and Horgan, 1984).

Plant material

Plants were propagated, grown and transferred from a heated glasshouse to a warm growth cabinet as above. After two weeks acclimatization in the warm growth cabinet, six

plants each of *B. megastigma* and *H. angustifolium* were transferred to cool conditions (17°C day/9°C night). Six plants of each species remained in warm conditions. After three weeks in these conditions, the roots and tops of the plants were separated at soil level. Roots were shaken, washed and dried as above. The material from all six plants was combined and the tissue ground in liquid nitrogen before being freeze dried and sealed in plastic for transport to Dr. Roger Horgan's laboratory at The University College of Wales, Aberystwyth.

Extraction

The equivalent of 20g fresh weight was homogenised in 200ml 80% ethanol to which was added approximately 500ng of the following compounds as internal standards, ^{15}N derivatives of Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP, [9R]iP, (OG)Z, (OG)[9R]Z, and ^{2}H derivatives of [9R-5'-P]Z and [9R-5'-P]iP. The homogenate was filtered through Whatman No. 1 filter paper using a Buchner funnel. The solids were re-homogenised in 200ml 80% ethanol, refiltered and washed through the funnel. The extract was rotary evaporated to water (20ml) and adjusted with acetic acid to pH 3 before being loaded on to a 20g cellulose phosphate column (Whatman floc cation exchanger cellulose phosphate). The 'acid wash' included all eluate from the loading of the extract and five column volumes of 0.1M acetic acid. The 'ammonia wash' included the eluate resulting from five column volumes of 2M ammonia.

Ammonia wash

The ammonia wash contained all cytokinins other than the 5'-monophosphates. This eluate was rotary evaporated to approximately 5ml and adjusted to pH 7 with sodium hydroxide (NaOH). The extract was then loaded on to a series of three Sep-Paks (Millipore, C_{18} cartridge). The Sep-Paks were washed with 20ml of distilled water and the extract eluted with 15ml 100% methanol. The methanol was evaporated in a vacuum centifuge and the remaining material redissolved in 2-3ml TEA buffer (2mM Triethyl-amine, pH 7 with formic acid) and filtered through glass-fibre filter paper (Whatman GF/F glass microfibre filter) ready for HPLC.

High pressure liquid chromatography - For initial HPLC separation, a 250 x 9.2mm, 5µm ODS-2 spherisorb C_{18} 'preparation' column was used. The HPLC pumped 5ml/min. at ambient room temperature using a linear gradient over fifty minutes of 5-30% Acetonitrile (CH_3CN) in TEA buffer (pH 7). The detector recorded absorbance at 265nm. Broad fractions were collected after using unlabelled standards to indicate where the cytokinins should elute. The following fractions were collected

17-19 minutes	(OG)Z
19-21 minutes	Z, (diH)Z, (OG)[9R]Z
21-25 minutes	[9R]Z, (diH)[9R]Z
31-40 minutes	iP, [9R]iP

The fractions were evaporated in a centrifuge evaporator, redissolved in 5% CH_3CN in TEA buffer and reinjected into the HPLC for further purification. The 'analytical' column used was 250 x 4.6mm, 5µm ODS-2 spherisorb C_{18}. The pump rate was 1.5ml/min. and the detector recorded UV absorbance at $\lambda = 265$nm. The two separate linear solvent gradients were run on the HPLC as shown in the following table. Broad fractions collected.

10-15% CH_3CN in TEA buffer over 25 min.		15-30% CH_3CN in TEA buffer over 30 min.	
8-10.5 min.	(OG)Z	13-17 min.	iP and [9R]iP
10.5-16 min.	Z, (diH)Z, (OG)[9R]Z		
16-20 min.	[9R]Z, (diH)[9R]Z		

Glucosidase - Those fractions containing the O-glucosides were treated to remove the glucose from the molecule. 5ml of a solution containing 0.1M tri-sodium citrate and 0.1M citric acid was added to the fractions and the pH adjusted to 5.0 with sodium hydroxide. A pin-head sized granule (approximately 0.5mg) of ß-glucosidase (Sigma,type 1) was added. This mixture was incubated at 37°C for 24 hours then loaded on to a Sep-Pak. The Sep-Pak was washed with 10ml of distilled water and the cytokinins eluted with 5ml 100% methanol which was then evaporated under vacuum.

Acid wash

This eluate contained the 5'-mono-phosphates of zeatin riboside and iso-pentyl adenosine, [9R-5'-P]Z and [9R-5'-P]iP. The acid wash was rotary evaporated to approximately 5ml and then loaded onto a Sep-Pak. The Sep-Pak was washed with 10ml of distilled water and the cytokinins eluted with 5ml 100% methanol. The samples were evaporated to dryness under vacuum and then dissolved in 5% methanol in 0.1M acetic acid ready for HPLC. The HPLC conditions and column used were as for the 'preparation' column above. A linear gradient from 10% to 50% methanol in 0.1M acetic acid was run over 30 minutes. Fractions were collected as follows and rotary evaporated to dryness.

8 - 12 minutes	[9R-5'-P]Z
19 - 25 minutes	[9R-5'-P]iP

Acid phosphatase - To each fraction was added 10ml of 0.5% bovine serum albumin (BSA) in 0.35M sodium acetate to bind to any non-specific inhibitors of phosphatase. The solution was adjusted to pH 4.8 with acetic acid and left for two hours. After the two hours approximately 2mg of acid phosphatase (from wheatgerm, 0.64 units/mg, Sigma) was added with 10000 - 20000 Bq of [^{3}H] AMP (adenosine 2'&3' mono-phosphate) as a standard. The solutions were incubated at 35°C for 24 hours. The pH was adjusted to 3.0 and the extracts loaded and washed through 5 x 1cm cellulose phosphate columns as described above. The radioactivity of the acid wash and ammonia wash were tested. If the phosphatase reaction was not inhibited by non-specific inhibitors then most of the radio-activity was found in the ammonia wash. In most cases less than 60% of the radioactivity was found in the ammonia wash and thus the base wash was rotary evaporated and the acid phosphatase procedure repeated. There was no need to add [^{3}H] AMP to the repeated procedure.

With the cleavage of the phosphate, [9R-5'-P]Z and [9R-5'-P]iP were now [9R]Z and [9R]iP respectively. After the repeat, the ammonia fractions were combined and rotary evaporated to 2-3ml. The solutions were loaded onto a Sep-Pak, washed with 10ml distilled water and eluted with 5ml methanol. The eluate was redissolved in 10% CH_3CN in TEA

buffer and filtered through a glass fibre filter. The extracts were loaded onto the 'analytical' HPLC column and fractions collected as described for [9R]Z and [9R]iP above.

Permethylation

After fractions were collected from the 'analytical' HPLC column and dried under vacuum, all samples were permethylated. Care was taken to ensure that all reagents, equipment and the samples were completely dry before use. The permethylation reagent was made immediately prior to use by dissolving 40mg potassium-tert-butoxide (97%, Aldrich Chem. Co.) in 1ml dimethyl-sulfoxide (DMSO) and stirring the mixture under nitrogen for 30 minutes. The samples were removed from the desiccator and dissolved in 100µl of DMSO before 100µl of the permethylation reagent was added. The solutions were left to react for 30 minutes in the desiccator before the addition of 100µl of methyl iodide. Again the solutions were left in the desiccator for one hour. The reaction was stopped by shaking the solution with 1ml distilled water. The samples were loaded on to a Sep-Pak, washed with 10ml distilled water and eluted with 5ml of 100% methanol which was evaporated under vacuum.

The permethyl derivatives of the cytokinins were further purified and separated by HPLC using the same 'analytical' column and pump rate as described above. Two separate linear solvent gradients were used as follows

22-35% CH_3CN in TEA buffer over 30 min.		33% CH_3CN in TEA buffer isocratic	
6-8 min.	Me-(diH)Z	12-14 min.	Me-iP and Me-[9R]iP
12-14 min.	Me-Z		
30-33 min.	Me-(diH)[9R]Z		
40-43 min.	Me-[9R]Z		

Two Me-Z and Me-[9R]iP fractions were collected, the Me-Z fractions from native Z and (OG)Z, and the Me-[9R]iP fractions from native [9R]iP and [9R-5'-P]iP. Three Me-[9R]Z fractions were collected, the native [9R]Z and [9R]Z from (OG)[9R]Z and [9R-5'-P]Z. The fractions were dried down in 1.5 x 1.0cm tapered vials ready for Gas Chromatography - Mass Spectrometry (GC-MS).

Gas chromatography-Mass spectrometry

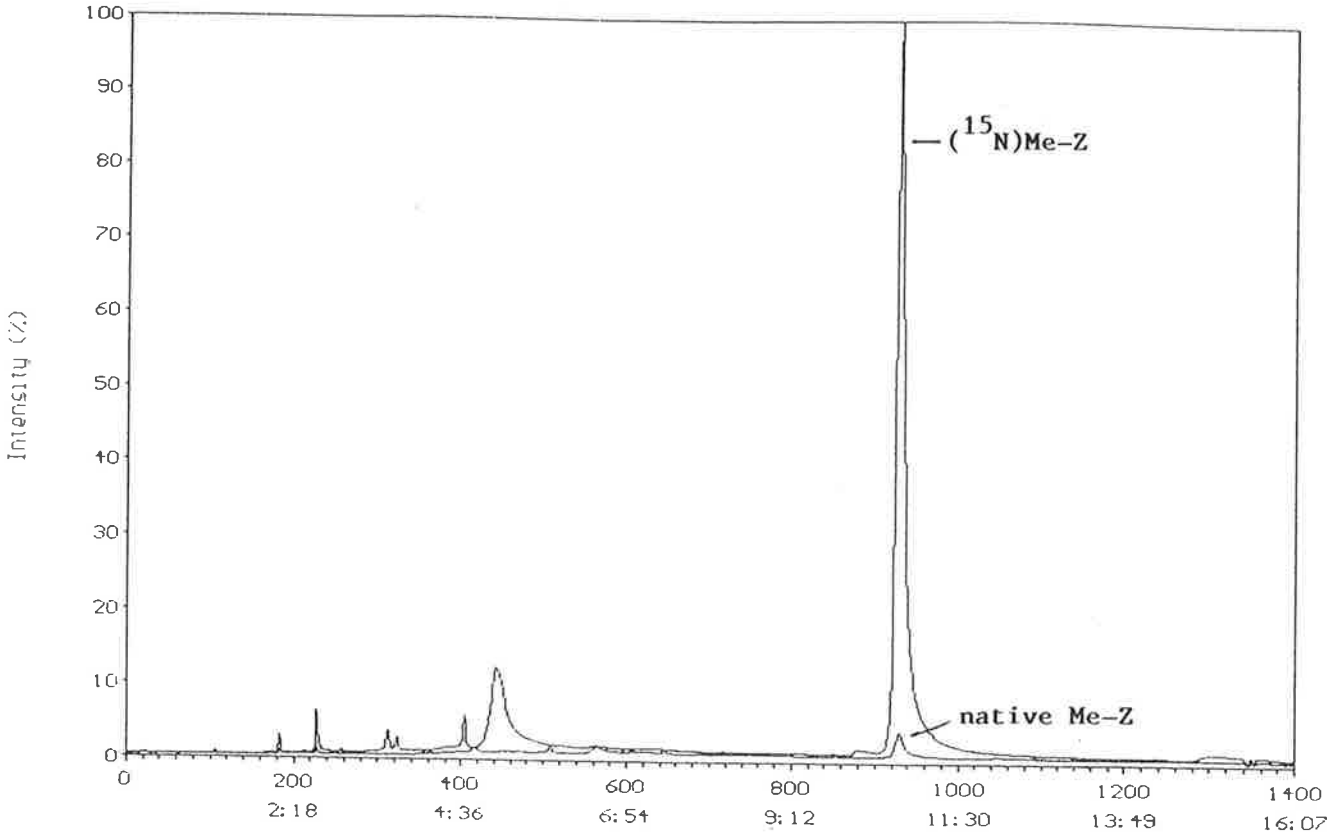
Samples were dissolved in 5.0µl ethyl acetate and 0.5µl injected (Grob splitless injector, temperature 260°C) onto the column of the Carlo Erba 4200 Gas Chromatograph (Crawley, United Kingdom). For the cytokinin bases, a capillary column (OV1, Phase Separations Ltd.), 25m X 0.32mm (internal diameter), 0.4µm film thickness, was heated ballistically from 35°C to 200°C then at 8°C per minute to 260°C and held at this temperature. The ribosides were injected onto a capillary column (OV1, S.A.C. Chromatography Ltd.), 25m X 0.32mm (internal diameter), 0.25µm film thickness, and heated ballistically from 35°C to 250°C and then at 8°C per minute to a final hold temperature of 300°C. The carrier gas was helium at 6000kg.m^{-2}. The gas chromatograph was directly linked to the mass spectrometer (Kratos Instruments MS 25, Manchester, United Kingdom) which employed electron impact with an ionising voltage of 70eV. The temperatures of the source, interface and inlet were 190°C, 190°C and 220°C respectively. Data was analysed using a Mass Spectrometry Services (MSS) data system. Compounds were detected using selected ion monitoring (SIM) (Figure 5.2a). Full electron impact Mass Spectrometry of the compounds displayed good correlation to the standards (Figure 5.2b). The retention times of the selected ions indicating the presence of the cytokinins are shown below

ions monitored	retention time (seconds)	cytokinins detected (permethyl derivatives)
230.1, 234.1	935	Me-Z, (Me-(^{15}N)Z)
390.2, 394.2	950	Me-[9R]Z, (Me-(^{15}N)[9R]Z)
232.1, 236.1	805	Me-(diH)Z, (Me-(^{15}N)(diH)Z)
392.2, 396.2	830	Me-(diH)[9R]Z, (Me-(^{15}N)(diH)[9R]Z)
231.1, 235.1	555	Me-iP, (Me-(^{15}N)iP)
391.2, 395.2	595	Me-[9R]iP, (Me-(^{15}N)[9R]iP)

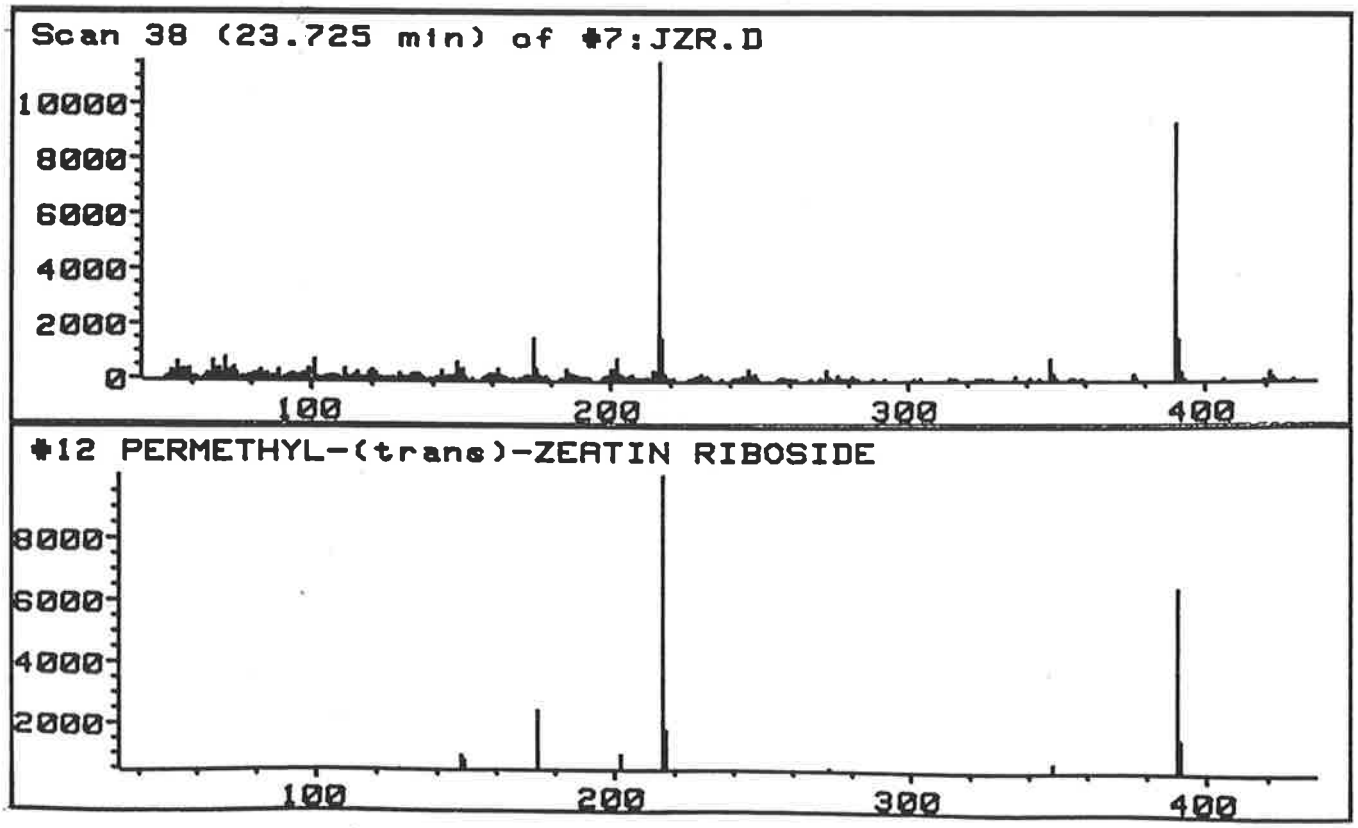
The gas chromatograph software determined the peak area of the native and ^{15}N cytokinin and calculated the ratio of these areas. The concentration of cytokinin in the original extract could be calculated using the following formula

Figure 5.2 - GC-MS measurement of cytokinins.

a) Selected ion monitor trace of permethyl-zeatin. Ions monitored were 234.1 (^{15}N Me-Z) and 230.1 (native Me-Z). The ratio of native to 15N Me-Z was 0.0367.



b) Full mass-spectrometry scan of native zeatin riboside (permethyl-[9R]Z) compared to a scan of standard zeatin riboside (permethyl-(trans)-zeatin riboside). The homology between the sample and standard traces was 98.4%.



$$\text{pmol.g}^{-1}\text{FW cytokinin} = \frac{\text{native cytokinin area}}{^{15}\text{N cytokinin area}} \times \text{initial } ^{15}\text{N cytokinin (ng)} \times \frac{1000}{\text{MW}} \times \frac{1}{\text{gFW}}$$

Carbohydrate measurements

Simon Robinson (pers. comm., 1989) devised the following unpublished method for the extraction and measurement of carbohydrates. Part of this method has been published by Grant *et al.*(1992).

Extraction

A section of stem with two nodes, four to six nodes basal to a vegetative shoot was used for the extraction of carbohydrates from benzyladenine treated plants (Figures 5.13 - 5.16) and all plants used for short-term experiments (Figures 5.8 & 5.9). This segment was ground with a teflon tipped plunger in a 1.5ml micro-centrifuge tube in 500µl 80% ethanol. After two hours at room temperature the supernatant containing the soluble sugars was removed and evaporated to dryness in a centrifuge evaporator (Savant). The sample was dissolved in 200µl of distilled water and 10µl 0.5N NaOH added. The microcentrifuge tube was capped and placed in an oven at 95°C for 30 minutes after which the sample was cooled, made up to 500µl with water and 20µl used for the assay.

The solid material containing the starch remaining in the micro-centrifuge tube after the removal of the supernatant above, was oven dried and weighed. 200µl of distilled water was added, the tube capped and placed in an oven at 95°C for 60 minutes. A series of standards containing 0, 5, 10, 20, 50 and 100 µg starch in 200µl of water were treated similarly. The samples and standards were allowed to cool before the addition of 200µl of amyloglucosidase (AMG, Sigma, grade II) in 0.2M sodium acetate (pH 4.8 with acetic acid). This enzyme converts the starch to glucose. Samples was incubated overnight at 55°C, made up to 500µl with distilled water, centrifuged and 50µl of the supernatant taken for the assay.

Carbohydrates were also extracted from the material used to measure cytokinins in the long-term experiment (Figures 5.13 to 5.16). 20µl was taken for the determination of soluble sugars before the butanol partition step in the RIA cytokinin extraction. 180µl of distilled water

was added and the solution treated as described above. For the starch measurements, the solid material remaining on the filter paper used during the RIA extraction of cytokinins was oven dried. Approximately 50mg dry weight was removed, 200µl of distilled water added and the extraction continued as described above.

Assay

Into a 1ml cuvettes was placed 500µl of assay medium (200mM imidazole, 10mM $MgCl_2$, 0.8mM NADP, 2mM ATP, 1mM dithiothreitol, 0.04% bovine serum albumin, pH to 7.0 with HCl), 20µl invertase (1550 units/ml in water), 20µl phospho-glucose isomerase (PGI, 154 units/ml in water) and water plus sample or standard to make up the total volume to 980µl. All chemicals were from Sigma except for dithiothreitol which was from Boehringer Mannheim. Starch measurement did not require the addition of invertase or PGI. 50µl of sample or standard were used for the starch assay (thus 430µl of water was added to starch assay cuvettes). 20µl of sample was used for the soluble sugar assay (thus 420µl of water was added). The soluble sugars were measured against a series of sucrose standards (0, 15, 30, 60, 150 nmol sucrose per assay) thus the soluble sugars were measured in sucrose equivalent. Soluble sugars were calculated on a tissue water (TW) basis since most of the soluble sugars would reside in solution. The tissue water was calculated by subtracting the dry weight of the tissue from the fresh weight.

The solution was mixed in the cuvettes and left at room temperature for 60 minutes. The absorbance was measured at 340nm (Pye unicam SP 8-200 spectrometer) before the addition of 20µl hexokinase/glucose-6-phosphate dehydrogenase (HK/G-6-PDH, Sigma, 1.7 units/ml and 0.7 unit/ml respectively in water). The assay was again mixed and left at room temperature for 60 minutes before the measurement of the final absorbance (340nm). The initial absorbance was subtracted from the final absorbance for both standards and samples and the resulting sample value compared to the standard curve for determination of soluble sugar and starch concentrations.

Rate of photosynthesis

The rate of uptake of carbon dioxide (or net rate of photosynthesis) was measured with *B. megastigma* plants in a cool growth cabinet (17°C day / 9°C night) and a warm growth cabinet (25°C day / 17°C night) with a Li-Cor LI-6200 portable photosynthesis system. The plants grew in warm conditions for one week before half the plants were transferred to cool conditions and measurements commenced. Three replicates in each temperature were measured. The photosynthesis system measured the depletion of CO_2 in a closed chamber. Because of the small size of the leaves, a stem with ten nodes was tagged and used for measurement over the three week period. The use of a stem rather than a leaf for determination of CO_2 uptake required the construction of a modified chamber with a volume of 4530cm^2. Leaf area was calculated by multiplying the total length of the enclosed leaves by 1mm (the approximate width of a leaflet). Measurements were taken between 10am and 11am on the days shown (Figure 5.10).

Results

GC-MS measurement of cytokinins

Z, iP and [9R]iP were present in both *B. megastigma* and *H. angustifolium* (Table 5.1). [9R]Z was found by GC-MS in *H. angustifolium* alone. In addition, (OG)Z, (OG)[9R]Z, [9R-5'-P]Z and [9R-5'-P]iP were found in both species. This is the first report of any cytokinins in *H. angustifolium* and the first report of iP, glucoside and mono-phosphate cytokinins in *B. megastigma.*

Short term changes

Figures 5.3 - 5.7 show the short term changes that occurred in Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP and [9R]iP concentration in leaf, stem and root tissue when plants were transferred to cool conditions (17°C day/9°C night) as measured by RIA. There were no

Table 5.1 - Cytokinins measured with Gas Chromatography / Mass Spectrometry.

All extractions were conducted on the same tissue. Values are pmol (cytokinin).g^{-1} FW. Where two parallel extractions were conducted, values are mean ± standard error. * - standard lost during extraction. --- not measured.

[1] - leaf and stem tissue from plants in warm conditions (25°C day / 17°C night) for three weeks.
[2] - leaf and stem tissue from plants in cool conditions (17°C day / 9°C night) for three weeks.
[3] - root tissue from plants in warm conditions (25°C day / 17°C night) for three weeks.

	warm - leaf and stem[1]	cool - leaf and stem[2]	warm - root[3]
B. megastigma			
Z	21.67 ± 8.75	3.65	---
[9R]Z	---	---	---
(OG)Z	8.65 ± 2.98	1.77 ± 0.17	0.43 ± 0.03
(OG)[9R]Z	0.53	0.43	---
(diH)Z	*	*	---
[9R-5'-P]Z	44.92	0.68	---
iP	50.44	---	---
[9R]iP	47.31	*	4.43
[9R-5'-P]iP	12.17	---	---
H. angustifolium			
Z	10.55	4.43	6.67 ± 1.83
[9R]Z	27.14	10.03	---
(OG)Z	*	*	3.65 ± 1.13
(OG)[9R]Z	---	---	0.59
(diH)Z	*	*	*
[9R-5'-P]Z	0.98	0.13	7.57
iP	1.92	68.90	---
[9R]iP	*	35.24	*
[9R-5'-P]iP	---	---	69.80

significant differences in Z between plants in warm (25°C day/17°C night) and cool conditions in any of the tissues measured (Figure 5.3). (diH)Z showed a decrease in concentration in root tissue during the first two days after transfer to cool conditions (Figure 5.5). The concentrations of [9R]Z, (diH)[9R]Z and iP + [9R]iP all increased in the roots of plants transferred to cool conditions (Figures 5.4, 5.6 & 5.7). The increase occurred rapidly, within one or two days, following which the concentration decreased to the level found in plants in warm conditions during the next three to five days. A similar increase and decrease occurred in the concentrations of (diH)[9R]Z and iP + [9R]iP in stem tissue of plants transferred to cool conditions (Figures 5.6 & 5.7).

The short term changes in starch and soluble sugar concentration (Figure 5.8 & 5.9) were measured in plants grown in the same conditions as those used for cytokinin measurements. There was an increase during the first day in the soluble sugar concentration of plants transferred to cool conditions (Figure 5.8). This higher level of soluble sugars was maintained throughout the three week period. Plants treated with benzyladenine experienced a decrease in soluble sugar concentration to a similar level to that in warm plants by the third week in cool conditions.

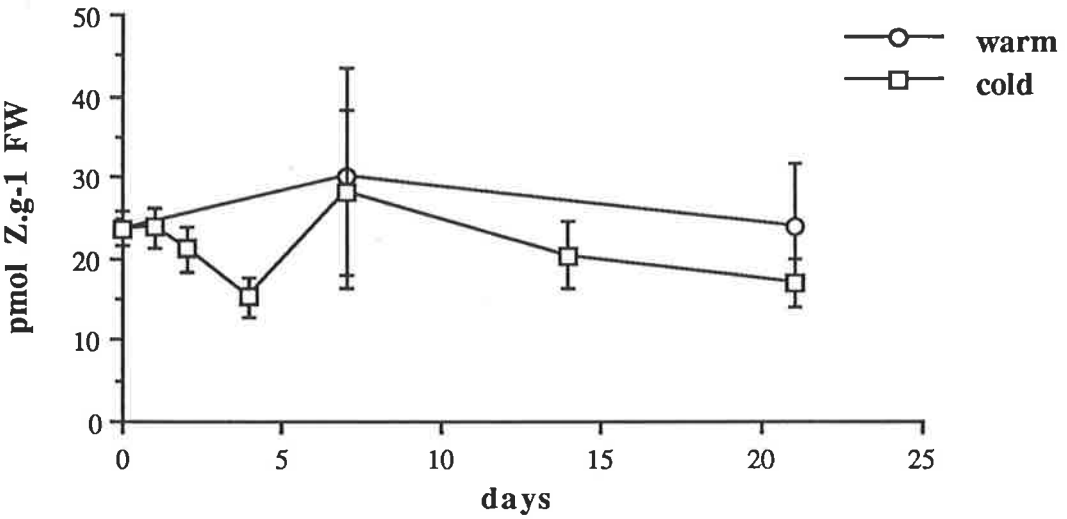
Transferring *B. megastigma* plants to cool condition resulted in an eight fold increase in starch concentration within the first days after transfer, however a continuous decrease in starch concentration occurred after day 8 in cool conditions until, by the third week, the concentration had decreased by approximately 50%. The starch content of benzyladenine treated plants was lower than non-benzyladenine treated plants after four days and fifteen days in cool conditions but was not significantly different at any other sample time in the first three weeks (Figure 5.9).

The rate of photosynthesis of *B. megastigma* plants decreased slowly in the two weeks after transfer to cool conditions (Figure 5.10). Between weeks two and three in cool conditions, the rate of photosynthesis was significantly lower in plants in cool conditions than that of plants in warm conditions.

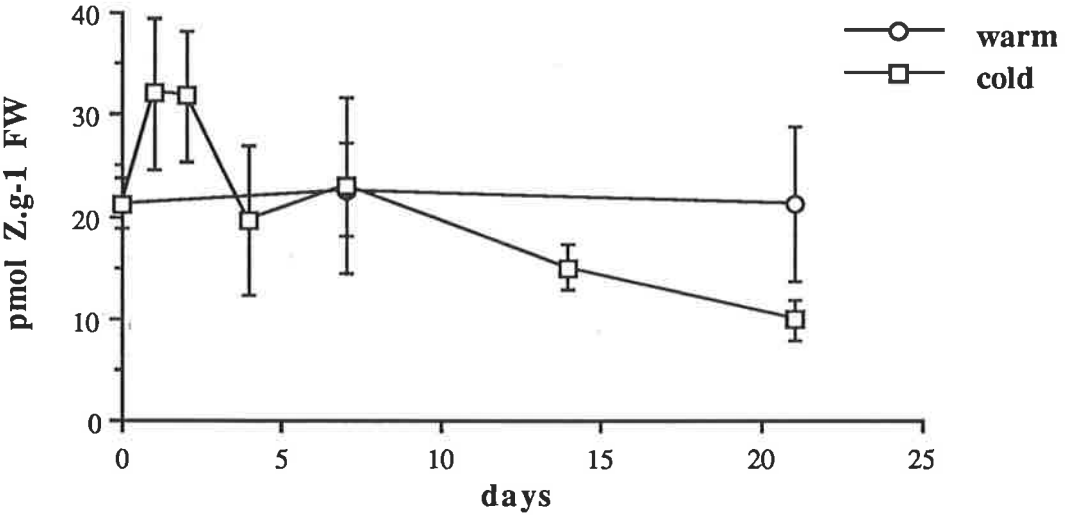
Figure 5.3 - Endogenous zeatin changes in *Boronia megastigma* during early flower development.

Plants were in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Values are mean ± standard error.

a) Zeatin in leaf tissue.



b) Zeatin in stem tissue.



c) Zeatin in root tissue.

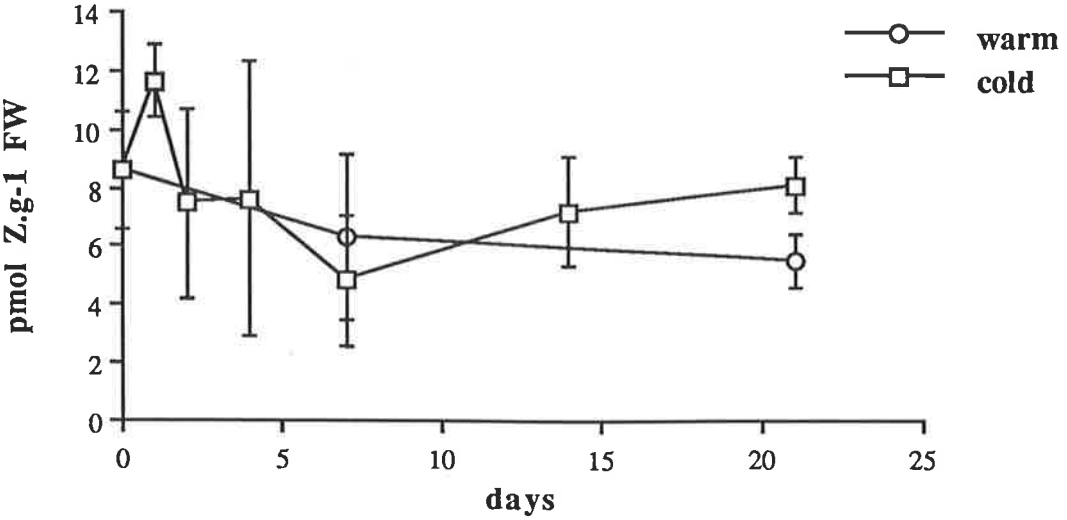
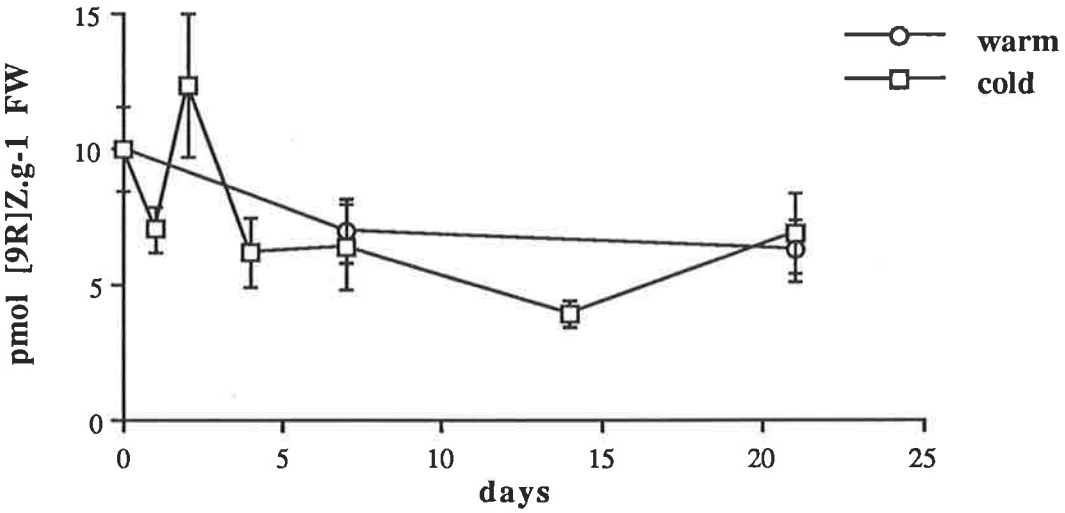


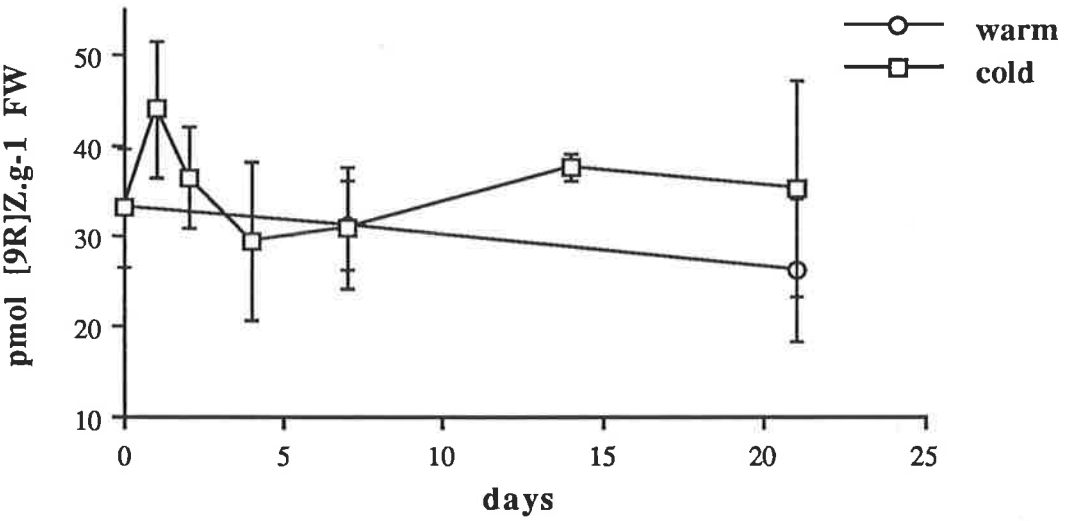
Figure 5.4 - Endogenous zeatin riboside changes in *Boronia megastigma* during early flower development.

Plants were in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Values are mean ± standard error. * - significantly different from plants in warm conditions, combined day 0 and 7 values (p = 0.05).

a) Zeatin riboside in leaf tissue.



b) Zeatin riboside in stem tissue.



c) Zeatin riboside in root tissue.

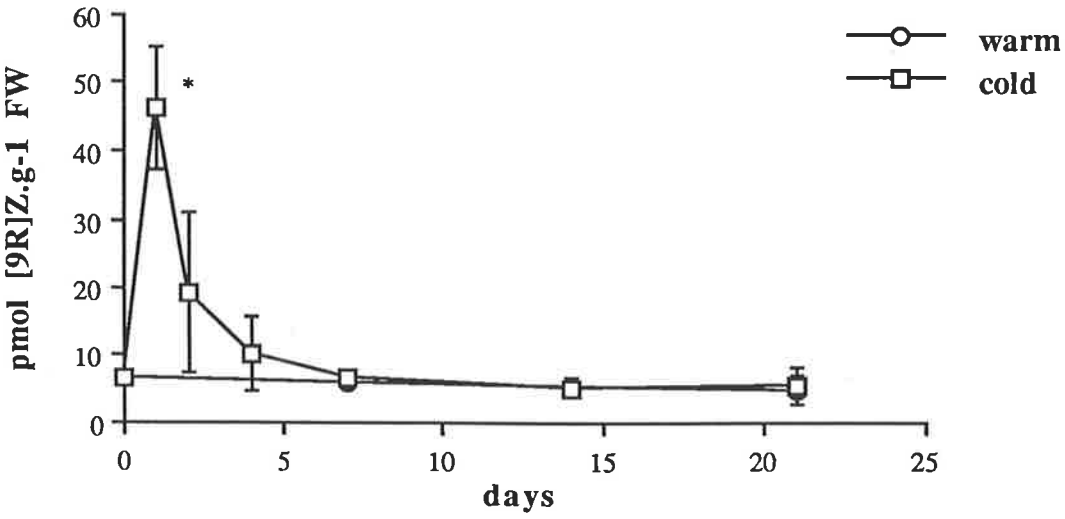
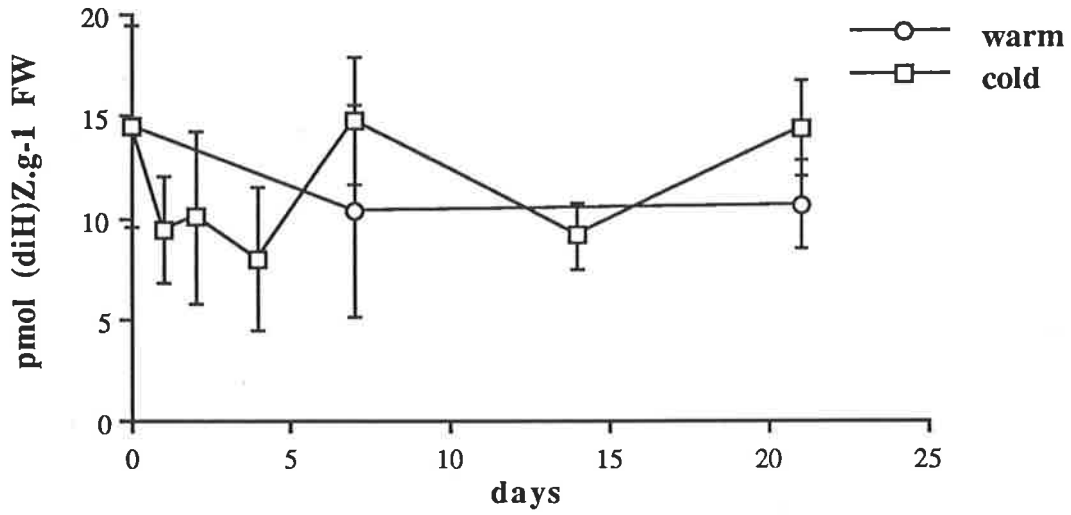


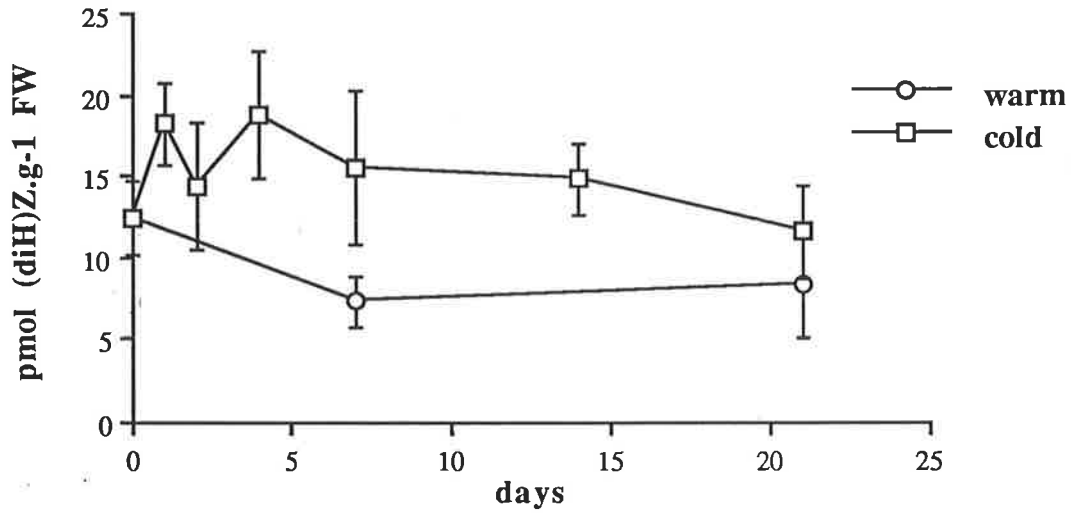
Figure 5.5 - Endogenous dihydro zeatin changes in *Boronia megastigma* during early flower development.

Plants were in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Values are mean ± standard error. * - significantly different from plants in warm conditions, combined day 0 and 7 values (p = 0.05).

a) Dihydro zeatin in leaf tissue.



b) Dihydro zeatin in stem tissue.



c) Dihydro zeatin in root tissue.

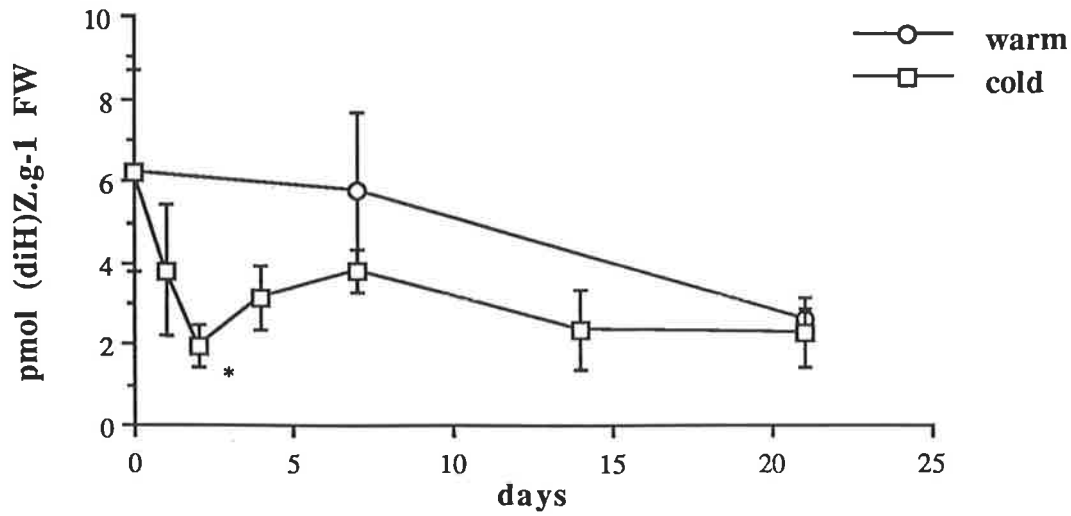
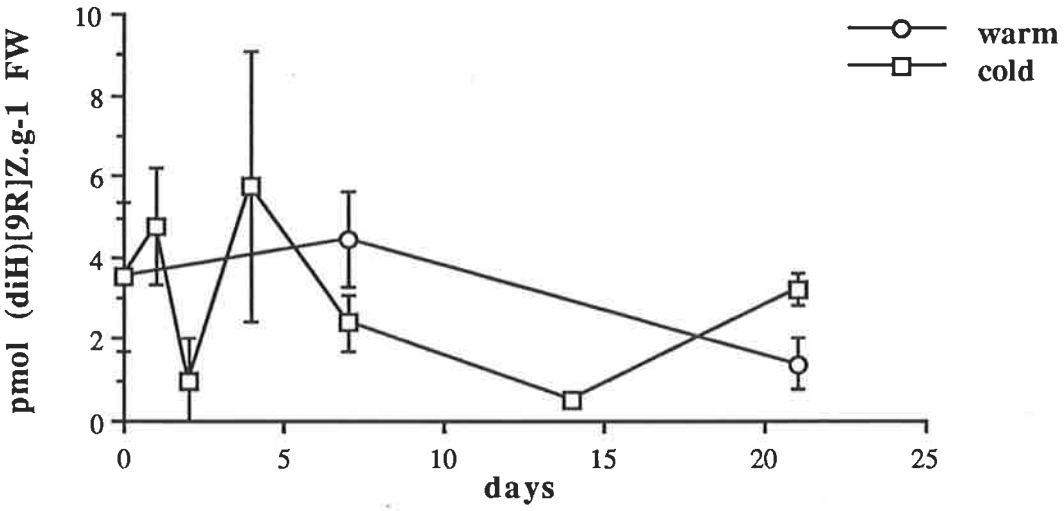


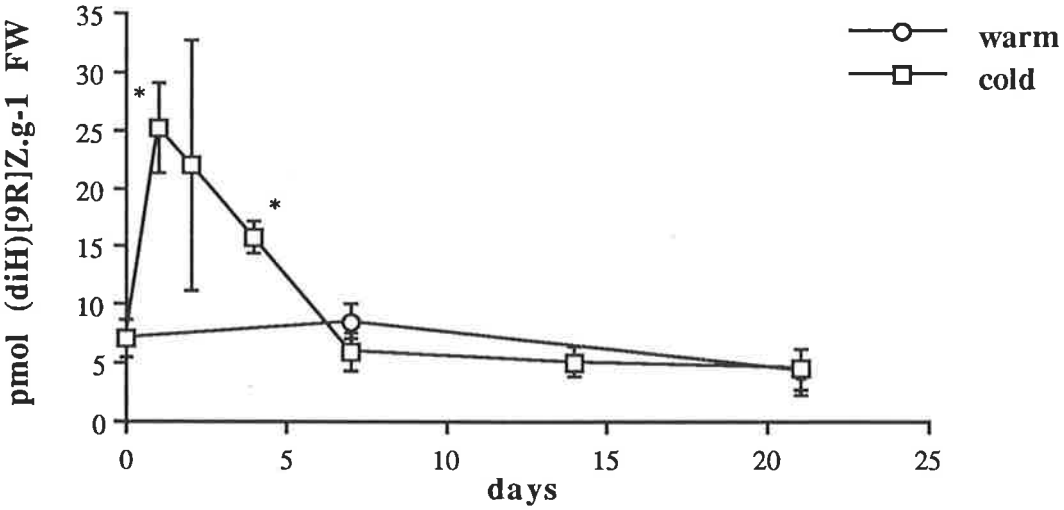
Figure 5.6 - Endogenous dihydro zeatin riboside changes in *Boronia megastigma* during early flower development.

Plants were in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Values are mean ± standard error. * - significantly different from plants in warm conditions, combined day 0 and 7 values (p = 0.05).

a) Dihydro zeatin riboside in leaf tissue.



b) Dihydro zeatin riboside in stem tissue.



c) Dihydro zeatin riboside in root tissue.

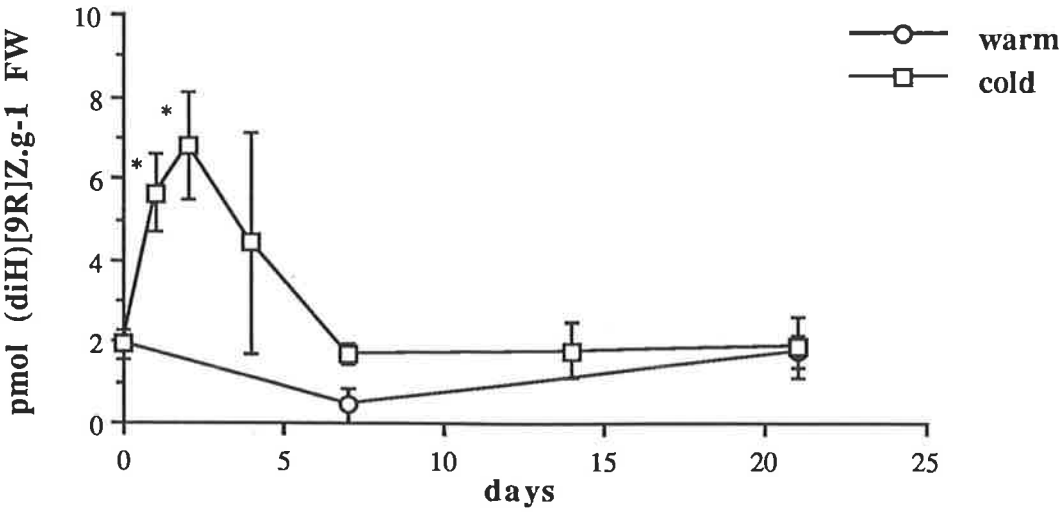
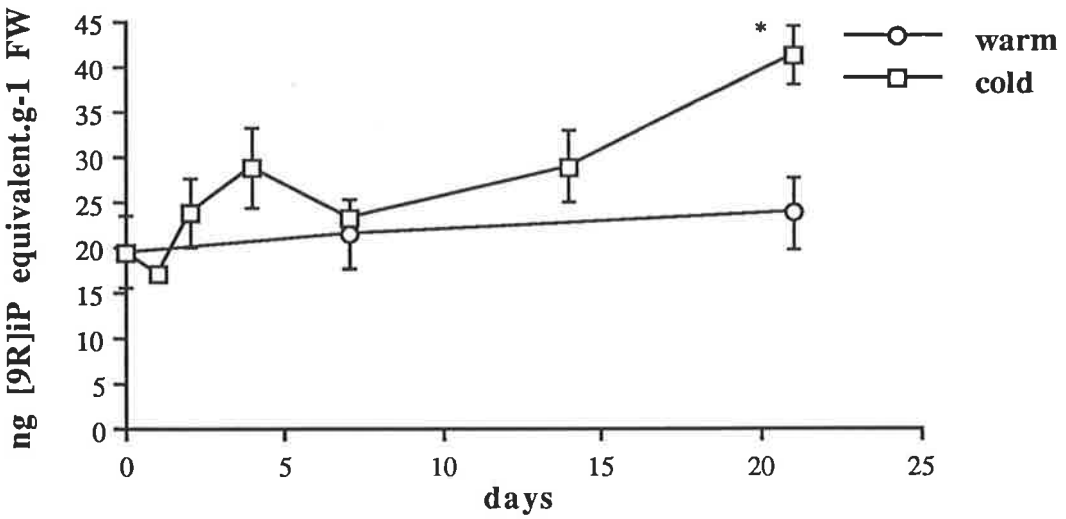


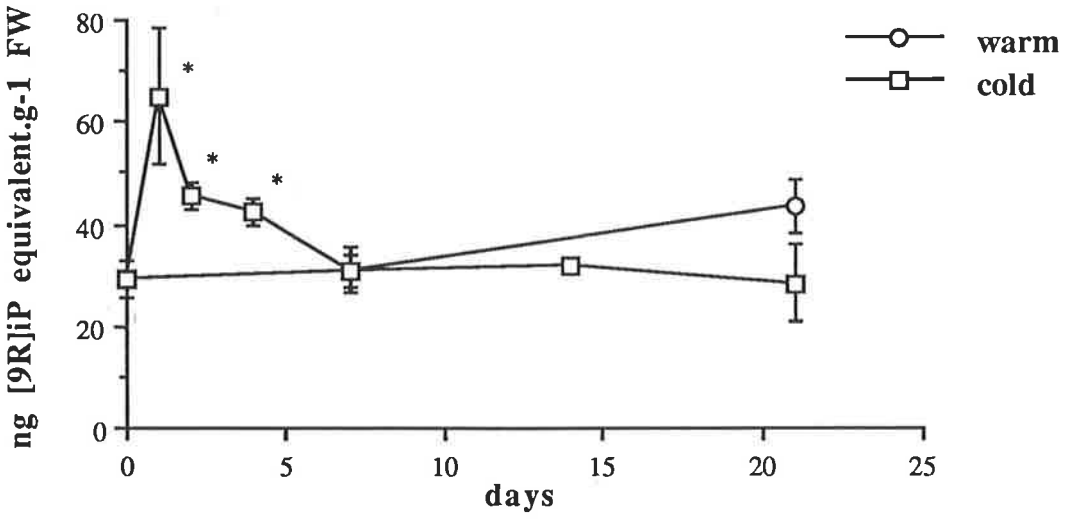
Figure 5.7 - Combined isopentyl adenine and isopentyl adenosine changes in *Boronia megastigma* during early flower development.

Plants were in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Values are mean ± standard error. * - significantly different from plants in warm conditions either at the same time period or combined day 0 and 7 values (p = 0.05).

a) Isopentyl adenine and isopentyl adenosine in leaf tissue.



b) Isopentyl adenine and isopentyl adenosine in stem tissue.



c) Isopentyl adenine and isopentyl adenosine in root tissue.

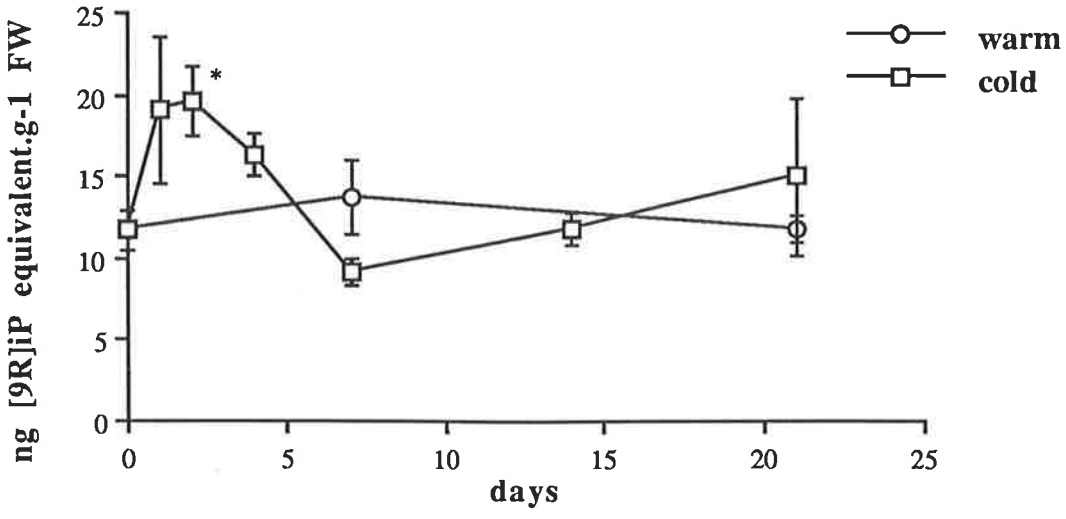


Figure 5.8 - Changes in the soluble sugar concentration of *Boronia megastigma* during early flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Benzyladenine treated plants grew in cool conditions. Total soluble sugars were measured in leaf and stem tissue against sucrose standards, thus measurement is in µmol(sucrose equivalent).g^{-1}TW, where TW (tissue water) = (fresh weight) - (dry weight). Values are mean ± standard error. * - significantly different from all other values of same time period (p = 0.05).

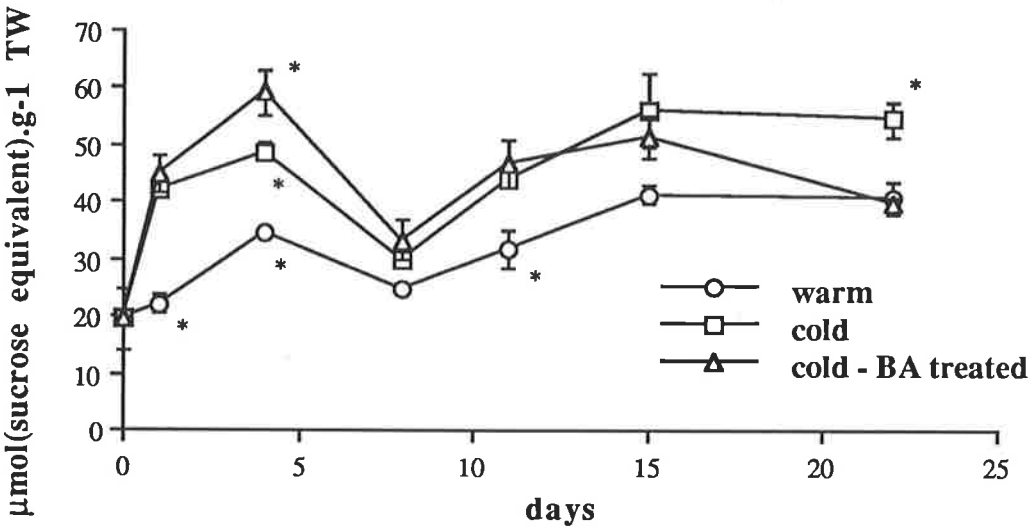


Figure 5.9 - Changes in starch concentration of *Boronia megastigma* during early flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Benzyladenine treated plants grew in cool conditions. DW = dry weight. Value are mean ± standard error. * - significantly different from all other values of same time period (p = 0.05). Starch concentration of plants in warm conditions was significantly different from plants in cool conditions at all time after day 0.

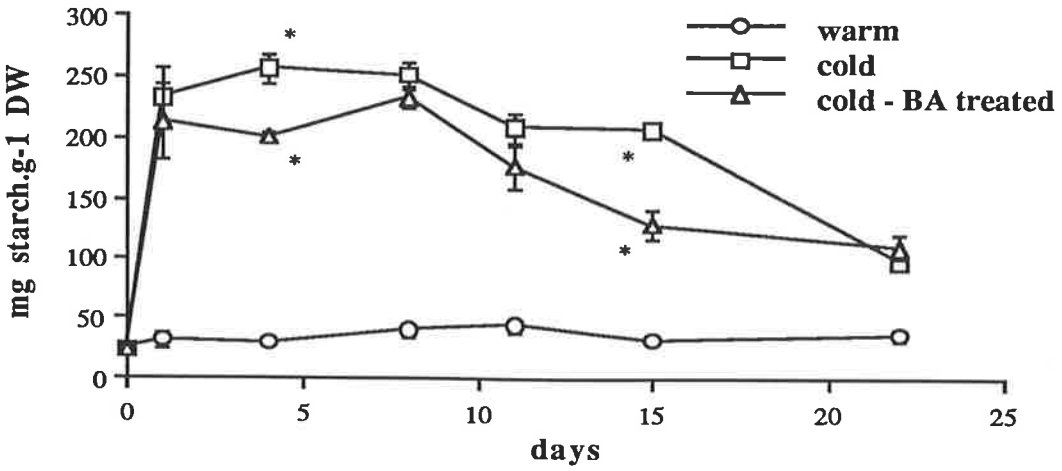
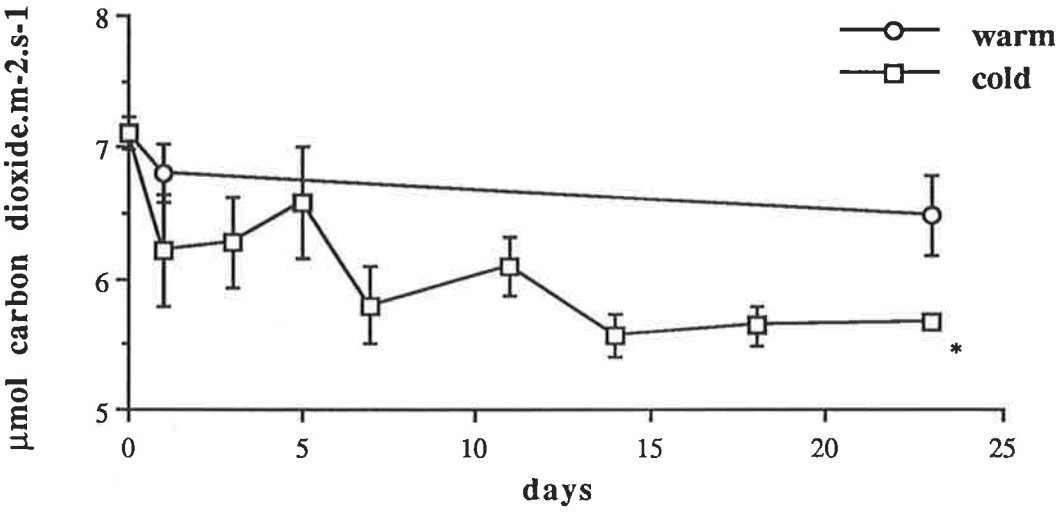


Figure 5.10 - Changes in net rate of photosynthesis or rate of carbon dioxide uptake of *Boronia megastigma* during early flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Value are mean ± standard error. * - significantly different from rate of CO_2 uptake of warm plants at same time period (p = 0.05).



Long term changes

The concentration of zeatin and zeatin riboside was measured in leaf and stem tissue throughout floral development (Figures 5.11 & 5.12). Zeatin and zeatin riboside concentrations were relatively constant in plants for the first ten weeks after transfer to cool conditions. *B. megastigma* plants are committed to flower after twelve to fourteen weeks in cool conditions (Chapter 3). This stage of flower development coincided both with the presence of flower buds large enough to enable measurements of cytokinins and carbohydrates separately from leaf and stem tissue, and the beginning of large changes in the endogenous concentration of cytokinins and carbohydrates. Thus after twelve weeks, carbohydrate and cytokinin concentration was measured in two tissue types, leaf and stem tissue and flower bud tissue, and in plants from three treatments, warm conditions, cool conditions and plants transferred from cool to warm conditions after commitment to flower (Figures 5.11 & 5.12).

There was a five fold increase in zeatin concentration in leaf and stem tissue between weeks ten and twelve in cool conditions and a similar increase at anthesis (Figure 5.11a). The

endogenous zeatin concentration was generally higher in flower bud tissue than leaf and stem tissue between fourteen weeks and anthesis both in plants remaining in cool conditions and those transferred to warm conditions (Figure 5.11a & b). In both transferred and non-transferred plants there was an increase in zeatin concentration in flower buds after commitment to flower followed by a decrease and then a further increase immediately prior to anthesis. At anthesis, there was no difference in the zeatin levels in flowers and leaf and stem tissue of plants in cool conditions.

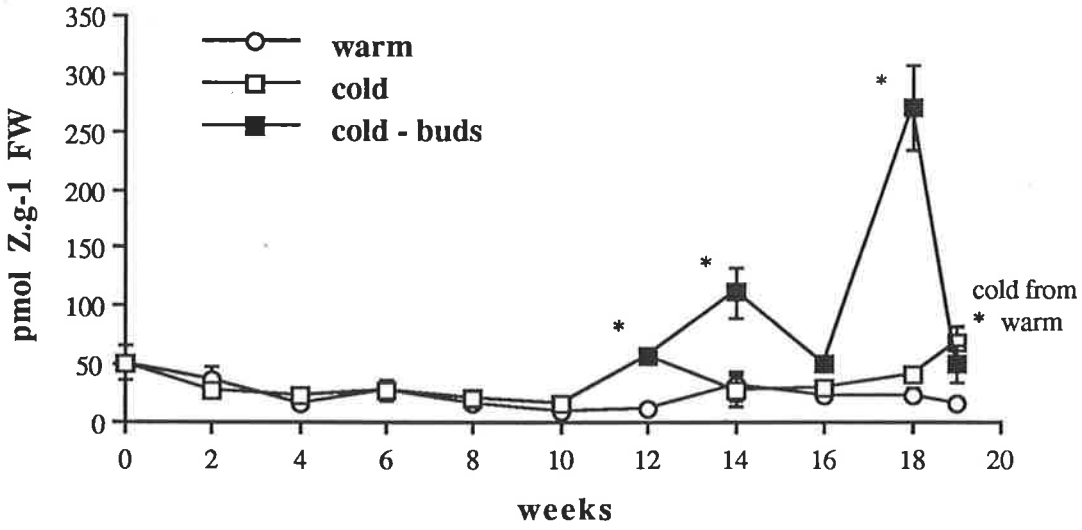
The level of zeatin riboside in leaf and stem tissue of plants in cool conditions increased by eight to ten fold between fourteen and sixteen weeks and subsequently declined over the next two weeks (Figure 5.12a). A similar increase and decrease occurred in the weeks prior to anthesis in leaf and stem tissue of plants transferred to warm conditions after commitment to flower (Figure 5.12b). The concentration of zeatin riboside remained low except at anthesis in flower buds of plants remaining in cool conditions. However, in flower buds of plants transferred to warm conditions after commitment to flower, the endogenous concentration of zeatin riboside was high from the stage of commitment to flower through to anthesis.

Soluble sugar concentration increased during the early stages of flower development in plants in cool conditions and remained at a high concentration for ten weeks, relative to plants in warm conditions (Figures 5.8 & 5.13a). A decrease to approximately 25% of the maximum concentration occurred between ten and fourteen weeks in cool conditions (Figure 5.13a). This decrease resulted in cool plants having approximately half the soluble sugar concentration of warm plants from fourteen weeks to anthesis. The same pattern of change in soluble sugar concentration was observed in plants in cool conditions treated with benzyladenine, however in this case the decrease in concentration occurred two to three weeks earlier then in plants not treated with benzyladenine. Figure 5.15 allows the pattern of change in the two treatments to be more easily seen by plotting concentration with respect to stage of flower development rather than time in cool conditions. The concentration of soluble sugars was low in all tissue, in both transferred and non-transferred plants, from the stage of commitment to flower through to anthesis (Figure 5.13a & b). At anthesis, the concentration of soluble sugars in leaf and stem

Figure 5.11 - Changes in zeatin concentration of *Boronia megastigma* throughout flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Plants were committed to flower after twelve weeks in cool conditions. After commitment, flower buds were large enough to allow measurement of the zeatin concentration separately from leaf and stem tissue. Value are mean ± standard error. * - significantly different from all other values at same time period (p = 0.05).

a) Plants remained in either warm or cool conditions. Anthesis occurred at nineteen weeks.



b) Plants were transferred from cool to warm conditions after commitment to flower at twelve weeks. Anthesis occurred at seventeen weeks.

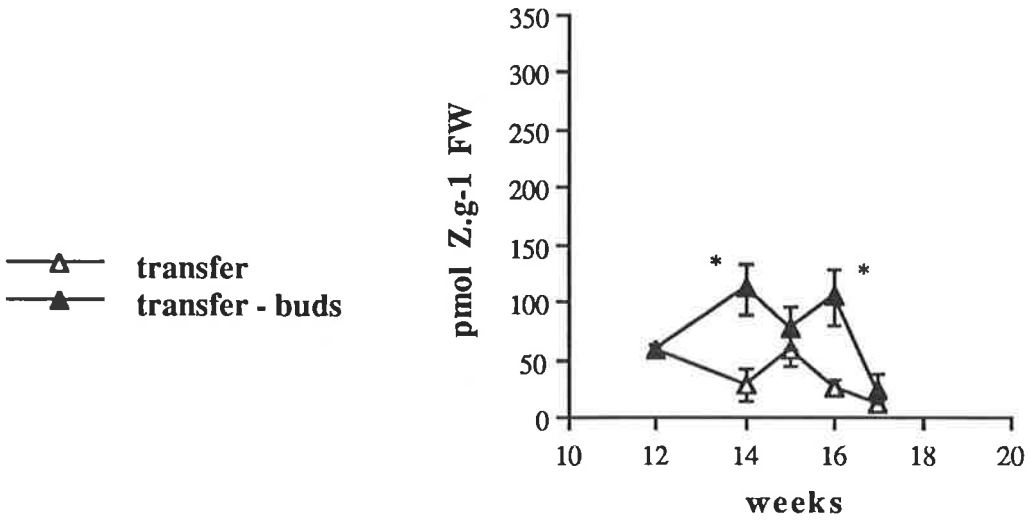
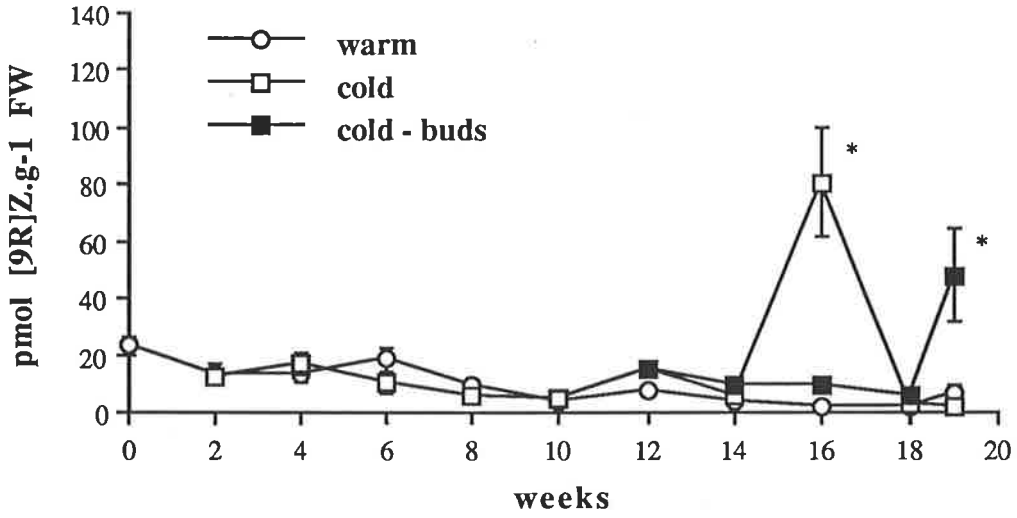


Figure 5.12 - Changes in zeatin riboside concentration of *Boronia megastigma* throughout flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Plants were committed to flower after twelve weeks in cool conditions. After commitment, flower buds were large enough to allow measurement of the zeatin riboside concentration separately from leaf and stem tissue. Value are mean ± standard error. * - significantly different from all other values at same time period (p = 0.05).

a) Plants remained in either warm or cool conditions. Anthesis occurred at nineteen weeks.



b) Plants were transferred from cool to warm conditions after commitment to flower at twelve weeks. Anthesis occurred at seventeen weeks.

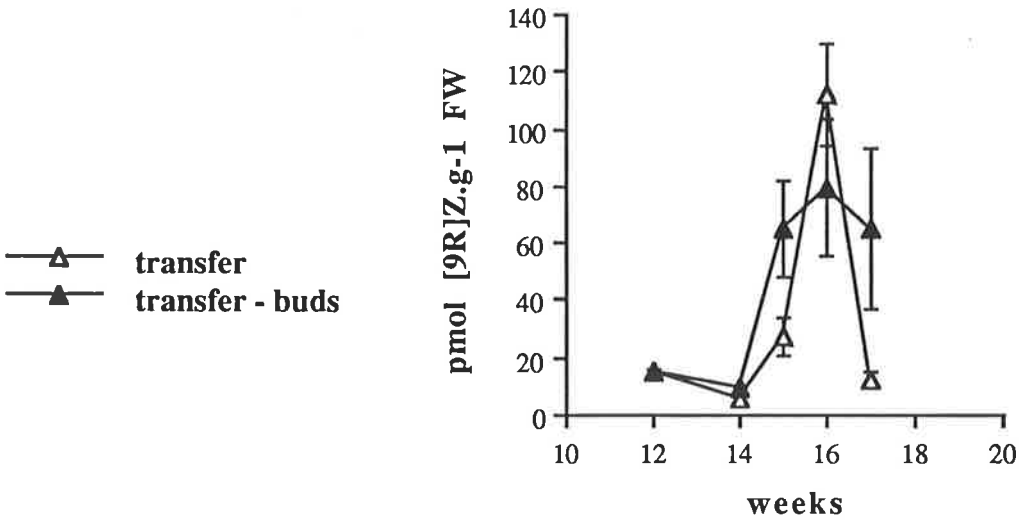
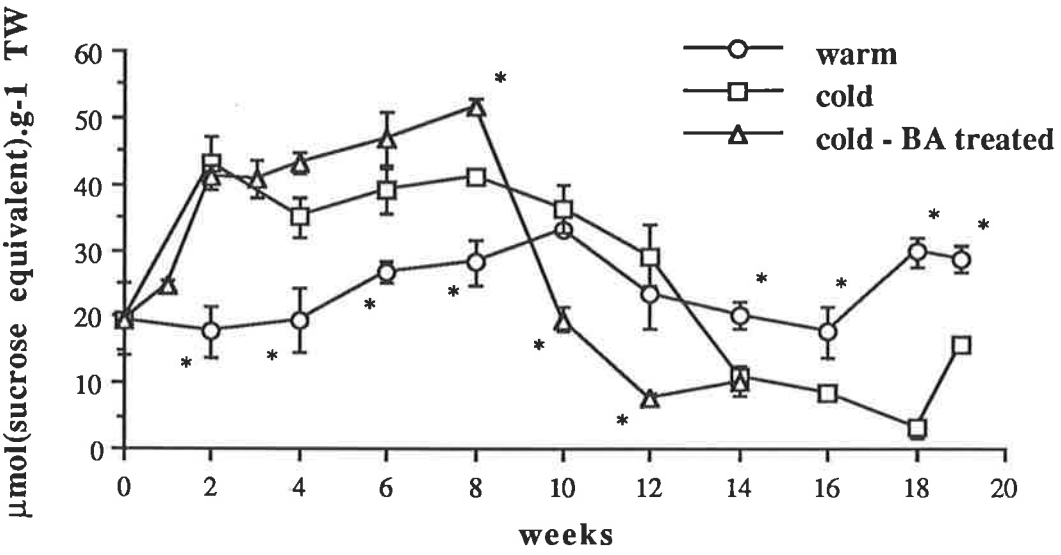


Figure 5.13 - Changes in soluble sugar concentration of *Boronia megastigma* throughout flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. Total soluble sugars were measured against sucrose standards, thus measurement is in µmol(sucrose equivalent) $.g^{-1}TW$, where TW (tissue water) = (fresh weight) - (dry weight). Values are mean ± standard error.

a) Plants remained in either warm or cool conditions. Benzyladenine treated plants grew in cool conditions. Anthesis occurred at nineteen weeks in non-benzyladenine treated plants and after fourteen weeks for benzyladenine treated plants. Measurements are from leaf and stem tissue. * - significantly different from all other values of same time period (p = 0.05).



b) Plants either remained in cool conditions or were transferred from cool to warm conditions after commitment to flower at twelve weeks. After commitment to flower, flower buds were large enough to allow measurement of the soluble sugar concentration separately from leaf and stem tissue. Anthesis occurred after nineteen weeks in cool conditions and seventeen weeks in plants transferred to warm conditions. * - significantly different from flower bud tissue at same time period (p = 0.05).

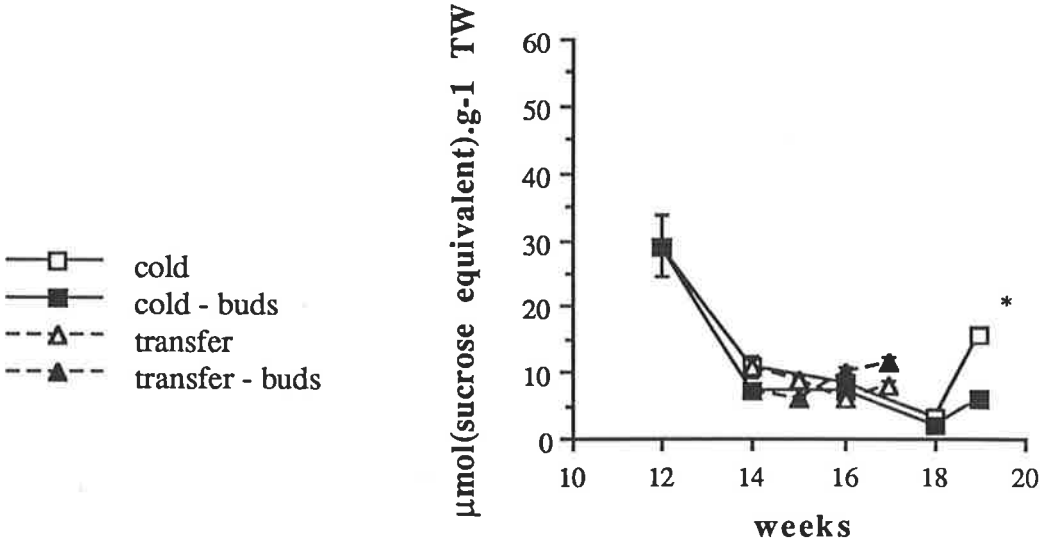
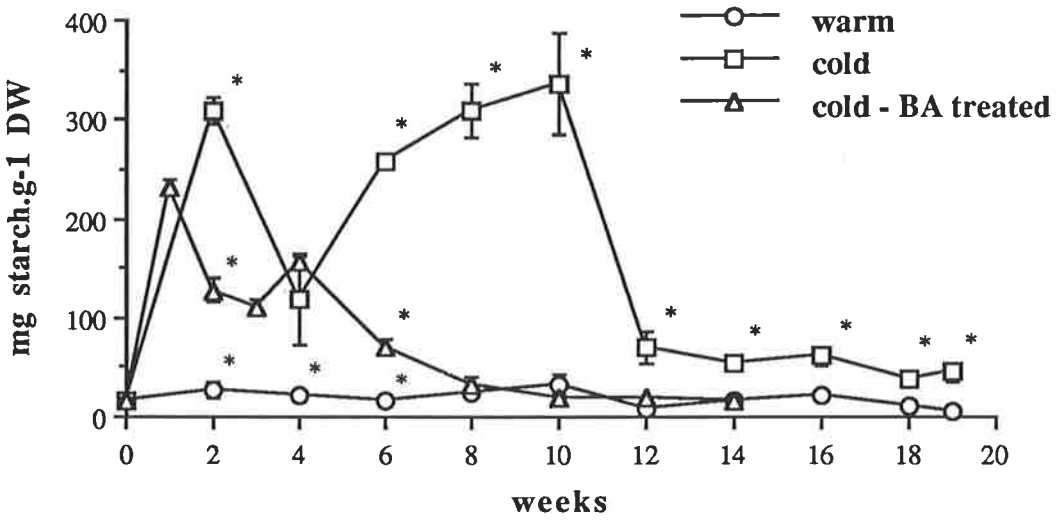


Figure 5.14 - Changes in starch concentration of *Boronia megastigma* throughout flower development.

Plants grew in warm (25°C day / 17°C night) or cool (17°C day / 9°C night) conditions. DW = dry weight. Value are mean ± standard error. * - significantly different from all other values of same time period (p = 0.05).

a) Plants remained in either warm or cool conditions. Benzyladenine treated plants grew in cool conditions. Anthesis occurred at nineteen weeks in non-benzyladenine treated plants and after fourteen weeks for benzyladenine treated plants. Measurements are from leaf and stem tissue. * - significantly different from all other values of same time period (p = 0.05).



b) Plants either remained in cool conditions or were transferred from cool to warm conditions after commitment to flower at twelve weeks. After commitment to flower, flower buds were large enough to allow measurement of the starch concentration separately from leaf and stem tissue. Anthesis occurred after nineteen weeks in cool conditions and seventeen weeks in plants transferred to warm conditions. * - significantly different from leaf and stem tissue at same time period (p = 0.05).

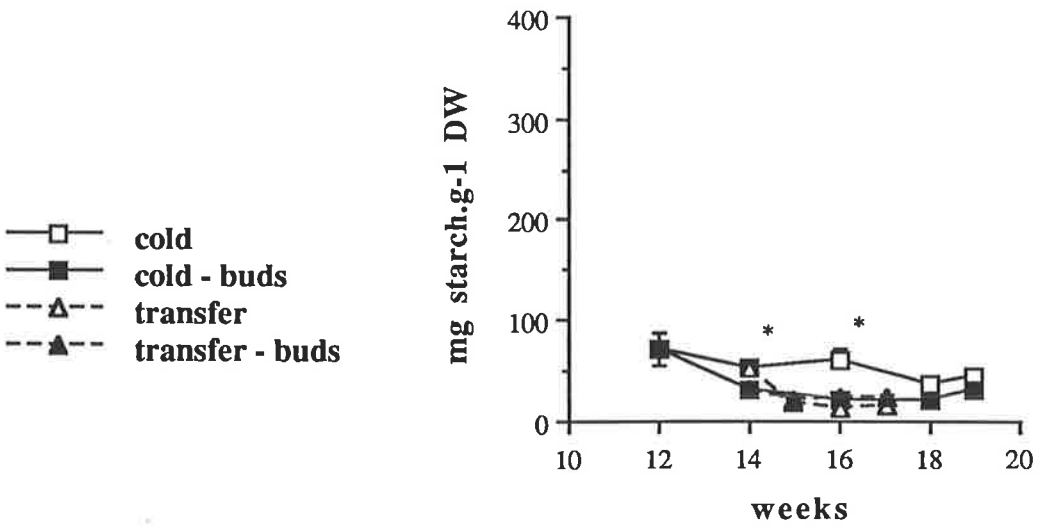


Figure 5.15 - Changes in soluble sugar concentration in leaf and stem tissue throughout *Boronia megastigma* flower development as defined by stage of flower development.

Plants grew in cool conditions (17°C day / 9°C night). Benzyladenine treated plants were compared with non-treated plants. Stages of flower development are defined in chapter 3. Total soluble sugars were measured against sucrose standards, thus measurement is in µg(sucrose equivalent).g^{-1}TW, where TW (tissue water) = (fresh weight) - (dry weight). Values are mean ± standard error.

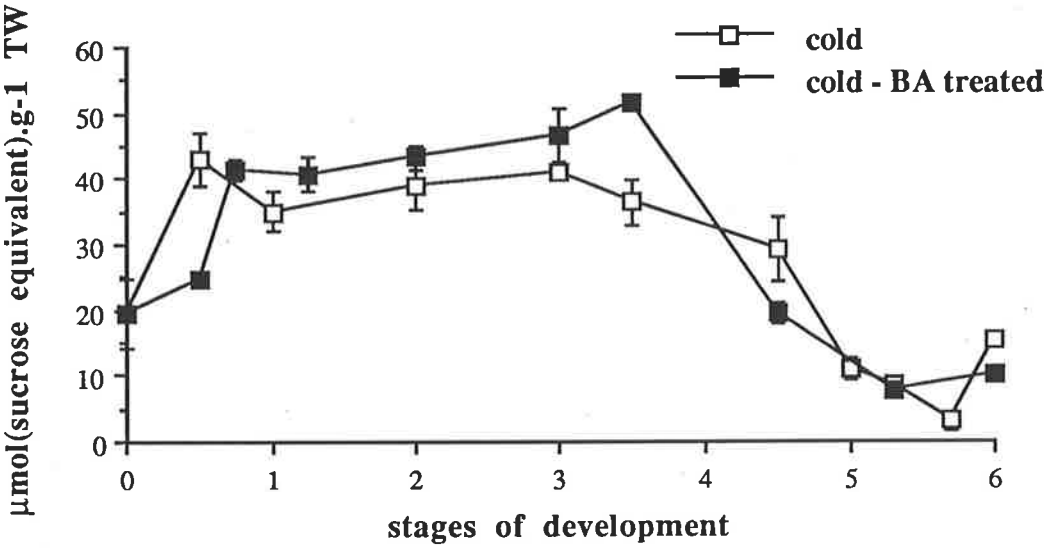
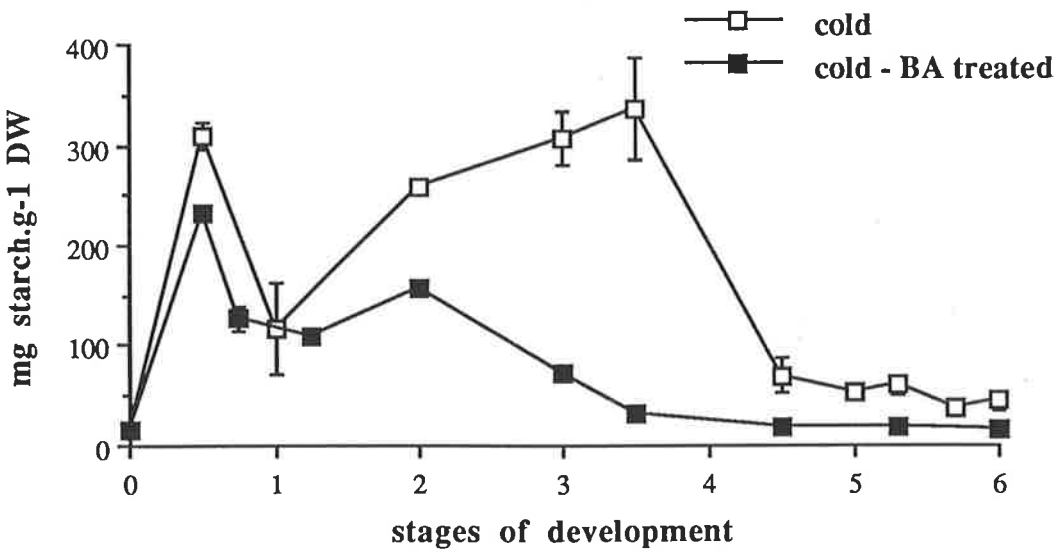


Figure 5.16 - Changes in starch concentration in leaf and stem tissue throughout *Boronia megastigma* flower development as defined by stage of flower development.

Plants grew in cool conditions (17°C day / 9°C night). Benzyladenine treated plants were compared with non-treated plants. Stages of flower development are defined in chapter 3. DW = dry weight. Value are mean ± standard error.



tissue of plants remaining in cool conditions was significantly greater than the level in flower bud tissue.

The changes in concentration of starch in benzyladenine and non-benzyladenine treated plants in cool conditions showed similar patterns of change although these changes were displaced in time with respect to one another (Figure 5.14a). As for soluble sugars, when starch concentration was plotted with respect to stage of flower development rather than weeks in cool conditions (Figure 5.16), the pattern of change can be more easily seen. Starch concentration in both treatments initially increased then decreased to between 30 - 50% of the maximum level by weeks three (for BA treated plants) and four (for non-treated plants) in cool conditions. This initial increase and decrease was more closely followed in figure 5.9. In non-treated plants, starch concentrations then increased again before decreasing dramatically between weeks ten and twelve at the stage of commitment to flower. Starch concentrations remained low from commitment to flower through to anthesis in all tissues in all plants (Figure 5.14b) although concentration remained higher in leaf and stem tissue of plants in cool conditions than in plants in warm conditions.

Discussion

Roberts *et al.* (1991) used RIA's to measure [9R]iP, and combined fractions of Z + [9R]Z and (diH)Z + (diH)[9R]Z in *B. megastigma.* Other than these measurements, I know of no measurements of cytokinins in *B. megastigma* or *H. angustifolium.* Table 5.1 extends our knowledge of the cytokinins present in these species. In addition to the presence of the more common cytokinins (Z, [9R]Z, iP & [9R]iP) there were dihydro, glucoside and mono-phosphate cytokinins present.

There was variation in the measurement of cytokinins using GC-MS (Table 5.1). There are a number of reasons for this variation, one being the low concentrations of most of the cytokinins in the tissue which resulted in a small peak for the native cytokinin (Figure 5.2). Consequently, any error in the determination of peak area would be large relative to the total

peak area leading to an error in the calculation of the ratio of native to labelled cytokinin. Further, the GC-MS study was not an exhaustive study and the number of replicates were few, if there were any replicates at all. There were not enough measurements to determine accurately the endogenous cytokinin concentrations using this technique and thus this study was primarily qualitative in nature. By contrast, the number of replicates and the sensitivity of the RIA method allowed more reliable estimates of cytokinins in *B. megastigma* (Figures 5.3 - 5.7, 5.11 & 5.12).

Short-term changes

During the short-term experiment, cytokinin and carbohydrate concentrations of plants in warm conditions were measured less frequently than of plant in cool conditions. All plants were allowed to adjust to warm conditions for two weeks prior to the commencement of the experiment. Thus, while not verified, it is reasonable to assume that concentrations of cytokinins and carbohydrates did not change during the course of the experiment in plants in warm conditions.

One day after plants were transferred to cool conditions, changes occurred in the cytokinin concentration in stem and root tissue (Figures 5.1 - 5.5). No significant changes were measured in leaf tissue after one day in cool conditions. Most of the changes were of cytokinin ribosides rather than the bases (Z, (diH)Z and iP). It is suggested that the free bases are the active form of cytokinins and the riboside are the transport form, and that the majority of cytokinin synthesis occurs in the roots from where they are transported to the rest of the plant (Letham and Palni, 1983). With this in mind, there are a number of explanations for the observed changes. Transferring plants to cold conditions may result in an increase in the production of cytokinin ribosides in the roots which are subsequently transported to the rest of the plant. Alternatively, cold temperature may affect cytokinin metabolism resulting in inhibition of cytokinin breakdown in root and stem tissue. Finally, there may be an inhibition of transport of cytokinins into the leaves of plants resulting in a build up of ribosides in the stem and root. Cold temperature does inhibit translocation of sugars out of the leaf in some

plants (Paul *et al.*, 1990), and may cause an inhibition of xylem translocation into the leaves. If this explanation is correct then a decrease in the cytokinin concentration in the leaf may be expected. However, plants have a large pool of protein bound cytokinin (Letham and Palni, 1983) which in the experiments may have maintained the free cytokinin at a constant level in the leaves if xylem translocation into the leaves was inhibited. Whatever the effect of cold on cytokinin transport or metabolism the return to pretreatment conditions was rapid and occurred within two to three days following transfer.

Transferring plants to cool conditions also resulted in a dramatic and rapid accumulation of starch and sugars in leaf and stem tissue (Figures 5.8 & 5.9). Accumulation of carbohydrates implies that the use of carbohydrates is inhibited more than their production (Paul *et al.*, 1990). Cool temperatures are known to affect sucrose metabolism enzymes (Zieslin and Khayat, 1990) and this may force the accumulation of carbohydrates. This is especially true since figure 5.10 shows that two weeks of cool conditions were required before the CO_2 uptake in cool plants was significantly less than in warm plants, and thus the production of assimilates presumably continued at a relatively constant level for the first two weeks in cool conditions. The accumulation of carbohydrates measured in *B. megastigma* plants probably resulted from a combination of the maintenance of photosynthesis, effects on sucrose metabolism enzymes and an inhibition of reactions that require carbohydrates.

A steady, lower rate of photosynthesis was reached by *B. megastigma* plants after two weeks in cool conditions. In some plants a steady lower photosynthesis rate is reached within thirty minutes of transfer to cool conditions (Lobate and Leegood, 1988), this being a straight temperature effect on photosynthesis. While there may have been a temperature effect on photosynthesis of *B. megastigma* plants within minutes of transfer to cool conditions, the long-term (weeks), stable, lower CO_2 assimilation rate (Figure 5.10) probably resulted from feedback inhibition of photosynthesis by high carbohydrate concentrations (Paul *et al.*, 1990).

Roberts and Menary (1990) state that starch is not the major storage form of carbohydrate when *B. megastigma* plants are transferred to cool conditions. Figure 5.9 shows that a great deal of carbohydrate is stored as starch. The proportion of carbon fed to plants as $^{14}CO_2$ and incorporated into starch was similar both before the transfer of plants and ten days

after transfer of plants to cool conditions (Roberts and Menary, 1990). The incorporation of ^{14}C into starch may have been low on day 0 because carbohydrate storage was not a priority in warm conditions when growth was favoured, and on day 10 because starch had accumulated to a maximum level (Figure 5.9). As a high concentration of starch would inhibit the further accumulation of starch, the comparison between days 0 and 10 by Roberts and Menary (1990) was inappropriate to evaluate the role of starch as the storage form for carbohydrates.

There is a possibility that both the cytokinin changes and the rapid accumulation of carbohydrates that occur within the first week after transfer to cool conditions are related to the beginning of differentiation of flowers in *B. megastigma* because the changes occur prior to this differentiation. However, it is unlikely that these changes are the 'signal' for flowering since the cytokinin and carbohydrate concentrations reach a stable state rapidly and remain relatively constant through to the stage of commitment to flower. It is most likely that the observed changes are a result of the transfer of plants to cool conditions *per se* rather than the signal for flower differentiation.

Long-term changes

Cytokinins

Figures 5.11 and 5.12 show that concentrations of Z and [9R]Z changed little during the first few stages of floral development, up to twelve weeks in cool conditions. This is in contrast to the situation in tomato where the [9R]Z level was found to decrease during flower development (Burch and Stuchbury, 1987), and in *Begonia* where the Z + [9R]Z concentration increased between zero and eight weeks in cool conditions during floral development (Hansen *et al.*, 1988). Roberts *et al.* (1991) found a general increase in Z + [9R]Z and (diH)Z + (diH)[9R]Z in non apical leaf and stem tissue of *B. megastigma* during the first ten weeks of floral development. The increase in Z + [9R]Z was not confirmed by our results.

During weeks ten to twenty in cool conditions there were many changes in the concentrations of zeatin and zeatin riboside (Figures 5.11 & 5.12). One hypothesis to account for these data is as follows. Between weeks ten and twelve, cytokinins may have been

transported from the roots to the aerial parts of the plant where they were converted to the active form Z and by week fourteen this cytokinin had reached the flower bud. Before week sixteen there may have been a large amount of [9R]Z transported to the top of the plant which by week eighteen was transported to the flower buds and converted to zeatin. Finally at anthesis there are at least two possibilities. Either zeatin was converted to the riboside form and transported out of the flower bud, possibly stimulating vegetative growth which occurs soon after anthesis (Table 4.7), or Z and [9R]Z may be active in the flower bud at different stages of development, zeatin at weeks 14 and 18 and zeatin riboside at week 19.

After flowers were committed, some plants were transferred to warm conditions to observe whether the measured changes in cytokinins could be related to the developmental stage of the flower rather than the environment of the plant. Both plants maintained in cool conditions and plants transferred to warm conditions, had a high Z concentration in flower buds immediately prior to anthesis and a high [9R]Z concentration both in the flower bud at anthesis and in leaf and stem tissue prior to anthesis. The only discrepancy in endogenous cytokinin concentration between these two treatments was that the [9R]Z concentration of flower buds in transferred plants was high well before anthesis, whereas the [9R]Z concentration in flower buds of plants that remained in cool conditions was low until anthesis (Figures 5.11b & 5.12b). This may suggest that there is some relationship between the concentration of [9R]Z and the increase in rate of flower development resulting from transfer of plants to warm conditions.

The cytokinin changes after commitment to flower can probably be related to the flower bud expansion that is occurring during this period. One of the major roles of cytokinins and the reason cytokinins were first recognised was by there ability to induce cell division (Letham and Palni, 1983). Thus during flower bud expansion, when cell division is rapid, it may be expected that the cytokinin concentrations would be high. Cytokinin increases are associated with the swelling of buds in apple (Tromp and Ovaa, 1990; Young, 1989), *Citrus* (Davenport, 1990) and *Pseudotsuga menziesii* (Pilate *et al.*, 1989). In these plants, bud swell occurs in warm conditions following a dormancy period in cool conditions, and the bud can be vegetative or floral. This situation is obviously different from flower bud expansion in *B. megastigma*,

however it has also been reported that maximum cytokinin concentrations in many *Citrus* species are found around the time of anthesis (Miñana and Primo-Millo, 1990), thus during the final stages of flower development.

Carbohydrates

B. megastigma plants treated with benzyladenine had a faster rate of flower development resulting in commitment to flower and anthesis after less weeks in cool conditions than was the case with plants not treated with benzyladenine. The pattern of changes in the concentrations of starch and soluble sugars between the two treatments showed good correlation when plotted with reference to the stages of flower development rather than the period of time in cool conditions (Figures 5.15 & 5.16). Figures 5.13 to 5.16 show an initial increase in both soluble sugars and starch, as has been reported in a range of species (Paul *et al.*, 1990; Schleppi *et al.*, 1990) including *Citrus* (Davenport, 1990). The soluble sugars remain at a relatively constant concentration for ten to twelve weeks in cool conditions (Figure 5.13) or until stage four of flower development (Figure 5.15). A decrease in soluble sugar concentration followed this until stage five of flower development after which a relatively constant low concentration of soluble sugars was maintained through to anthesis.

The starch concentrations followed a similar pattern to the soluble sugars, however there was a decrease in concentration prior to and during stage one of flower development (Figure 5.9) and a subsequent increase following this stage (Figures 5.14 & 5.16). Roberts and Menary (1990) found an increase in the percentage of carbon introduced to the plant as $^{14}CO_2$ partitioned into soluble sugars at a similar stage of *B. megastigma* flower development which they suggested was related to the beginning of floral differentiation. Both sets of evidence suggest that the beginning of floral differentiation in *B. megastigma* has a high demand for soluble sugars which are obtained both from photosynthesis and from carbohydrates stored as starch. In red clover, the carbohydrates required when plants are placed in conditions that induce flowering come from either an increase in the rate of photosynthesis or a mobilization of stored carbohydrates (Jones, 1990). These two sources for

carbohydrates were able to maintain a constant concentration of soluble sugars in *B. megastigma* plants throughout the period of flower differentiation (Figures 5.13 & 5.15).

The decrease in starch concentration between ten and twelve weeks in cool conditions or during stage four of flower development corresponds with the commitment to flower and the beginning of rapid flower bud expansion. This is a period of high energy use for the plant. The decrease in starch concentration preceded the decrease in soluble sugars as would be expected if the starch was being converted to soluble sugars during this period. When the carbohydrate stores were depleted, the soluble sugar concentration declined. The starch concentration in plants treated with benzyladenine declined long before stage four of flower development. The faster rate of flower development of these plants presumably increased the use of assimilates above the rate of photosynthesis thus resulting in a depletion of carbohydrate stores. This being so, it would be expected that the rate of flower bud expansion after stage four would be slower in benzyladenine treated plants due to the lower availability of carbohydrates.

After plants were committed to flower there was little change in the concentration of soluble sugars or starch. The expansion of flower buds presumably required all the carbohydrates produced by photosynthesis thus inhibited the accumulation of carbohydrates. Transferring plants to warm conditions after commitment to flower had little effect on the carbohydrate concentration. Higher concentrations of starch were measured in the leaf and stem tissue of plants maintained in the cold after commitment to flower than in other tissues or treatments (Figure 5.14b). In plants transferred to warm conditions after commitment to flower, vegetative growth was initiated before anthesis of flowers (Table 4.7). Carbohydrates in transferred plants would have been required for both floral development and vegetative growth thus depleting the starch concentration in these plants more than in non-transferred plants. Carbohydrates may also be involved in vegetative growth in plants maintained in cool conditions. The soluble sugar concentration in leaf and stem tissue of these plants increased immediately prior to anthesis (Figure 5.13b) and a flush of vegetative growth occurs after anthesis which suggests that the increase in soluble sugar concentration may be part of the signal or a result of the signal inducing vegetative growth.

Interaction

There is thought to be considerable interdependence between the distribution and concentration of cytokinins and carbohydrates because of the role of cytokinins in the mobilization of carbohydrates (Weaver and Johnson, 1985). During the latter stages of flower development in *B. megastigma* there was an association between the changes in carbohydrate and cytokinin concentrations. The stage of commitment to flowering is a time of dramatic change for the cytokinin and carbohydrate levels of the plant. The rise in Z concentration in leaf and stem tissue between ten and twelve weeks coincided with a large decline in starch concentration in the same tissue, thus the changes in zeatin concentration may be associated with the mobilization of carbohydrates from the starch reserves. There is an association between cytokinin concentration changes, changes in carbohydrate concentration and the developmental stage where flowers become committed at the beginning of a period of rapid bud expansion.

After the plants are committed to flower the changes in cytokinin concentration do not correlate with changes in carbohydrate concentration, although the concentration of cytokinins was generally high and the concentration of carbohydrates were maintained at a low level. It is possible there is some relationship between the concentrations of these substances. The high levels of cytokinins may mobilize the available carbohydrate ensuring that the concentration of carbohydrates remains low. Benzyladenine treatment of plants resulted in a lower starch concentration throughout most stages of flower development than in non-treated plants (Figures 5.14a & 5.16), suggesting that benzyladenine encouraged the mobilization of carbohydrates in these plants.

The results presented here indicate some of the associations that occur between the environment, cytokinin and carbohydrate concentrations and plant developmental changes. Temperature and cytokinins can influence the rate of flower development and this may be regulated by changes in endogenous cytokinin concentration which may then encourage the mobilization of carbohydrates necessary for the development of flowers.

CHAPTER SIX

GENERAL DISCUSSION

Plants growth regulator and carbohydrate interactions

Tables 4.5, 4.6 and 4.7 show that the effects of growth regulator treatment on floral development in *B. megastigma* varies with the time of application. Applied cytokinin increased the rate of floral development if treatment occurred at the time of transfer to cool conditions whereas cytokinin application after commitment to flower had no effect on the rate of development. Similarly, gibberellic acid application either decreased or increased the rate of development depending on the time of application in relation to commitment to flower. It is apparent that there are endogenous changes at the time of commitment to flower that affect the plant response to growth regulators.

One of the major differences between plants prior to and post commitment to flower was the carbohydrate status of the plant (Figures 5.13 - 5.16). Prior to commitment to flower the concentration of carbohydrates was high in *B. megastigma*. The application of gibberellins or cytokinins to plants at this stage of flower development would be expected to direct carbohydrates to the apical shoots or lateral floral buds respectively (Weaver and Johnson, 1985). It was suggested in chapter 4 that an increase in the rate of flower development resulted from direction of carbohydrates toward floral buds. This hypothesis rests on the assumption that the rate of floral development is determined by carbohydrate supply. In support, Roberts and Menary (1989b) found that the rate of *B. megastigma* flower development was faster in full sun than in 50% or 70% shade. Reducing the irradiance received by the plant presumably slowed the rate of development of flowers by decreasing the production of assimilates.

After commitment to flowering, the concentration of carbohydrates was low and the application of growth regulators had no significant effect on the rate of development of *B. megastigma* flowers (Table 4.7). However the rate of flower development of both *B.*

megastigma and *H. angustifolium* plants was increased by transfer to warmer conditions. These plants also initiated many vegetative shoots (Table 4.7), which would have competed with floral buds for the limited carbohydrate supply. The rapid rate of floral development and the initiation of vegetative shoots suggest that carbohydrate supplies were greater in plants in warm conditions than in cool conditions. There is an indication in figure 5.10 that more photosynthetic assimilation occurred in *B. megastigma* plants in warm conditions since the rate of photosynthesis of plants in warm conditions was greater than in cool conditions. Thus plants transferred from cool to warm conditions after they were committed to flower may have had more photosynthetic assimilate available for the rapid development of both flowers and vegetative shoots.

One explanation of the strategy employed by *B. megastigma* for the control of flower development is as follows. In evolutionary terms, since flower development occurs over many weeks, it is better not to be committed to flower until a later stage is reached. Then, if conditions change, flowers can be aborted rather than continue to develop in an unfavourable environment. The energy that would have been invested in the development of flowers could then be used for another purpose such as vegetative growth which could increase the number of sites at which flowers can develop at some later stage. Generally, if flowers may be aborted, it is best not to invest much energy during this early stage. *B. megastigma* flowers develop slowly at first and do not use much of the stored carbohydrates, however after all floral structures are present the flower becomes committed to further development and large amounts of the carbohydrate stores are invested (Figures 5.13a, 5.14a, 5.15 & 5.16).

H. angustifolium flowers were committed at an earlier stage of development than *B. megastigma* (Figure 3.3) and bud expansion began earlier (Chapter 2). It is likely that the carbohydrate stores would be mobilized at an earlier stage in this species. Alternatively there may be a less dramatic change in carbohydrate concentration associated with commitment to flower in *H. angustifolium* plants. *H. angustifolium* plants have the ability to flower on some stems and not others and flower development may occur at different rates on different parts of the plant. Thus, if conditions become unfavourable, *H. angustifolium* plants may not commit large amounts of energy to flowering because only a few flowers develop through to anthesis.

As stated above, commitment to flower represents a dramatic change for the plant. It is interesting to speculate on what brings about this change. In *B. megastigma*, changes in cytokinin and carbohydrate concentration are associated with the commitment to flower but are likely to be consequences of the change rather than the trigger. There is a change within the flower from differentiation of flower organs to bud expansion at the time of commitment (Chapter 2). In terms of the homeotic genes that control flower differentiation, commitment to flower in *B. megastigma* represents the end of floral organ determination by homeotic genes. There must be a regulator gene(s) defining an end to floral organ determination. This gene(s) or another regulator gene(s) may then 'switch on' genes which begin the changes that occur when a flower is committed. Alternatively, gene(s) specific not to the floral meristem but to tissue of one of the flower organs (eg: tapetum specific mRNA's, (Koltunow *et al.*, 1990)) may control commitment to flower. Thus commitment to flower would occur once this particular tissue containing the specific gene(s) was produced. Commitment to flower is likely to be a further example of a developmental process that is under multi-factorial control involving the interactions of genes, growth regulators and other substances.

Relevance to industry

Propagation

Because both *B. megastigma* and *H. angustifolium* root well from cuttings, the results in appendix 1 do not show how to improve rooting so much as they show what economies can be made in propagation procedures without sacrificing the rooting success. Maximum rooting of these species was obtained in those conditions where water loss from the cutting was reduced. However, rooting success was acceptable if tip rather than stem cuttings were used, if a pine-bark rather than a peat root medium was used and if cuttings were propagated in mist rather than fog. CO_2 enrichment did not aid propagation of these species and the application of auxin may only be necessary if less than ideal conditions are used for propagation. Many of the propagation conditions, while having little effect on the success of rooting, did affect the health

of cuttings and whether this would have a subsequent effect on the potting-on of rooted cuttings requires further testing.

Rooting success was affected by the time of year that cuttings were taken and care should be taken not to propagate from *B. megastigma* plant when they have flower buds in a late stage of development or from vigorously growing *H. angustifolium* plants. As suggested in appendix 1, a high endogenous concentration of gibberellins may be the reason for the measured reduction in rooting success of *H. angustifolium* cuttings taken during phases of vigorously vegetative growth. If this is the reason for poor rooting success, it would be interesting to test whether stem cuttings of vigorously growing plants root more successfully than tip cuttings since the majority of gibberellins are produced in shoot tips.

Potted plants

Growth regulators can be applied to alter plant shape and produce plants more attractive to the nursery industry (Keever and Foster, 1990). Both *B. megastigma* and *H. angustifolium* are not naturally bushy. Applications of TIBA and paclobutrazol to *H. angustifolium* plants was effective in controlling the length of internodes and producing a compact habit (Tables 4.1 & 4.2). Between 1mg and 2mg of paclobutrazol per pot was adequate for this purpose. An additional benefit of paclobutrazol was an enhancement of flowering in an environment unfavourable for flowering.

Paclobutrazol had little effect on the growth of *B. megastigma* plants unless very high doses were applied. Lewis and Warrington (1988) found *B. megastigma* 'Lutea' did not respond to either paclobutrazol or CCC applied as a soil drench. Benzyladenine and GA_{4+7} + BA treatment resulted in an increase in the branching of *B. megastigma* plants and produced a bushier plant (Table 4.3). Thus applications of growth regulators can be used to produce a more compact habit. Care must be taken to ensure that applications of growth regulators does not have a detrimental effect on the subsequent flowering of plants. For example, treatment with GA_3, BA or GA_{4+7} + BA two months prior to transfer of plants to flower promoting conditions resulted in a decrease in subsequent flowering (Table 4.6).

While growth retardants can be usefully applied to produce compact pot-plants, many countries have legislation banning the use of a number of these chemicals. Many chemicals that could enter ground water supplies and/or are slow to break down to non-active metabolites (eg: paclobutrazol) are banned in Germany and Scandinavian countries (von Hentig, pers. comm.,1991). It can be anticipated that a wider range of chemicals will be banned in many countries in the future. This provides additional incentive to increase our understanding of the natural controls of plant development, whether this be plant shape or flowering. Manipulation of plant growth and development using environmental conditions is much more desirable than using chemical means.

Control of flowering

Many of the species of plants from which cut-flowers are harvested are cultivated to produce flowers all year round by manipulating the growing environment or by treating plants with growth regulators (Goodwin, 1985). This form of intensive cultivation requires that the factors controlling flowering of these species be understood. For *B. megastigma* and *H. angustifolium*, the results in chapter 3 provide an appropriate understanding of the environmental control of flowering which would allow intensive cultivation of these crops. However, all year round flowering of these species would require the maintenance of cool temperatures for extended periods. More realistic control of flowering may be obtained by extending the natural flowering period both by increasing the length of time flowers remain open and extending the time of year over which anthesis occurs.

The period during which anthesis of *B. megastigma* and *H. angustifolium* plants occurred was extended by moving plants outside from a heated glasshouse throughout the winter (Tables 3.5 & 3.6). Treatment of *H. angustifolium* plants with paclobutrazol further extended the period of anthesis. In all treatments a decrease in the number of flowers did occur toward the end of the extended season (Table 3.6). Results from the application of benzyladenine to *B. megastigma* plants (Table 4.6) suggest that these plants may be able to be forced to flower earlier in the season. Care would be required to ensure that conditions were

cool enough for benzyladenine treatment, since in an intermediate temperature benzyladenine inhibited flowering rather than increasing the rate of flower development (Table 3.2).

Growth regulators and environmental treatments may be more usefully applied to time flowering of *B. megastigma* for specific days of the year. The rate of floral development can be increased and decreased both prior to and after commitment to flower (Tables 4.5, 4.6 & 4.7). Prior to commitment to flower, conditions that affect the distribution of carbohydrates may affect the rate of flower development, thus light conditions may be able to be used as well as growth regulator treatments to change the rate of floral development. After commitment to flower, temperature had most control over rate of flower development including the time flowers remained open. Anthesis may be manipulated to occur at any time from 17 to 44 days after commitment to flower by controlling the temperature of *B. megastigma* plants (19 to 42 days for *H. angustifolium*) (Table 4.7). Treatment of plants with growth retardants and other conditions that decrease the vigour of the post anthesis flush of vegetative growth may increase the time flowers remain open. Growth retardants may also inhibit ethylene synthesis (Grossmann, 1990) and thus these growth regulators plus ethylene synthesis inhibitors may be used to retard senescence and extend flower longevity.

In Tasmania there are plantations of *B. megastigma* grown for the extraction of essential oils from the flowers (Davies and Menary, 1983). The volume of essential oils extracted from *B. megastigma* flowers could be increased if a greater volume of flowers was harvested. A variety of environmental and growth regulator treatments failed to significantly increase the number of *B. megastigma* flowers (Chapters 3 & 4) however, after commitment to flower, the size of bud expansion that occurred was influenced by the application of growth regulators (Table 4.7). While benzyladenine and GA_3 treatments increased the size of flowers, they may not increase the amount of flower concrete or essential oils present and thus may not be usefully employed to increase the volume of extractable essential oils. In addition to increasing flower size, gibberellic acid treatment of plants after commitment to flower increased the length of the flower stalk. This may enhance the efficiency of harvesting flowers using hand combs (Davies and Menary, 1983) or mechanical harvesters.

Future work

Plant development and plant growth regulation are complex areas of study. The following section examines both some areas relating to this thesis that require further study, and some of the techniques that researchers are using and will use in the future to study plant development.

Areas of study

Flower evocation and initiation are processes that have been studied in a range of plants both because many plants require well-defined induction conditions for evocation and because evocation represents a dramatic change from vegetative to floral growth. In *B. megastigma*, dramatic endogenous changes occur when plants become committed to flower (Chapter 5). Commitment to flower in *B. megastigma* could be an area of increasing interest in the future because the changes can be well characterised. *H. angustifolium* would be less useful in this regard because the variability in timing of flower evocation and development hinder accurate characterisation of the changes that occur.

To more accurately determine the role of cytokinins in flower bud expansion in *B. megastigma*, measurement over time of a greater range of cytokinins would be required. This would allow further understanding of the role of those cytokinins detected with GC-MS (Table 5.1). Authors have shown that cytokinins that are considered to be less biologically active than Z and [9R]Z can be the most abundant in plant tissue (Scott and Horgan, 1984) and these cytokinins may play an important role in some developmental processes (McGaw *et al.*, 1985), thus the measurement of a wider range of cytokinins is often important. More accurate measurement of the changes that occur in Z and [9R]Z concentration may enable a greater understanding of the role of these cytokinins in the distribution of sugars or other substances. One technique that could greatly enhance this work is immuno-localization, which would allow a more accurate understanding of the distribution of cytokinins as well as changes in concentration.

In *H. angustifolium*, the role of gibberellins in controlling flower initiation and development is an obvious area that requires more study. An associated area of study would be the relationship between endogenous gibberellin concentration and vigorous vegetative growth. Both encouraging vegetative growth and treating plants with gibberellic acid are related to an inhibition of propagation (Figure A1.2d, Table A1.1) and flowering (Table 4.4). It would be interesting to compare *H. angustifolium* and *Chamelaucium uncinatum* (Shillo *et al.*, 1985) since in both plants gibberellins play some role in the initiation and development of flowers but cool temperatures and photoperiod respectively control floral induction. Two different systems of floral induction may use the same signal (eg: gibberellins) to evoke flowering.

An equally useful area of research would be wider investigation comparing the results presented here to results from other Australian native plants. Flowering in many other Australian plants is influenced by cool temperature. I have observed that other *Boronia* and *Hypocalymma* species, some *Verticordia* species and some *Thryptomene* species flowered in an unheated glasshouse but fail to flower in a heated glasshouse. It would be expected that there are similarities in the flowering of these species since the environmental control of flower induction may be the same and because the evolution of many of these species has been influenced by the same environmental conditions.

Current and future research techniques

In the last few years techniques have been developed that have greatly enhanced the study of plant development.

In plant growth regulator research the development of new separation and detection techniques using chromatography and spectrometry (eg: HPLC & GC-MS) have allowed the discovery of an extended range of compounds and smaller amounts of tissue are necessary for the detection of these compounds (Horgan and Scott, 1991). This has allowed greater accuracy in the determination of the synthesis, metabolism and distribution of plant growth regulators. The accuracy in measuring growth regulators has improved though the use of isotope dilution analysis in association with RIA and GC-MS techniques (Chapter 5). Isotope feeding

experiments, where radioactive chemicals are introduced into plants, have been used to study the endogenous metabolism of those chemicals.

Faster measurement of growth regulators in plant extracts has been possible using antibodies to the growth regulators. Radio-immuno assays and ELISA methods have been developed for many of the most common growth regulators (Weiler, 1984). Mono-clonal and poly-clonal antibodies have allowed simultaneous measurement of a range of plant growth regulators in the same plant extract which allows a greater understanding of the interactions between them.

With an increase in the number of available techniques for measuring growth regulator concentration has come controversy over how much information is required to positively identify an individual compound. Reeves and Crozier (1980) use information theory to define the number of 'bits' of information required to identify a compound from all others in a plant extract and conclude that chromatographic analysis is adequate. Scott (1982) argues that chromatographic analysis would only be adequate when the the compound in question is one of a group of known compounds. However in a plant extract, where not all compounds are known, structural information of the compound must be used in addition to chromatographic techniques to positively identify the compound. Thus immunological or spectrometric techniques that give some information about the structure of the compound must be used to provide enough 'bits' of information.

Further advances in antibody techniques will allow the development of reliable methods for the localization of plant growth regulators, for example the use of fluorescence or transmission electron microscopy (TEM) for the *in vivo* detection of growth regulator-mono-clonal antibody-marker complexes (Miginiac and Sossountzov, 1990). In this technique, mono-clonal antibodies which bind to a particular growth regulator are introduced to plant material. Markers which bind to the antibody are then introduced and the complex can be detected using a microscope. Researchers will be able to use these techniques to measure the amount of a growth regulator as well as it's position within the cell and within tissues, thus increasing our knowledge of where growth regulators reside and where there act. There are problems with immuno-localization techniques for growth regulators at the moment. Because

growth regulators are soluble, the fixatives currently used to fix tissue for immuno-localization removes the growth regulator from the tissue.

Associated with immuno-localization of growth regulators will be the localization of growth regulator receptors. Localization of receptors will again show where growth regulators act but also may give information about changes in growth regulator receptor numbers and the change in 'competence' of tissue. The immuno-localization of growth regulator receptors requires the isolation of these receptors. The most promising techniques for the isolation of receptors employ chemical or photo-affinity labelling. Photo-affinity labelling has been used to gain information about ABA binding site in protoplasts (Roberts and Hooley, 1988). These techniques use chemical or photometric means to alter a ligand (which may be a growth regulator) to form a reactive site which may co-valently bind to the receptor. The ligand-receptor complex can then undergo rigorous purification and separation procedures (Roberts and Hooley, 1988). In addition to allowing the isolation of binding sites, affinity labelling can give information on the molecular structure of the sites.

Cytokinin binding proteins (Romanov *et al.*, 1990), and possibly auxin receptors (Jones, 1990) have been found and the localization of receptors may occur in the near future. When the genes which control the production of growth regulator receptors are found they may be able to be manipulated to change the receptivity of tissues to growth regulators. If receptors specific to particular organs are found, great control over the development of particular organs may be achieved. For example, the longevity of flowers would be greatly enhanced if the receptivity of flower tissue to ethylene could be reduced and the use of chemicals for this purpose could be abandoned.

The availability and sophistication of molecular biology and molecular genetics techniques are increasing rapidly and the adaptation of these techniques to plant developmental study has proceeded at a similar pace. Dutcher (1989) states that herbicide resistance and flower colour are the most likely candidates for genetic manipulation because they only depend on single gene changes. Many other features of plant development (eg: flower evocation and development) will be much more difficult to manipulate because many genes interact to influence the feature of interest.

For many years experiments have been conducted with mutant plants. These plants provide and will continue to provide a unique opportunity to study the effect of different genes on plant development. For example, mutants that are deficient in the gene(s) responsible for the production of growth regulators provide useful plants from which those genes can be characterised. The genes can then be reinserted into plants with promoters that increase the expression of the genes in specific places or at specific times throughout development to determine the effect of that growth regulator. This has been accomplished by inserting an isopentenyl transferase gene into *Arabidopsis* (Medford *et al.*, 1989). The key to gaining reliable results using this technique is to use non-leaky promoters such that expression can be controlled in time and place.

Characterisation of a gene would also allow the construction of antisense DNA which when inserted in a plant can inhibit the expression of the natural gene. This technique has been used to alter the flower colour in Petunia's (Daggard *et al.*, 1990) and retard ripening in tomatoes (Speirs and Brady, 1991). One advantage of this technique is that genes with a specific action and residing in a specific tissue can be targeted and thus other tissues and functions of the plant can remain normal. As for the insertion of characterised genes, the anti-sense genes could be inserted with a specific promoter allowing the production of anti-sense mRNA at specific times and specific places (Speirs and Brady, 1991). For example, anti-sense mRNA for endopolygalacturonase has been inserted into tomato (Speirs and Brady, 1991). This insertion has no effect other than to reduce the softening process during fruit ripening.

Many of the molecular manipulation techniques require that the plant in question can be transformed and this is likely to be the next step toward the use of this techniques for the investigation of native plants.

APPENDIX ONE

PROPAGATION FROM CUTTINGS OF *BORONIA MEGASTIGMA* AND *HYPOCALYMMA ANGUSTIFOLIUM*

Introduction

Compared to traditional ornamentals such as roses and carnations, Australian plants have been cultivated for a relatively short period of time and methods of propagation are still being defined and refined. Many highly desirable species are extremely difficult to propagate vegetatively and their commercial potential is therefore restricted. Many factors can influence the number of cuttings that form roots. Factors such as time of year that cuttings are taken, treatment of cuttings with auxins, from where on the bush cuttings are taken, the propagation medium and the type of environment in which cuttings are placed can all influence propagation success (MacDonald, 1986). In addition, the treatment of stock plants in various ways can influence the number of cuttings taken subsequently that form roots (Moe, 1985). In the following chapter I have tested these factors to determine those conditions necessary to maximise the number of cuttings of *B. megastigma* and *H. angustifolium* that form roots.

Just as external conditions can influence the percent of cuttings that root, there are features unique to individual plant species that can affect propagation from cuttings. Williams (1984b) found that the amount of suberization of the cortex can be directly related to poor propagation in a range of Australian native plants. More cuttings had roots from species of plants with less suberin than plants with more suberin surrounding the cortex. The success of propagation in this example is genetically determined rather than being determined by the conditions of propagation. *B. megastigma* has been found to root easily (Sharma and Ollerenshaw, 1989) as does *H. angustifolium*, thus propagation from cuttings of these species will depend more on external conditions than on genetic factors.

Methods

Growth conditions

Stock plants were propagated and grown as described in chapter 2. Propagation houses were located in glasshouses where the average monthly minimum temperature ranged from 13.7°C to 18.3°C and the average monthly maximum temperature from 24.6°C to 28.1°C (for temperatures see table 3.1). During summer the glasshouses received 50% shade from shade cloth. The beds of the propagation houses were heated to 22°C during winter.

Monthly propagation

In the experiment where cuttings were taken monthly throughout the year (Figures A1.1 & A1.2) six stock plants of each species were kept outside in 50% shade. Twenty cuttings were taken from each of three plants per month and placed in pine-bark mix (three parts composted pine-bark and one part each of coarse river sand and fine horticultural sand, pH of 5.2) in three pots (1 litre) and propagated in mist, thus cuttings were taken from each stock plant every two months for fourteen months.

H. angustifolium cuttings were approximately 8 cm long with 5-6 internodes whereas *B. megastigma* cuttings were 5-6 cm long but also with 5-6 internodes. The basal third of all cuttings was stripped of leaves, branches and, in winter, flower buds. This part of the cutting was inserted into the propagation mix.

Pre-treatment of stock plants with growth regulators

Three stock plants per treatment were grown in a warm glasshouse and were treated with growth regulators two months before cuttings were taken. Gibberellic acid (GA_3) and benzyladenine were donated by Abbots Laboratories and were sprayed until run-off onto plants in three treatments one week apart at a concentration of $100mg.l^{-1}$. Paclobutrazol was donated by ICI Australia and was applied in one 50ml dose as a soil drench, at concentrations of $200mg.l^{-1}$ to *H. angustifolium* plants and $1000mg.l^{-1}$ to *B. megastigma* plants. Treatment of stock plants affected the length of internodes. Cuttings were taken from all treated plants with

the same number of internodes, thus cuttings from GA_3 treated plants were longer than control cuttings and cuttings from benzyladenine and paclobutrazol treated plants were shorter than the control. Fifteen cuttings were taken from each plant and placed in pine-bark mix in 30x25x5 cm trays, 90 cuttings per tray.

Other treatments

For all other experiments, cuttings were taken from stock plants in an unheated glasshouse during April before floral differentiation had begun. Three groups of fifteen cuttings were used for each treatment and placed in 30x25x5 cm trays, 90 cuttings per tray.

Auxin - The effect of auxin on the propagation of the two species was tested by dipping the basal third of treated cuttings for ten seconds in a 50% ethanol solution containing $1000mg.l^{-1}$ NAA and $1000mg.l^{-1}$ IBA. All cuttings in all treatments, unless otherwise stated, were treated in this way.

Carbon dioxide - In that experiment where the atmosphere was enriched with CO_2, 750ppm CO_2 was provided in a fog house identical to that described below.

Mist or fog propagation - Cuttings in mist were sprayed for 5 seconds every 35 minutes, delivering 1.25 litre/hour onto $1m^2$ of bench. In the fog house a 20 second spray with droplet size less than 50µm was used to maintain the humidity between 90% and 95%. An array of humidity sensors were connected to a computer controlling system which regulated the frequency of the fog. Benches in the fog house covered 12 m^2, approximately half the total ground area. Cuttings in the mist propagator were sprayed every two weeks with 1.5 g/l Rovral (May & Baker) to control fungal growth. This was not required in the fog houses.

Propagation medium - Cuttings were placed in one of two media, one using peat as a base the other using pine-bark. The peat mix had a pH of 4.4 and consisted of one part by volume each

of peat, perlite and coarse river sand. The pine-bark mix had a pH of 5.2 and consisted of three parts composted pine-bark and one part each of coarse river sand and fine horticultural sand.

Tip or stem cuttings - When tips of cuttings were removed this included the two visible immature whorls of leaves. For both *B. megastigma* and *H. angustifolium* the immature leaves are easily identified as those more or less parallel with the stem rather than perpendicular to it. The stem cuttings were taken from the stem immediately basal to the tip cutting, approximately 7-13 internodes back from the shoot tip. This section of stem, while being more rigid than the tip, was not woody.

Cultivar differences - *B. megastigma* 'Heaven Scent ' was used for all experiments except where *B. megastigma* 'Chandleri' cuttings were used as a comparison.

Measurements

H. angustifolium cuttings were propagated for eight weeks and *B. megastigma* cuttings for ten weeks. After this time the roots were shaken free of soil, counted and their maximum length measured. Cuttings were considered to have rooted if any roots were visible. The percent top growth represents the number of cuttings with new vegetative growth. This growth occurred from the axils of leaves rather than the tip of the cutting.

Where appropriate, data were initially analysed using Analysis of Variance (ANOVA). When significant differences were found ($p = 0.05$), LSD values were calculated using the Fisher's least significant difference test (Sokal and Rohlf, 1981). Duncan's multiple range tests were conducted on the data from cuttings taken monthly throughout the year to discover whether for any measurement, the data from any group of months could be distinguished from that of other months.

Results

Monthly propagation

Cuttings of *B. megastigma* propagated throughout the year showed significant changes between months in the number of roots per cutting, the length of the roots and percentage of cuttings with roots. There were no significant differences in the number of cuttings with top growth (Figure A1.1). It can be seen from the graphs and from the Duncan's multiple range test that for *B. megastigma*, the differences in number of roots and length of longest root between months were scattered throughout the year and showed no relationship to particular seasons of the year. For the percent of cuttings with roots there is a strong relationship with time of the year. The Duncan's multiple range test shows that during the months of late winter and early spring the percent of cuttings with roots was significantly lower than the percentage at all other times of the year.

All measurements of *H. angustifolium* cuttings showed significant differences between some months when propagated throughout the year. Figures A1.2a,b,c show that again the differences in number of roots per cutting, the length of the longest root and percentage of cuttings with top growth between months were scattered throughout the year and showed no relationship to particular seasons of the year. These conclusions are supported by the Duncan's multiple range tests. The time of the year cuttings were taken did affect the percentage of cuttings with roots, however the relationship was the inverse of that found with *B. megastigma*. During the colder months of the year, almost 100% of cuttings had roots, however during the warmer months there was much variation in the percentage of cuttings with roots. Not only was there variation between months of the year but also within months when the percentage of cuttings with roots was low, shown by the large standard errors in those months.

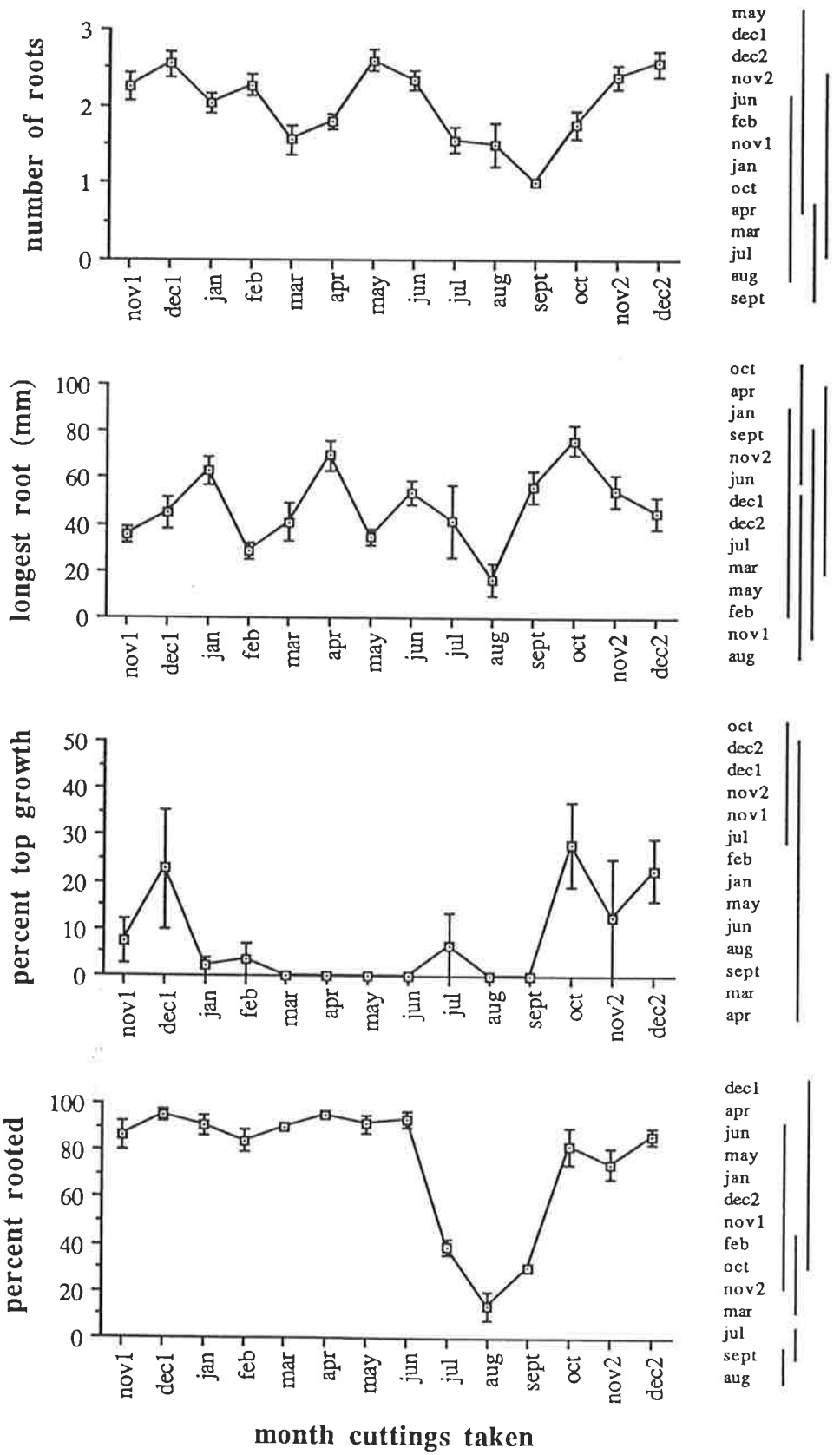


Figure A1.1 The number of roots (a), length of longest root (b), percentage of cuttings with top growth (c) and percentage of cuttings with roots (d) of *B. megastigma* cuttings taken throughout the year. The cuttings were taken from stock plants on the month shown. Values are mean ± standard error. For each parameter the Duncan's multiple range test is shown, the list of means in descending order. The bars align with those means which are not significantly different from one another ($p = 0.05$). Where bars do not overlap there is a significant difference between groups of means.

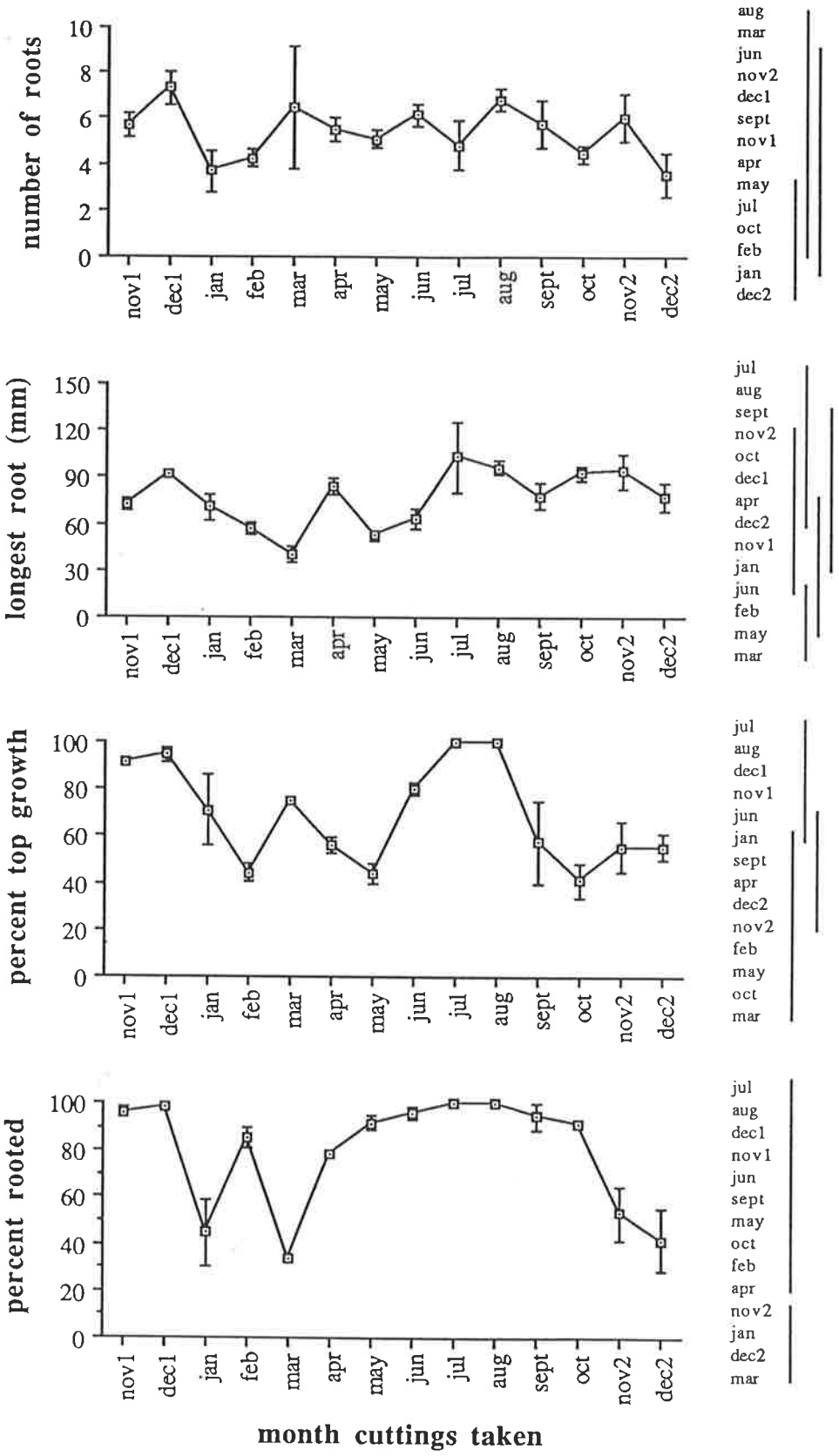


Figure A1.2. The number of roots (a), length of longest root (b), percentage of cuttings with top growth (c) and percentage of cuttings with roots (d) of *H. angustifolium* cuttings taken throughout the year. The cuttings were taken from stock plants on the month shown. Values are mean ± standard error. For each parameter the Duncan's multiple range test is shown, the list of means in descending order. The bars align with those means which are not significantly different from one another (p = 0.05). Where bars do not overlap there is a significant difference between groups of means.

Table A1.1 - Percentage of cuttings with roots when taken from stock plants treated two months previously with plant growth regulators.

Values are mean ± standard error. p = 0.05.

	percent of cuttings with roots	
treatment of stock plant	***B. megastigma***	***H. angustifolium***
control	82.54 ± 5.63	100
paclobutrazol	87.57 ± 4.91	94.63 ± 2.78
benzyladenine	0	13.89 ± 8.22
gibberellic acid	0	0
LSD	*9.67*	*11.43*

Pre-treatment of stock plants with growth regulators

The treatment of stock plants with growth regulators had a profound effect on the number of cuttings with roots taken subsequently from these plants (Table A1.1). Gibberellic acid treatment of stock plants totally inhibited the formation of roots and benzyladenine treatment greatly inhibited the percentage of cuttings with roots in both species. Paclobutrazol had no effect on the number of cuttings with roots.

Auxin

Tables A1.2 & A1.3 show the interactions between auxins and all other treatments. For Table A1.4, particular interactions which illustrate the effect of the various treatments have been chosen. The effect of treating *B. megastigma* and *H. angustifolium* cuttings with auxins was tested both in pine-bark mix in mist and in peat mix in fog.

The application of auxins increased the root length of *B. megastigma* cuttings propagated in pine-bark mix under mist (Table A1.2). In fog and peat mix only the percent of cuttings with top growth was enhanced by the application of auxins. No other measurements, including the percentage of *B. megastigma* cuttings with roots, varied significantly when cuttings were treated with auxins (Table A1.4).

Table A1.2 - Effect of auxin, mist and fog and propagation mix on the propagation of *B. megastigma* cuttings.

Values are mean ± standard error.

Propagation house	Propagation mix	Root number	Longest root (mm)	% top growth	% cuttings with roots
Auxin					
Mist	Pine-bark	1.81 ± 0.10	75.13 ± 9.71	0	91.11 ± 3.89
	Peat	3.41 ± 0.25	63.76 ± 5.15	44.72 ± 3.74	81.41 ± 12.13
Fog	Pine-bark	4.52 ± 0.41	92.22 ± 9.29	51.79 ± 5.74	78.33 ± 4.41
- CO_2	Peat	4.25 ± 0.32	108.42 ± 5.52	71.90 ± 4.54	96.67 ± 3.33
+CO_2		4.11 ± 0.22	95.41 ± 5.56	32.22 ± 7.78	87.78 ± 6.19
No auxin					
Mist	Pine-bark	1.77 ± 0.11	34.96 ± 4.98	0	64.32 ± 6.95
	Peat	4.42 ± 0.23	65.13 ± 4.90	15.48 ± 7.81	97.44 ± 2.56
Fog	Pine-bark	3.75 ± 0.51	79.50 ± 10.84	66.67 ± 16.67	58.33 ± 9.46
	Peat	5.03 ± 0.39	101.50 ± 7.58	41.67 ± 8.33	82.05 ± 5.13

Auxin application dramatically increased the number of roots produced by *H. angustifolium* cuttings both in pine-bark mix under mist and in peat mix in fog (Table A1.3). Auxins also induced more cuttings to have roots when in mist and pine-bark mix, however no difference was evident in fog and peat mix. Thus propagation success was enhanced by the application of auxins only when cuttings were placed in pine-bark mix in intermittent mist.

Because the application of auxins had some effect on the propagation of *B. megastigma* and *H. angustifolium* cuttings, all other interactions were compared using cuttings treated with auxin (Table A1.4).

Carbon dioxide

Comparing the propagation of cuttings between the two fog houses, one of which had its atmosphere enriched with CO_2, showed that CO_2 had little effect on propagation (Tables

Table A1.3 - Effect of auxin, mist and fog and propagation mix on propagation of *H. angustifolium* cuttings.

Values are mean ± standard error.

Propagation house	Propagation mix	Root number	Longest root (mm)	% top growth	% cuttings with roots
Auxin					
Mist	Pine-bark	5.53 ± 0.55	83.95 ± 5.64	44.12 ± 4.49	91.53 ± 2.91
	Peat	3.41 ± 0.25	132.86 ± 5.98	96.67 ± 3.33	97.54 ± 2.87
Fog	Pine-bark	8.40 ± 0.56	135.63 ± 4.69	97.77 ± 2.22	96.00 ± 3.06
-CO_2	Peat	4.95 ± 0.44	132.03 ± 6.95	90.43 ± 4.82	95.55 ± 4.44
+CO_2		4.22 ± 0.39	118.62 ± 5.46	64.64 ± 9.69	95.00 ± 2.89
No auxin					
Mist	Pine-bark	1.27 ± 0.14	70.60 ± 9.89	16.67 ± 16.67	18.89 ± 5.37
	Peat	3.10 ± 0.30	110.13 ± 4.72	95.83 ± 4.17	97.44 ± 2.56
Fog	Pine-bark	1.71 ± 0.18	159.43 ± 44.00	72.22 ± 14.70	23.33 ± 3.33
	Peat	2.56 ± 0.14	123.69 ± 5.83	76.59 ± 12.30	86.67 ± 3.85

A1.2, A1.3 & A1.4). The only significant difference attributable to CO_2 enrichment was a decrease in the number of *B. megastigma* cuttings with top growth. *H. angustifolium* cuttings show a non-significant trend toward a similar result. CO_2 enrichment did not enhance the propagation of *B. megastigma* or *H. angustifolium* cuttings although the percentage of cuttings with roots was high in both CO_2 enriched and non-CO_2 enriched fog houses.

Mist and Fog propagation

The differences between cuttings propagated in intermittent mist and fog were the most significant of any treatment. *B. megastigma* cuttings placed in pine-bark mix showed an increase in root number and percent of cuttings with top growth when propagated in fog rather

than in mist (Table A1.2). *H. angustifolium* cuttings under the same conditions showed increases in root number, length of roots and percent of cuttings with top growth (Table A1.3).

If peat mix was used instead of pine-bark mix, *B. megastigma* cuttings showed significant increases in number of roots, length of roots and percentage of cuttings with top growth attributable to fog in preference to mist propagation. *H. angustifolium* cuttings under these conditions showed an increase in the number of roots.

Propagation medium

Whether cuttings were placed in a pine-bark based or a peat based propagation medium made a significant difference to the propagation of *B. megastigma* and *H. angustifolium* cuttings (Tables A1.2, A1.3 & A1.4). Significantly more *B. megastigma* cuttings in peat medium in fog had roots than in pine-bark mix. There were no differences in the other measurements. In mist, the peat medium had no advantage over the pine-bark mix when comparing percentage of *B. megastigma* cuttings with roots, however cuttings had more roots and a greater percentage of cuttings had top growth.

The number of roots per *H. angustifolium* cutting was decreased by peat mix regardless of whether fog or mist was used for propagation. In mist the length of roots and the percentage of cuttings with top growth was increased by the peat medium. There was no difference in percentage of *H. angustifolium* cuttings with roots attributable to propagation medium.

Interaction of propagation conditions

Table A1.4 summarises the interactions between conditions and propagation success in *B. megastigma* and *H. angustifolium*. For *B. megastigma*, all parameters used to indicate propagation success, except for percent cuttings with roots, were increased by fog propagation in preference to mist. Both the number of roots and the percentage of cuttings with top growth were significantly increased regardless of which propagation medium was used. The number of roots on *H. angustifolium* cuttings were also significantly increased by propagation in fog rather than in mist regardless of propagation medium, however the other measurements only

Table A1.4 - Interactions between different conditions and the effect on propagation of cuttings.

* - significantly different at p = 0.05. ** - significantly different at p = 0.01.

		B. megastigma				*H. angustifolium*			
		Number of roots	Length of roots	% top growth	% rooted cuttings	Number of roots	Length of roots	% top growth	% rooted cuttings
Mist, pine-bark mix	± Auxin		**			**			**
Fog, peat mix	± Auxin			*		**			
Auxin, fog, peat mix	± CO_2			*					
Auxin, pine-bark mix	Mist V Fog	**		**	*	**	**	**	
Auxin, peat mix	Mist V Fog	*	**	**		**			
Auxin, mist	Pine-bark V Peat mix	**		**		**	**	**	
Auxin, fog	Pine-bark V Peat mix				*	**			

showed increases when cuttings were in pine-bark mix. The percentage of *B. megastigma* and *H. angustifolium* cuttings with roots were consistently high in both mist and fog.

Tip or stem cuttings

In the peat medium in fog *H. angustifolium* cuttings showed many differences between the propagation of stem cuttings, tip cuttings and cuttings without tips (Table A1.5). Stem cuttings had significantly longer roots than tip cuttings and a significantly greater number of stem cuttings had roots than both other treatments. There were significant differences in the percentage of *H. angustifolium* cuttings with top growth between all treatments with an ascending percentage from tip, to tip removed, to stem cuttings.

Table A1.5 - Propagation of *B. megastigma* and *H. angustifolium* stem cuttings, tip cuttings and cuttings with the tip removed.

Cuttings were treated with auxin and propagated in peat medium in the fog house. Values are mean ± standard error. N.S. - no significant difference between means. p = 0.05.

	root number	longest root (mm)	% top growth	% cuttings with roots
Boronia megastigma				
with tips	4.29 ± 0.34	99.75 ± 8.91	55.56 ± 9.11	71.80 ± 6.79
without tips	3.81 ± 0.27	98.44 ± 7.49	80.28 ± 6.81	79.85 ± 5.32
stem	3.41 ± 0.33	76.91 ± 5.28	58.33 ± 8.33	62.63 ± 4.04
LSD	*N.S.*	*N.S.*	*N.S.*	*N.S.*
Hypocalymma angustifolium				
with tips	5.89 ± 0.69	78.5 ± 10.50	54.17 ± 4.17	45.24 ± 8.19
without tips	5.29 ± 0.43	92.18 ± 5.67	76.19 ± 4.76	62.76 ± 7.24
stem	5.70 ± 0.41	96.63 ± 2.71	97.92 ± 2.08	100
LSD	*N.S.*	*16.96*	*13.31*	*21.84*

B. megastigma 'Heaven Scent' cuttings in the same conditions showed no significant differences in any of the features measured (Table A1.5). However in pine-bark mix in mist with no auxin treatment, stem cuttings had longer roots than other treatments and fewer cuttings with tips removed had roots (Table A1.6). These results show that the differences in propagation success and health of the cuttings from various parts of the plant may depend on other propagation conditions such as auxin treatment, propagation house and propagation medium.

Table A1.6 - Propagation of cuttings of two cultivars of *B. megastigma*.

Cuttings propagated in pine-bark mix in mist without auxin treatment. Value are mean ± standard error. N.S. - no significant difference between values. * significantly different from 'Heaven scent'. p = 0.05.

	root number	longest root (mm)	% top growth	% cuttings with roots
'Heaven Scent'				
with tips	1.86 ± 0.22	27.27 ± 6.94	0	75.93 ± 3.03
without tips	1.55 ± 0.21	20.45 ± 7.81	0	37.78 ± 6.19
stem	1.78 ± 0.15	49.26 ± 8.26	0	79.26 ± 0.74
LSD	*N.S.*	*21.42*	*N.S.*	*13.85*
combined	1.77 ± 0.11	34.96 ± 4.98	0	64.32 ± 6.95
'Chandleri'				
with tips	2.71 ± 0.26	25.62 ± 4.98	0	78.11 ± 6.74
without tips	2.83 ± 0.22	23.91 ± 4.23	0	76.67 ± 3.33
stem	3.04 ± 0.37	47.67 ± 5.89	74.74 ± 1.84	80.00 ± 5.78
LSD	*N.S.*	*14.72*	*3.62*	*N.S.*
combined	2.94* ± 0.22	36.04 ± 4.02	24.91 ± 12.47	78.33 ± 3.07

Results using *B. megastigma* 'Chandleri' show that stem cuttings in pine-bark mix under mist had significantly longer roots than tip cuttings (Table A1.6). In this variety the most significant difference was in the percent of cuttings with top growth. Almost 75% of stem cuttings had new vegetative growth whereas neither the tip or tip-removed cutting had any top growth. Table A1.6 shows that there was a strong similarity between the results from *B. megastigma* 'Chandleri' tip cuttings and cuttings with the tip removed. It was observed, in the conditions used, that the tip of tip cuttings tended to wilt and die thus rendering the tip cuttings and cuttings with tip removed virtually identical.

Cultivar differences

Comparing the propagation results of cuttings of *B. megastigma* 'Heaven Scent' and 'Chandleri' showed that there were strong similarities between the two cultivars (Table A1.6). The only obvious differences were the percent of 'Chandleri' stem cuttings with top growth and the overall greater number of roots per 'Chandleri' cutting. There was no significant difference in the percent of cuttings with roots between the cultivars.

Summary of results

Propagation of *B. megastigma* and *H. angustifolium* was affected by many of the conditions and treatments tested. The healthiest cuttings and greatest percentage of cuttings that formed roots was obtained with stem cuttings treated with auxin and placed in a peat-based medium in fog. For *H. angustifolium*, as long as auxin was supplied, the percentage of cuttings with roots was high in all conditions. *B. megastigma* cuttings required either a peat-based medium or fog to obtain a maximum percentage of cuttings with roots. Cuttings taken from *B. megastigma* during the late stages of flower development or from *H. angustifolium* during rapid growth periods had a low percentage of cuttings with roots.

Discussion

The realisation that many factors can contribute to the success of propagation has led to a utilization of a wide range of techniques and treatments to maximise propagation success. However, treatments that enhance the propagation from cuttings of one species may have no effect on the success of propagation of another species. This work examined the effect on propagation of *B. megastigma* and *H. angustifolium*, of a range of conditions and treatments that have been found to affect propagation of other species.

Removal of stem tips of *Pinus banksiana* caused a reduction in adventitious rooting of cuttings of this species (Haissig, 1989). *B. megastigma* and *H. angustifolium* cuttings which have their tips removed did not respond in the same way (Table A1.5). Our results parallel those of De Waart (1989) who reported an increase in the number of cuttings of *Boronia sp.* that formed roots when the tips were removed. Sharma and Ollerenshaw (1989) however, found tip cuttings of *Boronia sp.* to be superior to stem cuttings. It may be expected that tip cuttings root better than stem cuttings because basal tissue tends to have higher suberin levels as the phellem develops (Esau, 1977) and this can decrease the number of cuttings that form roots (Williams *et al.*, 1984b). The degree of suberization may play an important role in the propagation of *B. megastigma* and *H. angustifolium* if cuttings were selected from stem tissue more basal than that used.

Tip removal would decrease the rate of transpiration by removing that section of the cutting with a less developed epidermis. This is likely to be the reason why more stem cuttings of *H. angustifolium* had roots than tip cuttings (Table A1.5), and why stem cuttings of *B. megastigma* had longer roots than tip cuttings of this species (Table A1.6). This fits well with the observation that tip cuttings of *B. megastigma* (both varieties) and *H. angustifolium* in intermittent mist initially had a tendency to wilt and that the number of these cuttings that formed roots was enhanced when the tip was removed. Cuttings do not require an actively growing tip to successfully root in these species, in fact it can be detrimental. This may explain why cuttings taken monthly throughout the year successfully root virtually all year round. During winter it was difficult to obtain cuttings, especially ones with actively growing tips, yet

the percentage of cuttings with roots was still high. Further, in summer when tips were actively growing the percentage of *H. angustifolium* cuttings that formed roots was variable. Contrary to this, Clarke (1990) found that cuttings from *Calytrix*, a species from the same family and with a similar growth habit as *H. angustifolium*, root best if taken in summer.

The fluctuation in percentage of *H. angustifolium* cuttings forming roots when taken during the summer months (Figure A1.2d) may be due to a high endogenous gibberellin concentration. Gibberellins are synthesized in actively growing regions of the plant such as the shoot tip (Bidwell, 1977), and Smith (1990) reports that gibberellin treatment of *in vitro* grown *Chrysanthemum* resulted in an increase in transpirational loss and wilting when the plants were transferred out of *in vitro* conditions. Further, flower development of *H. angustifolium* did not inhibit the ability of cuttings to form roots (Figure A1.2d), and flowering of *H. angustifolium* may be associated with a decrease in gibberellin concentration (Chapter 4). Application of gibberellic acid to *H. angustifolium* plants inhibited flowering in cool conditions (Table 4.3). Gibberellic acid application also inhibited root formation of cuttings taken from treated stock plants (Table A1.1). Thus roots may only form on cuttings taken from *H. angustifolium* when the concentration of gibberellins are low (eg: during flowering). During periods of vigorous vegetative growth, the success of rooting was low and this may be associated with a high concentration of gibberellins.

In late winter, the percentage of *B. megastigma* cuttings with roots was low (Figure A1.1d). This time of year corresponded with a period of slow vegetative growth of the stock plant making it difficult to take cuttings with new vegetative shoots. However, as discussed above, *B. megastigma* cuttings with new vegetative growth are not necessary to obtain good propagation rates, stem cuttings will propagate equally as well. Something other than the quality of the cuttings must be determining the percentage of cuttings that form roots. Loach (1988) reported that the number of cuttings that form roots may be low in winter due to low irradiance or water-logging of the propagation medium. Our propagation media drained well and did not become water-logged. If low irradiance was the cause of fewer *B. megastigma* cuttings having roots, then propagation should have been poorest during the months of June and July, the months of least daylight hours and solar radiation in Adelaide. The propagation

of *B. megastigma* cuttings is most likely to be affected by flowering. During the late winter months, the endogenous carbohydrate concentrations of *B. megastigma* plants was low (Figures 5.13 - 5.16) and plants would be directing most nutrients to the floral buds which are in a late stage of development. Cuttings taken at this time of year would have limited carbohydrate supplies which may be detrimental to the success of rooting. It is also possible that the hormone balance of the stock plant may affect propagation at this time of the year. During the late stages of flower development of *B. megastigma* the endogenous cytokinin concentrations are variable but generally higher than at other stages of development (Figures 5.11 & 5.12). Cytokinin and/or low carbohydrate concentrations may be inhibiting the formation of roots on cuttings taken from plants at this time of year.

Table 4.5 showed that the application of gibberellins and benzyladenine decreased the starch concentration in *B. megastigma*, and in table A1.1 cuttings taken from similarly treated plants were less successful than untreated plants at forming roots. This suggests that one of the factors that may influence the success of rooting is the endogenous carbohydrate concentration. Evidence supporting this suggestion is provided by Moe (1985) who found that stock plants of a number of species treated with high CO_2 or high light irradiance, both of which increased the endogenous carbohydrate concentration, enhanced the subsequent rooting of cuttings. This does not exclude the possibility that growth regulators have a direct effect on the success of rooting rather than an indirect effect through the control of carbohydrate concentration.

Many authors have found that cuttings accumulate starch, and that an increase in starch concentration can be closely correlated with an increase in the number of cuttings that form roots (Grant *et al.*, 1992; Haissig, 1989). Carbon dioxide (CO_2) enrichment of the stock plant before cuttings were taken (Moe, 1985) or of cuttings during propagation has been found to encourage a faster formation of roots, more roots per cutting and an increase in dry weight of the cuttings (Molnar and Cumming, 1968). In *Correa schlechtendalii*, the increase in dry weight of cuttings propagated in a CO_2 enriched environment was primarily due to the accumulation of starch (Grant *et al.*, 1992). While *B. megastigma* and *H. angustifolium* cuttings enriched with CO_2 may have accumulated more starch than controls, there was no increase in the number of these cuttings that formed roots. Possibly in species where most

cuttings form roots, such as *B. megastigma* and *H. angustifolium*, CO_2 enrichment is of no benefit. Alternatively cuttings of some plant species do not respond to CO_2 enrichment (Lin and Molnar, 1980) and *B. megastigma* and *H. angustifolium* may belong to this group.

CO_2 enrichment also affects stomatal conductance, resulting in a decrease in transpiration from the cutting (Grant *et al.*, 1992), however, unlike the removal of shoot tips from cuttings which also may decrease the transpirational loss from the cutting, CO_2 enrichment did not enhance the propagation of cuttings.

One of the groups of plant hormones that greatly affect the propagation of cuttings are auxins (Williams *et al.*, 1985). The number of *Boronia pinnata* cuttings that form roots was greatly enhanced by auxin treatment, however auxins had little effect on propagation of *Boronia serrulata* cuttings (Sharma and Ollerenshaw, 1989). Our results concur with the suggestion by De Waart (1989) that *Boronia sp.* root quite well without auxins, although auxin treatment can result in *B. megastigma* and *H. angustifolium* cuttings with more or longer roots (Tables A1.2, A1.3 & A1.4). It was common in *H. angustifolium* cuttings treated with auxins, to observe short roots much of the way up the cutting on parts exposed to the air. These roots had characteristic red tips and grew no longer than a few millimetres. While auxin treatment resulted in an increase in the number of *H. angustifolium* cuttings with roots when propagated in pine-bark mix (Table A1.3), cuttings propagated in peat mix without auxin treatment produced roots quite successfully . Thus in this species when conditions were good for propagation, the percentage of cuttings that formed roots was independent from the application of auxin.

Loach (1988) reports that optimal conditions for the propagation of cuttings primarily depended on the water relations of the cutting. Water is provided to the cutting both from the aerial environment and the propagation medium. Table A1.4 shows that the propagation house and propagation medium affect *B. megastigma* and *H. angustifolium* cuttings in many ways. These treatments primarily resulted in changes to the health of the cuttings rather than to the percentage of cuttings with roots.

B. megastigma and *H. angustifolium* cuttings were generally healthier when propagated in fog. MacDonald (1986) reports that this is a general phenomenon, probably as a result of the

maintenance of a constant high humidity. The peat mix also generally resulted in healthier *B. megastigma* and *H. angustifolium* cuttings, which probably explains why peat based mediums are still widely used for propagation in the horticultural industry (MacDonald, 1986). *H. angustifolium* cuttings in pine-bark mix had more roots per cutting than in peat mix, however it was observed that *H. angustifolium* cuttings in peat mix had a greater number of lateral roots or root hairs which may have compensated for less primary roots. An increase in the number of lateral roots is a common feature of peat based mixes (MacDonald, 1986). *B. megastigma* did not display this feature.

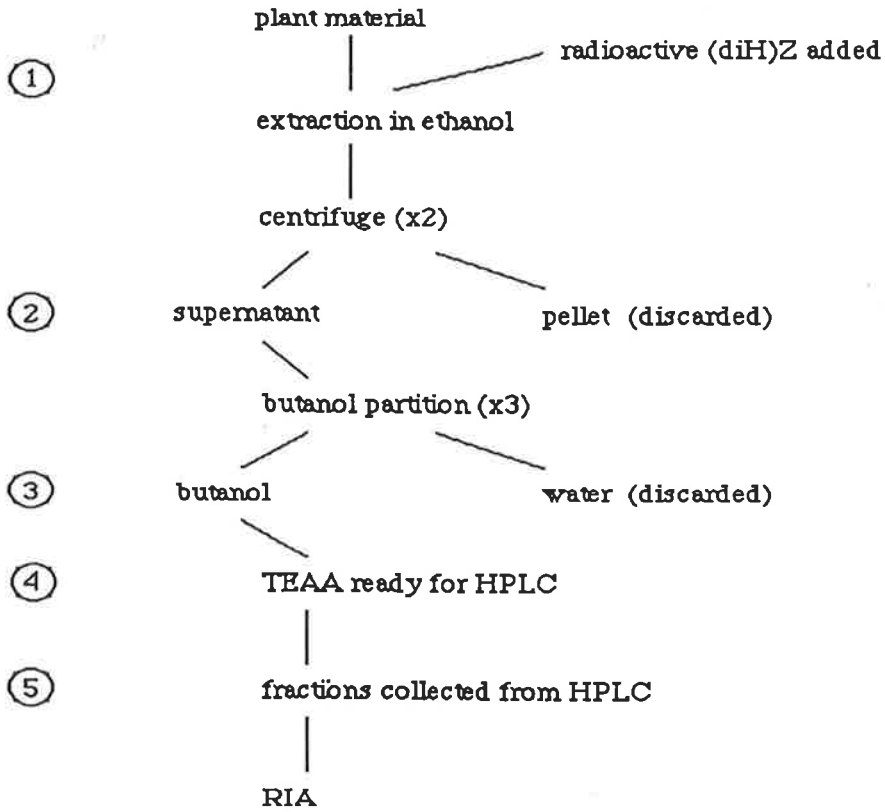
Pine-bark is gaining acceptance as a growing and propagation medium primarily because of its cost and reliability of supply. Growers of fuchsias tend to use the same pine-bark based mix for both propagation and subsequent growth (R. Marchant, pers. comm., 1990). This practice results in less stress on the plants when they are transferred from propagation trays to pots. Fuchsia cuttings root easily and our results suggest that, if fog is used, this practice could be used with other easy to root species with no decrease in health of the cutting. With mist a slight decrease in health of the cutting and a possible decrease in the percentage of cuttings that form roots may result (Table A1.4). Although mist treatment had some effect on the health of cuttings, this may have little effect on the subsequent potting-on of these cuttings. Healthier cuttings would be expected to have a greater survival rate and may begin growth faster after the transplant shock. Whether this is so requires further testing.

APPENDIX TWO

TESTS OF EXTRACTION AND MEASUREMENT PROCEDURES FOR RIA CYTOKININ ANALYSIS

Loss of internal standard [^{3}H-(diH)Z] during extraction

The extraction of cytokinins from plant tissue is a multi-stepped procedure as can be seen in the flow diagram below. At each stage of the method cytokinins are likely to be lost. 20000-30000 Bq of ^{3}H-(diH)Z was added to extracts prior to homogenization to allow quantification of the losses that occur. The recovery of ^{3}H-(diH)Z was measured at different stages throughout the extraction procedure to identify those stages during extraction where the major loss of cytokinin occurred. Two aliquots were taken from four sample and three separate parallel extractions were followed. Thus the following values represent combined results from duplicate aliquots of twelve separate samples.



	stage aliquot taken		percent of initial ^{3}H-(diH)Z recovered at specified stage of extraction	
1	initial			100%
2	centrifuge	first	53.6% ± 2.01	} 77.3% ± 1.87
		second	23.5% ± 1.55	
3	butanol	first partition	48.2% ± 3.63	
		second partition	20.4% ± 4.12	} 72.9% ± 3.76
		third partition	4.7% ± 2.09	
4	before HPLC			66.5% ± 5.76
5	HPLC fractions			54.8% ± 5.58

The greatest loss of cytokinins occurred during homogenization (approximately 23%) and a significant amount was unable to be recovered from the fractions resulting from HPLC separation (approximately 12%). For all extractions in chapter 5 the recovery of ^{3}H-(diH)Z prior to RIA measurement was consistently between 45% and 55%.

Separation of cytokinin standards using HPLC

The solvent used, the pH and the gradient of increase of the solvent all affect the peak shape, retention time and separation of cytokinins on the HPLC. A number of combinations of solvent, pH and gradient were tested before the adoption of the procedure in Chapter 5. Below is a selection of the methods tested.

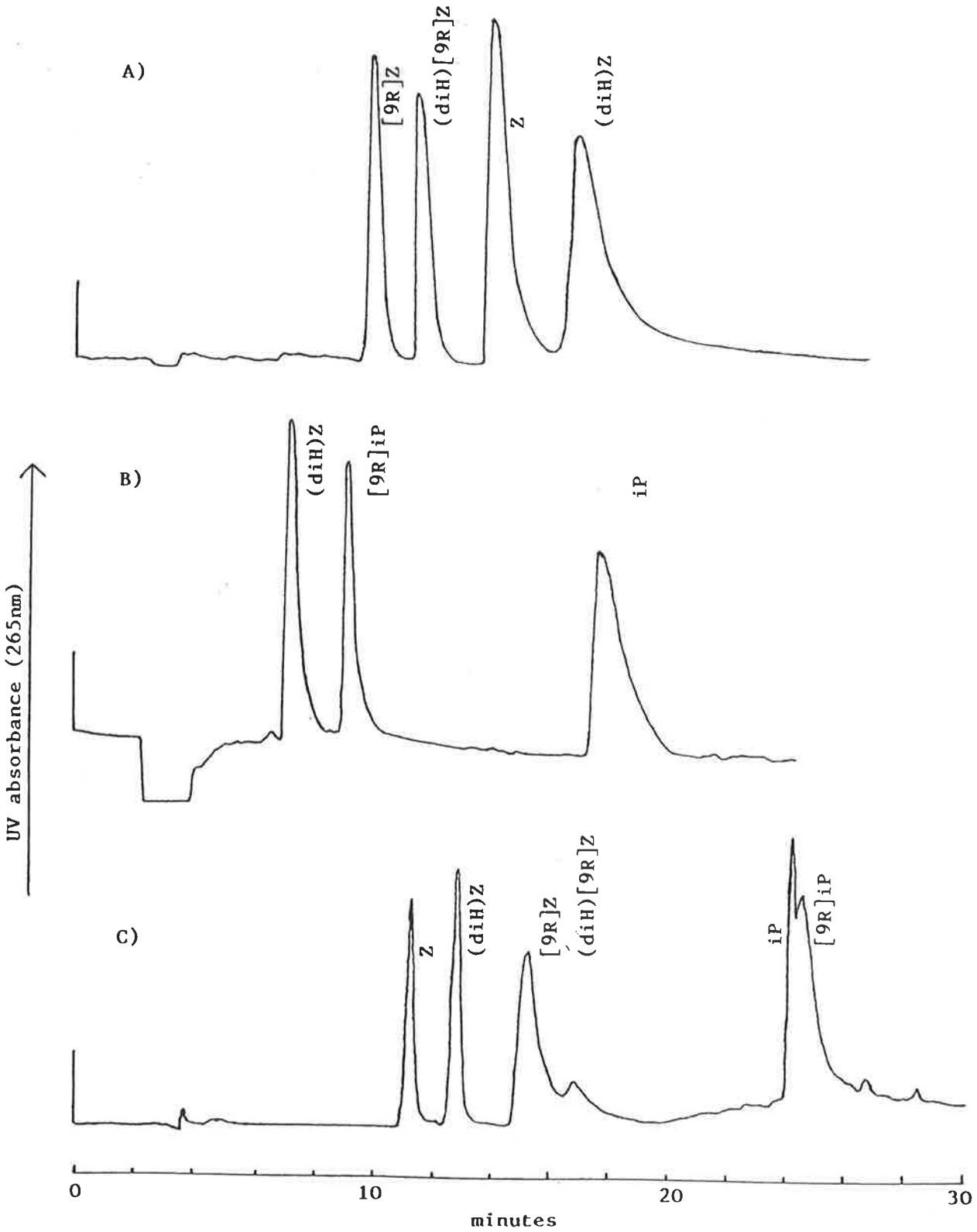
A Brownlee Labs, spherisorb, 250 x 4.6mm, 10µm C18 column was used. Column temperature was 30°C, pump rate was 1ml/min. and the detector recorded at 265nm. All injections were 250ng of each cytokinin. The solvents, gradient and pH are listed below

A selection of HPLC methods tested for separation of cytokinin standards

a) RO water[1], pH 3.35 (with acetic acid). Isocratic 40% methanol. ([1] - H_2O passed through reverse osmosis and ion exchange, with a conductivity of less than 12mΩ.cm[3])

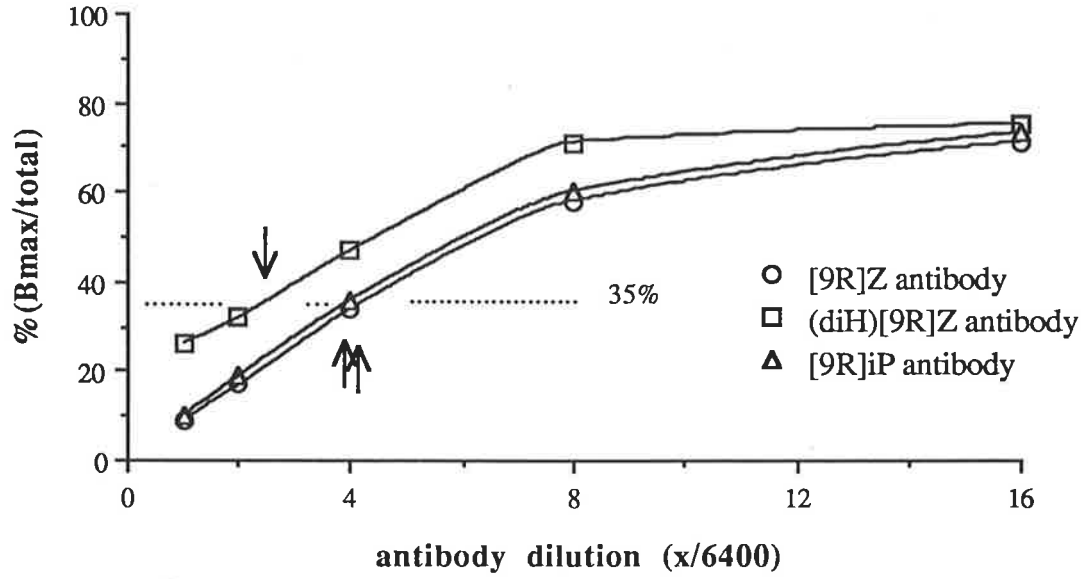
b) RO water, pH 3.35 (with acetic acid). Isocratic 55% methanol.

c) 2mM TEAA, pH 7.0. 0 to 15 minutes, 10 to 15% CH_3CN. 15 to 25 minutes, 15 to 60% CH_3CN.



Antibody dilution

Maximum efficiency of the radio immuno-assay requires that the antibody in the absence of cytokinin standard or sample will bind approximately 35% of the total radioactivity in the assay (Bmax/total = 35%). A range of antibody dilutions were tested and the appropriate dilution used throughout the RIA's. ↑ = concentration of antibody used for RIA ([9R]Z antibody $\frac{1}{2560}$ (w/v); [9R](diH)Z antibody $\frac{1}{1640}$ (w/v); [9R]iP antibody $\frac{1}{1560}$ (w/v)).



Cross reactivity of antibodies for cytokinins

The cross reactivities of the three antibodies for various cytokinins were calculated by Dr. Colin Turnbull using the method of Müller (1983). This method estimates cross reactivity by comparing the estimated concentration of cross reactant necessary to achieve a 50% inhibition of binding of the tracer in the assay. All measurements of cytokinin using RIA's were corrected for the cross reactivity of the cytokinin in question to the antibody used. --- cytokinin-antibody cross reactivity not tested.

cross reactant	Estimated cross reactivity		
	[9R]Z antibody	(diH)[9R]Z antibody	[9R]iP antibody
Z	44%	3%	---
[9R]Z	100% (set)	7%	0.3%
(diH)Z	---	46%	---
(diH)[9R]Z	2%	100% (set)	0.04%
iP	---	---	40%
[9R]iP	3%	---	100% (set)
[9R-5'-P]Z	87%	35%	---
[9G]Z	100%	6%	---
(diH)[9R-5'-P]Z	---	71%	---
(diH)[9G]Z	---	3%	---
[9R-5'-P]iP	---	---	45%
BA	2%	1.1%	---

Test of interference in RIA's by the internal standard [^{3}H-(diH)Z].

Dihydro-zeatin riboside has a 2% cross reactivity to the zeatin riboside antibody (compared to 100% for zeatin riboside). It would be expected that dihydro-zeatin has approximately half this cross reactivity. We see from Figure 5.1 that zeatin and dihydro-zeatin elute from the HPLC in adjacent fractions, thus the measurement of zeatin would be biased by the presence of dihydro-zeatin to some extent. The presence of the ^{3}H-(diH)Z internal standard would cause more interference than native (diH)Z because of the extra tritium added to the radio-immuno assay. Extra tritium in the assay would tend to result in an underestimation of the native cytokinin present.

A range of ^{3}H-(diH)Z standards were made as shown below. These standards were placed in assays with either no zeatin present or with 15ng zeatin per assay (an amount comparable to the amount of native cytokinin normally found in an assay of *B. megastigma*). An increase in the amount of unlabelled zeatin present should increase the competition for the antibody and thus should decrease the amount of ^{3}H-(diH)Z that binds in the assay. The measurements estimate the amount of increase to the bound radioactivity resulting from the addition of ^{3}H-(diH)Z standard relative to the radioactivity when no ^{3}H-(diH)Z was added.

dpm of ^{3}H-(diH)Z standard added to assay	**resulting additional dpm (no zeatin added)**	**resulting additional dpm (15ng zeatin added)**
0	0	0
220	76 ± 54	24 ± 35
790	363 ± 76	14 ± 67
1620	152 ± 45	101 ± 59
3260	273 ± 34	12 ± 91
6770	413 ± 86	89 ± 57
	r^2 = 0.78	**r^2 = 0.19**

Assuming an initial addition of 30000 dpm ^{3}H-(diH)Z and a recovery of 50%, we could expect a maximum of 2500dpm ^{3}H-(diH)Z in each assay [initial 3H-(diH)Z (30000dpm) x recovery after extraction (50%) x amount of initial extract in an RIA (1/6)]. If a normal amount of native zeatin was present there would be no significant interference between the internal standard and the assay. Further, the maximum amount of ^{3}H-(diH)Z would be present in a HPLC fraction at the tail end of the zeatin peak, thus the internal standard may result in an under estimation of zeatin in a fraction where the amount of zeatin would be small anyway. Interference between the internal standard and the assay would be negligible.

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