The effect of paclobutrazol on flowering activity and gibberellin levels in *Eucalyptus nitens* and *Eucalyptus globulus*

by

Omar Hasan B.Sc.

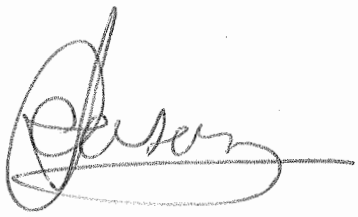
Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania, Hobart

October, 1993

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university and contains no copy or paraphrase of material previously published or written by another person, except where due reference is made in the text.



Omar Hasan

12th of October, 1993

For my grandmother,

A.E. Dawson.

Abstract

Experiments prior to this project demonstrated the capacity of the plant growth retardant paclobutrazol to enhance flowering in commercially important *Eucalyptus nitens* and *E. globulus* trees and produced anecdotal evidence of this material reducing time to first flowering. Paclobutrazol is known to reduce the levels of endogenous gibberellins (GAs) in several species and hence it was hypothesized that the effects of paclobutrazol on flowering in these *Eucalyptus* species may be mediated by an effect on GA levels. The lack of previous identification of GAs in this genus necessitated the development of extraction and purification procedures to identify and quantify GAs. The compounds identified suggested that the early 13-hydroxylation pathway was the dominant mechanism for production of GAs, with GA_1 as the likely biologically active compound. Persistence of a paclobutrazol induced increase in flowering of grafted *E. nitens* material was related to the continued depression of endogenous GA concentrations. Additionally, higher concentrations of GA_1 were found to be correlated with reduced flowering responses in this reproductively competent material. A lowering of endogenous GA levels, in combination with a co-requisite of a period of cold has been associated with the induction of first flowering in newly grafted *E. nitens* material. The effects of cold treatment were not mediated by an effect on levels of GA_1 or GA_{20}.

Soil applied paclobutrazol was shown to travel up stems and accumulate in leaf tissue. Breakdown in plant tissue was shown to be rapid, with a half-life likely to be substantially less than 21 d. Soil and foliar application methods were shown to produce different patterns of metabolism of labelled paclobutrazol, as demonstrated by HPLC separation of labelled metabolites extracted from growing apices. The rate of breakdown in the soil was observed to be variable, but slow in comparison to that within plant tissues and may be the source of the considerable persistence of effects of paclobutrazol application observed in some field trials.

Application of paclobutrazol to 6 month old *E. globulus* seedlings resulted in the production of flower buds at less than 18 months of age despite the retention of juvenile foliage. One year later, following normal bud development, anthesis and pollination, capsules were produced, while maintenance of material in a range of

growth conditions over the second winter again demonstrated the strong requirement for cold seen in grafted *E. nitens*, as well as revealing an apparent promotion of flowering associated with reduced pot size. The reduction in generation time achieved using commercial seedlings was ca. 50% (3 years) which should be of major benefit to tree breeders, given that the long generation time of eucalypts is a major determinant of the rate at which genetic gains can be made by conventional tree breeding methods. In reproductively competent seedlings and grafts, paclobutrazol application was confirmed to increase the average number of flower buds per seedling. This could be advantageous when seed requirements from an elite tree are high, or when yearly seed yield tends to be variable.

Acknowledgements

I would like to thank the following people for the help they have provided over the course of my post-graduate studies.

- Prof. Jim Reid for supervision and guidance through all aspects of post-graduate training.

- Dr John Ross for his plentiful assistance and the willingness with which it was given.

- Mr Mike Moncur of CSIRO Division of Forestry, Canberra for his cheerful help and his tireless work in our fruitful (sic) collaboration (see Chapter 2).

- Sandra Hetherington, formerly of Forest Resources and now with Australian Newsprint Mills, for providing me with plant material on very short notice on more than one occasion and for keeping me updated on the progress of her fine work.

- Peter Naughton of Forest Resources and David deLittle, Ian Ravenwood and Wayne Tibbits of Associated Pulp and Paper Mills for their friendly help and interest.

- Mr Noel Davies for his completely invaluable assistance with mass-spectrometry of gibberellins.

- Dr Brad Potts for providing eucalypt seed and helping with experimental design and statistical analyses.

- Dr Gregory Jordan for help with statistical analyses.

- Drs Robert Wiltshire and Dick Pharis for various helpful suggestions.

- Leigh Johnson, Peter Bobbi and Kathy McPherson in Hobart and John Turner in Canberra for maintaining experimental material.

- Heidi Dungey and Jon Marsden-Smedley for tolerating me as a room mate.

- Stephen Swain, Christine Beveridge, Jim Weller, Mike Battaglia, Kristen Williams and Naomi Lawrence for their help and company as fellow Ph.D. students.

- Peter Gore, Andrea Manson, Rhyl Opie, Paula Chalmers, Kate Booth and Dale Barron for help provided as part of their 3rd year Plant Science curriculum.

- Erik Wapstra for his competent help in preparation of this thesis.

- The Plant Science Tea Room cribbage players for many hours of entertainment.

- My parents for their support and encouragement throughout my years of schooling.

- And finally Lisa Stanton for her companionship and understanding.

Table of Contents

General Introduction

Eucalyptus globulus (Labill.) and *Eucalyptus nitens* (Deane and Maid.) Maid. are commercially important species of the myrtaceous genus *Eucalyptus*. They are heavily utilised for the production of wood pulp both in Australia (eg. Tibbits, 1986) and overseas (Zacharin, 1978) and are the most widely planted temperate species of eucalypts, which is in turn the most widely planted genus of angiosperm trees (Griffin, 1989). To obtain maximum gain from such a scale of planting requires large quantities of seed, preferably from improved trees. Work using plant growth retardants on orchard species such as apples (Tukey, 1981; Jones *et al.*, 1989a) and citrus species (Mauk *et al.*, 1986) was observed to increase bud numbers in the seasons following application, leading to an interest in assessing their effects on the reproductive output of eucalypt species. If fecundity of desirable trees could be increased within a seed orchard, a larger amount of improved seed would be available to satisfy the requirements of new/renewed plantations.

It was also of interest to assess the capacity of plant growth retardants to affect the relatively long generation times notable for species of this genus (Ashton, 1975; Turnbull and Pryor, 1978). These long generation times pose an important problem to eucalypt breeders (Griffin, 1989) due to the limitations on rates of incorporation of new traits they impose. A capacity for precocious (early) flowering under natural conditions has been noted for several species of eucalypts (Pryor, 1966). If a chemical or cultural treatment could be established to promote the expression of this capacity, breeding programs would benefit greatly, as long as the reproductive development of the treated plant continues to occur normally. In conifers, there are many reports of successful promotion of both heavier and precocious flowering through the use of cultural techniques ranging from stem girdling, to release from competition by stand thinning (Owens and Blake, 1985) and/or chemical treatments by application of growth retardants, especially non-polar gibberellin (GA) mixtures such as $GA_{4/7}$ (Pharis and King, 1985; Pharis *et al.*, 1987). Such a level of control over flowering processes in eucalypts and other woody angiosperms is the ultimate practical goal sought by tree breeders, while gaining an understanding of the control of flowering is a fundamental aim

from a theoretical standpoint.

As work to this stage has suggested that the use of plant growth retardants may be an effective means of promoting flowering in woody angiosperms including eucalypts, an understanding of the nature of these substances, along with an indication of the range of effects which could be expected from their application is an appropriate point of entry into this study.

What is a Plant Growth Retardant?

There are a range of chemical products which result in decreased internode elongation in plants. All such materials may be considered as plant growth retardants and these may be simply divided into two major groups, based on their mode of activity, or more specifically, on their ability or otherwise to inhibit GA biosynthesis. Those compounds which are thought to act alternatively to inhibition of the GA biosynthesis pathway include two commercially important plant growth retardants. The first consists of ethylene releasing compounds, such as ethephon (Reid, 1987), which have been widely used to induce a variety of effects, many of which are not related to growth retardation (Giafagna, 1987). One of the important uses of this compound is, however, the reduction of shoot growth in graminaceous species (Rademacher, 1991). However, despite the considerable knowledge of the effects of application of ethylene releasing compounds, very little is known about the means by which these effects are mediated. The second, daminozide (butane-dioic acid mono (2,2-dimethyl-hydrazide)) is a compound also used to reduce shoot growth, though this plant growth retardant has been mainly used for improving fruit colour, growth and storage properties of apples in a commercial production environment (Giafagna, 1987). Application of this plant growth retardant has been observed to actually increase the level of endogenous GAs despite growth being inhibited (see references in Graebe and Ropers, 1978; Pharis and King, 1985; Rademacher, 1991). The mode of action of this plant growth retardant is not clear, though some results suggest a deactivation of active GAs by some means (Menhenett, 1980; Takeno *et al.*, 1981). Other studies have indicated a possible mechanism based on the suppression of metabolic respiration (See and

Foy, 1982).

The second group of plant growth retardants includes those substances thought to act primarily through inhibition of the biosynthesis of the active GAs (eg. GA_1, Reid, 1990) which promote internode length elongation. It is this latter type of retardant which is of primary interest to this study.

It has been more than 40 years since the first substantial work on a range of GA biosynthesis inhibitors was completed (Warville and Mitchell, 1950) and since that time, knowledge of the increasingly diverse range of chemicals described as plant growth retardants has continued to grow. Plant growth retardants typically affect plant growth at very low concentrations, but do not induce phytotoxic effects even at levels considerably above the threshold of activity (Grossmann, 1990). While the effects of different inhibitors may vary, several characteristic changes to the morphology of treated individuals are usually evident. The most obvious of these is a reduction of all aspects of shoot growth including total height, internode elongation and leaf area, with leaves displaying an intensified green colouration (Grossmann, 1990). Root growth is maintained or even increased (eg. Curry and Reed, 1989), while adventitious root formation has been reported to be increased (Davis *et al.,* 1985). This alteration in the root-shoot ratio is recognised as perhaps the major effect of exogenously applied plant growth retardants. However, this is not without exception as Horrel *et al.* (1989, 1990) report shoot elongation of common ivy (*Hedera helix*) following treatment with a GA biosynthesis inhibitor.

Varieties of Plant Growth Retardants

While the broad range of effects of synthetic plant growth retardants may be said to be similar, the chemical compounds which constitute each variety are far from uniform. The majority of those which function by inhibition of GA biosynthesis can be divided into three groups based on the points at which they inhibit the GA biosynthetic pathway (see later).

The first group consists primarily of the growth retardants discovered early in the history of these materials. Its constituents are either amines, or compounds

containing sulfonium or phosphonium groups. The amine group includes the tertiary amine AMO 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine-carboxylate methyl chloride) (Cross and Myers, 1969) and quaternary amines such as chloremquat chloride or CCC (2-chloroethylmethylammonium chloride) (Cross and Myers, 1969), while Phosphon D (tributyl-2,4-dichloro-benzylphosphonium chloride) (Fall and West, 1971) is the most well known example of a phosphonium containing plant growth retardant.

There are four common chemical types which make up the second group of plant growth retardants. These include derivatives of triazoles and norbornanodiazetine, as well as substituted pyrimidines and 4-pyridines. The common element in each case is the lone pair of electrons on the sp^2-hybridized nitrogen atom in the heterocycle (Grossmann, 1990). Ancymidol (α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidine methanol) (Coolbaugh *et al.*, 1978), a substituted pyrimidine, was one of the earliest identified plant growth retardants from this second group, while inabenfide ([4'-chloro-2'-(α-hydroxybenzyl)]-isonicotinanilide) (Miki *et al.*, 1991) is probably the most notable 4-substituted pyridine. The triazole and norbornanodiazetine derivatives are relatively new. Tetcyclacis (5-[4-chlorophenyl]-3,4,5,9,10-pentaaza-tetracyclo-5,4,$10^{2,6}$,$0^{8,11}$-dodeca-3,9-diene) (Rademacher *et al.*, 1984) is the only norbornanodiazetine derivative of note. In comparison numerous derivatives of triazoles have been widely researched, the most important probably being paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl) pentan-3-ol] (Dalziel and Lawrence, 1984; Hedden and Graebe, 1985) and uniconazole (XE-1019 or S-3307) [(E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol] (Izumi *et al.*, 1985).

The third division of plant growth retardants has only been known for approximately five years. It consists of cyclohexanetriones such as calcium 3,5-dioxo-4-propionyl-cyclohexanecarboxylic acid (Nakayama *et al.*, 1991) as well as cimectacarb and LAB 198 999 (Rademacher *et al.*, 1992). While this type of compound has been demonstrated to be active in reducing vegetative growth (eg. Juntilla *et al.*, 1991; Nakayama *et al.*, 1992), relatively little has been published on novel uses for these inhibitors. However, their difference in form and function to other retardant varieties (Rademacher, *et al.*, 1992) suggests their potential

usefulness as tools for investigating the nature of GA metabolism and regulation of growth and other developmental processes may be great.

Effects of Plant Growth Retardants on GA Biosynthesis

The production of active GAs from the relatively non-specific precursor geranylgeranyl pyrophosphate requires many individual steps (Dalziel and Lawrence, 1984; Turnbull *et al.*, 1985; Rademacher, 1991) and hence a number of different enzymes are potentially susceptible to inhibition by plant growth retardants. The quite different structures and compositions of the wide variety of plant growth retardants results in action at different sites in the GA biosynthetic pathway, though sites tend to be common to each inhibitor type (Fig. 0.1). The amine containing plant growth retardant AMO 1618 was first found to act prior to *ent*-kaurene (Cross and Myers, 1969) until its site was isolated to kaurene synthetase A (Fall and West, 1971). Another amine containing compound, CCC, which is one of the most widely used of all plant growth retardants (Hedden, 1990), also acts primarily on kaurene synthetase A (Rademacher *et al.*, 1992). Phosphon D, on the other hand, blocks GA biosynthesis by inhibiting both cyclization steps between geranylgeranyl pyrophosphate and *ent*-kaurene, though some interspecific variation in the degree of effect on the activity of the second step (kaurene synthetase B) has been recorded (Coolbaugh, 1983).

The group of retardants which includes triazole derivatives such as paclobutrazol and uniconazole block the enzyme kaurene oxidase which catalyzes the 3 steps from *ent*-kaurene to *ent*-kaurenoic acid (Dalziel and Lawrence, 1984; Hedden and Graebe, 1985; Izumi *et al.*, 1985) (Fig. 0.1) or, more specifically, by binding to the protoheme iron of cytochrome P-450 in the enzyme (Rademacher *et al.*, 1984; Sugavanam, 1984). Despite their overall structural dissimilarity, the norbornanodiazetine derivative tetcyclacis (Rademacher *et al.*, 1984) and the substituted pyrimidine, ancymidol (Coolbaugh *et al.* 1978) act at the same enzymes, suggesting the possibility of a common active site with triazoles.

The cyclohexanetrione group of plant growth retardants block GA biosynthesis much later in the pathway (Fig. 0.1). These compounds inhibit 2-oxoglutarate

dependent dioxygenases (Rademacher, 1991a; Rademacher *et al.*, 1992) such as the 3ß-hydroxylase which metabolises GA_{20} to GA_1. In this manner, prohexadione-calcium (BX-112) has been shown to inhibit the conversion of inactive GA_{20} to the active GA_1 in rice seedlings (Nakayama *et al.*, 1992) and *Salix pentandra* (Juntilla *et al.*, 1991). Similarly, derivatives of these substances have also been demonstrated to block the activity of 2ß-hydroxylases, possibly by competitively inhibiting 2-oxoglutarate binding at low concentrations, while the method of inhibition at higher concentrations appears mixed (Griggs *et al.*, 1991). Other steps which utilise 2-oxoglutarate dependent dioxygenases to catalyse conversions between GA compounds are also affected, though not to the same extent (Rademacher, 1991). In recent work, a single C-20 oxidase, cloned via a cDNA library from *Cucurbita maxima*, was demonstrated to be capable of catalysing the 3 step conversions of GA_{53} to GA_{20} and GA_{12} to GA_9 (Fig. 1.6, Chapter 1) when the cofactor requirements for 2-oxoglutarate dependent dioxygenases were met (Lange *et al.*, 1993). This may explain the broad activity of cyclohexanetrione derivatives amongst the later steps of GA metabolism.

Effect of Plant Growth Retardants on other Biosynthetic Pathways

The majority of early plant growth retardants were initially recognised as fungicides which were thought to act through the inhibition of sterol biosynthesis, and the extraction of potential plant growth retardants from the ranks of the fungicides continues to the present (Koller, 1987). However, as described above, the effectiveness of these materials as plant growth retardants has largely been explained by their effects on GA biosynthesis. The possibility of effects on sterol biosynthesis have not been overlooked however, with a number of reports either mentioning (Rademacher *et al.*, 1984; Dalziel and Lawrence, 1984; Steffens *et al.*, 1985; Steffens, 1988) or actually testing (Douglas and Paleg, 1974; Grossmann *et al.*, 1985; Haughan *et al.*, 1989) other mechanisms for plant growth retardant activity. From the bulk of evidence provided by these reports, there can be little doubt that the majority of plant growth retardants do affect sterol biosynthesis to a greater or lesser degree, though the cyclohexanetriones are yet to

be assessed. Whether this change in sterol biosynthesis is active in producing some or all of the effects normally associated with plant growth retardant activity is difficult to discern, as is evidenced by the conflicting conclusions drawn by different researchers.

Grossmann *et al.* (1985) pursued the approach of Douglas and Paleg (1974) in investigating the effects of different sterols on the inhibition of growth caused by tetcyclacis. Perhaps the most significant outcome was the fact that the inhibition of growth of a cell suspension system caused by tetcyclacis could be totally overcome by addition of stigmasterol and cholesterol at 10^{-5} M and 10^{-4} M respectively, while addition of GA_3 and *ent*-kaurenoic acid at similar concentrations would not overcome the influence of the retardant. Koller (1987) also played down the role of GAs in concluding that growth inhibition in etiolated wheat seedlings following application of the azole fungicide triadimenol was mainly the result of sterol inhibition.

While it has long been realised that sterol and GA biosynthesis could both be affected by plant growth retardants, the possibility of the division of sterol and GA biosynthesis inhibition between different isomers of a single plant growth retardant, as confirmed by Sugavanam (1984), is a much more recent concept. The 2S,3S enantiomer of paclobutrazol was demonstrated to be active in reducing GA biosynthesis while the 2R,3R enantiomer had more fungicidal (sterol biosynthesis inhibition) effects. Through the use of computer modelling, structural similarities between the 2S,3S enantiomer and *ent*-kaurene, and the 2R,3R enantiomer and lanosterol were demonstrated (Fig. 0.2), rationalizing the site of activity of paclobutrazol in each pathway. As most commercial grade paclobutrazol contains a mixture of stereoisomers (Haughan *et al.*, 1989) which include both enantiomers discussed, both pathways are potentially affected, though particular preparations may contain a higher proportion of one isomer.

While this information may make the results generated using paclobutrazol or other inhibitors which have a similar combination of enantiomers such as uniconazole (Izumi *et al.*, 1985) more understandable, it does not explain the variations in effects reported for other varieties of plant growth retardants. In these cases (and possibly the former cases as well), it may be the dose of the retardant which is the important factor. Grossmann (1990) suggests that cell elongation is a sensitive process which appears to be closely linked to the synthesis of 3ß-

hydroxylated GAs. The relatively lower retardant concentrations required to affect GA biosynthesis thus affects cell elongation. Higher plant growth retardant concentrations are noted to cause inhibition of cell division in cell cultures and intercalary and basal meristems of whole plants, an effect which is thought to modulated by sterol synthesis. Zeevaart (1985) using tetcyclacis on *Agrostemma githago*, reported that as long as the tetcyclacis concentration did not exceed $5x10^{-5}$ M, the effects of the plant growth inhibitor could be totally overcome by addition of GA_1. Further to this, Nitsche *et al.* (1985) found that the inhibition of cell division became increasingly important in the inhibition of stem growth as concentrations of tetcyclacis rose, while at lower concentrations cell length reduction was the primary cause of internode length reduction. Steffens (1988) records several authors as postulating similar hypotheses. Perhaps between the effects of different isomers and of retardant concentration on GA and sterol biosynthesis it is possible to explain the majority of results recorded over the past forty years of plant growth retardant research.

The effect of these plant growth retardants on the endogenous phytohormone abscisic acid (ABA) is another means by which plant growth and development could be affected and has been considered to a limited extent (eg. Yadava and Lockard, 1977; Norman *et al.*, 1986; Hauser *et al.*, 1990; Hedden, 1990; Zeevaart *et al.*, 1990; Buta and Spaulding, 1991; Rademacher, 1991). These studies have indicated contradictory effects of growth retardant application on ABA levels with Hedden (1990) and Zeevaart *et al.* (1990) proposing the inhibition of ABA catabolism with the result of increased ABA levels (Hauser *et al.*, 1990), while Norman *et al.* (1986) and Buta and Spaulding (1991) claim ABA levels are reduced.

Plant Growth Retardant Effects

To try and describe, even briefly, the range of effects produced by each retardant on the array of plants to which it has been applied is well beyond the scope of this introduction. Instead, an overview of the effects of some triazole derivatives on the morphology and development of various woody angiosperms is given.

Effects on vegetative growth

Horticulturalists have been interested in the prospect of utilising exogenously applied plant growth retardants as a means of controlling non-productive vegetative growth in orchard trees for a considerable period of time. Similarly, the potential of plant growth retardants to drastically reduce the required frequency of pruning trees in close vicinity to power lines etc has been recognized. While some work on retardant induced vegetative growth inhibition has been performed on pears (Raese and Burts, 1983), peaches (Marini, 1986), sour cherries (Bukovac and Hull, 1985) and citrus trees (Aron *et al.*, 1985), as well as non-fruit trees such as silver maple (Arron, 1990), Californian privet, red maple, yellow poplar, white ash, American sycamore (Sterrett, 1985) and *Eucalyptus globulus* (Hetherington and Jones, 1990), most work has been done on apples (eg. Steffens *et al.*, 1985; Greene, 1986; Steffens, 1988; Mauk *et al.*, 1990a; Sterrett, 1990). In all cases appropriate retardant treatment resulted in a marked reduction of vegetative growth in conjunction with a reduction of leaf size and an intensifying of the green colouration of reduced leaves, the latter of which appears to be the result of an increased concentration of leaf chlorophyll (Wang *et al.*, 1985; Archbold and Houtz, 1988). Wang *et al.* (1985) demonstrated that paclobutrazol treatment of apple seedlings resulted in a shift of assimilate partitioning in favour of the roots along with an overall increase in carbohydrate concentration around the plant. Increased root weight has also been noted in triazole derivative (paclobutrazol, RSW 0411 and BAS 111) treated apple seedlings (Curry and Reed, 1989). In contrast to the plant growth retardant promoted increase in stress tolerance reported by other workers using herbaceous plants (eg. Upadhyaya *et al.*, 1990), Walser (1990) reports increased frost damage in paclobutrazol treated sour cherry trees. Some effects on tree architecture following paclobutrazol have been noted and while initially the lower, more open habit of treated trees was attributed to reduced lateral branching and growth, more recent observations suggest an increase in branch flexibility may be at least partially responsible for the almost pendulous appearance of some treated apple trees (Greene, 1986). Paclobutrazol application has also been noted to affect pest loading, with suppression of mite populations in

pears (Raese and Burts, 1983) and increasing scale infestation in eucalypts (Moncur *et al.*, 1993).

Effects on generative processes

Perhaps of the greatest interest to this study, however, are the effects growth retardants have been shown to have on reproductive activity. In comparison to the resounding agreement on the activity of growth retardants on shoot growth, effects on fruit production, and generative processes overall were much less clear. A combination of factors has probably led to this state of affairs. The seasonal nature of fruiting means that applications of growth retardants at different times may affect cropping in a different manner. The range of different retardants, possible doses, application methods and general dissimilarities in reproductive physiology between species will all play a role in the manifestation of plant growth retardant effects.

Despite the differences, some common elements can be extracted. An increase in fruit set of treated pear trees has been recorded (Raese and Burts, 1983). Similarly in apples, many workers (eg. Curry and Williams, 1983; Greene, 1986) have found that paclobutrazol treatment increased fruit set over control, while others (eg. Tukey, 1981) have found that enhanced flowering results from paclobutrazol treatment. A paclobutrazol stimulated enhancement of flower bud numbers has also been observed in citrus species (Mauk *et al.*, 1986), sour cherry (Bukovac and Hull, 1985) and eucalypts (Hetherington *et al.*, 1991; Griffin *et al.*, 1993) while CCC has been shown to induce flower bud formation in mature *Salix pentandra* (Junttila, 1980). Indeed, the bulk of evidence has lead to enhancement of flowering being proposed as a physiological effect of plant growth retardant treatment by Grossmann (1990).

While fruit set and number are usually increased by paclobutrazol application, fruit size is usually reduced. Such a case has been documented for citrus where ^{14}C-assimilate partitioning studies demonstrated that fruit sink intensity is reduced (Mauk *et al.*, 1986). In apples, morphological studies indicate a reduction in fruit size and pedicel length (Curry and Williams, 1983; Greene, 1986), though the flesh quality remained virtually unchanged in comparison to control.

Optimisation of Plant Growth Retardant Effects in Woody Angiosperms

General success of early use of retardant materials was limited because of the uncertainty of rate, timing and frequency of application (Mauk *et al.*, 1986). In order that appropriate quantities and values can be assigned to these aspects, modes of uptake and translocation of absorbed materials required investigation, as did rates of degradation. In concert, these parameters should allow the most efficient means and rates of application of a particular growth retardant to be calculated to produce the activity required.

Uptake and Translocation

In peach seedlings, transport of ^{14}C-labelled paclobutrazol applied as a root fed solution was found to occur exclusively in the xylem, and acropetal movement was demonstrated (Early and Martin, 1988). This result confirmed results of Wang *et al.* (1986) working with unlabelled paclobutrazol in apple seedlings who also stated that no basipetal movement of material occurs, suggesting that paclobutrazol transport in the phloem is negligible. Trunk injection of labelled paclobutrazol (eg. Sterrett, 1985) results in material moving rapidly to the apex via the xylem. Though uniconazole has been detected in phloem tissue of woody angiosperms after trunk injection, it was suggested that its appearance was the result of lateral movement from the xylem in relatively apical tissues (Sterrett, 1988). Foliar spraying of paclobutrazol was shown to retard terminal growth in 10 year old apple trees in the year of application, whereas a soil drench did not have an effect until the following year (Mauk *et al.*, 1990a). The rapid response of foliar application is most easily explained by suggesting that the point of application is very close to the point of response (the subapical meristem, Grossmann, 1990). However, foliar sprays have generally proven less effective in producing a consistent response (Curry and Reed, 1989), though indications are that more reliable control is possible through the use of repeated low doses (Hetherington and Jones, 1990).

Persistence

The persistence of growth retardants in the plant and/or soil after application has been recognized as playing a role in determining the effectiveness of the compound (Steffens, 1988). A study over four months, of apple trees trunk injected with uniconazole, determined that 16% of recovered label had moved into the shoot system, but of this only 51% remained as uniconazole (Sterrett, 1990). Arron (1990) demonstrated a similar proportion of degradation of uniconazole in silver maple, though in leaves only, where 49% of label remained unmetabolised after approximately four months. In comparison, a study utilizing peach seedlings (Early and Martin, 1988) records that after just nine days a maximum of 12.2% of labelled, root applied paclobutrazol in the leaves remained in an unmetabolised form. Paclobutrazol was also assessed in apple seedlings along with two other triazole plant growth retardants, RSW0411 and BAS111 (Curry and Reed, 1989). In this case the concentrations of paclobutrazol and RSW0411 recovered from leaf tissue eight weeks after soil drenching was at least 50 times greater than BAS111 which was applied at the same rate, though these results are somewhat complicated by the possibility of differential uptake from the soil. Collectively these results suggest a significant variation in persistence of triazole plant growth retardants in plant tissues between inhibitor types and/or between species.

Despite the differences, the rates of degradation of these triazole plant growth retardants appears to be relatively high, especially when considering the three or more seasons of growth inhibition which have been observed after soil drench applications of paclobutrazol (Steffens *et al.*, 1991; Griffin *et al.*, 1993). There are at least three ways in which growth inhibition may be maintained in the face of such apparently rapid retardant metabolism, with elements of each factor probably acting to produce the end effect. The first possibility is that concentrations of retardant required for activity at active sites may not need to be as high as first thought, allowing levels to remain above an active threshold for a considerable time despite high degradation rates. Secondly, the site(s) of breakdown may be important in determining the persistence of growth retardant effects. The work of Early and Martin (1988) on peach trees found that a higher percentage of root applied paclobutrazol was found in an unmetabolised form in the trunk while Arron

(1990) working with apples suggests that breakdown of uniconazole occurs mainly in the leaves. The implication of this fact is that breakdown in deciduous trees is effectively halted for a significant portion of each year. However, metabolism in many cases is still quite rapid and the persistence of plant growth retardant effects in evergreen trees (eg. Griffin *et al.*, 1993) cannot be satisfactorily explained. The third potential source of persistence of growth retardant effects is storage in the soil following soil drenching or foliar application, due in the latter case to irrigation and rainfall washing material from the shoot system onto the soil (Curry and Reed, 1989). Mauk *et al.* (1990a) performed a comprehensive study of paclobutrazol degradation in the soil and found that soil residues from either foliar or soil sprays decreased at a rate of approximately 50% per year, though Greene (1986) has noted the potential for variability in persistence of retardant effects resulting from differing soil types.

Though breakdown of plant growth retardants such as paclobutrazol appear to be rapid in leaf tissues, there is some indication of a reversible binding of this chemical to the vascular tissues of woody plants (Lever, 1986). This, possibly in conjunction with other factors discussed above, may explain the persistence of the effects of paclobutrazol treatment on growth and flowering observed following large trunk injections to *E. globulus* (Griffin *et al.*, 1993).

Plant Growth Retardants in Phytohormone Research

As well as offering important commercial and economic advantages, growth retardants have a role to play in theoretical research into plant physiology in general, and phytohormone study in particular. This is especially the case with GA research. These substances allow the activities of GAs in the growth and development of plants to be investigated. It is in work of this nature that the value of having a range of growth retardants with different sites of activity can be fully realised. By using several types of retardant, both separately or in combination, and monitoring their effects on a wide range of phytohormones and plant development, the degree of involvement of hormones in the modulation of particular plant responses can be determined. As an example, the activity of

applied GA_1 can be established in isolation from the effects of endogenous GAs by inhibiting the production of the latter at an early stage in the biosynthetic pathway. The cyclohexanetrione type inhibitors could be utilised in such a system to examine the possible *per se* activity of GA_1 precursors and/or whether pool size or rate of turnover is the important factor in modulation of GA effects. Elements of such investigations have been recently reported (Juntilla *et al.*, 1991; Nakayama *et al.*, 1992), with results in both cases suggesting that GA_1 is active in the control of stem growth, while the immediate precursors GA_{19} and GA_{20} are not.

Conclusion

Prudent use of plant growth retardants in industry and agriculture promises to provide significant advantages in plant management and productivity. Thoughtful use of retardants by plant physiologists will provide insight into the control of plant growth and development and responses of plants to changes in the environment. The continuing development of increasingly more specific inhibitors of the GA biosynthetic pathway should prove advantageous in both industrial and scientific areas. In each case, the finer level of control of the biosynthetic pathway will enable more straightforward interpretation of experimental results for plant physiologists. Within the commercial sector, a greater regulation of the effects of plant growth retardants will enable more accurate dosing and more reliable use of these materials in general.

Given the potential benefits, it seems that there is sufficient justification for significant effort to be directed toward the field of plant growth retardants in the future.

Aims of the present study

This brief review has indicated the range of effects of plant growth retardants on hormone and sterol levels and on plant development and growth. Early work by Griffin *et al.* (1993), used as the starting point for this study, utilised the triazole kaurene oxidase inhibitor paclobutrazol (Dalziel and Lawrence, 1984) to increase

bud production in eucalypt species. Plant growth retardants other than the GA biosynthesis inhibitor type have been assessed by Hetherington *et al.*, (1991) for ability to stimulate bud production, but neither ethephon or daminozide produced positive results in eucalypt species, despite their apparent effectiveness in apples (Jones *et al.*, 1989a). Given the success of Griffin *et al.* (1993) and the comparable success of Hetherington and Jones (1993) in increasing bud production in *E. globulus* and *E. nitens* through application of paclobutrazol, it is the effects of this plant growth retardant which are studied in this thesis. Specifically, it was hoped to determine some mechanism for paclobutrazol activity on flowering in *E. nitens* and *E. globulus*, and to assess the ability of this compound to induce precocious flowering, thereby reducing the large generation time of these species. In addition, some understanding of the rates of uptake, transport and subsequent metabolism of paclobutrazol was sought. It was hoped this would rationalise the persistence of paclobutrazol application effects observed in eucalypts (Griffin *et al.*, 1993) and other species such as apples (Steffens *et al.*, 1991) as well as providing an indication of optimal application methods.

As the effects of paclobutrazol treatment are likely to be mediated largely by an inhibition of biosynthesis of the phytohormone group known as the GAs, it seemed necessary and appropriate to examine the effects of paclobutrazol application on GA levels in eucalypt species and to subsequently relate these to any effects on reproductive activity observed. GAs have been previously recovered from a phylogenetically diverse range of species from the fungi in which they were first identified (Kurasowa, 1926), through ferns (Yamane *et al.*, 1985), gymnosperms (eg. *Picea sitchensis*, Moritz *et al.*, 1990), and angiosperms from herbaceous annual monocots (eg. *Sorghum*, Rood *et al.*, 1986) to arboreal perrenial dicots such as apricot (*Prunus armeniaca*, Bottini *et al.*, 1985). Thus, despite the fact that GAs had never been identified in eucalypts by modern analytical techniques, it was envisaged that a biosynthetic pathway for the production of GAs would exist and appropriate extraction, purification and assay procedures would confirm this. While the pathway of GA production from early precursors such as mevalonic acid to GA_{12}-aldehyde are common to all GAs in higher plants, a number of possible fates may befall this immediate precursor of true GAs (Fig. 1.6, Chapter 1). Once a selection of the GAs present are identified

and their levels quantified, they should indicate the likely dominant pathway of GA production. It is the levels of active GAs (Hoad, 1983) in the dominant pathway which are likely to be the most important, thus allowing future work to be less broad ranging in terms of number of GA compounds monitored, once these active compounds are identified.

Chapter 1 details the development of extraction, purification and assay techniques which are used to provide some indication of GA levels and subsequently identify the dominant pathway for biosynthesis of active GAs. Once established, these techniques are used to determine the effects of paclobutrazol, GA_3 and cold treatments on GA levels in grafted *E. nitens* material and these correlated with observed changes in reproductive activity (Chapter 2). Chapter 3 describes a series of experiments which utilise ^{14}C labelled paclobutrazol to determine the rates of uptake and metabolism of foliar and soil applied paclobutrazol. In addition, the rate of breakdown of ^{14}C paclobutrazol in the soil is assessed. Chapter 4 deals with the attempts to induce precocious flowering in potted, *E. globulus* seedlings using paclobutrazol. The effects of treatment on genetically precocious as well as 'normal' material are compared. The effects of different cultural treatments on bud production as well as the persistence of paclobutrazol effects in the second year subsequent to paclobutrazol application are assessed.

Figure 0.1. Sites of activity of the three major groups of plant growth retardants, with the effects of cyclohexanetriones shown in the early 13-hydroxylation pathway. Modified from Rademacher (1991).

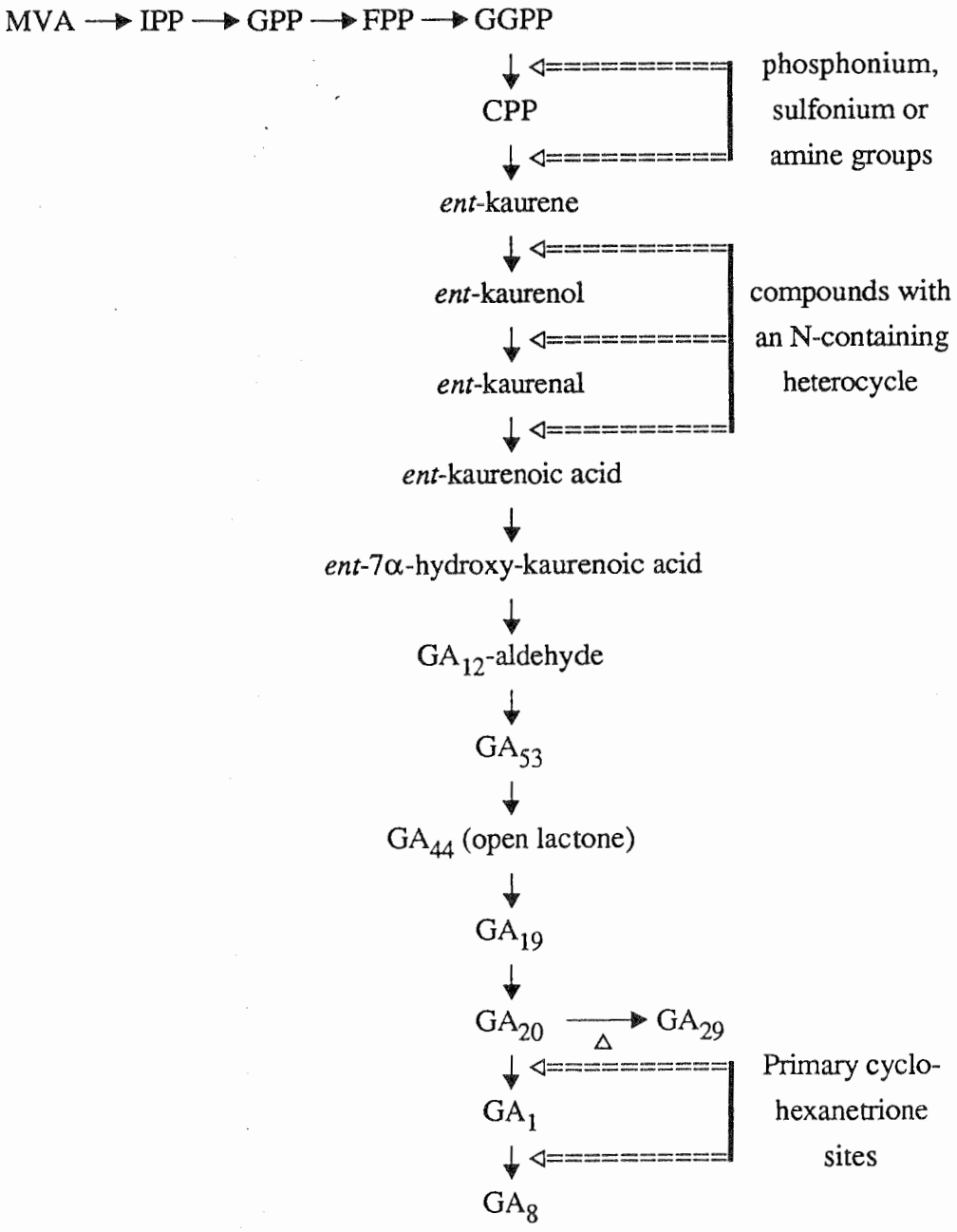
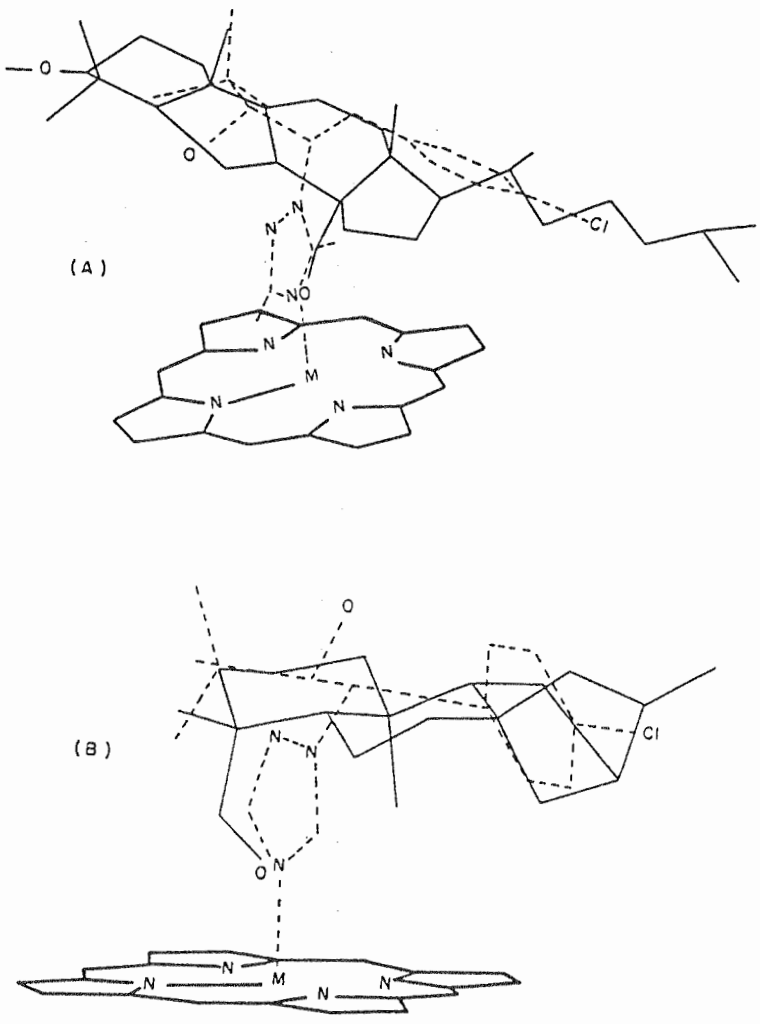


Figure 0.2. Computer graphics showing: (A) - the binding of lanosterol (——) and the (2R,3R)-enantiomer of paclobutrazol (----) to cytochrome P-450 porphyrin (part structure) (——) and (B) - the binding of kaurene (——) and the (2S,3S)-enantiomer of paclobutrazol (----) to cytochrome P-450 porphyrin (part structure) (——). Taken from Sugavanam (1984).



Chapter 1

Identification and Quantification of GAs in *E. nitens*

Introduction

The effects of paclobutrazol on the reproductive activity of eucalypts are postulated to be mediated via an effect on GA biosynthesis (General Introduction). While some previous work has demonstrated the existence of a group of hormone-like substances in some eucalypts (Dhawan *et al.*, 1979; Paton *et al.*, 1980; Paton *et al.*, 1981; Sharkey *et al.*, 1982; Taylor *et al.*, 1982), the presence of GAs has not been demonstrated in any eucalypt species. The initial aim of this study then, was to demonstrate the existence of a biosynthetic pathway for the production of this group of hormones.

The diverse range of species from which GAs have previously been identified (General Introduction) suggested a high likelihood of the existence of a chemical signalling system involving GAs. Despite this however, the goal of deriving a technique to extract, purify, identify and quantify GAs was preceded by a number of simple preliminary experiments to provide a straightforward indication of the likely presence or otherwise of GAs in eucalypts. Firstly, a methanolic extract of apical shoot tissue of seedling *E. nitens* was purified using a method based on that of Reid *et al.*, (1990), with the additional step of polyvinylpolypyrrolidone (PVPP) slurrying, and fractionated by high performance liquid chromatography (HPLC). The fractions produced were dried and tested for activity using the Tan-ginbozu dwarf rice seedling bioassay (Murakami, 1968). The results (Fig. 1.1) suggested the presence of both inhibitors and promoters of plant growth in the initial extract. This encouraging outcome was followed by a second experiment designed to assess the capacity of the plant material to metabolise GA_{20}, hence providing an indication of the presence or otherwise of some enzymes of GA biosynthesis and catabolism. [$^{13}C^{3}H$] GA_{20} was applied to the leaves of *E. nitens* seedlings and 2 days later material from above the treated leaves was harvested. Extraction and purification performed using the method of Reid *et al.* (1990) and HPLC fractionation as for the bioassay was followed by liquid scintillation

counting to assess the amount of tritium label in each fraction. The resulting chromatogram (Fig. 1.2) shows that a number of chromatographically separable tritium-labelled compounds were produced from the single GA applied. While this may simply be the result of the [$^{13}C^{3}H$] GA_{20} binding to different components of the purified extract such as phenols, it may alternatively represent specific metabolism of the applied compound to other GAs. Alternatively, some of the compounds produced may be the result of formation of GA glucosyl conjugates or other conjugated GA products (Schneider and Schmidt, 1990) which may also demonstrate specific metabolism of the applied [$^{13}C^{3}H$] GA_{20}.

The results of these preliminary experiments strongly suggested the presence of a GA biosynthetic pathway. Based on this assumption, efforts were directed towards the production of extraction and purification procedures which would enable the identification and quantification of GAs from apical shoot tissue of *E. nitens* using combined gas chromatography-mass spectrometry (GC-MS).

Materials and Methods

Extraction from plant material and addition of standards

Harvests of apical material, including both stem and unexpanded leaf tissue, were transferred to cold (-20° C) methanol at the rate of 5 mL.g fresh weight (FW)$^{-1}$ and homogenised with a rotary cutter. Samples requiring quantification of endogenous GAs had selections from [17, 17- $^{2}H_2$] GAs A_1, A_3, A_4, A_8, A_9, A_{19}, A_{20}, A_{29}, A_{53} and [17- $^{2}H_1$] GA_{44} (supplied by Prof. L. Mander, Research School of Chemistry, Australian Natl Univ., Canberra, Australia) added as internal standards. Tritiated GAs were utilised as tracers in samples used for both quantification and identification, with approximately 40000 dpm [^{3}H] GA_1 (1.39 TBq.mmol^{-1}) and [^{3}H] GA_{20} (1.11 TBq.mmol^{-1}) added to all samples. Similar quantities of [^{3}H] GA_4 (1.19.TBq mmol^{-1}) (Amersham International, Little Chalfort, UK) and/or [^{3}H] GA_9 (supplied by Dr R.P. Pharis, Dept of Biology, Univ. of Calgary, Canada) were added when a broader range of GAs was under analysis. The extracting solution was adjusted to 80% aqueous MeOH and

extraction proceeded for 24 h at 4° C.

Sample purification

After 24 h, tissue debris was removed by filtration, the extract reduced to an aqueous solution (*in vacuo*, <35° C) and an equal volume of pH 8.0, 0.5 M sodium phosphate buffer added. The buffered aqueous extracts were acidified to pH 2.9 with 4 M hydrochloric acid (HCl) and partitioned five times against 40% volumes of ethyl acetate. The ethyl acetate fractions were frozen, ice removed by filtration, then reduced to dryness with the remaining water removed azeotropically using toluene. The dried extracts were dissolved in ca. 10 mL of 60% methanol and the solution passed through a preconditioned Sep-Pak Plus C_{18} cartridge (Waters Assoc. Milford, MA, USA) at a rate of 5 mL.min^{-1}, followed by elution with 20 mL of 60% methanol. The combined eluates were reduced *in vacuo* until the volume of the extract had reached approximately 10 mL. A 15 x 100 mm anion exchange column was prepared by conditioning 2 g of QAE Sephadex A25 (Sigma Chemical Co. MO, USA) with 50 mL of 0.5 M sodium formate buffer followed by 40 mL of distilled water. The sample was adjusted to pH 7.3 with 1 M NaOH and introduced onto the column, which was then washed with 30 mL of pH 8.0 distilled water. Gibberellins were eluted with 40 mL of 0.2 M formic acid. The eluant was reduced to ca. 10 mL, adjusted to pH 7.3 and the anion exchange chromatography step repeated. The final eluate was taken to dryness in preparation for fractionation by HPLC.

HPLC

The HPLC system (Waters Assoc., Milford, MA, USA) consisted of two model 510 pumps delivering a mobile phase consisting of a linear gradient of 21 to 70% MeOH in 0.4% acetic acid at 2 mL.min^{-1} over 40 min. The sample was injected via a UK6 sample injection unit with a 2000 µL loop onto a 10 cm x 8 mm i.d. 10 µm Radial-Pak C_{18} cartridge fitted in a RCM 8 x 10 module. One minute fractions were collected and 5% aliquots were assayed for radioactivity by supplementing with ca. 4 mL of ReadySafe Liquid Scintillation Counting Cocktail (Beckman

Instruments Inc., Fullerton CA, USA) and subsequent analysis using a Beckman LS 5801 liquid scintillation counter. Based on the elution of radiolabelled GAs and known differences in polarity of GAs for which no suitably radiolabelled tracers were available (Jensen *et al.*, 1986; Lin and Stafford, 1988), fractions were grouped, dried, redissolved in MeOH and methylated using ethereal diazomethane. For the purposes of quantification these fractions were then analysed by GC-MS as described below. For identification of GAs, grouped fractions were further purified as methyl esters by HPLC using the same system components as described above. The solvents were MeOH and distilled water, and the flow rate was 1.5 mL.min^{-1}. For Me-GA_{19} and Me-GA_{20} the solvent program was a linear gradient from 40-80% MeOH over 35 minutes. For Me-GA_1 an isocratic program with 29% MeOH was utilised. One minute fractions were again collected, grouped on the basis of radioactivity, and dried.

GC-MS

The grouped fractions were trimethylsilylated by adding 2 µL of pyridine and 10 µL of bis (trimethylsilyl) trifluoroacetamide and heating at 60° C for 15 min. When Kovat's retention indices (KRIs) were to be determined, 0.1 µL of a mixture of n-alkanes in chloroform was added to the extract. Gas chromatography-mass spectrometry using selected ion monitoring at low resolution (LR-SIM) was performed using a Hewlett-Packard 5890 gas chromatograph linked via an open split interface to a Hewlett-Packard 5970 mass selective detector. One µL splitless injections were made at 250° C onto a 25 m x 0.22 mm i.d. BP1 column with a 0.25 µm film thickness (SGE Vic., Aust.). The helium carrier gas was supplied at an initial flow rate of 2 mL.min^{-1} at 190 kPa and the oven temperature was programmed to rise from from 60° C to 230° C at 30° C.min^{-1} then to 290° C at 3° C.min^{-1}. The interface temperature was 290° C and the ionisation potential was 70 eV.

Full scan mass spectrometry and high resolution GC-SIM (HR-SIM) were performed using a Hewlett-Packard 5890 Series II gas chromatograph linked via a direct inlet to a Kratos Concept ISQ mass spectrometer controlled by a Mach 3 data system. One µl splitless injections were made at 250° C onto the same SGE BP1

column. The carrier gas was helium with the head pressure programmed to maintain a flow rate of approximately 2 mL.min^{-1}. The oven temperature was programmed to rise from 60° C to 240° C at 30° C.min^{-1} then to 290° C at 3° C.min^{-1}. The interface temperature was 290° C and the ionisation potential was 70 eV. Where HR-SIM was used, the masses of characteristic ions were calculated to 4 decimal places and detection was achieved by voltage switching at a resolution of 10 000 (10% valley definition) and a cycle time of 0.6 seconds. Perflourokerosene was used to provide reference masses for HR-SIM.

GA identifications

Where possible, identifications were achieved by comparison of full scan mass spectra and KRI values of putative GAs with reference data (eg. Gaskin and MacMillan 1991). Where good quality full scan mass spectra were not obtainable due to the low level of putative GA or the presence of co-eluting impurities, LR- and HR-SIM in conjunction with KRI data were employed to aid identification. GC-SIM chromatograms of several characteristic ions were compared between the putative GA and a deuterated standard in order to establish an interference-free assessment of ratios of intensities of fragment ions to molecular ion intensity.

GA quantifications

Quantification of GAs was achieved as in Lawrence *et al.* (1992), whereby peak areas generated by GC-SIM for the endogenous and deuterated ions were compared. When LR-SIM was used, corrections for naturally occurring isotopes were calculated using a computer programme (Cooper *et al.*, 1975) and in conjunction with corrections for unlabelled material in the internal standards used to produce an adjusted ratio of endogenous to deuterated peak areas. This ratio was multiplied by the amount of internal standard added and divided by the weight of tissue harvested to produce a final GA level. Where HR-SIM was used, the correction for naturally occurring isotopes was determined empirically, while the remainder of the method was the same as that described for LR-SIM. The ion pairs monitored were 506/508 (GA_1 and GA_{29}), 504/506 (GA_3), 284/286 (GA_4),

594/596 (GA_8), 298/300 (GA_9), 434/436 (GA_{19}), 418/420 (GA_{20}), 432/433 (GA_{44}) and 448/450 (GA_{53}). Key additional ions monitored to support identifications of compounds being quantified were 448/450 and 377/379 (GA_1), 208/210 (GA_3), 289/291 and 418/420 (GA_4), 448/450 (GA_8), 270/272 (GA_9), 462/464 and 402/404 (GA_{19}), 375/377 (GA_{20}), 375/377 and 303/305 (GA_{29}), 238/239 (GA_{44}) and 251/253 and 389/391 (GA_{53}).

Results

Method development

Apical shoot material from *Eucalyptus nitens* seedlings has proven to be a nearly intractable tissue for the extraction and analysis of GAs when compared to similar tissues from more commonly studied domestic, herbaceous plant species (eg. *Pisum sativum*, Reid *et al.*, 1990). It contained a substance (or group of substances) which markedly affected the chromatographic behaviour of the GAs (especially as free acids) on C_{18} reverse-phase HPLC, possibly by overloading the column or otherwise affecting the interaction between GAs and the C_{18} column. This substance was not separated from the GAs by standard extraction procedures used successfully in this laboratory for relatively large harvests of pea and sweet pea material (eg. Reid *et al.*, 1990). The peak shape of tritiated GA_1 used as a tracer illustrates the problem (Fig. 1.3a). Radioactivity attributable to GA_1 was spread at least from fraction 10 to fraction 22. When these fractions were grouped, methylated and rechromatographed, a peak of just a few minutes duration was resolved, eluting only marginally earlier than the methylated tritiated GA_1 standard (Fig. 1.3b). The peaks on either side of the methylated GA_1 (Fig. 1.3b) may be the result of the formation of GA complexes which have altered chromatographic behaviour, when compared to that of the unbound Me-GA_1. The effects of the contaminant(s) on the chromatography of GA_1 were similar to the effects of phenols on the behaviour of GA_3 described by Nutbeam and Briggs (1982). However, the incorporation of a step using PVPP, an insoluble cross-linked form of polyvinylpyrrolidone thought to remove phenolic impurities (Glenn *et al.*,

1972), did not result in a significant improvement of chromatography, suggesting that perhaps some component of the extract other than a phenolic compound was responsible for the effects observed. The fractions constituting the relatively unaltered GA_{20} peak obtained after the first HPLC run as free acids (Fig 1.3a) when similarly grouped, methylated and rechromatographed, produced a single peak which corresponded precisely with the retention time of methylated GA_{20} (Fig. 1.3c). The identities of both the putative Me-GA_1 and Me-GA_{20} peaks were subsequently confirmed by GC-SIM (data not shown).

It is clear then, that in order to efficiently extract and purify GAs from *E. nitens* a means of removing the impurities which affect the chromatography of GAs is required. This aim was achieved through modification of the method of Reid *et al.* (1990) and the addition of an anion exchange chromatography procedure using QAE Sephadex A25 (Talon *et al.*, 1990a). The primary change to the former method was the reversal of the order of the first two steps, hence performing ethyl acetate partitioning prior to the Sep-Pak step. This was mainly due to the relatively small capacity of the Sep-Pak Plus C_{18} cartridges to hold pigmented contaminants from the eucalypt extracts. A large proportion of these contaminants are removed by the ethyl acetate partitioning step, the product of which can be rapidly dried, due to its high volatility, and taken up in a small volume of aqueous methanol thereby expediting the Sep-Pak step. The anion exchange step was incorporated after preliminary experiments demonstrated its effectiveness in removing visible pigments with little loss of all but the most non-polar GAs. The repetition of the anion exchange step achieved a noticeable increase in sample purity, as determined by the output of an in-line UV detector in the HPLC system. This level of sample purity could not be produced in a single step, despite assessing various changes in anion exchange column volume and elution volumes. Subsequent to the addition of this step, eucalypt extracts required only a single HPLC run to produce good separation and resolution of GA_1 and GA_{20} (Fig. 1.4) and to ensure the GAs were sufficiently pure for quantification work by GCMS-SIM.

While both immunoassay (Atzorn and Wieler, 1983a,b; Oden *et al.*, 1987; Hedden, 1993) and GC-MS (Hedden, 1987; Hedden and Croker, 1990; Hedden, 1993) techniques are potentially useful for quantification of GAs, a number of benefits provided by the latter procedure make it the one of choice. Perhaps the

greatest constraint on the use of immunoassay procedures is the difficulty in correcting for interference caused by non-GA extract components (eg. Oden *et al.*, 1987) and the related problem of producing highly specific assays (Hedden, 1993). To avoid these problems, considerable sample purification is required, necessitating an extra procedure to estimate GA recovery (eg. Nakajima *et al.*, 1991). The use of internal standards for the quantification of GAs by GC-MS techniques accounts for losses during the extraction and purification procedure (Hedden, 1987) and provides confirmation of the compound recovered.

The use in this study of dideuterated GA standards with very high isotopic substitution (produced by L. Mander - see Materials and Methods this chapter) has simplified the calculations required for quantification of endogenous GAs. The use of these standards with two deuterium atoms results in less interference from the molecular ion of the endogenous material in comparison to a singly ^{13}C substituted labelled standard. This is due to the two mass unit difference between the standard and endogenous hormone molecules reducing the overlap of the natural isotopes molecular ion 'cluster' with the heavier molecular ion of the standard. Some degree of correction is still required, however, due largely to the high abundance of natural heavy isotopes of silicon present in the trimethylsilyl derivatives of GAs which are required to make these hormones sufficiently volatile to enable GC-MS analysis (Gaskin and MacMillan, 1991). The use of extra deuterium substitutions could virtually eliminate the need to correct for the overlap of the endogenous molecular ion cluster onto the molecular ion of the internal standard. This may not be advisable however, as evidence of differential retention of $[^2H_5]$ indole acetic acid on a variety of HPLC systems has been produced (Brown *et al.*, 1986; Wodzicki *et al.*, 1987) suggesting altered chromatographic properties had resulted from the five deuterium substitutions. Given that the internal standard concept operates on the premise of no separation of labelled and unlabelled material during extraction and purification, such a situation must be avoided where possible. The use of ^{14}C labelled GAs is another possibility for isotope dilution type quantification studies. Such material offers the advantages of a dideuterated GA standard with the added advantage of the ability to trace the movement of the particular GA by means of its radioactivity, hence negating the requirement for added tritiated standards, as in the above method. However, it is difficult to

prepare [^{14}C] GA standards which are sufficiently free of unlabelled material to make their use practicable (Hedden, 1993). Additionally, the use of radioactive tracers of high specific activity and purity as internal standards demands considerably more stringent handling of samples, in order to avoid the potential hazards they pose. This may place unwanted restrictions on the analysis of such samples.

As noted by Hedden (1993), the deuterium substitutions in GA standards result in reduced retention times on GC in relation to their endogenous counterparts, requiring that the molecular ion abundances be averaged over their full GC elution times prior to comparison between endogenous and standard levels. This was performed and, as described, estimations of endogenous GA content were made by direct comparison of the corrected molecular ion pair intensities (Lawrence *et al.*, 1992). This is an accepted and frequently used method, despite the fact that it generates slight, though significant increases in calculated levels when compared with other methods used for analysis of such GC-MS-SIM data (Hedden, 1993). For comparisons of quantifications within this study, such a deviation between estimation methods was considered to be of little consequence.

GA identifications

Despite the use of a second HPLC fractionation step after GAs were derivatised to methyl esters, high levels of impurities with only moderate GA levels made the unambiguous identifications of GA compounds by subtraction spectra difficult. However, a mass spectrum was obtained for GA_{19}, while mass spectral data obtained for GA_1 and GA_{20} samples was adequate to discount the possibility that the major fragment ions were the result of breakdown of some larger unrelated molecule. Subsequent HR-SIM analysis of all 3 putative GA compounds produced results such as those for GA_{20} seen in Fig. 1.5. The background in each of the ion channels selected is relatively free of contaminating substances and the co-elution of 6 ions can be clearly observed, suggesting the detection of genuine endogenous GA_{20}. The clarity of ion chromatograms using this mass spectral technique make it ideal for quantification work where poor signal to noise ratios in the more usual LR-SIM mode make accurate estimations of peak areas difficult.

The major limitation with HR-SIM is the limited mass range over which scanning can be achieved. For this reason, final confirmation of the identity GA_1, GA_{19} and GA_{20} was achieved using LR-SIM to determine the relative abundances of characteristic ions, and KRI values (Tab. 1.1). Deuterated standards were used for comparisons and also for 'spiking' the extract to check co-elution of standard and putative GA.

The identifications of GAs achieved by GC-SIM from apical shoot tissue of *E. nitens* were subsequently supported by work utilising the procedures developed within this study, but not directly involved with it. This peripheral work also demonstrated the existence of a mechanism for GA biosynthesis in the second species of interest in this study, *E. globulus*. Analysis of the tissues of the cambial zone of *E. globulus* trees resulted in the identification of GA_{19}, GA_{20} and GA_{29} by full scan mass spectra and KRIs (Hasan *et al.*, 1994, Appendix A). *E. globulus* and *E. nitens* are closely related, both belonging to the *Symphyomyrtus* sub-genus, making it probable that they would share a common GA biosynthetic pathway.

GA quantifications

When deuterated internal standards were added at the beginning of the extraction procedure to enable quantifications of GAs, GC-SIM analysis of the molecular ion cluster of deuterated $[^2H_2]$ GA_{29} and $[^2H_2]$ GA_{53} indicated some dilution by endogenous material. Based on this evidence, levels of putative endogenous GA_{29} and GA_{53} are given in Tab. 1.2 along with the calculated levels of GA_1, GA_{19} and GA_{20}. There was no detectable dilution of $[^2H_2]$ GA_4, GA_8 and GA_9 or $[^2H_1]$ GA_{44} in the same extract (Tab. 1.2). Later work failed to detect dilution of $[^2H_2]$ GA_3 in extracts from similar material.

Discussion

The presence of those GAs detected suggests that the early 13-hydroxylation pathway of GA biosynthesis (Sponsel, 1983), with GA_1 as the species with

activity *per se* (Phinney, 1984; Reid, 1986), predominates over alternative pathways in vegetative shoot apices of *E. nitens* (Fig. 1.6). In particular, detected GAs A_1, A_{19}, A_{20}, A_{29} and A_{53} are members of the early 13-hydroxylation pathway while the unquantifiable GA_4 and GA_9 are important compounds of the early 3-hydroxylation and early non-hydroxylation pathways respectively. The GAs of the early 13-hydroxylation pathway are found in vegetative material across a phylogenetically diverse range of plant species. Amongst other tree species studied are willow (*Salix sp.*) (Davies *et al.* 1985; Junttila *et al.* 1988) and citrus species such as *Citrus sinensis* (Turnbull, 1989; Talon *et al.* 1990a). Turnbull (1989) identified GAs A_1, A_8, A_{20}, and A_{29} in vegetative tissues while Talon *et al.* (1990a) recovered GAs A_1, A_{19}, A_{20}, and A_{29} from similar material. Both workers also found traces of other GAs but suggested that the early 13-hydroxylation pathway is the major biosynthetic pathway in vegetative tissue of *Citrus*. Vegetative shoots of *Salix dasyclados* were shown to contain GAs A_1, A_4, A_8, A_9, A_{19}, A_{20}, and A_{29}, although levels of early 13-hydroxylation pathway GAs were much higher than those of GA_4 and GA_9 (Junttila *et al.* 1988). The authors suggest these results indicate the operation of the early 13-hydroxylation pathway with possible concurrent operation of the early non-hydroxylation pathway.

Interestingly, in a quite different species, *Thlaspi arvense*, an annual herbaceous angiosperm, a system in which the early 13-hydroxylation pathway and the early non-hydroxylation pathway control two separate aspects of vegetative growth has been postulated (Metzger and Mardaus 1986; Metzger 1990). In certain other herbaceous plants, for example maize (Fujioka *et al.* 1988), and wheat (Appleford and Lenton 1991), most diagnostic GAs of the early 13-hydroxylation pathways have been identified, though often in conjunction with members of the early non-hydroxylation pathway.

To compare actual pool sizes of particular GAs across plant groups from these reports is difficult due to the confounding influence of a number of factors including temporal and ontogenetic changes in levels and perhaps most importantly, the problems associated with selecting comparable tissue between different species. With these reservations in mind, some comparisons can be made between the levels of GAs in *E. nitens* and representatives of other plant groups.

In their quantitative study of the GAs of vegetative shoots of wild type maize, Fujioka *et al.* (1988) found that levels of GA_1 and GA_{29} were approximately 0.1 ng.g FW^{-1} and GA_{20} was higher at around 0.35 ng.g FW^{-1} whereas GA_{19} was considerably more abundant at levels of over 4 ng.g FW^{-1}. Shoot terminals of seedling *E. nitens* contained levels of GAs A_{29}, A_1 and A_{20} ranging from 0.61 to 0.76 ng.g FW^{-1} respectively, while in a similar fashion to maize, the level of GA_{19} was considerably higher at 2.1 ng.g FW^{-1} (Tab. 1.2). The trend of a high level of GA_{19} in comparison to that of GA_1 is also reflected in the study of willow (*Salix dasyclados*) (Junttila *et al.*, 1988) with levels determined to be 5.8 ng.g FW^{-1} and 0.13 ng.g FW^{-1} respectively. In this case, however, the level of GA_{20} (6.2 ng.g FW^{-1}) was also elevated in comparison to that of GA_1. Similar situations have been noted in the vegetative tissue of apple (Koshioka *et al.* 1985) and basal leaf segments of wheat (Appleford and Lenton 1991) (see the review of Sembdner *et al.* 1992 for further discussion of these trends).

Despite an apparent congruence in total GA levels across a wide range of species, the variation in the levels of particular GAs between species is significant. However, there does not appear to be any tangible link relating either phylogenetic or growth habit similarities, with similarities in GA levels. For example, in the herbaceous legume *Pisum sativum* (garden pea), cultivars resembling the wild type have been shown to contain levels of GA_1 and GA_{20} of around 10 and 20 ng.g FW^{-1} respectively (Reid *et al.* 1990), which are well over an order of magnitude greater than levels found in *E. nitens* and many grain crops. On the other hand, such a level of GA_1 has been shown to be mirrored in the shoots of the arboreal gymnosperm Sitka spruce, where GA_1 levels were found to be in the order of 10 ng.g FW^{-1} (Moritz *et al.* 1990).

In summary, the results obtained indicate that the early 13-hydroxylation pathway is likely to be the important source of active GAs in the expanding, vegetative shoot tissue of *E. nitens* seedlings. This pathway is common to vegetative material from the majority of plant taxa previously studied. The levels of GAs in this tissue appear to lie within the range shown to exist in similar material across a wide variety of plant species. Identification of GAs by full scan mass spectra was difficult, despite the assessment of many configurations not presented including changes to the number, order and pH of solvent partitioning steps.

However, a comparison of KRI and LR-SIM data between putative endogenous GAs and deuterated standards provided good evidence for the authenticity of the compounds which was further supported by HR-SIM analysis and low quality mass spectra. Though the purification procedure used did not produce optimal GA identifications in the case of material taken from apical shoots of *E. nitens*, it has proven very successful in other situations, as described for tissue of the cambial zone of *E. globulus* trees, resulting in the identification of GA_{19}, GA_{20} and GA_{29} by full scan mass spectra and KRIs (Hasan *et al.*, 1994, Appendix A). For quantification of GAs from apical shoot tissue, the methodology developed proved near ideal by producing reliable estimations of endogenous GA levels after a relatively simple and rapid sample purification. GAs have also been quantified using this technique from material of the cambial zone (Hasan *et al.*, 1994) and phloem tissue (Hasan, unpublished) of *E. globulus* trees. These results should allow investigation of the roles played by GAs in mediating the observed effects of GA biosynthesis inhibitors on the flowering and fruiting behaviour of *E. nitens* material.

Table 1.1. Relative ion abundances of deuterated GA standards and putative endogenous GAs in apical shoot tissue of ca. 1 year old, glasshouse grown *E. nitens* seedlings, as determined by LR-SIM.

GA detected	Source	KRI	Characteristic ions m/z (% relative intensity of base peak)							
GA_1	$^2H_2$ standard	2689	508	493	450	449	379	378	377	315
			(100)	(10)	(22)	(11)	(17)	(20)	(14)	(9)
	extract	2690	506	491	448	447	377	376	375	313
			(100)	(10)	(20)	(9)	(16)	(17)	(15)	(6)
GA_{19}	$^2H_2$ standard	2627	464	436	404	377	376			
			(6)	(100)	(36)	(67)	(79)			
	extract	2628	462	434	402	375	374			
			(6)	(100)	(29)	(81)	(90)			
GA_{20}	$^2H_2$ standard	2523	420	405	377	361	303	210	209	
			(100)	(14)	(59)	(32)	(13)	(9)	(27)	
	extract	2524	418	403	375	359	301	208	207	
			(100)	(12)	(53)	(15)	(16)	(tr[a])	(20)	

[a] trace

Table 1.2. Gibberellin levels in apical shoot tissue of ca. 1 year old, glasshouse grown *E. nitens* seedlings.

GA	KRI of $^2H_2$ standard	Endogenous Level (ng.g FW^{-1})
GA_1	2690	0.75
GA_4	2544	<0.08
GA_8	nd[a]	<0.08
GA_9	2372	<0.08
GA_{19}	2628	2.12
GA_{20}	2524	0.76
GA_{29}[b]	2698	0.61
GA_{44}[b]	nd[a]	<0.08
GA_{53}	2531	0.24

[a] not done

[b] putative GAs

Figure 1.1. Result of a rice seedling bioassay demonstrating the stimulation of growth of the second leaf sheath by fractionated components of an extract derived from the apical shoot tissue of *E. nitens* seedlings. The harvested apical shoot material was extracted for 24 h at 4° C and purified by PVPP slurrying, partitioning against acidic ethyl acetate and passing through a C_{18} Sep-Pak cartridge in 60% methanol. The purified sample was fractionated by reverse phase C_{18} HPLC (using the system of Reid *et al.*, 1990). The solvent programme began at 21% methanol in 0.4% acetic acid for 10 minutes, then rose linearly to 75% methanol over 60 minutes. Flow rate was maintained at 2 $mL.min^{-1}$ and 1 minute fractions were collected.

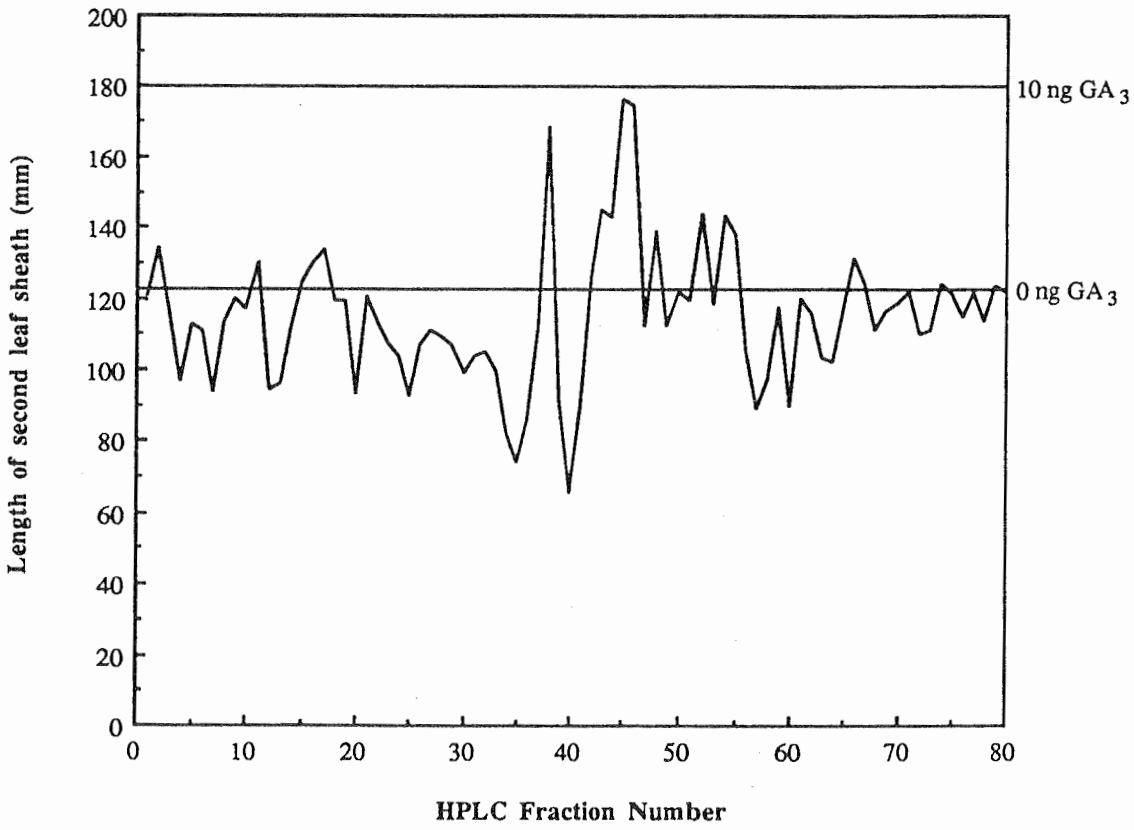


Figure 1.2. The production of tritiated metabolites of [$^{13}C^{3}H$] GA_{20} 55 h after application to the uppermost open leaf of 6 *E. nitens* seedlings (6-7 juvenile leaf pairs expanded). The harvested apical shoot was extracted and purified by the method of Reid *et al.* (1990) and subject to reverse phase C_{18} HPLC (using the system of Reid *et al.*, 1990). The solvent programme began at 21% methanol in 0.4% acetic acid for 10 minutes, then rose linearly to 75% methanol over 60 minutes. Flow rate was maintained at 2 $mL.min^{-1}$ and 1 minute fractions were collected. Radioactivity content of each fraction was assessed by liquid scintillation counting using a Beckman LS 5801 counter after supplementing each fraction with ca. 5 mL 'ReadySafe' LS counting cocktail. Unmetabolised [$^{13}C^{3}H$] GA_{20} material elutes in the zone shown by the open box.

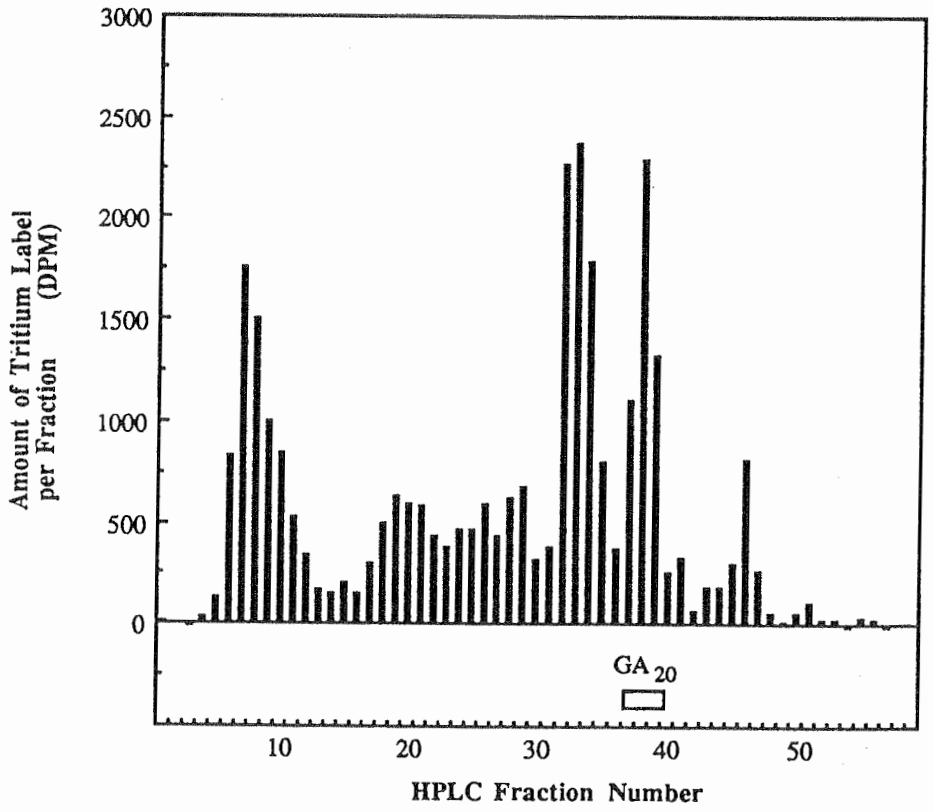


Figure 1.3. Fractionation by HPLC of an apical shoot tissue extract of ca. 1 year old, glasshouse grown *E. nitens* seedlings containing [^{3}H] GA_1 and [^{3}H] GA_{20} as tracers. HPLC profiles of free acids after initial purification by the method of Reid *et al.* (1990) (linear 21-70% methanol in 0.4% acetic acid over 40 minutes; flow 2.0 mL.min^{-1}) (a) and fractions grouped as shown in (a) refractionated as Me-esters (isocratic 29% methanol in distilled water; flow 1.5 mL.min^{-1}) (b), (linear 40-80% methanol in distilled water; flow 1.5 mL.min^{-1}) (c). [^{3}H] GA standards eluted in the zones indicated by open boxes.

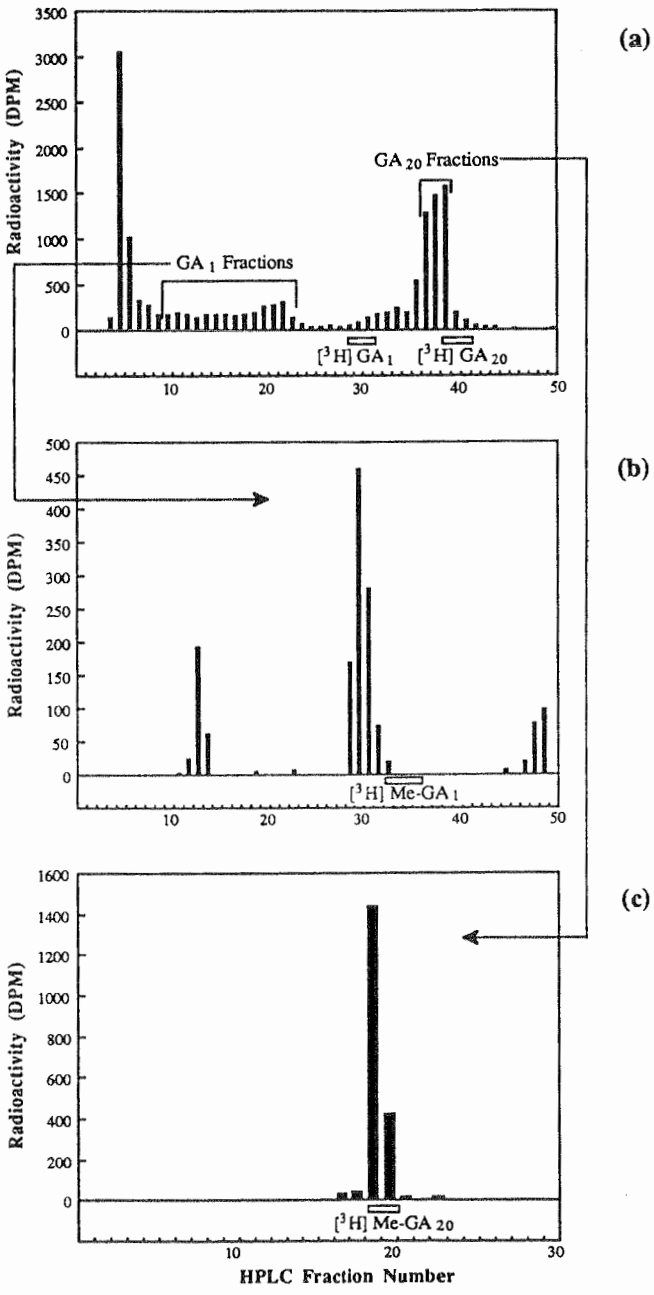


Figure 1.4. Fractionation by HPLC of an apical shoot tissue extract of ca. 1 year old, glasshouse grown *E. nitens* seedlings after purification including anion exchange chromatography. The linear methanol gradient used for HPLC rose from 21 to 70% methanol in 0.4% acetic acid over 40 minutes and a 2.0 mL.min^{-1} flow rate was maintained. [^{3}H] GA_1, A_4, A_9 and A_{20} tracers in the extract were resolved as shown. [^{3}H] GA standards eluted in the zones indicated by open boxes.

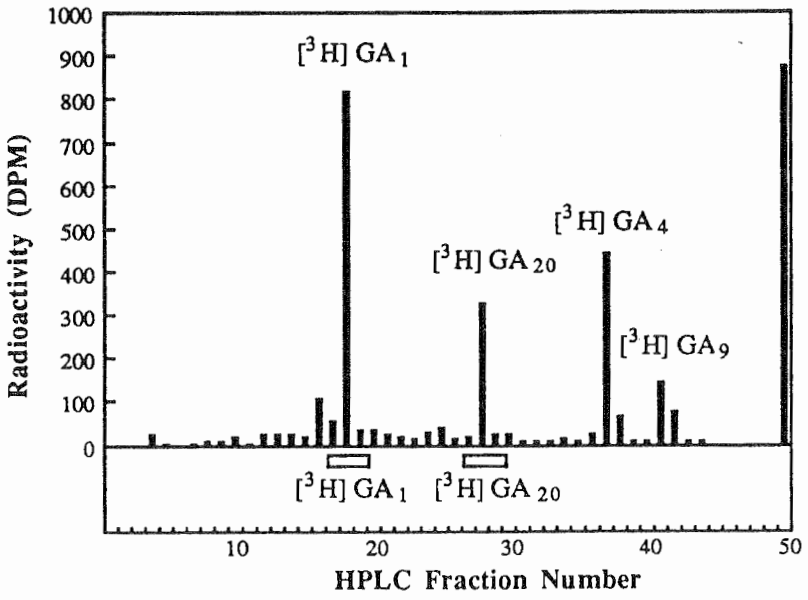


Figure 1.5. HR-SIM analysis of putative GA_{20} extracted and purified from the apical shoots of ca. 1 year old, glasshouse grown *E. nitens* seedlings. Co-eluting peaks in ion channels diagnostic for the GA provide a reasonable confirmation of identification.

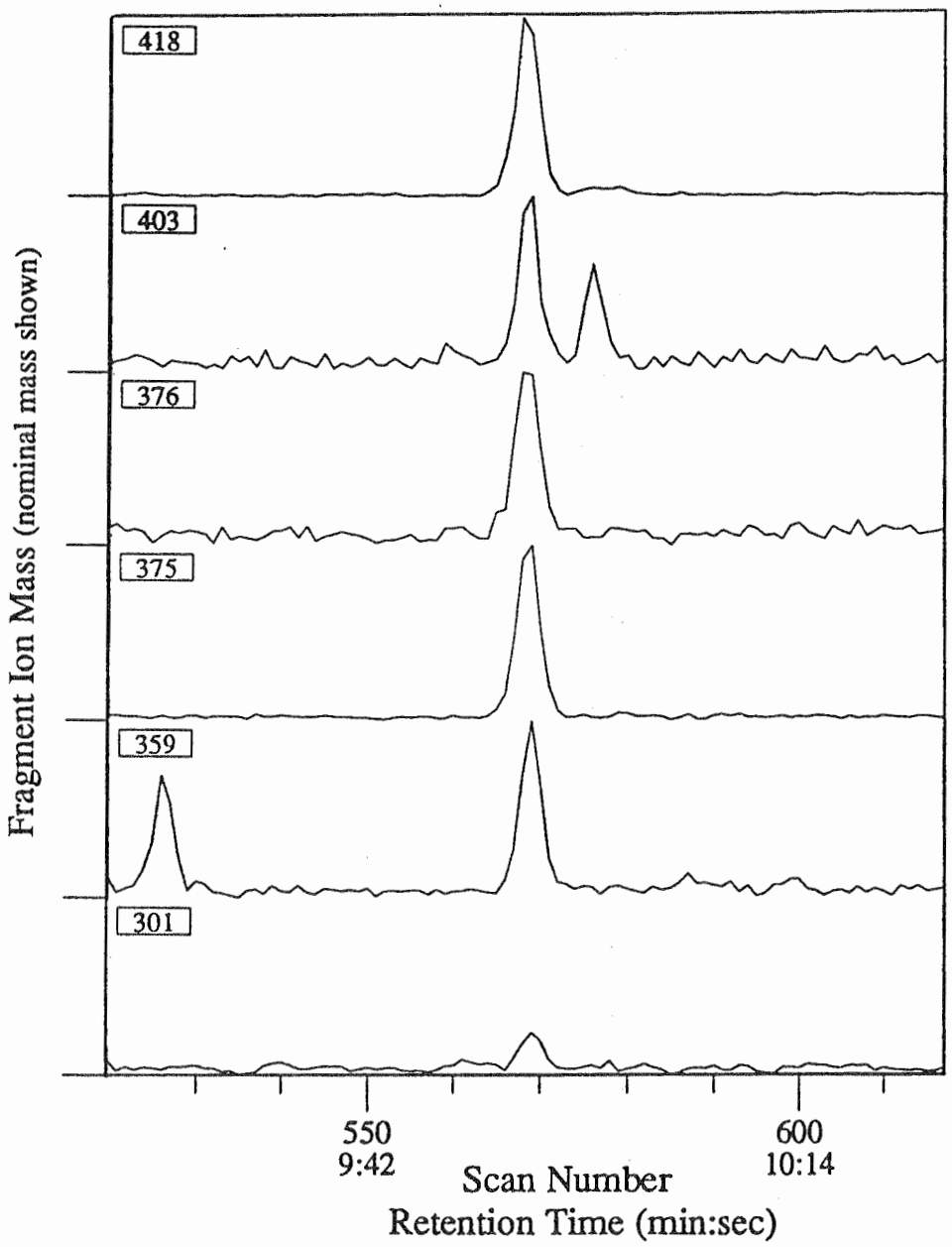


Figure 1.6. Parallel schemes of GA biosynthesis from GA_{12}-aldehyde shown in vertical rows.

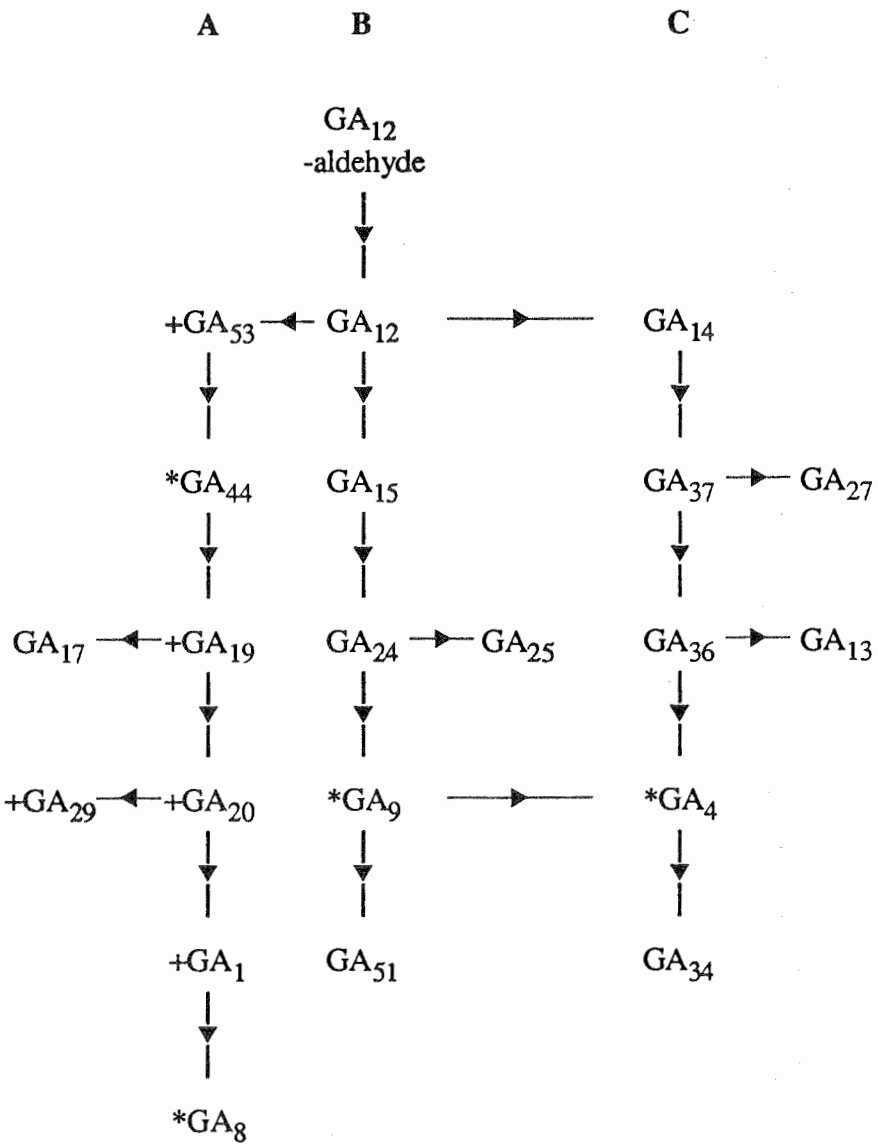
A = early 13α-hydroxylation pathway

B = early non-hydroxylation pathway

C = early 3β-hydroxylation pathway

GAs quantified from *E. nitens* apical shoots (+) and GAs not quantifiable (*) (see Tab. 1.2).

Modified from Talon *et al.* (1990b).



Chapter 2

Effects of Paclobutrazol and Cold on GA Levels and Flowering in Grafted *E. nitens*.

Introduction

The application of the plant growth retardant paclobutrazol (see General Introduction) has been observed to reduce vegetative growth (Hetherington *et al.*, 1991; Griffin *et al.*, 1993) and enhance flowering (Hetherington and Jones, 1993; Griffin *et al.*, 1993) in *Eucalyptus* species. A series of experiments attempting to produce further quantitative data on the effect of this plant growth retardant on the reproductive activity of grafted clonal material were performed by Mr Mike Moncur (CSIRO Division of Forestry, Canberra, Australia). Given the known effects of paclobutrazol in reducing the levels of endogenous GAs (eg. Dalziel and Lawrence, 1984; Hedden and Graebe, 1985) and the identification of these hormones in *E. nitens* (Chapter 1), the involvement of GAs in the mediation of the observed responses in eucalypts was hypothesized. To investigate this hypothesis the methodology for the quantification of GAs (Chapter 1) was utilised in an attempt to correlate paclobutrazol application with flowering responses and GA levels. Experiments described in this chapter are based on the material grown at the Canberra site of the CSIRO Division of Forestry, in experiments designed in collaboration with, and largely effected by, Mr Mike Moncur. Sampling of the appropriate material and the subsequent analysis of GA levels was performed in the Hobart laboratories of the Department of Plant Science and constituted the major contribution of this study to the overall project.

Initially it was considered that an assessment of the effect of paclobutrazol application to a whole plant on GA levels in apical shoot tissue was appropriate. Additionally, it was hoped that by comparing the levels of GAs in similar clones

harvested at different times post-treatment, some indication of the longevity of treatment effects could be obtained.

An apparent correlation between GA levels and flowering activity identified in this initial work suggested that paclobutrazol application may indeed play a role in flower bud production in grafted *E. nitens* material via its effect on the levels of endogenous GAs. To gain further insight into the association between paclobutrazol application, GA levels and flowering responses, a larger scale sampling of material from an espalier orchard (Cauvin, 1984; Moncur *et al.*, 1993, Appendix B) was performed. Sampling for the larger scale experiment occurred 12 months after the initial experiment to produce additional data on the persistence of paclobutrazol effects after application by different methods and at different doses. It was hoped that the analysis of a significant number of samples, covering a range of active GA_1 levels as a result of treatment with paclobutrazol by different methods and at different rates, would generate a more certain correlation between GA levels and flowering response.

E. nitens, like many other temperate eucalypt species, produces flower buds in early summer (Turnbull and Pryor, 1978), presumably after initiation in early spring following a period of relative dormancy during the colder months. This suggests that there is some mechanism by which the seasonal change is monitored, and the signals produced by this system may influence the flowering process. Photoperiodism and/or some period of cold treatment are possibly the most likely factors which are utilised in the monitoring of seasonal change. Bolotin (1975) reported the stimulation of precocious flowering in *E. occidentalis* when grown under a 16 hour photoperiod while Moncur (1992) demonstrated the ability to induce flower buds in *E. lansdowneana* through the administration of a 4-6 week period of cold, flanked by warm growth conditions. The results of the latter study, in combination with results of experimental work on inducing precocious flowering in *E. globulus* seedlings (Chapter 4), directed attention at this initial stage to an investigation of the effects of cold on floral induction and its association with paclobutrazol application and GA levels. To achieve this, the effects of varying lengths of cold treatment in conjunction with paclobutrazol treatment on both flowering response and GA levels in recently grafted clones of *E. nitens* was investigated.

A further means of establishing GAs as the mediating factor of the effects of paclobutrazol application on reproductive output in the clonal *E. nitens* grafts involves the introduction of an exogenous active GA in combination with paclobutrazol. In this manner any effects of paclobutrazol application on vegetative or reproductive growth which are not mediated by a reduction in the level of endogenous active GAs (i.e. GA_1) will continue to be expressed, while those effects which are GA mediated should tend to revert to the untreated state. The extent of success of such an experiment is dependent upon the timing, dose and rate of metabolism of the applied active GA and these factors can make interpretation of results difficult. However, such reversion studies have been performed in the past on orchard tree species, though mostly in relation to the counteraction of paclobutrazol effects on fruit characteristics (eg. in apple, Curry and Williams, 1983). Some some work has been carried out on GA_3 reversal of biochemical and physiological changes induced by paclobutrazol treatment (Steffens *et al.*, 1985). These then were the rationales behind an experiment carried out in Canberra in collaboration with Mr Mike Moncur in which the effects of GA_3 application to paclobutrazol treated grafts were assessed.

Materials and Methods

GA quantifications from espaliered *E. nitens* clones.

Plant Material and Paclobutrazol Treatments

Plants were maintained in a breeding arboretum in Canberra, ACT (Lat. 35° 10'S, Long. 149° 4'E; altitude: 600 m), where they were pruned to facilitate access to potential floral sites and regularly fertilised, irrigated and sprayed to control leaf eating insects. The '18 month' group of clonal grafted material was approximately 18 months old at the time of paclobutrazol treatment in March 1990. Plants constituting the '6 month' group were approximately six months old when treated with paclobutrazol in March 1991. Paclobutrazol was applied to the soil around the base of the stem as an aqueous 2 L collar drench (CD) at 0.5 g or 0.2 g active ingredient (a.i.).cm of stem circumference^{-1}, or as a trunk injection (TI) at 0.025 g

or 0.01 g (a.i.).cm of stem circumference^{-1}.

Harvesting and extraction.

The initial small scale harvesting took place in October 1991, which corresponded to the time at which bud initiation is thought to occur in *E. nitens* in Canberra, and was six or 18 months after paclobutrazol application. For the extended study, harvesting took place in late September 1992 and only the older material (now ca. 40 months since paclobutrazol application) used. In this case, tissue from four grafts of the untreated (control), high rate collar drench (CDH) and low rate collar drench (CDL) treatments was taken, while three trees treated by trunk injection at each of two dose rates (TIH and TIL) were sampled. Apical sections approximately 5 cm long were excised from branch terminals and leaves trimmed as necessary to maintain a constant ratio of leaf to shoot mass across all treatments. Gibberellin A_1 was quantified as described in Chapter 1, with the exception of lypholisation and dry weight determination of frozen material prior to extraction, due to contamination of samples with rainwater preventing measurement of fresh weights.

Effect of cold treatment on flowering and GA levels

Plant Material and Paclobutrazol Application

Forty grafts from a single clone were established in Canberra, in two groups of 20, one year apart. During April 1991 when grafts were either six or 18 months old, paclobutrazol was applied as a collar drench at 0.2 g (a.i.).cm of stem circumference^{-1}. At this juncture five grafts of each age were transferred to a naturally lit glasshouse with day/night temperatures of ca. 25° C/17° C. Remaining grafts were grown outside in ambient Canberra conditions (Fig. 2.1). In June 1991, then again during September 1991, five grafts of each age were transferred to the glasshouse while the remaining group of each age grafts was left outside. Material remained under these conditions until December 1991 when buds were large enough to allow scoring.

Harvesting of Samples and Extraction of GAs.

During October 1991 4-8 g of apical shoot material was sampled from each of the five plants constituting each treatment in the '18 month old' grafts. This material was combined to produce a single sample for each treatment. Gibberellin A_{20} and GA_1 were quantified as described in Chapter 1.

Reversion of paclobutrazol treatment effects by GA_3 application

Paclobutrazol was applied as an aqueous 200 mL collar drench at 0.2 g (a.i.).cm of stem circumference^{-1} to three groups of 15 six month-old, potted, grafted clones in April 1992, whilst an additional three groups of 15 were left untreated. The material was maintained outdoors in Canberra. At two and three months after paclobutrazol treatment, a stock solution of 100 mg.L^{-1} GA_3 was applied as an aqueous collar drench to two paclobutrazol treated groups, at either high rate (50 mL), or low rate (10 mL) and to two non-paclobutrazol treated groups (high/low). Height increments, stem diameters and flower bud production data were recorded over the subsequent months, to December 1992. The levels of endogenous GA_1 and GA_{20}, as well as the level of applied GA_3 which was present in new apical growth, were analysed from samples of apical shoot tissue taken during September 1992 by the methodology detailed previously (Chapter 1).

For further details consult the 'Materials and Methods' section of Moncur *et al.*, (1993) (Appendix B).

Results

GA quantifications from espaliered *E. nitens* clones.

The initial study showed that the effects of paclobutrazol treatment by collar drench on GA levels were fully evident in apical tissues after six months and persist for at least 18 months. The levels of GA_1 and GA_{20} were reduced between seven- and 10-fold in comparison to the control as a result of application of paclobutrazol as a collar drench (Tab. 2.1). The trunk injection treatment, on the other hand, appears ineffective in reducing GA levels 18 months after application.

The extended sampling of the espalier orchard material revealed a considerable degree of variation in the levels of GA_1 between grafts treated by the same method and the same rate (Tab. 2.2). However, taken together, a reasonable correlation between high (>0.5-0.6 ng.g DW^{-1}) GA_1 levels and low numbers of new buds initiated can be observed (Fig. 2.2; Plates 2.1, 2.2). The converse of this situation is not well correlated, with lower GA_1 levels associated with both high and low numbers of new flower buds.

The TIL treatment appears the least persistent, with the TIH treatment only marginally more active at the time of sampling, 30 months post-application. Two out of the 3 CDL applications exhibit a persistent ability to suppress the levels of GA_1, while the CDH samples demonstrate a consistent persistence of effect. However, this persistence is abating as demonstrated by a 3-fold rise in the level of GA_1 in the CDH treated 417-9 graft between the 1991 and 1992 harvests (compare Tabs 2.1 and 2.2).

Effect of cold treatment on flowering and GA levels

For flowering to occur in the material used, an obligate requirement for both complete overwintering in the outdoor environment and paclobutrazol treatment was demonstrated (Tab. 2.3). A greater proportion of the older material within the reproductively active treatments produced floral buds and the average number of buds per flowering graft was also greater (Tab. 2.3). However, the degree of age/size independency shown by the successful induction of flowering in the '6 month' material suggests that graft age may be a less important factor than paclobutrazol and cold treatment for the induction of early flowering. This is somewhat contradictory to the suggestions of Brindbergs *et al.* (1989) and Mullins and Snowball (1988) who indicate an involvement of size and age in the onset of flowering in grafted and seedling eucalypts and seedling citrus species respectively. In the untreated material, those grafts which received the maximum amount of cold had the highest GA levels, and as the amount of time spent under the colder outdoor regime decreased, GA levels appeared to decrease slightly (Tab. 2.4). These effects on GA levels (and flowering in the paclobutrazol treated material) may alternatively be the result of the timing of the transfer to warmer conditions,

rather than just the cumulative amount of cold received. In the paclobutrazol-treated material, levels of endogenous GA_1 and GA_{20} were reduced by approximately an order of magnitude, and the levels of these hormones in the group within which flowering had been promoted did not appear substantially different from non-flowering groups. This data suggests that the effect on flowering of cumulative cold or timing of transfer to warmer conditions is not mediated via a reduction of GA levels.

Reversion of paclobutrazol treatment effects by GA_3 application

Paclobutrazol application produced a three-fold reduction in the levels of endogenous GA_1 and GA_{20} and caused a substantial increase in the proportion of grafts flowering per treatment (31/45 across all paclobutrazol treatments in comparison to 2/45 for non-paclobutrazol treated grafts; $\chi^2_1=38.54$; $p<0.001$) and a significant ($p<0.001$) increase in the average number of buds produced per graft (Tab. 2.5; Plate 2.3). The effect on vegetative growth was equally apparent, with significant reductions in both height increment ($p<0.001$) and stem diameter ($p<0.001$) (Tab. 2.5; Plate 2.4). Gibberellin A_3 application by soil drench significantly increased stem diameter ($p<0.001$) (Tab. 2.5) indicating that applied GA_3 was assimilated by the grafts. The stem thickening response has been noted as the most dramatic effect of GA_3 application on vegetative tissues of apples (Mauk *et al.*, 1990a), which may explain the detection of this response in the absence of any significant effect on height increment ($p=0.863$) (Tab. 2.5). Gibberellin A_3 application by soil drench was associated with a decrease in average flower bud production of greater than 40%, though large inter-replicate variations resulted in non-significance ($p=0.293$) (Tab. 2.5). No GA_3 was found above the threshold of detection (approximately 80 pg.g FW^{-1}) in apical shoot samples from any group, despite the significant effect of GA_3 application on stem diameter. This suggests that the lack of recovery of the applied hormone is likely to be the result of catabolism within the plant tissues during the ca. 3 months which elapsed between application and subsequent sampling of tissue for analysis of GA levels.

Discussion

Gibberellins have been successfully identified from vegetative adult foliage of *E. nitens*, and paclobutrazol treatment of the grafted clonal material reduced the levels of endogenous GAs. The results show varying levels of effectiveness of application method and dose in reducing the levels of endogenous GAs at different times post-application. A substantial degree of within treatment variation in GA_1 levels is seen in the espalier material, 30 months post-paclobutrazol application (Tab. 2.2). The variability of GA_1 level seen within the untreated group indicates a level of natural fluctuation in these levels, while the CDH treatment, contrasting in its inter-replicate uniformity, provides some indication that the variation seen within other treatments may be a result of differring rates of release from the effects of paclobutrazol treatment. For collar-drenched individuals, local variation in soil structure, composition or drainage may play some role in producing inter-replicate variation in the persistence of effects, while the point of application and overall tree vigour may be more important factors in the case of grafts treated by trunk injection.

Divorcing the treatment method and rate from the results enabled a range of GA_1 levels at the time of flower bud initiation to be compared with the number of buds subsequently observed to be initiated some 3 months later (Fig. 2.2). The low level of bud production observed when GA_1 levels are above 0.5-0.6 ng.g DW^{-1} suggests that higher GA_1 levels are inhibitory to flowering. The lack of a good correlation between GA_1 level and bud production below the 'threshold' value of 0.5-0.6 ng GA_1.g DW^{-1} suggests that other factors must be involved in regulating the magnitude of reproductive activity each season.

There are a number of potential factors which may have disturbed the correlation between GA_1 level and flower bud production. The fact that all espaliered material used consisted of grafts generated from scions of only 2 trees, each of which exhibited both high and low flowering responses below the threshold GA_1 level, makes it difficult to assign variation in flowering behaviour to a genetic factor. However, a difference in the position on the donor tree from which scions were taken, or differences in graft growth cannot be ruled out as influencing factors. Another possible effect of grafting which may have produced variation in flowering is that the rootstocks used are not genetically identical and this may have some

effect on the physiology of the scion. Environmental factors cannot be completely ruled out as potential variables despite all espaliered grafts being grown under the same conditions on a gross scale. The possibility of microenvironment variation across the espalier orchard is suggested by the apparent involvement of a positional effect, with those grafts displaying an apparently reduced flowering response appearing at the southern-most end of rows of analysed material (Fig. 2.3). A further possibility for lack of complete correlation seen between GA_1 levels and bud production is variation in developmental stage at the time of tissue sampling. Steffens *et al.*, (1991) showed highly variable levels of GA_3 equivalents in both fruit and seed samples over the weeks following full bloom in apples. Perhaps then a variation in GA_1 levels occurs in the apical shoot tissue of *E. nitens* around the time of flower bud initiation, with the lack of correlation observed resulting from a common sampling time and asynchronous bud initiation. Finally, the pruning of the espaliered grafts must have some effect on reproductive output by reducing the number of potential floral sites. If pruning was not carried out consistently, this too could affect the correlation between GA_1 level and resulting crop size.

The action of cold has been demonstrated to be an important prerequisite to the onset of flowering in paclobutrazol treated material (Tab. 2.3). Conversely, the necessity of paclobutrazol treatment for the expression of a cold induced flowering response was indicated (Tab. 2.3). This was further demonstrated by an assessment of the year 2 flowering results for the cold requirement work, following the transfer of all grafts which did not flower to outside environs after year 1. All 40 grafts originally treated with paclobutrazol produced flower buds in the second year, after a full overwintering period. In contrast, only 1 graft of 40 not treated with the plant growth retardant produced flower buds after identical exposure to cold. The GA quantifications indicate that lowered GA_1 and GA_{20} levels alone are not sufficient for the induction of flowering in recently grafted *E. nitens* material (Tabs 2.3, 2.4). In addition, the GA quantifications suggest that any floral induction 'signal' produced by appropriate cold treatment is not mediated by a further reduction of these GA levels. Indeed the increase in the level of endogenous GA_1 and GA_{20} in the non-paclobutrazol treated 'outside' (maximum cold treatment) group suggests that cold treatment may actually increase the levels

of these GAs at the time floral induction is thought to be occurring.

Both the incomplete correlation between GA_1 levels and flowering and the lack of an obvious means of mediation of cold treatment could be seen as evidence for alternative signalling systems playing some role in controlling flowering responses. These systems could utilise alternative phytohormones, or even non-hormone mediated means to produce their effects. The involvement of an alternative pathway of GA biosynthesis, including the possible activity of 'florigenic' GAs (Evans *et al.*, 1990; King and Pharis, 1993; King *et al.*, 1993) cannot be completely ruled out, though it is difficult to reconcile a qualitative change or quantitative increase of alternative GAs in the presence of paclobutrazol, which inhibits the production of all GAs (General Introduction).

In many ways it is perhaps not surprising that the applied GA_3 is undetectable ca. 3 months after application, given the relatively transient nature of application effects in herbaceous angiosperms such as peas (Swain, 1993). The lack of a significant effect of GA_3 treatment on height increment in paclobutrazol treated individuals suggests that an insufficient quantity of GA_3 was present in the tissues at any stage following application to produce the desired reversion of vegetative growth to the non-paclobutrazol treated state. The stem diameter growth apparently stimulated by GA_3 application may have caused a diversion of assimilates away from reproductive activity (Sachs, 1977; Mauk *et al.*, 1986) at the time at which bud initiation is thought to occur, causing the non-significant indication of a reduction in bud production observed. Perhaps then a greater degree of reversion of paclobutrazol treated individuals to the stature of non-treated individuals would generate a statistically significant reduction in bud production. If an increase in GA_3 dose could produce such a reversion in stature, with a concurrent reduction in bud production, it would provide more convincing evidence of an involvement of 3ß,13-hydroxylated GAs in the control of flowering in eucalypts. This in turn would suggest that the increase in reproductive output of eucalypts observed following paclobutrazol application (eg. Griffin *et al.*, 1993; Hetherington and Jones, 1993; this chapter) may be mediated by the inhibition of GA biosynthesis which this plant growth retardant is known to cause (Dalziel and Lawrence, 1984; Hedden and Graebe, 1985).

Table 2.1. Bud scores and levels of GA_1 and GA_{20} (pg.g FW^{-1}) in the apical shoot tissue of clonal *E. nitens* grafts in a Canberra espalier orchard, 6 or 18 months after paclobutrazol treatment. CD, collar drench; TI, trunk injection. The grafts in the '6 month post-treatment' group were 13 months old and the grafts in the '18 month post-treatment' group were 37 months old at the time of sampling in October 1991.

	6 months post-treatment		18 months post-treatment		
	Control	CD High	Control	CD High	TI High
Graft			417-10	417-9	417-12
GA_1	780	90	670	90	750
GA_{20}	1850	190	1350	130	1270
floral bud no.	nd[a]	nd	165	1590	54

[a] not done

Table 2.2. Level of GA_1 in material harvested in October 1991 and resulting flower bud score (December 1991) in espaliered, clonal *E. nitens* grafts, 30 months after paclobutrazol treatment by trunk injection or collar drench. TIL, trunk injection low; TIH, trunk injection high; CDL, collar drench low; CDH collar drench high.

graft code	treatment	GA_1 conc. (ng.g DW^{-1})	number of buds (December 1991)
422-1	Control	0.79	270
417-2	Control	0.79	33
417-4	Control	1.93	20
417-10	Control	0.98	34
422-7	TIL	0.60	371
417-8	TIL	1.07	109
417-6	TIL	1.62	25
422-5	TIH	0.14	983
417-7	TIH	0.57	71
417-12	TIH	2.10	0
422-2	CDL	0.32	1510
422-3	CDL	0.20	1826
417-1	CDL	1.28	71
422-4	CDH	0.47	855
417-3	CDH	0.25	742
417-11	CDH	0.45	1972
417-9	CDH	0.29	586

Table 2.3. The effect of differing date of transfer from an outside (cold, winter) environment to a heated glasshouse.on the number of clonal *E. nitens* grafts flowering per treatment and the average number of floral buds per flowering graft.

Graft Age[a] /	Paclobutrazol Treated		Untreated	
Transfer Date[b]	flowering	mean floral bud no.[c]	flowering	mean floral bud no.[c]
18 month				
April	0/5	0	0/5	0
June	0/5	0	0/5	0
September	0/5	0	0/5	0
Kept Outside	4/5	55	0/5	0
6 month				
April	0/5	0	0/5	0
June	0/5	0	0/5	0
September	0/5	0	0/5	0
Kept Outside	2/5	15	0/5	0

[a] Graft age at time of paclobutrazol treatment

[b] Date transferred to heated glasshouse from outdoor environs

[c] Mean number of floral buds per flowering graft

Table 2.4. The effect of differring periods of cold treatment on the levels of GA_1 and GA_{20} in apical shoot tissue of '18 month old' group of clonal *E. nitens* grafts (see text).

	GA Levels ($pg.g\ FW^{-1}$)			
	Paclobutrazol Treated		Untreated	
Transfer Date[a]	GA_1	GA_{20}	GA_1	GA_{20}
April	64	20	597	765
June	117	63	639	893
September	74	63	726	918
Outside	141	59	1337	1903

[a] Date transferred to heated glasshouse from outdoor environs

Table 2.5. The effects on growth and reproductive performance (a) and endogenous GA_1 and GA_{20} levels (b) following GA_3 application (G) at either low (l) rate (2x10 mL of 100 $mg.L^{-1}$ GA_3) or high (h) rate (2x50 mL of 100 $mg.L^{-1}$ GA_3) to control and paclobutrazol-treated (P) (0.2 g ai.cm stem $circumference^{-1}$) clonal *E. nitens* grafts. GA levels were measured by GC-MS-SIM in apical shoot material grouped from each of the 15 plants in each treatment.

(a) Treatment	no. grafts with buds	flower buds/graft	Height inc. (mm)[a]	Stem diameter (mm)
Control	1/15	0.7	567	6.23
+G(l)	0/15	0.0	575	5.71
+G(h)	1/15	0.3	598	7.65
+P	10/15	33.3	149	3.39
+P+G(l)	12/15	18.7	145	3.61
+P+G(h)	9/15	17.8	151	4.36
S.E.D.[b]		8.1	48	0.54

[a] Height increment between the time of paclobutrazol application (April, 1992) and flower bud production (December, 1992)

[b] Standard error of difference of means

(b) Treatment	$[GA_1]$ ($ng.g\ FW^{-1}$)	$[GA_{20}]$ ($ng.g\ FW^{-1}$)
Control	1.71	0.58
+G(l)	1.57	0.56
+G(h)	1.76	0.93
+P	0.44	0.14
+P+G(l)	0.55	0.21
+P+G(h)	0.52	0.18

Figure 2.1. Average daily maximum and minimum temperatures each month for Canberra, 1991 and 1992.

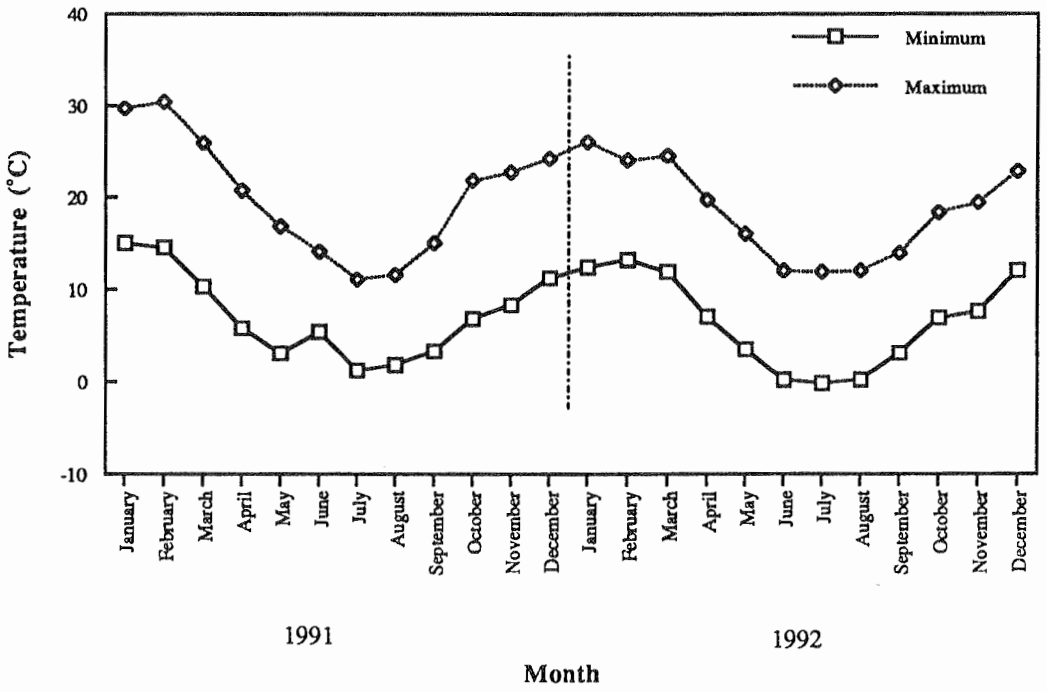


Figure 2.2. Relationship between GA_1 level of espaliered *E. nitens* grafts in September, 1992 and the number of flower buds subsequently initiated and visible in December, 1992. Those points marked with an asterisk have a relatively reduced flowering response for their GA_1 level.

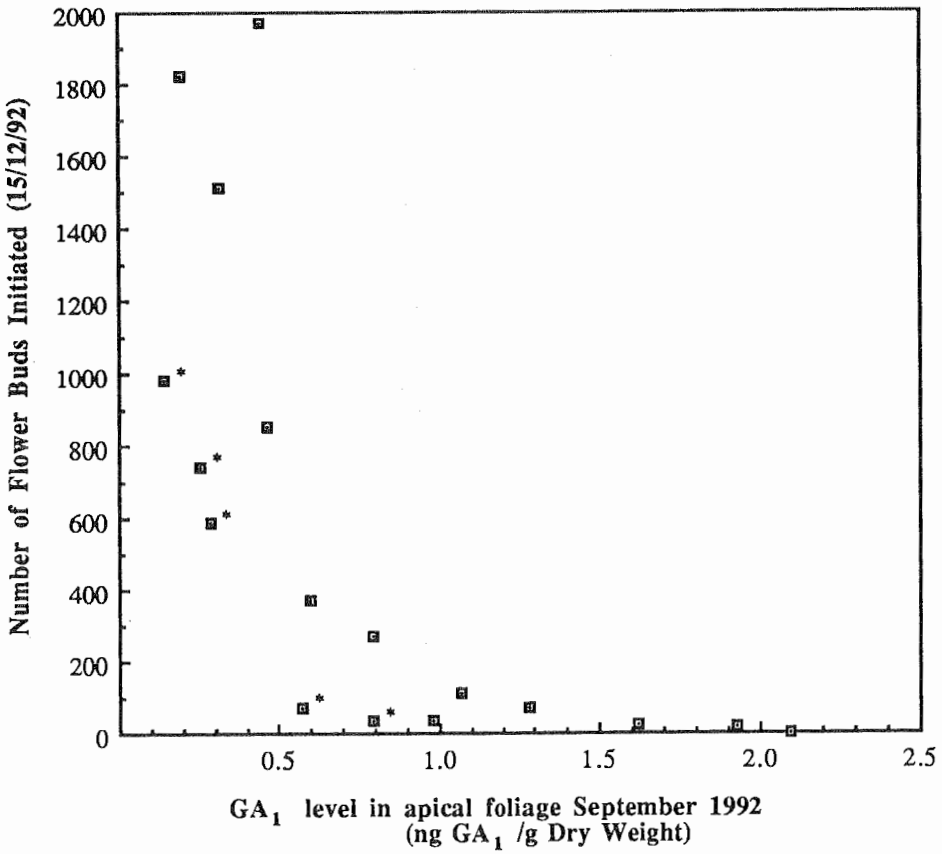


Figure 2.3. Positions of grafted trees sampled for GA_1 quantification within the espalier orchard, Canberra. Grafts marked as open circles (and corresponding to those individuals marked with an asterisk in Fig. 2.2) appear to have produced a lower flowering response than expected, based on their GA_1 concentrations. Grafts were grown 6 m apart in rows 3 m wide. Unlabelled row and column intersections contain grafted *E. nitens*, *E. bicostata* and *E. grandis* trees of similar age and stature to the *E. nitens* trees sampled.

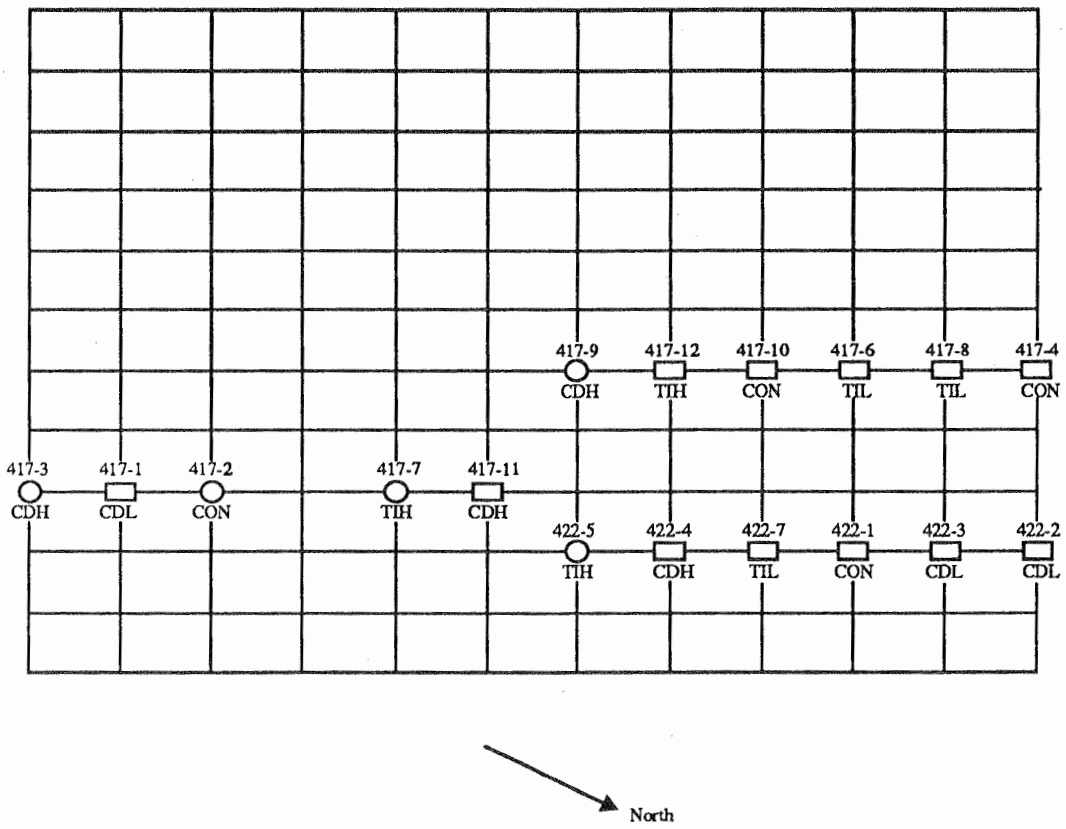


Plate 2.1. A graphic example of the relationship between GA_1 levels and flower bud production in grafted *E. nitens* clones grown in the Canberra espalier orchard. Gibberellin analyses of apical shoots harvested in September, 1992 suggest that at the time of bud initiation (September 1991), the level of GA_1 in the TIH treated (422-5) graft (top) would have been very low (<0.14 ng.g DW^{-1}), while the level of GA_1 in the control (422-1) graft (below) would be expected to be similar to that quantified (0.79 ng.g DW^{-1}). Photograph taken in September 1992.

Plate 2.2. The magnitude of effect on flower bud production achievable through application of paclobutrazol to grafted *E. nitens* clones grown in the Canberra espalier orchard. Note the very prominent bud crop in the paclobutrazol treated graft (top) in comparison to the virtual absence of reproductive activity of the non-treated graft (below).

Plate 2.3. The effectiveness of paclobutrazol applied as a collar drench (0.2g ai.cm stem circumference^{-1} in 200 mL of water) in promoting flowering on grafted *E. nitens* material in the pot system used.

Plate 2.4. The effect of paclobutrazol treatment on the height increment and growth habit of potted *E. nitens* grafts. Grafts were established in October, 1991. The treated graft (right) received a paclobutrazol collar drench (0.2g ai.cm stem circumference^{-1} in 200 mL of water) in April, 1992 while the control (left) remained untreated. The photograph was taken in December, 1992.

Chapter 3

Paclobutrazol Movement and Metabolism.

Introduction

The results of the previous chapter suggest that paclobutrazol can in increase the flowering intensity of reproductively competent *E. nitens* material as well as reduce the time lag between graft establishment and subsequent first flowering. This action appears to be related to a reduction in the level of endogenous GAs acting in combination with other stimuli such as cold treatment. The rapidity, extent and persistence of response to paclobutrazol treatment also appear to be affected by the method of application. It was hoped that through investigation of paclobutrazol movement through the plant after application by different methods, and observation of metabolism within plant tissues and in the immediate environment, some rationalisation of these variations in response would be possible.

Collar drenching, soil drenching, soil spraying or root drenching are all names for the practice of applying chemicals to plants via the immediately surrounding soil. Applications of this type result in uptake through the root system (Lever, 1986) and appear to be an effective means of applying paclobutrazol to both eucalypts (eg. Chapter 2, Hetherington and Jones, 1993), other commercial tree species such as apples, (Steffens, 1988; Curry and Reed, 1989; Mauk *et al.*, 1990a) and other plant species (eg. Barrett and Bartuska, 1982). It is both time and cost efficient and hence provides commercial users with a viable means of treating a large number of plants.

In order to assess the rate at which uptake and metabolism of soil applied paclobutrazol occurs in *E. nitens* seedlings, a number of experiments utilising ^{14}C labelled paclobutrazol were performed. Initially, it was necessary to establish an appropriate means of extraction of paclobutrazol from stem and leaf tissues of eucalypt seedlings and to gain some indication of the rate at which uptake occurred. The importance of the structure of paclobutrazol in performing its function of

inhibiting the synthesis of endogenous GAs (Sugavanam, 1984; Haughan *et al.*, 1989) suggests that any breakdown or other alteration of the molecular structure would at the minimum lead to a very severe reduction of activity. Thus it was proposed to use the separative power of HPLC coupled with liquid scintillation counting to discriminate between radioactivity from unmetabolised [^{14}C] paclobutrazol and radioactivity from its metabolites and breakdown products. A similar procedure was adopted by Early and Martin (1988) with [^{14}C] paclobutrazol, while other chromatographic separation methods such as thin layer chromatography (TLC) have been utilised for studying the degradation of the closely related [^{14}C] uniconazole (Sterret, 1990). Through the use of such methodology, the rates and patterns of metabolism and the proportion of unmetabolised and hence active material remaining could be ascertained for various samples. To achieve this, it was necessary to ensure extraction and purification procedures produced samples of sufficient purity for fractionation by HPLC without causing significant losses and additionally did not result in the artifactual degradation of unmetabolised paclobutrazol. Finally, an appropriate HPLC protocol was required in order to ensure the unmetabolised and metabolised components were sufficiently resolved to allow their quantification.

While soil drenching is a rapid and cost-effective means of treatment of field planted material, it may not be ideal in all circumstances. As uptake by the plant is preceded by percolation of the applied material through the soil, interactions with soil components may occur. This introduces soil chemistry, moisture content etc. as factors which may further influence the rate of uptake into the plant. Application of paclobutrazol to above ground stem and leaf tissue through the use of a foliar spray is an alternative application method. While less convenient to administer, it has the advantage of being a comparatively direct application method. Foliar spraying has been shown to generally produce a more transient effect (Hetherington and Jones, 1990; Mauk *et al.*, 1990a; Griffin *et al.*, 1993) and for users desiring rapid effects on flowering attributes, the closer site of application to the sub-apical meristem might be expected to produce a more rapid response. To assess the extent of advantages of rates of uptake and possible differences in metabolism resulting from foliar application rather than soil drenching, a comparative study was implemented.

Other workers have found that paclobutrazol can persist in an active form in the soil for a considerable period of time, with Lever (1986) estimating a half-life of 3-12 months. This was supported by Mauk *et al.* (1990a) with their estimate of a 50% per year reduction in paclobutrazol residues. However, whether or not these estimates are sufficient to explain the effects of paclobutrazol soil drenches persisting for five years or more in eucalypts (Griffin *et al.*, 1993), given the apparently rapid metabolism of the growth retardant in plant tissues (this chapter), is not easily answered. Through a brief investigation of the rate of metabolism of paclobutrazol in the soil of potted eucalypt seedlings, it was hoped that an estimate of the rate of breakdown of paclobutrazol specific for this situation could be gained. Additionally, it was of interest to determine if there were any breakdown products of the labelled paclobutrazol generated in the soil which could be found in the suite of products observed in plant tissues following soil drenching, but which were not observed in material receiving a foliar application of the retardant. This would indicate if any proportion of the breakdown products seen in the case of soil drench applications of [^{14}C] paclobutrazol to *E. nitens* seedlings were formed in the soil prior to assimilation, or during uptake into the roots.

Materials and Methods

Plant material

All plant material used consisted of *E. nitens* seedlings grown in a eucalypt potting mix consisting of sandy loam, peat and river sand in a 5:4:1 ratio, with 60 mL of osmocote, 60 mL of dolomite and 120 mL of blood and bone added to each 40 L of mix. The plants were raised in 5 cm x 5 cm x 15 cm plastic bags and maintained in a naturally lit, heated glasshouse (25° C day / 15° C night) with a daily watering regime, until required. At the time of use, seedlings were between six and 12 months of age and 60-100 cm in height. Replicates of comparable age and size were chosen for each experiment.

^{14}C paclobutrazol application

The [^{14}C] paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl) pentan-3-ol] used was a gift from S. Pettenon (ICI Australia) and had a specific activity of 1.975 GBq.mmol^{-1}. The purity of the [^{14}C] paclobutrazol standard was 94% as determined by HPLC fractionation (Fig. 3.1). The full drying, extraction and purification procedure (described below) used to assess the metabolism did not affect the pattern of impurities, though a decrease in the proportion of label associated with unmetabolised paclobutrazol (to 79%) was experienced. As all work involving an assessment of either levels or proportions of unmetabolised paclobutrazol to be performed were of a comparative nature, this degree of degradation during analysis was considered acceptable. No corrections for this degradation or the initial proportion of label not associated with unmetabolised [^{14}C] paclobutrazol were made unless otherwise specified.

For the initial estimations of the rate of labelled paclobutrazol uptake from the soil, 12 µg (42 n*M*) of [^{14}C] paclobutrazol was applied in 20 mL of water to the soil surface of each of 3 potted seedlings. The seedlings remained in the heated glasshouse and were watered daily. For the investigation of rates of uptake and metabolism following collar drench application, 24 seedlings were similarly treated. To compare the rates of uptake and metabolism of [^{14}C] paclobutrazol following collar drench and foliar application methods, six seedlings were collar drenched as described. Foliar application of 12 µg (42 n*M*) of labelled paclobutrazol to each of a further six seedlings was achieved by brushing the material in a 7 mL aqueous solution containing 0.05% Tween 20 as a surfactant onto all leaf and stem tissue other than the apical bud consisting of unexpanded stem and leaf tissue. This alternative was used to simulate a thorough and quantifiable foliar spray while minimising the risk associated with overspray of the potentially harmful [^{14}C] label. The node above the last opened leaf pair on the stem was marked on all 12 seedlings.

Sample harvesting, extraction, purification and ^{14}C label quantification

Initial determination of rates of paclobutrazol uptake

At 22 d, 40 d and 50 d after application, one plant was harvested by cutting the

stem at soil level. Leaf tissue from each node was separated and dried at 60° C for 3 d, followed by weighing and grinding to a fine powder in a mortar and pestle. Three aliquots of approximately 5 mg were taken and suspended in a 1:1 mixture of distilled water and Instagel (Canberra Packard, St Kilda, Vic, Australia) totalling 6 mL. The harvested stem tissue was frozen in liquid nitrogen and individual internodes were ground in a mortar and pestle, placed into a glass vial and supplemented with 10 mL of 80% methanol. This was allowed to extract for 24 h at 4° C before 10% (1 mL) aliquots were taken from each and supplemented with ca. 5 mL of Readysafe Liquid Scintillation Counting Cocktail (Beckman, Fullerton, CA, USA) for liquid scintillation counting. Assessment of the quantity of ^{14}C label in both stem and leaf tissue samples was performed on a Beckman LS 5801 Liquid Scintillation Counter.

Determination of rates of labelled paclobutrazol uptake and metabolism

Material to be assessed for the extent of label uptake and metabolism was placed immediately into liquid nitrogen and stored at -20° C for a minimum of one hour. Samples were then dried by heating for 2 d at 60° C, ground in a mortar and pestle and weighed. An aliquot of known mass was taken and extracted in 20 mL of 80% methanol for 24 h at 4° C. The extracted sample was filtered through Whatman Number 1 filter paper and the solids washed with further 80% methanol. The combined filtrates in 60% methanol were passed through a C_{18} Sep-Pak classic cartridge (Waters Associates), pre-conditioned with 5 mL of 100% methanol and 10 mL of 60% methanol. The Sep-Pak cartridge was subsequently rinsed with 5 mL of 60% methanol and the combined eluates dried *in vacuuo* at 60° C, dissolved in HPLC initial conditions and fractionated by reverse-phase C_{18} HPLC using one of two Waters Assoc. (Milford, MA, USA) systems. High performance liquid chromatography System one consisted of a 10 cm x 8 mm i.d. 10 µm Radial-Pak C_{18} cartridge contained within a Z-Module cartridge compression system, a Model 660 Solvent Programmer, two Model M-45 solvent delivery pumps and a UK6 sample injector. HPLC System two utilised the same C_{18} column contained within an RCM 8 x 10 cartridge compression system, two model 510 solvent delivery pumps, a UK6 injector and 'Maxima' solvent control software run on an IBM compatible computer with appropriate pump control interfacing. For both systems

the solvent program ran from 20-80% methanol in 0.4% acetic acid over 60 minutes with a linear increase in methanol concentration, while the flow rate was set at 2 $mL.min^{-1}$. Two mL aliquots were collected and these analysed by liquid scintillation counting as above.

Rates of uptake and metabolism following collar drenching

Groups of six plants treated with labelled paclobutrazol were harvested at 22, 43, 71 and 155 d after application. Leaf and shoot material growing from stem nodes was grouped into incremental height classes in 25 cm divisions up to 100 cm with a final height class of 100 cm+. Extraction and purification were achieved as described above utilising HPLC System one for separation of the labelled components of the extract.

Comparison of the rates of uptake and metabolism of ^{14}C labelled paclobutrazol in seedlings treated by collar drench or foliar application.

After nine weeks the apical tissue above the marked node was harvested and material from each treatment method grouped. Extraction and purification were performed as detailed above with HPLC System two utilised for separation of the labelled components of the extract.

Metabolism of labelled paclobutrazol in the soil.

Soil samples were taken from potted *E. nitens* seedlings to which doses of 12 µg (42 n*M*) [^{14}C] paclobutrazol had been applied either 90 d or 176 d previously. The soil was separated from the root material by mechanical sieving. Aliquots of sampled soils were extracted and purified as described. HPLC separation was performed using System two for the 90 d harvest and System one for the 176 d harvest.

Results

Determination of initial rates of ^{14}C paclobutrazol uptake following soil application

After just 22 d, labelled paclobutrazol was positively recovered from internode segments of the lower ca. 75% of the stem, while decreasing radioactivity in samples exhibiting high chemiluminescence made the exact extent of paclobutrazol movement through the stem impossible to determine (Fig. 3.2a). The level of ^{14}C material remained relatively constant through the lower stem segments where accurate quantification was possible, with only the lowest few internodes recording significantly higher levels, at a maximum of approximately twice the mean value (Fig. 3.2a). This pattern was repeated in the 36 d (Fig. 3.2b) and 50 d (Fig. 3.2c) harvests, with increased levels of ^{14}C material in the higher internodes enabling accurate quantifications to very near the apex in the latter case. In this 50 d sample, the upper few internodes revealed a steeply falling gradient in the level of labelled material in contrast to the constancy seen over the vast majority of the stem.

In comparison, the situation found with leaf and shoot tissue occurring at nodes was quite different, with a strong negative correlation between height from soil level and paclobutrazol concentration occurring throughout the length of the seedling. This appeared to be an almost linear function in the 22 d harvest (Fig. 3.3a) though the correlation became progressively weaker in the 36 d (Fig. 3.3b) and 50 d (Fig. 3.3c) harvests. The differences in rates of uptake between plants sampled at each time period is the likely cause for the lack of a consistent increase in the total amount of radioactivity taken up by a node of a particular height with increasing harvest time.

Uptake and metabolism of [^{14}C] paclobutrazol following soil application

In a similar manner to the initial rates of uptake demonstrated above, the level of labelled material in nodal leaf tissue consistently decreased as height increased for each harvest time (Fig. 3.4 a-d), when averaged by pooling material from the six seedlings sampled at each harvest. Over the first three harvesting times (22 d, 43 d and 71 d) the rate of uptake of radioactive label continued to climb, with lower segments demonstrating a higher rate of uptake than their more distal counterparts (Fig. 3.5). The increasing rate in these early harvests may have been associated with increasing temperatures and photoperiod from the winter application time (late July) to the spring months of the second and third harvests. However, between the

final two harvest dates (71 d and 155 d) a decrease in uptake rate, which could not be explained by seasonal photoperiod or temperature changes, was observed across all harvested segments (Fig. 3.5). In conjunction with this decrease in uptake rate, the level of unmetabolised paclobutrazol in each stem segment also fell, after a similar rise through the first three harvest dates (Fig. 3.6) Both these trends are obvious in the graphical portrayal of the level of total ^{14}C label and unmetabolised [^{14}C] paclobutrazol in tissue from each harvest date (Fig. 3.7). This suggests that the rate of uptake of unmetabolised paclobutrazol has fallen below the rate of metabolism within the eucalypt seedlings. The proportion of unmetabolised paclobutrazol in the leaves of seedlings appears to fall in a logarithmic manner with time (Fig. 3.8), though harvest after longer post-application times may make the nature of the descriptive function clearer. Such a decrease in the face of continued uptake of unmetabolised material and the rise (through early harvests) of the overall level of unmetabolised paclobutrazol in the same leaf tissues supports the findings of Wang *et al.*, (1986) who suggest that leaves may act as the final sink for paclobutrazol and its metabolites.

Comparison of collar drench and foliar application methods

The patterns of metabolism of labelled paclobutrazol as illustrated by HPLC profiles are generally similar in both treatments, with a large metabolic product centering on fractions 27-28 and a smaller peak around fractions 37-38 (Fig. 3.9a,b). However, collar drench application has resulted in the production of a larger number of minor metabolites, with an additional peak in fraction 34, the hint of a peak as a shoulder in fraction 30, and a general increase in the level of early (polar) metabolites producing an elevated baseline through the first 20 fractions (Fig. 3.9b). This is likely to be the result of the more complex path taken by the applied paclobutrazol in reaching the apex following soil application.

Though there has been no attempt to correct for losses during the extraction and purification procedure, the similarity of sample size, extraction and purification procedures etc between the collar drenched and foliar treated sample material should have resulted in similar recovery rates. Based on this supposition, the levels of label as both unmetabolised paclobutrazol and as its breakdown products

are compared. Collar drenching proved more effective in moving labelled substances to the subapical meristem, over the nine week duration of the experiment, with a level of 748 DPM.g DW^{-1} of ^{14}C label in the tissue sampled, in comparison to the 453 DPM.g DW^{-1} observed in the case of foliar application. However, this advantage was reduced when comparing the levels of unmetabolised (and hence active) paclobutrazol in the apical tissue of the two treatments, with 329 DPM.g DW^{-1} for collar drenched material and 261 DPM.g DW^{-1} for the material treated by foliar application. This suggested 56% and 44% degradation of the parent structure respectively, supporting the contention of increased metabolism resulting from the soil application method. The similarity of effectiveness between the two application methods was somewhat unexpected, given the longer path which paclobutrazol applied to the soil must take in reaching the apex. However, in herbaceous angiosperms such as *Phaseolus vulgaris* and *Chrysanthemum morifolium* similar efficiencies have been noted in the height growth retarding effects of paclobutrazol after foliar and soil applications (Barrett and Bartuska, 1982), suggesting that the apparent situation in eucalypt seedlings may not be atypical.

Metabolism of [^{14}C] paclobutrazol in soil

Despite the differences in sampling time, the chromatograms generated from the two soil samples after 90 d (Fig. 3.10a) and 176 d (Fig. 3.10b) were very similar. They revealed 64% and 69% of ^{14}C labelled components coinciding with unmetabolised labelled paclobutrazol after 90 d and 176 d respectively, while a range of putative breakdown products appear on the more polar (left) side of each chromatogram. Labelled paclobutrazol standard passed through the full extraction and purification procedure produced a chromatogram in which approximately 79% of radioactivity remained associated with natively configured paclobutrazol, thus requiring the extent of metabolism observed within the soil to be corrected to avoid artificially inflating the results. Such correction suggests the proportion of unmetabolised paclobutrazol in the first soil extract has been reduced by approximately 19%, 90 d post-application, while in the 176 d post-application soil sample only 13% of active paclobutrazol has been metabolized to products which

are likely to be weakly active or inactive. The results of these soil metabolism assays demonstrate the variability of rates of metabolism of paclobutrazol in the soil, with the 90 d post-application sample producing a higher proportion of metabolites than the 176 d post-application sample. Such estimations may only be relevant to the particular soil type used and its interaction with the physical environment. The large number of variables, such as soil pH, porosity, organic content etc, which may affect the rate of breakdown of paclobutrazol whilst in the soil may explain the highly specific nature of decay coefficients between different soil samples.

Discussion

Rates of paclobutrazol uptake

The rapidity of uptake of labelled paclobutrazol following application to the soil was clearly demonstrated in the two experiments in which this application method was exclusively used. The small size of the bags in which the seedlings were grown, and the resultant high density of roots will almost certainly have created an artificially high rate of uptake in comparison to a situation which would be expected with outplanted material. However, this study provides a worthwhile indication of the upper limits of the rate of uptake from this application method.

The relative constancy of concentration of [^{14}C] paclobutrazol through the stem tissue (Fig. 3.2a-c) suggests that only limited regions are suitable for binding and storage of paclobutrazol or its metabolites and these are rapidly filled after application of the material as a root drench. Both the apparent rapidity of translocation and the low level of movement into stem storage reservoirs suggests transport is occurring within the xylem, as part of the transpiration stream. This concurs with previous studies which have utilised microautoradiography to isolate the tissue within which root applied labelled paclobutrazol was being carried in peach seedlings (Early and Martin, 1988). The strongly acropetal movement of paclobutrazol noted in other studies (eg. Wang *et al.*, 1986) add further confirmation to this finding. The increased level of label in the lower few nodes

(Fig. 3.2a-c) may have been due to these lower nodes having had the longest exposure to the labelled substances, within which time some transport from the xylem to the phloem (Sterret, 1990) may have occurred by means of radial rays. Some degree of further accumulation in the phloem tissue may be possible, leading to a gradual increase in the level of ^{14}C label in the internodes.

The clearly linear decrease in radioactivity with increasing distance from the roots seen in the foliage arising from nodes in the 22 d post-application seedling (Fig. 3.3a) is likely to have arisen from the varying exposures to labelled paclobutrazol carried in the xylem fluid reveived by leaf material from different heights. This, in concert with the observed manner of movement of radioactivity through internodes suggests that some mechanism has effectively reduced the transport rate of paclobutrazol in internodes to below that of material carried freely in the transpiration stream. This reduction in transport rate may be involved with the binding of labelled compounds to the xylem walls or alternatively may be the result of some other movement retarding mechanism. The path to leaves near the apex passes many more potential binding or movement retarding sites than the path from the roots to leaves closer to ground level. Until these sites are filled this will result in a reduced concentration of labelled material in the xylem stream which reaches the upper leaves, which in turn results in the negative correlation between ^{14}C content and height above ground level. The slope and intercept of the function describing the level of labelled paclobutrazol in nodal tissue with height will be affected by the parameters determining rate of uptake of the growth retardant into the plant. These will include factors which affect transport into the roots, such as root density, soil composition and water content, and presumably those which affect transpiration rate as well. The varying coefficients of the slope functions from the three individuals used for the three harvests of the preliminary experiment (Fig. 3.3a-c) suggest that some of these factors were varying between individuals.

These variations in uptake rate between individuals within each harvest make the pattern of uptake of ^{14}C label from the soil less clear in the case of later work in which six individuals were taken at each harvest time. However, the overall pattern of decreasing label concentration with increasing height remains clear (Fig. 3.4a-d). The observed decrease in the rate of uptake of label from the soil (Figs 3.5, 3.7) suggests that at some time after 76 d the level of ^{14}C material in the soil

and available to the roots was reduced. The cause of this reduction is either continued uptake into the plant depleting its concentration, or loss through leaching and/or metabolism within the soil to form compounds which cannot be assimilated by the root system. Sporadic monitoring of the runoff from the polythene bags containing the soil drench treated seedlings revealed no evidence of the leaching of labelled paclobutrazol or labelled paclobutrazol metabolites, concurring with the low soil mobility of paclobutrazol observed in other studies (Lever, 1986; Mauk *et al.*, 1990a). Soil metabolism can largely be discounted as a major mechanism of loss of effective paclobutrazol, given the low rates of breakdown in the soil demonstrated in this study. This then suggests that the level of labelled paclobutrazol in the soil is being significantly reduced as a result of uptake into the seedling.

The comparison of soil drench and foliar application provides further evidence of the rapidity of uptake of applied paclobutrazol. However, when considering the proportion of applied growth retardant which has reached the actively growing apical shoot tissue, it is evident that neither treatment method has been particularly effective. Even if losses through the extraction and purification procedures were of an order of magnitude or more, the proportion of [^{14}C] paclobutrazol incorporated into the harvested segment is still less than a fraction of one percent. Despite this poor uptake and transport, both methods have been proven to be effective for increasing the flowering response and/or reducing shoot growth in eucalypts (Chapter 2; Chapter 4; Hetherington *et al.*, 1991; Griffin *et al.*, 1993; Hetherington and Jones, 1993; Moncur *et al.*, 1993; Moncur and Hasan, unpublished) as well as other arboreal angiosperms such as apples (Mauk *et al.*, 1990a) and citrus species (Aron *et al.*, 1985). This suggests that the levels of paclobutrazol in tissue necessary to produce effects on flowering and growth are relatively low. This may explain why in some circumstances foliar sprays are effective in the first year of application (Mauk *et al.*, 1990a) while collar drenching may not be effective until the second year. Foliar spray deposited paclobutrazol requires only minimal transport to reach the potential sites of floral induction. Though only a small fraction of the growth retardant may move into the tissue, this may be enough to be effective, resulting in paclobutrazol activity being rapidly expressed. The much simpler collar drenching method of application may not

produce the required level of paclobutrazol in the appropriate plant tissues rapidly enough when applied at the same time as a foliar spray, causing the effect to be missed until the subsequent growing or flowering season. Perhaps the former method could be made effective in the first year by earlier application, or by increasing the dose size, though the latter means may result in an excessive level of growth reduction, or an unwanted persistence of treatment effects.

[^{14}C] paclobutrazol metabolism

The similar pattern of rise prior to fall of unmetabolised paclobutrazol level in all of the height zones of seedlings following a collar drench application of labelled paclobutrazol (Fig. 3.6) provided little evidence of any preferential sites of metabolism amongst these zones. However, it was apparent that breakdown of the labelled paclobutrazol was occurring and the fall in the level of unmetabolised material between 71 d and 155 d (Figs 3.6, 3.7) suggests that the point at which uptake of paclobutrazol fell below the rate of breakdown within the plant was reached at that stage. Such a situation will eventually lead to the release of treated seedlings from the inhibitory effects of paclobutrazol, as uptake slows to zero while metabolism continues.

A comparison between individual experiments however, demonstrates that the proportion of unmetabolised paclobutrazol is not necessarily similar in all tissues. In the expanding apical tissue, nine weeks after collar drench application the proportion of labelled material associated with natively configured paclobutrazol was approximately 44% (Fig. 3.9b). In mature leaves however, after 10 weeks the average proportion of unmetabolised ^{14}C paclobutrazol was only half this level at 22% (Fig. 3.8). If leaf tissue is one of the end-point sinks of paclobutrazol and its metabolites, as well as a major site of paclobutrazol breakdown (Early and Martin, 1988; Arron, 1990; Sterret, 1990), the difference in proportions of unmetabolised paclobutrazol seen are likely to be the result of the accumulation of stored paclobutrazol metabolites in the older leaf tissue possibly in combination with an increased rate of breakdown. Such a situation would see similar levels of label associated with unmetabolised paclobutrazol in the two tissues producing a lower percentage of total radioactivity in the case of the samples of mature leaves.

The radioactivity profiles of [^{14}C] paclobutrazol standards (Fig. 3.1), and those of tissue and soil extracts of material treated with ^{14}C paclobutrazol (Figs 3.9a,b and eg. Fig. 3.10b respectively) possess a number of similarities in their patterns of labelled products. The dominant peak in all of the profiles is the unmetabolised paclobutrazol, appearing around fractions 42-43. Two other peaks, one some five to six fractions earlier than the standard and the second, a further nine fractions earlier (around fraction 27) are common to all the profiles. Given these facts, it is tempting to speculate that these corresponding peaks in the various profiles represent identical breakdown structures of the pure labelled paclobutrazol standard, while the presence of the peaks in the bottled standard (Fig. 3.1) suggests that a degree of non-enzymatic breakdown to these products is possible. The considerable size of the peak five to six fractions to the left of the unmetabolised material in the soil and tissue samples suggest that this compound may be a primary metabolite of the unlabelled material. However, without further analysis of the structures of the compounds which constitute these common peaks, such associations are purely speculative.

The extra structures indicated by peaks seen in the chromatogram of the collar drenched material (Fig. 3.9b) relative to the material treated by foliar application (Fig. 3.9a) are likely to have been due to the more complex path taken by the applied material in reaching the apex from the soil. It was of interest to note that the peak which appeared eight fractions to the left of the unmetabolised peak in both of the soil extracts (Fig. 3.10a, 3.10b) also appears in the apical extract from the collar drenched treatment (Fig. 3.9b) but not in the corresponding extract from the group treated by foliar application of [^{14}C] paclobutrazol (Fig. 3.9a). Perhaps this soil-produced metabolite is assimilated via the root system, to eventually appear in the apical tissue. Alternatively, some part of the uptake and transport process may produce the extra metabolite, while the chromatographically similar metabolite produced within the soil may not be taken up at all. Speculation of this nature could also be made in regard to other peaks in the chromatographic radioactivity profile of the soil extracts after comparison with the profile of the collar drenched seedlings.

Predicting persistence of paclobutrazol activity

Predicting at which point the activity of paclobutrazol will cease, with any degree of accuracy, is a matter which requires the consideration of many variables. Assuming a relatively simple enzymatic breakdown of paclobutrazol within the plant, without complicating factors such as inducible enzymes, a logarithmic rate of decay for a given level of paclobutrazol within the plant could be expected. Such a rate could be determined by introducing a dose of paclobutrazol by a means such as trunk injection which would ensure that the entire dose was assimilated by the seedling. The rate of degradation within the plant could then be calculated by sampling foliage at various times post-application and determining the level of unmetabolised paclobutrazol at each time interval. However, over a long period of time, the calculation may be complicated by an increase in plant tissue mass, with a corresponding increase in capacity to metabolise the inhibitor, accompanied by a diluting effect resulting from the increase in plant tissue volume.

In the case of a soil drench application of paclobutrazol, further complicating factors for such a calculation would include the rate of breakdown of paclobutrazol in the soil and the plants continuing ability to take up the inhibitor and possibly its metabolites from the soil. Despite the considerable variability in the rate of [^{14}C] paclobutrazol breakdown in the soil (this chapter), it seems apparent that the rate of metabolism in the soil is much lower than that seen within plant tissues. A reduction of 13% in the level of unmetabolised paclobutrazol in the soil over a 176 d period suggests the half-life of paclobutrazol in the soil would be 2 years or more. This is considerably longer than the 3 to 12 month period suggested by Lever (1986) or 1 year as suggested by Mauk *et al.* (1990a). Soil drench application of paclobutrazol revealed ca. 50% metabolism (after correcting for degradation through the extraction and purification procedures) of the retardant in leaf tissue after just 21 d, despite the continued uptake of unmetabolised material from the soil. This suggests relatively rapid breakdown of paclobutrazol within the seedling, with a half-life in leaf tissue substantially less than 21 d, making it seem logical to assume that the persistence of effects seen after soil applications of paclobutrazol is mainly due to the stability of the material in soil. Continued uptake from the soil could occur as the root system proliferated or as a result of irrigation water transferring the paclobutrazol to areas within the existing root system. In the majority of cases then, the most convenient and probably accurate manner of

determining the likely maximum extent of paclobutrazol metabolism would be to perform a test assay of paclobutrazol metabolism in the soil at the potential site of use.

No matter what the application method, a means of establishing the minimum active level of paclobutrazol in the particular plant tissues of interest is required. This is perhaps the most useful next step for experiments of this nature to take and would involve the quantification of paclobutrazol levels in plant material showing the effects of paclobutrazol treatment. This line of experimentation could be extended to include a joint assay of paclobutrazol and GA levels, hence determining the effectiveness of the former in reducing the latter and allowing the levels of both to be correlated with effects on vegetative growth and flowering. Previously, gas chromatography (Curry and Reed, 1989) and HPLC (Mauk *et al.*, 1990b) have been used to quantify paclobutrazol. However, the high level of pigmented material in eucalypt extracts may favour a GC-MS based approach similar to that used by Wang *et al.* (1986) if suitable internal standards were available.

Figure 3.1. HPLC chromatogram of the [^{14}C] paclobutrazol standard fractionated by HPLC System 2 (see text). The large peak is the unmetabolised [^{14}C] paclobutrazol which constitutes ca. 94% of all labelled material detected.

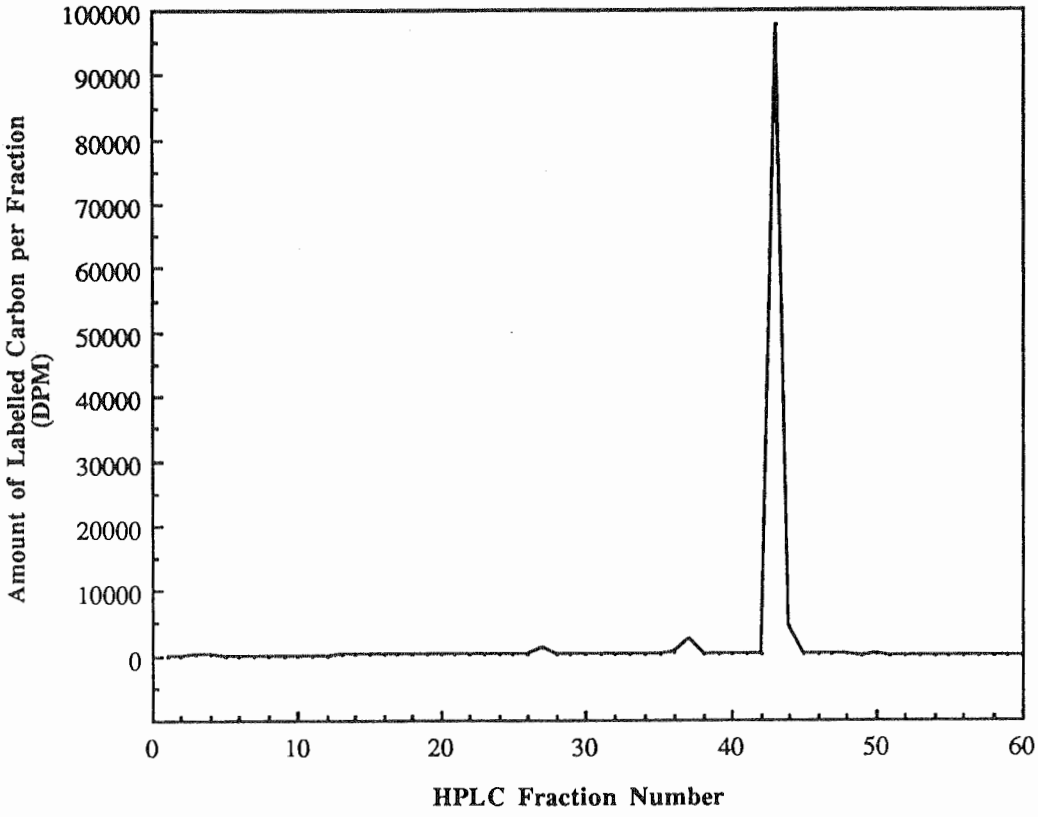


Figure 3.2. Methanol extractable ^{14}C material recovered from internode (stem) tissue segments 22 d (a), 36 d (b) and 50 d (c) after soil drenching with [^{14}C] paclobutrazol. Note that beyond the point marked by the vertical line and arrow in the 22 d graph (a) there is some overestimation of radioactivity due to the effects of chemiluminescence in the sample.

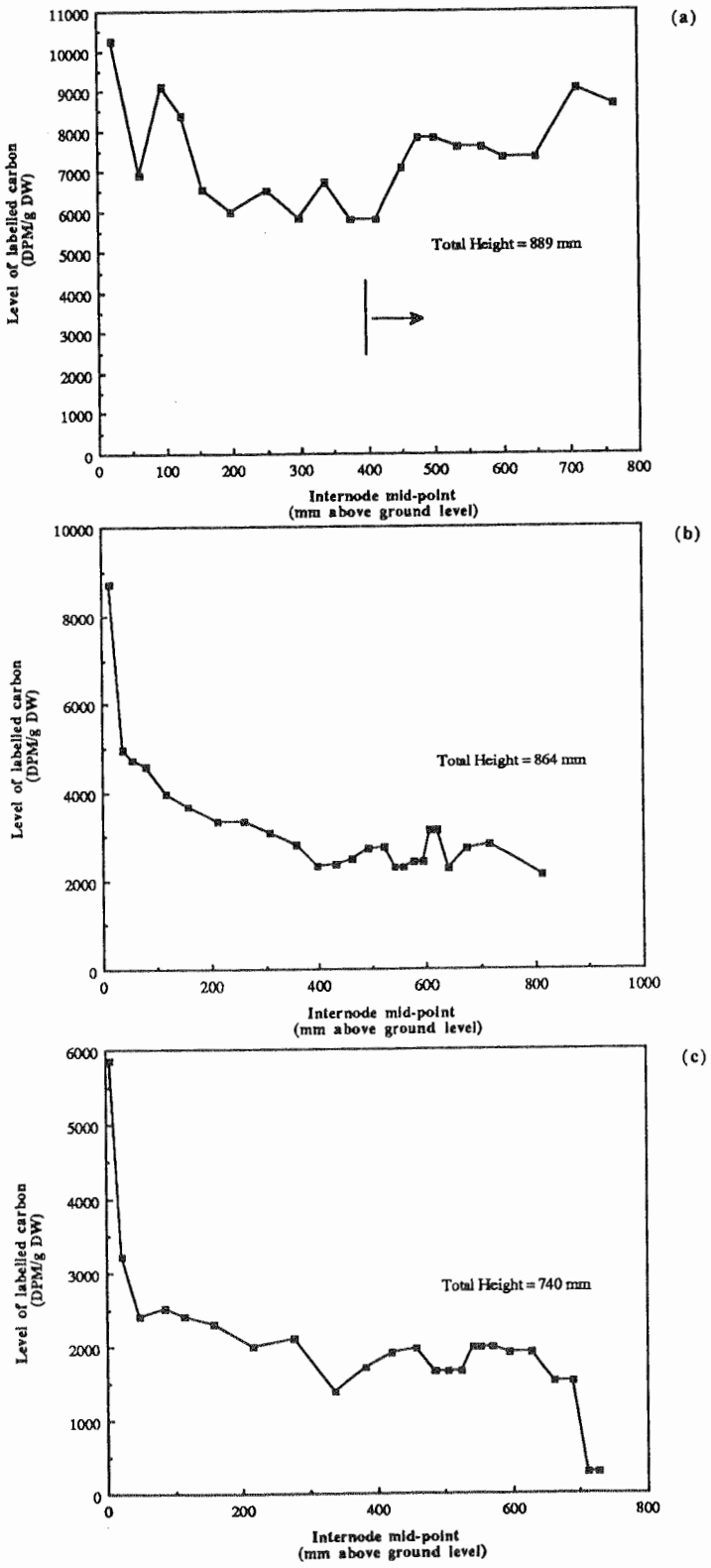


Figure 3.3. Correlation between height of node above the ground and level of ^{14}C label in leaf tissue 22 d (a), 36 d (b) and 50 d (c) after soil drenching seedlings with [^{14}C] paclobutrazol.

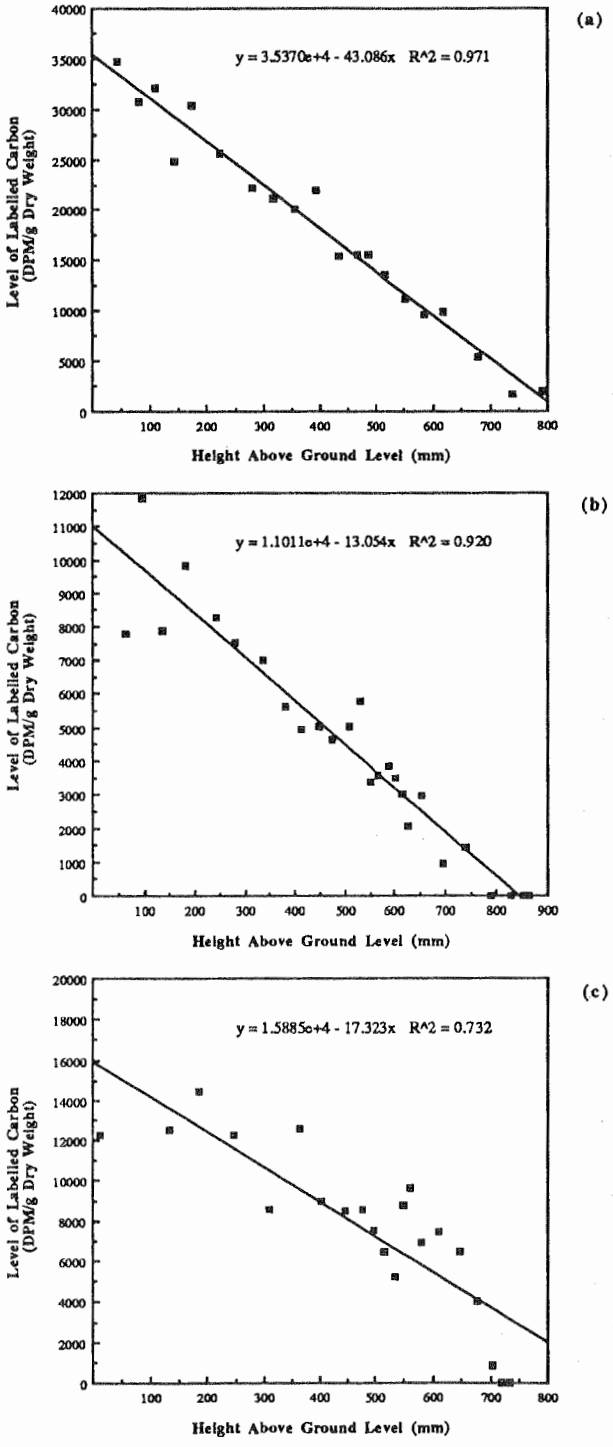


Figure 3.4. Correlation between height of leaf material above the ground and the level of ^{14}C label in leaf tissue 22 d (a), 43 d (b), 71 d (c) and 155 d (d) after collar drenching seedlings with [^{14}C] paclobutrazol. Leaf tissue from 6 individuals at each harvest date was grouped into height classes of 25 cm increments from ground level, with the fifth class being >100 cm.

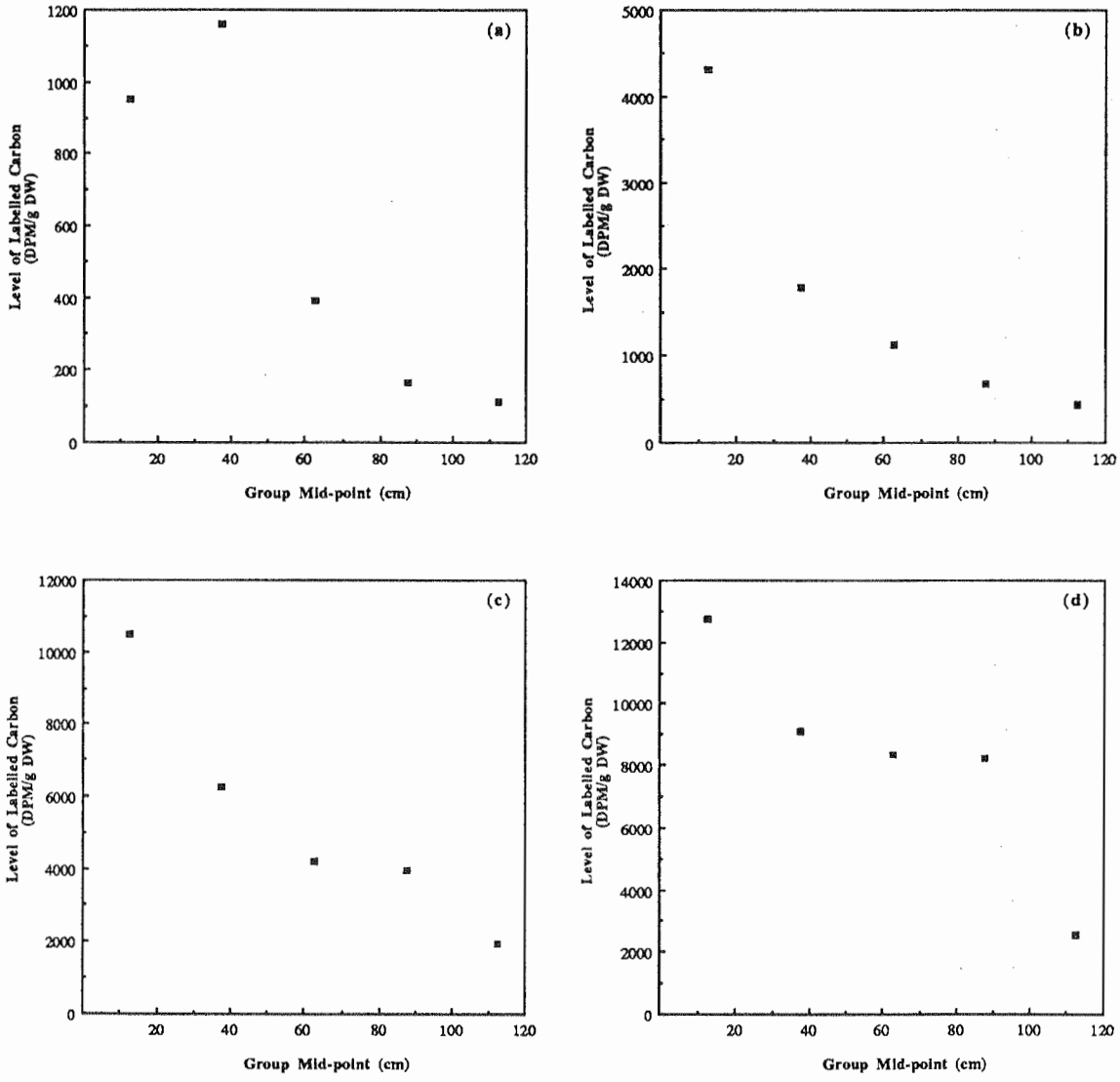


Figure 3.5. Uptake of ^{14}C label into leaf tissue of each height class over the successive harvest dates following ^{14}C paclobutrazol application by soil drench to seedlings on day zero. Leaf tissue from six individuals at each harvest date was grouped into height classes of 25 cm increments from ground level, with the fifth class being >100 cm.

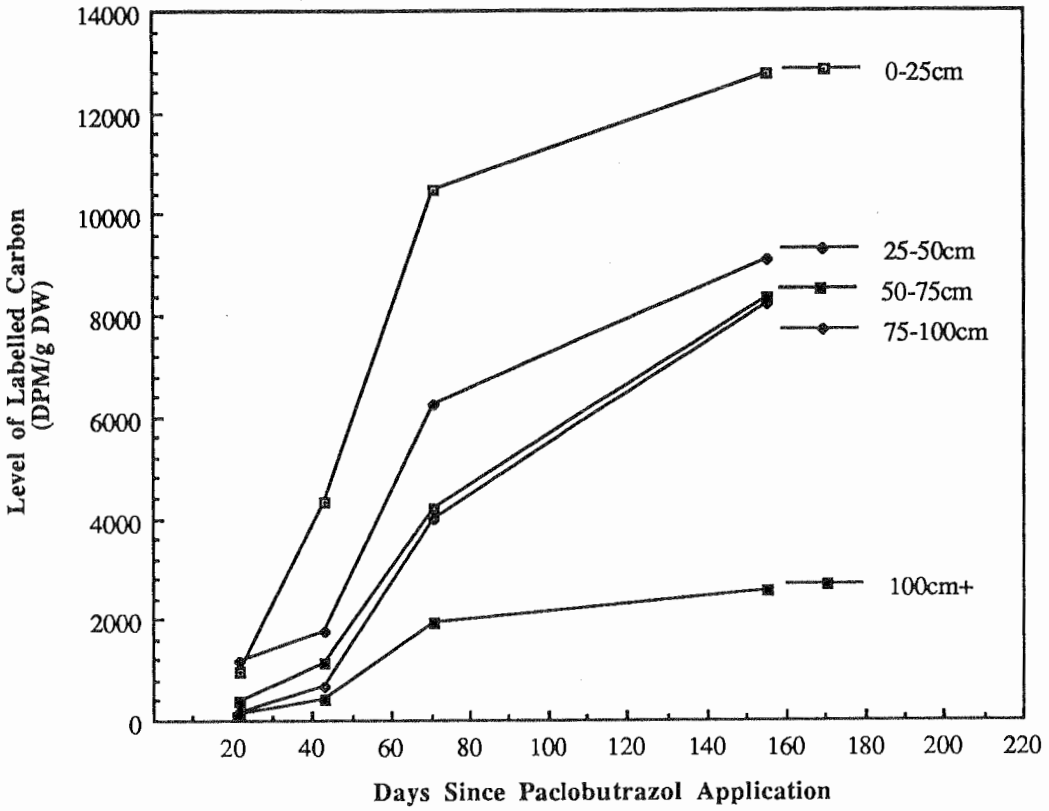


Figure 3.6. The level of unmetabolised ^{14}C paclobutrazol in leaf tissue of each height class over the successive harvest dates following ^{14}C paclobutrazol application by soil drench on day zero. Leaf tissue from six individuals at each harvest date was grouped into height classes of 25 cm increments from ground level, with the fifth class being >100 cm.

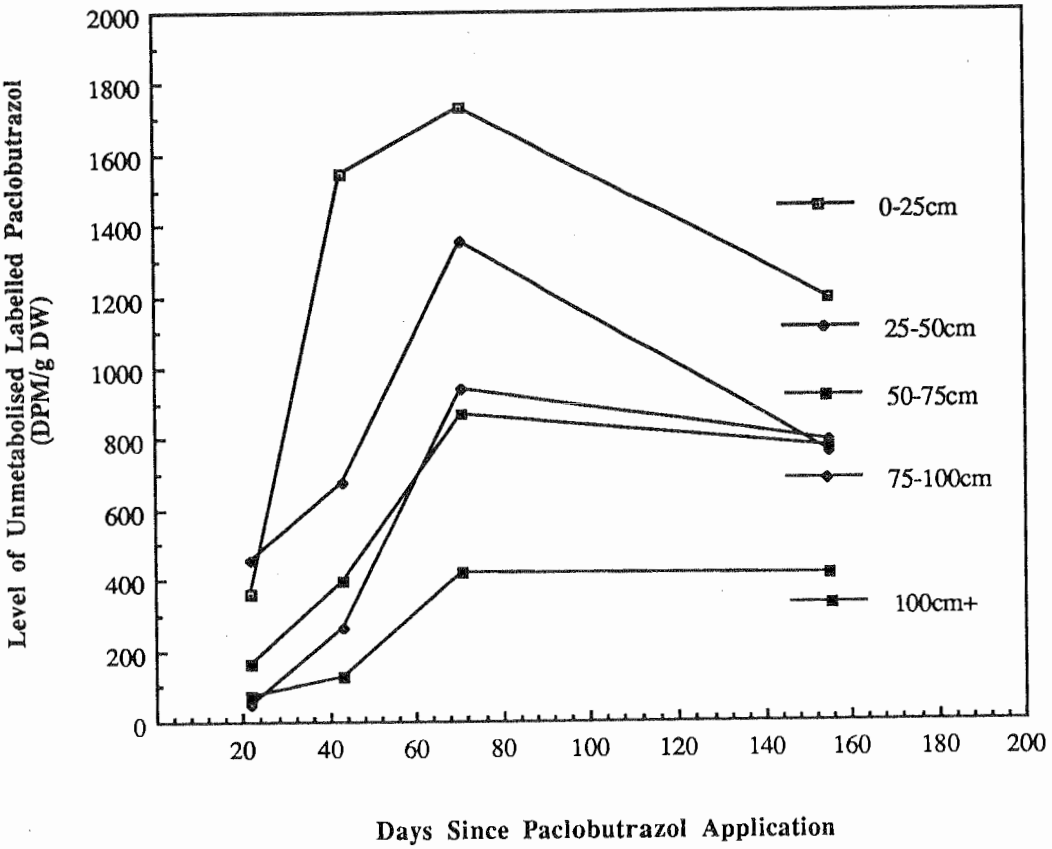


Figure 3.7. Changes in the level of unmetabolised ^{14}C paclobutrazol (open boxes) or total ^{14}C label (closed boxes) in leaf tissue pooled from all height classes, over the successive harvest dates following application of ^{14}C paclobutrazol by soil drench on day zero.

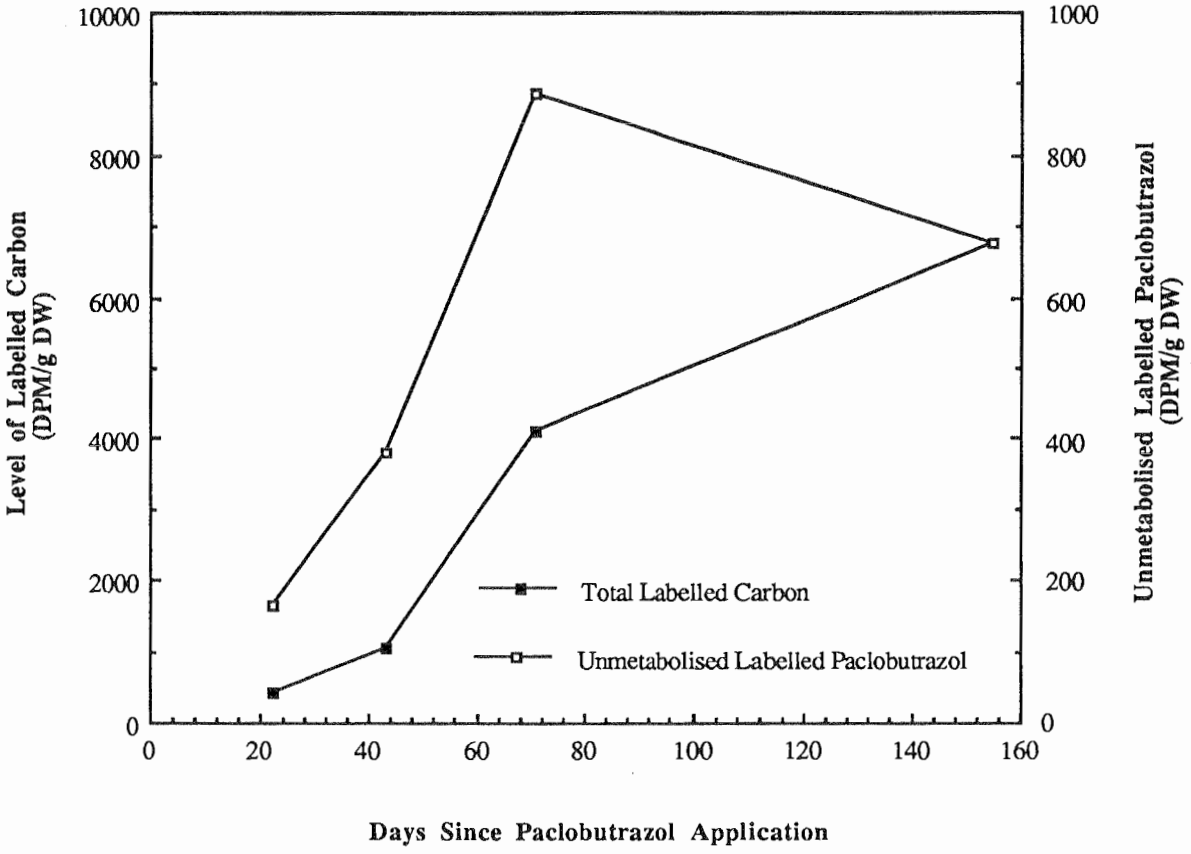


Figure 3.8. The proportion of unmetabolised [^{14}C] paclobutrazol in leaf tissue pooled from all height classes, over successive harvest dates following application of [^{14}C] paclobutrazol by soil drench on day 0.

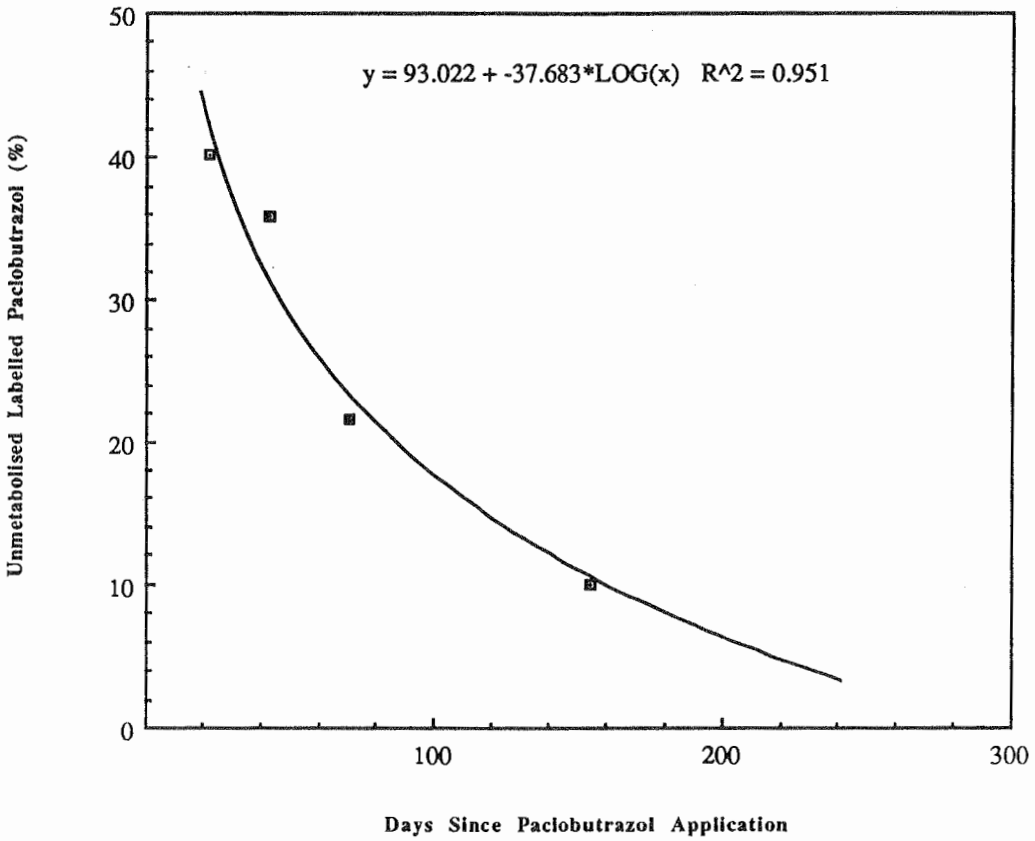


Figure 3.9. HPLC chromatograms showing the extent of metabolism of [^{14}C] paclobutrazol 9 weeks after application to groups of 6 seedlings by either foliar application (a) or collar drench (b). [^{14}C] paclobutrazol standard elutes in the zone indicated by the open box.

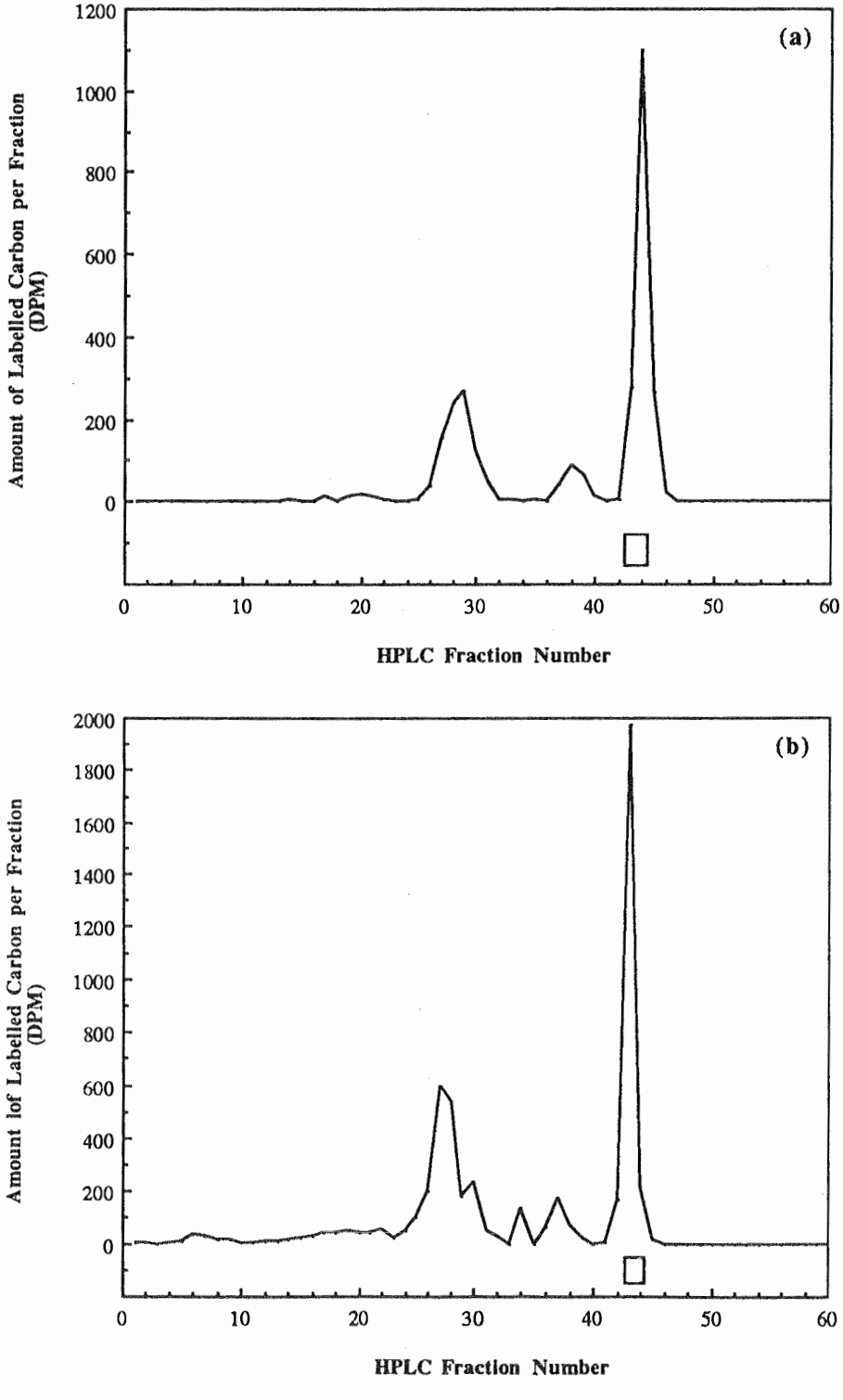
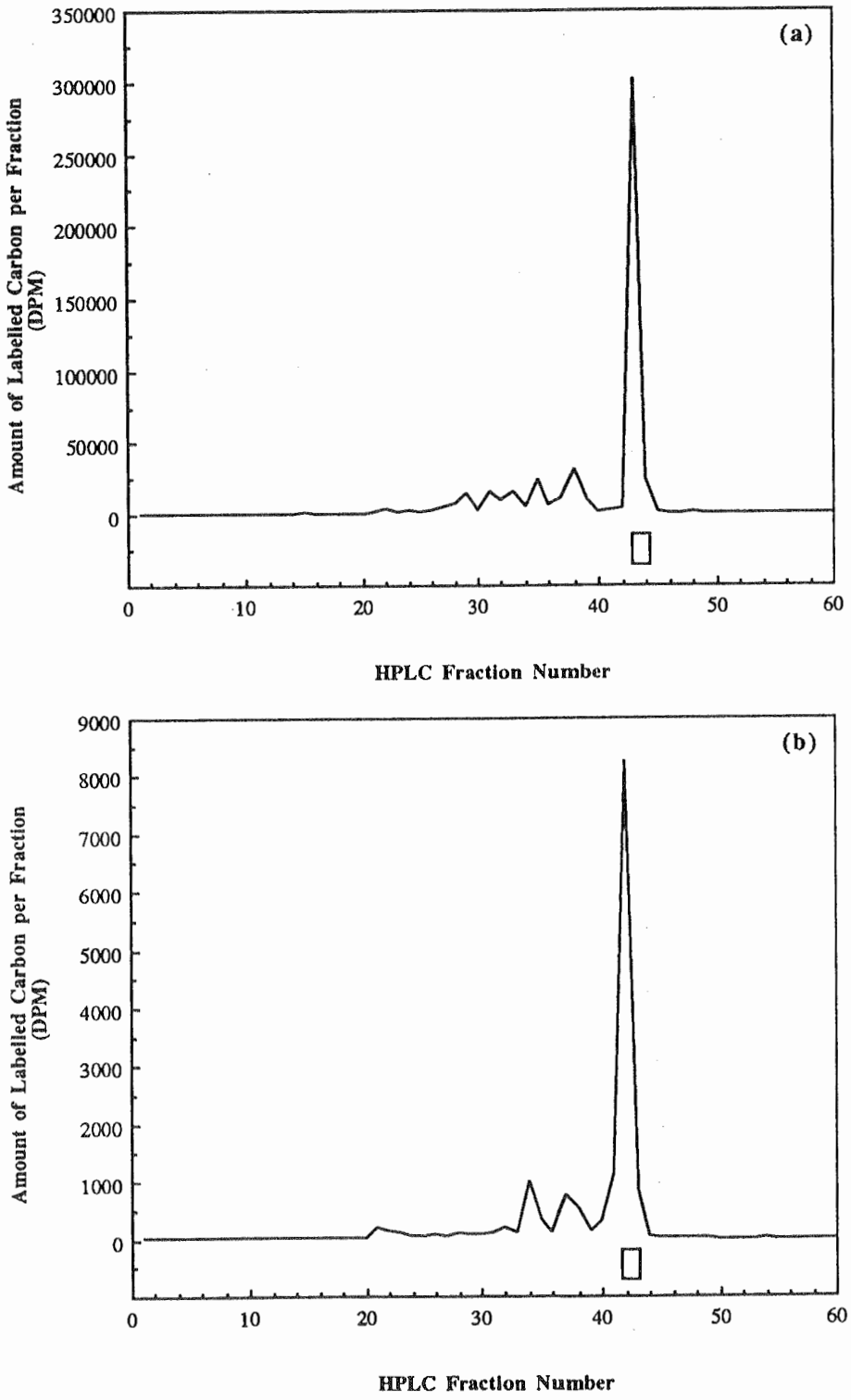


Figure 3.10. High performance liquid chromatography generated chromatograms showing the extent of metabolism of [^{14}C] paclobutrazol in the soil either 90 d (a) or 176 d (b) after application to the soil by collar drench. [^{14}C] paclobutrazol standard elutes in the zone indicated by the open box. The large differences in total ^{14}C content are the result of different soil sample sizes.



Chapter 4

Induction of Flowering Precocity in *E. globulus* Seedlings

Introduction

In the past, native forest has been an important source of eucalypt wood for pulp production, but as demand grows, only a well managed plantation-based production scheme will provide a renewable resource capable of producing the required quantity of material. As a result, plantations have been widely established through suitable areas (eg. Tibbits, 1986), making eucalypts the most widely planted genus of angiosperm tree in the world (Griffin, 1989). However, until relatively recently, seed used for establishment and regrowth of such plantations has been obtained from unimproved trees sourced from selected provenances or stands (Boland *et al.*, 1980).

The potential gains which could be accrued from an improvement programme seem large, given the variability in various useful traits seen in natural stands. If the positive factors of each of these traits could be combined, increases in productivity and efficiency of production could come from three major sources. Firstly growth traits such as rate of volume production, or pulping traits such as pulp yield could be increased. Secondly, improved material may be more resistant to biotic and abiotic stress factors which would usually result in a loss of productivity. If tolerance to various stresses was sufficiently developed, a third benefit which could result from the genetic improvement of eucalypts may entail an increase in the range of conditions (and hence localities) over which material could be planted. This may permit planting of material closer to mill sites, reducing the substantial transport costs involved, or may alternatively allow planting on sites which are less attractive to other industries.

In an effort to increase productivity, a limited amount of eucalypt tree breeding has taken place in the past with the earliest work of this nature recorded in the 1940s (Krug and Alves, 1949a,b in Boland, 1980). In recent times, however, large investments of resources and new technologies have been directed at this effort (Griffin, 1989), once the extent of potential benefits began to become clear.

As desirable traits in stand attributes and wood quality are identified and the relevant genetic parameters of breeding become established (heritabilities of various traits etc.) breeding programs involving crosses between various families, provenances, sub-species and even species are being undertaken (eg. Tibbits, 1986; Griffin, 1989a; Dungey, 1991).

Amidst this wave of effort in tree improvement, the long generation time in commercially important *Eucalyptus* species looms as a potentially major factor constraining the rate at which genetic improvement programs can progress. As isozyme (Sykes, 1988) and/or molecular markers (Michelmore *et al.*, 1991; Paterson *et al.*, 1991; Grattapaglia *et al.*, 1992; Strauss *et al.*, 1992) for desirable traits are characterised, marker-aided selection should allow selection of desirable plants from a very young age. This early selection is obviously of little benefit if generation times are large, such as that of *E. globulus* which is a minimum of around five years in fertilised plantations (Turnbull and Pryor, 1978; Tibbits, 1986; Griffin, 1989a), or even longer in natural environments.

The induction of earlier (precocious) flowering in seedlings could increase the rate of incorporation of desirable traits by providing a means of reducing the generation time of breeding stock. While some genetic precocity has been observed in *E. globulus* (Hasan *et al.*, 1992) and *E. nitens* (A.R. Griffin, *pers. comm.*) as well as other eucalypt species (Pryor, 1966) and this trait may be sufficiently heritable to breed into desirable stock, such an approach is probably not ideal if there are other alternatives. The major drawbacks of encouraging a genetic approach to the induction of early flowering arise from the continuing expression of the character in subsequent generations. While this may be desirable in fruiting crops such as peach (Hansche, 1986), it may be detrimental for crops where vegetative growth is the sole human objective, given a proposed negative correlation between reproductive output and vegetative growth in both fruit trees (Shearing *et al.*, 1986; Jones *et al.*, 1989a) and eucalypts (Griffin, 1989; Hetherington and Jones, 1993). Thus, genetically precocious flowering breeding stock could introduce genetic material which will continue to affect overall vegetative growth performance. In addition the lack of a mechanism (using current breeding techniques) to incorporate the early flowering traits from a genetically precocious individual in isolation from the remainder of the genome will inevitably

result in a dilution of genetic gains made up to that point.

In his review, Grossmann (1990) maintains that the plant growth retardant paclobutrazol increases reproductive output and controls vegetative growth in fruit crops. Examples of at least short term increases in the number of reproductive structures per treated tree have been produced for a number of species (General Introduction). In accordance with this, applications of paclobutrazol have been observed to increase the fruit and seed output of *E. globulus* and *E. nitens* (eg. Hetherington and Jones, 1993; Griffin *et al.*, 1993) and the possibility of overriding the almost notorious variability in annual bearing of species of this genus (Turnbull and Pryor, 1978; Boland *et al.*, 1980) recognised. Anecdotal evidence of a reduction in generation time following paclobutrazol application to some eucalypt species aroused considerable interest in testing its effect on *E. globulus* seedlings. In the first instance, the most desirable effect which paclobutrazol could have on seedling eucalypts would be to induce flowering prior to the time it would occur in untreated plants. With this goal in mind, the effects of paclobutrazol application on vegetative growth and first season bud production of *E. globulus* seedlings were assessed.

In conjunction with the above work, it was hoped to assess the effectiveness of paclobutrazol in increasing reproductive output on a per plant basis. It was thus necessary to ensure that paclobutrazol treatment did not have any detrimental effects on bud development and/or rates of capsule set. It was similarly of interest to examine the possible persistence of paclobutrazol treatment effects into the second bud producing season and also to assess the effects of various cultural treatments on reproductive activity.

Materials and Methods

Plant material

For the primary study, four distinct groups of seedlings were used. The seed used was provided by Dr B.M. Potts (Univ. of Tasmania, Australia). One group was from a precocious flowering family from a harsh environment at Wilson's

Promontory (39° 12'S, 146° 30'E; altitude: 20 m) on the south-eastern coast of Australia (APPM Provenance 733) while the second was from a family in a similar area (APPM Provenance 734) which did not express the high level of precocious flowering of the previous material. The third group (PerM) was from seed collected at another marginal site, though a different area (Perpendicular Mountain, Tasmania; 42° 40'S, 148° 8'E; altitude: 240 m), while the fourth group (Bulk) was derived from a bulk collection of seed collected from south-eastern areas of Tasmania. The latter group was intended to serve as a control group, which represented an average of this species' performance in Tasmania.

A secondary study was performed with seedlings grown from a single commercial seedlot not known to have any precocious flowering tendencies (Sandra Hetherington, *pers. comm.*). This material was assumed to be less genetically heterogeneous than the material constituting the 'Bulk' group in the primary study, as commercial seedlots are generally collected from a single provenance, stand or even individual tree. In conjunction with the seedling trial, clones rooted from cuttings of a single tree were tested for response to paclobutrazol application. This latter case produced a trial in which there was no genetic variation between individual plants across all treatments.

Growing conditions (Season 1)

In the primary study, *E. globulus* plants were grown from seed, which was germinated on moist vermiculite prior to planting out in slimline 12.5 cm pots filled with a eucalypt potting mix consisting of sandy loam, peat and river sand in a 5:4:1 ratio (v/v) with added osmocote (60 mL), dolomite (60 mL) and blood and bone (120 mL) for every 40 L of mix. For the secondary study, seven month old *E. globulus* seedlings and clones which first developed roots nine months previously were received as gifts (S. Hetherington, Forest Resources, Launceston, Tasmania, Australia). This material had been grown in a shade-house as part of commercial production stock. At the time of receipt, the material was growing in small plastic pots (ca. 6 cm diameter and 12 cm depth) and individual plants were ca. 50 cm in height with 8-10 nodes expanded. These seedlings were immediately repotted into slimline 12.5 cm pots with extra eucalypt potting mix added as required. All

material was grown outdoors under Hobart (42° 54'S, 147° 19'E; altitude: 150 m) conditions (Fig. 4.1) from early March 1991. Seedlings were watered lightly twice per day using timed sprinklers.

Paclobutrazol application

For the primary study, paclobutrazol treatments were administered in March 1991 when seedlings were five to six months of age. Each type of seedling, other than those grown from the 734 seedlot, were divided into three groups which received either no paclobutrazol treatment (Zero), 1 x 1000 ppm (w/v) paclobutrazol foliar spray (Low), or 2 x 1000 ppm (w/v) paclobutrazol foliar sprays approximately two weeks apart (High) (Tab. 4.1). For the secondary study, paclobutrazol was administered in mid-April 1992, one month after repotting. The seedling and clonal material was divided into three equal groups of 33 and 26 respectively and groups received zero, low or high doses as described for the initial study. Foliar sprays consisted of an aqueous solution of 'Cultar' (ICI Australia Operations Pty Ltd) with 0.05% Tween 20 added as a surfactant (Hetherington and Jones, 1990). Plants were sprayed to run-off whilst in a horizontal position, to minimize solution running into the potted soil.

Statistical analysis

Scoring of the primary experiment after one reproductive season occurred during March, 1992. The primary study involved the assessment of each seedling for a number of characters (Tab. 4.2). After suitable transformations to correct for non-normality, analysis of variance was carried out using the GLM (General Linear Modelling) procedure of SAS/GRAPH (1988). Scoring of bud production, nodes expanded and height data for the secondary experiment was completed during April, 1993. Untransformed data of the two latter parameters was subjected to analysis of variance, again using the GLM procedure of SAS/GRAPH (1988).

Growing conditions (Season 2)

Plants from the primary study were utilised in an assessment of the activity of paclobutrazol and various environmental treatments on bud retention, second season flower bud production and pollen viability. After the time at which new flower buds ceased becoming visible (February/March 1992) seedlings from each seedlot and paclobutrazol treatment level were divided into further groups and placed under a small range of growth conditions to assess their effects on second season flower bud production. The primary division involved the repotting of some material and not others. Seedlings were repotted into black polythene bags (20 cm diameter, 25 cm depth) with extra eucalypt potting mix. Care was taken to ensure the root mass from the slimline pots was sufficiently broken up to promote good root growth into the new mix. A further division was made by growing some material within a glasshouse while the remainder was maintained under outdoor conditions (Fig. 4.1). Where glasshouse conditions were prescribed, plants were grown in a naturally lit, heated glasshouse which maintained minimum temperatures ca. 5° C above ambient during the cooler months.

No seedlings from the 734 seedlot had been treated with paclobutrazol at this stage (February/March 1992). All were repotted and the total divided into three equal groups. One remained untreated, while the two others received low and high paclobutrazol doses in the manner previously described. This trial was to assess the effects of paclobutrazol application on *E. globulus* seedlings which had reached phase change.

Scoring of reproductive output after the second flower bud producing season was accomplished during July, 1993.

Pollen viability testing

During 1992 pollen was collected from opened flowers of material of the primary study and stored in gelatin capsules surrounded by silica gel, at -20° C. Testing was carried out within a maximum of eight weeks of collection. Media consisted of an agar base (5 $g.L^{-1}$) with sucrose (200 $g.L^{-1}$) and boric acid (200 $mg.L^{-1}$) (Potts and Marsden-Smedley, 1989). Testing was accomplished by spreading small amounts of pollen onto individual agar blocks contained within a plate, using a damp, clean, fine-bristled paint brush. The plates were incubated at 20° C for 2 d

by which time viable pollen had germinated, but pollen tubes were still sufficiently small to allow easy counting.

Results

Vegetative growth

In the primary study, paclobutrazol significantly reduced total seedling height when analysed across all seedling types ($p<0.0001$) with average heights for Zero, Low and High treatments calculated to be 117.2 cm, 106.3 cm and 98.5 cm respectively in March, 1992. The maximum reduction in height relative to control of 29% (34 cm) occurred in the case of the High treatment in the 733 (precocious flowering) provenance (Fig. 4.2), while a similar comparison in the case of the Bulk material revealed a height difference of 15% (18 cm). The PerM material was not responsive at a significant level to paclobutrazol treatment in terms of this character, though this is likely to be a result of small sample size.

When analysed across all seedling types, the effect of paclobutrazol treatment on the number of expanded nodes was non-significant ($p>0.4$; Fig. 4.3). The reduction in height is thus the result of a reduction in internode length. The family effect on the number of nodes expanded was significant ($p<0.003$), with averages ranging from 29.3 in the 733 group to 32.4 in the bulk group, suggesting a difference in the rate of development between the families.

In the secondary study, paclobutrazol again had a significant ($p<0.0001$) effect on plant growth, with increasing dose causing increasing reduction in height for both the clonal and seedling material in April, 1993 (Fig. 4.4). There was no significant effect (at the $\alpha=0.05$ level) of paclobutrazol treatment on the number of nodes expanded in the clonal material (Fig. 4.5). The seedling material did not show any general effect of paclobutrazol treatment, though the number of nodes expanded by seedlings in the low treatment group did show a slight increase (Fig. 4.5). These results support those of the primary experiment which suggested an overall reduction in height results from a decrease in internode length. An overall difference in the total height ($p<0.0001$) and number of expanded nodes ($p<0.0001$) between the seedling and clonal material was observed across all

treatment levels. This may be the result of a genotypic difference in intrinsic growth rates, or alernatively may be a maturation effect resulting from the growth of the clonal material from cuttings.

Vegetative maturity

By March 1992, when material of the primary experiment was ca. 18 months old, the seedlings of the 733 and 734 provenances had undergone significant morphogenesis towards the vegetatively adult foliage form (Plate 4.1), as indicated by the results for maximum leaf maturity of lateral and main shoots (Tab. 4.3). Despite a non-significant indication of paclobutrazol application reducing the level of vegetative maturity in the 733 group (Tab 4.3), the developmental stage at which phase change occurred as indicated by the node at which the first intranode appeared ($p>0.58$), or the node at which the first petiolate leaf ($p>0.37$) appeared was not affected (Tab. 4.4). In conjunction with the reduction of overall plant height following paclobutrazol treatment, there has been a similar reduction in the height at which phase change occurs (Tab. 4.4). For example, the maximum reduction in height of 29% (34 cm) seen in the 733 High treatment was associated with a similar 29% (29 cm) reduction in height to the first petiole and a 27% (31 cm) reduction in height to the first node where leaves are inserted alternately rather than opposite one another (i.e. where the first intranode is found).

Both the clonal and seedling material of the secondary experiment remained fully juvenile, regardless of paclobutrazol treatment or otherwise. A similar situation was observed in the primary experiment, with neither the seedlings from the Bulk seedlot or the PerM provenance demonstrating any tendency to change from the vegetatively juvenile phase at the first scoring time (Tab. 4.3). In both experiments this juvenilityof foliage type remained despite the production of flower buds in some cases (Plate 4.2).

First season reproductive activity

In the primary study, all groups of material had some seedlings which produced flower buds regardless of whether they had been treated with paclobutrazol or not

(Fig. 4.6). However when averaged across all seedling types, paclobutrazol markedly increased reproductive output as demonstrated by the increase in the proportion of seedlings producing flower buds (χ^2_2=44.87; p<0.001) (Fig. 4.6) and the average number of flower buds per flowering seedling (p<0.0001) (Fig. 4.7) as a result of paclobutrazol application. The proportion of treated seedlings producing flower buds rose with increasing paclobutrazol dose in the precocious flowering (733) group and the PerM group, while the high treatment resulted in a lower proportion of treated seedlings flowering than the low treatment for the bulk material (Fig. 4.6).

As expected paclobutrazol application resulted in a significant (p<0.0001) effect on the height of the first flower bud on the main shoot (Tab. 4.5) when analysed across all treatments, related to the overall effect on internode length. The 733 group, which accounted for most of the significance of treatment effect on this character, showed a reduction between the high and zero treatments of 26% (28 cm) which compares well with the 29% (34 cm) decrease seen in total height. For other factors demonstrative of the developmental stage at which reproductive maturity was beginning, such as the node of the first bud on the main shoot (Tab. 4.5), there was some evidence of a significant (p<0.021) effect of paclobutrazol treatment, averaged across all seedling types. However, the average number of nodes to the first flower bud on the main shoot for each treatment level averaged across all treated seedling types revealed the order Low>Zero>High for this character. This suggests that there is no general effect of paclobutrazol treatment on this character, as was observed to be the case for the total number of nodes expanded. There was a significant (p<0.0001) family effect on the number of nodes to the first flower bud on the main shoot, similar to that seen for the total number of nodes previously.

In the secondary study, neither untreated clonal or seedling material produced flower buds in April 1993, when material was 21 or 19 months old respectively. Paclobutrazol induced the production of flower buds in seedling material during this first reproductive season following application, with 24% of low dose-treated seedlings flowering and 39% of high dose-treated seedlings flowering (Tab. 4.6.; Plate 4.3), but was ineffective in the clonal material. The average number of flower buds per flowering seedling did not vary significantly between low and high

dose treatments (t_{19}=2.00; 0.1>*p*>0.05) (Tab. 4.6), though this may be the result of small sample sizes. The absence of a significant difference in the number of nodes to the first flower bud on the main shoot between low and high dose treatments (t_{11}=0.70; 0.7>*p*>0.5) (Tab. 4.6) was indicative of the apparent lack of a general effect of paclobutrazol on the developmental stage at which flowering begins observed in the primary study (Tab. 4.5). Though non-significant (t_{11}=1.91; 0.1>*p*>0.05), the 10% (9.8 cm) reduction in average height to the first flower bud on the main shoot between low and high treatment levels reflected the trend of the 20% (23.8 cm) reduction seen in total height (Fig. 4.4).

Second season reproductive activity

Effects on Flower Bud Maturation

There was no noticeable affect of paclobutrazol treatment on the process of floral development, with flowers on all seedlings apparently developing normally (Plate 4.4). Pollination occurred naturally on material maintained outside, while a mixture of outcross pollen was used to achieve pollination of flowers reaching anthesis whilst in the glasshouse. Testing of pollen taken from a small number of both paclobutrazol treated and untreated plants did not indicate any general effect of treatment on germination rates (Tab. 4.7). Capsule maturation appears to be occurring normally, and recent dissection of a developing capsule from a paclobutrazol treated PerM group plant suggests seed maturation will be complete in less than 3 years since planting.

Retention of flower buds produced in the first season

The effect of paclobutrazol on the retention of first season flower buds and their subsequent development into capsules was assessed using only the material maintained outdoors. Glasshouse grown material could not be assessed due to the lack of pollinators in this indoor environment producing a lower than normal rate of capsule set. As indicated mainly by the results of the 733 provenance seedlings, paclobutrazol dose did not appear to have any effect on the proportion of seedlings which lost 100% of their first (1992) season flower buds (χ^2_2=1.44; 0.5>*p*>0.3), or the proportion of buds lost, where this was less than 100%, as determined by a

capsule count in July 1993. While the untreated seedlings of this family saw a 70% (16/23) rate of complete loss of flower bud crop, the low and high paclobutrazol treatments showed rates of 54% (20/37) and 61% (25/41) respectively. Again for the 733 group, proportions of first season flower buds lost, where this was less than 100%, ranged from 63% (n=7) in the case of no paclobutrazol treatment to 72% (n=17) for low treatment to 73% (n=16) for the high paclobutrazol dose. The numbers of repotted Bulk and PerM seedlings which were maintained outdoors was not sufficiently high to draw any valid comparisons between untreated and treated groups, though no evidence of a paclobutrazol effect on these characters was shown. All the 734 provenance seedlings were repotted and paclobutrazol treated (where prescribed) after the period of first season flower bud production. As a result, paclobutrazol concentration in treated seedlings would be likely to be considerably higher than in seedlings of the other provenance types which were treated 12 months previously. Despite this, however, no general effect of paclobutrazol treatment on retention of first season flower buds was observed, with the proportion of 100% flower bud loss in the untreated group of 40% (4/10) comparing to 64% (7/11) and 33% (2/6) for low and high doses respectively. The average proportion of flower buds lost (where this was less than 100%) for untreated material was 33% (n=6), while increasing paclobutrazol dose saw averages of 76% (n=4) and 45% (n=4).

The result of the lack of an apparent paclobutrazol effect on the proportion of the first season flower bud crop lost, coupled with the increase in the proportion of paclobutrazol treated seedlings bearing first season flower buds has resulted in a general increase in the proportion of paclobutrazol-treated individuals bearing capsules (χ^2_1=7.56; 0.01>p>0.001), relative to their untreated controls. In the 733 group, untreated seedlings bore capsules in 16% (7/43) of cases, averaging 3.7±0.97 (n=7) capsules.seedling^{-1} while low and high paclobutrazol treatment saw 43% (17/40) and 38% (16/42) of seedlings bearing averages of 5.8±1.17 (n=17) and 6.3±1.08 (n=16) capsules respectively. No evidence against this trend was found in the Bulk or PerM material.

Second season bud production

Two years after paclobutrazol treatment, second season bud production appeared to

be affected by three factors, these being level of paclobutrazol application prior to first season flower bud production, pot size and growth temperatures.

Within each seedling type, when outdoor-grown material in small and large pots was considered together, increasing paclobutrazol treatment in April 1991 increased second season flower bud production in terms of both the proportion of seedlings in each group which bore second season flower buds (Fig. 4.8) and the average number of flower buds per flowering seedling (Fig. 4.9).

Maintaining seedlings in a heated glasshouse from early 1992 appeared to have an inhibitory effect on the production of second season flower buds. Of the 51 seedlings grown in the glasshouse environment after early 1992, only three produced flower buds. Between the paclobutrazol treated, repotted Bulk and PerM families maintained within the glasshouse, 0% (0/15) produced second season flower buds. In comparison, the same material held outdoors saw 25% (6/24) of treated seedling bearing second season flower buds. The effect was more pronounced in the case of a comparison between the same groups of material amongst plants which were not repotted. In this case the summation of paclobutrazol treated Bulk and PerM material held in the glasshouse saw 5% (1/20) of seedlings bearing flower buds. The Bulk and PerM material grown outdoors in the original small pots on the other hand, produced a 59% (16/27) rate of second season flower bud bearing.

There appears to have been an effect of pot size on second season flower bud production, with seedlings in smaller pots showing a greater reproductive response in terms of both proportions of seedlings bearing flower buds and bearing rate. Untreated 733 provenance material repotted in early 1992 produced 12% (5/43) of seedlings bearing an average of 2.0±0.63 second season flower buds per flowering seedling, while material which was not repotted saw new reproductive activity in 35% (6/17) of seedlings, with an average bearing rate of 4.5±0.99. Examination of first and second season bud production in untreated seedlings of the 734 seedlot provides additional evidence of the response. In this case, 34% (30/89) of seedlings produced an average of 7.0±1.29 first season flower buds in 1992. Subsequent repotting of all material reduced the incidence of second season bud production in untreated material to 12% (3/26), and bud bearing to 2.7±0.33 buds per flowering seedling, despite the material now being one year more mature.

Further evidence can be drawn from untreated and treated groups of the PerM and Bulk groups (data not shown). It seems that some factor associated with reduced pot size is effectively a promoter of floral induction. The promotory effect of this factor appears to be additive to the promotory effect of paclobutrazol application.

The summer of 1992/1993 was the first season of bud production which could have been influenced by paclobutrazol treatment in the 734 provenance material, following its application in March 1992. In this case, increasing levels of paclobutrazol treatment applied to these vegetatively mature seedlings were associated with a clear increase in flowering response in July of the following year. This response was obvious in both the proportion of seedlings in each treatment which bore second season flower buds (Fig. 4.8) and the average number of buds per reproductively active seedling (Fig. 4.9).

Discussion

First season bud production

In addition to an increase in the production of flower buds on seedlings genetically predisposed to rapid development (provenance 733 and 734 material), a chemical stimulation of flowering precocity to a level useful for breeding purposes has been produced in 18 month-old seedlings. These seedling were still bearing juvenile foliage, and would not be expected to flower for three to four more years under natural stand conditions (Turnbull and Pryor, 1978). This is a novel result, which suggests a separation of the vegetative and reproductive juvenility which could not be achieved by Griffin *et al.* (1993) in *E.nitens*, though it was not attempted in *E. globulus*. A similar situation, with flower buds being produced on vegetatively juvenile trees has also been observed in field grown *E. nitens*, following collar drenching with paclobutrazol (S. Hetherington, unpublished). Together, these results suggest that the factors which regulate the developmental sequences of vegetative and reproductive phase change are independently controlled.

In the primary study paclobutrazol acted as a facultative promoter, causing an increase in the proportion of seedlings producing flower buds and the average

number of flower buds per flowering seedlings, when averaged across all seedling types. In the majority of cases, the most significant results were recorded in seedlings of the 733 provenance. For characters of reproductive development scored, the small sample sizes of the PerM and Bulk groups, as well as the comparative genetic heterogeneity of the latter group were likely factors in diminishing the significance of any trends. In the secondary study, paclobutrazol appeared to be an obligate promoter of precocious flowering in the material used, with no untreated seedlings producing flower buds. The apparent difference in the degree of paclobutrazol requirement between the two experiments was likely to stem from two non-exclusive sources. First, maintaining both treated and non-treated seedlings together over a polythene cover to prevent interaction with external soil, may have resulted in some of the foliar applied paclobutrazol washing off live and dead leaves (Williams, 1984; Mauk *et al.*, 1990a) and then being taken up through the pot base of some untreated seedlings. Great care was taken in the secondary study to eliminate this possibility. The second potential source of difference of paclobutrazol requirement for flowering activity may stem from the greater heterogeneity of the Bulk material in the primary study. This mixture may have included seed which was genetically predisposed to early flowering. The PerM seedlot may also have contained seed with precocious flowering tendencies.

The results produced by both the primary and secondary experiments suggest that a variation in the genetic predisposition to early flowering is likely to exist. This conclusion can be drawn from the fact that all seedlings within various treatments received the same paclobutrazol dose and were maintained under the same environmental conditions, yet individuals displayed variation in their reproductive response. The clonal material of the secondary experiment was the only genetically homogenous material used. However the failure to induce a reproductive response at any paclobutrazol treatment level prevented the assessment of the effects genetic homogeneity. There is a possibility that small differences in microenvironment which affect the growth of seedlings or perhaps have an indirect effect on the rate of paclobutrazol uptake may have some role in producing the variation in flowering response observed within a single treatment.

Paclobutrazol is active in reducing height growth through a reduction of internode length, rather than any substantial effect on the rate of vegetative

development as indicated by the number of nodes produced. A similar reduction of internode length as a major contributor to the overall reduction of vegetative growth has been reported for other plant groups such as *Citrus* (Aron *et al.*, 1985). In general, height growth and reproductive output were observed to be negatively correlated (compare Figs 4.2, 4.6 and 4.7). Whether the diversion of assimilates from growth to the production of reproductive structures can be isolated as the major cause of some or all of the effects of paclobutrazol application on reproductive output observed cannot be readily determined. This possibility is considered further in the following chapter (General Discussion).

Second season bud production

The general effects of paclobutrazol treatment on first season flower bud production have carried over to second season bud production. The relatively large number of seedlings bearing second season flower buds coming from the 733 material has meant that the data from this group forms the basis of this generalisation, though further support was found in the data derived from the Bulk and PerM material. Given the evidence of a short half-life of paclobutrazol in plant tissue (Chapter 3) and the utilisation of a foliar application method, the persistence of the effects of paclobutrazol over some 20 months may seem unlikely. However, as material applied to the leaves and stems can be washed down into the soil by rainfall and irrigation water (Williams, 1984; Mauk *et al.*, 1990a) where it may become reversibly bound (Lever, 1986), its relative stability (Chapter 3; Mauk *et al.*, 1990a) may result in persistence for a number of years. The length of time over which it will be active in affecting the growth and development of seedlings in the vicinity depends upon its initial concentration in the soil and subsequent rate of breakdown.

Increasing the pot size between first and second year flower bud production seasons was shown to reduce reproductive output, both in terms of the proportion of seedlings in each treatment bearing flower buds and the average number of flower buds per flowering seedling. It seems that some factor associated with reduced pot size, whether water or nutrient stress, root growth stress or some other parameter is effectively promoting floral induction. As a difference between the reproductive output of seedlings in small and large pots was noted in the case of

untreated material, it appears that the effect is not associated with a loss or dilution of paclobutrazol activity with the introduction of new soil. Whatever its source, the promotory effect on second season flower bud production achieved by maintaining seedlings in small pots appears to be additive to the promotory effect of paclobutrazol application. Such a promotion of flowering resulting from the use of small pots has been observed in the case of apples (see Tydeman, 1937 in Doorenbos, 1965).

The transfer of some seedlings to a heated glasshouse after the first season of flower bud production appears to have been a stronger inhibitor of reproductive activity than repotting. It seems likely that this effect is similar or identical to the mechanism which prevented precocious flowering in paclobutrazol-treated grafted material which did not receive a full overwintering period (Chapter 2). Whether this temperature effect and the effects of changing pot size are all active through an influence on growth rate and a proposed negative correlation between vegetative and reproductive output as a result of assimilate diversion is considered further in the following chapter (General Discussion).

Floral development, anthesis and capsule production

Paclobutrazol treatment did not outwardly affect the mechanisms of floral development, anthesis, pollen viability, pollination or subsequent capsule production under any environmental treatment. Thus, paclobutrazol treatment seems to be an ideal means of inducing flowering for breeding purposes in far less time than would be possible in normal planting regimes. The only remaining step is to ensure that paclobutrazol treatment has no detrimental effects on the final stages of reproduction or seedling development. At this stage an estimation of the numbers of seeds per capsule, followed by seed viability testing can finalise the overall effect of paclobutrazol treatment on the generative processes of material tested in this group of experiments.

The lack of any effects of paclobutrazol treatment on rates of bud loss, in combination with a paclobutrazol-stimulated increase in both the proportion of seedlings bearing flower buds and the average number of flower buds per flowering seedling, has produced a net increase in bud production in paclobutrazol-

treated material. This has produced an overall increase in capsule production in the paclobutrazol-treated seedlings, relative to their untreated controls. This is in agreement with the results of previous work on *Eucalyptus* species (Hetherington *et al.*, 1991; Griffin *et al.*, 1993, Hetherington and Jones, 1993) and extensive work carried out with other fruit bearing arboreal angiosperm species such as apples (Curry and Williams, 1983) and pears (Raese and Burts, 1983). In a similar manner to the confirmation required in capsules produced preciously on *E. globulus* seedlings, the numbers of viable seeds per capsule for the paclobutrazol enhanced crops requires quantification. This is especially important in light of the work of Griffin *et al.* (1993) who felt there was a non-significant decrease in numbers of seeds per capsule as a result of paclobutrazol treatment. A large scale assessment of this character is required in order that the net activity of paclobutrazol on the magnitude of reproductive responses in important *Eucalyptus* species may be determined.

Conclusion

The potential for the use of paclobutrazol to reduce the generation times of *E. globulus* seedlings, grown from commercial seedlots, has been demonstrated. A reduction in the order of 50% (three years) in comparison to natural stands was achieved. Paclobutrazol treatment, pot size and growth temperatures have all been shown to play roles in determining the extent of new reproductive activity each season in seedling *E. globulus*. Presumably, seedling maturity also has some effect, though this factor cannot be extracted in isolation from the data gathered. Paclobutrazol treatment does not appear to have any negative effects on the retention of floral buds through to the development of mature capsules, while having a distinct positive effect on reproductive output, in terms of both flower bud and capsule numbers. Foliar spraying during autumn has been shown to be an effective means and timing of paclobutrazol application, with results evident in the following fruiting season. Considerable persistence of paclobutrazol effects were noted in the second season after application.

Table 4.1. The number of seedlings in each treatment of the primary experiment at time of scoring in March, 1992. Paclobutrazol treatments were applied in March, 1991 when seedlings were 5-6 months of age. Seedlings from seedlot 734 were not paclobutrazol treated until after the first scoring of the primary experiment (see text) in order that the effects of the retardant on vegetatively adult material could be assessed.

Paclobutrazol Dose	Seedlot Source			
	Bulk	PerM	733	734
Zero	19	23	80	89
Low (1x1000 ppm)	32	15	43	0
High (2x1000 ppm)	29	17	47	0

Table 4.2. Characters assessed in seedlings of the primary experiment in March 1993, ca. one year after paclobutrazol application.

Abbreviation	Character
HT	height in cm
NE	number of expanded nodes
BUDnum	number of flower buds per flowering seedling
HBUDms	Height of first flower bud on the main shoot
NBUDms	node of first flower bud on the main shoot
ShootMat	assessment of maturity of the main shoot
LatMat	assessment of maturity of the most mature lateral shoot
HPCpet	height in cm of the first node showing a petiole
HPCintra	height in cm of the first node exhibiting an intranode
NPCpet	node number of the first node showing a petiole
NPCintra	node number of the first node exhibiting an intranode

Table 4.3. Maximum leaf maturity of main shoots and laterals in seedlings of the primary experiment in March 1992, ca. 1 year after paclobutrazol application. Maturity was based on a subjective assessment of leaf shape and petiole insertion (1 = opposite, sessile, oblong-ovate leaves 2 = ovate leaves, 3 = ovate, petiolate leaves 4 = lanceolate, petiolate leaves with visible intranode).

Seedlot	Paclobutrazol Level	Maximum Leaf Maturity	
		Main Shoot	Lateral Shoots
Bulk	Zero	1	1
	Low	1	1
	High	1	1
PerM	Zero	1	1
	Low	1	1
	High	1	1
733	Zero	2.32±0.12	2.44±0.11
	Low	2.09±0.13	2.28±0.13
	High	2.10±0.10	2.19±0.09
734	Zero	2.48±0.08	2.39±0.08

Table 4.4. The effect of paclobutrazol treatment on the node and height of phase change in provenance 733 seedlings of the primary experiment. Scoring was performed in March 1992, ca. 1 year after paclobutrazol application.

Character	Paclobutrazol Dose		
	Zero	Low	High
Node number of first intranode	26.2±0.53	25.5±0.83	26.7±0.90
Node number of first petiole	22.9±0.48	22.2±0.63	21.8±0.62
Height to first intranode (cm)	112.9±1.99	92.8±3.26	82.1±3.45
Height to first petiole (cm)	101.4±1.86	83.4±2.68	72.2±2.75

Table 4.5. The effect of paclobutrazol application on height to the first bud on the main shoot (HBUDms) and the node of the first bud on the main shoot (NBUDms) in the three types of paclobutrazol treated seedlings in the primary experiment. Scoring was accomplished in March 1992, ca. 1 year after paclobutrazol application.

Paclobutrazol		HBUDms	NBUDms
Seedlot	Dose	(cm)	
Bulk	Zero	101.0±14.4	28.0±3.69
	Low	107.5±4.30	33.8±1.10
	High	not-est[a]	not-est[a]
PerM	Zero	112.7±7.54	26.5±2.71
	Low	119.6±5.34	33.1±1.73
	High	98.9±4.81	29.1±1.19
733	Zero	105.8±3.70	26.7±1.17
	Low	91.8±2.99	25.0±0.76
	High	78.3±3.77	23.8±0.72

[a] not-estimatable as no high dose treatment Bulk seedlings produced flowers on the main shoot

Table 4.6. The effect of paclobutrazol treatment on the percentage of seedlings flowering, average number of floral buds per flowering seedling (BUDnum), node of the first bud on the main shoot (NBUDms) and height to the first bud on the main shoot (HBUDms) for seedlings grown from a commercial seedlot for the secondary experiment. Characters were scored in April 1993, ca. one year after paclobutrazol application. The increase in the proportion of seedlings flowering after low and high dose paclobutrazol treatment are highly significant ($p<0.01$) in both cases.

	Paclobutrazol Dose		
Character	Zero	Low	High
% flowering[a]	0% (0/36)	24% (8/33)	39% (13/33)
BUDnum	not-est[b]	19.9±7.4	14.0±2.5
NBUDms	not-est[b]	32.5±0.9	33.6±1.3
HBUDms (cm)	not-est[b]	98.0±4.9	88.3±1.3

[a] Significances for % flowering :

Zero vs Low $\chi^2_1=9.87$; $p<0.01$

Zero vs High $\chi^2_1=17.46$; $p<0.01$

Zero vs Low+High $\chi^2_1=14.43$; $p<0.01$

[b] not-estimatable due to the lack of flowering in the untreated seedlings

Table 4.7. Germination rates of pollen produced by seedlings of the primary experiment during the first flowering season following paclobutrazol application. Averages shown were generated from ca. three trials for each individual.

Seedling Type	Paclobutrazol Dose	Plant Number	% germinating ± SE
Bulk	Low	5	43±12.0
		18	42±10.5
		22	28±9.9
		35	20±19.5
PerM	Low	1	53±7.0
		9	47±14.5
		27	24±8.0
	High	1	48±8.5
		33	48±14.4
		45	29±10.0
733	Zero	9	41±19.0
	Low	17	9±3.5
734	Zero	7	40.5±12.7
		21	10±4.0
mix[a]	Zero	-	40±12.7

[a] A mixture of pollen collected from two untreated trees growing at sites in south-eastern Tasmania. This represents an average performance of *E. globulus* pollen from field grown material in the germination system used.

Figure 4.1. Average daily maximum and minimum temperatures each month for Hobart, 1991 and 1992.

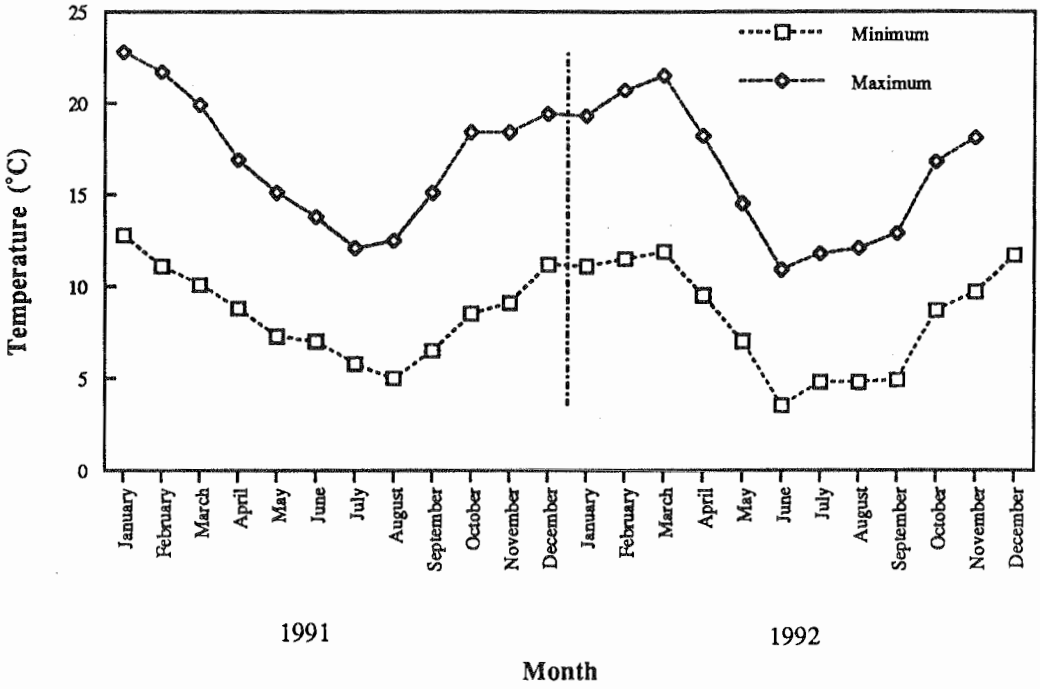


Figure 4.2. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on total height across the 3 groups of material assessed in the primary study. Paclobutrazol applications were administered in March 1991 when seedlings were 5-6 months of age and 50-60 cm in height. Measurements were taken ca. 12 months later in April 1992.

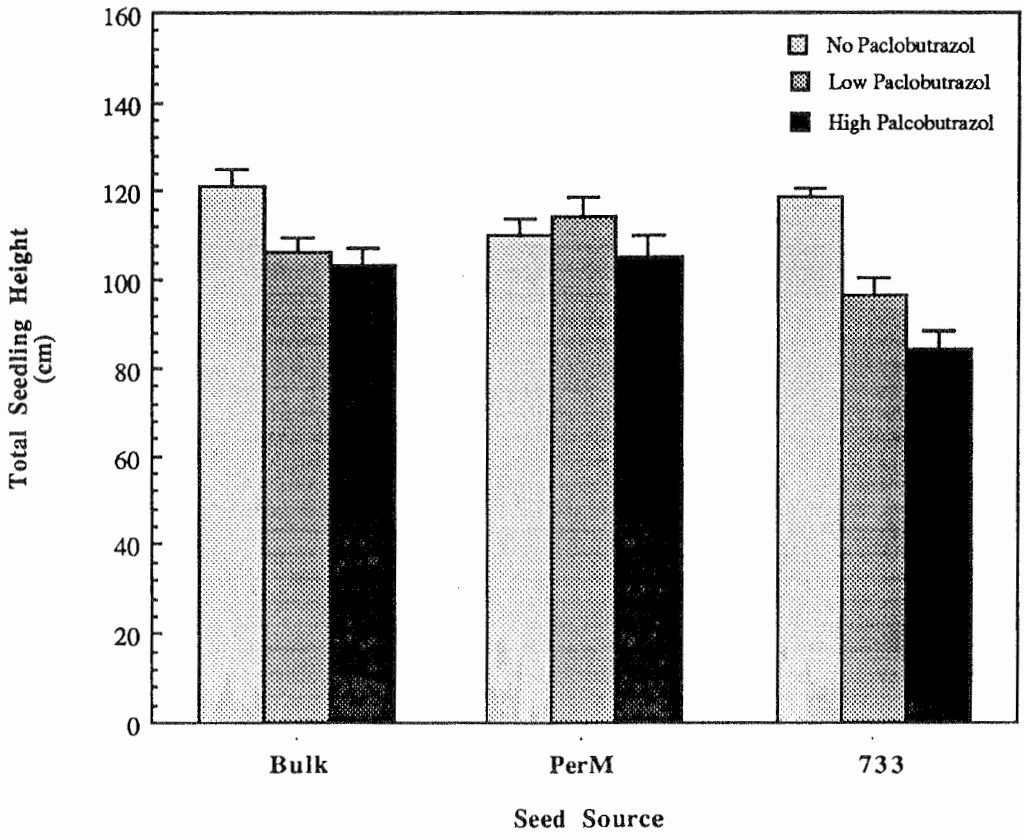


Figure 4.3. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on the number of expanded nodes (±SE) across the three groups of material assessed in the primary study. Paclobutrazol applications were administered when seedlings were five to six months of age in March 1991. Node counts were performed ca. 12 months later in April 1992.

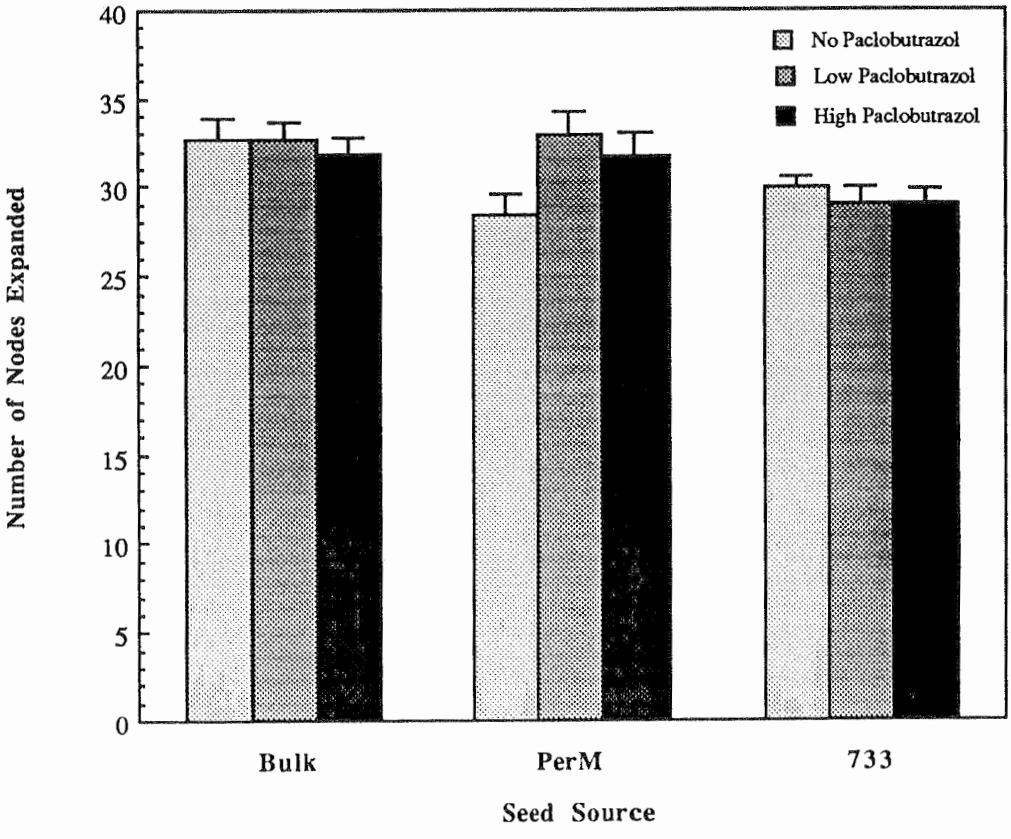


Figure 4.4. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on total height in seedling and clonal material used in the secondary study. Paclobutrazol applications were administered in April 1992 when seedlings were 8 months of age and clones were 10 months of age. Measurements were taken ca. 12 months later in April 1993.

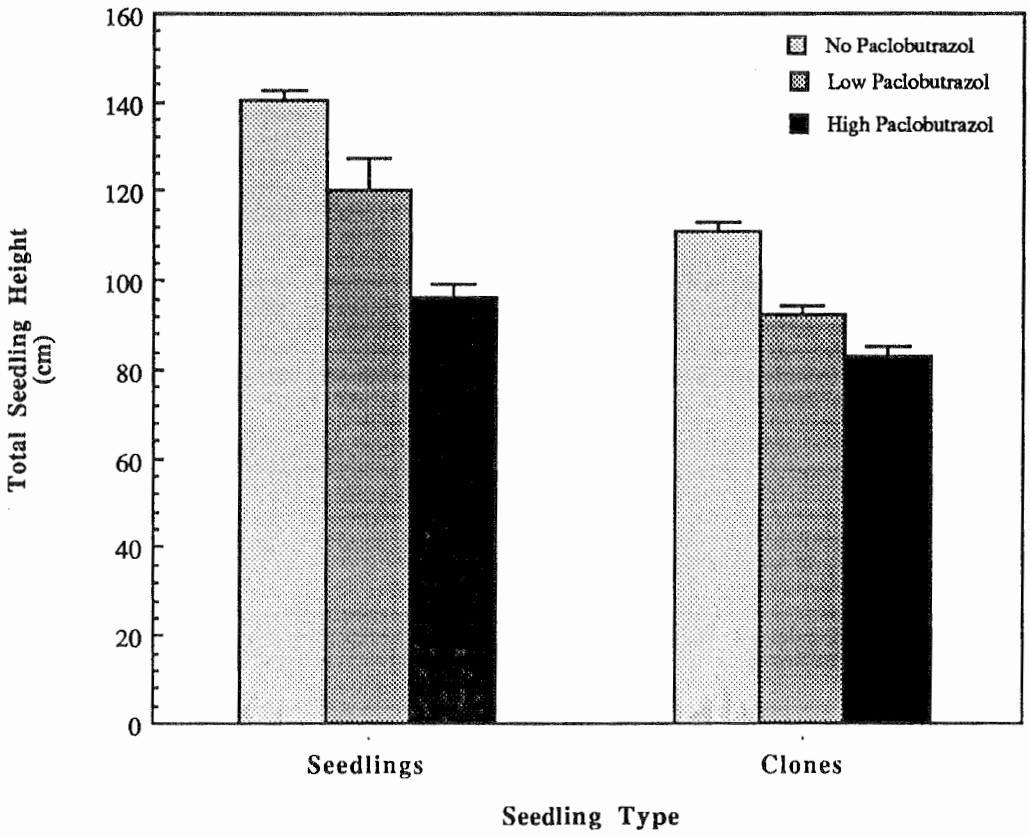


Figure 4.5. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on the number of expanded nodes in seedling and clonal material used in the secondary study. Paclobutrazol applications were administered in April 1992 when seedlings were eight months of age and clones were 10 months of age. Node counts were performed ca. 12 months later in April 1993. Columns topped with different letters are significantly different at the (α=0.05) level as determined by Ryan-Einot-Gabriel-Welsch multiple range testing (SAS/GRAPH, 1988).

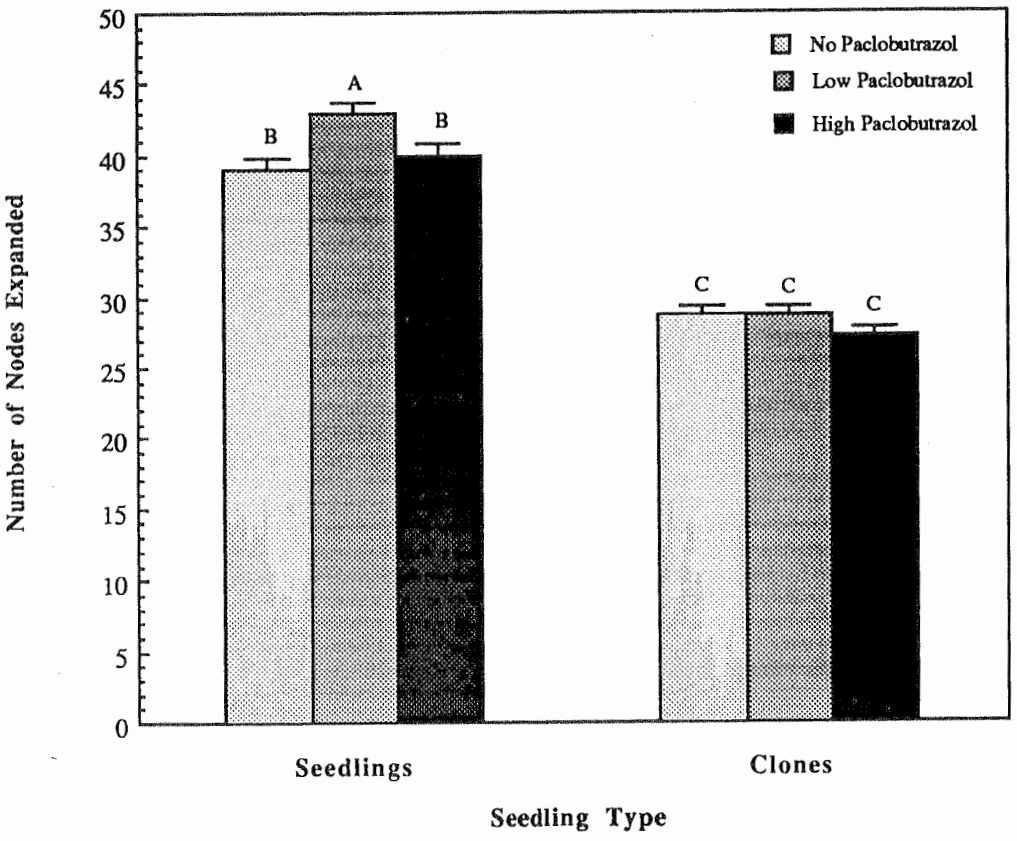


Figure 4.6. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on the percentage of seedlings bearing flower buds across the three groups of material assessed in the primary study. Paclobutrazol applications were administered when seedlings were 5-6 months of age in March 1991. Assessment of flowering was made ca. 12 months later in April 1992. The number in brackets above each bar indicates the number of seedlings in each sample.

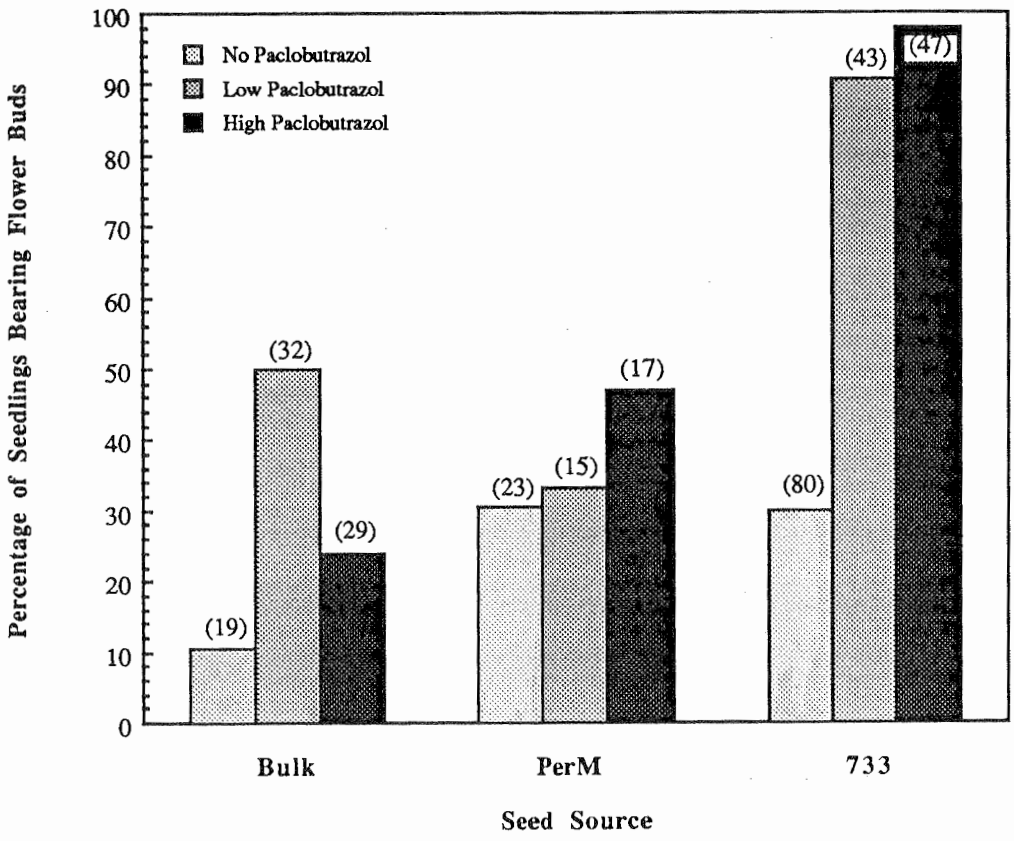


Figure 4.7. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on the average number of flower buds per flowering seedling (±SE) across the three groups of material assessed in the primary study. Paclobutrazol applications were administered when seedlings were five to six months of age in March 1991. Flower bud counts were performed ca. 12 months later in April 1992. Bars within a single seed source group topped by different letters are significantly different at the $p<0.05$ level.

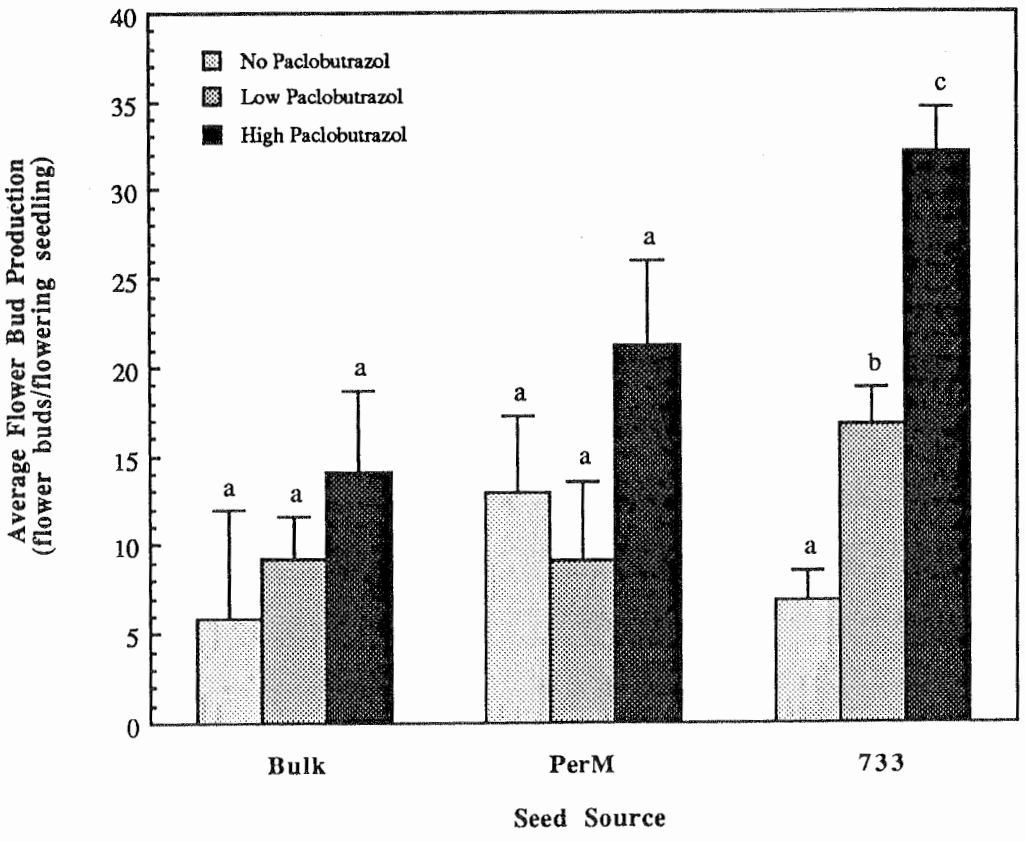


Figure 4.8. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on the percentage of seedlings bearing second season flower buds across the four groups of material assessed in the primary study. Paclobutrazol applications, other than for the 734 group, were administered when seedlings were five to six months of age in March 1991. The 734 material was not paclobutrazol treated until March 1992 in order that the effects of paclobutrazol could be assessed on vegetatively mature material. Assessment of bud production was made ca. 16 months later in July 1993. The number in brackets above each bar indicates the number seedlings in each sample.

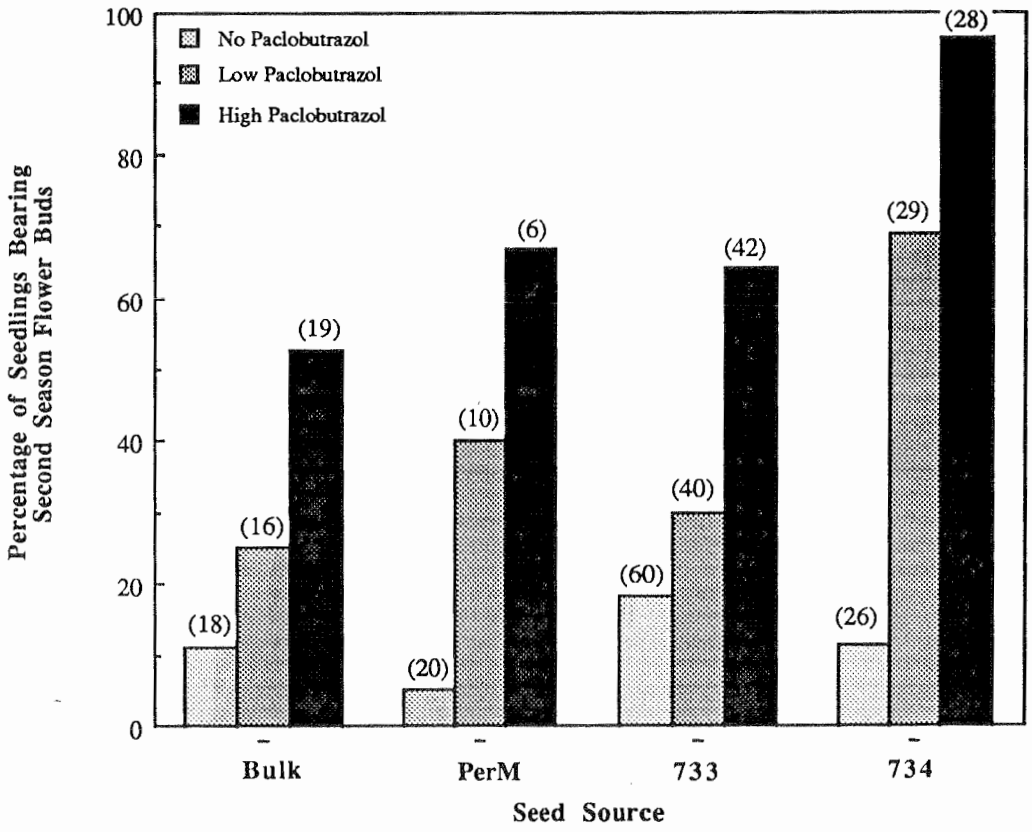


Figure 4.9. The effect of low (1x1000 ppm) and high (2x1000 ppm) rates of paclobutrazol foliar spray on the average production of second season flower buds per flowering seedling across the four groups of material assessed in the primary study. Paclobutrazol applications, other than for the 734 group, were administered when seedlings were five to six months of age in March 1991. The 734 material was not paclobutrazol treated until March 1992 in order that the effects of paclobutrazol could be assessed on vegetatively mature material. Bud counts were performed ca. 16 months later in July 1993.

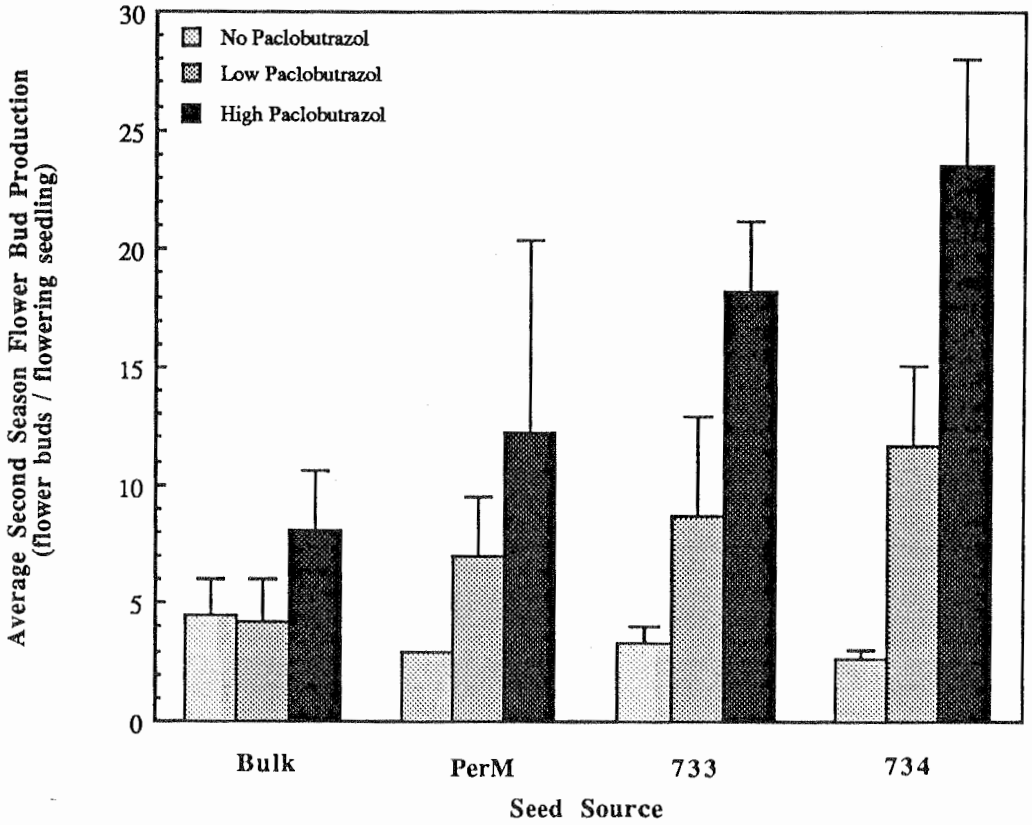


Plate 4.1. An 18 month old '733' provenance seedling showing the transition to adult foliage. Note the green, elongate, petiolate leaves of the upper stem in comparison to the obovate and sessile leaves lower on the plant. Along with the attainment of vegetative phase change, reproductive competence is also demonstrated by the presence of flower buds on this seedling which received a low dose (1x1000 ppm) paclobutrazol foliar spray at age 6 months.

Plate 4.2. A 'PerM' group seedling showing the paclobutrazol stimulated production of flower buds despite the continued production of juvenile leaves at 18 months of age. This seedling received a high dose (2x1000 ppm) paclobutrazol foliar spray at age 6 months.

Plate 4.3. Paclobutrazol stimulated flower bud production in seedling material of the secondary experiment (see text). While untreated seedlings failed to produce flower buds at 18 months of age, low (1x1000 ppm) and high (2x1000 ppm) rate foliar sprays generated flowering precocity in a proportion of treated seedlings. Note the continued production of vegetatively juvenile foliage in the low dose paclobutrazol treated seedling pictured.

Plate 4.4. Despite the much reduced stature of plants flowering after paclobutrazol treatment such as the high (2x1000 ppm) dose treated 'PerM' seedling shown (left), bud development and flowering appeared normal (right).

General Discussion

Techniques were developed for purifying GAs from apical shoot tissue samples of *E. nitens* seedlings (Chapter 1). The range of GAs which were subsequently identified suggested that the early 13-hydroxylation pathway is the major pathway for the production of endogenous GAs in this seedling tissue. Similar GAs have also been identified in the cambial tissue of *E. globulus* (Hasan *et al.*, 1994) indicating the operation of the early 13-hydroxylation pathway is likely to be common to vegetative tissues of these two eucalypt species. Both *E. globulus* and *E. nitens* are members of the same sub-genus (*Symphyomyrtus*), suggesting a possible commonality of this pathway amongst species of this sub-genus. The apparent ability of *E. risdonii*, a member of one of the six alternative sub-genera, to metabolise GAs of the early 13-hydroxylation pathway (data not shown) suggests that the enzymes required for GA metabolism are present in species of other *Eucalyptus* sub-genera. This may be viewed as additional evidence of the operation of the early 13-hydroxylation pathway throughout the genus. The GAs of this pathway are also found in the majority of plant species thus far studied (Chapter 1).

This work has demonstrated that paclobutrazol treatment can promote flowering of both vegetatively juvenile and mature seedlings (Chapter 4) as well as grafts produced from reproductively competent scions (Chapter 2). Thus, in vegetatively juvenile *E. globulus* seedlings, precocious flower bud production was induced, with no flowering occurring in non-paclobutrazol treated seedlings grown from a commercial seedlot. The flower buds appear normal in all respects and are expected to produce viable seed, given the experiences of Griffin *et al.* (1993) and Moncur (unpublished) with seed produced from other paclobutrazol treated eucalypts. In these cases, the seed to seed generation time has been reduced by more than 50% in comparison to field-grown material. This is perhaps the most novel result of this work, with relatively few previous examples of a growth retardant promoting the induction of flowering in apparently juvenile seedlings (eg. Snowball *et al.*, 1990). Instead, most attempts at reducing generation times have utilised either a genetic basis, by breeding for a reduced period of juvenility as in peach and nectarine seedlings (Hansche, 1986), or have been concerned with

increasing the rate at which perceived height / age / developmental stages are reached by manipulation of growth conditions (Longman and Wareing, 1959; Mullins and Snowball, 1988; Brindbergs *et al.*, 1989).

In the case of seedling and grafted material which was already reproductively competent, paclobutrazol application increased the size of the flower bud crop on seedlings of *E. globulus*, again without detrimental effects on bud development, anthesis or capsule production. Such a result is in agreement with previous work both on eucalypts (Griffin *et al.*, 1993; Hetherington and Jones, 1993; Moncur *et al.*, 1993) and other species such as apples (Tukey, 1981), citrus species (Mauk *et al.*, 1986) and sour cherry (Bukovac and Hull, 1985). Together these examples provide ample confirmation of this particular effect of paclobutrazol treatment.

Using the GA analysis techniques developed, paclobutrazol was demonstrated to reduce the levels of endogenous GAs in grafted *E. nitens* material (Chapter 2). Quantification of paclobutrazol treated, espalier grown, *E. nitens* grafts revealed a range of GA_1 concentrations in apical shoot tissue samples taken from different individuals. The variability was related to both treatment method and dose, and was thus interpreted to be the result of the differing persistence of the effect of paclobutrazol on GA biosynthesis. Relating the concentration of GA_1 in apical shoot tissue at the time of flower bud initiation with the level of flowering response evident some months later suggested higher concentrations (>0.6 ng.g DW^{-1}) of GA_1 were inhibitory to large flowering responses. In addition, there was some indication of a reduction of the effects of paclobutrazol on flower bud production as a result of GA_3 application. Together these findings provide at least circumstantial evidence for some GA mediated role of paclobutrazol in the regulation of the reproductive processes of eucalypts.

Flowering in woody angiosperms is often inhibited by the application of endogenous active GAs, such as GA_3 (see references in Pharis and King, 1985). Studies with mature trees of *Prunus avium* compared the varying capacity of a number of GAs to inhibit flowering and found a considerable range of levels of inhibition (Oliveira and Browning, 1993). Indeed much detailed work investigating the relationship between the structure and activity of applied GAs has been completed in herbaceous plants such as *Lolium temulentum* (eg. Evans *et al.*, 1990; King *et al.*, 1993). However, this work can only be considered partial

evidence of a role for endogenous GA hormones in the regulation of floral induction, as in many cases the quantities applied may be supra-physiological, or the applied GA may not have been demonstrated to be native to the plant. In such a case, Plummer (1987 from Mullins and Snowball, 1988) found that flowering in adult *Citrus sinensis* and *Poncirus trifoliata* was inhibited by application of exogenous GA_3 and GA_4. However in addition to this they found that high concentrations of endogenous GA_1- and GA_4-like substances in shoot tips were associated with inhibition of floral induction. This supports the findings of this thesis that increased concentrations of endogenous GA_1 are associated with a depression in flowering of mature *E. nitens* grafts.

Despite the evidence for GA mediation of paclobutrazol effects, it is difficult to completely rule out the possibility that some of the activity of paclobutrazol on flowering in *E. globulus* and *E. nitens* is due to the effect it is known to have on sterol biosynthesis (Dalziel and Lawrence, 1984). One means of ascertaining if it is the effects on GAs which are active in affecting flowering would be to use a plant growth retardant which does not affect sterol metabolism. The appropriate purification of paclobutrazol or some other triazole growth retardant compound to remove the stereoisomers responsible for activity on sterols (Sugavanam, 1984; Izumi *et al.*, 1985; Koller, 1987) might yield such an inhibitor. Alternatively, cyclohexanetrione type inhibitors, with their quite different mechanisms and sites of activity (Rademacher *et al.*, 1992), could be assessed for their effectiveness in promoting reproductive activity in eucalypt species. In either case, an appropriate application of an active GA compound should be able to reverse all the effects of growth retardant treatment. This would provide the most straightforward means of establishing if the lowering of active GAs is the mechanism by which growth retardant chemicals affect flowering activity.

The stimulating effect of paclobutrazol on flowering can be inhibited by the substitution of normal outdoor growth during winter with a period of growth in a heated glasshouse (Chapters 2 and 4). Perhaps the most significant evidence of this was the failure of paclobutrazol treatment to induce flower bud formation in grafted material not fully overwintered in Canberra (Chapter 2). This failure occurred despite the fact that application of paclobutrazol effectively reduced GA levels in the non-flowering grafts, while similarly treated grafts of the same age

which were fully overwintered produced flower buds. In fact, some of the paclobutrazol treated, fully overwintered grafts which flowered were 12 months younger than material which failed to initiate as a result of not receiving a full overwintering period. This suggests an obligate requirement for an additional factor associated with the normal outdoor environment of a Canberra winter. The natural light regime received by the material after transfer to the glasshouse rule out an effect of differential photoperiod, leaving increased exposure to cold as the likely factor involved. This strong requirement for a period of cold to trigger (or allow) flowering was also demonstrated in the seedling *E. globulus* material in Hobart, where second season bud production was severely inhibited by growth in a heated glasshouse over winter. While a relationship between temperature and flowering has been observed previously in woody angiosperms, it is usually high temperatures which increase flowering (Jackson and Sweet, 1972), though olive trees require a period of winter chilling to form flowers in the following spring (Hackett and Hartman, 1963) and Moncur (1992) has shown that flowering in *E. lansdowneana* can be reliably induced by a period of cold treatment. The longer generation time of field planted *E. globulus* in Western Australia (L. Barbour, *pers. comm.*) may thus be the result of the more mild winter conditions in that state failing to produce the necessary degree of cold treatment for the initiation of flowering processes.

Pot size was shown to affect flowering in seedling *E. globulus* at Hobart (Chapter 4), with small pots promoting flowering in material bearing either adult or juvenile foliage in both paclobutrazol treated and untreated material. Reduced pot size is likely to cause physiological stress in seedlings, with water shortage and lack of nutrients the most probable factors involved. The former has long been recognised as a means of stimulating flowering in many forest tree species (Jackson and Sweet, 1972; Owens and Blake, 1985). This then may be the source of the promotive effect on flowering which was associated with continued growth in small pots. There has similarly been a long history of research demonstrating stimulation of flowering in herbaceous angiosperms as a result of nutrient deficiency (see Lang, 1965 for references). However, the effects of nutrient stress on flowering in forest trees is much more sparsely covered in the literature, though in a similar fashion to the case for herbceous angiosperms, Pharis *et al.* (1987) cite

papers showing that nutrient deficiency can stimulate flowering in some conifers. The converse situation has also been recorded in both angiospermous and gymnospermous trees, with high nutrient status following the application of nitrogenous fertilisers often resulting in the promotion of an increase in reproductive activity (Jackson and Sweet, 1972; Owens and Blake, 1985; Pharis *et al.*, 1987; Jackson, 1989). An experiment using root pruning of grafted *E. nitens* as a means of inducing a controllable amount of water and nutrient stress is in progress (M. Moncur, *pers. comm.*), and such work may help to isolate these effects from others which may be associated with reduced pot size.

It is difficult to assess if the effects of various cultural treatments tested are active in regulating normal annual flowering of espaliered, field planted or natural stands, though it may be possible to correlate exact meteorological data and soil water potentials with flowering output in an attempt to investigate any association. The expression of both cold treatment and pot size effects is influenced by the action of paclobutrazol, with very little evidence of the induction of precocious flowering in non-paclobutrazol treated material (Chapter 4). Paclobutrazol then may be seen to be performing a potentiating role, allowing the promotory effects of overwintering and small pot size on flowering to be expressed.

While the interaction of paclobutrazol, cold treatment and pot size and their resultant effects on flowering can be rationalised by a crude model (Fig. 5.1), the physiological means by which these three factors are associated is not yet clear. While paclobutrazol has been shown to reduce GA levels, this alone is not sufficient to induce a floral response in recently grafted *E. nitens* if cold treatment is not given. Cold treatment in conjunction with paclobutrazol did not significantly alter concentrations of GA_1 or GA_{20} despite the induction of flowering by paclobutrazol. However, while the levels of these GAs did not vary significantly between flowering and non-flowering treatments, the levels of other GAs may have been affected. Metzger (1990) showed that thermoinduced stem growth in *Thlaspi arvense* was mediated by an activation of the early non-hydroxylation pathway resulting in the accumulation of GA_9. The existence of parallel pathways controlling different functions within the same plant (Metzger and Mardaus, 1986; Metzger, 1990) suggests that while no GA_9 was found in the apical shoot tissue of vegetative *E. nitens* seedlings (Chapter 1), the onset of reproductive activity may

alter the suite of GAs present. This necessitates further study to confirm or discount this possibility. Whether qualitative or quantitative changes in the levels of GAs of an alternative pathway sufficient to account for the onset of flowering could occur in a plant which has had its capacity to synthesize GAs suppressed by paclobutrazol treatment is not known.

No attempt has yet been made to assess any effect of pot size on GA levels, so the means by which this factor induces flowering can only be speculated upon. The effect of low soil moisture in many species has been studied in some detail. Perhaps its primary activity in terms of effects on endogenous hormone levels is an increase in abscisic acid (ABA) levels (Wright and Hiron, 1972), though enhanced levels of certain GAs have been recorded following water stress in conifers (Pharis *et al.*, 1987). Abscisic acid is generally thought to antagonise the effect of GAs causing a similar effect to lowering GA levels, a process which has been shown to stimulate flowering in the eucalypt species studied. However some studies have suggested a synergism of ABA and active GA effects at lower ABA concentrations (Yadava and Lockard, 1977; Horrell *et al.*, 1990). In a similar fashion, the effects of paclobutrazol on ABA levels are not clearly defined. Some studies have indicated that ABA catabolism may be inhibited by blocking its conversion to 8'-hydroxy abscisic acid (Hedden, 1990; Zeevaart *et al.*, 1990) as this step is catalysed by a monooxygenase-like enzyme which is thus susceptible to N-heterocyclic plant growth retardants, such as paclobutrazol. If paclobutrazol does decrease the rate of catabolism of ABA and this phytohormone was antagonistic to the effects of active GAs, a mediation of the effects of water stress resulting from reduced pot size via GAs could be postulated. However, a further lowering of GA levels already reduced by paclobutrazol application would have to be demonstrated to be effective in promoting flowering in order that such a scheme could be accepted. Contradictory results on the effects of paclobutrazol on ABA levels complicate matters, with reductions in ABA levels shown following paclobutrazol application to the fungus *Cercospora rosicola* (Norman *et al.*, 1986) and wheat (Buta and Spaulding, 1991). If this was the case in eucalypts, the proposed mechanism would not be effective.

If the effects of pot size and cold treatment are not mediated via effects on GA levels, they may have some influence on other factors affected by depression of

active GA levels by paclobutrazol. Exactly how GAs inhibit flowering in woody angiosperms is still unknown, but given the lack of identification of a specific promotory or inhibitory substance controlling flowering it is tempting to assign a balance between vegetative and reproductive growth (Shearing *et al.*, 1986) as a means of explaining the effects of various cultural and chemical treatments on flowering. Such a nutrient diversion hypothesis, as discussed by Sachs (1977), receives considerable support from observations such as those of Jones *et al.* (1989b) who found a negative correlation between flowering and vegetative growth in apples. In general, treatments to woody angiosperms which promote flowering also inhibit vegetative growth. In the case of the work of this thesis, the three factors which appear to promote precocious flowering (i.e. paclobutrazol application, reduced pot size and outdoor overwintering), at least some of which appear to influence the flowering of reproductively competent material, all reduce vegetative growth. Paclobutrazol application to apple trees has been shown to increase the levels of carbohydrates in all plant parts (Wang *et al.*, 1985), a situation which may make an increased concentration of assimilates available to the plant for production of reproductive structures. However, the many cases in woody angiosperms where cessation or even significant reduction of shoot growth is not a prerequisite to the onset of flowering (eg. Juntilla, 1980) cast doubt upon the robustness of this theory. Similarly, the situation in conifers (Pharis and King, 1985; Pharis *et al.*, 1987) where the application of less polar GAs such as GA_4 and GA_7 can promote both growth and flowering suggests that a nutrient diversion theory on its own is not sufficient to explain the control of flowering. Almost certainly the concentration of assimilates within tissue will have some effect on reproductive activity, but it seems unlikely that this can be the controlling factor in the induction and subsequent initiation and differentiation of floral primordia.

Further evidence which favours a more direct mediation of a flowering response by GAs has been generated by other workers. Spruce trees of different coning ability have been shown to metabolise GAs differently (Moritz *et al.*, 1989) and the use of cultural conditions inducive to flowering have been shown to affect GA metabolism (Dunberg *et al.*, 1983; Moritz *et al.*, 1989), again providing at least circumstantial evidence for a role of GAs in the flowering processes of some tree species. Despite the very many differences between gymnosperms and angiosperms, there are a few studies which indicate the possibility of a

commonality of GA effects on flowering. Pharis and King (1985) cite two cases of exogenous GA_3 application promoting flowering in other woody angiosperms, one of which is a species of the family Myrtaceae, in common with the eucalypt species studied within this thesis. Looney *et al.* (1985) demonstrated a promotion of flowering in apple trees with GA_4 and C-3 epi-GA_4, while the results of Luckwill (1970) suggested that GA_3 may enhance flower bud production in apples at a high dose. There is some doubt regarding the latter piece of evidence however, with the high dose failing to produce a growth response consistent with those resulting from lower doses assessed.

While the role of GAs in flowering is still not clear, the reduction in their levels through the use of paclobutrazol has other effects on plant growth and development in eucalypts. The reduction of internode length (Plate 5.1) has already been mentioned, while effects on leaf size (Plate 5.2), epicormic shoot production (Plate 5.3) and stem size (Plate 5.4) have received some attention from Griffin *et al.* (1993). In particular, the unusual production of epicormic shoots bearing flower buds on paclobutrazol treated *E. globulus* trees noted by other workers (Cauvin, 1991; Griffin *et al.*, 1993) were observed (Plate 5.5). Other effects on the stature of treated trees, which tend to present a characteristic weeping appearance (Plate 5.6) may be associated with the reduction in fibre length and/or the alteration of the proportions of the various xylem elements caused by paclobutrazol treatment (Hasan, unpublished). An apparent loss of apical dominance (Plate 5.7) has also been observed in many cases. Several of these characteristics are worthy of further study.

While the theoretical aspects of the control of flowering in plants in general and forest tree species in particular requires significant further work before a mode of action can be identified, some of the means of producing precocious flowering or increased seed production are already utilised on a routine basis (Owens and Blake, 1985). With respect to eucalypts, the experimental work composing the bulk of this thesis has identified such means, though further work should be done to maximise these practical advantages, thereby increasing the proportion of individuals responsive to treatments to enhance reproductive activity.

First, as paclobutrazol treatment is the mainstay of all successful treatments shown, further work could be performed on application methods. While foliar

spraying has been demonstrated to be effective for inducing precocity in seedling *E. globulus*, the relatively rapid uptake of paclobutrazol following soil drenching (Chapter 3) suggests that this less labour intensive method may be a viable alternative if the appropriate timing of application, which takes into account the possible latency of effect while inhibitor concentration builds, can be established. With regard to the possibility of using a foliar application method on larger trees, the inability of paclobutrazol to penetrate mature bark (Hawkins, 1985 from Lever, 1986) in conjunction with the results of studies which indicate that the majority of effect of foliar applied paclobutrazol is caused by material absorbed by the stems (Barrett and Bartuska, 1982; Lever, 1986), suggests foliar application may be inefficient. In addition, the practical problems associated with applying a foliar spray to large trees render this method inviable unless aerial spraying is used. This would have to be undertaken with care to avoid inadvertent contamination of surrounding land. As the extent of effects on flowering varied for the two paclobutrazol rates used for foliar sprays (Chapter 4), additional experimentation with other doses may establish an optimum rate for a particular situation.

A further alternative application method is trunk injection. This mode of application has the benefits of complete dose assimilation, rapid transport to the likely sites of activity via the transpiration stream and the least environmental contamination of the three application methods discussed. Effects are likely to be more predictable, due to the the first two characteristics and the relatively rapid metabolism within plant material (Chapter 3) tends to produce more transient effects (Moncur *et al.*, 1993; Moncur and Hasan, 1994) though in apple trees reversible binding to vascular tissue has been shown to produce some persistence of growth effects into the season following application (Lever, 1986). While uptake of material into small seedlings may be too slow to be practicable (M. Moncur, *pers. comm.*), it may be ideally suited to applications to larger trees in a breeding arboretum where size precludes foliar spraying and soil drenching would entail application of large quantities of material to the soil where its effects may persist for many years (Chapter 3). The use of newer and more sophisticated injection equipment has significantly increased the feasibility of this method through reducing the time spent on each application (I. Ravenwood, *pers. comm.*).

The other factor related to the application of plant growth retardants to promote early or heavy flowering is the composition of the plant growth retardant itself. While paclobutrazol has proven effective in this study, it is likely that other GA biosynthesis inhibitors may be equally or possibly superior in their suitability for the task. Juntilla (1980) has demonstrated the effectiveness of the quaternary amine, CCC in inducing flower bud formation, while other studies have compared growth control effects of a number of alternative growth retardants and found both their persistence and effectiveness to vary over a considerable range (Steffens, 1988; Curry and Reed, 1989). The plant growth retardant uniconazole (XE-1019 / S-3307), which is structurally very similar to paclobutrazol, was trunk injected into California privet and American sycamore trees and showed less than 9% degradation after 28 days in any tissue (Sterrett, 1988), suggesting higher stability than that shown by paclobutrazol in eucalypts. The plant growth retardant daminozide (Alar / B-995 / SADH) (succinic acid-2-2-dimethylhydrazide) has been shown to increase flower initiation in apples both alone (Jones *et al.*, 1989) and in combination with ethephon (Williams, 1972). Careful assessment of these and other growth retardants may identify a compound with less persistence in the environment than paclobutrazol but with an equal or enhanced ability to stimulate flowering in eucalypts. This would allow a plant breeder a greater freedom of choice of application methods and may even produce a more controllable and predictable response. As stated previously, the use of plant growth retardants which are not known to affect sterol synthesis in such a screening for effectiveness may resolve the possibility that paclobutrazol is having some effect on flowering via an inhibition of this alternative pathway.

In a similar fashion to the optimisation of exogenously applied growth retardant treatments, further investigation into the mechanisms by which reduced pot size and overwintering treatments promote flowering could be performed to make these treatments more effective. The elucidation of their "active factors" may give a more clear indication of the means by which they affect flowering and interact with paclobutrazol treatment. Other treatments demonstrated to enhance flowering in forest and fruit tree species such as girdling, banding, photoperiod effects etc (Owens and Blake, 1985) could be assessed for their effectiveness in enhancing flowering and/or inducing precocious flowering in eucalypt species. After bud

production has been induced, treatments may be altered to speed the maturation of flowers, then capsules. The use of a containerised planting system (Brindbergs *et al.*, 1989) may facilitate such changes in growth environment, especially as trees increase in size. Schuch and Fuchigami (1990) found that application of GA_3 to flower buds of coffee trees reduced the time to anthesis with no effect on fruit set, fresh weight of fruits or vegetative shoot growth. The effectiveness of such treatment in the case of eucalypt buds may be worth assessing, given the extended period of bud maturation in species such as *E. globulus*.

The ideal outcome of any further work would be the arming of eucalypt breeders with a battery of treatments, both cultural and chemical, which could induce precocious flowering and enhance seed production in any seedling or tree which contained potentially advantageous genetic material. A situation where a tree must be selected on the basis of its genetic qualities and its responsiveness to treatments to induce precocious or heavy flowering is definitely not desirable, as further selection criteria late in the selection process could severely limit the number of useful individuals in a breeding programme.

The work described within this thesis should begin to give breeders of some commercial eucalypt species the basis of an ability to increase bud numbers and promote precocious flowering in material with desirable characteristics. Such an ability promises to be highly advantageous, allowing the rapid turnover of generations and hence accelerated incorporation of desirable traits into breeding stock, followed by the rapid production of large quantities of improved seed for plantation stocking. This work has also identified treatments which influence flowering. As the factors which cause the responses observed are extracted, this may eventually lead to an understanding of flowering in this significant genus, which will hopefully contribute to an overall understanding of the regulation of reproductive growth in perrenial, woody angiosperms.

Figure 5.1. A model of the proposed interactions between paclobutrazol treatment, reduced pot size and cold on the magnitude of reproductive responses each flowering season in *E. globulus* and *E. nitens*. The magnitude of a proposed 'flowering signal' is determined by the height of the promoter curve above the flowering threshold. Paclobutrazol treatment is shown to lower the flowering threshold, though it may alternatively act by increasing the magnitude of promotory factors. Under normal overwintering conditions, the promoter curve drawn with a bold line represents the magnitude of promotory factors in material grown with non-limiting soil conditions (N) while the curve drawn with a fine line demonstrates the promotory effect of small pot size (P). A warm overwintering reduces the size of the promoter peak as shown (W), though with increasing age this reduction may become less effective, with flowering eventually occurring. The alternating peak heights are representative of a biennial bearing pattern while the narrow peak widths indicate the strongly seasonal nature of flowering. Note that this model assumes a constant concentration of active paclobutrazol, and the expression of the effects of the promotory factors breaching the flowering threshold are limited by the physical size and structure of the plant, due to the limitation on the number of potential flowering sites these parameters impose.

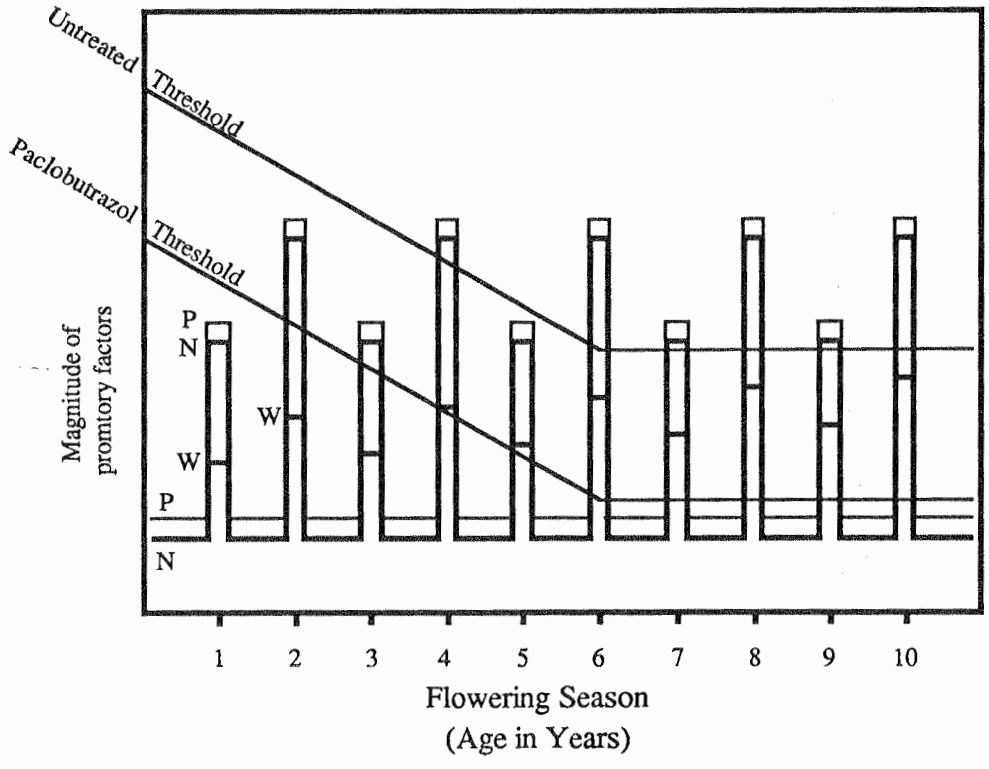


Plate 5.1. The reduction of internode length of *E. nitens* foliage resulting from paclobutrazol treatment. The branch on the left is from an untreated tree while the branch on the right was taken from a tree which received a soil drench of paclobutrazol (2.7 g ai.cm of stem circumference^{-1}) ca. 18 months previously. Branches were taken from the trunk at the same proportion of total tree height in both cases.

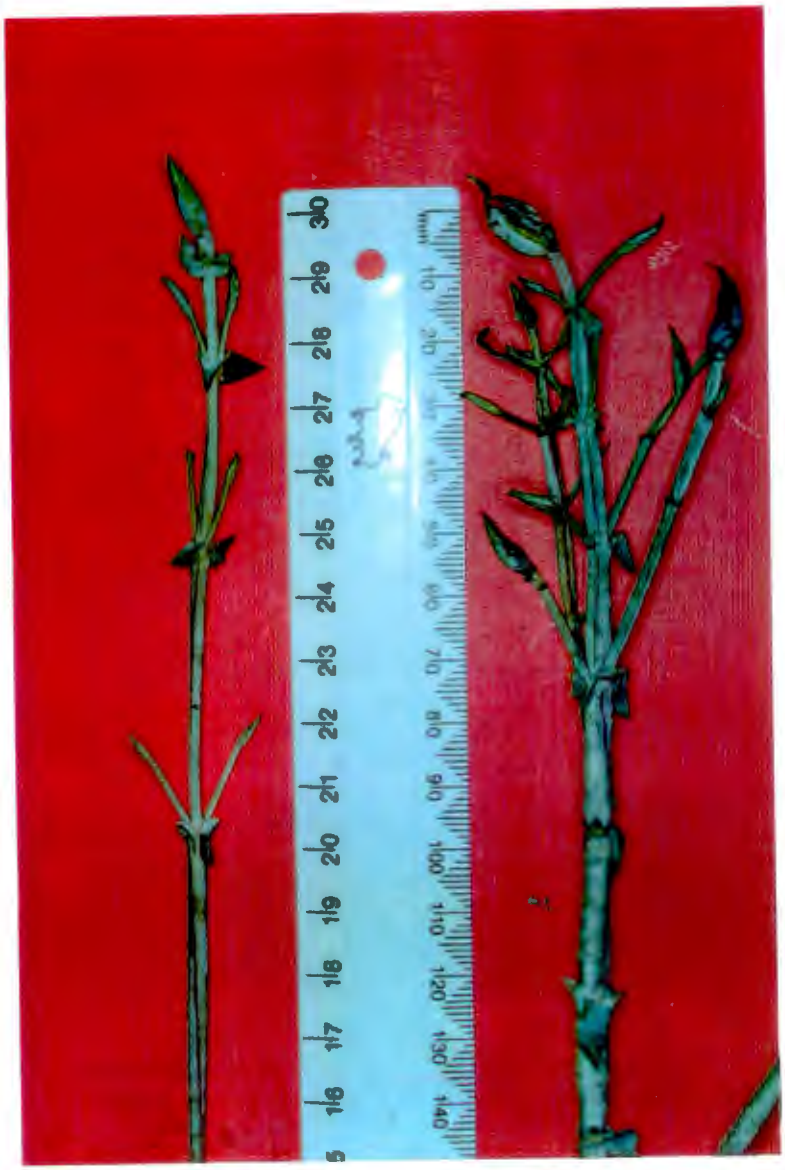


Plate 5.2. The reduction of leaf size in *E. nitens* resulting from paclobutrazol treatment. The branch in the upper panel is from an untreated tree while the branch in the lower panel was taken from a tree which received a soil drench of paclobutrazol (2.7 g ai.cm of stem circumference^{-1}) ca. 18 months previously. Branches were taken from the trunk at the same proportion of total tree height in both cases.

Plate 5.3. Profuse epicormic shoot production in *E. globulus* seedlings caused by a 2x1000 ppm paclobutrazol foliar spray ca. 30 months previously.

Plate 5.4. The reduction of stem diameter in *E. nitens* trees resulting from paclobutrazol treatment. The trunk section in the upper panel is from an untreated tree while the trunk section in the lower panel was taken from a tree which received a soil drench of paclobutrazol (2.7 g ai.cm of stem circumference^{-1}) ca. 18 months previously. Trunk sections were taken from the same proportion of total tree height in both cases.

Plate 5.5. Production of flower buds on epicormic shoots of an *E. globulus* seedling (APPM provenance 733 - see Chapter 4) which received a 2x1000 ppm paclobutrazol foliar spray ca. 30 months previously. The seedling was ca. 7 months of age at the time of paclobutrazol application.

Plate 5.6. The effects of paclobutrazol treatment on branch structure in *E. nitens* seedlings. The tree on the left is indicative of the normal structure of *E. nitens* while the tree on the right is showing the characteristic weeping appearance of a paclobutrazol treated tree (soil drench at 2.7 g ai.cm of stem circumference^{-1} applied ca. 18 months previously).

Plate 5.7. The effects of paclobutrazol treatment on apical dominance in *E. globulus* seedlings. A paclobutrazol foliar spray (2x1000 ppm) at 6 months of age produced the apparent loss of apical dominance observed below.

References

Appleford, N.E.J. and Lenton, J.R. (1991) Gibberellins and leaf expansion in near-isogenic wheat lines containing *Rht1* and *Rht3* dwarfing alleles. Planta **183**: 229-236.

Archbold, D.D. and Houtz, R.L. (1988) Photosynthetic characteristics of strawberry plants treated with paclobutrazol or flurprimidol. Hortscience **23**: 200-202.

Aron, Y., Monselise, S.P., Goren, R. and Costo, J. (1985) Chemical control of vegetative growth in citrus trees by paclobutrazol. Hortscience **20**: 96-98.

Arron, G.P. (1990) Translocation of uniconazole after trunk injection of silver maple saplings. J. Plant Growth Regul. **9**: 141-146.

Ashton, D.H. (1975) Studies of flowering behaviour in *Eucalyptus regnans* F. Muell. Aust. J. Bot. **23**: 399-411.

Atzorn, R. and Weiler, E.W. (1983a) The immunoassay of gibberellins. I. Radioimmunoassay for the gibberellins A_1, A_3, A_4, A_7, A_9, A_{20}. Planta **159**: 1-6.

Atzorn, R. and Weiler, E.W. (1983b) The immunoassay of gibberellins. II. Quantification of GA_3, GA_3 and GA_3 by ultra-sensitive solid-phase enzyme immunoassays. Planta **159**: 7-11.

Barrett, J.E. and Bartuska, C.A. (1982) PP333 effects on stem elongation dependent on site of application. Hortscience **17**: 737-738.

Boland, D.J., Brooker, M.I.H., Turnbull, J.W. and Kleinig, D.A. (1980). '*Eucalyptus* seed.' Division of Forest Research, CSIRO, Canberra.

Bolotin, M. (1975) Photoperiodic induction of precocious flowering in a woody species *Eucalyptus occidentalis* Endl. Bot. Gaz. **136**: 358-365.

Bottini, R., De Bottini, G., Koshioka, M., Pharis, R.P. and Coombe, B.G. (1985) Identification of gibberellins A_1, A_5, A_{29}, and A_{32} from immature seeds of Apricot (*Prunus armeniaca* L.). Plant Physiol. **78**: 417-419.

Brindbergs, M., Borralho, N. and Leal, A.C. (1989). Accelerating flowering in *Eucalyptus In* 'Breeding tropical trees: Population structure and genetic improvement strategies in clonal seedling forestry.' (Eds G.L. Gibson, A.R. Griffin and A.C. Matheson) pp. 459-460. Oxford Forestry Institute, Oxford.

Brown, B.H., Neill, S.J. and Horgan, R. (1986) Partial isotope fractionation during high-performance liquid chromatography of deuterium-labelled internal standards in plant hormone analysis: a cautionary note. Planta **167**: 421-423.

Bukovac, M.J. and Hull, J. (1985). Control of vegetative growth and enhancement of flowering in sour cherry with paclobutrazol and flurprimidol (abstract). HortScience **20**: 595.

Buta, J.G. and Spaulding, D.W. (1991) Effect of paclobutrazol on abscisic acid levels in wheat seedlings. J. Plant Growth Regul. **10**: 59-61.

Cauvin, B. (1984). Les effets du paclobutrazole applicables a la gestion intensive des vergers a hybridation d'eucalyptus *In* 'Proceedings of the IUFRO Int. Conference on Frost Resistant Eucalypts.' pp. 509-523. Bordeaux, France.

Coolbaugh, R.C. (1983). Early stages of gibberellin biosynthesis *In* 'The Biochemistry and Physiology of Gibberellins.' (Ed. A. Crozier) pp. 53-98. Praeger, New York.

Cooper, M.K., Garman, D.E.J. and Nelson, D. (1975) Mass spectrometric method for the determination of the ratio of uranium isotopes using the AEI MS9 -

an organic machine. *In* 'The Australian and New Zealand Society for Mass Spectrometry, Third Conference Programme and Abstracts, January 1975' p. 4B.4. Canberra, Australia.

Cross, B.E. and Myers, P.L. (1969) The effect of plant growth retardants on the biosynthesis of diterpenes by *Gibberella fujikuroi*. Phytochemistry **8**: 79-83.

Curry, E.A. and Williams, M.W. (1983) Promalin or GA_3 increase pedicel and fruit length and leaf size of 'delicious' apples treated with paclobutrazol. Hortscience **18**: 214-215.

Curry, E.A. and Reed, A.N. (1989) Transitory growth control of apple seedlings with less persistent triazole derivatives. J. Plant Growth Regul. **8**: 167-174.

Dalziel, J. and Lawrence, D.K. (1984). Biochemical and biological effects of kaurene oxidase inhibitors, such as paclobutrazol *In* 'Biochemical Aspects of Synthetic and Naturally Occurring Plant Growth Regulators - Monograph 11.' (Eds R. Menhenett and D.K. Lawrence) pp. 43-57. British Plant Growth Regulator Group, Wantage, Oxfordshire, England.

Davies, J.K., Jensen, E., Junttila, O., Rivier, L. and Crozier, A. (1985) Identification of endogenous gibberellins from *Salix pentandra*. Plant Physiol. **78**: 473-476.

Davis, T.D., Sankhla, N., Walser, R.H. and Upadhyaya, A. (1985) Promotion of adventitious root formation on cuttings by paclobutrazol. Hortscience **20**: 883-884.

Dhawan, A.K., Paton, D.M. and Wiling, R.R. (1979) Occurrence and bioassay responses of G. Planta **146**: 419-422.

Doorenbos, J. (1965) Juvenile and adult phases in woody plants. *In* 'Encyclopedia of Plant Physiology' (Ed. W. Ruhland) **15**: 1222-1235.

Douglas, T.J. and Paleg, L.G. (1974) Plant growth retardants as inhibitors of sterol biosynthesis in tobacco seedlings. Plant Physiol. **54**: 238-245.

Dunberg, A., Malmberg, G., Sassa, T. and Pharis, R.P. (1983) Metabolism of tritiated gibberellins A_4 and A_9 in Norway Spruce, *Picea abies* (L.) Karst. Plant Physiol. **71**: 257-262.

Dungey, H.S. (1991). 'Hybridisation as a tree breeding strategy.' Honours Thesis, University of Tasmania.

Early, J.D. and Martin, G.C. (1988) Translocation and breakdown of ^{14}C-labeled paclobutrazol in "Nemaguard" peach seedlings. Hortscience **23**: 196-200.

Evans, L.T., King, R.W., Chu, A., Mander, L.N. and Pharis, R.P. (1990) Gibberellin structure and florigenic activity in *Lolium temulentum*, a long-day plant. Planta **182**: 97-106.

Echols, L.C., Maier, V.P., Poling, S.M. and Sterling, P.R. (1981) New bioregulators of gibberellin biosynthesis in *Gibberella fujikuroi*. Phytochemistry **20**: 433-437.

Fall, R.R. and West, C.A. (1971) Purification and properties of kaurene synthetase from *Fusarium moniliforme*. J. Biol. Chem. **22**: 6913-6928.

Fujioka, S., Yamane, H., Spray, C.R., Gaskin, P., MacMillan, J., Phinney, B.O. and Takahashi, N. (1988) Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1, dwarf-2, dwarf-3,* and *dwarf-5* seedlings of *Zea mays* L. Plant Physiol. **88**: 1367-1372.

Gaskin, P. and MacMillan, J. (1991). 'GC-MS of the gibberellins and related compounds: Methodology and a library of spectra.' Cantock's Enterprises, Bristol.

Gianfagna, T.J. (1987). Natural and synthetic growth regulators and their use in horticultural and agronomic crops *In* 'Plant Hormones and their Role in Growth and Development.' (Ed. P.J. Davies) pp. 614-635. Martinus Nijhoff Publishers, Dordrecht.

Glenn, J.L., Kuo, C.C., Durley, R.C. and Pharis, R.P. (1972) Use of insoluble polyvinylpyrrolidone for purification of plant extracts and chromatography of plant hormones. Phytochemistry **11**: 345-351.

Graebe, J.E. and Ropers, J.J. (1978) Gibberellins. *In* 'Phytohormones and Related Compounds: A Comprehensive Treatise' (Eds D.S. Leatham, P.B. Goodwin and T.J.V. Higgins) pp, 107-173. Elsevier, Amsterdam.

Grattapaglia, D., Chaparro, J., Wilcox, P., McCord, S., Werner, D., Amerson, H., McKeand, S., Bridgwater, F., Whetten, R., O'Malley, D. and Sederoff, R. (1992) Mapping in woody plants with RAPD markers: application to breeding in forestry and horticulture. *In* 'Applications of RAPD Technology to Plant Breeding', Minneapolis, Minnesota. (Crop Science Society of America / American Society for Horticultural Science / American Genetic Association). pp 37-40.

Greene, D.W. (1986) Effect of paclobutrazol and analogs on growth, yield, fruit quality, and storage potential of 'Delicious' apples. J. Amer. Soc. Hort. Sci. **111**: 328-332.

Griffin, A.R. (1989). Sexual reproduction and tree improvement strategy- with particular reference to *Eucalyptus In* 'Breeding tropical trees: Population structure and genetic improvement strategies in clonal seedling forestry.' (Eds G.L. Gibson, A.R. Griffin and A.C. Matheson) pp. 52-67. Oxford Forestry Institute, Oxford.

Griffin, A.R. (1989a). Strategies for the genetic improvement of yield in *Eucalyptus In* 'Biomass production by fast-growing trees.' (Eds J.S. Pereira

and J.J. Landsberg) pp. 247-265. Kluwer Academic Publishers, Dordrecht.

Griffin, A.R., Whiteman, P., Rudge, T., Burgess, I.P. and Moncur, M. (1993) Effect of paclobutrazol on flower-bud production and vegetative growth in two species of *Eucalyptus*. Can. J. For. Res. **23**: 640-647.

Griggs, D.L., Hedden, P., Temple-Smith, K.E. and Rademacher, W. (1991) Inhibition of gibberellin 2ß-hydroxylases by acylcyclohexanedione derivatives. Phytochemistry **30**: 2513-2517.

Grossman, K., Weiler, E.W. and Jung, J. (1985) Effects of different sterols on the inhibition of cell culture growth caused by the growth retardant tetcyclacis. Planta **164**: 370-375.

Grossmann, K. (1990) Plant growth retardants as tools in physiological research. Physiol. Plant. **78**: 640-648.

Hackett, W.P. and Hartman, H.T. (1963) Morphological development of buds of olive as related to low-temperature requirement for inflorescence formation. Bot. Gaz. **124**: 383-387.

Hansche, P.E. (1986) Heritability of juvenility in peach. Hortscience **21**: 1197-1198.

Hasan, O., Potts, B.M. and Reid, J.B. (1992). Chemical promotion of flower bud initiation in *E. globulus In* 'Proceedings of a Symposium on Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species.' (Eds J.N. Marnier, C.L. Hubert and P. Alazard) pp. 444. AFOCEL/IUFRO, Bordeaux.

Hasan, O., Ridoutt, B.G., Ross, J.J., Davies, N.W. and Reid, J.B. (1994) Identification and quantification of endogenous gibberellins in apical buds and the cambial region of Eucalyptus. Physiol. Plant. Physiol. Plant. (in press).

Haughan, P.A., Burden, R.S., Lenton, J.R. and Goad, L.J. (1989) Inhibition of celery cell growth and sterol biosynthesis by the enantiomers of paclobutrazol. Phytochem. **28**: 781-787.

Häuser, C., Kwiatowski, J., Rademacher, W. and Grossman, K. (1990) Regulation of endogenous abscisic acid levels and transpiration in oilseed rape by plant growth retardants. J. Plant Physiol. **137**: 201-207.

Hedden, P. and Graebe, J.E. (1985) Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm and *Malus pumila* embryos. Journal of Plant Growth and Regulation **44**: 111-122.

Hedden, P. (1987). Gibberellins *In* 'The principles and practice of plant hormone analysis.' (Eds L. Rivier and A. Crozier) pp. 19-67. Academic Press., London.

Hedden, P. and Croker, S.J. (1990). GC-MS analysis of gibberellins in plant tissues *In* 'Molecular Aspects of hormonal Regulation of Plant Development.' (Eds M. Kutácek, M.C. Elliot and I. Machácková) pp. 19-30. Academic Publishing, The Hague.

Hedden, P. (1990). The action of plant growth retardants at the biochemical level *In* 'Plant Growth Substances 1988.' (Eds R.P. Pharis and S.B. Rood) pp. 322-332. Springer-Verlag, Berlin, Heidelberg, Germany.

Hedden, P. (1993) Modern methods for quantitative analysis of plant hormones. Ann. Rev. Plant Physiol. Plant. Mol. Biol. **44**: 107-129.

Hetherington, S. and Jones, K.M. (1990) Effectiveness of paclobutrazol in retarding height growth of *Eucalyptus globulus* seedlings. Can. J. For. Res. **20**: 1811-1813.

Hetherington, S., Jones, K.M. and Koen, T.B. (1991). Stimulation of bud

production in *Eucalypt globulus* by paclobutrazol application *In* 'Intensive Forestry: The Role of Eucalypts.' (Ed. A.P.G. Schonau) pp. 104-109. IUFRO, Durban, South Africa.

Hetherington, S. and Jones, K.M. (1993) Seed stimulation in *Eucalyptus* by paclobutrazol application. Proc. Int. Plant Prop. Society. (in press).

Hoad, G.V. (1983). Gibberellin bioassay and structure-activity relationships *In* 'The Biochemistry and Physiology of Gibberellins.' (Ed. A. Crozier) pp. 57-94. Praeger, New York.

Horrell, B.A., Jameson, P.E. and Bannister, P. (1989) Growth promotion of ivy (*Hedera helix* L.) by paclobutrazol. Plant Growth Regulation **8**: 309-314.

Horrell, B.A., Jameson, P.E. and Bannister, P. (1990) Responses of ivy (*Hedera helix* L.) to combinations of gibberellic acid, paclobutrazol and abscisic acid. Plant Growth Regulation **9**: 107-117.

Izumi, K., Kamiya, Y., Sakuri, A., Oshio, H. and Takahashi, N. (1985) Studies of sites of action of a new plant growth retardant (e)-1-(4-Chlorophenyl) - 4, 4-dimethyl-2-(1, 2, 4-triazol-1-yl)-1-penten-3-ol (S-3307) and comparative effects of its stereoisomers in a cell-free system from *Cucurbita maxima*. Plant Cell Physiology **26**: 821-827.

Jackson, D.I. and Sweet, G.B. (1972) Flower initiation in temperate woody plants- a review based largely on the literature of conifers and deciduous fruit trees. Hortic. Abs. **42**: 9-24.

Jackson, J.E. (1989) The manipulation of fruiting. *In* 'Manipulation of Fruiting' (Ed. C.J. Wright) pp. 3-12. Butterworths, London.

Jensen, E., Crozier, A. and Monteiro, A.M. (1986) Analysis of gibberellins and gibberellin conjugates by ion-suppression reversed-phase high-performance liquid

chromatography. J. Chromatography **367**: 377-384.

Jones, K.M., Koen, T.B., Oakford, M.J. and Longley, S.J. (1989a) Using ethephon and daminozide to regulate growth and initiate flower buds on bearing red delicious trees. Acta Hortic. **240**: 185-188.

Jones, K.M., Koen, T.B., Wilson, S.J., Oakford, M.J. and Longley, S.J. (1989b) A re-examination of the relationship between vegetative growth and flower bud initiation in apples. Acta Hortic. **239**: 363-366.

Junttila, O. (1980) Flower bud differentiation in *Salix pentandra* as affected by photoperiod, temperature and growth regulators. Physiol. Plant **49**: 127-134.

Junttila, O., Abe, H. and Pharis, R.P. (1988) Endogenous gibberellins in elongating shoots of clones of *Salix dasyclados* and *Salix viminalis*. Plant Physiol. **87**: 781-784.

Junttila, O., Jensen, E. and Ernstsen, A. (1991) Effects of prohexadione (BX-112) and gibberellins on shoot growth in seedlings of *Salix pentandra*. Physiol. Plant. **83**: 17-21.

King, R.W., Evans, L.T., Mander, L.N. and Pharis, R.P. (1993) Gibberellin structure and florigenic activity in *Lolium temulentum* long-day plant. *In* 'Frontiers of Gibberellin Research, Program and Abstracts, Wako-shi, Japan.

King and Pharis (1993) Searching for florigen(s): gibberellins and other growth regulators. *In* 'XV International Botanical Congress, Abstracts' pp. 143 Yokohama, Japan.

Koller, W. (1987) Plant growth regulator activities of stereochemical isomers of triadimenol. Physiol. Plant. **71**: 309-315.

Koshioka, M., Taylor, J.S., Edwards, G.R., Looney, N.E. and Pharis, R.P.

(1985) Identification of gibberellin A19 and A20 in vegetative tissue of apple. Agric. Biol. Chem (Tokyo) **49**: 1223-1226.

Kurasowa, E. (1926) Experimental studies on the nature of the substances excreted by the "Bakanae" fungus. Trans. Nat. His. Soc. Formosa. **16**: 213-227.

Lang, A. (1965) Physiology of flower initiation. *In* 'Encyclopedia of Plant Physiology' (Ed. W. Ruhland) **15**: 1380-1536.

Lange, T., Hedden, P. and Graebe, J.E. (1993) Molecular cloning and expression of a gibberellin C-20 oxidase gene from pumpkin. *In* 'XV International Botanical Congress', Japan.

Lawrence, N.L., Ross, J.J., Mander, L.N. and Reid, J.B. (1992) Internode length in *Pisum*. Mutants *lk, lka* and *lkb* do not accumulate gibberellins. Journal of Plant Growth Regulation **11**: 35-38.

Lever, B.G. (1986) 'Cultar'- a technical overview. Acta Hortic. **179**: 459-466.

Lin, J.-T. and Stafford, A.E. (1988) Reversed phase C_{18} and normal-phase silica high performance liquid chromatography of gibberellins and their methyl esters. J. Chromatography **452**: 519-525.

Longman, K.A. and Wareing, P.F. (1959) Early induction of flowering in birch seedlings. Nature **184**: 2037-2038.

Looney, N.E., Pharis, R.P. and Noma, M. (1985) Promotion of flowering in apple trees with gibberellin A_4 and C-3 epi-gibberellin A_4. Planta **165**: 292-294.

Luckwill, L.S. (1970). The control of growth and fruitfulness of apple trees *In* 'Physiology of Tree Crops.' (Eds L.S. Luckwill and C.V. Cutting) pp. 237-254. Academic Press, London.

Marini, R.P. (1986) Growth and cropping of 'Redhaven' peach trees following foliar applications of flurprimidol and paclobutrazol. J. Amer. Soc. Hort. Sci. **111**: 849-853.

Mauk, C.S., Bausher, M.G. and Yelenosky, G. (1986) Influence of growth regulator treatments on dry matter production, fruit abscission, and ^{14}C-assimilate partitioning in citrus. J. Plant Growth Regul. **5**: 111-120.

Mauk, C.S., Unrath, C.R., Blankenship, S.M. and Lehman, L.J. (1990a) Influence of method of application of paclobutrazol on soil residues and growth retardation in a "Starkrimson-Delicious" apple orchard. Plant Growth Regul. **9**: 27-35.

Mauk, C.S., Unrath, R.C. and Blankenship, S.M. (1990b) Rapid quantitative analysis of a gibberellin-sterol inhibitor using high-performance liquid chromatographic cartridge columns. J. Plant Growth Regul. **9**: 181-184.

Menhenett, R. (1981) Interactions of the growth retardants daminozide and piproctanyl bromide, and gibberellins A_1, A_3, A_{4-7}, A_5 and A_{13} in stem extension and inflorescence development in *Chrysanthemum morifolium* Ramat. Ann. Bot. **47**: 359-369.

Metzger, J.D. and Mardaus, M.C. (1986) Identification of endogenous gibberellins in the winter annual weed *Thlaspi arvense* L. Plant Physiol. **80**: 396-402.

Metzger, J.D. (1990) Vernalization, gibberellins, and thermoinduced stem growth in *Thlaspi arvense* L. British Society for Plant Growth Regulation, Monographs **20**: 69-83.

Michelmore, R.W., Paran, I. and Kessell, R.V. (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to

detect markers in specific genomic regions using segregating populations. Proc. Natl. Acad. Sci. **88**: 9828-9832.

Miki, T., Ichikawa, T., Kamiya, Y., Kobayashi, M. and Sakurai, A. (1991) Studies on Sites of Action of the Plant Growth Retardant 4'-Chloro-2'-(α-Hydroxybenzyl)isonicotinanilide (Inabenfide) in Gibberelin Biosynthesis *In* 'Gibberellins' (Eds N. Takahashi, B. Phinney and J. MacMillan) pp. 311-319. Springer-Verlag, New York.

Moncur, M.W. and Boland, D.J. (1989) Floral morphology of *Eucalyptus melliodora* A. Cunn. ex Schau. and comparisons with other eucalypt species. Aust. J. Bot. **37**: 125-135.

Moncur, M.W. (1992) Effect of low temperature on floral induction of *Eucalyptus lansdowneana* F. Muell. & J. Brown subsp. *lansdowneana*. Aust. J. Bot. **40**: 157-167.

Moncur, M.W., Rasmussen, G.F. and Hasan, O. (1993) Effect of paclobutrazol on flower-bud production in *Eucalyptus nitens* (Deane & Maiden) Maiden espalier seed orchards. Can. J. For. Res. (in press).

Moncur, M.W. and Hasan, O. (1994) Floral induction in *Eucalyptus nitens* (Deane & Maiden) Maiden. Tree Physiology (under consideration).

Moritz, T., Philipson, J.J. and Odén, P.C. (1989) Detection and identification of gibberellins in Sitka spruce (*Picea sitchensis*) of different ages and coning ability by bioassay, radioimmunoassay and gas chromatography-mass spectrometry. Physiol. Plant. **75**: 325-332.

Moritz, T., Philipson, J.J. and Odén, P.C. (1990) Quantification of gibberellins A_1, A_3, A_4, A_9, and an A_9-conjugate in good- and poor-flowering clones of Sitka spruce (*Picea sitchensis*) during the period of flower-bud differentiation. Planta **181**: 538-542.

Mullins, M.G. and Snowball, A.M. (1988). Speeding the generations in citrus breeding *In* 'Citrus Breeding Workshop.' (Ed. R.R. Walker) pp. 101-110. CSIRO, Canberra.

Murakami, Y. (1968) A new rice seedling bioassay for gibberellins, "microdrop method" and its use for testing extracts of rice and morning glory. Bot. Mag. **81**: 33-43.

Nakajima, M., Yamaguchi, I., Kizawa, S., Murofushi, N. and Takahashi, N. (1991) Preparation and validation of an antiserum specific for non-derivatised gibberellins. Plant Cell Physiol. **32**: 505-510.

Nakayama, I. (1989) Mode of action of novel plant growth regulators, dioxocylohexanecarboxylic acid derivatives. *In* 'Gibberellin Symposium', Tokyo.

Nakayama, I., Kobayashi, M., Kamiya, Y., Abe, H. and Sakurai, A. (1992) Effects of a plant-growth regulator, prohexadione-calcium (BX-112), on the endogenous levels of gibberellins in rice. Plant Cell Physiology **33**: 59-62.

Nitsche, K., Grossmann, K., Sauerbrey, E. and Jung, J. (1985) Influence of the growth retardant tetcyclacis on cell division and cell elongation in plants and cell cultures of sunflower, soybean, and maize. J. Plant Physiol. **118**: 209-218.

Norman, S.M., Bennett, R.D., Poling, S.M., Maier, V.P. and Nelson, M.D. (1986) Paclobutrazol inhibits abscisic acid biosynthesis in *Cercospora rosicola.* Plant Physiol. **80**: 122-125.

Nutbeam, A.R. and Briggs, D.E. (1982) Gibberellin-phenol interactions in plant extracts. Phytochemistry **21**: 2217-2223.

Odén, P.C., Weiler, E.W., Schwenen, L. and Graebe, J.E. (1987) Comparison

of gas chromatography-mass spectrometry, radioimmunoassay and bioassay for the quantification of gibberellin A_9 in Norway Spruce (*Picea abies* (L.) Karst.). Planta **171**: 212-219.

Oliveira, C.M. and Browning, G. (1993) Gibberellin structure-activity effects on flower initiation in mature trees and on shoot growth in mature and juvenile *Prunus avium*. Plant Growth Regul. **13**: 55-63.

Owens, J.N. and Blake, M.D. (1985). 'Forest Tree Seed Production.' Canadian Forestry Service, Ontario, Canada.

Paterson, A.H., Tanksley, S.D. and Sorrells, M.E. (1991) DNA markers in plant improvement. Advances in Agronomy **46**: 39-90.

Paton, D.M., Dhawan, A.K. and Willing, R.R. (1980) Effect of *Eucalyptus* growth regulators on the water loss from plant leaves. Plant Physiol. **66**: 254-256.

Paton, D.M., Willing, R.R. and Pryor, L.D. (1981) Root-shoot gradients in *Eucalyptus* ontogeny. Ann. Bot. **47**: 835-838.

Pharis, R.P. and King, R.W. (1985) Gibberellins and reproductive development in seed plants. Annu. Rev. Plant Physiol. **36**: 517-568.

Pharis, R.P., Webber, J.E. and Ross, S.D. (1987) The promotion of flowering in forest trees by gibberellin $A_{4/7}$ and cultural treatments: a review of the possible mechanisms. For. Ecol. Manage. **19**: 65-84.

Phinney, B.O. (1984). Gibberellin A_1, dwarfism and the control of shoot elongation in higher plants *In* 'The biosynthesis and metabolism of plant hormones.' (Eds A. Crozier and J.R. Hillman) pp. 17-45. Cambridge University Press, New York.

Potts, B.M. and Marsden-Smedley, J.B. (1989) *In vitro* germination of

Eucalyptus pollen: response to variation in boric acid and sucrose. Aust. J. Bot. **37**: 429-441.

Pryor, L.D. (1966) Ornamental Eucalypts. Breeding to secure early flowering in *Eucalyptus*. Australian Plants- Eucalypts **3**: 357-358.

Rademacher, W., Jung, J., Graebe, J.E. and Schwenen, L. (1984). On the mode of action of tetcyclacis and triazole growth retardants *In* 'Biochemical Aspects of Synthetic and Naturally Occurring Plant Growth Regulators - Monograph 11.' (Eds R. Menhenett and D.K. Lawrence) pp. 1-11. British Plant Growth Regulator Group, Wantage, Oxfordshire, England.

Rademacher, W. (1991). Biochemical effects of plant growth retardants *In* 'Plant Biochemical Regulators.' (Ed. H.W. Gausman) pp. 169-200. Marcel Dekker, Inc., New York.

Rademacher, W. (1991a) Inhibitors of gibberellin biosynthesis: applications in agriculture and horticulture. *In* 'Gibberellin Symposium: Program and Abstracts', Tokyo.

Rademacher, W., Temple-Smith, K.E., Griggs, D.L. and Hedden, P. (1992). The mode of action of acylcyclohexanediones - a new type of growth retardant *In* 'Progress in Plant Growth Regulation: proceedings of the 14th International Conference on Plant Growth Substances, Amsterdam, 21-26 July, 1991.' (Eds C.M. Karssen, L.C. Van Loon and D. Vreugdenhil) pp. 571-577. Kluwer Academic Publishers, Dordrecht.

Raese, J.T. and Burts, E.C. (1983) Increased yield and suppression of shoot growth and mite populations of 'd' Anjou' pear trees with nitrogen and paclobutrazol. Hortscience **18**: 212-214.

Reid, J.B. (1986). Gibberellin mutants *In* 'Plant Gene Research.' (Eds A.D. Blonstein and P.J. King) pp. 1-34. Springer-Verlag, Wien.

Reid, J.B. (1990) Phytohormone mutants in plant research. J. of Plant Growth Regul. **9**: 97-111.

Reid, J.B., Hasan, O. and Ross, J.J. (1990) Internode length in *Pisum*. Gibberellins and the response to far-red-rich light. Plant Physiol. **137**: 46-52.

Reid, M.S. (1987). Ethylene in plant growth, development, and senescence *In* 'Plant Hormones and their Role in Growth and Development.' (Ed. P.J. Davies) pp. 257-279. Martinus Nijhoff Publishers, Dordrecht.

Rood, S.B., Larsen, K.M., Mander, L.N., Abe, H. and Pharis, R.P. (1986) Identification of endogenous gibberellins from *Sorghum*. Plant Physiol. **82**: 330-332.

Sachs, R.M. (1977) Nutrient diversion: an hypothesis to explain the chemical control of flowering. Hortscience **12**: 220-222.

SAS/GRAPH (1988). 'SAS Institute Inc. SAS/GRAPH User's Guide, Release 6.03 Edition.' SAS Institute Inc., Cary.

Schneider, G. and Schmidt, J. (1990). Conjugation of gibberellins in *Zea mays* *In* 'Plant Growth Substances 1988.' (Eds R.P. Pharis and S.B. Rood) pp. 300-306. Springer-Verlag, Berlin.

Schuch, U.K., Fuchigami, L.H. and Nagao, M.A. (1990) Gibberellic acid causes earlier flowering and synchronises fruit ripening of coffee. Plant Growth Regul. **9**: 59-64.

See, R.M. and Foy, C.L. (1982) Effect of butanedioic acid mono (2,2-dimethylhydrazide) on the activity of membrane-bound succinate dehydrogenase. Plant Physiol. **70**: 350-352.

Sembdner, G., Hermann, G. and Schliemann, W. (1992) Growth. Progress in Botany **53**: 99-125.

Sharkey, T.D., Stevenson, G.F. and Paton, D.M. (1982) Effects of G, a growth regulator from *Eucalyptus grandis,* on photosynthesis. Plant Physiol. **69**: 935-938.

Shearing, S.J., Quinlan, J.D. and Webster, A.D. (1986) The management of orchard crops using paclobutrazol. Acta Hortic. **160**: 185-188.

Snowball, A.M., Mullins, M.G., Warrington, I.J. and Goodwin, P.B. (1990) Shortening the juvenile period in citrus seedlings. *In* 'Australian Society of Plant Physiologists- thirtieth annual general meeting', Sydney.

Sponsel, V.M. (1983b) The localization, metabolism and biological activity of gibberellins in maturing and germinating seeds of *Pisum sativum* cv. Progress No. 9. Planta **159**: 454-468.

Steffens, G.L., Byun, J.K. and Wang, S.Y. (1985) Controlling plant growth via the gibberellin biosynthesis system- I. Growth parameter alterations in apple seedlings. Physiol. Plant. **63**: 163-168.

Steffens, G.L. (1988) Gibberellin biosynthesis inhibitors: comparing growth-retarding effectiveness on apple. J. Plant Growth Regul. **7**: 27-36.

Steffens, G.L., Stafford, A.E. and Lin, J.T. (1991) Influence of an inhibitor of gibberellin biosynthesis, paclobutrazol, on apple seed gibberellin content. Physiol. Plant. **83**: 366-372.

Sterrett, J.P. (1985) Paclobutrazol: A promising growth inhibitor for injection into woody plants. J. Amer. Soc. Hort. Sci. **110**: 4-8.

Sterrett, J.P. (1988) XE-1019: Plant responses, translocation, and metabolism. J.

Plant Growth Regul. **7**: 19-26.

Sterrett, J.P. (1990) Translocation and degradation of injected uniconazole in apple during a 4-month growing period. J. Plant Growth Regul. **9**: 147-150.

Strauss, S.H., Lande, R. and Namkoong, G. (1992) Limitations of molecular-marker-aided selection in forest tree breeding. Can. J. For. Res. **22**: 1050-1061.

Sugavanam, B. (1984) Diastereoisomers and enantiomers of paclobutrazol: Their preparation and biological activity. Pest. Sci. **15**: 296-302.

Swain, S.M. (1993). 'Gibberellins and seed development in *Pisum*.' Ph.D. Thesis, University of Tasmania.

Sykes, S.R. (1988). Application of isozyme techniques to citrus breeding *In* 'Citrus Breeding Workshop.' (Ed. R.R. Walker) pp. 111-119. CSIRO, Canberra.

Takeno, K., Legge, R.L. and Pharis, R.P. (1981) Effect of the growth retardant B-9 (SADH) on endogenous GA level, and the transport and conversion of exogenously applied [3H] GA_{20} in Alaska Pea. Plant Physiol. **67** (supplement): 103.

Talón, M., Hedden, P. and Primo-Millo, E. (1990a) Gibberellins in *Citrus sinensis:* A comparison between seeded and seedless varieties. J. Plant Growth Regul. **9**: 201-206.

Talón, M., Koornneef, M. and Zeevaart, J.A.D. (1990b) Endogenous gibberellins in *Arabidopsis thaliana* and possible steps blocked in the biosynthetic pathways of the semidwarf *ga4* and *ga5* mutants. Proc. Natl. Acad. Sci. **87**: 7983-7987.

Taylor, J.S., Blake, T.J. and Pharis, R.P. (1982) The role of plant hormones in the growth and survival of coppiced *Eucalyptus* seedlings. Physiol. Plant. **55**: 407-485.

Tibbits, W.N. (1986) Eucalypt plantations in Tasmania. Aust. For. **49**: 219-225.

Tukey, L.D. (1981). The growth regulator PP333 on apples *In* 'Abstracts of contributed papers: Seventy-eighth annual meeting American Society for Horticultural Science.' Atlanta, Georgia.

Turnbull, C.G.N., Crozier, A., Schwenen, L. and Graebe, J.E. (1985) Conversion of [^{14}C]gibberellin A_{12}-aldehyde to C_{19}- and C_{20}-gibberellins in a cell-free system from immature seed of *Phaseolus coccineus* L. Planta **165**: 108-113.

Turnbull, C.G.N. (1989) Identification and quantitative analysis of gibberellins in *Citrus*. J. Plant Growth Regul. **8**: 273-282.

Turnbull, J.W. and Pryor, L.D. (1978). Choices of species and seed sources *In* 'Eucalypts for wood production.' (Eds W.E. Hillis and A.G. Brown) pp. 6-65. CSIRO, Adelaide.

Upadhyaya, A., Davis, T.D., Larsen, M.H., Walser, R.H. and Sankhla, N. (1990) Uniconazole-induced thermotolerance in soybean seedling root tissue. Physiol. Plant. **79**: 78-84.

Walser, R.H. (1990) Reproductive development and dormancy characteristics of paclobutrazol-treated tart cherry trees. *In* 'Australian Society of Plant Physiologists- thirtieth annual general meeting', Sydney.

Wang, S.Y., Byun, J.K. and Steffens, G.L. (1985) Controlling plant growth via the gibberellin biosynthesis system- II. Biochemical and physical alterations in apple seedlings. Physiol. Plant. **63**: 169-175.

Wang, S.Y., Sun, T. and Faust, M. (1986) Translocation of paclobutrazol, a

gibberellin biosynthesis inhibitor, in apple seedlings. Plant Physiol. **82**: 11-14.

Warville, J.W. and Mitchell, J.W. (1950) Six new plant-growth inhibiting compounds. Bot. Gaz. **111**: 491-494.

Williams, M.W. (1972) Induction of spur and flower bud formation in young apple trees with chemical growth retardants. J. Amer. Soc. Hort. Sci. **97**: 210-212.

Williams, M.W. (1984) Use of bioregulators to control vegetative growth of fruit trees and improve fruiting efficiency. Acta Hort. **146**: 97-104.

Wodzicki, T.J., Abe, H., Wodzicki, A.B., Pharis, R.P. and Cohen, J.D. (1987) Investigations on the nature of the auxin-wave in the cambial region of pine stems. Plant Physiol. **84**: 135-143.

Wright, S.T.C. and Hiron, R.W.P. (1972). The accumulation of abscisic acid in plants during wilting and under other stress conditions *In* 'Plant Growth Substances 1970.' (Ed. D.J. Carr) pp. 291-298. Springer-Verlag, Berlin.

Yadava, U.L. and Lockard, R.G. (1977) Abscisic acid and gibberellin in three ungrafted apple (*Malus sylvestris*) rootstock clones. Physiol. Plant. **40**: 225-229.

Yamane, H., Yamaguchi, I., Kobayashi, M., Takahashi, M., Sato, Y., Takahashi, N., Iwatsuki, K., Phinney, B.O., Spray, C.R., Gaskin, P. and MacMillan, J. (1985) Identification of ten gibberellins from sporophytes of the tree fern, *Cyathea australis*. Plant Physiol. **78**: 899-903.

Zacharin, R.F. (1978). 'Emigrant Eucalypts.' Melbourne University Press, Melbourne.

Zeevaart, J.A.D. (1983). Gibberellins and Flowering *In* 'The Biochemistry and Physiology of Gibberellins.' (Ed. A. Crozier) pp. 333-374. Praeger Publishers,

New York.

Zeevaart, J.A.D. (1985) Inhibition of stem growth and gibberellin production in *Agrostemma githago* L. by the growth retardant tetcyclacis. Planta **166**: 276-279.

Zeevaart, J.A.D., Gage, D.A. and Creelman, R.A. (1990). Recent studies of the metabolism of abscisic acid *In* 'Plant Growth Substances 1988.' (Eds R.P. Pharis and S.B. Rood) pp. 233-240. Springer-Verlag, Berlin.

Appendix A

Hasan *et al.* (1994)

This paper has been accepted for publication by Physiologia Plantarum.

Identification and quantification of endogenous gibberellins in apical buds and the cambial region of Eucalyptus

Corresponding Author: J. B. Reid

Address for Correspondence: Cooperative Research Centre for Temperate Hardwood Forestry and Department of Plant Science, The University of Tasmania, Hobart, Tasmania 7001, Australia

Fax: +61 02 202698

Identification and quantification of endogenous gibberellins in apical buds and the cambial region of Eucalyptus

O. Hasan, B.G. Ridoutt, J.J. Ross, N.W. Davies and J.B. Reid

Hasan, O., Ridoutt, B.G., Ross, J.J., Davies, N.W. and Reid, J.B. 1994. Identification and quantification of endogenous gibberellins in apical buds and the cambial region of Eucalyptus - Physiol. Plant. 00: 000-000.

Endogenous gibberellins (GAs) were extracted and purified from apical buds of Eucalyptus nitens (Deane and Maid.) Maid. and the cambial region of E. globulus (Labill.), then analysed by capillary gas chromatography-mass spectrometry. GA_1, GA_{19}, GA_{20} and GA_{29} were identified by full scan mass spectra, Kovat's retention indices and high resolution selected ion monitoring. Using deuterated internal standards, GA_1, GA_{19}, GA_{20} and putative GA_{29} and GA_{53} were quantified in the apical buds, while GA_4, GA_8, GA_9 and GA_{44} were shown to be either absent or present at very low levels. From the cambial region, GA_1 and GA_{20} were quantified at levels of 0.30 ng [g fresh weight]$^{-1}$ and 8.8 ng [g fresh weight]$^{-1}$ respectively. These data suggest that the early 13-hydroxylation pathway is the dominant pathway for GA biosynthesis in Eucalyptus.

Key words- Eucalyptus globulus, Eucalyptus nitens, Gibberellin A_1, A_4, A_8, A_9, A_{19}, A_{20}, A_{29}, A_{44}, A_{53}, gibberellin biosynthesis, high resolution selected ion monitoring, vascular cambium.

O. Hasan and J.B. Reid (corresponding author), Cooperative Research Centre for Temperate Hardwood Forestry and Department of Plant Science, The University of Tasmania, Hobart, Tasmania 7001, Australia; B.G. Ridoutt, Cooperative Research Centre for Hardwood Fibre and Paper Science, School of Forestry, The University of Melbourne, Creswick, Victoria 3363, Australia; J.J. Ross, Department of Plant Science, N.W. Davies, Central Science Laboratory, The University of Tasmania, Hobart, Tasmania 7001, Australia

Introduction

Eucalypt forests are important for the manufacture of pulp and paper products and are the most extensively planted of all hardwood genera. As a consequence, there is interest in understanding the mechanisms that control important developmental processes that would enable the quantity and quality of wood produced to be increased. One example is the need to reduce the relatively long generation time of eucalypts and thereby accelerate the rate at which breeding programs can provide genetically improved planting stock. The plant growth regulator paclobutrazol has been observed to increase the fruit and seed output of E. globulus (Hetherington et al. 1991, Hetherington and Jones 1993) and E. nitens (Hetherington and Jones 1993, Moncur et al. 1993) as well as reduce the time taken for the first seed crop to be produced (Hasan et al. 1992). Paclobutrazol is known to reduce the level of endogenous gibberellins (GAs) in many plants (eg. Hedden and Graebe 1985) and in whole plants this is usually obvious by a reduction in both the internode length and overall plant stature, as shown for E. globulus by Hetherington and Jones (1990).

A second example is the need to determine the mechanisms responsible for producing variability in fibre development such that fibre properties might be manipulated

by silvicultural or genetic management. Ridoutt and Sands (1993) have demonstrated that the elongation of secondary xylem fibres that occurs during differentiation, increases with decreasing height within the stem of E. globulus. Aloni (1992) has suggested that gradients in secondary fibre development along the plant axis are related to gradients in plant growth regulator levels, namely GA and indole acetic acid (IAA). Application experiments have also demonstrated that fibre length can be manipulated with GA and IAA (Little and Savidge 1987).

To date there are no accounts of the isolation, identification or quantification of endogenous GAs from any species of the genus Eucalyptus or from the cambial region of any hardwood species. The aim of this study was therefore to determine which GAs are present in apical buds and the cambial region of E. nitens and E. globulus and to use these identifications to ascertain the dominant pathway for GA biosynthesis. This is a necessary prerequisite for monitoring the effects of GA biosynthesis inhibitors on the levels of active GAs, and for determining the involvement of GAs in vegetative and reproductive development.

Abbreviations- KRI, Kovat's retention index; Me, methyl; SIM, selected ion monitoring; HR-SIM, high resolution selected ion monitoring; LR-SIM, low resolution selected ion monitoring; TMS, trimethylsilyl.

Materials and methods

Extraction from plant material

For extraction of GAs from E. nitens apical buds, harvests of approximately 10-15 g of actively growing apical material, including both stem and unexpanded leaf tissue, were

removed from approximately 1-year-old glasshouse-grown seedlings, transferred to cold (-20°C) methanol at the rate of 5 ml [g fresh weight]$^{-1}$ and homogenised with a rotary cutter. To samples used for quantification, 30 ng of [17, 17-$^{2}H_2$] GA_1, GA_4, GA_8, GA_9, GA_{19}, GA_{20}, GA_{29} GA_{53} and [20-$^{2}H_1$] GA_{44} (supplied by Prof. L. Mander, Research School of Chemistry, Australian Natl. Univ., Canberra, Australia) were added as internal standards. To samples used for both quantification and identification, approximately 670 Bq of [^{3}H] GA_1 (1.39 PBq mol^{-1}), GA_{20} (1.11 PBq mol^{-1}), GA_4 (1.19 PBq mol^{-1}) (Amersham International, Little Chalfort, UK) and GA_9 (supplied by Prof. R.P. Pharis, Dept of Biology, Univ. of Calgary, Canada) tracers were added. The extracting solution was adjusted to 80% aqueous methanol and extraction proceeded for 24 h at 4°C, after which the tissue debris was removed by filtration, the extract reduced to an aqueous solution (in vacuo, <35°C) and an equal volume of 0.5 M Na-phosphate buffer, pH 8.0 was added.

GAs were extracted from E. globulus cambial region tissue as follows. In May, during a period of active growth, three 3-year-old trees growing in Creswick, Victoria were felled and cut into approximately 30 cm long stem segments. These segments were rapidly (ca 5 min) transported to a -15°C freezer. Further processing proceeded immediately. Individual segments were removed from the freezer, the bark peeled and the soft tissue underlying the differentiated phloem and adhering to the differentiated xylem was scraped into a mortar containing liquid nitrogen. Microscopy confirmed that this material consisted primarily of vascular cambium and differentiating xylem with a minor component of recently differentiated xylem. These tissues (in excess of 600 g) were homogenised in liquid nitrogen using a mortar and pestle and extracted for 24 h in 4°C 80% aqueous methanol (5 ml [g fresh weight]$^{-1}$). This extract was reduced to an aqueous solution (in vacuo, <35°C) and an equal volume of 0.1 M Na-phosphate buffer, pH 8.0

was added. This extract was divided into two. To one sample containing the equivalent of approximately 100 g fresh weight of tissue was added 50 ng of [$^2H_2$] GA_1 and 50 ng of [$^2H_2$] GA_{20} as internal standards for quantification and 670 Bq of [^3H] GA_1 and [^3H] GA_{20}. To the other extract containing the equivalent of approximately 500 g fresh weight of tissue was added 330 Bq of [^3H] GA_1, GA_{19} and GA_{20}. Part of the fresh sample was also dried at 70°C for 24 h to determine the dry weight and water content.

Preliminary purification

Methodology used routinely for the purification of GAs in extracts of Pisum sativum (Reid et al. 1990) proved ineffective when applied to extracts of Eucalyptus sp. These extracts contained contaminants which markedly affected the chromatographic behaviour of the GAs on C_{18} reversed phase HPLC (high performance liquid chromatography), possibly by interacting with the stationary phase. GA_1 was significantly affected, typically eluting in a broad zone over fractions 10-22, rather than in fractions 29 to 30 as seen when the tritiated standard is run alone (Fig. 1). This resembled the effects of phenols described by Nutbeam and Briggs (1982). However, slurrying with polyvinylpolypyrrolidone, a cross-linked form of the polyvinylpyrrolidone presumed to selectively remove phenols (Glenn et al. 1972), did not significantly improve subsequent chromatography. Removal of the interacting substance was achieved by the addition of a step utilising the anion exchanger QAE Sephadex A25, as indicated in the purification procedure described below.

The buffered aqueous extracts were partitioned five times at pH 2.9 against 40% volumes of ethyl acetate. The ethyl acetate fractions were frozen, ice removed by filtration, then reduced to dryness with the remaining water removed azeotropically using toluene. The dried extracts were dissolved in ca 10 ml of 60% methanol and the solution

passed through a preconditioned Waters Sep-Pak Plus C_{18} cartridge (Waters Assoc. Milford, MA, USA) at a rate of 5 ml min^{-1}, followed by elution with 20 ml of 60% methanol. The combined eluates were reduced in vacuo until the volume of the extract had reached approximately 10 ml. A 15 x 100 mm anion exchange column was prepared by conditioning 2 g of QAE Sephadex A25 (Sigma Chemical Co. MO, USA) with 50 ml of 0.5 M sodium formate buffer followed by 40 ml of distilled water. The sample was adjusted to pH 7.3 and introduced onto the column, which was then washed with 30 ml of pH 8.0 distilled water. GAs were eluted with 40 ml of 0.2 M formic acid. The extract was reduced to ca 10 ml, adjusted to pH 7.3 and the anion exchange chromatography was repeated. The final eluate was taken to dryness in preparation for HPLC.

HPLC

The HPLC system consisted of two Waters model 510 pumps (Waters Assoc. Milford, MA, USA) delivering a mobile phase consisting of a linear gradient of 21 to 70% methanol in 0.4% acetic acid at 2 ml min^{-1} over 40 min. The sample was injected via a Waters UK6 sample injection unit with a 2 ml loop onto a 10 cm x 8 mm i.d. 10 µm Radial- Pak C_{18} cartridge fitted in a RCM 8 x 10 module (Waters Assoc. Milford, MA, USA). Two-ml fractions were collected and 5% aliquots were assayed for radioactivity by supplementing with ca 4 ml of ReadySafe Liquid Scintillation Counting Cocktail (Beckman Instruments Inc., Fullerton, CA, USA) and subsequent analysis using a Beckman LS 5801 liquid scintillation counter. Based on the elution of radiolabelled GAs, fractions were grouped, dried, redissolved in methanol and methylated using ethereal diazomethane. For the purposes of quantification these fractions were then analysed by GC-MS as described below. Analysis of GA_{29} for the purpose of identification was also performed at this stage. For identification of remaining GAs, grouped fractions were

further purified as methyl esters by HPLC using the same system components as described above. The solvents were methanol and distilled water, and the flow rate was 1.5 ml min^{-1}. For Me-GA_{19} and Me-GA_{20} the solvent program was a linear gradient from 40 to 80% methanol over 35 min. For Me-GA_1 an isocratic program with 29% methanol was utilised. One min fractions were again collected, grouped on the basis of radioactivity and dried.

GC-MS

The grouped fractions were trimethylsilylated by adding 2 µl of pyridine and 10 µl of bis (trimethylsilyl) trifluoroacetamide and heating at 60°C for 15 min. When Kovat's retention index (KRI) was to be determined, 0.1 µl of a mixture of n-alkanes in chloroform was added to the extract. GC-MS-SIM (low resolution) was performed using a Hewlett-Packard 5890 gas chromatograph linked via an open split interface to a Hewlett- Packard 5970 mass selective detector. One µl splitless injections were made at 250°C onto a 25 m x 0.22 mm i.d. SGE BP1 column with a 0.25 µm film thickness (SGE Vic., Aust.). The helium carrier gas was supplied at an initial flow rate of 2 ml min^{-1} at 190 kPa and the oven temperature was programmed from 60 to 230°C at 30°C min^{-1} then to 290°C at 3°C min^{-1}. The interface temperature was 290°C and the ionisation potential was 70eV.

Full scan mass spectrometry and high resolution selected ion monitoring (HR-SIM) were performed using a Hewlett- Packard 5890 Series II gas chromatograph linked via a direct inlet into a Kratos Concept ISQ mass spectrometer controlled by a Mach 3 data system. One µl splitless injections were made at 60°C onto the same SGE BP1 column. The carrier gas was helium with the head pressure programmed to maintain a flow rate of approximately 2 ml min^{-1}. The oven temperature was programmed from 60

to 240°C at 30°C min^{-1} then to 290°C at 3°C min^{-1}. The interface temperature was 290°C and the ionisation potential was 70eV. Where HR-SIM was used, the masses of characteristic ions were calculated to four decimal places and detection was achieved by voltage switching at a resolution of 10 000 (10% valley definition) and a cycle time of 0.6 s. Perflourokerosene was used to provide lock masses for HR-SIM.

Quantification of GAs was achieved as in Lawrence et al. (1992) including corrections made for naturally occurring isotopes and for unlabelled material in the internal standards. The ion pairs monitored were 506/508 (GA_1 and GA_{29}), 418/420 (GA_{20}), 434/436 (GA_{19}) and 448/450 (GA_{53}). In most cases identifications were achieved by comparison of full scan mass spectra and KRI values of putative GAs with reference data (Gaskin and MacMillan 1991). Where good quality full scan mass spectra were not possible due to the low concentration of putative GA or the presence of co-eluting impurities, HR-SIM in conjunction with KRI data was employed as a means of identification. HR-SIM chromatograms of several characteristic ions were compared between the putative GA and a deuterated standard in order to establish an interference free assessment of ratios of intensities of fragment ions to molecular ion intensity.

Results and discussion

When extracts from the cambial region of E. globulus, purified by acidic ethyl acetate partitioning, anion exchange chromatography and HPLC of both the free acids and methyl esters, were analysed by GC-MS, GA_{19}, GA_{20} and GA_{29} were identified on the basis of KRI and full scan mass spectra. Figure 2 presents a mass spectrum of the GA_{20} isolated from E. globulus analysed as the Me ester TMS ether. GA_1 was identified by KRI and HR-SIM. Extracts from apical buds of E. nitens yielded identification of GA_{19} based on mass spectra and KRI while identification of GA_1 and GA_{20} was based on KRI

and HR- and LR-SIM of characteristic ions. Tab. 1 lists the identified GAs, KRI values and the relative abundance of characteristic ions monitored.

From apical buds of E. nitens GA_1, GA_{19}, GA_{20} and putative GA_{29} and GA_{53} were quantified using deuterated internal standards while GA_4, GA_8, GA_9 and GA_{44} were not present at detectable levels (Tab. 2). From the cambial region of E. globulus, GA_1 and GA_{20} were quantified in terms of both concentration per unit weight (fresh and dry) and per unit area scraped (Tab. 3).

In the case of GA_1, HR-SIM was used to confirm the identity of the putative compound. This technique is useful in resolving ions derived from the putative GA from ions of similar mass derived from other closely eluting compounds, particularly where the concentration of the latter is large in comparison to the putative GA. The clarity of ion chromatograms using this technique also makes it valuable for quantification of GAs where chemical noise in the LR-SIM mode renders difficult the accurate determination of peak areas.

The identity of GAs recovered suggests that in eucalypts the early 13-hydroxylation pathway (Sponsel 1983) is the dominant pathway of GA biosynthesis, with GA_1 as the compound with activity per se (Phinney 1984, Reid 1986). Similar conclusions have been made in regard to Salix (Davies et al. 1985, Junttila et al. 1988), Citrus (Poling and Maier 1988, Turnbull 1989, Talon et al. 1990) and Populus (Rood et al. 1988). Although conclusive identification of GAs in vegetative tissues of woody dicotyledons is limited to only a few species, it is evident that the early 13-hydroxylation pathway is likely to be the main biosynthetic pathway in these plants.

While it is difficult to compare between studies, the present results suggest that the apical buds of E. nitens contain levels of GAs within the range found in the shoots of other hardwood species. In this tissue GAs were present at levels below 1 ng [g fresh weight]$^{-1}$, except for GA_{19} (which may represent a GA pool). This study is also

the first in which GAs have been identified and quantified in the cambial region of a hardwood. The high levels of GA_{20} (8.8 ng [g fresh weight]$^{-1}$) in this tissue relative to GA_1 (0.30 ng [g fresh weight]$^{-1}$) indicates that the 3-ß-hydroxylation of GA_{20} may limit GA_1 availability. While it may be postulated that this is part of a GA-mediated control of some developmental factor, the significance of high GA_{20} levels in the cambial region is yet to be fully understood.

The development of effective techniques for the isolation, identification and quantification of GAs from E. nitens and E. globulus now permits examination of the possible roles played by GAs in the control of important developmental processes such as flowering and fibre development.

Acknowledgments- The authors thank Prof. L.N. Mander for providing deuterated GA standards and Prof. R.P. Pharis for providing tritiated GA9. Financial assistance from the Tasmanian Forest Research Council is acknowledged and Mr L. Johnson, Mr P. Bobbi, Ms K. McPherson and Ms C. Sandercock are thanked for their technical assistance.

References

Aloni, R. 1992. The control of vascular differentiation.- Int. J. Plant Sci. 153: S90-S92.

Davies, J.K., Jensen, E., Junttila, O., Rivier, L. & Crozier, A. 1985. Identification of endogenous gibberellins from Salix pentandra.- Plant Physiol. 78: 473-476.

Gaskin, P. & MacMillan, J. 1991. GC-MS of Gibberellins and Related Compounds: Methodolgy and a Library of Spectra. University of Bristol, UK. ISBN 0-86292-388-3.

Glenn, J.L., Kuo, C.C., Durley, R.C. and Pharis, R.P. 1972. Use of insoluble polyvinylpyrrolidone for purification of plant extracts and chromatography of plant hormones.- Phytochemistry 11: 345-351.

Hasan, O., Potts, B.M. & Reid, J.B. 1992. Chemical promotion of flower bud initiation in E. globulus.- In Proceedings of a Symposium on Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species (J.N. Marier, C.L. Hubert & P. Alazard, eds) p. 444. AFOCEL / IUFRO, Bordeaux.

Hedden, P. & Graebe, J.E. 1985. Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of Cucurbita maxima endosperm and Malus pumila embryos.- Plant Growth Regul. 4: 111-122.

Hetherington, S. & Jones, K.M. 1990. Effectiveness of paclobutrazol in retarding height growth of Eucalyptus globulus seedlings.- Can. J. For. Res. 20: 1811-1813.

Hetherington, S. & Jones, K.M. 1993. Seed stimulation in eucalypts by paclobutrazol application.- Proc. Int. Plant Prop. Society (In press).

Hetherington, S., Jones, K.M. & Koen, T.B. 1991. Stimulation of bud production in Eucalyptus globulus by paclobutrazol application.- In Intensive Forestry: The Role of Eucalypts (A.P.G. Schonau, ed.) pp. 104-109. IUFRO, Durban. ISBN 0-621-15961-8.

Junttila, O., Abe, H. & Pharis, R.P. 1988. Endogenous gibberellins in elongating shoots of clones of Salix dasyclados and Salix viminalis.- Plant Physiol. 87: 781-784.

Lawrence, N.L., Ross, J.J., Mander, L.N. & Reid, J.B. 1992. Internode length in Pisum. Mutants lk, lka and lkb do not accumulate gibberellins.- Plant Growth Regul. 11: 35-37.

Little, C.H.A. & Savidge, R.A. 1987. The role of plant growth regulators in forest tree cambial growth.- Plant Growth Regul. 6: 137-169.

Moncur, M.W., Rasmussun, G. & Hasan, O. 1993. Effect of paclobutrazol on flower-bud production in Eucalyptus nitens (Dean and Maid.)Maid. espalier seed orchards.-Can. J. For. Res. (In press).

Nutbeam, A.R. & Briggs, D.E. 1982. Gibberellin-phenol interactions in plant extracts.- Phytochemistry 21: 2217-2223.

Phinney, B.O. 1984. Gibberellin A_1, dwarfism and the control of shoot elongation in higher plants.- In The Biosynthesis and Metabolism of Plant Hormones (A. Crozier & J.R. Hillman, eds), pp. 17-41. Society of Experimental Biology Seminar Series 23, Cambridge University Press, Cambridge. ISBN 0521-26424-3.

Poling, S.M. & Maier, V.P. 1988. Identification of endogenous gibberellins in navel orange shoots.- Plant Physiol. 88: 639-642.

Reid, J.B. 1986. Gibberellin mutants.- In Plant Gene Research: A Genetic Approach to Plant Biochemistry (A.D. Blonstein & P.J. King, eds), pp. 1-34. Springer-Verlag, Wien. ISBN 3-211-81912-6.

Reid, J.B., Hasan, O. & Ross, J.J. 1990. Internode length in Pisum: Gibberellins and the response to far-red-rich light.- J. Plant Physiol. 137: 46-52.

Ridoutt, B.G. & Sands, R. 1993. Within-tree variation in cambial anatomy and xylem cell differentiation in Eucalyptus globulus.- Trees. (In press).

Rood, S.B., Bate, N.J. & Mander, L.N., Pharis, R.P. 1988. Identification of gibberellins A_1 and A_{19} from Populus balsamifera x P. deltoides. Phytochemistry 27: 11-14.

Sponsel, V.M. 1983. In vivo gibberellin metabolism in higher plants.- In The Biochemistry and Physiology of Gibberellins (A. Crozier, ed.), Vol. 1, pp. 151-250. Praeger, New York, NY. ISBN 0-03059054-X.

Talon, M., Hedden, P. & Primo-Millo, E. 1990. Gibberellins in Citrus sinensis: A comparison between seeded and seedless varieties.- Plant Growth Regul. 9: 201-206.

Turnbull, C.G.N. 1989. Identification and quantitative analysis of gibberellins in Citrus.- Plant Growth Regul. 8: 273-282.

Tables

Tab. 1. Sample identifications of endogenous GAs isolated from Eucalyptus spp. All four GAs were identified from extracts of the cambial region of 3-year-old E. globulus while GA_1, GA_{19} and GA_{20} were identified from extracts of the apical buds of 1-year-old E. nitens. The identification of GA_1 shown was achieved by selected ion monitoring while the remaining GAs were identified by full scan mass spectra.

GA	Tissue (Species)	KRI Sample/$^2H_2$ standard	Relative abundance of characteristic ions				
GA_1	Apical buds (E. nitens)	2690/2689	506(100) 376(17)	491(10) 375(15)	448(20) 313(6)	447(9)	377(16)
GA_{19}	Cambial region (E. globulus)	2627/2626	462(trace) 374(67)	434(100) 345(31)	405(25) 315(31)	402(48)	375(56)
GA_{20}	Cambial region (E. globulus)	2521/2520	418(100) 235(3)	403(17) 207(17)	375(51)	359(15)	301(11)
GA_{29}	Cambial region (E. globulus)	2696/2695	506(100) 207(17)	491(16)	447(5)	375(16)	303(24)

Tab. 2. Endogenous GA levels in apical buds of 1-year-old Eucalyptus nitens.

GA	ng g FW^{-1}
GA_1	0.75
GA_4	<0.08
GA_8	<0.08
GA_9	<0.08
GA_{19}	2.1
GA_{20}	0.76
GA_{29}	0.61
GA_{44}	<0.08
GA_{53}	0.24

Tab. 3. Endogenous GA levels in the cambial region of 3-year-old Eucalyptus globulus.

GA	ng g FW^{-1}	ng g DW^{-1}	ng cm^{-2}
GA_1	0.30	2.8	0.013
GA_{20}	8.8	81	0.37

Figure legends

Fig. 1. Fractionation by HPLC of an extract of E. nitens containing [^{3}H] GA_1 and [^{3}H] GA_{20} as tracers. HPLC profiles of free acids after initial purification by the method of Reid et al., (1990) (a) and fractions grouped as shown in (a) refractionated as methyl esters (b-c). [^{3}H] GA standards eluted in the zones indicated by open boxes.

Fig. 2. Full scan mass spectra of putative GA_{20} Me ester TMS ether isolated from the cambial region of 3-year-old Eucalyptus globulus.

Fig. 1.

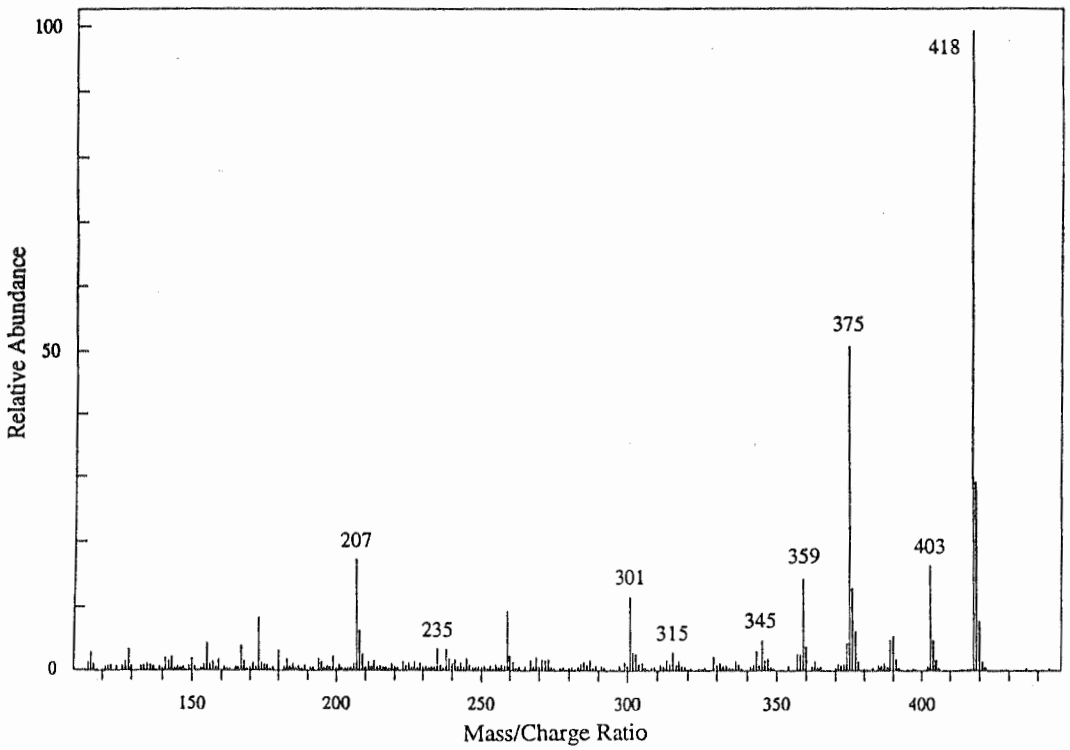
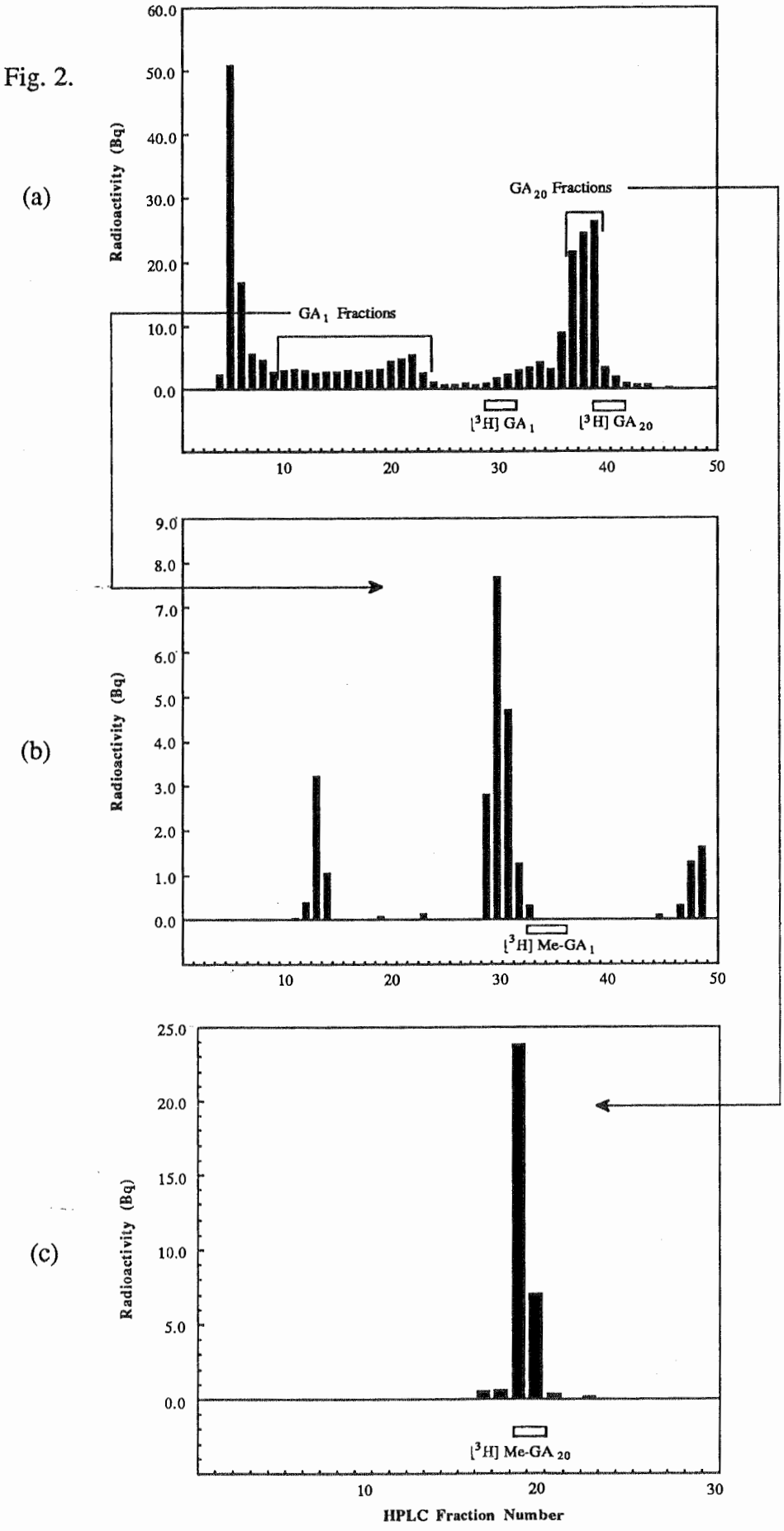

100
50
0
Relative Abundance
418
375
207
359
403
301
235
315
345
150
200
250
300
350
400
Mass/Charge Ratio

Fig. 2.


(a)
Radioactivity (Bq)
GA20 Fractions
GA1 Fractions
[3H] GA1
[3H] GA20
(b)
Radioactivity (Bq)
[3H] Me-GA1
(c)
Radioactivity (Bq)
[3H] Me-GA20
HPLC Fraction Number

Appendix B

Methods section of Moncur *et al.* (1993)

This paper has been accepted for publication by the Canadian Journal of Forest Research.

Methods

Experiment 1. Ridgley, Tasmania; Lat 41° 15' S, Long 145° 83' E, Alt 280m

One year old *E. nitens* grafts were outplanted in a breeding arboretum in 1988. Grafts were planted 6m apart in rows 3m wide. Branches were trained by tying down to wires running 1 and 2m above ground level. Pruning was used to restrict the number of branches to one or two per wire and to shape the graft to fit within an oblong shape, the upper surface of which lay just above the top wire and expanded approximately 0.5m on each side. Pruning to this configuration occurred regularly until 1991 and was subsequently discontinued. Grafted trees (grafts) were fertilised, sprayed to control leaf eating insects and irrigated as required.

In August 1989 groups of 6 grafts received a collar drench (CD) of paclobutrazol in the form of "Cultar" (ICI Australian Operations Pty Ltd) at either 1g or 2g active ingredient (ai)/cm stem circumference. Rate of CD treatment was varied by using different formulations and diluting to 2L with water per tree before pouring around the base of the stem. Six grafts were not treated and served as controls.

The inflorescence in eucalypts is an umbel, consisting of between 1 and 30 flowers (*E. nitens* has 7 flowers). Umbels initiate in the leaf axils of this years shoot terminals. A large proportion of terminals do not initiate umbels (see Moncur and Boland (1989) for more detailed descriptions).

Each year the total number of umbels, flowers and capsules were counted on each graft. 50 terminals which had initiated umbels from each graft were randomly

selected and the number of umbels on each terminal counted. In Year 3, capsules were harvested and mature seed extracted. 100 seeds per graft were germinated using the method of Boland *et al.*, (1980).

Experiment 2. Canberra, Australian Commonwealth Territory; Lat 35° 10'S, Long 149° 4'E, Alt 600m.

One year old *E. nitens* grafts were outplanted in a breeding arboretum in 1989. Grafts were spaced 6m apart in rows 3m wide and trained as at Ridgley (Experiment 1). Grafts have been regularly fertilised, sprayed to control leaf eating insects and irrigated.

In March 1990 "Cultar" was applied as a 2 L collar drench (see experiment 1) to groups of 4 grafts at either 0.5g ai/cm stem circumference or 0.2g ai/cm stem circumference. Again to two groups of 4 grafts, paclobutrazol as "Clipper" (ICI Australian Operations Pty Ltd) (10g/L in ethanol) was injected into the stems with a pressure gun approximately 0.5m above ground level at either of two rates; 0.025g ai/cm circumference or 0.01g ai/cm circumference. 4 grafts were not treated and served as controls.

The dose rates used in these trials were based on the rates used by Griffin *et al.* (1993). Trunk injection was applied at lower rates than collar drench because of the persistence of paclobutrazol in the soil (Mauk *et al.*, 1990) and as Hetherington *et al.* (1991) have pointed out trunk injection is the most precise way of applying a specified dose rate to a tree.

Endogenous gibberellin quantifications.

Gibberellin (GA) levels were determined for controls, collar drench and stem injection treatments for the Canberra experiment. In October 1991, 18 months after paclobutrazol application, material was sampled from a single grafted tree from each treatment in the Canberra espalier orchard. In addition, material was sampled from grafts planted one year later and treated with paclobutrazol when 6 months old and are therefore not directly comparable with the main experiment.

Apical sections approximately 5cm long were excised from branch terminals and leaves were trimmed as necessary to maintain a constant ratio of leaf to shoot mass across all treatments. Sections were then frozen in liquid nitrogen and transferred (at 0° C for 8 hours) to storage at -20° C for 4-8 weeks when processing took place. Samples supplemented with (17,17 $^2H_2$) GA_1 and (17,17 $^2H_2$) GA_{20} (L. Mander, Australian Natl Univ., Canberra, Australia) internal standards were extracted and purified as in Reid *et al.* (1990) with the additional purification step of anion exchange chromatography (Talon *et al.*, 1990). Levels of GA_1 and GA_{20} were estimated via gas chromatography-mass spectrometry in the selected ion mode (GCMS-SIM) of appropriate portions of the fractionated, purified extract (Reid *et al.*, 1990).

Appendix C

Works published by Omar Hasan during the course of Ph.D. candidature

Refereed Papers

Hasan, O., Ridoutt, B.G., Ross, J.J., Davies, N.W. and Reid, J.B. (1994) Identification and quantification of endogenous gibberellins in apical buds and the cambial region of *Eucalyptus*. Physiol. Plant. (in press).

Moncur, M.W. and Hasan, O. (1994) Floral induction in *Eucalyptus nitens* (Deane & Maiden) Maiden. Tree Physiology (under consideration).

Moncur, M.W., Rasmussen, G.F. and Hasan, O. (1993) Effect of paclobutrazol on flower-bud production in *Eucalyptus nitens* (Deane & Maiden) Maiden espalier seed orchards. Can. J. For. Res. (in press).

Reid, J.B., Hasan, O. and Ross, J.J. (1990) Internode length in *Pisum*. Gibberellins and the response to far-red-rich light. J. Plant Physiol. **137**: 46-52.

Reid, J.B., Ross, J.J. and Hasan, O. (1991) Internode length in *Pisum*: Gene *lkc*. J. Plant Growth Regul. **10**: 11-16.

Unrefereed Material

Hasan, O., Potts, B.M. and Reid, J.B. (1992). Chemical promotion of flower bud initiation in *E. globulus*. *In* 'Proceedings of a Symposium on Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species.' (Eds J.N. Marnier, C.L. Hubert and P. Alazard) p. 444. AFOCEL/IUFRO, Bordeaux.

Hasan, O., Reid, J.B. and Potts, B.M. (1993) 'Paclobutrazol induced precocious flowering in eucalypts: a possible role for gibberellins.' Research Report No. 8. Tasmanian Forest Research Council, Inc., Hobart. 50 pp.