Aspects of the Physiological and Chemical Control of Adventitious Root Formation in *Eucalyptus nitens* (Deane & Maiden) Maiden.

by

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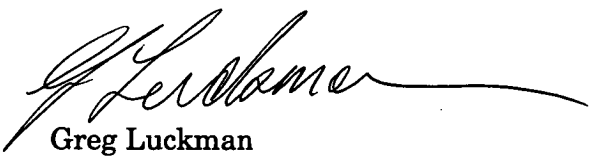

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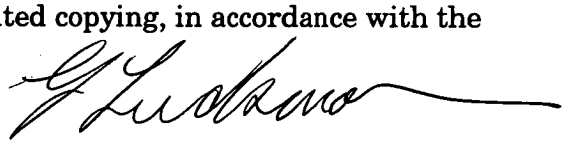

Greg Luckman
1 November 1996

Science moves, but slowly slowly, creeping on from point to point.

Alfred, Lord Tennyson 1809 - 1892.
Locksley Hall (1842

If a man will begin with certainties, he shall end in doubts;
but if he will be content to begin with doubts, he shall end in certainties.

Francis Bacon 1561 - 1626
Advancement of Learning

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Abbreviations

ATP	- Adenosine triphosphate
BAP	- Benzylaminopurine (Benzyladenine)
BSA	- Bovine serum albumin
DES	- Diethyl stilbestrol
2,4-D	- 2, 4 dichlorophenoxyacetic acid
FAA	- Formalin Acetic Alcohol, fixative
GA	- Gibberellic acid
IAA	- Indole-3-acetic acid
IAAsp	- Indole-3-acetyl L aspartate
IAN	- Indole-3-acetonitrile
IBA	- Indole-3-butyric acid
NAA	- α-Naphthaleneacetic acid
NET	- North Eucalypt Technologies
NPA	- 1-N-naphthylphthalamic acid
PBA	- 1-Pyrenoylbenzoic acid
PPO	- Poly phenol oxidase
TIBA	- Tri-iodobenzoic acid
2,4,5-T	- 2, 4, 5 trichlorophenoxyacetic acid

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Summary

Eucalyptus nitens is an important species in plantation forests in Tasmania and is becoming important in other cool temperate regions of the world. Reliable initiation of roots in cuttings is needed, to enhance forest productivity through the establishment of clonal plantations.

Adventitious root initiation is dependent on the re-polarisation of cells to form a new root meristem. Failure to initiate roots may be due to the inability of the plant to undergo re-polarisation of cells to form a new meristem. The research undertaken has been directed at the manipulation of some of the factors that are implicated in the establishment of cellular polarity in plants. Several new techniques for enhancing root initiation have been investigated and shown to promote root formation in cuttings of *E. nitens*. The principle conclusions of the research are outlined below.

It was demonstrated that a high proportion of *E. nitens* seedlings have the ability to initiate adventitious roots in cuttings collected from very young seedlings. This ability is lost quickly as the plant ages. The loss of rooting ability does not appear to be related to any specific changes in the stem morphology. Histological examination demonstrated that the loss of rooting ability is not correlated with the formation of structural barriers within the stem or callus. Root primordium formation appears to occur in regions of undifferentiated callus in most cuttings but can also occur directly from the cambium, with little or no intervening callus phase.

Root initiation was found to be sensitive to the timing and method of auxin applications. It was possible to increase the proportion of cuttings that initiate roots by delaying the application of auxin until several weeks after the cuttings were first placed on the mist-bed. Calcium ions are part of the mechanism by which auxin signals are translated into cellular actions. Attempts to increase the rate of root initiation in cuttings by altering calcium levels in the surrounding medium were inconclusive.

Electric potential differences are known to play a role in the establishment of cellular polarity and in organogenesis from callus. It was demonstrated that low voltage electric currents could be used to stimulate root initiation in some

cuttings. It was hypothesised that cuttings with substantial basal callus would be most receptive to this treatment, but this was found not to be the case. The experiments using electric currents were difficult to replicate and were subject to unexplained variation in results. Further development of the technique is required to quantify the effects of such currents and to identify the mode of action.

Stigmasterol and vitamin D were demonstrated to act as auxin synergists in the promotion of root initiation in cuttings and also to stimulate the growth of tissue cultures. ATPase extracts were prepared to investigate whether the action of sterol potentiation of auxin is associated with changes in membrane bound H^+-ATPase activity.

The treatment of cuttings with G compounds, as a method of promoting root initiation, was investigated. G compounds are a group of naturally occurring chemicals found in some eucalypt species and are known to inhibit and to boost root initiation in some species. In cuttings of *E. nitens*, they were found to have no significant effects on root initiation.

The results obtained are discussed in terms of the role that the treatments might play in root morphogenesis through the re-polarisation of cells to create a new axis. The results support the general hypothesis that treatments aimed at enhancing the development of cellular polarity are useful in stimulating root initiation in cuttings. Some suggestions are made for future research, to develop these initial findings into practical treatments for the propagation of *E. nitens*.

1. GENERAL INTRODUCTION

There are large, well established clonal eucalypt forestry plantations in a number of countries around the world (Zobel, 1993). These plantations have amply demonstrated the superior productivity and consistency that can be obtained from clonal eucalypt forests. The species used include *Eucalyptus grandis, E. urophylla* and hybrids (Zobel, 1993), species which are readily propagated from cuttings. This success has provided a stimulus to research on the propagation of cool temperate eucalypts, such as *Eucalyptus nitens*(Deane & Maiden) Maiden and *Eucalyptus globulus*. These species have proved to be much more difficult to propagate, both from cuttings and *in vitro* (Hartney and Svensson, 1992). The inability to propagate clones of these species at commercially acceptable rates is an impediment to accessing the potential yield increases which have resulted from breeding and selection programs (Jamieson, 1995). The objective of the research contained in this thesis was to investigate methods by which the rate of root initiation in cuttings of *Eucalyptus nitens* can be improved.

Despite advances that have been made in both macropropagation and micropropagation techniques over the past ten to fifteen years, there remain significant obstacles to routine propagation of some species and clones of eucalypts. It has been demonstrated that there is a capacity to initiate adventitious roots within all, or most, *E. nitens* seedlings. This ability is rapidly lost with the growth and development of the seedling, although it may be retained in some basal stem tissues (Hartney, 1980). There is currently no clear understanding of why the ability to initiate adventitious roots is lost so quickly.

The present understanding of the mechanisms controlling adventitious root initiation in cuttings is inadequate. Despite there having been substantial advances in knowledge of hormone physiology, there is still an incomplete understanding of what causes and controls the formation of adventitious roots. It is generally accepted that the primary stimulus for root initiation is provided by auxin but the connection between auxin action and organogenesis is not fully understood. Haissig, Davis and Reimenschneider (1992) have proposed that the past concentration of research on the correlation of physiological processes with

root initiation has impeded understanding of root initiation. They have also proposed that there must be a greater emphasis on establishing the causal physiology of root initiation.

Adventitious root initiation can be considered to be a form of organogenesis. A new apical meristem is formed where previously none existed. This can occur either from some differentiated, specialised tissues, such as the cambium, or from unspecialised cells, such parenchyma or callus (Lovell and White, 1986). The process of organogenesis can be separated into three phases, as described by Thorpe and Kumar (1993). The first phase is the pre-induction phase, where cells attain the competence to undergo organogenesis. Induction of the new meristematic locus from competent tissues occurs during the second phase. The differentiation of meristematic cells leads to the formation of a region of co-ordinated, polar growth. During the third phase, the pattern of differentiation is expressed and the new meristem develops to form a root primordium.

One of the features that distinguishes organised tissues from undifferentiated cells is that they possess an axis of polarity. That is, there is a specific orientation of activity within space for all differentiated tissues (Nick and Furuya, 1992). The establishment of polarity must be the first part of the induction phase of organogenesis. The formation of co-ordinated growth cannot occur until the axis of polarity has been established within cells. This leads to the conclusion that the mechanisms by which polarity is established within cells are critical to the induction of new meristems. Thus, the formation of a meristem depends on the ability of the tissues to respond to those signals which establish polarity within those tissues.

Adoption of this view of organogenesis leads to the conclusion that the initiation of new root meristems depends on the establishment of a new axis of polarity. This polarity may be induced within the existing cells of the cutting or in undifferentiated tissues that arise from the cutting. Much of the work contained in this thesis is based on the hypothesis that promotion of root initiation in *E. nitens* can best be achieved by gaining an understanding of the factors that are known to be, or thought to be, important in the establishment of cellular or tissue polarity. Application of that knowledge to the particular problems associated with the induction of new root primordia in cuttings will result in some new approaches and techniques for use in commercial situations.

2. LITERATURE REVIEW

2.1 INTRODUCTION

Adventitious root initiation has been the subject of considerable research over a wide range of species. There are a number of recent books that review much of this research (Davis and Haissig, 1994; Davis, Haissig and Sankhla, 1988; Haissig *et al*, 1992; Hartmann, Kester and Davies, 1990). The research has demonstrated that there is a matrix of interacting and counteracting factors that can affect a plant's ability to initiate roots. It seems that the relative importance of factors can vary between species of the same genus and even between varieties of the same species. This can result in quite contradictory results being obtained when the same treatments are applied to different species or clones.

It is also necessary to distinguish between treatments that affect a cutting's intrinsic ability to initiate roots and those treatments that improve the apparent root initiation rate, perhaps by enhanced survival or faster appearance of root initials. The latter category, while important, may not ultimately be the most useful in a commercial application.

2.1.1 Scope of the review

This review will cover some of the more important facets of current and past research into the control of adventitious rooting. Some of the theories that have been advanced in the past to explain apparent variations in rooting ability no longer seem to fit current theories on basic plant physiology, which have been developed with the aid of molecular biology and improved methods of hormone measurement. Theories about how and why particular rooting treatments work could be refined if more attention was given to recent findings on the basic physiological factors influencing the process of organogenesis.

Adventitious root initiation is ultimately dependent on the fact that a group of cells re-orient their growth polarity and begin to differentiate into a new meristematic region. The root primordium arises from this meristem which then controls the growth and development of the primordium. Whether the

differentiation occurs after a callus phase, or within existing non-meristematic tissues, the primary event must be the re-orientation and co-ordination of a new growth centre.

To understand why some cuttings fail to undergo root initiation requires knowledge of the factors controlling morphogenesis and an understanding of which of those factors are of greatest significance in preventing the meristem formation.

Thorpe and Kumar (1993) suggest that morphogenesis should be studied at different levels of organisation, for example cell, tissue, organ and whole plant levels. They believe it is likely that there are control points at each level.

The remainder of this review will briefly consider the following questions:

1) What are the economic and scientific imperatives that provided the impetus for this study to be undertaken?
2) What are the anatomical and physical features associated with root initiation?
3) What are the key events in the differentiation of cells that lead to the formation of an adventitious root?
4) How is morphogenesis initiated and controlled at a sub-cellular and molecular level?
5) How can the whole plant and cutting be manipulated to enhance the rooting ability of the cutting?

2.1.2 Definition of terms used

For the purposes of this thesis, a cutting is defined as any part of a plant that has been excised from its parent plant for the purpose of producing a clone of that parent plant. The plant from which the cutting has been excised is referred to as the stock-plant. The term cutting is intended to include all types of propagule used for clonal multiplication of plants. Where a specific type of cutting is is being referred to, such as leaf cuttings, it is explicitly named.

Macropropagation is considered to be the process of clonal multiplication using cuttings, as distinct from micropropagation which is the propagation of plant material using *in vitro* growth and multiplication of propagules.

2.2 SCIENTIFIC AND ECONOMIC RATIONALE

2.2.1 Advantages of clonal eucalypt forestry

Some tree species have been successfully cloned for over a thousand years (Haissig and Davis, 1994; Ritchie, 1994; Zobel, 1993). During the last 20 years or so, eucalypts have become the most commonly propagated species for clonal forestry around the world. Several companies have very large operations which each produce several thousands of hectares of new plantings each year (Mtarji, 1992; Ruiz, 1992; Souvannavong, 1992; Vieira *et al*, 1992).

Clonal propagation provides more immediate access to the genetic gains made from intensive selection or breeding programs (Borralho, 1992). This technique provides a more uniform product than seedling based production, which imparts significant cost advantages (Wright, 1992).

Clonal propagation techniques provide many benefits to tree improvement schemes generally. Four main benefits are listed by Maynard (1988). These are:

a) Speeding the release of superior stock;
b) Capture of specific combining ability;
c) Capture of epinastic gene activity; and
d) Precision deployment of genotypes.

In clonal eucalypt forestry, achieving greater uniformity of wood and fibre characteristics is described as one of the most important advantages (Zobel, 1993). Selection can be based on fibre length and processing characteristics, which result in decreased processing costs. This is in addition to selection on the basis of more traditional agronomic features, such as growth rate, yield and insect or disease resistance.

Seedling populations, even those from seed orchards, are heterogeneous in growth habit and growth rate, factors that result in lower yields in comparison to monoclonal plantings (Eldridge, 1993). In addition, the speed and uniformity of growth that can be obtained from selected clones results in much faster canopy closure. This reduces the need for herbicide use and decreases establishment costs (Zobel, 1993). Enhanced frost tolerance has also been reported for *E. globulus* plants grown from cuttings over seed grown plants receiving the same hardening procedures prior to planting out (Sasse, 1995).

An additional future benefit is the potential to take advantage of genetically engineered plants. Guaranteed sterility will be needed to ensure new genes do not mix with the wild gene pool. Efficient vegetative propagation will be essential for such plants to be exploited (Jamieson, 1995).

2.2.2 *Eucalyptus nitens* and its role in plantation forestry

Eucalyptus nitens (Deane & Maiden) Maiden is described as a fast-growing and relatively uncommon species found in a number of quite separate populations in Victoria and New South Wales (Eldridge, 1993). They grow at altitudes between 670 to 1280 metres in Victoria and up to 1600 metres in northern New South Wales. These are areas that are also populated by *E. regnans*, *E. delagatensis* and in New South Wales, *E. fastigata*. In Tasmania, *E. nitens* is grown as a plantation species for its frost hardiness and vigour in moist, cool sites (Tibbits, 1986).

There are in excess of 56,000 ha of eucalypt plantations in Tasmania, which is approximately 35 % of the total area of plantation forests (ABARE, 1996). Annual plantings of eucalypt species in Tasmania were approximately 7000 ha in 1992/93, the last year for which there are statistics are available (Forestry Commission of Tasmania, 1994). Most of these plantings are of *E. nitens*, although no statistics are collected to indicate the proportions.

2.2.3 The advantages of macropropagation

The development of clonal eucalypt plantations has paralleled the development of plant tissue culture techniques to the stage where large scale micro-propagation of plants is routine and cost competitive for many species. However, micropropagation has generally not been adopted for clonal eucalypt production, although some reports suggest an imminent change to this situation (Lakshmi Sita, 1993).

Micropropagation of eucalypts has proved to be more difficult and significantly less productive than for many other species. Some of the problems have been, low proliferation rates (Wilson, 1995), inconsistent root initiation (Bennet *et al*, 1994) and difficulty in establishing the plantlets in soil (Hamill *et al*, 1995).

The success of commercial plantations of *Eucalyptus grandis*, *Eucalyptus urophylla* and other hybrids (Eldridge, 1993) established in Brazil, the Congo and elsewhere have provided a model that demonstrates the projected economic benefits that can be realised from clonal forestry through macropropagation in the field. These plantations use field grown stock plants that are felled close to the ground to provide basal coppice shoots. The shoots are harvested for preparation of cuttings consisting of two leaf, softwood cuttings. These usually root within a few weeks (Souvannavong, 1992).

2.2.4 Macropropagation of *Eucalyptus nitens*

Adventitious root initiation in eucalypts is usually only successful in young seedlings or from coppice shoots derived from basal tissues (Hackett, 1988). Exceptions to this are *E. deglupta* and *E. robusta*, (Eldridge, 1993).

At present, cuttings propagation of *E. nitens* is only possible on a limited scale. There is a strong seasonal variation in rooting ability recorded for both *E. nitens* and *E. globulus*. Peak rooting ability occurs in cuttings collected in early spring with the lowest success rates being recorded in mid winter (de Little *et al*, 1992).

Fewer than 10% of plants have commercially viable rooting rates (Willyams *et al*, 1992). In addition, *E. nitens* trees do not produce basal coppice shoots as predictably as some of the other species (Jacobs, 1955). The frequency and extent of basal coppice shoot production has been enhanced by treatment with plant growth regulators in *E. ficifolia* (Mazalewski and Hackett, 1979) but there is no record of such treatments being successfully applied to *E. nitens*.

Only a small percentage of trees in a breeding and selection program that have the desired yield and processing characteristics can actually be propagated at commercially viable rates. To be commercially viable, rooting percentages in excess of 70% are required. This has been achieved on a commercial scale with *E. grandis* cuttings propagation (Wright, 1992). In addition, a greater proportion of the high yielding trees from the breeding and selection programs need to be propagated successfully.

Achieving the desired efficiency in propagation of *E. nitens* requires a greater understanding of the factors that affect adventitious root initiation in general. It will then be possible to determine which of these factors can be manipulated to improve the root initiation of *E. nitens* cuttings.

2.3 ROOT INITIATION IN CUTTINGS

Careful study of the pattern of cell development and the anatomy of adventitious root formation in cuttings is highly desirable. It provides information on the timing and origins of callus and root primordia formation. It can also show whether theories developed about particular control points in rooting are likely to be relevant to that plant species.

Some of the factors which can affect root initiation include stem selection, the effect of juvenility and severance, and stem damage. Endogenous auxin levels in cuttings and how they vary during the root initiation process are also of critical importance to the initiation of cell divisions, callus formation and the development of root initials.

There have been extensive studies published about the anatomy of root formation in some woody species. The review by Lovell and White (1986) lists many such studies but none of these refer to eucalypts. They also note that the current understanding of anatomical development of root initials in woody plants may be distorted by the concentration of this research on a relatively small number of species, particularly conifers, but also apples and some other fruit trees.

2.3.1 Selection of the stem used for cutting

Roots are known to be able to initiate from almost all plant parts in one species or another (Barlow, 1986). In eucalypts, leafy stem cuttings from juvenile type shoots arising from basal coppice are held to be the most viable propagules, although not all eucalypts can be relied upon to produce basal coppice shoots (Hartney, 1980). Stem cuttings from young seedlings are also able to root readily in most species.

The origin of the stem within the stockplant, the size of the cutting and extent of leaf retained on the cutting all have the potential to affect rooting ability. This has been studied in great detail in *E. grandis* and *E. globulus* (Wilson, 1992; Wilson, 1993; Wilson, 1994a; Wilson, 1994b; Wilson, 1995) and in *E. nitens* (Joyce and de Little, North Eucalypt Technologies (NET), Pers. Comm.).

2.3.1a Juvenility and rejuvenation

It is common for plants exhibiting juvenile characteristics to have a much greater rooting ability than the mature form of the same species. In a number of eucalypt species, adventitious root initiation can be demonstrated in young seedlings whereas cuttings from mature plants show little or no rooting ability (Hartney, 1980). However, in eucalypts, the transition in rooting ability is not well correlated with other markers of its juvenile phase, such as leaf shape or absence of flowering (Hackett, 1988). The loss of rooting ability with age appears to be correlated to leaf node number and is well documented across many different species of eucalypts. According to Paton, cited by Hackett (Hackett, 1988) rooting ability in *E. viminalis* and *E. grandis* is lost in cuttings taken at nodes 4 and 15 respectively, while in *E. camalduensis,* root initiation rates decline to 50% at node 100. *E. deglupta* is able to form aerial roots 6 metres above the ground (Eldridge, 1993). *E. ficifolia* loses its rooting ability at about node 7 (Salmon, 1990). In *E. nitens* seedlings, rapid loss of rooting ability has been demonstrated, but the link to node number was not made (Willyams *et al*, 1992). In that experiment, eight weeks after germination the percentage of rooting had declined to 16%.

Commercial forestry relies extensively on the enhanced rooting potential that is observed in shoots arising from basal coppices of mature trees (Wright, 1992; Zobel, 1993). The enhanced rooting observed from coppice shoots is correlated with retention of some juvenile characteristics and is referred to as apparent rejuvenation. The shoots are believed to have arisen from basal accessory buds, laid down while the plant was still in its juvenile growth phase and which have retained their juvenile phenotype (Hartney, 1980).

In shoots that have arisen from mature tissues it is still possible to cause rejuvenation to occur. The use of micropropagation, serial production of cuttings over several generations or cascade grafting onto juvenile rootstocks will produce plants with increasing degrees of juvenile characteristics and increasing rooting ability (Eldridge, 1993). The pattern of rejuvenation achieved in grafting mature scions of *E. grandis* onto seedling rootstocks was explained by Paton in terms of endogenous concentrations of G compounds (Paton, Willing and Pryor, 1981). G compounds are naturally occurring rooting inhibitors, originally extracted from *E. grandis*. The importance of G compounds in root initiation of eucalypts is discussed further in section 2.7.2.

Consideration also needs to be given to how reversal of juvenility is achieved. It has been demonstrated that retention of juvenile characteristics is closely correlated to the size of the apical meristem. When the juvenile growth phase is over, the meristem becomes enlarged but juvenility can be restored by the surgical removal of part of that enlarged meristem (Moncousin, 1991). Moncousin also claimed that the rejuvenation achieved by a period of *in vitro* culture is also related to a reduction in the size of the apical meristem, caused by prolonged exposure to high levels of cytokinin.

It has not been determined how a change in meristem size can cause the substantial growth differences associated with a phase change to a mature growth pattern. Increased auxin production is one possible method by which an enlarged meristem might exert such an effect. Heuser and Hess (1972) attributed the greater rooting ability of juvenile forms of *Hedera* to the presence of lipid-like co-factors. Hackett (1988) claims the controlling factor is a characteristic of the cells at the site of root initiation rather than a translocated promotor or inhibitor.

Regardless of its identity, the apical meristem is able to alter rooting ability in all of the stem material that is subsequently derived from that meristem by a mechanism that is yet to be identified.

2.3.2 Physical and chemical factors in the wound response

According to Lovell and White (1986) the act of severing a stem produces a whole range of effects and responses - some of which will be directly related to new root formation and some of which are ' noise' that are not directly relevant to the processes under study.

The extent of damage caused to the base of the stem is important to the subsequent success of root initiation in a wide range of species. Intentionally inflicting additional damage to the base of the cutting is often used to improve rooting, apparently on the basis of enhancing the wound response of the cutting and providing a greater volume of callus from which roots can initiate (Imaseki, 1985).

The act of severance also allows for the release of a host of chemicals, some from damaged cells and others newly synthesised from surrounding cells as part of

the response to wounding (Wilson and van Staden, 1990). The main chemicals released are phenolics, which are thought to be oxidised and polymerised to form tannins, part of the plant's defences against infection from wounding. Phenolics are thought by some to play a role in stimulating root initiation, and are discussed in section 2.5.

During wounding there is also liberation of Indole-3-acetic acid (IAA) oxidase, which is largely membrane bound in intact cells (Bandurski *et al*, 1995) and whose release is probably dependent on the degree of damage to individual cells. The existence and liberation of IAA oxidase during wounding is central to many models of how variations in rooting ability occur (Bansal and Nanda, 1981; Bhattacharya, 1988; Jarvis, 1986).

Ethylene production is a feature of the wound response in many plants. There is a substantial increase in ethylene production shortly after wounding. This, in turn, stimulates the activity of peroxidase and poly phenol oxidase enzymes (Imaseki, 1985). Ethylene production is also under the influence of auxin. Later increases in ethylene have been attributed to the effect of increased levels of free auxin at the wound site (Moncousin, 1991).

The interaction between ethylene production, auxin, peroxidases and polyphenol oxidases has lead many to consider that wound induced ethylene production plays a crucial role in the early stages of root formation (González, Rodríguez and Tamés, 1993; Moncousin, 1991; Mudge, 1988). However, the exact mechanism by which ethylene might exert that effect is not yet defined.

2.3.3 Endogenous auxin levels

Since the discovery of the critical role of auxin in root initiation, there has naturally been a great deal of research on the relationship between endogenous auxins and rooting ability (Hackett, 1988). Endogenous auxin levels vary substantially in response to seasonal and physiological factors. Some of this variation correlates well with rooting ability, in other instances the association is less clear (Jarvis, 1986).

2.3.3a Auxin levels prior to collection and at excision

Despite the key role that auxin plays in root initiation, relatively little work has been published on the estimation of endogenous levels of auxin in shoots. Much of what has been published relies on the use of bioassays and has been found to produce contradictory results (Blakesley, Weston and Hall, 1991). More recently, Blakesley, Weston and Elliott (1991) measured endogenous IAA levels in *Cotinus* cuttings collected over several months. They found that a seasonal decline in rooting ability correlated with a decline in free IAA levels in stem tissues. It would have been informative to know if the application of exogenous IAA was able to totally or partially compensate for the decline in auxin. This has been shown in herbaceous plants, where removal of leaves from a cutting reduces root initiation. The rooting ability was restored by supplying auxin (Jarvis, 1986). The existence of a correlation does not, of itself, prove a causal relationship. There may be other factors that change at the same time which are playing an equal or greater role in the loss of rooting ability. That auxin treatments have much less effect on cuttings from mature plants of some species leads to the conclusion that endogenous auxin levels are not the sole limiting factor (Hackett, 1988).

Excision of the cutting has two effects on auxin levels. First, there is a release of auxin as a result of the wound response of the plant (Imaseki, 1985). Secondly, there is an accumulation of auxin in the basal region of the cutting as a result of continued basipetal auxin transport. This accumulation of auxin in the basal region is thought to be central to root initiation.

2.3.3b Auxin levels during root initiation

During root initiation, auxin levels rise initially then start to decline until, in a number of species, they are lower than the starting point (Blakesley, 1994). This has lead to the hypothesis that the peak in auxin levels coincides or precedes the onset of cell divisions leading to the formation of the primordia, which assumes that the endogenous auxin level declines because roots are initiating (Blakesley, 1994; Moncousin, 1991). However, it is possible that roots initiate because the hormone levels start to decline for other reasons. In many plants, the number of cells in the base of the cutting that are directly involved in the early stages of root initiation is very low (Lovell and White, 1986). If auxin levels decrease in response to the early stages of root primordia formation

it is not clear how this small number of cells alter the hormone levels so rapidly in the mass of surrounding, non-initiating cells.

2.3.4 Initiation of cell divisions and the formation of callus

Severing the stem to produce the cutting initiates a series of processes in response to the wounding. Early wound responses are predominantly part of the plant's protection against desiccation and pathogens (Hartmann *et al*, 1990). They also result in the formation of a wound callus. According to Sachs (1991), callus formation is a direct consequence of the disruption of normal tissue organisation and the loss of growth co-ordination signals within that tissue. Callus growth is likely to continue until roots or shoots are initiated to restore tissue co-ordination of cell growth.

Most callus initiates from the vascular cambium, close to the base of the cutting, and occasionally from the pith or cells in the cortex (Hartmann *et al*, 1990). It forms as an irregular mass of mostly parenchymous cells, although Sachs (1991), notes that there is usually a degree of organisation and structure within callus. Tracheary elements are often found embedded within the callus and there are often groups of cells with some meristematic characteristics. The key feature of the growth of callus is its lack of co-ordination. If root initiation is to occur from within the callus, a cell or group of cells must re-establish and maintain ordered, co-ordinated growth.

2.3.5 Formation of root initials

Regardless of whether root initiation occurs from callus at the base of a cutting or roots initiate directly from pre-existing tissues, it must start with the formation of a meristematic locus. This locus is described as consisting of a group of small densely staining cells with enlarged nuclei (Blakesley, 1994). In woody plants, the initial cell divisions can arise from a wide range of tissues (Lovell and White, 1986). They are predominantly associated with living parenchyma cells near secondary phloem, vascular cambium or rays cells (Hartmann *et al*, 1990).

In *Pinus radiata* it has been claimed that the development of this locus can be traced from a single cell (Smith and Thorpe, 1975). In some species there are pre-existing meristematic cells that are the 'pre-formed primordia'. In other

species, the meristematic locus is not discernable until later, when it consists of a few dozen to a few hundred cells (Lovell and White, 1986). This may be because the meristematic locus is induced by division and differentiation of a group of cells, as appears to happen in *Agathis*(White and Lovell, 1984b). In many species the formation of such a locus has not been identified. This may be because its formation and differentiation into a root primordia is quite rapid (Lovell and White, 1986).

2.3.6 Formation of root initials from callus

According to Hartmann, Kester and Davis (1990), the emergence of roots from callus has lead to the erroneous conclusion that callus is the site of root initiation. However, callus has been shown to be the site of root initiation in some difficult-to-root species (Lovell and White, 1986).

Adventitious roots initiating from callus have been described as being associated with 'tracheary nests', regions of xylem differentiating within the callus (Cameron and Thomson, 1969; Davies, Lazarte and Joiner, 1982; Sutter and Luza, 1993). Lovell and White (1986) describe the initiation of roots from induced vascular tissue as being a characteristic of woody plant root initiation. The vascular tissue is usually induced within callus which differentiates to form thin strands of tracheids. A root primordium forms below but some distance from the strand, close to the lower boundary of the callus (White and Lovell, 1984a; White and Lovell, 1984b). This pattern of root formation is very similar to that described in lettuce pith by Warren Wilson and Warren Wilson (1991). In lettuce, the formation of vascular strands and associated root initiation has been demonstrated to be due to the channelling of auxin flows through cells within the pith (Warren Wilson and Warren Wilson, 1993; Warren Wilson, Warren Wilson and Walker, 1991). It is likely that a similar pattern of auxin flows are occurring in the callus of other species, where this pattern of root initiation has also been observed.

The anatomy and morphology of root formation in *Malus pumila* transformed with *Agrobacterium rhizogenes* was studied by Sutter (1993). *A. rhizogenes* caused roots to form in two different ways. In the first type, roots formed from small balls of callus which had an outer layer of smaller densely staining cells. These balls of callus formed in the cortex and often developed roots and began to extend. Connection to the vascular bundles occurred much later and 2 to 3 mm further up the stem. Transformed cultures of *M. pumila* also underwent

root initiation in basal callus. "Growth centres" formed in the callus, with vascular elements visible at an early stage. These may be similar to the 'tracheid nests' referred to by Hartmann, Kester and Davies (1990). These vascular elements appear to form into roots, which later develop vascular connections to the main stem. In this instance it appears that the roots are forming in association with partially differentiated vascular tissues.

2.3.7 Further development of the root initial

Following the formation of the root meristem initial cells, the development of the root primordium appears to proceed along a similar pattern in most species studied (Lovell and White, 1986; Harbage, Stimart and Evert, 1993). The root primordium develops the typical pattern of cell divisions, including a root cap and vascular connections to the stem, then extension growth commences.

2.3.8 Theories about stem barriers

The research by Beakbane (1961; 1969) into physical barriers within stem structures has, at times, been cited as indicating a cause of low rooting ability. Beakbane proposed that there is a relationship between anatomical structures and rooting ability over a range of species, and within species between easy-to-root and difficult-to-root clones. Most attention was focused on the schlerenchymous sheath that is often derived from the primary phloem in many species. Beakbane claimed that this sheath may be either a mechanical barrier to root emergence or a physiological barrier to root formation. Most subsequent research and discussion has concentrated on the possible existence of mechanical barriers to root emergence (Hartmann *et al*, 1990; Hasan and Dodd, 1989).

Beakbane also proposed that phellogen layers formed by some plants may be some form of barrier, particularly as it forms over the distal end of the ray cells, providing a continuous layer of non-living cells around the stem. Secretory canals are formed from primary phloem in some species. It was suggested that these could break the cytoplasmic connections with the secondary phloem and that this may interfere with root initiation.

There are a number of species where the pattern of root initiation and the distribution of structural tissues within the cutting has been studied. In most instances, root inhibition has been identified as occurring at the stage of root initiation rather than root emergence (Lovell and White, 1986). This makes the concept of physical restrictions on root emergence seem less plausible.

Little research has been devoted to the second part of Beakbane's hypothesis: that physical structures, such as lignin, suberin bands or resin canals in stems, may be part of a physiological restriction on root initiation. This is despite the observation by White and Lovell (1984b) that such structures are correlated with reduced root initiation. Williams, Taji and Bolton (1984) discount the possibility of physical restrictions being a significant factor in preventing root initiation in a range of Australian native plants. However, they point to poor rooting being well correlated with suberisation of the cortex. No explanation of a possible mechanism for this observation was offered.

2.3.9 Summary

The studies of the effect of auxin on root initiation have been hampered because relatively little is known about exactly how the presence of auxin in some cells triggers the start of a host of metabolic changes and cell divisions that result in the formation of a root primordium.

Similarly, there is much known about the patterns of cell division and the anatomical changes that occur during root initiation but little about how auxin triggers the process of root initiation or what drives the process once triggered.

More information about the process of root initiation can be obtained from studying how manipulation of the growth, environment and physical attributes of a cutting affect its root initiation potential.

2.4 MANIPULATION OF ROOT INITIATION

2.4.1 Introduction

A wide array of treatments have been applied in an attempt to manipulate the 'natural' rooting response of a cutting. Treatments ranging from centrifugation (Cristoferi, Filiti and Rossi, 1988) to coating cuttings with cow dung, cited in Eldridge (1993), p 234. Many of the treatments that have been developed over the years are specific to a particular variety or species (Couvillon, 1988) and results are often highly inconsistent (Loach, 1988).

Manipulation of root initiation is achieved in one of two ways; by applying treatments to the stock plant prior to collection of the cutting or applying a treatment to the cutting after it has been collected. Most treatments that can be applied to stock plants have been adopted from the practical experiences of horticulturalists. According to Davies and Hartmann (1988), by applying a treatment the stock plants are being physiologically conditioned to develop competent cells that will be able to de-differentiate and develop into meristematic regions within the cutting.

2.4.2 Stock plant environment

Seasonal differences have been noted in the capacity of cuttings to initiate roots. Such variations are often seen even if cuttings are taken from greenhouse grown plants (Moe and Andersen, 1988). This fluctuation may reflect the seasonal variation in temperature, irradiance level and photoperiod which in turn affect the physiological status of the plant such as, flowering vs non-flowering, dormant vs not dormant. Seasonal effects are also important in propagation of many *Eucalyptus* species (Hartney, 1980) including *E. nitens* (de Little *et al*, 1992).

In many of the studies it is difficult to determine the extent to which temperature and irradiance are affecting rooting capacity directly or are acting via a general growth promotion or stimulatory effects of higher carbohydrate levels.

2.4.2a Temperature

There are apparently relatively few studies that relate stock plant temperature to root initiation success. Of the work that has been carried out, much of it relates to softwood plants where it is suggested that lower temperatures may favour root initiation in subsequent cuttings. Moe and Anderson (1988) suggest there is little difference in the rooting ability of cuttings collected from plants growing in air temperatures between 12°C and 27°C.

One factor noted by Anderson (1986) is that different temperatures almost certainly means a change in growth rate with consequences for C/N ratios, meristematic activity and possibly even nutrient uptake and use. All of these may be more important than the actual temperature at which the plant is growing.

2.4.2b Irradiance

According to Moe "Increasing stock plant irradiance can have a positive, negative or no effect on rooting"! (Moe and Andersen, 1988) Much of the research refers to qualitative changes rather than quantitative changes in light intensity, for example, shaded vs unshaded.

Moe divides the experimental effects into four types.

Group 1: Stock plant shading. Has been found to be beneficial in some cases, probably because light intensities are above optimal, during summer, resulting in plant stress. Shading reduces this stress.

Group 2: Low natural light + supplemental light. Winter light levels are below those optimal for growth, hence supplemental lighting enhances growth and root initiation.

Group 3: Growth cabinet experiments, where variations in light quality have a greater effect than light intensity.

Group 4: Etiolation of stock plants. Mostly confined to woody plants. Probably most usefully considered as a cutting effect rather than general stock plant physiology effect as it can be localised to a small region on the potential cutting, as in stem banding.

Marczynski and Joustra (1989) suggested that their results on the effects of high and low light levels on stock plants and cuttings could be interpreted in

terms of there being both supra and sub-optimal levels of carbohydrate. Certainly this would explain some of the results particularly with respect to low light levels on the cuttings.

It is difficult to explain all the reported effects of irradiance solely in terms of altered carbohydrate levels in the cutting (Veierskov, 1990). Altered auxin levels due to higher rates of auxin metabolism and production of essential co-factors in high light levels have also been proposed for some species (Christensen, Eriksen and Andersen, 1990), as has altered auxin sensitivity (Hansen, 1987).

2.4.2c Photoperiod

There is a diversity of response to alterations in photoperiod in stock plants. Some species root better after exposure to long days and some to short days (Hansen, 1987). Effects of photoperiod on rooting ability are assumed to relate to induction of flowering and dormancy.

Generally, it is considered that cuttings should be free from flower buds when selecting cutting material as there is an antagonistic effect between flower initiation and rooting ability, particularly in herbaceous plants. This has been linked to enhanced levels of gibberellins (Moe and Andersen, 1988).

2.4.2d Light quality

There is little work on the effects of light quality on rooting ability except where it relates to the onset of dormancy (Moe, 1988). Some reports on the effects of light quality in vitro have suggested a role for a phytochrome in shoot and root initiation (Economou and Read, 1987).

Increasing the ratio of red to far red light in *E. grandis* stockplants increased the internode length and improved root initiation rates (Hoad and Leakey, 1992). There was no difference in total dry weight accumulation between the different treatments. However there was an increased proportion of the total dry weight in the stems (Hoad and Leakey, 1994). It is also known that red light affects auxin sensitivity in some plants (Jones *et al*, 1991; Libbenga and Mennes, 1995).

2.4.2e Etiolation and banding

Etiolation of shoots or application of opaque bands to the stem prior to taking cuttings is a technique that is practised extensively in propagation of woody plants, particularly fruit trees and ornamentals (Maynard and Bassuk, 1990). Its use has not been recorded for eucalypt propagation (Maynard, 1988).

Banding, the localised exclusion of light from a stem section prior to collection of the cutting is achieved by applying an opaque material such as pvc tape to a stem. It is most effective when the band is placed on the stem as close to the growing tip as possible but is generally less effective than etiolation (Howard, 1994). Increased effects have been reported when Indole-3-butyric acid (IBA) was impregnated in the band applied (Avanzato and Cappellini, 1988).

Absence of light seems to be the critical factor in the treatments. Various experiments have excluded environmental effects such as increased temperature or humidity inside the banded zone (Maynard and Bassuk, 1988). Research in several species suggests that the effect of light exclusion is not transmissible and that the enhanced effect on root initiation is only expressed in those tissues where light has been excluded (Bassuk and Maynard, 1987).

It is believed that the effects of both etiolation and banding are related to a great increase in auxin sensitivity of the stem and anatomical changes that occur as the stem matures (Hartmann *et al*, 1990). The anatomical changes that have been noted includes reduced chlorophyll, increases in internode length, decreased lignification and sclerification, increased proportions of undifferentiated parenchyma and decreased suberisation (Maynard and Bassuk, 1988; Schmidt, 1989). Howard (1994) has recorded that there is an inverse relationship between stem diameter and rooting success in etiolated stems. It is proposed that this alters the carbohydrate relations within the stem, resulting in greater root initiation.

Etiolation may increase the level of auxin found in tissues after cuttings are taken. However some of the studies published on this aspect were using bio-assays, some with questionable reliability (Bassuk and Maynard, 1987). More recent experiments have shown that etiolation may increase auxin activity or sensitivity to auxin in the etiolated tissues (Sun and Bassuk, 1991).

The effects of etiolation on the presence or absence of secondary metabolites has also been studied. Poly phenol oxidase (PPO) activity has been found to increase in dark grown stems while IAA oxidase activity declined. Decreased IAA oxidase activity might be attributed to changes in the phenolic acid biosynthesis pattern (Maynard and Bassuk, 1988). However the role of phenolics, IAA oxidase and PPO is open to question, see section 2.5

2.4.2f Carbohydrate levels and adventitious root formation

Carbohydrates have long been thought of as a possible controlling influence in adventitious root formation, originating from work in 1918 using tomato stem segments (Veierskov, 1988). Despite numerous attempts, the validity of this hypothesis is still open to doubt. This is due to the difficulty of producing stock plants with altered carbohydrate levels without altering other critical factors in root initiation. The best evidence comes from peas where temperature sensitive mutants were use to alter carbohydrate levels at constant light levels. This showed that there was correlation between root number and carbohydrate level at one light intensity but not at a lower light intensity (Veierskov, 1990).

It seems logical that very low tissue carbohydrate levels are correlated with low root initiation since the cuttings are likely to have such a slow rate of development (if any at all) in the generally low light, low photosynthesis conditions of mist-beds. Haissig (1986) offers the view that there is a relationship between carbohydrate status of stock plants and rooting ability of cuttings but that the means by which that relationship is effected has not yet been determined. Sugars have been proposed to be auxin synergists (Jarvis, 1986).

2.4.2g Summary of temperature and light effects

There is no doubt that the light conditions in which the stock plant is grown can have significant effect on the ability of cuttings to initiate roots. It is also clear that the results that have been obtained do not allow the effects of light on stock plants to be attributed to any one mode of action.

It is probable that light interacts with rooting ability at several levels

a Seasonal effects of dormancy and flower initiation

b General effects of photosynthate accumulation on cutting vigour and carbohydrate reserves

c An etiolation/light exclusion effect that seems to result in altered stem anatomy, increased auxin sensitivity and other changes mentioned above.

All of these have the potential to interact in quite complex ways to produce a host of different root initiation responses. The responses would be expected to vary between species and even between clones. Of the three effects the etiolation/light exclusion effect may be the most important in terms of understanding how root initiation is controlled.

2.4.3 Stock plant nutrition

Stock plant nutrition research has generally focused on carbon to nitrogen ratios, calcium and boron. Some nutrition effects may also relate to enhanced stock plant growth rates that allow maximal cutting production and vigour.

2.4.3a Carbohydrate levels and carbon/nitrogen ratios

There have been numerous studies on the effect of nitrogen nutrition and how carbon to nitrogen ratios affect root initiation. Some of these are concentrated on how carbohydrate and nitrogen levels affect subsequent growth rates of the isolated cutting. This ensures that there is nitrogen within the cutting available for protein synthesis and metabolic processes relating to rapid cell growth in the new primordium. There are also effects of leaching of solutes in the period before new roots begin to actively absorb (Hartmann *et al*, 1990).

It has been demonstrated several times that varying carbon to nitrogen (C/N) ratios is correlated to rooting percentage. Plants with high C/N ratios , obtained by low N fertilising, generally have higher rooting potential than plant with low C/N ratios. Veierskov (1988), suggests that the effect of low nitrogen nutrition might be due to its effect in increasing carbohydrate reserves within the cutting. The total tissue nitrogen level may not be the key factor and specific pools of nitrogen within the plant are likely to be more critical. There is also some question about the importance of different amino acids during root initiation, but the picture is by no means clear.

Leakey (1983) demonstrated that *Triplochiton scleroxylon* shoots containing large quantities of nitrogen relative to carbohydrate are likely to root more readily. Some of this may be attributable to changed patterns of assimilate transport and perhaps apical dominance. The upper shoots on low nitrogen status plants root best but in high nitrogen plants lower shoots have improved rooting ability with no noticeable effect on the rooting ability of upper shoots.

It is necessary to identify the effect of ensuring that cuttings have adequate carbohydrate reserves to allow rapid cellular processes during the critical root initiation phases. These may be separate from direct influence that the carbohydrates may have on the degree of root initiation that takes place. For example, if there is a very short critical period during which auxin levels are such that initiation can occur and carbohydrate levels are low at that time the cutting metabolic rate may be insufficient for all the necessary cell divisions to occur before the auxin levels change. This is probably a different role for carbohydrates to that proposed by Warren Wilson, *et al* (1994) where sucrose is proposed to play a regulatory role in differentiation.

2.4.3b Mineral nutrition

Very little work seems to have been done on the effects of other minerals on the rooting ability of cuttings. In particular, the effects of stock plant nutrition and the potential to affect the "hardness" of the cutting has not been researched. This must have implications for the ability of the cutting to undergo initiation. Does it affect the onset of lignification?. It will also have an effect on the ease with which a cutting is affected by pathogens during the root initiation phase

Calcium is often cited as being important in terms of general stock plant nutrition but is mostly claimed to be immobile and therefore not important once the cutting is taken (Blazich, 1988b). This ignores the critical role Ca plays in auxin transport, wound responses and general signal transduction in plant cells and is termed a simplistic view by Trewavas, Read and Knight (1994) who claim that "Ca^{2+} stands at the heart of life itself".

There has been some consideration of the effects of NPK levels in the cutting but again this seems to be mostly linked to the need for those elements to be available for the metabolic processes that occur during root initiation, rather than of inductive competence. Mn and Zn have been studied a little because of their role in auxin metabolism.

Boron has been demonstrated to be important in root initiation (Haissig, 1986) but the main influence seems to be after root initiation. Large numbers of roots can initiate in peas but do not develop without boron being present (Jarvis, 1986). It has also been demonstrated that boron is necessary for a second phase of root development in *Phaseolus* after an initiation phase using either IBA or vitamin D_2 (Jarvis and Booth, 1981). This would seem to fit well with the observation that inhibition of meristematic cell division is one of the first symptoms of boron deficiency (Salisbury and Ross, 1992), possibly due to a role in nucleic acid synthesis (Lovatt, 1985; Ali and Jarvis, 1988).

2.4.4 Pruning, hedging and grafting

The observation that adventitious rooting ability of many woody plants decreases markedly with ontogenetic age has lead to the adoption of strategies that give the appearance of reversing or retarding the maturation process. This process is used extensively in ornamental horticulture to produce the desired type of material when it is needed (Scott, 1987). There needs to be some qualification about giving the appearance of retaining or regaining juvenility as in most cases the shoots that result from such treatments have some mixture of juvenile and mature plant characteristics. For example, apple root stock clones grown in stool beds are reported to retain the rooting ability of juvenile plants but are able to flower from one year old wood (Hackett, 1988).

It has been held that much of the benefit to be obtained from hedging is due to rejuvenation, the production of shoots with enhanced shoot vigour (Howard, 1994). While others claim that the effect is more to do with regaining some features of juvenility (Paton *et al*, 1981). Severe pruning or hedging is used extensively in the production of *Pinus*, *Malus* and *Eucalyptus* cuttings to enhance rooting ability. In *Eucalyptus* and *Malus* the beneficial effects appear to be ongoing while in *Pinus* the benefits are lost after a few years production necessitating the establishment of fresh hedges (Haines, 1992).

2.4.5 Growth regulators

There have been relatively few studies on the effects of applications of plant growth regulators to stock plants to improve the root initiation ability of subsequently collected cuttings. Applications of auxins and ethephon have been

found to increase root initiation, while applications of cytokinins decreased root initiation (Preece, 1987). Close timing of application of auxin and collection of the cuttings was found to be critical. It seems likely that the effect of the auxin sprays was simply to raise the endogenous auxin level.

Cytokinin applications have also been used to induce lignotuber bud break in *Eucalyptus ficifolia* (Mazalewski and Hackett, 1979; Salmon, 1990). The shoots produced from such treatments have been found to have a similar root initiation rate to those obtained from basal coppice shoots.

A number of anti-gibberellin growth retardants such as paclobutrazol have been tested for their ability to enhance rooting. It has been found that decreasing the endogenous gibberellin content of tissues can increase the rooting response of some species, but may also decrease the rate of root extension (Davis and Sankhla, 1988). It has been noted that the effects of these growth retardants is not restricted to gibberellin synthesis and may also affect auxin , cytokinin and sterol metabolism.

2.4.6 Size type and timing of cutting collection

Most plants root best from young and vigorous tissues but in some species softwood or semi-hard wood cuttings do not appear to root as readily as hardwood cuttings (Couvillon, 1988). Ability to withstand moisture stress and pathogen attack are factors that can influence the type of plant material used for propagation.

In eucalypts, the relative importance of subtending leaves, stem diameter and shape and position of the node with respect to the apical shoot have all been found to affect the success of root initiation (Wignall, Brown and Purse, 1992; Wilson, 1993; Wilson, 1994a; Wilson, 1994b). It is likely that much of this relates to the accumulation of carbohydrate reserves within tissues as they mature. There is some evidence that carbohydrate reserves in relation to the volume of cells which must be sustained by those reserves is an important consideration in rooting ability (Leakey, Dick and Newton, 1992; Dick, Dewar and Leakey, 1992).

2.4.7 Auxin applications to cuttings

Rooting in most woody plants is improved by application of additional auxin in the form of either a powder or liquid dip. The duration of exposure and concentration are both important and problematic. Hartmann, Kester and Davis (1990) report that there are some instances where powder dips outperform liquid application but these are less common. Auxin application by dips produces highly variable results even under the most carefully controlled conditions. A replicated trial to compare the efficacy of powder vs liquid dips was arranged with 11 nurseries using identical clonal material. Of the 44 comparisons, 22 favoured liquid dips, 21 favoured powder and one found them equivalent. Only two of the nurseries were able to obtain consistent results over two years but found in favour of different treatments (Loach, 1988). Other substantial variations in the repeatability of auxin dip experiments are reported in the same paper.

There is an implicit assumption that some of the success from IBA and α-Naphthalene acetic acid (NAA) might be due to its greater stability and assumed persistence in the plant tissues (Jarvis, 1986). When measured, most of the applied IBA has been found to have been metabolised, probably conjugated, within 24 hours of application (Epstein and Ackerman, 1993).

It is also frequently reported that the K^+ salts are more effective at promoting root initiation than the free acids, although little use is made of this knowledge (Blazich, 1988a). Mixtures of auxins are claimed to be more effective at promoting root initiation (George, 1993), a fact which is reflected in the formulation of some commercial rooting powders (Hartmann *et al*, 1990).

The amount of auxin that a cutting needs will be a function of its current endogenous auxin content, its tissue sensitivity and the rate at which it metabolises or conjugates auxin. None of these are easy to measure and may vary in response to a number of environmental and cultural factors. The fate of applied auxin has been studied in only a few instances but has been found to be rapidly transported throughout the cutting (Hartmann *et al*, 1990). Rooting ability was not correlated with IBA transport rates in *Leucodendron* cuttings in that both easy to root and difficult to root varieties had similar transport rates. In basal dip IBA applications the amount of IBA accumulated in *Leucodendron discolor* leaves was 34-45% in easy to root cuttings and 10%

in difficult to root varieties (Epstein and Ackerman, 1993). This was despite the fact that both types of cuttings were found to have similar auxin absorption rates.

Cytokinins have occasionally been applied to stem and leaf cuttings with positive results. Although usually considered as inhibitors of root initiation, van Staden and Harty (1988), consider that there is room for re-evaluation of their role in root initiation. This is particularly in view of the higher levels of endogenous cytokinins that are generally measured in juvenile tissues, that have higher rooting potential.

Anti-gibberellin growth retardants such as paclobutrazol, chlormequat chloride and morphactins have been used to promote root initiation. However it appears the results are variable and may be species dependent (Davis and Sankhla, 1988). It is assumed that the effect of the growth retardants is to reduce gibberellin levels in tissues (Pan and Zhao, 1994). Gibberellins are also thought to inhibit the earliest cell divisions of the root initiation (Jarvis, 1986).

Abscisic acid (ABA) applications have shown to both enhance and inhibit root formation, to the extent that it has been claimed that any rooting response is possible to ABA, merely by adjusting the experimental procedure (Rassmussen and Anderson, 1980). ABA counteracts the effects of gibberellins in many physiological processes, which may provide an explanation for its effects on root initiation. It also induces stomatal closure and thus may reduce moisture stress on cuttings (Davies, 1995b), which could also have a positive effect on root initiation.

2.4.8 Propagation conditions

Maintaining optimum propagation conditions is obviously important to obtaining maximum root initiation. This will consist of two sets of factors, minimisation of stress and optimisation of physiological processes.

Reduction of physiological stress, principally moisture stress, is necessary to ensure the survival of the cutting in the period between severance and the establishment of functional roots. High humidity is required to reduce transpirational loads on the cutting. Maintaining low light levels and low temperatures also assist in this.

Protection from pathogen attack is important because the high humidity, low light environment in which cuttings are maintained is highly suitable for pathogen attacks. The act of severance also provides a wound through which pathogens can enter the plant.

The need for a low stress environment must be balanced against the need to maintain an environment that will allow the cell divisions leading up to root initiation. The temperature of the root initiation zone is believed to be the most important factor. Temperatures between 18°C and 25°C are considered adequate for temperate species but the optimum for a particular species needs to be determined. Hartmann, Kester and Davies (1990) note that there is some evidence that the initiation phase has a different optimum temperature to that for optimum root development. Light and moisture conditions are mostly determined by the need to reduce stress on the cutting. Light levels must be high enough to allow some photosynthesis lest carbohydrate levels become depleted but there is evidence that root initiation is negatively correlated with light intensity (Loach, 1979).

Optimal propagation conditions are important to the ultimate rate at which root initiation is expressed by a group of cuttings. However there is nothing to suggest that it is possible to alter a cutting's intrinsic rooting ability once it is on a mist-bed.

2.4.9 Summary

Most of the manipulations applied to the stock plant and to cuttings have been derived from practical observation and nursery practice. Many of these practices have subsequently been tested experimentally (Haissig and Davis, 1994) but in many cases the physiological basis is yet to be determined.

Optimisation of environmental variables is capable of improving root initiation in most cases. It seems unlikely that major advances in the understanding of root initiation will arise from such studies. It is the view of Hassig, Davis and Reimenschneider (1992) that such manipulations are also unlikely to provide the answer to the problem of root initiation in recalcitrant species. As they point out, '*these environmental factors seem to influence rooting but there is little or no evidence that any of them control them*'.

Considerable effort has been devoted to identifying what controls root initiation. In particular there have been a number of theories about the role played by rooting inhibitors promoters and co-factors.

2.5 NON-HORMONE ROOTING PROMOTERS AND INHIBITORS

2.5.1 Introduction

The ability of cuttings to initiate roots varies with species and clones and within clones over time. The fundamental biology of the development sequence in root initiation is not yet fully understood. Perhaps as a consequence, there have been many theories developed that attempt to explain all or most of the variation in rooting ability in terms of the existence of specific rooting inhibitors or the absence of essential promoters (Haissig and Davis, 1994).

A number of compounds have been proposed as inhibitors and/or promoters of root initiation, for example G compounds (Paton *et al*, 1970), phloroglucinol (Welander and Huntrieser, 1981), derivatives of cinnamic acid (Curir *et al*, 1993) and derivatives of ellagic acid (Vieitez and Ballester, 1988) while the existence of others has been speculated upon (Haissig and Davis, 1994). As noted by Wilson and van Staden (1990) ,the list of compounds proposed as rooting co-factors with auxin or promoters of root initiation in their own right is extensive, and appears to be still growing.

According to Wilson and van Staden (1990), many of the studies have relied on bioassay techniques in species other than those from which the compounds under investigation were originally extracted. Others have made assumptions about endogenous hormone levels without measurement of hormone levels or by use of bioassay measurement. This is a technique which has its own limitations, particularly if auxin inhibitors are inferred to be present. Other studies make assumptions about the physiology of hormone action, for which there is only limited supporting evidence.

Given the extensive reporting of the existence of rooting promoters and inhibitors, it would be surprising if all of them were eventually proven to have no role in the promotion or inhibition of root initiation. What seems to be lacking at the moment is a clear understanding of which substances are actually functioning as promoters or inhibitors. Correlation between the occurrence of a compound and a particular rooting response does not prove that there is a causal link between the two. This can only come with evidence of the biochemical mechanism by which the compound can exert its effect.

G compounds isolated from *Eucalyptus grandis* are claimed to present the strongest evidence for the existence of rooting inhibitors that can play a controlling role in adventitious rooting (Hackett, 1988). Phenolic compounds are regarded as being of importance in root initiation and are thought by many to influence endogenous auxin levels (Jarvis, 1986). The evidence for a specific role for phenolics is not clear but is claimed to be via an inhibitory influence on IAA oxidase, resulting in increased auxin levels (George, 1993).

Vitamins D_3 and D_2, cholecalciferol and ergocalciferol, and some of the related degradation products of sterols have been shown to be able to promote root initiation in a number of species (Buchala and Pythoud, 1988). They act primarily as synergists of applied IBA but can promote root initiation when applied alone.

2.5.2 G compounds

G compounds were first isolated from *Eucalyptus grandis* as part of an investigation into the rooting inhibition associated with ontogenic aging in that species. They were isolated by extensive use of bio-assays to detect and monitor the presence of the inhibitors at each stage of the extraction and identification process (Paton *et al*, 1970). The compounds were identified as being three isomers of ß-triketone (Nicholls, Crow and Paton, 1970).
Haissig and Davis (1994) proposed that G compounds remain the most convincing evidence for the existence of rooting inhibitors that play a controlling role in adventitious rooting. However, it was noted by Haissig and Davis (1994) and also by Paton *et al* (1970) that the influence of G compounds in *Eucalyptus* species may be an exceptional instance rather than pointing to the general applicability of rooting inhibitors.

2.5.2a Occurrence of G compounds

The rooting inhibitor "G" was found to increase in concentration in successively older leaves of *E. grandis*. This increase correlated well with a decline in the ability of cuttings to form roots when taken from successively higher internodes (Paton *et al*, 1970). The inhibitor was subsequently found to consist of three closely related compounds. They are apparently found in most of the tissues of mature plants of *E. grandis*, although most research has been conducted using leaf extracted compounds. The relevance of concentrations of G found in leaves

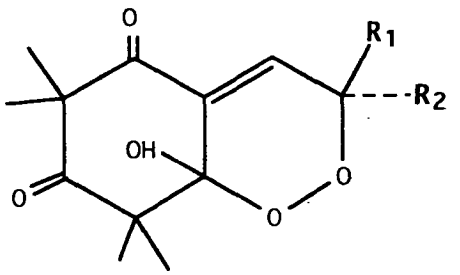
to root initiation occurring from stem tissues needs to be clarified. It has not yet been determined how closely the leaf concentrations of G correlate with levels found in stem tissue. Nor has the rate or extent of transport from leaves to other tissues been quantified.

Concentrations of G compounds have been claimed to reach 2×10^{-2} mol/kg in mature leaves, equal to approximately 6% of fresh weight (Bolte, Crow and Yoshida, 1982b). No evidence was cited for this claim. Dhawan, Paton & Willing (1979) measured maximum levels of 0.75%. This represents a concentration of $2x\ 10^{-3}$ mol/l, at which they have demonstrable inhibitory properties. Another compound, grandinol, has also been identified as an inhibitor (Crow *et al*, 1977). The effects of grandinol are less well characterised and it has not been the subject of as much scrutiny. All are believed to be derived from phloroglucinol (Crow *et al*, 1976).

2.5.2b The chemistry of G compounds

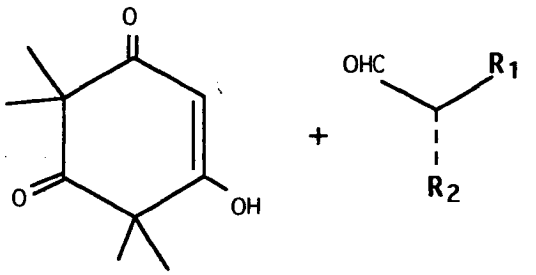
The structure of the three G compounds involved (ß–triketones), Figure 2.5a, was first published by Nicholls, Crow & Paton (1970). Subsequent studies confirmed the identity of the compounds (Crow, Nicholls and Sterns, 1971). Synthesis of the compounds from syncarpic acid has been demonstrated (Crow *et al*, 1976; Baltas, Benbakkar and Gorrichon, 1992). Analysis of intermediates in the biosynthetic pathway has discounted the possibility that the structures are artefacts of the extraction and purification process (Crow *et al*, 1976). Structural analogues have also been synthesised which have been found to possess similar biological activity (Bolte, Crow and Yoshida, 1982a).

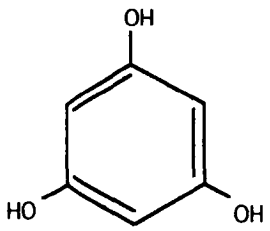
Potential inactive storage forms of the compounds have been identified, which would provide a plausible mechanism for the accumulation of very high concentrations that have been claimed for the compounds (Bolte *et al*, 1982b). At such high concentrations, the G compounds are highly inhibitory and would be quite damaging to tissues if some storage form did not exist or if the storage was not compartmented.



	R1	R2
G1	Et	Me
G2	Me	Et
G3	Me	Me

G compounds





Syncarpic acid and aldehyde precursor

Phloroglucinol

Figure 2.5a Structures of G compounds and precursors, adapted from Crow et al (1976).

2.5.2c Effects on rooting of G compound applications

Although the effects of exogenous applications of G compounds have been tested in a number of systems, the results obtained seem inconsistent. This is perhaps due to differences in test systems.

Nicholls, Crow and Paton (1970), showed that G compounds inhibited rooting in *E. grandis* seedling cuttings at 10^{-4} M but had little or no effect at 10^{-5} M. There was no firm evidence found of any promotory effects of the compounds at lower concentrations.

Dhawan, Paton & Willing's experiments (1979), using a mung bean bio-assay, detected inhibition of root initiation using a 5 x 10^{-4} M solution and promotion of root initiation at 10^{-5} M. The compounds were claimed to be twice as effective as the optimal IAA concentration, also 10^{-5} M. The concentration at which promotion of root initiation occurred was claimed to be equivalent to the level of G compounds found in juvenile *E. grandis* tissues. Each of the three

compounds have similar effects. No synergistic effects with IAA were detected using the oat coleoptile assay. G compounds were thought to be acting as a replacement for IAA. Promotion of root initiation was also observed in *Azalea mollis* and *Lagestroemia indica*, when G compounds were applied as a dry powder dip at a concentration of 0.5 mg/g.

In contrast, Menary (1992) found promotion of root initiation at 10^{-4} M in mung bean hypocotyls and no inhibition of root initiation was noted, except at 10^{-3} M, where physiological damage was believed to have occurred. Promotion of growth by G compounds was found to be synergistic with that of IAA when measured by the barley coleoptile assay.

2.5.2d Other reported effects of G compounds

Paton (1981) claims G compounds play a role in frost resistance in *E. grandis*. Frost resistance was well correlated with G compound levels at different node numbers. Leaves below node 7 had low frost resistance and G compound levels, those above had higher levels of G compounds and were better able to cope with frost. Seasonal variation in G content in two metre high saplings was also correlated with frost resistance. Maximum content (5 mg/g fresh weight) was measured in winter with levels of 3 mg/g found during spring and summer. There was significant diurnal variation measured in this experiment, with G compound levels decreasing by 50% during the first few hours of daylight.

G compounds also decreased water loss and increased stomal resistance in mung bean hypocotyls and in *Eucalyptus rupicola* cuttings (Paton, Dhawan and Willing, 1980). At the highest concentration used, 5×10^{-4} M, the rate of water loss was more than halved and was similar in effect to a 1×10^{-4} M solution of abscisic acid. A similar effect was noted on stomatal resistance.

Some anti-transpirant effects were also reported for *E. grandis* by Sharkey, Stevenson & Paton (1982), who showed that the reduced stomatal conductance in detached leaves was associated with decreased photosynthetic activity. This was claimed to be due to reduced photosystem II activity, as has been demonstrated for spinach chloroplasts treated with G compounds.

2.5.2e Role of G in basal shoots

Close proximity of mature epicormic buds to a grafted juvenile stock can reduce the level of G to a level sufficient to increase root initiation. The mechanism for this change appears to be via reduced synthesis rather than increased degradation or transport. This indicates that juvenile tissues that may be able to reduce G compound synthesis (Paton *et al*, 1970). This is proposed as the explanation for the high rooting capacity of juvenile and juvenile-derived shoots. They have a reduced G synthesis capacity that results in the level of G remaining in the concentration range that promotes rooting (Paton *et al*, 1970).

2.5.2f G inhibitors in other eucalypt species

G compounds have been isolated from *Eucalyptus grandis, E. delegatensis* and *E. melliodora*. Menary (1992) extracted compounds with G - like activity from *E. globulus* but was not able to obtain positive identification of the extract. They appeared to have different mobility in thin layer chromatography (TLC) separation and were thought to be a different compound. Harrison, Chandler & Hamill (1993) were unable to detect the presence of significant levels of G compounds in *E.nitens* using reverse phase HPLC over a range of clones with varying rooting ability.

2.5.2g Mode of action of G compounds

Dhawan, Paton & Willing (1979) have proposed that G compounds are actually growth regulators, with biological activity that is directly comparable to that of IAA. They cite evidence that it can directly replace IAA in promoting rooting initiation. However this does not coincide well with the observations of other researchers where auxin synergism has been found to be an effect at least as important.

The effects of G compounds on photosynthesis have been proposed to be a consequence of the role the G might play in frost tolerance via altered membrane organisation (Sharkey *et al*, 1982). Without direct measurements these proposals are only speculative.

Three possible modes of action were proposed by Menary (1992), that G compounds may:

a) be a competitive inhibitor for IAA;
b) interfere with IAA/IAA oxidase interaction and cause IAA to be above optimum for root initiation; or
c) act as a synergist for IAA oxidase and cause sub-optimal levels of IAA.

The first option is difficult to sustain as there is no evidence of competitive inhibition of auxin action from any of the studies so far and the G compounds do not appear to have any structural similarities that would allow for such inhibition. The difficulty with the latter two hypotheses is that there are relatively few direct measurements of auxin levels in eucalypts generally and, so far, none have been published in conjunction with analysis of G compound levels. There is also no clear understanding of what optimal, sub-optimal or super-optimal levels of auxin might be in eucalypts, or how the cuttings would react to them.

It is difficult to develop a mode of action that would explain all of the observed effects of G compounds on physiology the of plants. None of the models proposed so far have overcome this problem. It is also difficult to attribute too central a role to the compounds when they are not detectable in all difficult to root species of *Eucalyptus*.

2.5.3 Other promoters and inhibitors of root initiation

A degree of scepticism is developing about the relevance or importance of some reports on the presence of inhibitors and promoters of root initiation (Hand, 1994; Wilson and van Staden, 1990). In part, this stems from disagreement about whether treatments have an effect by removal of inhibition or by stimulation of rooting. Similar treatments have been variously reported as evidence for the blocking of inhibitors or acting as promoters of root initiation in different species.

2.5.4 Phenolics

It seems clear that applied phenolics are able to influence root initiation (Haissig, 1986; Jarvis, 1986) but the mode of action seems much less clear. Most attention has centred around a role for phenolics in the oxidation of auxin. It has long been assumed that there is active removal of free IAA from the metabolic pool by "IAA oxidase", peroxidases that decarboxylate the side chain. Phenolics have been clearly shown to be central to the metabolism of IAA oxidase. Monophenols and *m*-diphenols stimulate IAA oxidation, *p*-diphenols, *o*-diphenols, coumarins and polyphenols inhibit the enzyme (Bandurski *et al*, 1995). This knowledge has been coupled with many studies demonstrating a correlation between the occurrence of various phenolics in plant tissues and variations in the ability to initiate roots. The difficulty with these studies is that although there is a clearly understood metabolic pathway, there is very little evidence that this pathway plays any significant role in modifying IAA levels *in vivo*. The products of auxin oxidation do not appear to be present in plant tissues in significant amounts and transgenic plants with vastly altered peroxidase levels show no variation in IAA levels (Normanly *et al*, 1995).

The existence of auxin-phenolic conjugates that might play a significant role in root initiation has also been postulated (Bansal and Nanda, 1981; Hartmann *et al*, 1990). Such conjugates are held to protect IAA from the effects of IAA oxidase. However, there has been little evidence presented to demonstrate their existence *in vivo*.

Wilson and van Staden (1990) reviewed the role of phenolics and other promoters and came to the conclusion that the evidence for a defined role for phenolics in root initiation was lacking. Their favoured explanation was that high, but sub-lethal, levels of a range of chemicals tend to induce changes in cells that are a response to the chemical injury suffered by the cells but which mimic a wound response. They have also noted that the mung bean assay is not a reliable indicator of promotion of root initiation, an observation that is supported by the work of Al Barazi and Schwabe (1985)

Phloroglucinol and phloridzin have been demonstrated to be a synergist of IBA in the induction of roots in cuttings and tissue cultures of apples and some other species (George, 1993; Gur, Gad and Haas, 1988; Welander and Huntrieser, 1981). However it is only effective on some varieties and is harmful to some others. Both phloroglucinol and phloridzin were found to be most

effective in clones with high levels of PPO activity in their bark (Gur *et al*, 1988). They proposed that it was actually some oxidation product of phloroglucinol that was having the rhizogenic effect. G compounds found in eucalypts are also obtained by oxidation of phloroglucinol to syncarpic acid (Crow *et al*, 1976). Objections to the role proposed for poly phenol oxidases in rhizogenesis have been raised by Hand (1994). He has noted that PPO is localised within the plastids in intact cells and is only available to oxidise phenolics in wounded or damaged tissues. The mechanism by which phloroglucinol or its oxidation products obtain their synergistic effect has not been established.

The study of Curir *et al* (1993) was able to identify inhibitors of root initiation from hardwood cuttings of *Chamaelaucium uncinatum* Schauer. The inhibitors, derivatives of cinnamic acid were found to be present in hardwood cuttings possessing low rooting potential but not present in soft wood cuttings. The inhibitors were tested using mung bean bioassay. It was also found that the inhibitors were effective in softwood cuttings. However these results may be another example of co-existence not being related to causation. *Trans*-cinnamic acid is a precursor in lignin biosynthesis so it would not be at all surprising if hardwood cuttings were found to have higher levels of it. Flavonoids were assumed to be acting as IAA oxidase inhibitors (Curir *et al*, 1993). However the flavonoid quercetin has been demonstrated to play a role in auxin transport regulation that is similar to that of 1-N-naphthylphthalamic acid (NPA) (Faulkner and Rubery, 1992; Jacobs and Rubery, 1988). Quercetin and several other flavonoids have been proposed as natural auxin transport regulators. This may represent a more sustainable approach to defining a role for flavonoids

During the initiation of *in vitro* cultures of *Eucalyptus* species, accumulation of phenolics in the medium is often claimed to be the reason for cultures failing to grow (Le Roux and van Staden, 1991). Production of phenolics *in vitro* was believed to be due to a combination of wound response and exudates from senescing cells. Cauvin and Massoc (1992) found that rootability of clones was negatively correlated to phenolics concentration, but also showed that individual clones showed a much greater variation in phenolics concentration over time. Whether root initiation rates also varied with time was not recorded. Even if a correlation was found, it may simply indicate the rooting ability and phenolics concentration vary in response to the same factors, such as temperature or stress.

2.5.5 Vitamin D promotes root initiation

Root initiation has been shown to be promoted by vitamin D_2 and D_3 in *Populus temula*, *Vigna radiata* and *Cynara scolymus* (Buchala and Schmid, 1979; Jarvis and Booth, 1981; Moncousin and Gaspar, 1983). The evidence was reviewed by Buchala and Pythoud (1988). The effect of vitamin D is to increase the number of roots formed per cutting. There are no reports of an increase in the proportion of cuttings forming roots.

Buchala and Schmid (1979), first demonstrated that vitamin D_2, ergocalciferol, applied in the range of 10-50 mg/l is effective in promoting root initiation in seedlings and woody cuttings from *Populus tremula*. Ergosterol, the sterol precursor of ergocalciferol, was found to be ineffective. This seems curious as the inter-conversion between the two is by a reversible photo-isomerisation (Miller and Norman, 1984), see Figure 2.5b. Buchala and Schmid (1979) do not specify the growth conditions used for the experiment so there may not have been sufficient UV light to allow for the conversion to the seco-steroid. The effects of cholesterol applications on root initiation were not tested despite cholesterol being a precursor of vitamin D_3 and a common plant sterol (Hartmann and Pierre, 1987).

2.5.5a Metabolic functions of vitamin D

Beyond the effects on root initiation, there are no established metabolic functions for vitamin D in plants. Vitamin D plays a central role in calcium and phosphorus homoeostasis in animals. Vitamin D deficiency causes the disorder rickets. The occurrence of vitamin D in plants has been studied sporadically, mainly in association with reports of hypervitaminosis and anti-rachitic activity in animals (Buchala and Pythoud, 1988).

1,25 $(OH)_2$ vitamin D has been demonstrated to be the active form which reaches the nucleus in animals (Miller and Norman, 1984). This form of vitamin D was not detectable in the study by Pythoud and Buchala (1989). The enzyme needed for hydroxylation of 1, 25$(OH)_2$ vitamin D_3 is known in some plant species but no evidence of the product could be detected in aspen plants where vitamin D was promoting root initiation (Buchala and Pythoud, 1988).

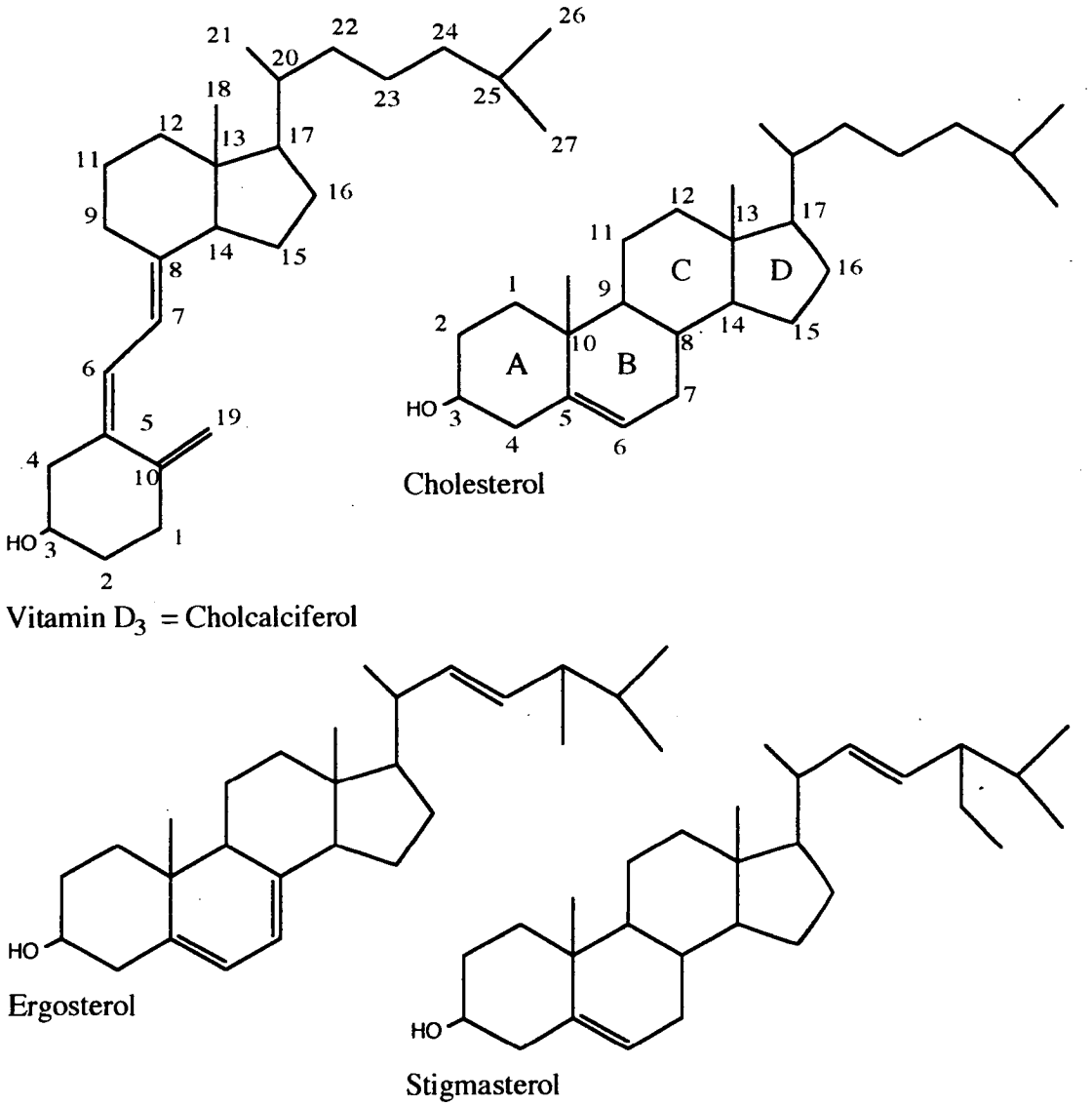


Figure 2.5b Structure of vitamin D_3 and related sterols

2.5.5b Possible mode of action of vitamin D in plants

Despite the work that has been done on the effects of Vitamin D on plants there is no clear evidence of a mode of action (Buchala and Pythoud, 1988). It is unlikely to be similar to the effects of vitamin D on animals, as few of the more active metabolites can be detected and they are less effective in plants. The synergism with auxin is not due to a transport effect as neither IBA nor vitamin D has an effect on the distribution of the other, nor does auxin metabolism seem altered (Pythoud and Buchala, 1989).

65% of labelled vitamin D was found to be in the membrane fraction of cells, with 89% being in the xylem and pith with a small amount found in the vascular cambium. Labelled vitamin D_3 is believed *"to become localised in the membrane system of of the parenchyma cells of the xylem"* (Buchala and Pythoud, 1988). They note that root initials in *Populus spp* form in the cambial ray initials. Buchala and Pythoud also speculate on a possible role in calcium metabolism, noting that vitamin D increases calmodulin synthesis without affecting degradation. Calcium uptake is stimulated in root sections, an effect that is prevented by protein synthesis inhibitors (Vega *et al*, 1985).

In *Phaseolus* (Buchala and Schmid, 1979) and *Populus* (Pythoud, Buchala and Schmid, 1986) it was also noted that a mixture of degradation products, largely devoid of vitamin D_3, appeared to have a similar effect as vitamin D_3 on its own.

2.5.5c Problems with practical use

One of the practical problems in interpreting the results of some of the previously published work on vitamin D is that several of the papers do not specify the light conditions under which the tests were carried out. This is an important omission given that vitamin D and its associated seco-steroids undergo rapid, reversible photo-isomerisation (Miller and Norman, 1984). It is possible that the vitamin D supplied to plants might be converted back to a provitamin, sterol form if the cuttings were exposed to natural light.

Vitamin D_3 is also a highly toxic compound, readily absorbed through the skin and is used as a rodenticide (Ware, 1989). It is readily oxidised in air, which restricts its potential for use as a practical treatment. Other, less toxic, sterols may be more useful if they have a similar effect on root initiation. In this respect, Talmon, Vega, Mujica and Boland (1989) showed that stigmasterol had a similar effect to vitamin D_3 on the stimulation of root extension. Stigmasterol is the sterol pre-cursor of vitamin D_6. , (see figure 2.5b). It is possible that stigmasterol is being converted to vitamin D_6, however this vitamin is claimed to be 80 to 200 times less effective than vitamin D_3 in animals as a calcium regulator (Hennessey, 1992).

2.5.5d Vitamin D and other sterols

Vitamin D_3, cholecalciferol, is derived from cholesterol, while vitamin D_2, ergocalciferol is derived from ergosterol (Miller and Norman, 1984). Cholesterol or its precursors have been demonstrated to be the starting material for the synthesis of all other plant steroids. Ergosterol, sitosterol and stigmasterol are the the most common sterols found in plants (Heftmann, 1977). Cholesterol is also widespread in plants but only is only a minor proportion of the total sterol content (Hartmann and Pierre, 1987).

Sterols normally compose about 5% of the dry weight of membranes, which is normally composed of sitosterol (ca 70%) stigmasterol (ca 20%) Campesterol (ca 5%) and cholesterol (ca 5%) (Harwood and Russell, 1984). In addition there may be sterol esters, glycosides and acylated glycosides, all formed via a 3 hydroxyl linkage. Ergosterol, the pre-cursor to ergocalciferol, vitamin D_2, is the main sterol produced by fungi but it is not normally found in plants (Harwood and Russell, 1984). Sterol biosynthesis is affected by pH, light and temperature, altering the proportions of different sterols occurring within a plant and their distribution throughout the plant.

According to Hennessey (1992) the primary function of sterols in plants is their ability to affect membrane structure and water permeability. Sterols affect the packing of membrane bilayers to increase the fluidity of bilayers which are below their phase transition temperature and decrease the fluidity of bilayers that are above their phase temperatures. The side chain of plant sterols is important in determining the degree of ordering of the bilayer that occurs.

The amounts and relative proportions of free sterols and sterol esters, glycosides and acylated glycosides vary substantially between plant species and within plants, although the total sterol content of plants is much less than in animal membranes. Because of this, it has been claimed that the action of sterols in modulating lipid fluidity and strengthening membranes is of much less importance in plant than in animals (Harwood and Russell, 1984).

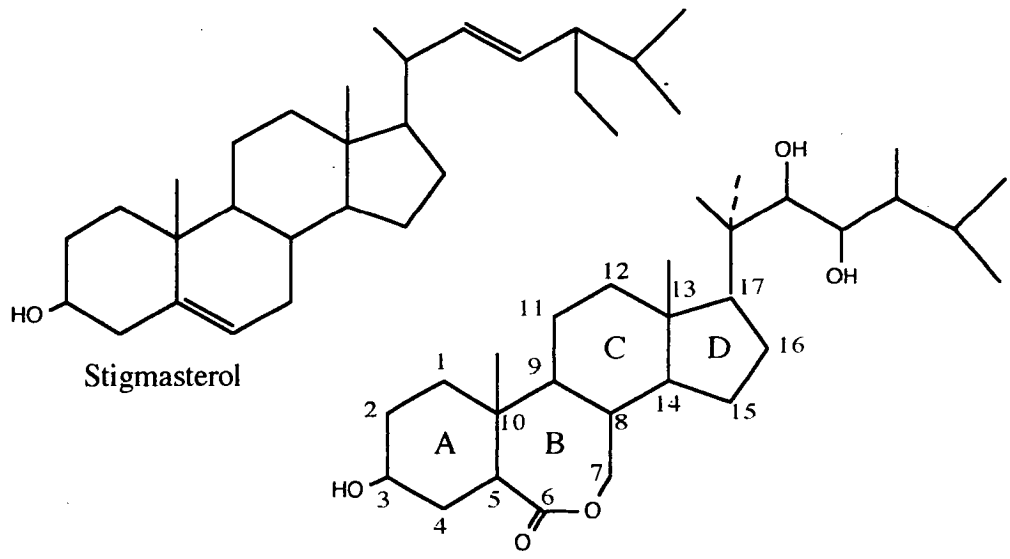
Sterols are also believed to play an important role in modulation of plasma membrane ATPase (adenosine triphosphatase) activity (Cooke *et al*, 1994). Cooke and Burdon (1990) also believe that the relationship between ATPase activity and membrane fluidity is inconsistent and that modulation of ATPase activity is more likely to occur by a more direct means. An inverse relationship

was found between ATPase activity and phospholipid to free sterol ratios in two studies (Burgos and Donaire, 1996; Douglas and Walker, 1984). Douglas and Walker (1984) speculated that this might be due to differences in membrane fluidity but no such correlation was found by Burgos and Donaire (1996).

2.5.6 Similarities to Brassinolides

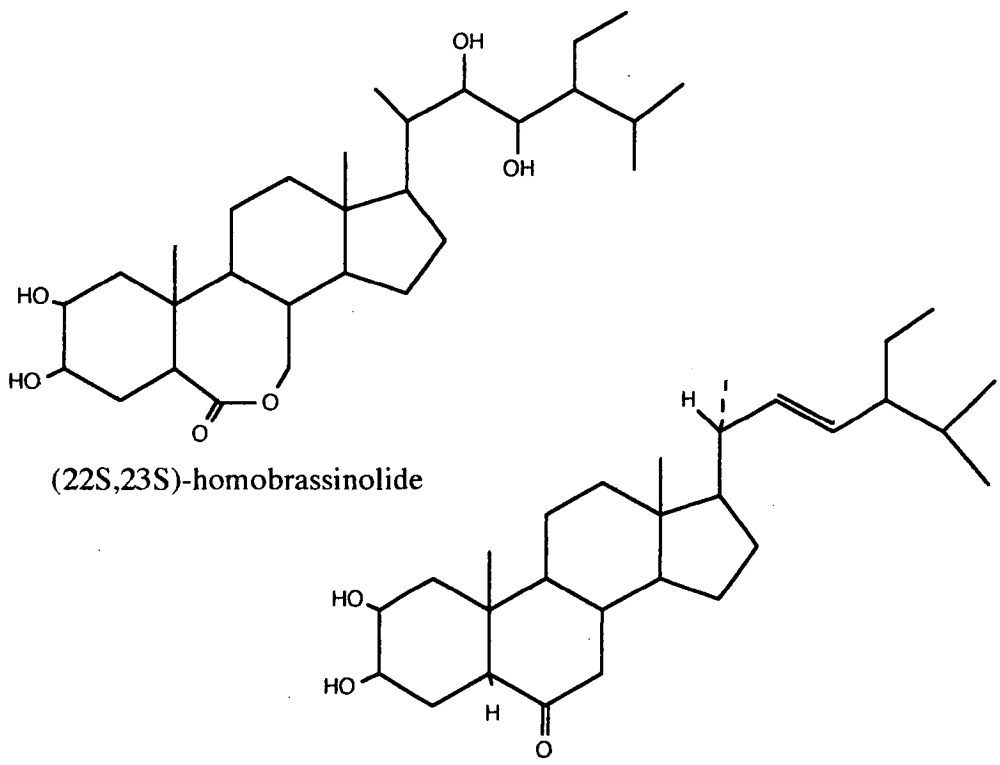
There are many similarities between the effects reported for vitamin D on plants and those reported for brassinolides. A review of the effects of brassinolides on plants (Arteca, 1995) lists many effects that are similar to those reported for vitamin D by Buchala and Pythoud (1988). Specifically, both Vitamin D and Brassinolide are claimed to be : auxin synergists; root extension inhibitors; promoters of ethylene biosynthesis; promoters of protein synthesis; and stimulators RNA synthesis.

There are also reports of brassinolides being able to promote root initiation. Gross (1994) demonstrated that cuttings of Norway spruce *Picea abies* had enhanced formation of adventitious roots when treated with 3 to 60 ppm (22S, 23S)-28-homobrassinolide (SSHB), which is a synthetic analogue of brassinolide. This compound has also been reported to stimulate the proton pump mechanism in the plasmalemma (Dahse *et al*, 1990) Interestingly, these researchers also showed that stigmasterol also had a similar effect, but only in the dark. It is known that stigmasterol is readily converted by photo-isomerisation to the seco-steroid and it might be concluded that this is the reason for stigmasterol's inactivity in light. 2α-3α-dihydroxy-5α-stigmast-22-en-6-one (DHS), a brassinolide derived from stigmasterol, was active in light as well as in dark. See Figure 2.5c.



Stigmasterol

Brassinolide



(22S,23S)-homobrassinolide

2α–3α–dihydroxy-5α–stigmast-22-en-6-one (DHS)

Figure 2.5c Chemical structure of stigmasterol and related brassinolides

Brassinolide decreased root initiation in mung bean bio-assays performed by Yopp, Mandava and Sasse (1981). The authors did not report the effect of brassinolide plus IAA in their assay despite other tests performed by them that showed a strong synergistic effect between brassinolide and IAA.

Brassinolides are usually thought to act at the level of regulation of gene expression (Arteça, 1995) , although Ronsch *et al* appears to favour the possibility that SSHB might be functioning as an anti-stress agent. Dahse et al (1990) are firmly of the opinion that SSHB and the sterols that they tested were exerting their effect by direct stimulation of proton
pumping. They also concluded that the mechanism by which the proton pumping is stimulated is different to those of both IAA and of fusicoccin. They concluded that it was most unlikely that the stimulation resulted from changes in membrane fluidity.

2.5.7 Conclusions

It is clear that G compounds do possess significant biological activity and their presence is well correlated with loss of rooting ability in *E. grandis*. However, until their production in the plant can be linked to some functionally significant, clearly defined mode of action, their exact relevance will be open to question.

Regardless of the significance of G compounds to *E. grandis*, the fact remains that there are a number of other species of *Eucalyptus* that exhibit similar patterns of loss of rooting ability with age where very little or no G compounds have been detected (Willyams *et al*, 1992; Harrison *et al*, 1993). There are three possible explanations for this: there may be different mechanisms inhibiting root initiation in different species of *Eucalyptus*; there may be other compounds that fulfil the same role in species other than *E. grandis*; or G compounds are not as significant to root initiation *in vivo* as has been supposed.

Other examples of rooting promoters and inhibitors are less convincing. Correlation between the occurrence of a particular compound and rooting ability is, in itself, not evidence of a direct role in root initiation. All are likely to remain difficult to prove until there is a better understanding of the mechanism of root initiation, at which time it will become apparent whether the modes of action proposed for inhibitors are relevant, or whether other roles must be found for them.

There seems to be enough evidence to support the theory that phenolics and flavonoids play some role in root initiation. The claim that phenolics behave as fine growth regulators able to act directly on the IAA level and influence tissue physiological state seems premature (Curir *et al*, 1993), particularly as IAA was not measured directly.

There is good evidence that vitamin D and some sterols are able to enhance root initiation in some plants but the range of species in which they act has not been tested. The prospects for defining a metabolic role for vitamin D in root initiation and auxin action seem higher than for other rooting promoters. There are similarities between the vitamin D and other bio-active compounds such as brassinosteroids. The crucial role played by steroids in animal physiology has lead to speculation of plant steroids playing similar roles (Heftmann, 1977). The mode of action is yet to be established. Possibilities include acting though altered bulk membrane properties or by a more direct and specific effect on ATPase activity. Either of these could affect auxin action and auxin signal transduction, which might affect any of the membrane associated cell functions.

Part of the problem in reviewing the mode of action of any of the proposed co-factors or inhibitors is that they are all assumed to be acting on, or in conjunction with, auxin. While auxin may be the primary determinant in root initiation, there are likely to be other steps in the root initiation process at which rhizogenic compounds or inhibitors can have their effect. Such compounds could act by direct inhibition of cellular processes or by diverting or disrupting cell divisions, causing fresh callus production. It may be the lack of knowledge of these other steps in the root initiation process that is holding back a proper understanding of the role of rooting co-factors and inhibitors.

2.6 CELLULAR PROCESSES AND ROOT MORPHOGENESIS

2.6.1 Root initiation at the cellular level

Study of the processes of root initiation relies on an understanding of the mechanisms by which undifferentiated cells and meristematic tissues are able to form into complex organisational units with varied but distinct tissue types growing in a co-ordinated, regular pattern. Such spatial ordering and co-ordination depends on the existence of a polarity within the tissues, a defined axis of orientation, usually the root shoot axis (Nick and Furuya, 1992). It is the establishment of polarity of growth that is the crux of all morphogenesis. Regrettably, current knowledge of this crucial area is "fragmentary" (Thorpe and Kumar, 1993).

Over the last thirty years, plant cell, tissue and organ culture research has provided most of the advances in the understanding of cellular control of morphogenesis that have occurred. Much of what is known has been derived from studies of shoot initiation but seems to be applicable to all organogenesis.

That the processes of root initiation and shoot initiation are closely allied is illustrated by two examples. One concerns pre-existing root primordia found on stem internodes, which convert into shoot primordia. This occurs readily in the species *Sesbania rostrata* without additional hormones and without an intervening callus phase (Spenser-Barreto and Duhoux, 1994). In this species, all that is needed to stimulate the meristems to change from root to shoot primordia is the isolation of the internodes from apical or axillary meristems by excision.

The second example is from studies of the *rolB* gene of *Agrobacterium rhizogenes*. It has been demonstrated that the effect of the *rolB* gene is not simply to cause greater initiation of roots but to generate a greater number of meristems, from which, under normal circumstances, roots develop (Altamura *et al*, 1994). Using thin cell layers, they showed that there was a substantial increase in the number of neo-formed meristems, regardless of the differentiative commitment and destiny of the meristems later. Both rooting and flowering were enhanced by the RolB protein, which promoted meristem organisation. The newly formed meristems then evolved into either root or

flower primordia, depending on the hormonal conditions in the culture medium and possibly on the origin of the explant.

These cases demonstrate that the control of root and shoot morphogenesis share some common features. Full understanding of the processes controlling morphogenesis requires a detailed knowledge of the phases of differentiation that lead to the formation of new meristems. This general information on differentiation can then be related to the specific case of root primordia formation and its failure to occur in some situations.

2.6.2 Phases of organogenesis

Thorpe and Kumar (1993), describe three stages in organogenesis which consists of: attainment of cellular competence; induction of the new meristematic locus and post-initiation expression and development.
A four stage process is defined by Blakesley (1994), namely: induction of a new meristematic locus; early cell divisions; later cell divisions to form an organised, determined meristem; development of the root by extension growth and the establishment of vascular connections to the stem. This latter scheme seems to place less emphasis on the effects of tissue sensitivity and pre-conditioning on the ability to undergo morphogenesis.

According to Thorpe and Kumar (1993), the attainment of competence by cells is similar to dedifferentiation but does not always involve proliferation of cells to produce callus. It may not involve any cell division at all. In many plants, initiation of new meristems occurs directly from pre-existing parenchymous or cambial tissues (Lovell and White, 1986). In other plants, particularly woody plants, there may be extensive callus development from which primordia may develop directly or in association with xylem elements that are induced within the callus (White and Lovell, 1984a; White and Lovell, 1984b; White and Lovell, 1984c; White and Lovell, 1984d).

It is generally believed that the first discernable event in the formation of a new meristem is the development of a small group of cells with an enlarged nucleus, dense cytoplasm lacking vacuoles and lacking polarity of cell division (Lovell and White, 1986; Moncousin, 1991). As few as two cells have been identified as the beginnings of meristems (Smith and Thorpe, 1975) but all develop a common structure during subsequent phases.

2.6.3 Meristem organisation and function

Root and shoot meristems have the same fundamental appearance and operation, apart from the shoot meristem forming leaf primordia, having a different vascular pattern and lacking a root cap (Sachs, 1991). In both, the meristem consists of two regions of cells: the promeristem and the primary meristem. In roots, the promeristem consists of a small region of cells lying immediately underneath the root cap. Growth of the promeristem in both root and shoot primordia is very slow, undergoing only occasional divisions. The root primordia also possesses a 'quiescent centre', a region that may not undergo any divisions at all unless the meristem is damaged. However it is the 'quiescent centre' of the promeristem that is the source of cell divisions when roots regenerate from damage or when new roots initiate (Barlow, 1993). Such new root primordia are regularly differentiating in growing plants in the form of new lateral root primordia.

The primary meristem lies beneath the promeristem and is the origin of almost all growth, both in terms of cell numbers and cell volume. It is the primary meristem that is responsible for differentiation of cell types within the root. However, physical isolation of this region from the promeristem reveals that it has a determinate growth pattern and must have an associated promeristem for continued growth (Rost and Jones, 1988).

The promeristem appears to determine the pattern of cell divisions in the primary meristem but the cells of the promeristem are not clearly polar. All of the cells are of even size and the orientation of cell divisions is not co-ordinated (Sachs, 1991).

It is the establishment of the promeristem that determines the orientation and co-ordination of new root primordia. It would appear from this that the remaining stages in organogenesis as outlined in section 2.6.2 are primarily a consequence of the establishment of the promeristem.

2.6.4 Differentiation of primordia

The processes that underlie the establishment of new primordia have been extensively studied but remain obscure (Nick and Furuya, 1992). According to Sachs (1991) the control of differentiation can be divided into three parts: intracellular controls, which depend on the past development history of the cell;

environmental conditions, which regulate the development of the plant as a whole; and intercellular controls, which includes all of the signal and spatial cues that a cell receives from the surrounding tissues. It is this third group of signals that is of greatest importance in the initiation and development of meristems.

Meristematic cells are often equated with undifferentiated cells and able to undergo any pattern of cell development and differentiation. Meristematic cells are in fact highly specialised and restricted in their responses to signals and they are limited in the types of differentiation they can undergo (Sachs, 1993). The vast array of tissues and organs are in fact derived from these limited cell types by further differentiation and specialisation of cells. The formation of a meristem involves the establishment of a defined group of cells that are able to control spatial organisation of cells and pattern formation within tissues.

Callus is frequently referred to as undifferentiated parenchyma and is assumed to lack structure, however callus often contains tracheary elements and sieve cells, though the latter are more difficult to discern (Sachs, 1991). It is the absence of normal organisation rather than the absence of differentiation that is the main feature of callus (Murashige, 1974). The induction of tracheary canals as described by Warren Wilson and Warren Wilson (1993) is one manifestation of the differentiation that occurs within callus. The formation of meristematic cells is another.

Cell division is not required for the differentiation. Root lateral primordia can develop in root sections that have been treated with colchicine to prevent cell divisions (Barlow, 1989; Lyndon, 1990). In this case lateral extension of a small group of cells in the pericycle gives rise to the new meristem.

Meristems are complex, organisational units growing in a co-ordinated, regular pattern which can arise either from callus or from organised tissues already possessing some degree of organisation and orientation. Spatial ordering and co-ordination of cell divisions depends on the existence of a defined axis of orientation within the tissue (Nick and Furuya, 1992). Hence the primary requirement for the differentiation of a new meristem is the establishment of polarity (Thorpe and Kumar, 1993).

2.6.5 Polarity and its role in morphogenesis

Establishment of polarity leads to spatial organisation of cells and pattern formation that characterises the organ that derives from those cells. There are three types of gradients that are recognised as important in the establishment of cellular polarity: auxin; electrical potential difference and mechanical stress (Cyr, 1994).

2.6.5a Auxin gradients

Auxin can be regarded as a "general correlative signal, conveying information about the presence, development rates and location of developing shoot tissues" (Sachs, 1993). In this scenario, the responses that are elicited by auxin are dependant on other factors, such as the prior state of differentiation and the rate of auxin transport or accumulation.

Auxin has been clearly established as being able to provide the gradient to establish tissue and cellular polarity. Tracheary element formation from pith cultures has been used as a system to study the processes controlling cell differentiation and morphogenesis. The ease of induction of differentiation and the distinct morphological features make detection easy (Fukuda, 1992). The orderly pattern of vascular differentiation is determined by the canalisation of auxin flux (Sachs, 1993; Warren Wilson *et al*, 1991). Auxin moves from sources initially by diffusion. The auxin flux induces cell differentiation and differentiated cells promote further auxin transport, strengthening the flow along a narrow file of cells. Formation of root primordia at the end of such vascular traces has frequently been recorded (Warren Wilson *et al*, 1991). Localised auxin applications to stem tissues has also been shown to induce new polar shoot like structures (Sachs, 1993).

In many plants, the wound response prior to root initiation in cuttings can be seen to follow a similar pattern. New tracheary elements differentiate from callus or vascular cambium. The differentiation of tracheids results in the formation of a vascular strand, which then develops by a combination of differentiation and division to arch down towards the base of the cutting. In some instances, a root primordium forms below, but some distance from the strand. By the time the root initial has emerged and begun to extend, there is a complete vascular connection which has arisen from the root primordium.

The mechanism by which the developing vascular strand is able to cause the differentiation of cells into a root primordium at a distance is obviously a key to understanding how root initiation occurs (Lovell and White, 1986; White and Lovell, 1984d).

Warren Wilson and Warren Wilson (1993) describe experiments that have demonstrated a gradual decrease in pH in the same direction as auxin flow. This fits well with the chemi-osmotic theory of auxin transport, driven by asymmetric distribution of auxin efflux carriers. They have summarised the hypothesis:

> *"In tissues that have little or no polarity, diffusive transport of auxin from a local source to sinks can gradually induce polar transport of auxin in the same direction, with an associated proton current, through development of asymmetry in the distribution of auxin efflux carriers in individual cells."*
>
> (Warren Wilson and Warren Wilson, 1993), page 558

Others have proposed that the mechanism by which auxin flows are directed and enhanced may involve a more elaborate and precise control. There is evidence that suggests that auxin flows through undifferentiated tissues follow and enhance polarity that is first manifested by the establishment of localised electric currents (Goldsworthy and Lago, 1992; Rathore, Hodges and Robinson, 1988; Sachs, 1991).

2.6.5b The role of electrical currents in morphogenesis

The existence of small, measurable electric currents surrounding and emanating from developing plant tissue has been known for many years (Harold, 1986). The currents are created by the differential transport of ions from tissues, leading to an accumulation of one ion species at one point and a consequential electrical potential difference. The role these currents play in differentiation and maintenance of growth polarity has been the object of considerable study (Nuccitelli, 1986).

In higher plants, steady state ionic currents have been studied in most detail in tobacco and carrot tissues, although they have been demonstrated to exist in many other species. An example of these currents occurs during somatic embryogenesis, where the development of polar currents precedes the development of vascular tissues and cotyledons. (Rathore *et al*, 1988). Cell clusters, thought to be the progenitors of somatic embryos, also possess such a current (Gorst, Overall and Wernicke, 1987; Overall and Wernicke, 1986). Similar electric currents have been demonstrated in other developing tissues such as roots and tip growing organisms (Harold, 1986), organogenic callus (Goldsworthy and Lago, 1992) and detached leaf petioles undergoing organogenesis (Hush, Overall and Newman, 1991).

It has been proposed that the ion currents which precede organogenesis play a major role in the determination of growth polarity in higher plant tissues (Goldsworthy and Rathore, 1985; Harold, 1986; Hush *et al,* 1991). Measurement of surface potential in callus growing on regenerative media and non-regenerative media, shows that electrical potentials in tobacco callus are much higher on regenerative media than non-regenerative (Goldsworthy and Lago, 1992). It is suggested that areas of voltage difference provide a mechanism for co-ordinated orientation of cell division that leads to bud formation.

It has also been demonstrated that applied potential differences can induce sustained currents to be generated and that polarity can be imposed or altered in some tissues (Goldsworthy and Lago, 1992; Rathore and Goldsworthy, 1985a; Rathore and Goldsworthy, 1985b).

2.6.5c Pressure

Mechanical stress is a particularly important factor in the orientation of cell division. A number of simple experiments have been developed to demonstrate that mechanical stress is a controlling factor in orienting cell divisions in callus, cambium and in meristems (Hush and Overall, 1991; Lyndon, 1990). It is suggested that the orientation of the plane of cell division is in the plane of least shear stress. In this hypothesis, even the orientation of cell divisions in meristems can be modelled based on the effects of increased turgor pressure (Green and Selker, 1991; Lyndon, 1990; Sachs, 1991).

Mechanical stress may also be linked to the establishment of ionic currents. The existence of stretch activated ion channels has been demonstrated in a number of situations (Ramahaleo, Alexandre and Lassalles, 1996). They have been proposed as being a means by which osmotic pressure changes and mechanical stress might result in changes in cellular function or orientation.

2.6.6 Translation of polarity signals into meristems

The basis of organogenesis is the co-ordinated cell division and cell extension. The direction of cell division and extension co-ordination is under the influence of microtubules through their ability to control the orientation of cell divisions and cell extension (Warren Wilson and Warren Wilson, 1993). Microtubules exert direct influence on the direction of cell division by controlling the orientation of cell division planes. The orientation of the cell plate between the two daughter cells is predicted by the development of the pre-prophase band, which is formed from cortical microtubules (Wick, 1991).

Microtubules also control the direction of cell extension growth by controlling the orientation of cellulose microfibril deposition. As the microfibrils are themselves not extensible, cell extension growth can only occur by partial re-alignment and slipping between microfibrils. This is assumed to occur during auxin mediated extension growth, by means of acidification of the cell wall allowing for slippage. By aligning cellulose microfibrils in a non-random pattern the microtubules limit the directions in which cell extension can occur (Preston, 1988).

The first cytological changes that are observed in differentiation of cells involves the microtubules (Fukuda, 1992). Cells form a sparse, apparently random network of microtubules but the number increases rapidly during the early processes of differentiation. In association with the increase, the arrays of microtubules change from random to an ordered pattern, especially to a pattern oriented parallel to the long axis of the cell. This has been linked to the pattern of cellulose microfibril deposition (Emons, Derksen and Sassen, 1992) and calmodulin localisation on the microtubules (Fisher and Cyr, 1993). Tubulin synthesis also increases at this time, while degradation is decreased. This is taken as evidence of specific gene expression occurring very early in the differentiation process. If microtubule formation is disrupted with the herbicide

oryzalin, cells are unable to re-polarise in response to wound stimuli, however removal of the herbicide restores the ability to re-polarise (Hush and Overall, 1992).

2.6.7 Mechanisms of microtubule alignment

The alignment of the cortical array clearly plays a critical role during the initiation of polar growth. The microtubules must receive the relevant spatial information to allow for their consistent positioning. Any signal perception must be constant, to allow for stable positioning during the rapid turnover of tubulin that occurs within microtubules (Hush *et al*, 1994). Once such spatial orientation information has been perceived by the plants it can be retained by the cell, independently from the microtubules. The information is not retained by the microtubules themselves, as cold induced dispersion of microtubules does not disrupt cell division patterns in root cells (Barlow, 1993).

Three mechanisms have been proposed by which such spatial information could be received by cells and translated into alignment and co-ordination of cell divisions (Cyr, 1994).

2.6.7a Hormones

It is clear the auxin stimulation directs cell extension and that the directional control of cell extension is under the control of microtubules. What is not clear is the mechanism by which auxin and other hormones influence microtubule orientation. Cyr (1994) proposes that hormones are unlikely to provide sufficiently accurate spatial information to directly co-ordinate microtubule arrangement within the cortical array. This is in conflict with the theories of others who have proposed significant interaction between auxin gradients and microtubule alignment (Nick *et al*, 1994; Sachs, 1993; Warren Wilson and Warren Wilson, 1993).

Gibberellins have also been shown to cause a change in microtubule alignment and an increase in their stability (Shibaoka, 1993). The close association between the microtubules and the plasma membrane might provide a mechanism by which hormone induced membrane changes could be transmitted to the cortical array (Shibaoka, 1994).

2.6.7b Electric Fields

It has been demonstrated that microtubules do re-orient when placed into an electric field (Hush and Overall, 1991). This would seem to indicate that electric fields are a potential mechanism by which cortical microtubules might orient during morphogenesis. However, Cyr (1994) raises two objections to this. The first is that there is no certainty that the electric fields measured outside a cell are reproduced within the protoplast. The electrical potential of the cytoplasm is often quite high, which would have the effect of producing an inward directed field all around the cell. This would be expected to negate any spatial information that might otherwise be conveyed.

The second difficulty is that the microtubules orient at right angles to the imposed field. Tubulin in isolation is known to align parallel to fields. Hush and Overall (1991) have concluded that a plasma membrane-microtubule linking protein might behave as a linear dipole, allowing for an indirect re-alignment of tubules via the plasma membrane. This proposal would also satisfactorily account for the objections regarding the direction of internal fields, raised above.

2.6.7c Pressure

There is good evidence that microtubules are able to realign in reaction to applied mechanical force. Non-injurious application of compression resulted in the re-orientation of microtubules in roots (Hush and Overall, 1991). Similar results have been obtained from brief centrifugation (Wymer *et al*, 1996). It has also been suggested that realignment of microtubules in cells surrounding mechanical injury sites could be in part due to altered stress patterns within the cells (Hush, Hawes and Overall, 1990). There is also evidence that the cell division patterns within apical meristems may be aligned with strain lines within the domed meristem (Sachs, 1991; Green and Selker, 1991).

2.6.8 Post initiation phase

The processes leading to the establishment of polarity for new root and shoot primordia are assumed to be largely the same. This is based on the similarity of structure and the fact that they rely on the same polarity signals.

If root and shoot morphogenesis have a common induction process then there must be two sets of subsidiary signals that lead the developing promeristem to commit to root or shoot meristematic organisation. The evidence outlined in section 2.6.1, from *Sesbania* and tobacco cultures, suggests that there is a common primary induction phase which is followed by, or overlayed by, a subsidiary set of cues which direct the promeristem toward root or shoot organisation.

The alternative view must be that the induction processes are fundamentally different. However it is difficult to see how such differences can be accommodated using the same polarity signals.

There is little evidence as to what cues are used by the meristem to commit to root initiation rather than to becoming a shoot. Some information on this can be obtained by considering the physiological conditions that exist in tissues where root meristem formation occurs most frequently. Lateral root formation occurs exclusively by the initiation of new primordia from pre-existing root tissues but the mechanism by which this is controlled is considered to be poorly understood (Torrey, 1986). Consideration of some of the physiological and growth regulatory conditions existing at the time of lateral root formation may provide some clues.

Absence of light is one of the most obvious features of lateral root initiation. The exclusion of light has been shown to improve *in vitro* root initiation in many species (George, 1993). The beneficial effects of etiolation and banding of stems prior to cutting have been fairly clearly demonstrated to be due to the exclusion of light (Maynard and Bassuk, 1988), probably by altering some aspect of auxin sensitivity. However there are many instances where the exclusion of light is not necessary for the formation of root initials (Lovell and White, 1986).

Cytokinin applications are considered to be inhibitory to new root formation and to lateral root formation (Torrey, 1986). Lateral root formation has been shown quite clearly to be inhibited by cytokinins produced by the root apical meristem and usually occurs at some distance from the root tip or after the removal of the root meristem (van Staden and Harty, 1988; Wightman, Schneider and Thimann, 1980). Both are situations where the differentiation occurs after a decline in cytokinin levels. The type of cytokinin used in proliferation media in *in vitro* cultures of *E. globulus* has been found to affect

the rate of root initiation in the subsequent root initiation phase. This lead to the conclusion that BAP benzylaminopurine had a deleterious effect on root initiation (Bennet *et al*, 1992; Bennet *et al*, 1994). Interpretation of the effects of cytokinins on root initiation and the effects of auxin to cytokinin ratios needs some caution due to the greater auxin sensitivity exhibited by root tissues (Davies, 1995a).

The differences between commitment to a root meristem and shoot meristem is an area that has not received much study. Instances where induction of a meristematic locus and its commitment to root or shoot morphology are distinct and identifiable are rare. In *Begonia* tissue cultures, the requirements for commitment to root or shoot formation have been studied in cells that are able to form either organ (Burritt and Leung, 1996). It has been found that commitment to form either root initials or shoot meristems becomes fixed in the tissues before there is any organ specific differentiation visible in the cells. Commitment to root initial formation occurred more quickly than shoot induction after organogenesis had commenced. This may indicate that the signal and the commitment cues are separate and distinct phases of organogenesis.

2.6.9 Other influences on morphogenesis

Of the host of factors other than auxin that are thought or known to influence root initiation, only a few are factors that are known to have a direct role in morphogenesis.

2.6.9a Nutritional factors

Sachs (1991) claimed that there is little likelihood of metabolites or ions playing a determinative role in morphogenesis. He claimed that their main role would be via effects on overall growth rate and auxin synthesis, metabolism and transport.

In *in vitro* studies it has been found that morphogenesis can be affected by the concentration and form of nitrogen in the medium (Moncousin, 1991). This may, in part, be due to selective ion absorption resulting in an altered media pH, more conducive to organogenesis (Luckman, 1989). For somatic embryogenesis in callus cultures, the requirement for a reduced form of

nitrogen is more clearly established (Nomura and Komamine, 1995). Ammonium, or an organic form of nitrogen, such as glutamine, is a requirement for formation of somatic embryos in carrot cultures and a number of other species. According to Kamada and Harada (1984), the concentration and form of nitrogen is more important to the development of the embryo after its formation has been induced by 2, 4 dichlorophenoxyacetic acid (2,4-D). This suggests a role in nutrition of the developing embryo rather than its induction.

2.6.9b Polyamines

Polyamines are believed to play a role in root morphogenesis (Baraldi *et al*, 1995; Rugini, 1992). Geneve and Kester (1991) showed that inhibition of putrescine production promoted root meristem formation when applied during the inductive phase of root initiation. This seems to be evidence for a role for polyamines at some point in the organisation and development of new meristems. Inhibition of polyamine synthesis has the effect of decreasing root meristem formation, an effect that was attributed to inhibition of cell division, possibly by disruption of the cytoskeleton (Torrigiani *et al*, 1993).

2.6.9c Ethylene

Ethylene is often implicated in events surrounding morphogenesis although it is usually held that it cannot be part of the primary stimulus, because as a gas, it is too diffuse to provide a correlative signal. It is often suggested that ethylene plays a role in the depolarisation of pre-existing cellular orientation. Kumar, et al (1987) produced evidence that the role of ethylene in conjunction with CO_2 is in the early stages of differentiation in *Pinus radiata* cotyledon cultures. Ethylene is able to disrupt various aspects of tissue polarity and may be able to cause re-orientation of microtubules (Fukuda, 1992).

Ethylene's ability to induce partial synchrony in cell suspension cultures has been used to supported the hypothesis that ethylene may be involved in morphogenesis through modification of cell division cycle (Constabel *et al*, 1977). However this effect was achieved by pulsing ethylene levels above the culture medium and might easily be attributed to inhibition of the cell cycle at some point.

2.6.9d Gibberellins

Gibberellins are considered to be inhibitory to induction of root primordia but their presence promotes root extension (Thorpe, 1980). Cellular re-orientation is the first stage in tuber formation in potato stolons, gibberellins have been demonstrated to be able to prevent this re-orientation, possibly by preventing microtubule re-organisation; Fujino, Koda and Kikuta, 1995). There is also evidence from gibberellin biosynthesis mutants that gibberellins are needed for proper orientation of cell division within the meristem. Gibberellins are known to stabilise microtubule arrangement. The absence of gibberellins may be the cause of the disrupted development in such mutants (Barlow, 1993).

2.6.10 Summary

Meristems are an expression of a stabilised tissue polarity. The formation of a root meristem from undifferentiated callus, parenchyma or other tissues is centred around the repolarisation of cells to establish a new axis of polarity.

Auxin gradients and electric potential gradients are the two best known ways of repolarising cells to form new meristems. Pressure, although clearly an important polarity gradient, seems to be an unlikely method by which meristems might initiate. It is possible however that pressure gradients might interfere with either auxin or electrical signals to prevent the formation of meristems.

The effects of other factors on repolarisation, such as nutrition and other hormones although important, seem subsidiary to the central role played by auxin, electrical currents and mechanical stress. These factors would be more important if it was shown that polarity signals existed within the tissues and were being perceived by the cells but failing to produce the normal response of meristem formation.

The formation of auxin and electric current induced polarity depends on the existence of specific cellular mechanisms that are able to direct and maintain differentials in the concentration of auxin and ions. Understanding the nature of the channels and the links between polar auxin flows and endogenous electric currents is fundamental to comprehending how meristem formation and organogenesis is controlled.

2.7 AUXIN SIGNALS, AUXIN RECEPTORS AND ROOT FORMATION

2.7.1 Introduction

This section reviews some of the literature on how auxin polarity and electric currents might be established and maintained to provide the polarity signals necessary for organogenesis.

Polarity signals are the primary stimulus by which organogenesis is initiated. Auxin flows and electrical currents are the main mechanisms for the transmission of polarity signals to individual cells and tissues. However, the mechanism by which polarity signals are generated and become translated into ordered, co-ordinated cell growth is not clearly understood. Such signals must first be transmitted across the plasma membrane in order for them to activate intracellular response mechanism (Felle, 1993). The interaction between auxin and its receptors in the plasma membrane is the primary method by which such regulation occurs. Similarly, electric currents must also interact with the plasma membrane since the plasma membrane are central to the establishment of polarity.

Calcium ions play a role as part of the auxin signal transduction system and are also an important component in the establishment and maintenance of intercellular electric currents. The regulation of calcium ions within the cell via membrane pumps and efflux channels appears to be a link between auxin signals and electric currents.

2.7.2 Auxin signalling and receptors

The system for maintenance and detection of auxin polarity signals within tissues must consist of two components. Firstly, there must be a means of generating and controlling the auxin flows. There must also be a signal perception system that can detect the direction and strength of the auxin flow.

The identification of auxin channels and receptors, their location and how they are able to control such a wide range of responses has been the subject of much research and speculation. The identification and localisation of a number of possible auxin binding proteins has advanced over the last few years but there

remain many unanswered questions and unresolved ambiguities. Regrettably, the mode of action of auxin is still not clear and opinions vary on the the importance of various components of the system. An example of this is the recent 'debate' on the importance of type 1 auxin binding proteins (ABP1). Some researchers now believe that ABP1 may not be a significant part of the auxin reception system (Hertel, 1995), while others remain convinced of its importance (Venis, 1995).

2.7.2.a Auxin transport

Polar auxin transport has been studied by a number of techniques since the chemi-osmotic theory of auxin transport was first advanced by Rubery and Sheldrake (1974). Polar auxin transport is an energy requiring process that is specific for IAA and synthetic auxins. The direction of flow is basipetal in shoot tissues but both basipetal and acropetal transport has been identified in roots (Lomax, Muday and Rubery, 1995). Such auxin flows are separate from the movement of auxin and auxin conjugates that can occur by mass flow within the plant's vascular system both in the phloem and in the xylem (Lomax *et al*, 1995).

There is strong evidence for the existence of a membrane bound, auxin efflux carrier protein (Palme, 1993; Hertel, 1995). The carrier is believed to be complex and consists of at least two binding sites and may have as many as five different binding sites associated with it (Hertel, 1994). An important feature of the efflux carrier is the existence of at least one separate regulatory component. Phytotropins are able to regulate auxin efflux and bind to a protein separate to that responsible for auxin transport (Lomax *et al*, 1995). The auxin efflux carriers are also inhibited by some naturally occurring flavonoids and flavonoid esters (Faulkner and Rubery, 1992; Jacobs and Rubery, 1988) making flavonoids possible endogenous regulators of auxin transport (Lomax *et al*, 1995). The phytotropin NPA has been used to inhibit root initiation, when applied in the first few days after the cutting is made, however this inhibition was not related to changes in auxin concentration at the rooting site (Diaz-Sala *et al*, 1996).

The existence of an auxin uptake carrier was first demonstrated by Rubery and Sheldrake (1974), who found that the carrier was inhibited by 2,4 D but was unaffected by the efflux inhibitor TIBA. More recently two proteins have been

identified that are believed to be part of the carrier protein complex responsible for auxin uptake. It has also been shown that tomato plants deficient in some aspects of auxin transport lack these proteins (Jones, 1994).

Changes in the concentration of endogenous regulators of auxin efflux is one of several methods by which auxin transport could be regulated. There is also evidence that changes in the distribution of carrier proteins and in the activity of the carrier proteins could regulate auxin flows within tissues (Lomax *et al*, 1995). This provides a mechanism by which differential auxin flows can be established and controlled.

2.7.2.b Auxin binding proteins

The state of research into auxin binding proteins has been the subject of a number of recent reviews (Palme, 1993); (Napier and Venis, 1995); (Goldsmith, 1993); (Hertel, 1994); (Jones, 1994); (Libbenga and Mennes, 1995). However, the terminology is not consistent and there is no consensus on how many of the proteins are functional auxin receptors. Hertel (1994) and Goldsmith (1993) review the evidence for three types of auxin binding proteins as auxin receptors. They point to difficulties and inconsistencies in experimental data obtained so far, for all three types of auxin binding proteins. Jones (1994) offers a different classification, pointing to the likely existence of multiple receptor proteins. Napier and Venis (1995), appear to favour the existence of a single receptor but do not exclude the possibility of other auxin receptors being identified.

Type 1 auxin binding protein (ABP1) is the most studied. Over 90% of this protein is found in the endoplasmic reticulum, with a much smaller amount being found on the outside of the plasma membrane. Its presence has been demonstrated in a wide variety of plants and tissues (Palme *et al*, 1994). Its site of action is on the plasma membrane, where it has been linked to an ATPase driven transmembrane potential difference (Barbier-Brygoo *et al*, 1992) and to modulation of anion channels (Venis, Napier and Oliver, 1996). There are discrepancies that remain to be resolved between some of the measurements, made in evacuolated protoplasts, and auxin responses measured in intact cells (Napier and Venis, 1995; Hertel, 1995).
A correlation is claimed between ABP1 abundance on the plasma membrane and rooting ability in tobacco plants transformed using *Agrobacterium rhizogenes* (Hertel, 1995). There are also suggestions that there may be organ

specific isoforms that result in variations in auxin action within plants (Jones, 1994). Transmission of the protein from the endoplasmic reticulum to the plasma membrane has been proposed to be linked to alterations to intracellular calcium levels (Palme *et al*, 1994).

Auxin stimulation of ABP1 results in clustering of ABP1 on the surface of the protoplast (Diekmann, Venis and Robinson, 1995), but no change in the concentration of ABP1 on the membrane and no change in the protein turnover rate (Oliver *et al*, 1995). There is probably some form of docking protein that contains a transmembrane domain to allow signal transduction through the membrane (Diekmann *et al*, 1995). Two poly-peptides, which may fill such a role, have been identified in association with ABP1 (Oliver *et al*, 1995).

André and Scherer (1991) have proposed that phospholipase activation is a primary step in signal transduction from ABP1 to growth response. They demonstrated that auxin stimulation of phospholipase A was via a 21 KDa auxin binding protein that is very similar to the 22 KDa ABP1, with a possible G protein intermediate step. The phospholipase is believed to stimulate ATPase either directly or by activation of a protein kinase.

Several membrane associated auxin-binding proteins have also been studied in tobacco callus cultures. One of the auxin-binding proteins detected is only found in cultures that are capable of regenerating roots (Nakamura and Ono, 1988). In 2,4 D dependent cultures this protein is not present but it reappears after culture on NAA+kinetin media, which also restores the rhizogenic potential of the culture (Nakamura *et al*, 1988). This is taken as evidence that this auxin binding protein, which is only capable of detecting high concentrations of auxin, is in some way responsible for programming the cells to acquire the ability to initiate roots. In this respect, it is interesting that the protein is present in reduced quantities in cytokinin synthesis deficient cell lines but increased synthesis can be triggered by the addition of kinetin to the culture medium (Nakamura *et al*, 1988). In another cell line tested the level of protein was decreased when cultures were grown in the dark. A separate auxin binding protein, similar to the maize ABP1, has also been found in the same cultures (Libbenga and Mennes, 1995).

2.7.2c *Agrobacterium rhizogenes* and auxin action

Transformations by *Agrobacterium rhizogenes* have been produced in a wide variety of plant species (Hamill and Chandler, 1994). In eucalypts, transformation has resulted in enhanced adventitious root formation *in vitro* (Hamill *et al*, 1995; MacRae and Staden, 1993).

In addition to its potential to boost adventitious root formation, *A. rhizogenes* has been used to study auxin action. One of the effects of transformation with Ri T DNA is to cause an alteration in the source/sink relationship within transformed plants leading to a much larger percentage of the plant biomass being in the roots. This appears to be the result of a much higher frequency of lateral root differentiation (Hamill and Chandler, 1994). In terms of root initiation, the *rolB* gene has been demonstrated to be the most important of those identified from *A. rhizogenes*. The *rolB* gene was thought to be involved in either increased IAA synthesis or release. However the work of Nilson *et al* (1994), has discounted this option.

Filippini et al (1994), studied the effects of the *rolB* gene on auxin binding proteins in tobacco. They demonstrated that transformed plants had a much higher level of an auxin-inducible auxin binding protein. They found the protein to be less tightly bound to membranes than ABP1 and could be washed from membranes with 0.5 M KCl. *rolB* transformed tissues show higher levels of this protein and much greater auxin induced production of the protein. It was not possible to determine whether the RolB protein, the protein produced by the *rolB* gene, was directly involved in auxin binding or whether it induces the formation of other, related proteins. It is believed, however, that the enhanced auxin binding capacity could account for at least some of the increased auxin sensitivity found in transformed cells.

2.7.3 Electric currents in morphogenesis

The ion currents which precede organogenesis are believed to play a major role in the initiation and determination of growth polarity in higher plant tissues. How the ion currents are established and maintained, and the way they direct tissue polarity provides important information on the control of organogenesis.

2.7.3a Where currents have been measured

The evidence of transcellular ion currents in plants has been studied for many years (Harold, 1986). In recent years, advances in the design and sensitivity of micro-electrodes has provided much more information on the size, direction and ion composition of the currents. This has led to a clearer understanding of their role in plants.

The strength and direction of ion currents in higher plants has been most closely studied in somatic embryo cultures. Overall and Wernicke (1986) used vibrating probes to measure the size and direction of currents in tobacco callus cultures. They found that a definite pattern of ion flow preceded the development of a visible growth polarity of the small clusters of embryogenic callus. Similar currents were present in carrot somatic embryos (Rathore *et al*, 1988). Gorst, Overall and Wernicke (1987) found that such currents could even be detected in those cell clusters where somatic embryo formation was prevented by the presence of 2, 4 D.

Currents have also been demonstrated to exist around cells and cell clumps in tobacco callus suspension cultures. The currents were strongest when measured during the active growth phase of the cultures. Cells with strong electrical polarity were found to be elongated and tended to divide at right angles to their electrical axis, resulting in long filaments of cells (Goldsworthy and Mina, 1991). Tobacco callus growing on solid organogenic media developed areas of much higher surface potential than did callus on de-differentiation media. Shoot regeneration was closely associated with the regions of highest surface potential (Goldsworthy and Lago, 1992). Strong ionic currents found on detached leaves of *Graptopetalum* prior to shoot regeneration (Hush *et al*, 1991).

Root tips, root laterals and immature laterals have all been demonstrated to have a current which enters the growing portion of the root tip and leaves through the non-growing regions (Miller, Shand and Gow, 1988). Wounding of roots caused strong currents to be induced (Hush and Overall, 1989), which may provide a mechanism for some fungal zoospores to target suitable infection sites (Miller *et al*, 1988). Up to ten hours prior to the emergence of lateral roots there is a substantial change in the surface potential of the primary root surface at the point at which the new root will emerge (Hamada *et al*, 1992).

The appearance of the changes in surface electric potential has been correlated with anatomical studies. This revealed that the earliest detectable cell divisions leading to lateral root development coincide with the changes in surface potentials (Watanabe *et al*, 1995).

2.7.3b Composition of ion currents

The electric currents that are measured by probes at the cell surface represent the sum of ion flows across a particular region of the cell membranes. Ion substitution and ion transport inhibitors in the surrounding medium and ion-selective micro-electrodes have been used to develop an understanding of the composition of these ion fluxes.

Rathore *et al* (1988), demonstrated a slight variation in the pH level of the medium adjacent to embryos from which currents were emanating. The pH was lower than the bulk medium at both the top and the bottom of the embryo by 0.02 and 0.07 units respectively. The current was consistent with K^+ influx and active H^+ efflux being the main carriers of the current. They also demonstrated that removal of calcium from the medium resulted in a doubling of the currents. This is consistent with the work of Mina and Goldsworthy (1992), who also found that the current was higher in the absence of calcium. This has led to the conclusion that Ca^{2+} is unlikely to be a major component of steady state ion fluxes.

Hush, Newman and Overall (1992) and Hush and Overall (1989) have used ion-selective electrodes to measure substantial Ca^{2+} and K^+ and H^+ fluxes in wounded pea roots and *Graptopetalum*. It was concluded that these were not the only ion species involved, as the sum of these ion fluxes did not match the total current. During the early stages of organogenesis in detached leaves of *Graptopetalum*, Ca^{2+}, H^+ and K^+ were the dominant ions in the fluxes. There is a strong influx of Ca^{2+} immediately after detachment. This later became an efflux, while K^+ maintained a steady efflux throughout. The authors also noted that 24 hours after detachment, other ions, which were not measured, were contributing significantly to the net current (Hush *et al*, 1991).

2.7.4 The role of auxin in currents

It has been established that auxin plays a significant role in establishing and stabilising cell polarities in callus in the lead up to organogenesis (Goldsworthy and Lago, 1992).

Tobacco callus grown in media containing IAA were found to be much more likely to exhibit natural electrical polarity than those grown in media containing 2,4-D and more likely to retain a stable pattern of polarity (Goldsworthy and Mina, 1991). The currents were strongest when the cultures were actively growing. In cultures grown with IAA, electrical polarity was associated with cell elongation and formation of filaments, whereas with 2,4-D in the medium, there was formation of irregular clusters of cells. Goldsworthy and Mina concluded that IAA is much better at promoting and maintaining electrical polarity in cells than 2,4-D. It was suggested that this is the reason IAA is better at promoting organogenesis in cultures.

Contrary evidence was found in embryogenic cultures of carrot (Gorst *et al*, 1987). In this report, small cell clusters, obtained from media containing 2,4-D, were found to have similar patterns of currents to equivalent sized cell clusters obtained from embryogenic media not containing any auxin. The effect of the presence of 2,4-D in these cultures was to prevent the early cell clusters from retaining and enhancing an organised morphology and polarity. This occurred because the the cell clusters continue to fragment, an effect assumed to be an effect of auxin mediated changes to cell walls.

2.7.5 The effects of applied electric currents

It has been known for some years that the application of constant electric fields can polarize plant cells and control their direction of growth. This has been demonstrated in tissues of a number of higher plant species and also in animal tissues (Robinson, 1985)

Goldsworthy and Rathore (1985a) obtained 60 to 70% stimulation of growth of tobacco callus. This was achieved by exposing the callus to either a 1 or 2 μAmp current, which passed between the callus and the medium for 21 days. Callus growth was promoted only in the presence of IAA. The effect could be negated by the addition of Tri-iodobenzoic acid (TIBA), as an auxin transport inhibitor,

or the use of 2,4-D or Indole-3-acetonitrile (IAN), auxins not showing polar transport. This growth promotion was polarity sensitive and only occurred when the callus was positive to the medium. This is the opposite polarity to that required for direct electrophoresis of auxin. The authors claim that the effect is due to the induction of cellular polarity and co-ordinated growth within the callus, although they do not offer any evidence that this is occurring.

Rathore and Goldsworthy (1985b) also reported up to five fold stimulation of adventitious shoot formation in tobacco callus when grown on organogenic media. This was achieved by passing a 1 or 2 μAmp current between the callus and the medium for 45 days. In contrast to the effect on callus growth, organogenesis was stimulated by currents flowing in either direction. Root initiation was also stimulated by similar currents applied to embryogenic wheat callus cultures.

Dijak, Smith, Wilson and Brown (1986), used a general field on *Medicago* protoplasts to induce somatic embryogenesis. They found that both the frequency of successful regeneration and the number of embryos obtained were boosted by applying a potential difference of 0.02 Volts across the radius of the petri dish. 0.05 Volts and 0.15 Volts were also used successfully but resulted in cell death at longer exposure times. In a clone where somatic embryogenesis was not known, exposure to the same fields also caused cell aggregation and the development of early stages of somatic embryos. However, the development did not continue to completion. It is unfortunate that the current flowing in this system was not reported, as this would make comparison with the previous reports easier.

One factor which needs to be considered in these experiments is the possibility of electrophoretic ion transport leading to pH changes in the media surrounding the electrodes. This is important as it is known that variation in pH of media can have a significant effect on callus growth. pH changes induced by currents have in the past been proposed as the mode by which endogenous currents might enhance growth (Brawley, Wetherell and Robinson, 1984). Rathore and Goldsworthy (1985b), considered that electrophoretic effects were unlikely to be the cause of the stimulation of organogenesis because stimulation occurs with currents of either polarity. Any charged substance drawn towards the callus under the effects of a current of one polarity would be repelled by the reversed current.

Mina and Goldsworthy (1991), have also studied the effect of the application of DC currents to individual cells. Currents of 3 μA and 100μA cm^{-2} were applied as a point source close to individual cells from tobacco callus suspension cultures. The effect was to reverse the electrical polarity that had been measured prior to treatment.

Re-polarisation occurred after 10 minutes at the higher currents and after 2-3 hours at the lower currents. This is direct evidence that the effect of applied currents is to alter the pre-existing patterns of electrical polarity within a tissue, It also implies that such currents are responsible for the organogenesis subsequently observed. The effect was more pronounced in cells growing in a medium containing 2,4-D medium than IAA. This suggests that cells growing in 2,4-D are more susceptible to re-polarisation and that natural polarity is less firmly established. This may be related to the reduced rate at which 2,4D is able to undergo polar transport in cells (Lomax *et al*, 1995)

Hush and Overall (1991), showed that application of a field of 0.36 Volt cm^{-1} for six hours caused an alteration to the cortical microtubule arrays in the cells within the field. Previous experiments had demonstrated that the pattern of microtubule alignment was an accurate predictor of cellular polarity (Hush *et al*, 1990). This is consistent with the hypothesis that applied currents orient cell extension and cell division planes by controlling the orientation of microtubules.

Attempts to discover the effect of applying currents to organised tissues and organs have met with varied results. Diprose, Benson and Willis (1984) cite a number of early experiments where the application of electric fields to plants and crops has been claimed to have growth promoting effects, although the results have often been inconsistent. Newman (1958) tried unsuccessfully to reverse normal cellular growth polarity in flax hypocotyls, using longitudinal currents. He found that a current flowing down the hypocotyl was more detrimental than one flowing up (tip negative). This is consistent with the work of Desrosiers and Bandurski (1988) who demonstrated that shoot growth could be inhibited by a voltage of 5 to 40 Volts passed through a stem and resulting in a current of 6 to 40 μAmp. At low voltages, (5 to 10 Volts) there was only growth inhibition when the tip was positive but for 15 Volts or higher, treatments with the tip negative were also affected. The authors proposed that a negative effect on auxin transport was at least part of the mechanism of the inhibition.

The subtlety of the influence of electric fields is illustrated by one report which showed that the growth of *Quercus rubra*, *Pinus resinosa* and *Populus tremuloides* trees was increased by the presence of a 1 to 7 milliGauss electromagnetic field. The field was generated by a U S Navy low frequency (76Hz) communications antenna (Reed *et al*, 1993)

There do not appear to be any reports of the use of electric currents to stimulate organogenesis from stems or other plant tissues that possess any degree of differentiation or organisation. This suggests that such organised tissues are not susceptible to the influence of currents or that the right combination of suitable plant material, current and experimental conditions has not yet been found.

2.7.6 The role of calcium in initiation

Calcium gradients and the information they convey are crucial to a whole host of cellular responses (Trewavas *et al*, 1994). Calcium ions play a central role in most of the signal transduction responses to plant hormones including those responsible for induction of polarity (Bethke, Gilroy and Jones, 1995).

2.7.6a Calcium in polar currents

Ca^{2+} is also central to the establishment and maintenance of electric polarity and currents (Mina and Goldsworthy, 1992). Re-polarisation could be achieved by application of a current of 250 $\mu A\ cm^{-2}$, as a point source, close to individual cells. When calcium was omitted from the bathing medium pre-existing currents continued but the cells could not be re-polarised. Similar effects were obtained with the addition of cobalt to the bathing medium, where cobalt ions were believed to act as calcium channel blockers. This experiment indicates that although calcium channels are not part of the maintenance of polar currents they are essential for their establishment or re-orientation.

The presence of calcium channel blockers La^{2+} or Gd^{2+} substantially reduced wound induced currents in peas (Hush and Overall, 1989). This suggests that calcium ions are an essential part of the cellular re-orientation prior to formation of meristems or wound tissues. Similar results were obtained by Malhó, Read, Pais, and Trewavas (1994), who were able to control the orientation of pollen tube growth in an electric field of 5 Volts cm^{-1}. This effect

could be negated by the presence of La^{3+}, which is a Ca^{2+} channel blocker, indicating that calcium ions also play a key role in determining orientation of growth in this system.

Mina and Goldsworthy (1992) noted that cells contain very little free calcium as the ions are actively pumped out of cells and adsorbed to negatively charged macromolecules. Most of the free Ca^{2+} is concentrated around the point of entry, such local accumulations seem to control polar growth.

> *"The importance of calcium in the initial establishment of electrical polarity in the fucoid zygote has also been implied from the observation by Robinson and Jaffe that calcium ions form a substantial but declining proportion of the early transcellular current entering the presumptive growing region. This may be interpreted as an initial local entry of calcium stimulating the activity of channels for other ions in the same region. Perhaps the local entry of calcium ions was stimulating the activity of channels for other ions in our tobacco cells too. If so, it seemed possible that the necessary calcium might be entering via specific calcium channels. If this was the case, supplying substances such as cobalt to block the calcium channels should inhibit the electrical re-polarisation of the cells."*
>
> Mina and Goldsworthy (1992)

The strong asymmetry in calcium flux during cell polarisation may be the result of calcium channel and calcium pump re-distribution as the net calcium flux in cells is claimed to be unchanged from that occurring before polarisation (Weisenseel and Kicherer, 1981). Hush and Overall (1989), also suggest that an intracellular calcium gradient may be set up by the calcium influx measured following wounding. Other experiments have indicated that more than one type of calcium channel may be involved (Hush *et al*, 1992).

Another example of the importance of calcium transport in initiation and orientation of growth is provided by the use of ionophores, which when placed close to zygotes, have induced growth in the direction of the source (Weisenseel and Kicherer, 1981).

Interactions between calcium levels and auxin have been noted on many occasions (Hepler and Wayne, 1985). Most of these seem to concern the role calcium plays in polar auxin transport but it is noted that calcium at physiologically high concentrations (1-10 mM) inhibits growth in antagonism to auxin. The concentration of calcium in the extracellular medium has also been demonstrated to alter the properties of some ion channels (Kourie, 1996).

2.7.6b Calcium signalling in cells

There is a large body of evidence that calcium acts as one of the key intracellular messengers and that it, and a small group of other compounds, play a pivotal role in signal transduction. This is achieved by transmitting signals perceived at localised receptors to other relevant parts of the cell, where the effectors of cellular responses are found. While there is still debate about the nature of primary receptors and their role in plant signal transduction, there is considerably more known about the secondary messengers. Felle (1993), lists six secondary messengers involved in signal amplification in plants, namely phosphoinositides, Ca^{2+}, fatty-acid derived compounds, sphingolipids, pH and electric fields.

Regulation of Ca^{2+} levels is closely interlinked to plasma membrane H^+ ATPase activity. Calcium levels in the cytoplasm are, in part, regulated by the transmembrane proton gradient, maintained by plasma membrane H^+ ATPases. In turn, the activity of plasma membrane H^+ ATPase is regulated by Ca^{2+} levels via the effects of calcium dependent protein kinases (Bethke *et al*, 1995).

2.7.7 ATPases as part of the auxin reception chain

The currents around carrot somatic embryos is maintained by active H^+ efflux (Rathore *et al*, 1988). This has been taken to imply that H^+ ATPases are a major part of the currents that are required for the initiation of organogenesis.

The plasma membrane ATPase has been described as the "master enzyme" able to control a range of cell functions (Serrano, 1989). By pumping protons out of the cell it is able to create pH and electrical potential differences across the plasma membrane. These allow the plant to undertake a host of physiological functions. ATPases play a crucial role in activating the transport of minerals

and solutes against the osmotic gradients and in maintaining cell turgor. ATPase is also central to the acid growth model of auxin induced cell extension, where ATPase is believed to be stimulated by auxin to lower the pH of the cell wall. pH sensitive loosening of cell wall components allows the turgor of the cell to stretch the cell in the direction of cellulose microfibril alignment (Cleland, 1995; Raven, 1979). The *rol A* gene from *Agrobacterium rhizogenes* has been demonstrated to increase the sensitivity of auxin induced ATPase activity in some situations (Vansuyt *et al*, 1992). This points to a link between the product of the *rol A* gene and an altered signal transduction mechanism at the plasma membrane.

Auxin stimulation of ATPase is claimed to be via the auxin binding protein 1, possibly by activation of an ion channel leading to a decrease in cytoplasmic pH (Barbier-Brygoo *et al*, 1992; Rück *et al*, 1993). As the pH of the cytoplasm is normally higher than the optimum for ATPases this would cause an increase in ATPase activity. Other possible mechanisms for auxin stimulation of ATPase involve either protein kinase stimulation following an increase in cytoplasmic calcium concentration, or auxin activation of phosopholipases (André and Scherer, 1991; Cleland, 1995).

ATPases also maintain cell turgor, which can be used to control organ movements and the changes to stomatal guard cells. The cells are sensitive to a variety of stimuli, including auxin, which affect the H^+ ATPase as well as ion channels. Once activated, the H^+ ATPase causes plasma membrane hyperpolarisation, ion channel activation and consequent turgor increase, which results in stomatal opening (Michelet and Boutry, 1995).
Such a pattern of responses to an auxin signal may be replicated in other auxin controlled cellular processes, such as cell extension. Associated with the auxin induced cell extension are rapid increases in H^+ ATPase levels. These increases begin to be apparent 10 minutes after auxin stimulation, reach a maximum after 30 minutes and are believed to be the result of new enzyme synthesis (Hager *et al*, 1991).

2.7.8 Summary

The polar auxin flows required for organogenesis and differentiation are almost certainly the result of re-distribution of membrane bound carrier proteins and auxin efflux pump proteins. ABP1 on the other hand appears to to play a role in the establishment of electrical polarity by stimulation on H^+ ATPases.

Small electric currents are ubiquitous around most cells. They are a reliable marker and predictor of growth polarity. Polar currents have been measured as part of organogenesis and lateral root formation.

Application of currents to plant tissues can be used to impose polarity where none existed and to change pre-existing cellular polarities. This technique has been used to induce somatic embryogenesis, shoot formation and root formation from callus cultures. The mode of action is almost certainly to cause the re-orientation of microtubules and thus control the orientation of cell division and extension. There does not seem to be any reason why such currents could not be used to induce the formation of new roots from suitable organogenic tissues.

Ca^{2+} and H^+ ATPase regulation within cells are both directly affected by auxins. They represents a link between auxin signals and the generation of electric currents around cells and developing organs This may be the means by which ionic currents are initiated and controlled.

This is a more complete picture of the processes underlying organogenesis. Some parts of that process are still the subject of debate and need further research to confirm them, others are more generally accepted. Research into the factors controlling root initiation needs to be framed with these fundamental processes in mind.

2.8 LITERATURE REVIEW SUMMARY - DIRECTIONS FOR INVESTIGATIONS

Adventitious root initiation is a process of organogenesis; a new apical meristem is formed with a new axis of cellular polarity. There are many other factors which impinge on the success or otherwise of root initiation. These may relate to the physiology of the parent plant, the selection of the cutting, the treatments applied to the cutting and the environment maintained around the cutting. However, the essential feature that must occur is the induction of a new meristem. Research that is directed at obtaining a more detailed understanding of this central process will provide clarification of the factors controlling root initiation. Once the process is properly understood, that knowledge can be applied to devise practical improvements in propagation techniques.

The establishment of a new axis of polarity is the critical first stage of organogenesis. All later developments depend on its existence. Once polarity has been established, it provides the co-ordination framework for the remaining stages of meristem formation.

The cell divisions and cytological changes that mark the earliest visible signs of differentiation must be responding to some polarity signal. The development of that polarity signal must precede those changes. There are two mechanisms by which such a polarity signal could be established. Auxin flux and ionic currents through cells and tissues are both mechanisms by which tissue polarity can be established and maintained. There is a strong likelihood that both are involved in the early stages of meristem formation. Both appear to be able to exert control on the direction of cell growth and the orientation of cell division.

The development of a new root from the pro-meristem is largely under the control of that meristem. The stage at which the meristem commits to root formation rather than to shoot formation is unclear. However, all subsequent development of the root initial is under the direction of hormonal and other development controls generated within the meristem.

If this view of the processes underlying root initiation is accepted, then some of the factors that are known to influence root initiation need to be re-assessed. It is desirable to identify how those factors can be interpreted in terms of their ability to affect the induction or expression of polarity, leading to organogenesis.

It is possible to describe a number of ways in which polarity signals might be controlled or modified to alter the frequency or extent of organogenesis. Firstly, the polarity signal must be generated within the tissues that are to undergo organogenesis. Secondly, those signals must be received by the tissues and transduced to provide the appropriate metabolic responses.

Three factors are likely to be important in the establishment and maintenance of polarity signals:

(1) Strength of the signal.
There must be a threshold level below which the polarity signals generated within tissues are insufficient to initiate organogenesis. The presence of adequate levels of auxin and a means of directing and controlling its movement must be an important part of the strength of that polarity signal. With regard to ionic currents, the requirements are less clear but must involve maintenance of intracellular ionic balance and the means to regulate and direct the ion channels through which the ions move.

(2) Duration of the signal.
There is little evidence of how long a polarity signal needs to be maintained to induce meristem formation. In some species, there is evidence is that the whole process of root initiation occurs within a few days at most. (Harbage *et al*, 1993).

(3) Clarity of the signal.
Since meristems are formed as discrete organs originating from just a few cells, it seems reasonable to expect that the polarity signals leading to their formation must also be spatially restricted. The existence of competing sources of polarity signals or circumstances that cause a polarity signal to become dispersed or confused will restrict the chances of that signal being properly perceived.

The attributes which control cells' responses to those signals are the signal reception and transduction mechanisms. The identification of auxin receptors in recent years has improved our knowledge in this area. Advances are being made in understanding how auxin receptors invoke some cellular responses and the link between receptors and re-orientation of cells. How currents are perceived and acted upon is less clear. A role in the orientation of microtubules is indicated but there must be an intervening step, since the fields have the wrong orientation to exert their effects on microtubules directly.

It should be also be possible to explain the mode of action of some inhibitors and promoters of root initiation in terms of them having a role in the generation or perception of polarity signals. Chemicals or treatments that affect auxin transport or the ease with which cells can re-polarise have the potential to disrupt signals or reduce the tissue response to them. Structural features, such as suberin bands or discontinuities in tissues, also have the potential to disrupt the generation of signals.

Not every factor that is important to root initiation directly affects the induction of root initials. There are conditions and treatments that relate to the general physiology of the cuttings and the parent plant but which affect the cuttings' ability to initiate roots. Examples of this are treatments that affect the accumulation of carbohydrate reserves or the physiological conditioning needed to withstand moisture stress after the cuttings have been collected. These are quite separate from root induction factors but their absence will tend to obscure their importance.

The objective of this research program was to apply the theory of organogenesis outlined above to the practical problem of finding ways to improve root initiation in *E. nitens*. The most obvious strategy is to study *E. nitens* seedlings and cloned plants with a range of rooting abilities to attempt to identify changes which occur during the development of the seedlings which lead to the loss of rooting ability. From this, it should be possible to identify where organogenesis is impeded or disrupted. Once this knowledge has been gained, treatments that are designed to overcome these impediments can be tested.

3. GENERAL METHODS

3.1 PLANT AND CUTTING CULTURAL PROCEDURES

Small seedlings were used as the source of all cuttings. Seedlings were raised in the glasshouse under a standardised culture regime that resulted in uniform sized, easily grown seedlings. Cuttings were harvested from these plants at a growth stage defined by the number of nodes with fully expanded leaves.

3.1.1 Pots and potting mix

The same pot and potting mix combination was used for raising seedlings and setting cuttings. Plants were grown in Kwik Pots, a large celled, plug tray (Rite Gro, Kwik Pots, Arthur Yates and Co Pty Ltd, Laverton Victoria). Kwik Pots consist of a 6 x 8 array of 43 mm x 38 mm tapered cells, 50 mm deep that fit into a standard 350 mm x 280 mm seedling tray. The volume of each cell is 62.5 ml. These were filled with the standard potting mix.

The potting mix consisted of equal parts of milled horticultural peat and horticultural grade perlite with the following additions per 100 litres:

Crushed Dolomite	1,000 g
Osmocote (3-4 month)	250 g
$FeSO_4$	50 g
Super Phosphate	75 g
Micromax	100 g

The soil mixture was steam pasteurised after mixing and stored in covered containers until used. The pH of the mixture after pasteurisation was approximately 5.2.

3.1.2 Seed germination

Seed was germinated in a liquid culture system and grown for two weeks in a growth cabinet, prior to being transferred to soil.

Seed was supplied from North Eucalypt Technologies, Ridgley Tasmania. Two batches were used, EXT.112 and EXT.113. Seedlings were grown in batches of 1,000 to 2,000 plants at a time, using 2 to 3.5 g of seed.

Seed was initially surface sterilised by soaking for 30 minutes in 25% v/v commercial bleach solution containing detergent, such as White King, giving a final ClO_4 concentration of 1%. The seed was washed with several changes of distilled water, until all traces of the detergent and bleach were removed. After transferring the seed to a growth cabinet at 22°C it was allowed to imbibe for 48 hrs in aerated distilled water with $CaSO_4$ added. After imbibition, the seed was spread thinly over a double layer of muslin covering a stainless steel mesh screen. The edges of the muslin draped over the edge of the screen into a 0.1 Normal Hoaglands solution, maintaining moisture to the seed by capillary action. The level of the Hoaglands solution was maintained just below the screen, at a depth of 10 cm in a plastic tray lined with agricultural polythene. The level of the liquid was maintained by the regular addition of distilled water. The seed was allowed to germinate for seven days in the dark followed by up to five days in 16 hr light. The temperature was maintained at 22°C throughout germination.

Seedlings 2 to 4 cm high with similar length roots were pricked out into Kwik Pots filled with potting mix. The trays were drenched with Previcur (Schering Pty Ltd), 1.5 ml/L, and placed on a bottom heated mist-bed for two to three weeks before transferring to a glasshouse. Once in the glasshouse, the plants were watered daily by overhead sprinklers and they received weekly applications of 2 Normal Hoaglands solution. Insecticides were applied, as required, to control aphids and white flies, as part of the normal glasshouse operations.

3.1.3 Cutting preparation

Cuttings were prepared from three node stem sections of seedlings, using the highest nodes with fully expanded leaves (Figure 3.1a). The lowest pair of leaves were removed at the base and the immature leaves and stem above the third node were also removed. Cuttings were designated as being Node 1, Node 2, Node 3, etc, cuttings based on the position on the seedling of the lowest node in the cutting (since this is the node that is most involved in root initiation). The cotyledonary node was designated as Node 0, hence a Node 1 cutting consisted of the first three nodes above the cotyledons.

All cuttings were prepared in the shade and they were immediately placed into a container of water before setting into trays. Unless affected by disease or watering defects, approximately 80% of a batch of seedlings were sufficiently uniform to be used in a single experiment. Most of the remainder were discarded due to poorer growth and uneven stem extension as part of an edge effect.

3.1.4 Application of treatments to cuttings

All treatment solutions were prepared from distilled water, IBA stock solutions were prepared in 100% ethanol to provide a final ethanol concentration of 1%.

Treatments were supplied as 15 ml aliquots in a 50 ml, polycarbonate tube (Bunzyl). Single cuttings were placed in each tube, the pressure of the leaves on the side of the tube holding them in place. Treatment tubes were packed in disposable plastic tubs, eight tubes per tub, with a single treatment in each tub. Fifteen tubs were placed in a tray, in randomised blocks. Humidity and air circulation was maintained in the tray by covering the tray with a sheet of glass, filling the tray with water to a depth of approx 2 cm and then bubbling air from an aquarium tank pump into this water. This maintained gentle circulation of humidified air inside the tray. There was no significant change in the volume of treatment solutions over the 48 hours for which most treatments were applied. Treatments were all applied in the dark, in a controlled temperature room at 20°C ± 2°.

Figure 3.1a

Cutting preparation

At the completion of the treatment period the cuttings were rinsed under running water to remove the treatment solution. They were then replanted into Kwik Pots in a randomised complete block design, with one cutting per treatment, per block. The filled trays were immediately returned to the mist-bed and drenched with Previcur.

3.1.5 Mist-bed conditions

Cuttings were rooted in trays on a heated sand bed. The temperature was maintained at 24°C ± 2°. The bed was covered by a shaded polythene cover, allowing less than 50% light penetration inside a temperature controlled glasshouse. Humidity was maintained at a very high level by a centrifugal humidifier (Defensor AG, Zurich, Model 2002), which maintained a fine mist over the heated bed. Additional watering was provided by four, low volume spray nozzles connected to a mist-leaf.

All cuttings were drenched with Previcur (Schering Pty Ltd), 1.5 ml/l, immediately after being placed on the mist-bed. This was followed by weekly applications of one of five fungicides, used in rotation, see table 3.1.

Table 3.1 Fungicidal drenches

Fungicide	Manufacturer	Concentration
Benlate	DuPont	1 g/L
Ronilan	BASF	1 g/L
Fongarid 250WP	Ciba Geigy	1 g/L
Bavistin	BASF	1 g/L
Thiram 80	Roche Maag	2 g/L
Ridomil MZWP	Ciba Geigy	1 g/L

3.1.6 Assessment of cuttings

Rooting was assessed after an appropriate period for each experiment. This was usually five weeks. Cuttings were carefully removed from the tray and the adhering potting mix washed off. The roots were counted and a visual assessment was made of callus development relative to the diameter of the stem of the cutting (Figure 3.1b). An eight point rating scale was used, although in practice most cuttings were found to rate at 5 or less. Cuttings were assessed without reference to the treatment applied to reduce the possibility of operator bias affecting the assessment of cuttings.

In all experiments, the percentage rooting refers to the percentage of surviving cuttings with one or more roots visible. Because of this, the number of replicates per treatmet often varies between treatments due to variations in survival. The data for the mean number of roots per cutting is calculated on a per rooted cutting basis. Thus, in treatments where there is a low percent root initiation, the number of replicates for this statistic is also low. The number of replicates for each treatment and measurement combination is supplied in the appropriate appendix table in the columns labelled 'Count'

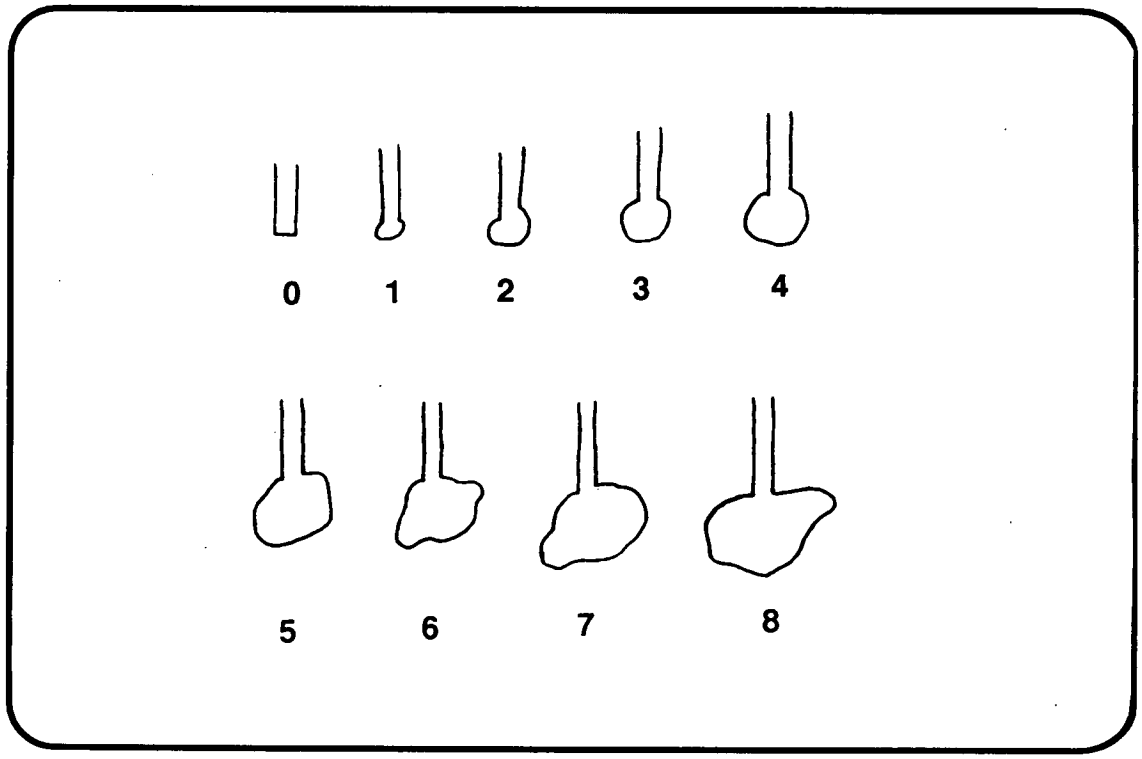


Figure 3.1b

Callus rating scale.

3.2 HISTOLOGICAL TECHNIQUES

The basal portion of freshly prepared cuttings and callused cuttings were collected, fixed, embedded and sectioned for histological examination.

3.2.1 Sample collection

Stem samples for histological examination were collected from freshly prepared stem material or from cuttings removed from the mist-bed. The majority of samples were collected from cuttings in the seedling age experiment. The cuttings were chosen at random from within all treatments. After the cuttings had been set in the mist-bed, fresh samples were collected every two days, for the first 14 days. A final sample was collected 28 days after cuttings were set. This provided samples with a range of callus development and from four different nodes.

Additional samples were collected from time to time as needed either from cuttings surplus to experiment plans or from specific stock plants.

3.2.2 Fixing, dehydration and clearing

Cuttings were briefly rinsed in tap water. The lower 2 to 3 cm of the cutting was removed and immediately fixed in FAA (5 ml commercial formalin, 5 ml glacial acetic acid, 90 ml 50% ethanol). Samples were stored in the dark until dehydration and clearing.

The samples were dehydrated in 25 ml sample tubes using 10 to 15 mls per 5 to 10 stem pieces. Samples were first rinsed twice in 70% ethanol for 30 minutes each, then stored overnight or until needed. The remainder of the dehydration series consisted of:

95% ethanol for 2 hours,
100% ethanol, two quick rinses then 2 hours,
Cleared in 50 : 50 ethanol : xylene for 1 hour,
100% xylene for 1 hour.

3.2.3 Paraffin Infiltration and Sectioning

Paraffin infiltration was achieved by removing part of the xylene from the tube and adding molten paraffin to give a 50:50 ratio, then mixing. The remaining xylene was evaporated off in a heated water bath under vacuum for several days until volume changes ceased, indicating that all the xylene had been removed. The samples were transferred to fresh, melted paraffin, with two more changes of paraffin with several hours minimum between each change. Samples were either immediately embedded in paraffin blocks or stored in solidified wax for later use.

Blocks were sliced into 12 micron sections, using a Jung rotary microtome. Sections were relaxed in a waterbath and floated onto slides, 20 to 40 sections per slide. Mayers adhesive was used to hold the sections in place (Prakash, 1986). For transverse sections, up to 150 serial sections per sample were stained. For longitudinal sections, all were mounted and stained. Only blocks with complete or nearly complete series of sections were fixed onto slides.

3.2.4 Staining

Sections were stained in Safranin and Fast Green, as described by Jensen (1962). Paraffin removal and rehydration was achieved by 5 minute rinses in a rehydration series consisting of: xylene, 50/50 xylene/ethanol, absolute ethanol, 95% ethanol, 70% ethanol, 50% ethanol.

Sections were stained in Safranin overnight (0.5% in 50% ethanol for 18 hours), rinsed in water, destained in acidified 70% ethanol for 5 seconds, rinsed in 95% ethanol then absolute ethanol, for 30 seconds each. Counter staining was with 0.5% Fast Green dissolved in 50% ethanol/ 50% clove oil for 20 seconds. Three, 15 minute rinses in a differentiation solution (50% clove oil, 25% ethanol, 25% xylene) were followed by three rinses in xylene.

Stained sections were covered with glass cover slips using Canada balsam, dried to remove the xylene and stored for later examination.

3.2.5 Clearing

Attempts were made to clear stem samples for examination of material prior to sectioning, as described by Gardner (1975)

Fresh stem samples were placed directly into 70 % ethanol but rapidly blackened from the formation of phenolics. Sections were rinsed in warmed 70 % ethanol for up to a week with several changes of ethanol in an attempt to remove phenolics. Soaking in 3% chromic acid for 24 hours and longer failed to decrease the phenolic content of the samples.

3.2.6 Photography

Permanently mounted sections were viewed on a Zeiss Axioskop microscope. They were photographed using Kodak Kodachrome print film and an Olympus OM-10 camera.

3.2.7 Image processing

Computer image processing was used to generate images from serial sections to demonstrate the connection between root initial formation and associated vascular tissues.

3.2.7a Scanning of sections

Sections were photographed using a digital video camera to provide separate red, green and blue images for each section. Each image captured using a Data Translation 2955 frame grabber card and Global Lab Image application to average the image over 50 frames. The set of three images for each section were saved as TIFF format files and transferred to a Macintosh computer for image processing. The images obtained were at a resolution of 72 x 72 dpi and an image size of 768 x 512 pixels.

3.2.7b Processing of sections

Image processing was carried out using the public domain NIH-Image program, version 1.58, (written by Wayne Rasband at the US National Institutes of Health and available by anonymous ftp from zippy.nimh.nih.gov). This generated a stack of images from the serial sections. Each successive section was aligned to its predecessor and stacked to form a 40 slice stack of images.

Full colour images were first re constituted from three colour RGB stacks. The sections were then aligned using the image alignment algorithm AlignSlice developed by Carl Gustafson and Oleh Tretiak which is available as an add-on to NIH Image. The best fit to reduce translational and rotational movement of features between sections was transferred to an image stack.

3.3 APPLICATION OF ELECTRIC POTENTIALS

A tank was constructed in which low voltage currents could be applied to the base of cuttings. A flow-through liquid system was devised to prevent the accumulation of electrolytes around the electrodes. Electrical isolation of the unit was maintained by making the nutrient inflow and outflows run as steady dripping into funnels.

3.3.1 Equipment

The tank used for these experiments was constructed after discussion with Dr I Newman, of the Physics Department at the University of Tasmania. The aim was to maintain a constant electrolyte concentration around the base of the stem in opposition to the tendency for ion migration to occur under the influence of the applied electrical potential difference.

The tank was constructed from a one metre length of rectangular section PVC pipe, laid on its side (see Figures 3.3a and 3.3b). The ends were made from the same material and the joints sealed with silicon rubber. 8 mm diameter drain holes were drilled into the ends 35 mm above the base of the tank. Each drain hole was connected to a short length of flexible PVC tubing resting on a vertical screw mechanism. This allowed the tubes to be raised or lowered to make fine adjustments to equilibrate the flow rates from each drain. Electrical isolation was maintained by ensuring that the drain tubes emptied by dripping into funnels. To overcome surface tension in the drain tubes and to assist in equilibration of flow rates, a length of cotton wool was laid in the bottom of each drain tube. The electrodes lay on top of the cotton wool.

The two electrodes were made from 2 mm diameter, 80 mm lengths of stainless steel wire, bent into an L shape. They were inserted into slots, cut into the upper surface of the flexible PVC tubes, so that one end of each electrode just protruded into the tank and the other end protruded above the tube. Power was supplied by either two or six 1.5 Volt, Eveready size C Super Heavy Duty batteries connected in series. Current and potential difference measurements were made using a digital multimeter (Model Q-1442, Dick Smith Electronics, North Ryde, NSW), rated as accurate to ± 1%.

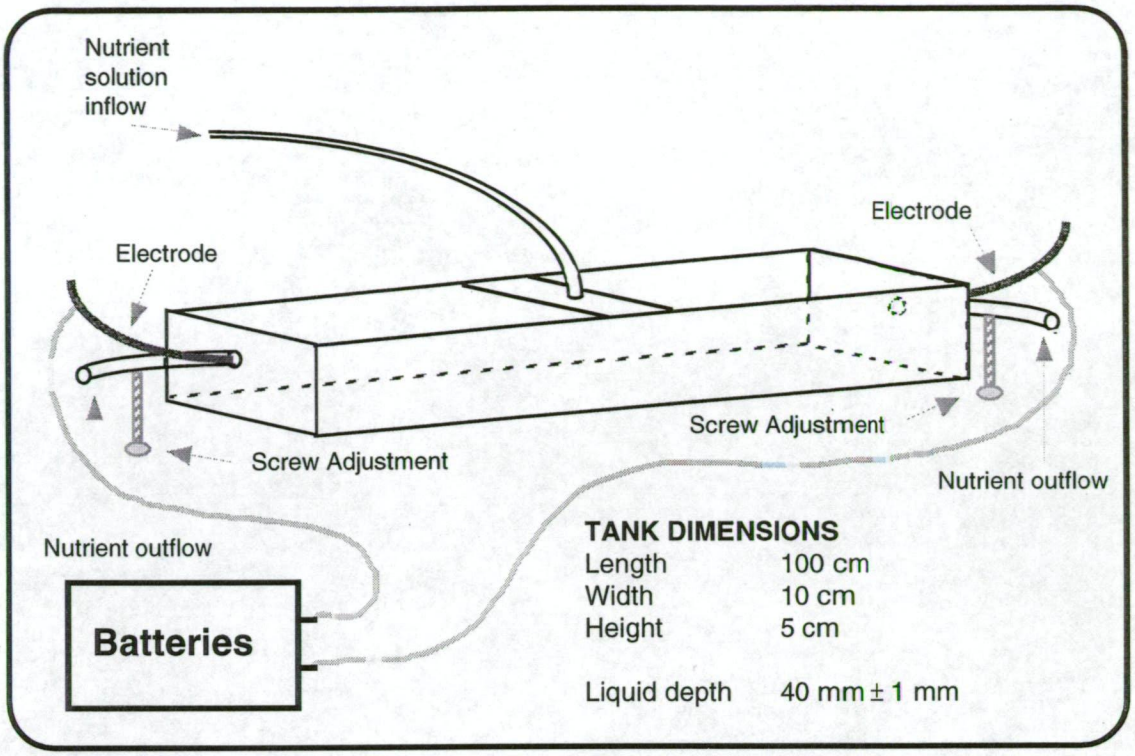


Figure 3.3a

Tank construction details

Figure 3.3b

Photograph of tank *in situ*.

A 1 metre length of clear plastic ridge capping covered the tank. This provided a close fit along the length of the tank. The ends of the cover were loosely capped with aluminium foil. Humidity was maintained by bubbling air through a side chamber on the tank. This air was then directed into the centre of the tank cover, maintaining a gentle flow of humidified air towards each end of the tank.

3.3.2 Nutrient solution

The electrolyte solution consisted of 0.1 Normal Hoaglands solution (Hoagland and Arnon, 1950) or a modified, calcium free solution, as detailed in Table 3.3. This was prepared using distilled water and 10 litres of Normal Hoaglands solution as a stock for each experiment. This was also prepared with distilled water from analytical reagents. Electrolyte solutions were supplied to the treatment tank by means of a peristaltic pump (Cole Palmer, Masterflex) connected to a 50 litre reservoir. Solution flow rates were maintained at 2 litres per hour. The outflow from the two ends were equilibrated to within 10% prior to the commencement of experiments, this equilibration was checked during the treatments and occasionally adjusted. To maintain electrical isolation, the nutrient supply dripped into a funnel whose outlet ran into the centre of the tank.

	Normal Hoaglands (ml/l)	Ca free modification (ml/l)
KH_2PO_4 (1 M)	1	1
KNO_3 (1 M)	5	5
$Ca(NO_3)_2$ (1 M)	5	
$MgSO_4$ (1 M)	2	2
NH_4NO_3 (1 M)		5
FeEDTA (3.28 g/l)	1	1
Micro nutrients	1	1

Table 3.3

Normal Hoaglands solution and Calcium free solution used at 1 in 10 dilution for the bathing medium in potential difference treatments.

The flow pattern of solution within the tank was found to be remarkably even. An ink solution was fed into the tank via the nutrient inflow pump while the current was turned off. The dye travelled in two even fronts along to each end of the tank and reached the outlets within two minutes of each other.

Prior to use, the apparatus was cleaned with a 4% bleach solution, followed by several rinses in distilled water. As most of the experiments were carried out in the dark, algal growth was not usually a problem. In the experiments using IBA in the electrolyte solution, the ethanol used to dissolve the IBA (1% or 0.5 % final concentration) was sufficient to provide a carbon source . This resulted in some slight blocking of the tank outlets after two or three days operation. Later experiments used the K salt of IBA to overcome this problem.

3.3.3 Cuttings

Whilst not undergoing treatment in the tank, cuttings were held in the same controlled environment room, in PVC trays filled with the same treatment solution. They were covered with a glass sheet and aquarium air pumps provided aeration, humidity and air circulation by pumping air through the bathing medium via aquarium air-stones.

Cuttings were exposed to the treatments by mounting them vertically in a polystyrene raft, floating on the surface of the treatment solution. The rafts were made from 10 mm thick sheets of expanded polystyrene. Each raft was 8 cm x 35 cm, with three rows of holes in the raft of a size to support the cuttings when pushed into the raft. At least 1 cm of each cutting protruded below the raft. Up to 40 cuttings could be mounted in each raft. The tank could hold two rafts at a time. None of the cuttings were closer than 10 cm to the electrodes. This system worked very well for fresh cuttings or lightly callused cuttings but had a tendency to damage some of the more heavily callused cuttings in experiments using pre-callused cuttings.

Once treatments were completed, the cuttings were returned to the mist-bed and treated as per normal.

3.4 PLASMA MEMBRANE EXTRACTION and ATPase ASSAY

Plasma membrane extraction procedures were adapted from Douglas and Walker (1984). All extraction procedures were carried out on ice. All buffers, glassware and rotors were chilled to 4°C before use, and kept on ice during use and transport.

3.4.1 Sample collection

Treatments were applied to node 4 cuttings, four weeks after collection and placing on the mist-bed. Rooted cuttings and cuttings showing excessive fungal damage were discarded prior to treatment.

Callused cuttings were rinsed briefly in tap water. The lower 1 cm of the stem and attached callus was cut off and immediately placed on ice. Any roots that had formed in the period since application of treatments were removed. The harvested material was given a second rinse in distilled water, patted dry and weighed and returned to ice until processed.

3.4.2 Membrane extraction

All stages of processing were carried out with the samples in an ice bath. Each sample was processed for 30 seconds in an extraction buffer (Buffer A) using an Ultra Turrax (20,000 rpm). A ratio of 5 mls of buffer for every 2 grams of callus and stem material was used throughout. After processing, the homogenate was immediately filtered through four layers of cheese cloth and rinsed through with two washes of 5 ml of buffer.

The filtrate was centrifuged for 5 minutes at 13,000 g, (10,000 rpm in JA-20). The supernatant was transferred to a fresh centrifuge tube and centrifuged at 90,000 g for 30 minutes (35,000 rpm in Beckman 70 Ti rotor). The supernatant was discarded and the pellet was re-suspended using a camel hair brush in 5 ml of Buffer B.

The re-suspended pellet was layered on top of previously prepared discontinuous sucrose gradient, made from 20%, 34%, 45%(w/v) sucrose in Buffer B. The sucrose gradient was centrifuged at 74,000 g for 80 min (24,000 rpm in a Beckman SW25.1 rotor), using slow acceleration and deceleration programs.

The 34%/45% interface was collected using a pasteur pipette. The position of the interface was marked on tubes during preparation and was checked against the appearance of a visible liquid boundary. Approximately 4 mls of the gradient was collected and diluted with 15 mls of buffer B, which was centrifuged at 90,000 g for 30 min, (35,000 rpm in Beckman 70 Ti rotor).

The supernatant was discarded and the pellet re-suspended in either 2 or 3 mls of buffer B. Each sample was divided, part kept for immediate use and the remainder placed into cryo tubes, frozen in liquid nitrogen and stored at -80°C. This storage method should allow retention of ATPase activity for several months (Briskin, Leonard and Hodges, 1987).

3.4.3 ATPase assay

The ATPase assay was based on the method of Douglas and Walker (1984), with minor modifications.

The assay was carried out in 1.5 ml micro cuvettes (Bio-Rad Laboratories), in triplicate. 0.3 ml of assay buffer, containing appropriate inhibitors, was placed in a cuvette. This was warmed to 30 °C for five minutes. To this, was added a 50 µL aliquot of membrane fraction. The mixture was incubated at 30 °C for 30 minutes.

The assay was stopped by the addition of 0.8 ml Ames' solution, for phosphate determination Ames (1966). Phosphate evolution from ATP was calculated and converted to a nMol PO_4 per mg of protein basis

3.4.4 Phosphate determinations

Phosphate determinations were based on the method of Ames (1966). Assays were either carried out in disposable microcuvettes directly or in 1.5 ml micro-centrifuge tubes. To 0.35 ml test solution was added 0.8 ml of Ames' solution and mixed. A water + Ames' solution was used as a blank. Assays were incubated at 37 °C for one hour, poured into micro cuvettes and absorbance measured at 820 nm.

3.4.5 Protein determinations

Protein determinations were made using the Bio Rad Protein Assay kit (Bio-Rad Laboratories (Bradford method). The kit was used according to the manufacturer's instructions, using a protein standard curve prepared with bovine serum albumin (BSA), as supplied with the kit. Assays were performed in triplicate, using freshly extracted membrane preparations. Assays were later re-run using frozen samples diluted with buffer, to allow all samples to be measured using the micro assay procedure. Absorbance was measured at 595 nm.

3.4.6 Tables of extraction buffers and assay reagents

Table 3.4.6a Extraction buffers

Buffer A ,	Buffer B ,
0.25 M Sucrose	20% Sucrose
6 mM DTT	6 mM DTT
1.0 mM $CaCl_2.2H_20$	25 mM Tris-MES buffer, pH 7.2
25 mM Tris-MES buffer, pH 7.2	

All buffers were prepared the day before use and stored in a refrigerator.

Table 3.4.6b ATPase assay solutions

Fresh assay buffer was prepared on the day of use from 20 mls of stock solution + 1 ml Tris ATP solution + 1 ml vanadate or molybdate stock solution as appropriate.

ATPase assay solutions

To make 25 ml	A	B	C
Assay Buffer Stock	20 ml	20 ml	20 ml
Tris-ATP	1 ml	1 ml	1 ml
$(NH_4)_6Mo_7O_{24}.4H_2O$		1 ml	
Na_3VO_4			1 ml

Prepared on day of use from stock solutions

Assay Buffer Stock Solution

to make	250 ml
37 mM MES	2.2875 g
3 mM $MgSO_4$	0.2312 g
50 mM KCl	1.165 g

Adjusted to pH 6.5 with 1M Tris , stored refrigerated

$(NH_4)_6Mo_7O_{24}.4H_2O$ stock solution:

$(NH_4)_6Mo_7O_{24}.4H_2$ 0.7444 g dissolved in 100 ml, stored at room temp used 1 ml in 25 ml assay solution

Na_3VO_4 stock solution:

Na_3VO_4 0.4597g dissolved in 100 ml water and stored at room temperature.

Tris-ATP stock solution:

Tris-ATP (Sigma, VO_4 free) 0.380g dissolved in 10 mls water. Dispensed in 1 ml aliquots and stored frozen.

Table 3.4.6 c Phosphate determination reagents

Reagent A: 10%(w/v) ascorbic acid in water, made as 100 ml lots, stored refrigerated, replaced monthly.

Reagent B: 4.2 g $(NH_4)_6Mo_7O_{24}.4H_2O$ + 28.6 ml concentrated H_2SO_4 in 1000 ml H_2O, stored at room temperature.

Ames' reagent:Mix 10 ml reagent A+ 60 ml reagent B,
Prepared on the day of use and stored on ice.

Phosphate standards containing 0, 3 to 30 µM phosphate per ml were prepared from KH_2PO_4.

3.5 G COMPOUND EXTRACTIONS

G compounds were extracted from leaves from *Eucalyptus grandis* trees growing in southern Tasmania using established extraction procedures. The identity of the compounds was checked against known standards.

3.5.1 Collection and Extraction

Leaves of *Eucalyptus grandis* were obtained from a C.S.I.R.O. provenance trial in Southern Tasmania. The ten year old trees were grown from seed obtained near Coffs Harbour in New South Wales. Small branches from the lower half of the canopy were taken at random, all leaves from a selected branch were used in samples.

The extraction procedure was derived from the work of Menary (1992); Nicholls, Crow and Paton (1970) and also Dhawan, Paton and Willing (1979).
250 g leaves was homogenised in 300 ml methanol, using a Semak vitamizer. This material was filtered through 3 layers of muslin, washed with a further 100 ml of methanol and squeezed to recover as much methanol as possible (about 300 ml). 300 mls of the extracted methanol was used to homogenise a further 250 g leaves and filtered through muslin and again washed with extra methanol and squeezed.

The resulting extract was filtered using Whatman #4 filter paper under vacuum. This took some time.

The extract was evaporated down to about 100 ml in a rotary vacuum evaporator. 100 ml distilled water added and the mixture returned to the rotary vacuum evaporator to remove the remaining methanol.

The remaining aqueous phase was partitioned with 50 mls petroleum ether (40-60° boiling range). The partitioning was repeated twice with 25 ml pet ether, discarding the aqueous phase. The pet ether extracts were combined and then evaporated to dryness in weighed flask on a rotary vacuum evaporator.

The crude extract was washed with cyclohexane (5 mls). Small quantities of white crystals were observed to form in the cyclohexane over a 48 hour period at room temperature. The crude extract was dried and stored at 4°C in the dark.

3.5.2 Thin Layer Chromatography (TLC)

The crude extracts were compared by TLC against known G compound samples (Menary, 1992). Small quantities of extract and G compounds were dissolved in chloroform and spotted onto 0.25 mm thick silica gel plates 3 cm x 8 cm, cut from Merck Silica Gel 60 F-254 plates (Merck, Darmstadt, Germany).

The plates were developed in 5% diethyl ether in chloroform for about 30 minutes. The plates were examined under a UV light and absorbing spots marked. The Rf values for these and the G compounds were compared against previously published results.

3.5.3 Mass Spectroscopy

The identity of the extracts was confirmed by mass spectroscopy carried out on a Kratos Concept ISQ mass spectrometer, using the direct probe insertion technique. Operating parameters for the instrument were as follows: scanning from m/2 35 to 800 at 2 secs/decade; source temperature 180°C; accelerating voltage 5.3 kV; 70 eV electron beam energy and resolution 2000. The mass pectrum produced is contained in Figure 3.6a The spectra from samples were compared to that obtained by Menary (1992).

3.5.4 Application to cuttings

For the preparation of treatment solutions it was assumed that the molecular weight of the mixed compounds was approx 280 and that the sample was approximately 90% pure. A G compounds stock solution was prepared containing 0.0310 g crystals = $\pm 10^{-3}$ M, dissolved in 10 ml ethanol. All of this solution was used to prepare 1 litre of 10^{-4} M solution, serial dilutions were prepared from this treatment. The control solution also contained 1% ethanol. 5 drops of Tween 80 was added to each treatment solution. All treatment solutions were prepared on the day of use.

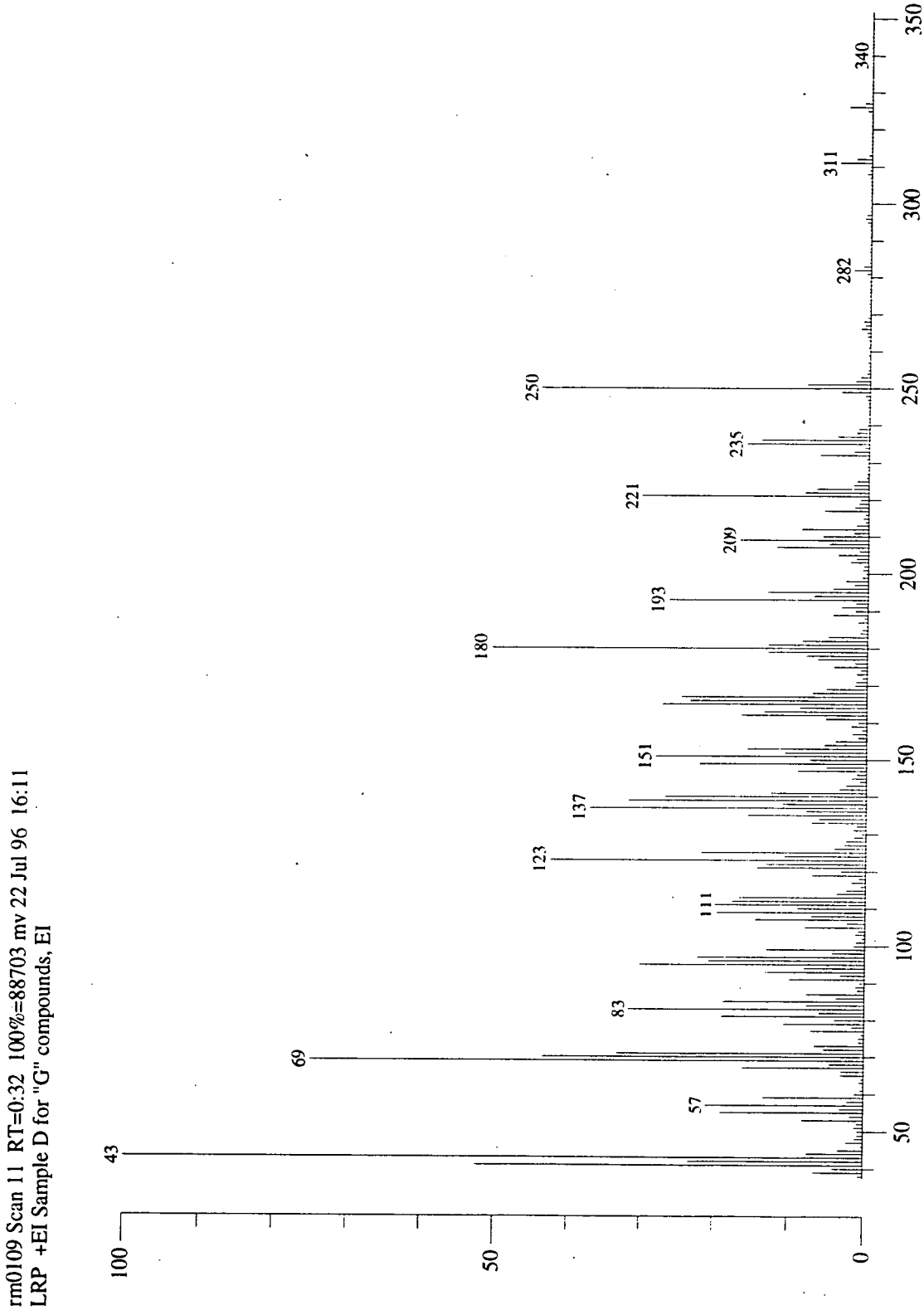


Figure 3.6a

Mass spectrum of G compound extract used in experiments.

3.6 *IN VITRO* EXPERIMENTS

The effects of the addition of a range of sterols to *in vitro* media was tested in an experiment carried out at the North Eucalypt Technologies research centre, at Ridgley in northern Tasmania.

3.6.1 Media

Cultures were grown on proprietary culture media developed by North Eucalypt Technologies, for use in *E. nitens* micropropagation (de Little *et al*, 1992). Proliferation and root initiation media were prepared to standard formulae and dispensed into 250 ml Bunzyl polycarbonate culture vessels in 50 ml aliquots. The treatment compounds were dissolved in re-distilled ethanol. After autoclaving, the flasks were opened inside a laminar flow cabinet while media was still liquid, at a temperature of approx 60°C. 0.5 ml of sterol solution was dispensed directly into the media and swirled briefly to mix. The resulting flasks had a theoretical ethanol concentration of 1%, but would actually be less due to evaporation from the still warm media.

Treatments	Final concentration	
Control		100 ml ethanol
Stigmasterol	20 mg/l	200 mg in 100 ml ethanol
Stigmasterol	50 mg/l	500 mg in 100 ml ethanol
Cholecalciferol	20 mg/l	200 mg in 100 ml ethanol
Cholesterol	20 mg/l	200 mg in 100 ml ethanol
Stigmasterol acetate	20 mg/l	200 mg in 100 ml ethanol

3.6.2 Explants

Cultures were transferred to the media the same day. For the proliferation media, five clumps per flask, approximately 5 mm x 5 mm were cut from stock proliferation cultures. Ten clone lines were used, 1 replicate per clone, from five to six week old cultures which had been grown on standard proliferation medium.

For the rooting media there were two types of explants used. Type 1 explants were microcuttings of two node stem sections taken from plantlets growing on hormone free medium, five cuttings per flask. The apical shoot of each stem was excised. The second type of explant were single stems, 1 to 1.5 cm long taken from 5 to 6 week old proliferation cultures, stem apex intact.

Cultures were placed in a growth chamber maintained at a temperature of 22°C and a 16 hour photoperiod. Light was provided by cool white fluorescent tubes. Light intensity at the level of the culture flasks has been measured at approximately 40 μEinsteins m^{-2} s^{-1}.

3.6.3 Measurement

Experiments were measured after 6 weeks growth. For the proliferation medium, individual clumps were removed, cleaned of adhering agar and weighed. To avoid competition effects from the fungi, in flasks where contamination was visible only those clumps that were more than 5 mm from the edge of the contaminating growth were assessed.

For root initiation cultures, measurement of the number and length of roots per micro-cutting cutting was intended. However only one root was found.

3.7 GENERAL MATERIALS AND EQUIPMENT

3.7.1 Equipment, reagents and solvents

The following general laboratory equipment was used.

Blenders:	Semak Vitamizer, Model CVS-3L
	Ultra Turrax, probe TP18/2, 20,000 rpm, Janke and Kunkle KG
Centrifuge:	Beckman J2-21ME, Beckman Instruments Australia, Gladesville NSW.
Ultra centrifuge	Beckman L8 - 70M
Spectrophotometer:	Shimadzu UV 160
Water stills:	Model O3D, LabGlass, Brisbane.
Air Pumps:	President Model Pi - 6000 Aquarium air pump, Pets International Pty Ltd, Chester Hill, NSW.

All laboratory chemicals were analytical grade obtained from Sigma Aldrich Pty Ltd, Castle Hill, NSW, unless otherwise stated. Petroleum ether, ethanol and methanol were technical grade, re-distilled prior to use.

3.7.2 Statistical analysis

All statistical analyses were carried out using Statview, version 1.2, from Abacus Concepts Inc.

The significance of differences between mean rooting percentages were tested using the χ^2 test with differences accepted at $p = 0.05$. Root number and callus rating means were subjected to an analysis of variance, using untransformed data. Unless stated otherwise, tests of statistical significance are made between the treatment and the relevant control. Summary data and statistical analysis for all experiments are included in the Appendix.

4. EXPERIMENTAL

4.1 INTRODUCTION

The experiments described in this chapter of the thesis are intended to cover a wide range of techniques that may have application to the propagation of cuttings. The emphasis was to attempt to develop some alternative strategies on the problem of macropropagation of a "difficult to root" species, such as *E. nitens,* since existing techniques have been found wanting.

The experiments in this thesis are based on the hypothesis that the most important factor in root initiation is the induction of polarity in tissues, as the first stage of the formation of a new meristem. Investigations were designed to provide information on the factors controlling cellular polarity and to demonstrate how those factors might be influenced to promote root initiation. This has led to the development of experiments using previously untried techniques and some techniques that have not been widely adopted for the propagation of cuttings.

Many of the experiments described in the following sections are preliminary. These are intended to demonstrate the potential of some new approaches to the study of root initiation. The results of some of these experiments are, therefore, also of a preliminary nature. The need for additional data and clarification of the results obtained so far is considered in the discussion of the results of each section.

4.2 CHANGES IN ROOTING ABILITY WITH ONTOGENETIC DEVELOPMENT

4.2.1 Introduction

This experiment was designed to quantify the loss of rooting ability that occurs in seedlings as they grow and develop. Cuttings were collected at several stages of the growth of a single batch of seedlings. The rooting ability of the cuttings collected at each stage was compared.

4.2.2 Rationale

It is well documented that the ability of eucalypt cuttings to initiate roots is less when they are taken from higher nodes in a developing seedling (Hartney, 1980). A similar pattern has been documented for loblolly pine, *Pinus taeda*, (Diaz-Sala *et al*, 1996). In some instances, this loss of rooting ability has been linked to particular node numbers (Paton *et al*, 1981).

In *E. nitens*, Willyams *et al,* (1992) assessed the rooting ability of micro-cuttings from *E. nitens* seedlings raised *in vitro*. They found that the cuttings lost the ability to initiate roots within a few weeks of germination. In that research, no attempt was made to link this change in rooting ability to any marker of development, such as the number of leaves or nodes on the seedling.

This experiment was designed to quantify the change in rooting ability of *E. nitens* seedlings by determining if rooting ability changes at a specific node number or gradually declines with seedling growth. It was also intended to determine if rooting ability is correlated in any way to the ability of a cutting to form callus.

Samples were collected from this experiment for later histological examination to determine if there were any changes in the anatomy of the stem that correlate with changes in rooting ability (see Section 4.3).

4.2.3 Procedures

All of the cuttings were collected from seedlings grown as a single batch of cuttings over a period of seven weeks in mid to late winter. The seedlings were placed in a glasshouse on 30 June. The first setting of cuttings were collected six weeks later. Seedlings were grown in tapered plastic pots 5 cm x 5 cm x 15 cm, with an approximate volume of 350 ml, using standard potting media. The pots used for growing seedlings in this experiment were larger than those used for all others and gave greater plant spacing, less shading as plants grew larger and additional soil volumes. To ensure that all the cuttings were as uniform as possible, the plants were moved in the glasshouse at regular intervals to minimise edge effects.

All cuttings consisted of the three highest nodes with fully expanded leaves. This ensured that the basal tissues of the cuttings were all at a similar stage of development. Four batches of cuttings were collected at different stages of growth of the seedlings. This resulted in cuttings with base nodes at node 1, node 2, node 3 and node 5, as described in the General Methods section.

Treatments were applied by inserting cuttings through a sheet of polystyrene foam, so that 1 cm of each stem was protruding below the sheet. The foam sheets were floated in plastic tanks containing 10 litres of treatment solution. The treatment solution was either 20 mg/l IBA in distilled water or just distilled water as a control. The IBA was dissolved in a few ml of 1 N KOH, similar quantities were added to the control treatment. The tanks of treatment solution and cuttings were kept on a shaded mist-bed for 48 hours. At the conclusion of the treatments, cuttings were rinsed in running water and transferred to standard Qwik Pot trays, containing standard potting medium and drenched as per normal. There were four replicates per treatment, consisting of 48 cuttings per replicate. All cuttings were assessed 30 days after treatments commenced.

Even though the method of applying auxin is quite different from traditional quick dips in ethanolic IBA solutions, an IBA treatment of 20 mg/l for 48 hours was chosen for several reasons:

a. It is a treatment known to be effective in promoting root initiation in *in vitro* cultures of *E. nitens* (Willyams *et al*, 1992);
b. There are problems getting reproducible results from ethanolic quick dips, where there is poor control over how much IBA adheres to the cutting, how quickly it is absorbed and how much gets transferred to surrounding soil (Blazich, 1988a; Loach, 1988); and
c. It was intended to avoid getting any wounding effect from high ethanol concentrations, which might affect the results from the control treatments (Wilson and van Staden, 1990). There was no testing of this within the experiment.

The design of this experiment did not account for the effects of seasonal variations in rooting ability. This would normally show that cuttings collected in September have a higher rooting ability than those collected in August (de Little *et al*, 1992). The reverse of this was found in this experiment.

Node 4 cuttings were not treated due to a limit in the number of plants available for treatment. Problems with humidity control for a few days towards the end of the experiment resulted in some node 5 cuttings receiving a degree of moisture stress, mostly in cuttings close to the edge of the mist-bed. This resulted in some partial leaf desiccation but no apparent stem damage. It is believed to be unlikely that this had an effect on root initiation.

This experiment was measured at 4 weeks, partly to allow comparison with the results of Willyams et al (1992) who claimed that all root initiation was complete within this period. Later experience demonstrated that under the growth conditions used for this research, some root initiation is likely to have continued to occur for at least another two weeks. There is no evidence to suggest that this later root initiation would alter the general trend of declining rooting ability in older, larger seedlings.

4.2.4 Results and interpretation

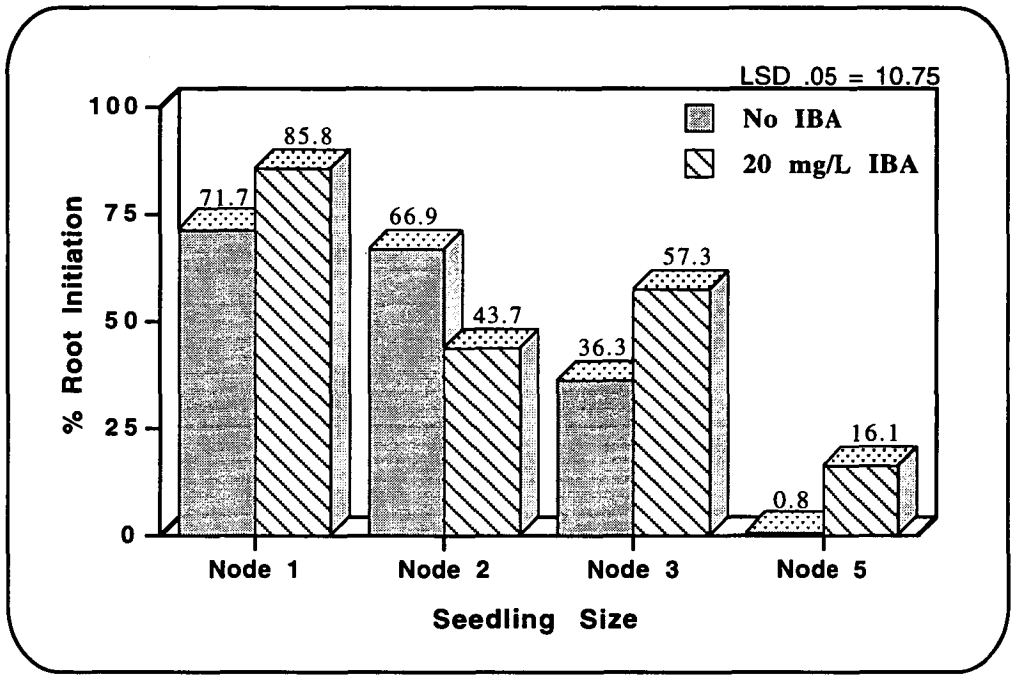


Figure 4.2a

Root initiation in cuttings originating from different sized seedlings, showing the effect of IBA treatment for 48 hours.

The change in root initiation rate observed between node 1 cuttings and node 5 cuttings is dramatic(Figure 4.2a). This is in line with the pattern demonstrated for other species of *Eucalyptus*. The actual level of root initiation is substantially higher than measured in *E. nitens* by Willyams *et al* (1992), who recorded 16% root initiation in cuttings from 8 week old seedlings receiving a similar IBA treatment. In this experiment, the node 1 cuttings were set in the mist-bed some 9 weeks after germination commenced. This variation may be explained, in part, by differences due to the *in vitro* test system used by Willyams *et al* (1992), which would result in different light conditions and mineral nutrition of the parent plants.

In general, the effect of IBA treatment seems to be to boost root initiation, however there is still an apparent substantial decline in rooting ability between node 1 nd node 5. Beyond this, it is difficult to draw any conclusions about endogenous hormone levels or hormone sensitivity from a single set of treatments, such as this.

The result for node 2 cuttings seems somewhat anomalous, there is no readily apparent reason for the decrease in percent root initiation caused by application of IBA to these cuttings. This pattern was not repeated in the measurement of the number of roots per cutting, see below.

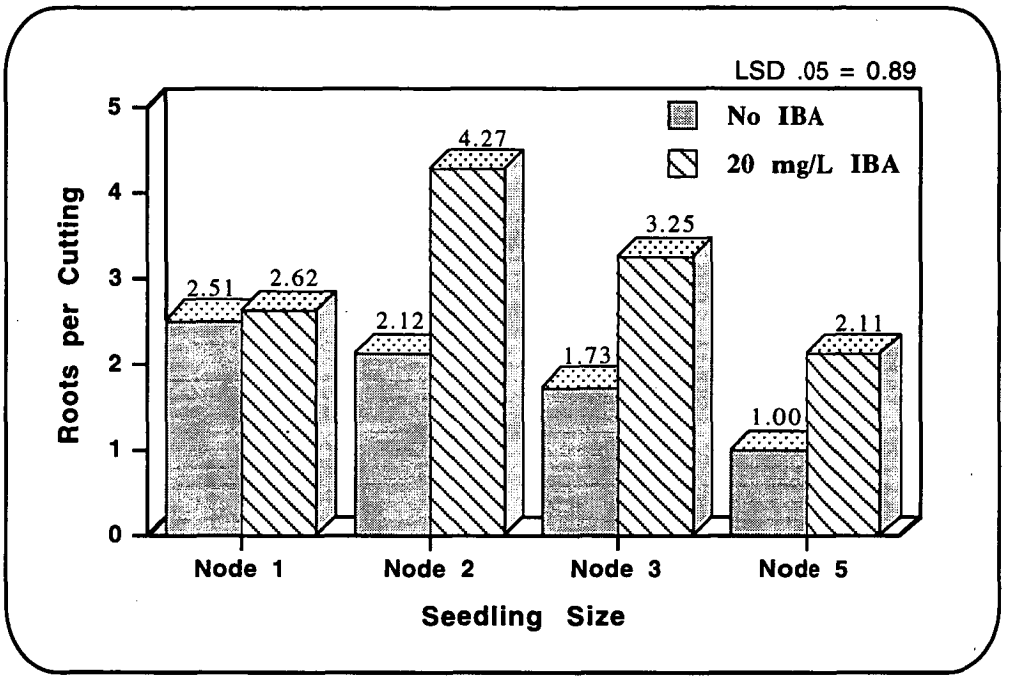


Figure 4.2b

Roots formed per rooted cutting in cuttings originating from different sized seedlings, showing the effect of IBA treatment for 48 hours.

In cuttings not treated with IBA, the mean number of roots formed per rooted cutting also declined steadily with increasing node number (Figure 4.2b). This is another expression of the higher degree of rooting "competence" found in small seedlings. In the IBA treated cuttings, the pattern is less clear. In contrast to the result for percent root initiation, the number of roots formed per cutting increased in node 2 cuttings with IBA treatment. In addition, there would appear to have been an increase in the number of roots produced per cutting.

There was substantial callus development in all cuttings, although there is a slight but significant decline in the callus rating at node 5 in untreated cuttings (see Figure 4.2c). Auxin treatment increased callus production marginally, this increase being significant at node 5, which might suggest increased sensitivity to applied auxin.

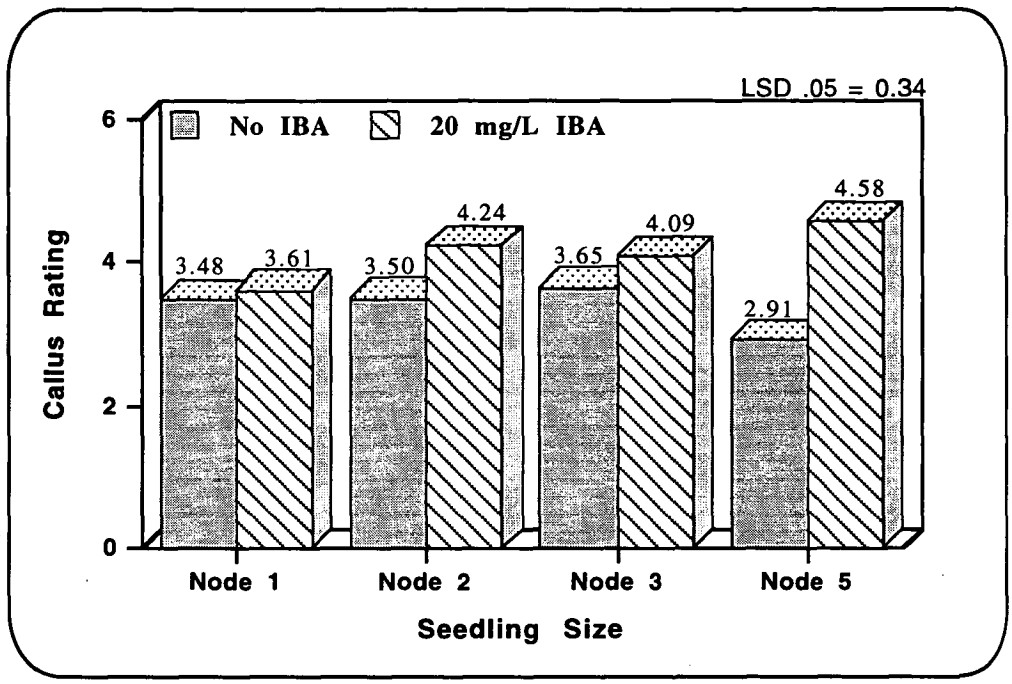


Figure 4.2c

Callus formation in cuttings originating from different sized seedlings showing the effect of IBA treatment for 48 hours.

Some caution needs to be adopted in interpreting variations in callus ratings between different node numbers. There are two problems that need to be taken into account. The first is that callus development seems to decline once roots have initiated. This is an expected result, since roots would be likely to be a stronger sink for carbohydrates than callus. The effect of this is that generally, treatments that have a higher rooting percentage will tend to have lower callus ratings than cuttings with low rates of root initiation.

The other limitation on the callus results is that the callus rating system used, which is described in section 3.1.6, is only partly successful in compensating for the variation in stem diameter. Direct quantitative measurement of callus would not improve the situation either as a larger cutting would normally be expected to produce a larger volume of callus. There is no simple method of making relative measurements of callus production unless callus volume were measured, perhaps by volume displacement in a measuring cylinder, and divided by the stem volume of the original cutting. It is unlikely that the increased effort required to make such measurements would be justified by any great increase in reliability of results.

This experiment was designed to compare rooting ability of tissues with a similar maturity or physiological age. That is, all of the cuttings were collected when the node at the base of the cutting was a similar distance from the apex of the seedling. The experiment did not attempt to assess how rooting ability changes in any given node as its tissues mature. There is evidence from other species of *Eucalyptus* to suggest that individual nodes retain some of their rooting ability as they mature (Paton *et al*, 1981).

The results from this experiment were used to provide a model for all subsequent experiments. Cuttings for subsequent experiments were collected at either node 3 or 4. From the results of this experiment, these cuttings were thought to have a 'natural' level of rooting ability so as to be able to respond clearly to either stimulatory or inhibitory treatments.

4.2.5 Discussion

In *E nitens* seedling development there is a substantial and significant loss of rooting ability in cuttings in the first few nodes of the seedlings. Without additional auxin, rooting ability declines, both in terms of the percentage of cuttings initiating roots and in terms of the number of roots formed per cutting. Callus formation may also decline, but this trend is much less substantial. On this basis, it seems unlikely that the decline in root initiation rates is related to the extent to which cuttings initiate callus.

The decline in rooting ability can only be partially compensated for by the addition of auxin. This suggests that the decline in rooting ability is not related to decreased endogenous auxin levels. However, this could only be ascertained by direct measurement. In the work of Diaz-Sala *et al* (1996) it was found that there was little difference in ability to absorb, transport or metabolise auxin, between cuttings of high and low rooting ability.

4.3 STEM AND ROOT INITIAL MORPHOLOGY

4.3.1 Introduction

Histological investigations of *E. nitens* stems were undertaken to examine the tissues involved in root initiation. There is little information available about how and when roots initiate in eucalypts generally and particularly in *E. nitens*. These investigations were designed to identify where callus initiation occurs, how it develops, and when and where root initials form.

Comparative studies of root initiation in very young seedlings and older seedling were undertaken to give information on how or why rooting ability changes. Cuttings derived from very young seedlings have a very high root initiation rate. This declines as the seedling grows. It was hoped to identify changes in stem anatomy and morphological processes of root formation which are linked to rooting ability. To achieve this, sections of cuttings at different stages of growth and with different levels of rooting ability were studied.

4.3.2 Sectioning of stems

4.3.2a Rationale

Stem pieces from the base of freshly collected cuttings were removed and fixed for later sectioning and examination. The aim was to investigate if there were any discernable differences in the stems of cuttings collected at different node numbers, which correlate with different rooting ability. It has been hypothesised that the physical and anatomical characteristics of the stem may be a factor in rooting ability (Beakbane, 1969).

4.3.2b Procedures

Most of stem sections used were collected from cuttings prepared for the experiment described in section 4.2. These were collected and prepared as described in the General Methods section.

Additional samples were collected from plants from clonal selections used by North Eucalypt Technologies. The plants were in use as stock plants for cuttings propagation at Ridgley, Tasmania. All were growing in pots in a glasshouse and had themselves originated from cuttings or *in vitro* cultures of

selected clones. Cuttings were collected from these plants, the lower 2 cm of each cutting was removed and immediately placed in FAA. All the other stages of tissue preparation were as described in the General Methods section.

4.3.2c Data and interpretation

In all of the stems sectioned and examined, the vascular system was found to be a complete ring, broken only by the emergence of leaf and axillary bud traces, just below the nodes. Many of the stems tended towards a square or rectangular profile.

There were no identifiable differences between the stems of cuttings from different nodes, apart from differences in stem diameter (Figure 4.3a, 4.3b). Sections through stems below node 1 had the same pattern of arrangement of cells as those at node 5, even though they have quite different rooting ability. Stem diameter increased with increasing node number. This increase in stem diameter appears to be a reflection of increased cell diameter, as well as an increased number of cells. There was no evidence of the presence of structural or physiological differences in the stems that would provide an explanation for the variation in rooting ability that was observed in the experiment outlined in Section 4.2. There did not appear to be any substantial differences between the stems of seedling derived cuttings and those collected from clonal stock plants, (Figure 4.3c).

Sections obtained from close to the base of the cutting were found to have a disrupted vascular cambium (Figures 4.3a, 4.3b,4.3c). The damage appears to have been caused by the crushing action of the secateurs, used to sever the cuttings from the stock plant. This feature was found to a greater or lesser extent in all the cuttings examined. It appears that the cortex and phloem of the cutting is pushed away from the underlying xylem, which is more rigid. The stress that results from this procedure results in the cells of the vascular cambium being ruptured for some distance up the stem from the base. This damage may play a significant role in determining where callus growth originates from and may also affect root initiation patterns. The extent of the disruption varied substantially in the stems examined, possibly in proportion to the diameter of the stem or the degree of lignification of the stem, although no measurements were attempted.

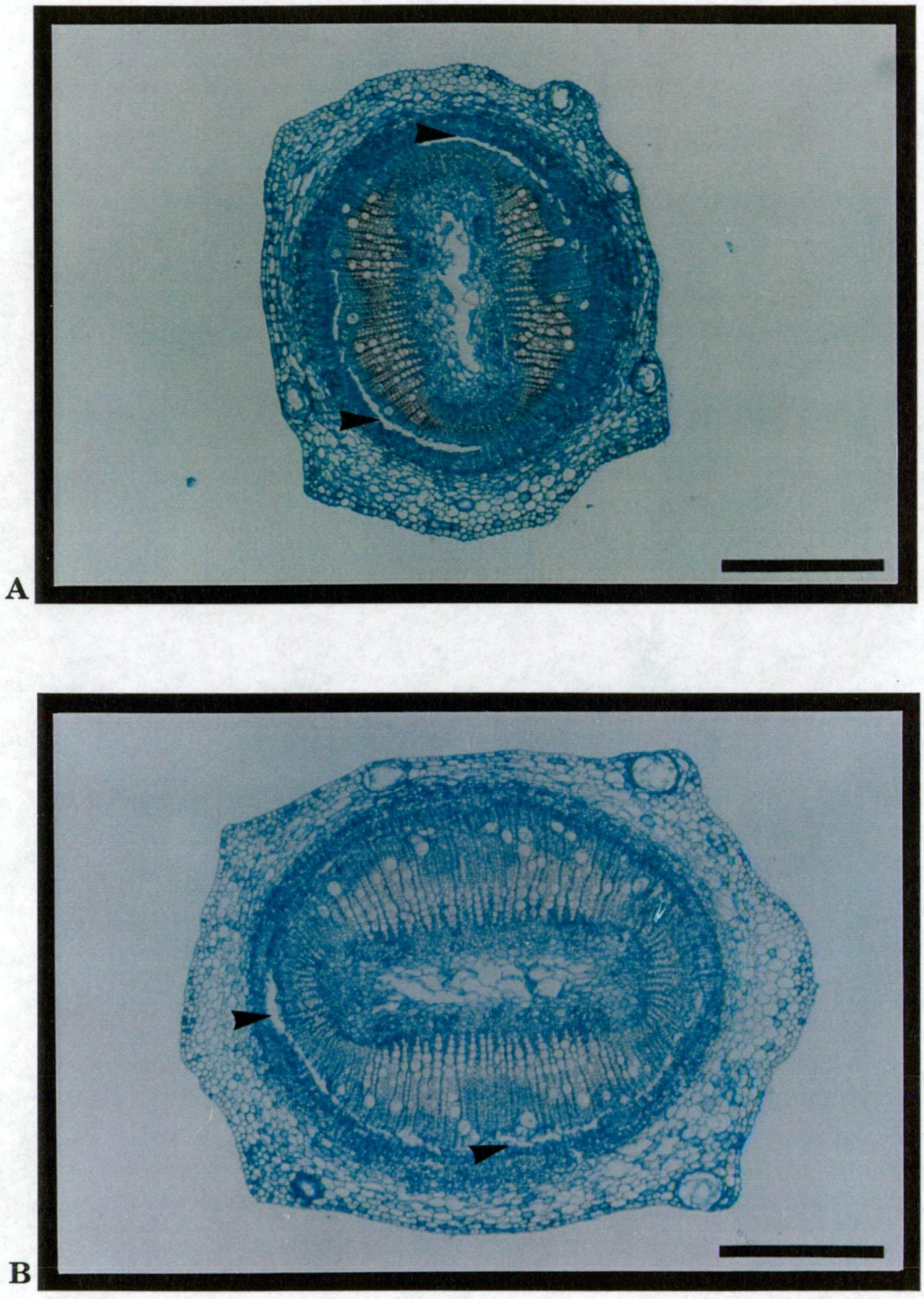


Figure 4.3a

Transverse section through stem of node one (A) and node two (B) cuttings. Note splitting of stem which results from severing the cutting (arrow). Bar = 500μ.

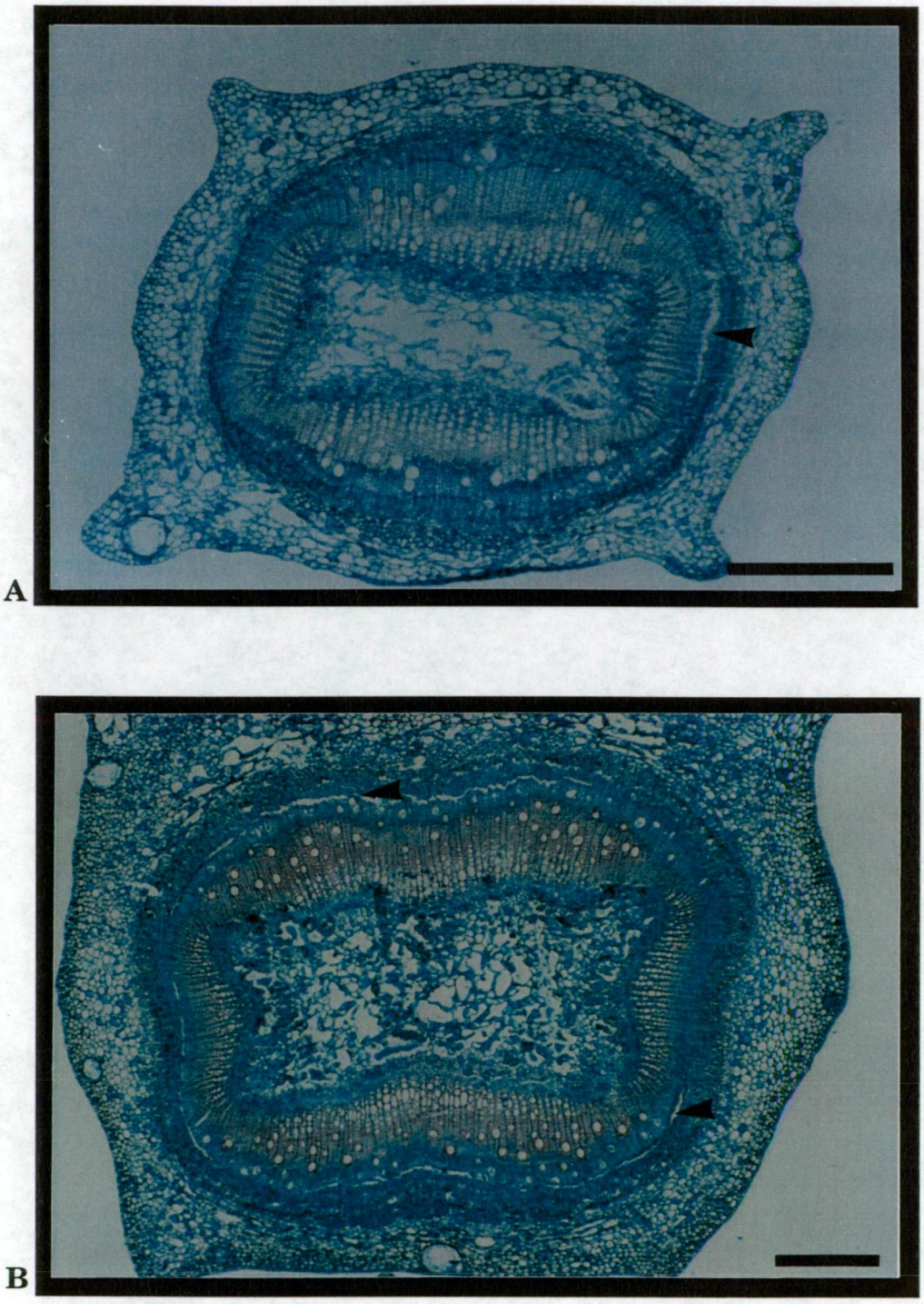


Figure 4.3b

Transverse section through stem of node three (A) and node five (B) cuttings. Note splitting of stem which results from severing of stem (arrow). Bar = 500μ.

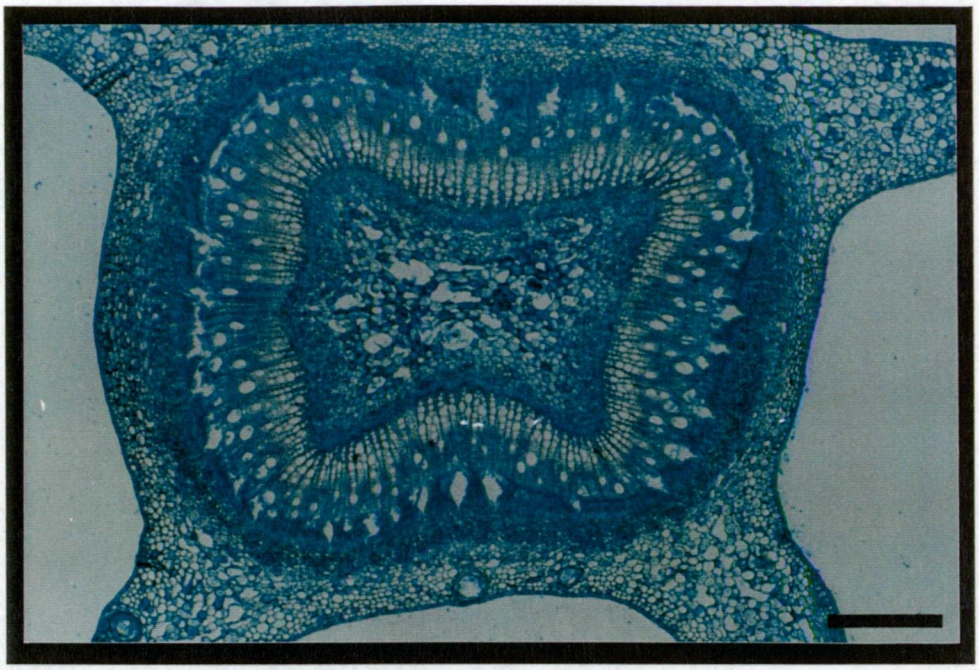


Figure 4.3c

Transverse section through stem of a cutting from a container grown stock plant, approximately six months old. Bar = 500μ.

The secateurs used to collect these samples were near new, with sharp blades. Preparation of cuttings with less efficient secateurs would, presumably, result in greater crushing injury to the cuttings and more extensive disruption of the vascular cambium. It is also possible that the practice of placing collected cuttings into water immediately after collection, may exacerbate the damage to the cambium by allowing the cut base of the cutting to absorb more water, causing it to swell and curl away from the base of the stem. The practice of placing a cutting into water immediately after severing it from the stem is standard practice in commercial propagation and is believed to improve root initiation, as well as preventing damage caused by dehydration (K. Joyce, pers. comm.).

4.3.3 Serial sections of callused stems

4.3.3a Rationale

Stem pieces from the base of cuttings were collected at regular intervals after setting the cuttings on the mist-bed. These were fixed for later sectioning and examination. The aim was to investigate if there were any discernable differences in the development of callus in stems of cuttings with different rooting ability. This could provide an explanation for the ability of some cuttings to initiate roots more frequently than others.

4.3.3b Procedures

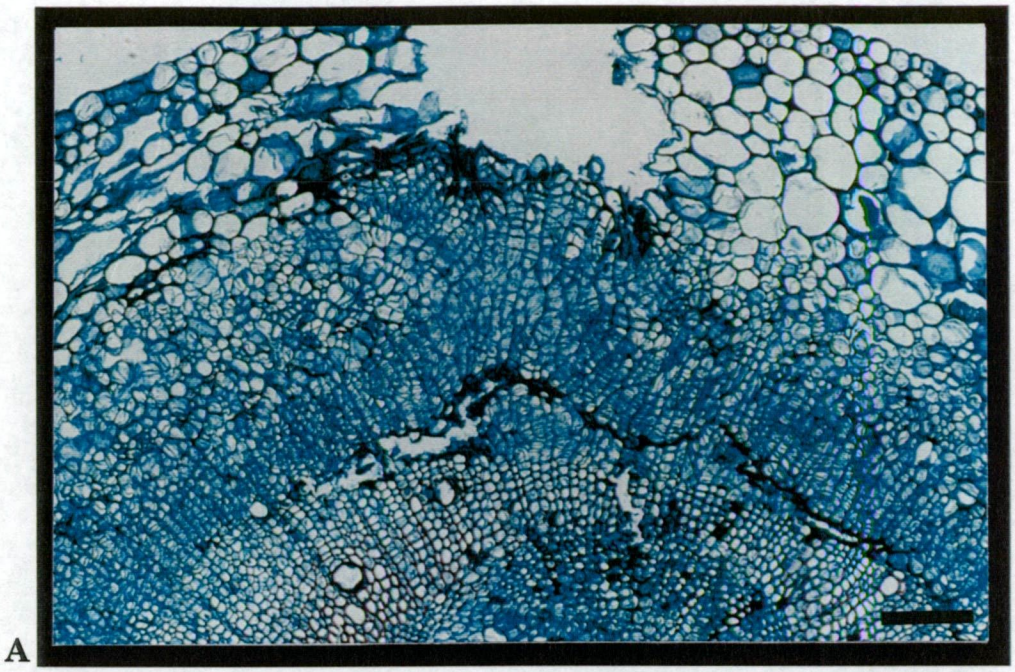
All procedures are as detailed in the General Methods section.

4.3.3c Data and interpretation

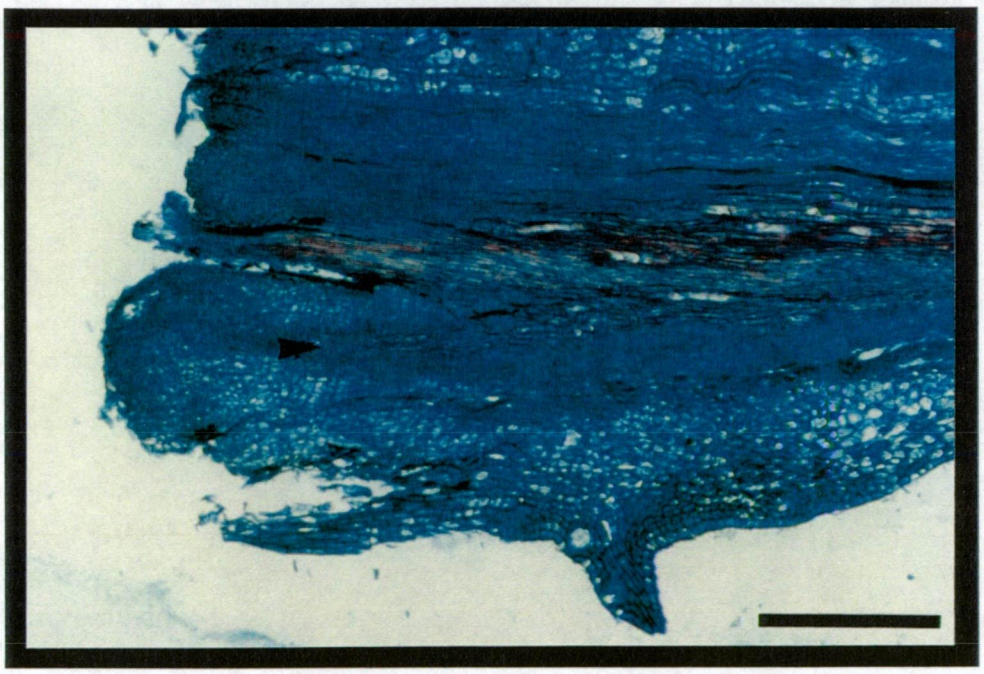
Callus initiation occurs rapidly after the collection of the cutting. The callus appears to arise in the region of the vascular cambium, close to the upper edge of the zone of splitting in the cambium, which occurs following collection of the cutting (Figure 4.3d).

There were no differences apparent in the way in which callus initiated or developed in cuttings collected at different nodes. The callus that grew all had a similar appearance. Most callus that was examined was found to have substantial areas of vascular tissue, which ramified into strands in the callus closest to the base of the cutting (Figure 4.3e). The strands of xylem were often quite long and usually had a strong vertical orientation, only achieving a horizontal orientation in larger callus sections taken from older cuttings.

There was no difference discernable in the extent of vascularisation that occurred in cuttings of different rooting ability. The pattern of callus initiation and vascularisation of callus in cuttings from node one was similar to that observed for node three cuttings. Strands of xylem formed in callus were carefully followed through successive sections. In most cases, the xylem strand was found to gradually dissipate as it got closer to the lower edges of the callus mass.



A



B

Figure 4.3d

A Transverse section through stem of cutting, after eight days on the mist-bed, showing early stages of callus formation. Bar = 100μ.

B Longitudinal section of stem, after ten days on mist-bed, showing development of xylem in newly formed callus (arrow). Bar = 500μ.

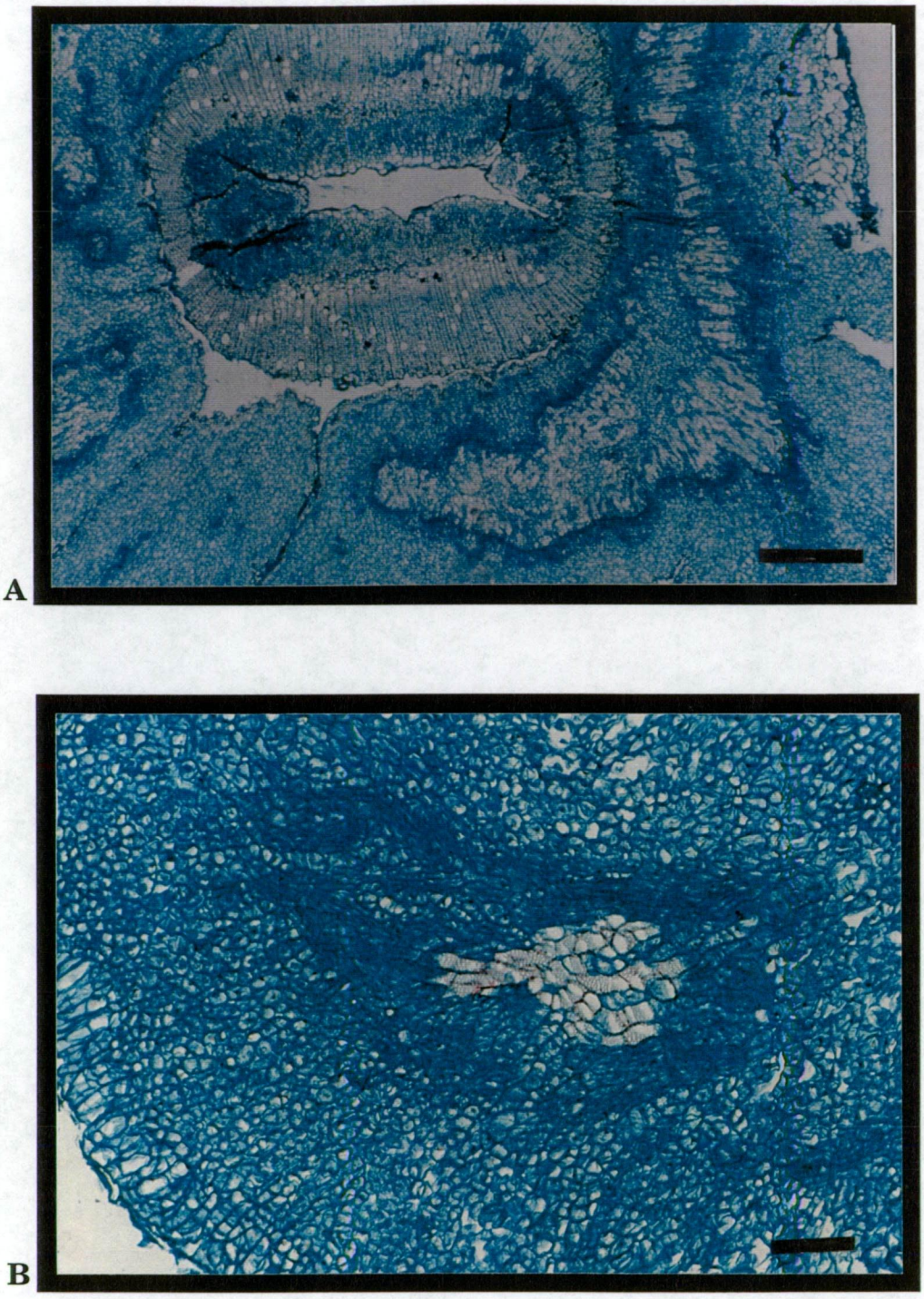


Figure 4.3e

A Transverse section through stem, after 14 days on the mist-bed, showing extensive callus and vascularisation of callus. Bar = 500μ.

B Close detail of xylem differentiation in callus mass. Bar = 100μ.

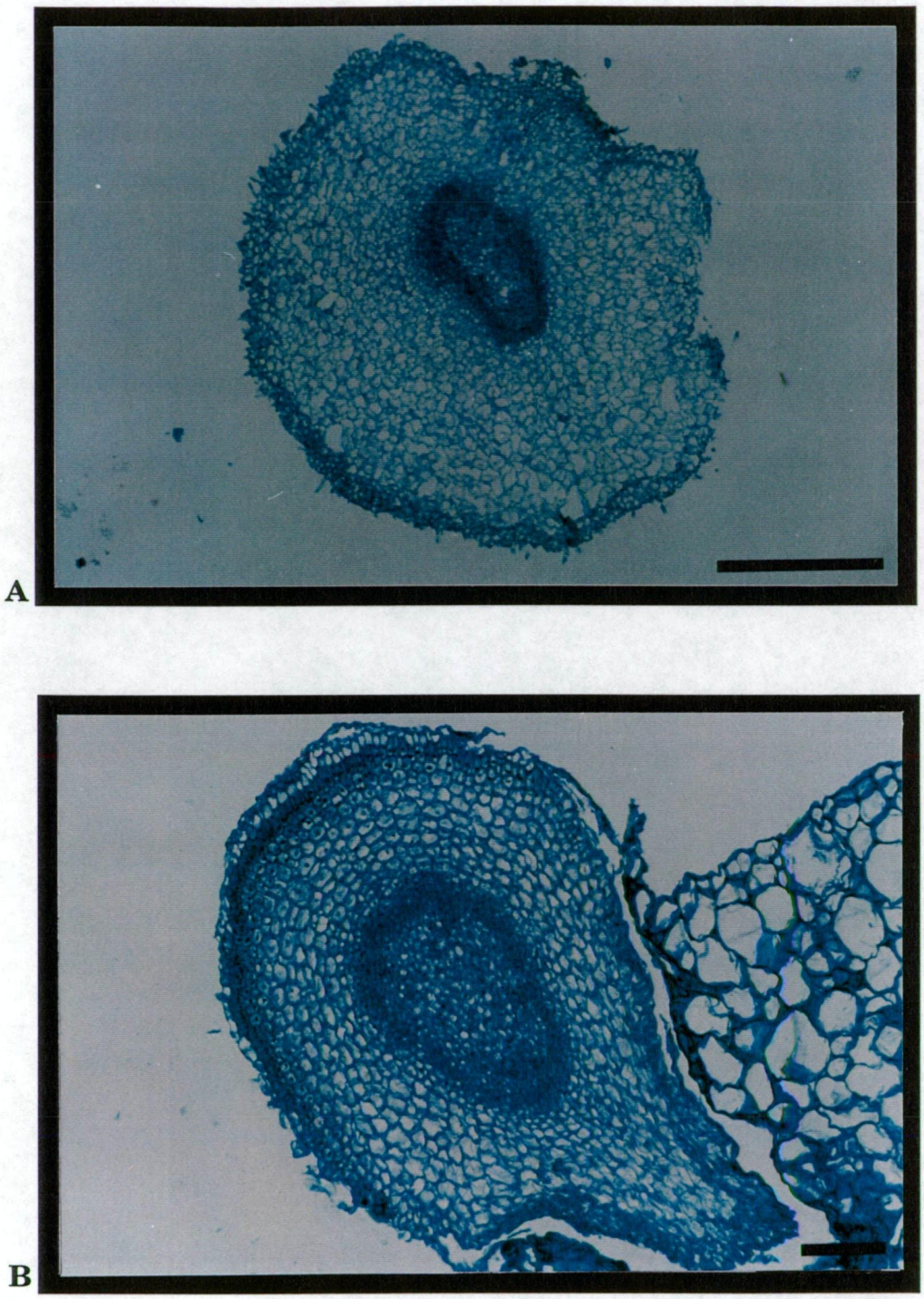


Figure 4.3f

A Transverse section through root-like projection from main callus mass. Bar = 500μ.

B Transverse section through a root which is projecting from the cutting, note similarities of structure with A. Bar = 100μ.

Large callus masses often had protrusions of a root-like appearance. Sections through one of these extensions showed that it had a degree of organisation but was composed of loosely packed parenchyma, which surrounded a core of smaller, dense staining cells (Figure 4.3f A). This pattern of organisation is, in some ways, similar to that of a primary root, (Figure 4.3f b) however functional roots were not found in association with these structures. These are presumed to be similar to the "anomalous roots" mentioned by Salmon (1990), although in that instance, they were claimed to have no internal structure.

It possible that some of the xylem formed in the callus may have formed due to pressure from the surrounding cortical tissues, particularly at the upper part of the callus, where the cortex was least disrupted. However, much of the vascularisation has occurred in callus that does not have such physical restriction.

4.3.4 Serial sections of root initials

4.3.4a Rationale

Serial sections through root initials were examined to determine if they could offer some clues on what is controlling or preventing root initiation in *E. nitens*. In particular, evidence was sought to demonstrate the mode of root initiation claimed by Warren Wilson and Warren Wilson (1991). They found that roots initiated in association with the formation of strands of vascular tissue that form in lettuce pith parenchyma. It was claimed that the induction of such strands of vascular tissue through the parenchyma was a key requisite for root induction in this situation.

4.3.4b Procedures

The procedures used in this section are all as outlined in the General Methods section.

A total of eighty callused stems were sectioned, fixed and examined. A minimum of 100 sections were inspected in those stems sectioned transversely. All sections were examined in those stems sectioned longitudinally.

4.3.4c Data and interpretation

Only a small number of root primordia were found at the stage of development where it was possible to determine their relationship to the surrounding tissues. From these samples, two distinct patterns of root initiation were observed. Root primordia were observed to be "direct"; formed on the vascular cambium, or "indirect"; formed in basal callus spatially separated from the vascular cambium.

'Direct' roots appear to initiate from the surface of the vascular ring, with little or no callus development appearing to have occurred prior to their induction, Figure 4.3g. 'Direct' roots were observed to form at a distance of 5 mm or more above the base of the cutting. Such roots also lack visible vascular connections to the cutting. There was no evidence of any vascularisation of the root primordium. Xylem vessels were not visible until the roots had extended to be over 5 mm in length. All of the examples of this type of root initiation examined were from node one samples. However, the same pattern of root initiation was

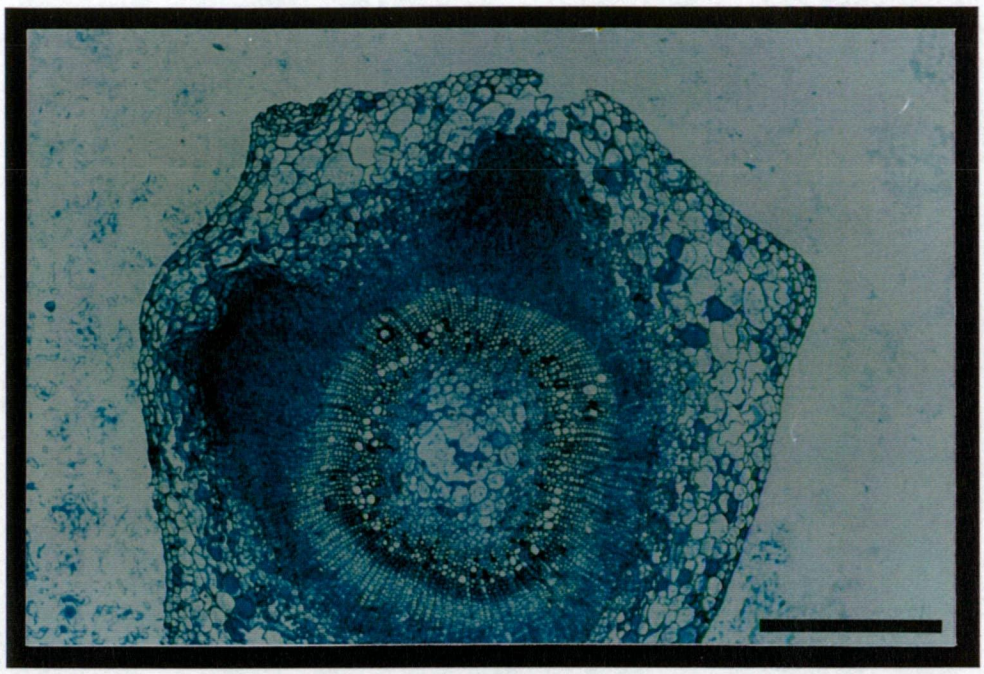
also observed in node two and node three cuttings. That is, cuttings were found with roots emerging from the stem at a point a centimetre or more from the base of the cutting and with little visible callus development at the point of root emergence.

'Indirect' root initials were found in areas of callus which are spatially separated from the original stem tissues, Figure 4.3h. The root initials all appear to have formed in association with vascular tissues that have formed in the callus. In all cases of 'indirect' root formation the root primordia was found a short distance from the end of a strand of xylem vessels Figures 4.3i, 4.3j and 4.3k. In all but the smallest primordium, there is evidence of lignification, perhaps indicating that the formation of the xylem cells occurred prior to the initiation of root initials.

It is not possible to say that the formation of such root primordia is caused by the presence of these strands of vascular tissue. Co-existence of the two structures at a particular stage of development does not prove a causal relationship. However, their co-existence is consistent with the hypothesis of Warren Wilson and Warren Wilson (1991), that there is some causal relationship between channelled auxin flow and root initiation.

When roots initiate from callus, the earliest stages of initiation probably occur very quickly. No root initials could be definitively identified that were at an earlier stage of development than those shown. In particular it was not possible to discern the earliest stages of root primordium formation to demonstrate that vascular strands are present prior to initiation. This could be demonstrated by finding a series of sections showing all stages of initiation through to recognisable primordia. Regrettably, this has not proved possible from the material sampled in this research. There may be some earlier stages present in some of the sections examined, but they are not recognisable as root primordia. Many regions of densely staining callus cells were observed but it is not possible to say that they would have developed into root primordia.

A



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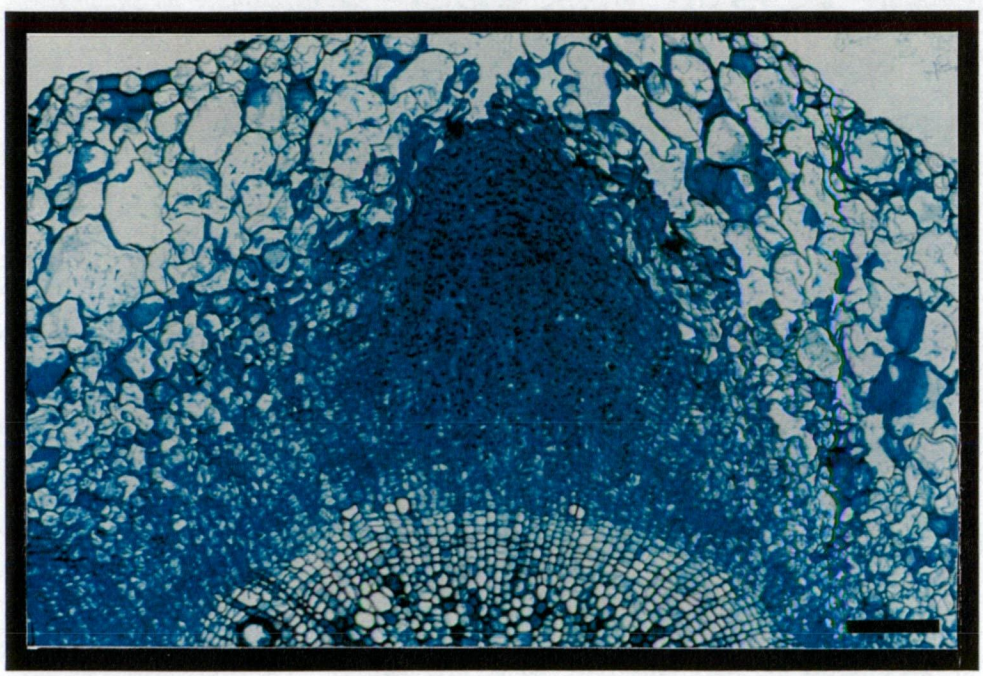


Figure 4.3g

A) Section through a 'direct' root initial, note lack of callus surrounding root initial. Bar = 500μ.

B) Enlarged view of A, note that xylem formation has not yet commenced in this root. Bar = 100μ.

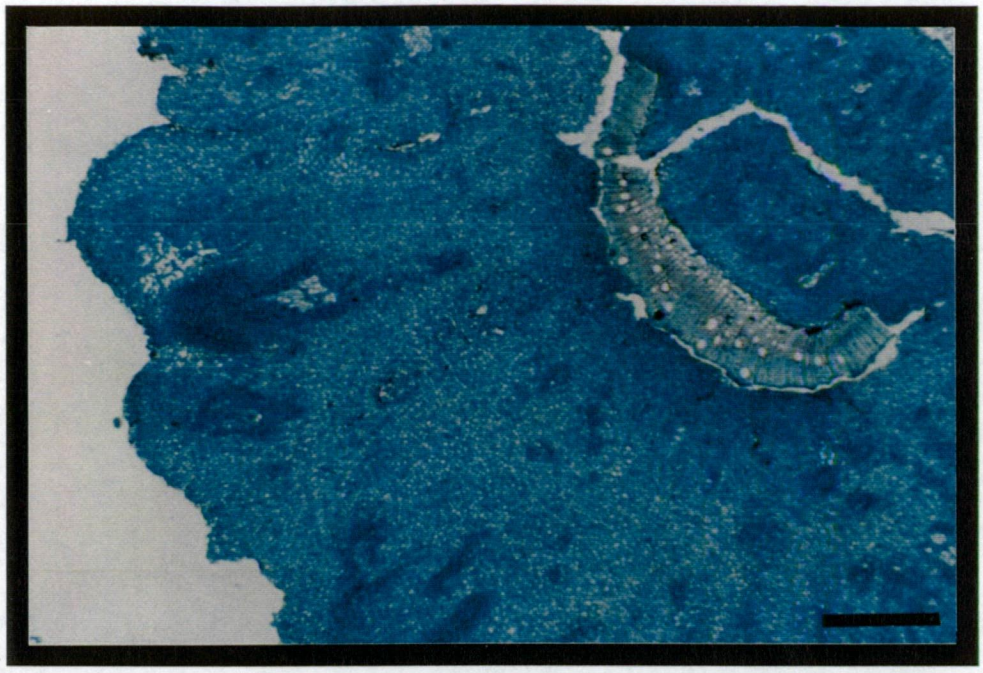
For more detailed study of the morphology of root initiation, sample collection needs to be timed to the early stages of root initiation. This is difficult, as root initiation appears to be a quick event that can occur at any time over several weeks. It would be possible to use treatments that enhance root initiation rates to provide samples with a higher percentage root initiation and possibly a larger number of root initials per cutting. However, before such treatments could be used, it would be necessary to demonstrate that the treatments do not alter the pattern of root initiation in some way. That is, some base data on root initiation in untreated cuttings is still required.

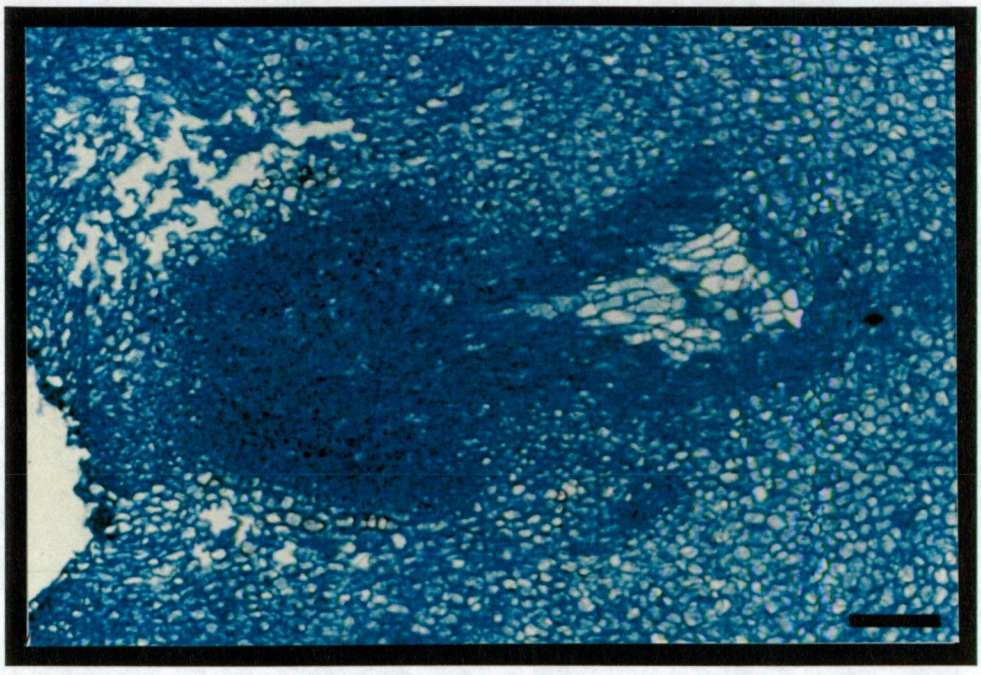
4.3.4d Discussion

The "direct" pattern of root initiation appears to be similar to that described for a range of other species, including herbaceous plants (Lovell and White, 1986). In *E. nitens,* it seems to be associated mainly with young seedlings, the roots appear to form further up the stem away from the base. This is a pattern of root initiation that is not seen with cuttings collected from larger plants or in serial cutting production (K Joyce, North Eucalypt Technologies, pers. comm.).

Root initiation observed in callus is rare, according to Hartmann, Kester and Davis (1994). It is likely that the indirect pattern of root initiation is the one that occurs most often in higher node cuttings. This type of root initiation is, therefore, probably of the greatest importance in practical terms. Concentration on treatments that enhance this pattern of root initiation is likely to provide the greatest commercial benefit.

A





B

Figure 4.3h

A) Section through a root primordium formed in a large callus mass. Note small region of xylem vessels at base of primordium. Bar = 500μ.

B) Enlarged view of A, showing xylem vessels. Bar = 100μ.

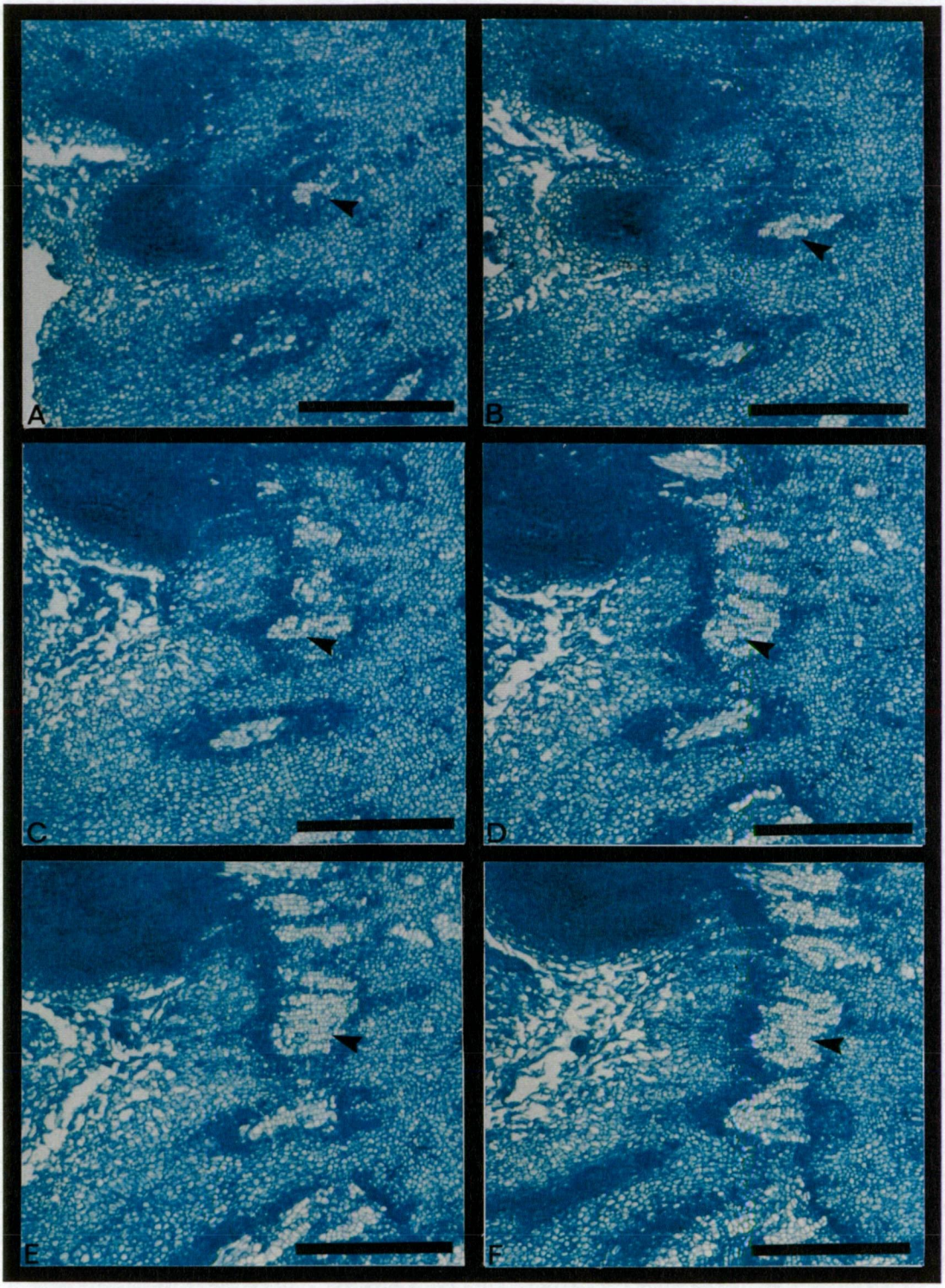


Figure 4.3 i

Non-consecutive sections of a root primordium and the callus above it. The sections show the relationship between the xylem cells at the base of the root and the remainder of the cutting. Sections shown are 72 to 96 μ apart. Arrows mark contiguous xylem. Bar = 500μ.

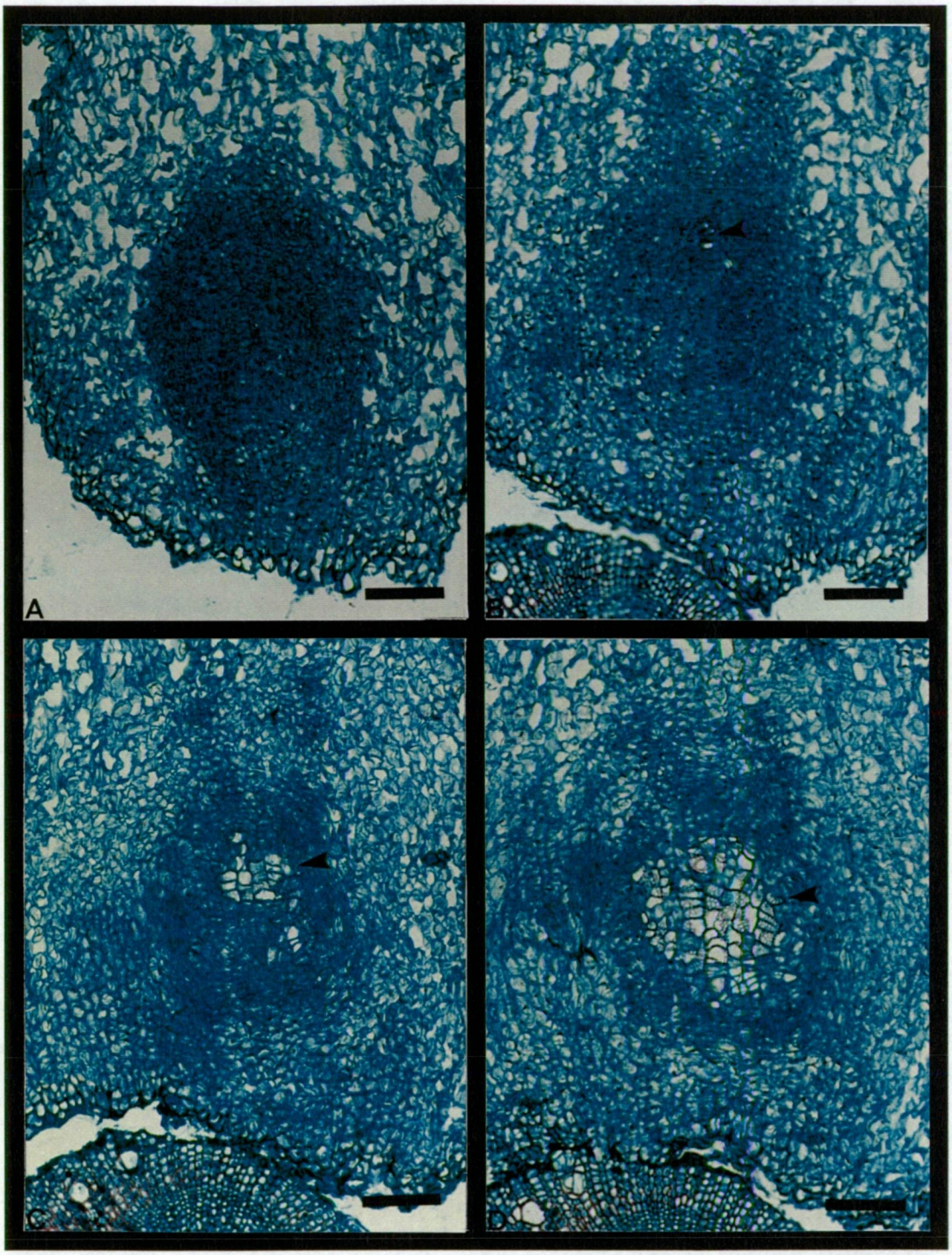


Figure 4.3j

Early stage root primordium and associated xylem vessels. The sections are transverse to the orientation of the root primordium. Sections shown are 72 to 96 μ apart. Arrows mark contiguous xylem. Bar = 100μ.

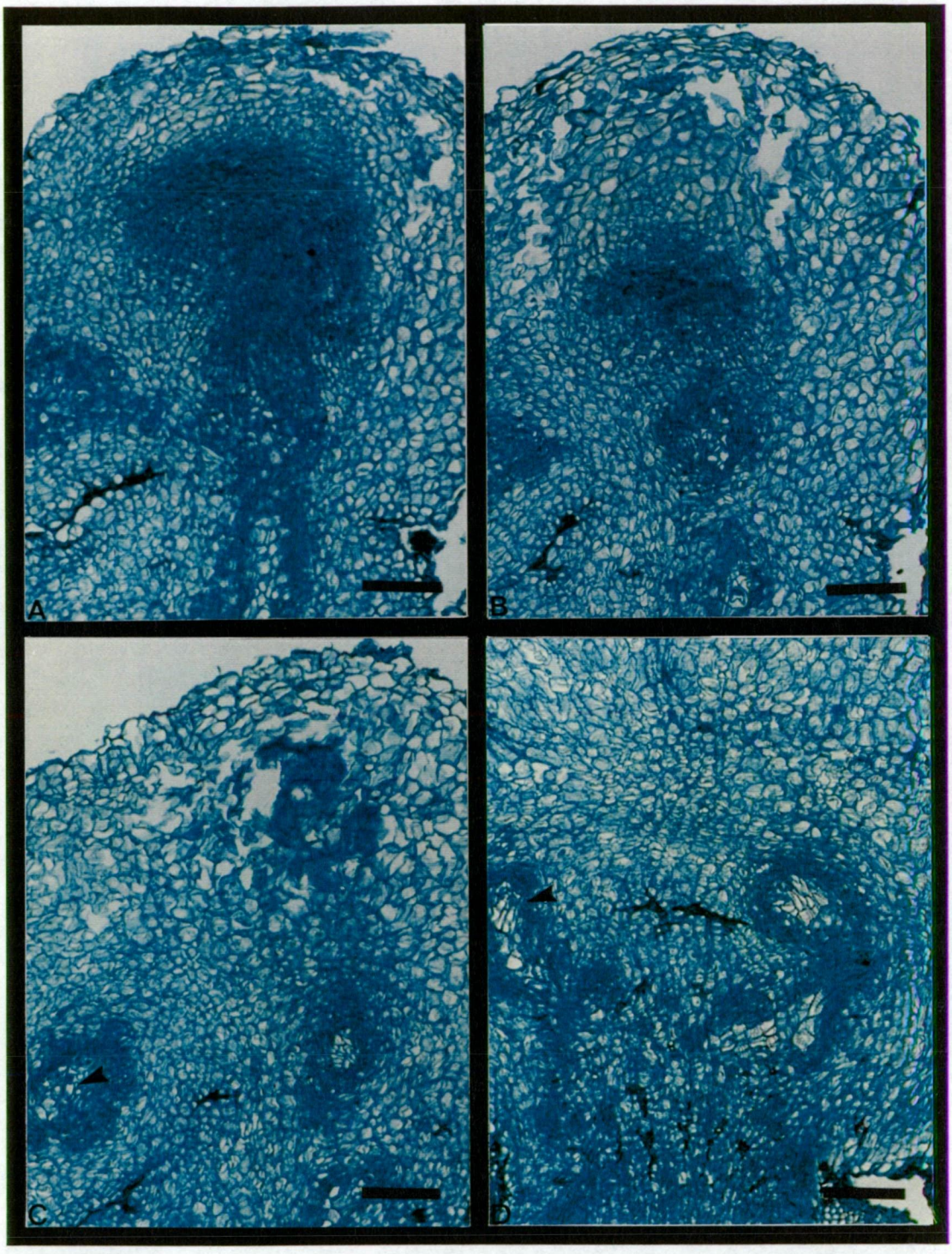


Figure 4.3k

Sections through a small root primordium, probably at an earlier stage of development than those shown in Figures 4.3i and 4.3j. Note nearby strands of vascular tissues, (arrows) which did not have roots associated with them. Bar = 100μ.

Its occurrence would appear to depend on the existence of regions of callus which are not already partially differentiated into vascular tissues. Auxin is presumed by Warren Wilson and Warren Wilson (1991) to move through such undifferentiated tissues and become channelled through a few cells, which differentiate into xylem vessels. It is proposed that root primordia form at the end of such strands, in response to an auxin gradient caused by the rapid oxidation of auxin in cells at the edge of the callus mass.

It should be noted that the two patterns of root initiation discussed appear to represent different responses to the assumed direction of auxin flow in those tissues. In the case of indirect root initiation, the hypothesis of Warren Wilson and Warren Wilson (1991) is that the root primordia are formed at the end of the strand of vascular tissues, which are formed by the channelling of auxin flows. In other words, the root primordia initiate in response to axial auxin flow. However, in the case of direct root initiation, the direction of auxin flow is most likely to be parallel to the existing vascular tissues and at right angles to the root initial. Lateral root formation also occurs under similar conditions. This latter pattern of root initiation is difficult to reconcile with a theory of root initiation which depends on auxin flows inducing polarity.

4.3.5 Digital image processing

The use of a Macintosh computer based, digital image processing application was investigated to generate images which might be useful in identifying morphological features within stems and basal callus.

4.3.5a Rationale

This work aimed to generate images of morphological features that were spread over several sections. This can be done by "stacking" images and "reslicing" the image stack in an orientation that shows the desired features. Image stacks can also be rotated and projected to provide a representation of the internal structures of the object.

Using this technology it was anticipated that it would be possible to demonstrate the relationship between root initials and nearby vascular strands.

4.3.5b Procedures

Images of a root primordium were obtained from serial sections using the procedures outlined in the General Methods section.

4.3.5c Data and interpretation

Images of successive sections were aligned to their predecessor, using an alignment macro that is included in the program. It was found that there were not sufficient common features between successive sections to allow for accurate alignment of sections. The resulting image stacks were not of sufficient quality to provide a useful three dimensional view of the stem material. In addition, the 72 dpi resolution resulted in a picture quality that tended to obscure fine details of sections that were visible by normal light microscopy (see Figure 4.3l).

Figure 4.3l

Images from image processor package.

Colour picture regenerated from red, blue and green images.

4.3.5d Discussion

The image processing package used in this work is designed to process image stacks collected from CAT scans and other medical image collection systems. It is not able to easily accommodate the rotational and translational movement that is encountered in processing wax embedded serial sections. Microtomed ribbons, which have been formed from serial sections are rarely completely straight. They are easily rotated or otherwise moved during the process of relaxing the wax ribbon and fixing the sections to the slide. Without some common reference points, it is almost impossible to realign successive sections accurately enough to allow them to form a usable image stack.

If a technique for image alignment can be perfected, it should be possible to use such programs and isolate structural features within the image stack to produce pseudo three dimensional images of the internal structure of plant tissues. Spatial relationships could be demonstrated by rotation of the image stack.

Image processing, using desk top computers, has reached the capacity to be used routinely to provide additional data from serial sections of plant material. To achieve its full potential will require the sections to be prepared with several datum points which are common to all sections. This would allow for automated or semi-automated orientation and alignment of successive images. It would be possible to construct meaningful stacks of images having regular and common boundaries, such as seeds or stem sections with regular boundaries. However, in the material used for this trial, the boundaries of the sections were not regular and changed between sections, making the alignment process too difficult.

Preliminary attempts to view the internal structures using cuttings that had been cleared using chromic acid were also unsuccessful, due to the accumulation of phenolic compounds, which made the stem pieces opaque (see General Methods, Section 3.2.5).

4.3.6 Conclusions from histological studies

The histological studies have provided some useful basic data on the stem and the morphology of root initiation. The patterns of root initiation and callus development observed do not provide any direct explanation of the variation in rooting ability observed.

The studies were intended to gather evidence on two specific propositions relating to how morphological features might affect root initiation. The propositions to be examined were that:

a) Physiological barriers to root formation or development have been proposed as a significant impediment to root initiation in some species, (Beakbane, 1969);

b) Root initiation could be shown to be associated with the formation of strands of vascular tissue which form in callus as a pre-cursor of root initiation. and that the rate of formation of such vascular tissue might be associated with root initiation rates.

On examination, only one of the propositions was supported by the evidence obtained from the sections that have been examined. In particular, there was no evidence found of the stem barriers hypothesised by Beakbane (1969) to be the cause of variation in rooting ability. However, there was an association found between the initiation of roots in callus and the presence of strands of vascular tissue. It is clear that the mere existence of strands of vascular tissue is not sufficient for root initiation to occur as there were many region of vascularisation where no root primordia could be discerned. No evidence was obtained as to what determines whether a strand of xylem will result in a root or not. This may be some feature of the strand of xylem, or more likely, some attribute of the callus tissues as a whole.

The differences in cell size that are found in stems originating from different nodes may be relevant to determining the rooting ability of the cutting. Larger diameter cells in the cambium of cuttings from older plants may simply have greater difficulty in re-polarising. This would make direct initiation of roots fromthe region of the vascular cambium less frequent in larger diameter stems. However, such a proposition does not explain why roots form less frequently in callus of higher node cuttings.

4.4 METHODS OF AUXIN APPLICATION

4.4.1 Introduction

In Section 4.2, auxin applications were shown to boost root initiation and to a lesser extent, increase callus production. The concentration of auxin used and duration of application in that experiment were based on *in vitro* experiments (Willyams *et al*, 1992). It is unlikely that auxin treatments found to be optimal for *in vitro* root initiation would be the optimum for root initiation in cuttings.

The concentration and method of auxin application have a significant effect on the root initiation rate of cuttings. There have been few reports published that have examined the effects of applying auxin to cuttings at times other than immediately after the collection of the cutting (Jarvis, Ali and Shaheed, 1983).

A series of experiments was undertaken to provide a more strategic approach to the application of auxin to cuttings. These experiments tested the effect of applying auxin pre-treatments at times other than immediately after the collection of the cutting, varying the concentration of auxin applied and duration of its application, applying auxin to the apex of the cutting rather than to the base and applying auxin in the presence of varying concentrations of calcium.

4.4.2 Timing of auxin application

This experiment was used to investigate what effect the timing of auxin applications has on root initiation. 48 hour IBA treatments were applied to cuttings up to five weeks after the cuttings were first collected and placed onto the mist-bed.

4.4.2a Rationale

In most species, the period between root initiation and root emergence is quite short (Lovell and White, 1986). However, during the collection of samples for use in histological examination in Section 4.3 it became obvious that root initiation does not occur quickly, as very few roots were visible before 14 days.

This led to the hypothesis that the most critical period for stimulation of root initiation might be at some time well after the cutting is collected. In *E. nitens,* significant root initiation has been observed to continue for up to seven weeks after collection of the cuttings (de Little, North Eucalypt Technologies, pers. comm.).

There is no published evidence about how long applied IBA stays in the base of a eucalypt cutting. Evidence from other genera suggests that much of the free IBA will be conjugated or transported out of the root formation zone quite quickly (Garcia-Gómez *et al*, 1994; Hartmann *et al*, 1990).

This experiment was designed to test the hypothesis that an auxin application would be more effective in stimulating root initiation if it was applied nearer to the time when root initials are actually forming. This is based on the assumption that there is a period of de-differentiation and callus formation which occurs before root initiation can take place and that this early phase is not auxin dependent. The alternative is that treatment with IBA in the first few days stimulates callus production, which boosts subsequent root initiation by providing more sites from which root initials can form.

4.4.2b Procedures

A large batch of cuttings was prepared and placed onto the mist-bed. Each week, some of the cuttings were removed from the mist-bed and treated with an auxin dip. Standard conditions and procedures were used, as described in the General Methods section, with variations as outlined below.

Node 4 cuttings were used, prepared from a single batch of seedlings.
The treatments consisted of a 48 hour dip in a 20 mg/l solution of IBA.
Treatments were applied to cuttings each week by removing some cuttings from the mist-bed, rinsing off adhering soil with water, shaking excess water off and then immediately placing the cuttings into the treatment solution. Application of treatments occurred in the dark in a growth cabinet at 22°C for 48 hours. After treatment, the cuttings were rinsed in running water to remove auxin and returned to the same position in the tray and returned to the mist-bed. The cuttings were then watered gently to re-embed them in the potting soil.

Treatments were applied to groups of six cuttings, with each treatment represented in each tray. Cuttings found to have already initiated roots were still treated, as it was thought that the additional auxin might have an effect on the number of roots initiated and on callus production. Root initiation was assessed three weeks after the final auxin application, that is, eight weeks after the cuttings were first collected.

IBA treatment solutions were prepared from a stock solution of IBA dissolved in re-distilled, absolute ethanol, stored at 4°C in the dark. The final concentration of ethanol in treatment solutions was 0.5%. The control treatment was a 0.5% ethanol solution and was applied at week 0.

This experiment was more affected by fungal damage than any other experiments carried out. Only 50% of cuttings survived to be measured. Some of the damage occurred at the commencement of the experiment, when the initial protective drench with Previcur was delayed for two days until after the first two treatments (Control and Week 0) were re-planted. This delay was to avoid giving all the other cuttings in the experiment a second drench of the fungicide. A χ^2 analysis showed that there was no significant difference in survival between the treatments.

4.4.2c Data and interpretation

The results from this experiment show that the proportion of cuttings undergoing root initiation is affected by the timing of the application of auxin, Figure 4.4a. Cuttings receiving later IBA applications (Weeks 4 and 5) have significantly more roots than either the Control (no IBA) or Week 0 treatments.

The results of this experiment show that Week 0 treatment has no effect on root initiation, Figure 4.4a. This contrasts with the results obtained in an earlier experiment (Section 4.2) where treatment with IBA immediately after collection of cuttings did boost root initiation.

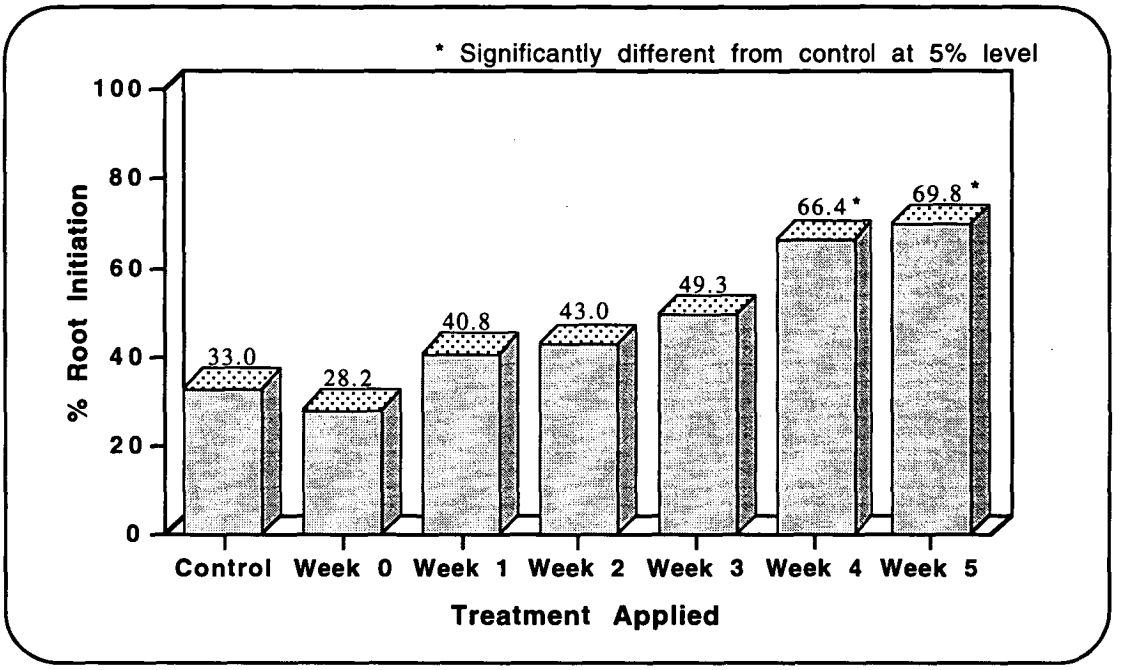


Figure 4.4a

The effect of timing of IBA applications on the proportion of cuttings initiating roots, IBA applied as 20 mg/l for 48 hours.

There was an additional control treatment in this experiment where the cuttings were undisturbed for the duration and were not removed for application of treatment solutions. These untreated cuttings had an identical root initiation rate to that seen in the Control treatment, where cuttings received a blank (no IBA) treatment.

An analysis of variance shows that there was no significant difference between the mean number of roots per cutting from different treatments. However a trend seems evident, see Figure 4.4b. The trend indicates that there was an optimum timing of auxin applications to achieve maximum root number and that this may be different to that required to obtain maximum root initiation. This suggests that many of the cuttings stimulated to initiate roots by later treatment with IBA do so with a single root or a low number of roots.

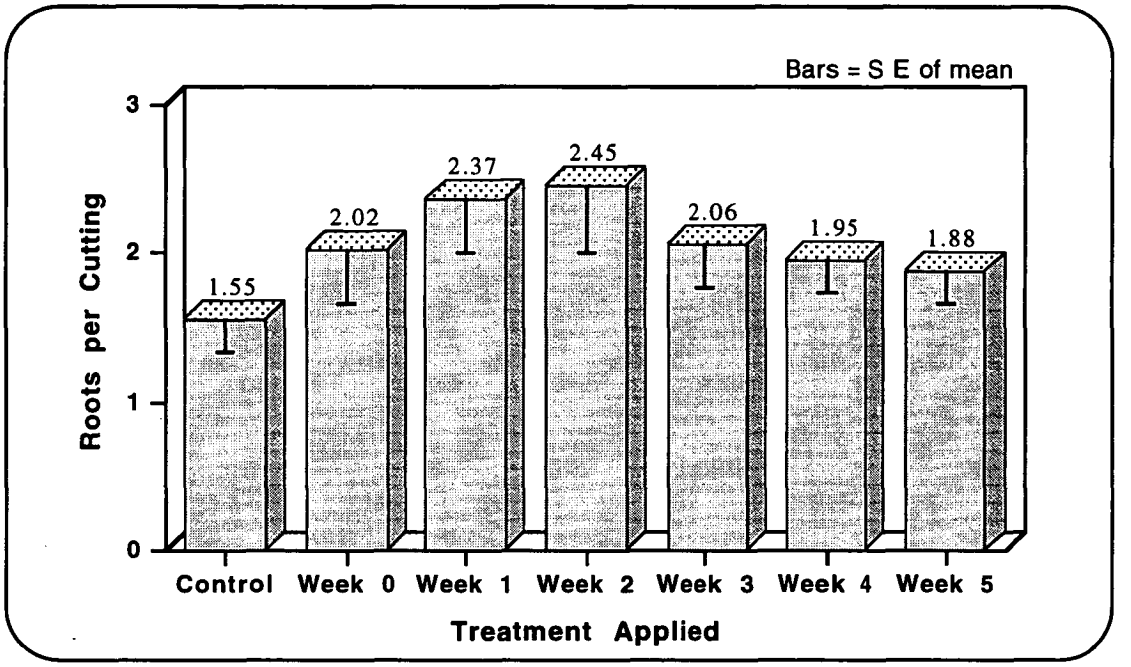


Figure 4.4b

The effect of timing of IBA applications on the mean number of roots per rooted cutting, IBA applied as 20 mg/l for 48 hours.

Callus production is only significantly affected in the first two auxin treatments (Figure 4.4c). It is not surprising that maximum callus production occurs in those treatments where there is the longest period for callus to grow after treatment. Auxin treatments had no apparent effect on callus production when applied later, when callus growth was well established in the cuttings. This may be due to root initiation preventing excess callus formation in these treatments.

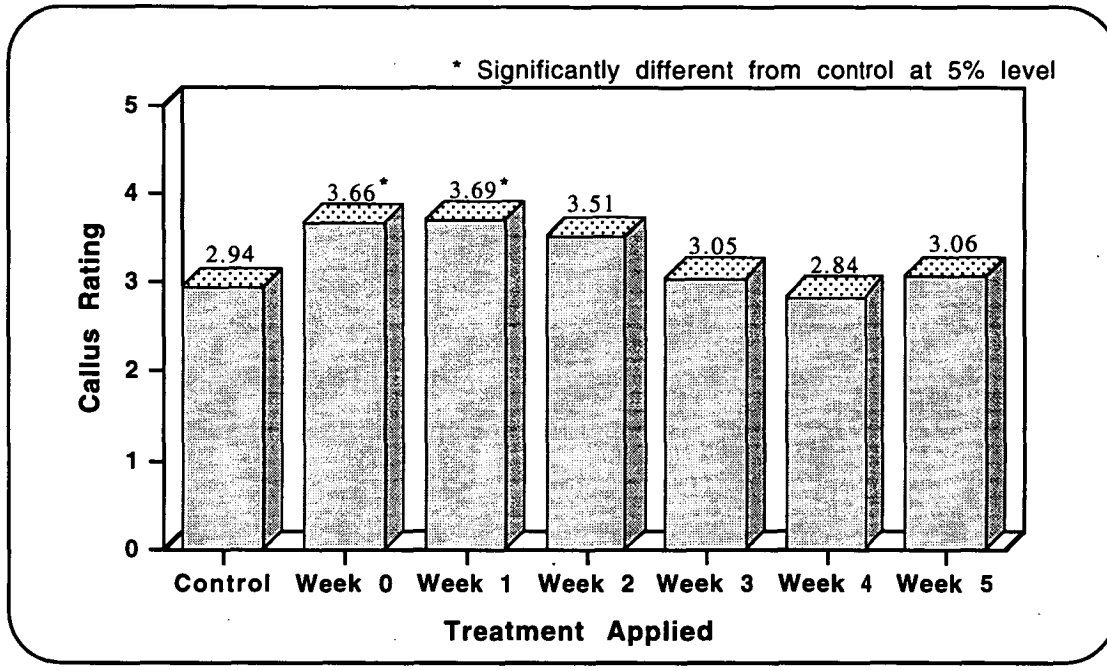


Figure 4.4c

The effect of timing of IBA applications on callus rating, IBA applied as 20 mg/l for 48 hours.

4.4.2d Discussion

Taken overall, the results from this experiment suggest that it is possible to improve the rate of root initiation in cuttings by altering the time at which auxin is applied. This is not surprising in cuttings where root initiation is believed to occur in basal callus and to occur several weeks after the cuttings are first collected. Such an approach to auxin application to improve rooting in woody cuttings does not appear in recent reviews of the subject (Blazich, 1988a; Haissig, 1986; Hartmann *et al*, 1990; Jarvis, 1986). There is one report of a negative effect from later applications of IBA used on mung beans (Jarvis *et al*, 1983). They found that root initiation declined sharply with ageing of cuttings.

The observation that root numbers are not affected in the same way by the timing of IBA applications is of interest. If the number of roots per cutting is of practical importance, as is suggested by Sasse (1995), these results may demonstrate the need for compromise between obtaining higher number of roots per cutting and maximum rooting percentage.

Some reservations have to be placed on the significance of these results because

of the failure of the early auxin treatment in Week 0 to have any effect on root initiation. This is clearly contrary to the results of earlier experiments (see Section 4.2). This could be due to reduced auxin sensitivity or increased metabolism or conjugation during that time but there is no real evidence to support either of these propositions. An alternative explanation is that the longer time that the early treatments had for root initiation has masked an initial boosting effect from IBA applied in the first few weeks. That is, IBA applications at this time might make the roots appear earlier but have no effect on total root initiation if the cuttings are all allowed sufficient time to initiate.

These results generally support the hypothesis that root initiation would be improved by later applications of auxin. The most probable explanation for this is that there would be a greater volume of callus from which the roots could initiate.

The concentration of IBA used in this type of experiment may not have any direct commercial or practical significance. The treatments are time consuming and expensive to apply and require that the cuttings be handled twice, once to collect the cutting and once to apply the treatments.

4.4.3 Optimum auxin concentration

This experiment was undertaken to identify optimum auxin treatment rates for cuttings that have been allowed to callus for four weeks.

4.4.3a Rationale

In the previous experiments, in Section 4.2 and Section 4.4.1, IBA treatments of 48 hours exposure to 20 mg/l were used. These rates were chosen on the basis of work on *E. nitens* using *in vitro* micro cuttings (Willyams *et al*, 1992). However, *in vitro* experiments on *E. globulus* micro-cuttings showed that the duration of exposure to IBA was quite critical to the number of root primordia formed (Pelosi *et al*, 1995). In contrast, Carter and Slee (1993) found no effect from the duration of exposure of *E. grandis* cuttings to high concentrations of IBA.

It is not clear how relevant the results obtained *in vitro* and using hypocotyls are to experiments using callused macro-cuttings. To ensure that the results of these and subsequent experiments are able to be interpreted accurately it was thought desirable to test if the treatments being used were close to those giving maximum root initiation.

4.4.3b Procedures

All treatment conditions were as described in the General Methods section. Treatment solutions were prepared from IBA dissolved in absolute ethanol and diluted to give a final concentration of 0.5%. The control treatment consisted of 0.5% ethanol.

The cuttings used were standard, node 4 cuttings. Treatments were applied 4 weeks after the cuttings were first collected. Approximately 4% of cuttings had rooted prior to treatment and were discarded. Root initiation was assessed a further three weeks after treatments were concluded.

4.4.3c Data and Interpretation

When cuttings were treated with IBA for 48 hours there were significant increases to root initiation for the 20 and 40 mg/l treatments, with 40 mg/l appearing to be close to the optimum (Figure 4.4d). Higher concentrations than these gave lower root initiation rates, indicating excess auxin causing some inhibition of root initiation.

With 12 hours of exposure to IBA, a higher concentration was needed to achieve a significant increase in root initiation, with an optimum concentration appearing to be near 120 mg/l.

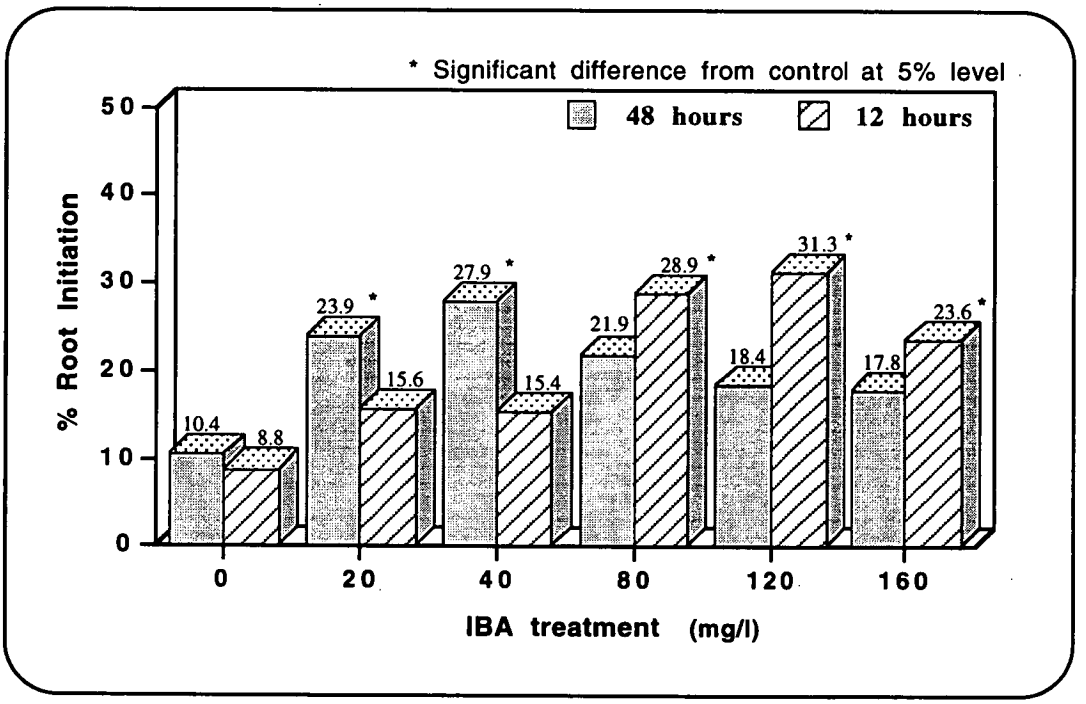


Figure 4.4d

The effect of varying concentration of IBA on the proportion of cuttings initiating roots, IBA applied for 48 hours and 12 hours.

The number of roots per cutting showed a different response to increasing IBA concentration for both exposure times (Figure 4.4e). In both cases, root numbers appear to increase at IBA levels above the optimum concentration for root initiation for that duration of treatment.

Callus production showed no significant differences in either 12 hour or 48 hour treatments. This is consistent with the observation in Section 4.4.2 that callus production is largely insensitive to later auxin applications.

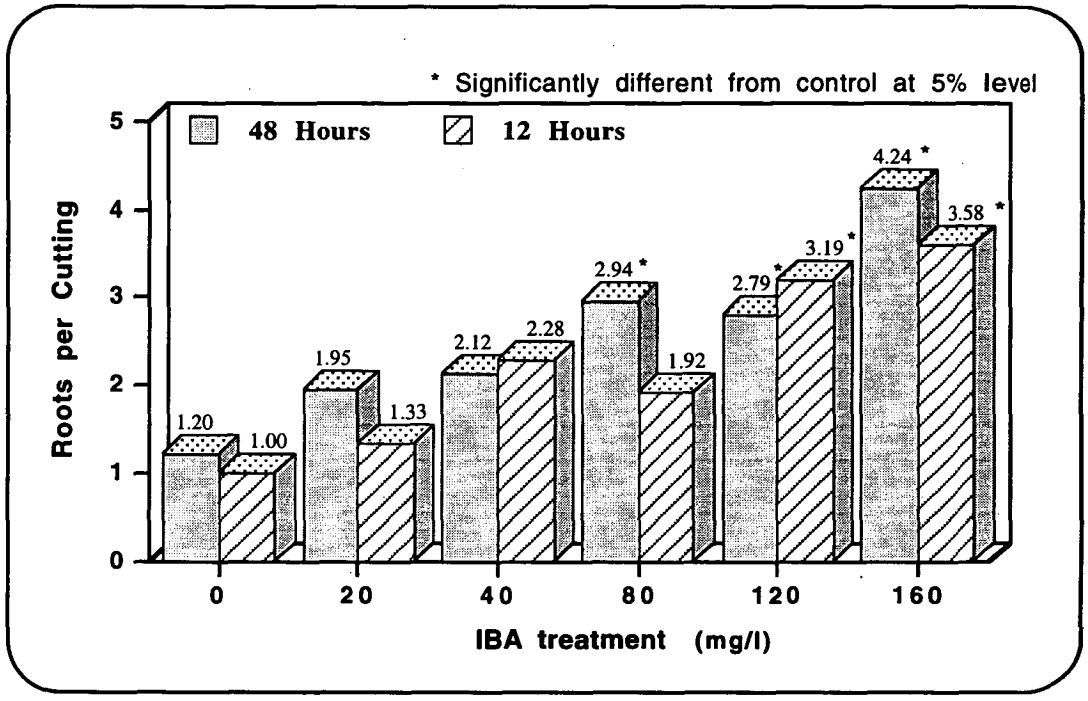


Figure 4.4e

The effect of concentration of IBA on the mean number of roots per rooted cutting, IBA applied for 48 hours and 12 hours.

The cuttings in this experiment showed substantial amounts of damage to both the stem and callus. Much of the damage was due to fungal infections. However there was also a clear treatment effect (Figure 4.4f). It was not possible to distinguish between cuttings damaged or killed by fungal infections and those where the fungal infections were secondary to treatment damage. The effect of treatments on the proportion of cuttings assessed as damaged was analysed using the χ^2 test. This showed clearly that IBA treatments have a significant effect on the proportion of cuttings with damage to the stem and callus and that increasing either the concentration of IBA or the duration of treatment causes an increase in tissue damage. For the 48 hour treatments, even the lowest rate of IBA application caused significant damage. Such a treatment effect had not been observed in the experiments described previously.

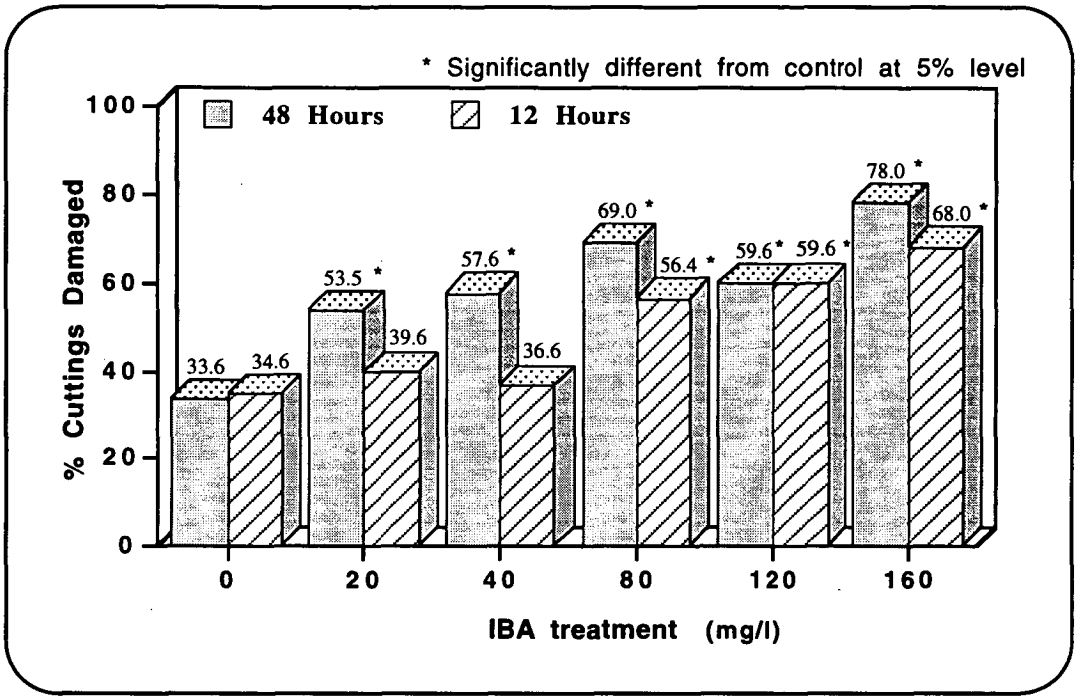


Figure 4.4f

The effect of concentration of IBA on the proportion of cuttings showing some necrosis of callus or basal stem, IBA applied for 48 hours and 12 hours.

The damage caused to cuttings by IBA treatments need not have an entirely negative effect on the proportion of cuttings initiating roots. Some of the external regions of the callus may absorb excess IBA, causing cell death. This would result in the formation of a reservoir of IBA, which could be subsequently absorbed by the underlying tissues to promote root initiation. Root initiation would be promoted in such a situation, provided the damaged tissues were not those required for root initiation. Such essential tissues would be more likely to be damaged by higher concentrations of IBA or longer exposure times, both of which allow for greater accumulation. This gives a picture of a stem with a basal callus composed of a core of live, active cells surrounded by a layer of dead, spongy, IBA-loaded cells.

4.4.3d Discussion

The results from this experiment are largely as expected and demonstrate that concentration and duration of exposure to IBA are important in controlling root initiation. The root initiation rate obtained in this experiment was substantially below that obtained in the experiment in Section 4.4.2. This is believed to be due to seasonal variation in rooting ability.

The relationship between optimum auxin concentration and the duration of exposure will depend on the rate of absorption of auxin by the cutting. It is not crucial to know what the optimum treatments are to understand how auxin affects root initiation but it is important to use a treatment that is close to the optimum. Cuttings treated with an above optimum concentration of IBA are likely to suffer from auxin induced inhibition of growth, while treatments significantly below the optimum may be difficult to measure and are more likely to be affected by endogenous auxin levels.

This experiment also seems to indicate that callus growth is either unaffected by exogenous auxin or requires much higher concentrations of IBA to show significant differences. The latter seems unlikely, as the higher treatments in this experiment caused damage to stems and callus.

There was no indication from these experiments of how much of the IBA was actually absorbed by any of the treatments. To do this would require that the experiments be run in conjunction with uptake studies, using radioactive labelled IBA. However, labelled IBA is not readily available. Such studies would be useful in indicating not only the rate of uptake but the degree of metabolism, conjugation and transport of the exogenous IBA within the cutting.

The data from this experiment confirms that the IBA treatment used in previous experiments is close to the optimum concentration for that duration of treatment. It is possible that longer treatments with lower concentrations, or shorter treatments with higher concentrations could deliver final rooting percentages which are greater than those obtained in this experiment. However, it is doubtful if such experiments would provide any additional information on what prevents root initiation occurring in many other similar cuttings.

4.4.4 Apical applications of auxin

This experiment was undertaken to determine if application of auxin to the apical region of the cutting could be used to stimulate root initiation. This could be a preferable method of applying auxin, as it avoids the need to remove the cutting from the potting mix.

4.4.4a Rationale

Application of auxin is more effective when it occurs three or four weeks after cuttings have been collected. The method of applying auxin used in previous experiments, using IBA in a relatively low concentration dip for 48 hours, is not practical for large scale use. It is labour intensive and has the potential to disrupt those cuttings that have already initiated roots.

The natural direction of auxin flow is basipetal, which suggests that it should be possible to apply auxin to the top of a cutting and have it naturally transported to the base, to enhance root initiation. Spray applications of auxin on the stock plants prior to collection of cuttings have been demonstrated to be effective in promoting root initiation in some species (Preece, 1987). Controlled foliar or spray applications of IBA to cuttings would be difficult to achieve in a mist-bed. After the cuttings have been collected, the leaves of the cuttings need to be kept constantly wet, to prevent moisture stress. This could remove auxin treatments from the leaves before they were fully absorbed.

It was proposed that dissolving IBA in lanolin would provide a means of applying auxin to leaf or stem surfaces in a wet environment. A simple system was devised to apply controlled doses of IBA to cuttings allowing for a clearly defined termination of treatment.

4.4.4b Procedures

Auxin was applied as a lanolin paste, in a small aluminium foil cap, which formed a waterproof, opaque container to limit auxin degradation. The cap was made by shaping a small piece of aluminium foil (30mm x 40mm) over a 2.5 ml test tube, 10 mm in diameter. The IBA was applied at a concentration of 20 mg/l, in lanolin. The lanolin pastes were prepared by weighing 300 g ± 0.2 g of warm lanolin into jars. 6.0 mg IBA was added to the jars and stirred. Prior to

dispensing, the contents of the jars were remelted over boiling water, with regular stirring. An Eppendorf multipette was used to dispense 0.75ml of lanolin into each foil cap. The caps were stored at 4°C, in the dark, until used.

Standard, node 3 cuttings were chosen for this experiment as they have a slightly higher root initiation rate. A higher rate of rooting was thought desirable for this experiment to allow for easier detection of inhibition of root initiation, should that occur. The cuttings were prepared with 1.5 cm of the stem extending beyond the top leaf pair of the cutting. The foil caps were pushed down onto this extra piece of stem. At the conclusion of each treatment, the IBA was removed by cutting off the top 1 cm of stem together with the lanolin capsule. This ensured that no residual auxin was left on the stems.

Treatments were applied after 21 days growth on the mist-bed and consisted of:

Control, no auxin application;
Blank, lanolin paste applied but no auxin, cap removed after 2 days;
20 mg/l IBA applied, cap removed after 2 days;
20 mg/l IBA applied, cap removed after 4 days; and
20 mg/l IBA applied, cap removed after 6 days

The treatments were applied by placing an inverted capsule over the top of the stem and pushing it down firmly, ensuring that the lanolin was in good contact with the stem. The blank treatment was included to detect any effect on root initiation from cutting off the top section of the stem.

4.4.4c Data and interpretation

Treatment	Control	Blank	2 days	4 days	6 days
% Root Initiation	61.9	69.2	69.5	75.0*	72.2
Roots/Cutting	2.41	2.53	2.57	2.86	2.57
Callus Rating	3.98	3.78	3.83	3.87	3.69

* Significantly different from control at 5% level

Table 4.4g

The effect of application of IBA to the apical region of cuttings., IBA applied as a lanolin paste, 20 mg/l.

The only significant difference measured was a small increase in root initiation after treatment for 4 days (Table 4.4g). The fact that treatment for 6 days gave a lower root initiation rate may indicate that there is some inhibition beginning to be demonstrated but the difference was not significant. The positive result obtained from treatment for 4 days demonstrates that this method of delivering auxin to cuttings may be a viable alternative to basal dips. However, the small size of the increase in root initiation obtained indicates that the procedure needs some refinement.

4.4.4d Discussion

A more substantial boost in root initiation might have been obtained if a higher IBA concentration was used. The total IBA available to each cutting is much less than in the basal dips (0.015mg vs 0.30 mg) but there is no evidence on how much of the IBA is absorbed in either situation. IAA may be a more appropriate auxin to use in this situation, as it is absorbed more readily and transported more rapidly (Blazich, 1988a), both of which would reduce the likelihood of damage to the stem from high concentrations.

The transfer of IBA from the lanolin paste to the stem may not be rapid enough to raise auxin levels substantially. This could only really be checked using radioactive labelled IBA to measure the extent of IBA movement into cuttings.

The results from this experiment could lead to the conclusion that applying auxin to the upper parts of a cutting is less effective than applications to the base. Several possibilities could explain this result. Basipetal auxin transport rates in the cuttings may be insufficient to raise auxin levels in the basal tissues to the level needed to initiate roots. It is known that IBA transport rates are generally lower than those for IAA (Epstein and Ludwig-Muller, 1993), however there have been no studies done on the rate of auxin transport in *E. nitens* or any other *Eucalyptus* species.

It is possible that directional auxin flow is a disadvantage to stimulation of root initiation. In basal applications, all of the external tissues are surrounded by auxin and it is likely that auxin would be able to diffuse quite readily through the lower region of the stem. However, with apical applications, all the auxin that is delivered to the base will be delivered via the phloem. Diffusion into other tissues such as parenchyma and callus may be restricted or channelled, preventing it reaching potential sites for root initiation in undifferentiated callus.

4.4.5 Calcium auxin interactions

There is no doubt that calcium ions are involved in hormonal signal transduction but there have been few studies looking at the implications of calcium concentration on root initiation. Two experiments were designed to quantify variation in root initiation rates due to changes in the calcium concentration in the auxin treatment solution.

4.4.5a Rationale

Calcium ions are central to auxin signalling and the induction of polarity (Bethke *et al*, 1995) and yet the effect of calcium on root initiation has received only cursory study (Blazich, 1988b; Haissig, 1986).

Interactions between calcium levels and auxin in whole plants and tissues has been studied on many occasions (Hepler and Wayne, 1985). According to Hepler and Wayne (1985), most studies have investigated the role calcium plays in polar auxin transport, where calcium supplied at physiologically high concentrations, in the range 1-10 mM, inhibits growth in antagonism to auxin. Hepler and Wayne (1985) also note that many studies which examine the effect of calcium nutrition on plant development need to be considered carefully because they are carried out at calcium levels that are far in excess of normal physiological concentrations. Intracellular calcium concentrations are 'sub-micromolar'. Cellular processes are activated by raising levels to 1-10 μM, whereas most of the studies on the interaction between calcium and plant growth regulators have been carried out with mM levels of calcium, that is, 100 to 1000 times those occurring in normal tissues.

It has been demonstrated that calcium must be present in the external medium for re-polarisation of individual cells to occur in cell suspension cultures (Mina and Goldsworthy, 1992). The implications of this result for re-polarisation of tissues during organogenesis have not been studied. It was hypothesised that treatment of cuttings with IBA, in the presence of various concentrations of calcium, may affect the re-polarisation required for root organogenesis. This could result in variation in root initiation rates for different calcium concentrations.

4.4.5b Procedures

Two separate experiments were undertaken, using the same calcium treatments. Treatment solutions contained 20 mg/l IBA and $CaCl_2$ in concentrations ranging from 1 μM to 500 μM. IBA was dissolved in ethanol to give a final concentration of 0.25% ethanol in water. $CaCl_2$ stock solutions were prepared from oven dried $CaCl_2 . 2H_20$, which was stored in a vacuum desiccator prior to the preparation of solutions. The control treatment consisted of distilled water plus 0.25% ethanol.

In both experiments, the cuttings were node 4 cuttings, which had been allowed to callus for four weeks prior to treatment. Prior to transfer to treatment solutions, the cuttings were rinsed three times in tap water and then twice in distilled water to remove adhering potting mixture and calcium from soil solution.

Treatments were applied for 48 hours in the dark, under standard conditions, as described in the General Methods section. Root initiation rates were assessed three weeks after the completion of treatments.

The second experiment was conducted with identical treatment levels but with greater replication. In this experiment, vacuum infiltration was used to provide rapid equilibration of the calcium levels in the extracellular spaces of the callus and basal tissues. In addition, the calcium treatments were continued for a further 2 days after IBA treatment ceased, to maintain tissue calcium levels during what was assumed to be the critical period for auxin absorption.

Vacuum infiltration was achieved by placing the rinsed cuttings into calcium treatment solution to a depth of 2.5 cm. The cuttings plus treatment solution were then placed in a vacuum desiccator. A vacuum was applied twice for 5 seconds at 25 inch of mercury, with the cuttings returned to atmospheric pressure between applications. After treatment in $CaCl_2$ + IBA solution for 48 hours, the cuttings were transferred directly to $CaCl_2$ solutions for a further 48 hours.

4.4.5c Data and interpretation

Treatment (μM Ca)	0	1	10	50	100	500
Root Initiation %	54.9	39.3	46.9	53.7	63.8	52.9
Roots/Cutting	3.64	3.64	3.69	3.89	3.93	3.33
Callus Rating	3.43	3.25	3.57	3.81	3.45	3.73

Table 4.4h

Effect of calcium concentration in IBA treatment solution, IBA 20 mg/L. First experiment, no vacuum infiltration.

None of the means for calcium treatment are significantly different from that of the 0 μM calcium control, (Table 4.4h). There is the appearance of a trend in the effect of different levels of calcium that is worthy of further investigation, see Figure 4.4i, however the difference in root initiation between the 0 and 1 μM treatments is difficult to explain.

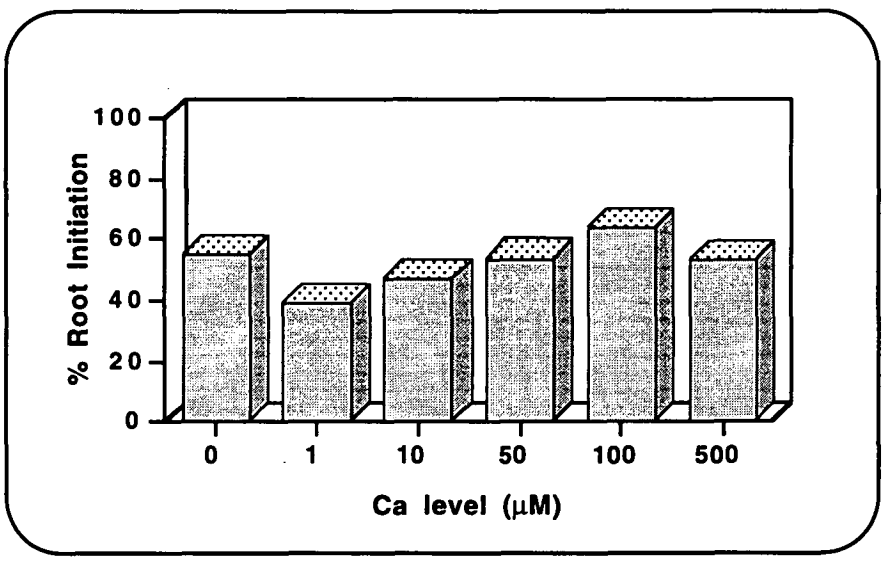


Figure 4.4i

Effect on root initiation of the calcium concentration in IBA treatment solution. First experiment , no vacuum infiltration.

If the 0 calcium treatment is excluded from the analysis then the difference in root initiation between the 100 μM treatment and the 1 μM treatments is statistically significant at the 5% level. This may indicate that variation in

external calcium concentration does affect root initiation at non-zero concentrations and that another mechanism is in operation when the external calcium concentration is zero.

The second experiment failed to produce any significant differences in root initiation rates see Table 4.4j. All treatments showed clear evidence of damage to the basal callus. This appears to have been caused by the vacuum infiltration process. The callus damage may also have caused the higher level of fungal infection that was noted in this experiment. There is no obvious explanation why the callus rating for the highest calcium treatment was significantly different from that of the control.

Treatment (μM Ca)	0	1	10	50	100	500
% Root Initiation	34.9	38.9	34.8	31.4	40.7	41.0
Roots/Cutting	3.13	2.05	3.37	3.06	4.41	2.70
Callus Rating	1.66	1.76	1.48	1.90	1.65	2.02 *

* Significantly different from control at 5% level

Table 4.4j

Effect of calcium level in IBA treatment solution. Second experiment, with vacuum infiltration.

4.4.5d Discussion

Neither of the two experiments have provided clear evidence of the effect of apoplastic calcium concentration on root initiation. There are some indications that such an effect may exist but the experiments lack the replication needed to detect small differences between treatments.

The treatment solutions contained only water, $CaCl_2$, IBA and ethanol. A less dilute treatment solution, containing other inorganic ions may have been more appropriate. This would make it possible to maintain a constant osmotic potential between treatments. It is possible that the conductivity of the solution surrounding tissues undergoing organogenesis may affect the success of that organogenesis.

An underlying assumption of both experiments is that the critical phase of root initiation occurs within the period that the auxin and calcium treatments are applied. In fact, there is little or no evidence to support this assumption. It is quite possible that initiation of the root primordium occurs later than this, driven by the absorbed auxin. If that is true, then the only effect that calcium treatments such as these could have is on the rate of auxin absorption by the stem and callus.

There was no attempt to limit or control calcium levels in the surrounding medium during the rest of the time that the cuttings were on the mist-bed. This would be difficult to control for eucalypt cuttings, as they need to be maintained in a very high humidity environment. Calcium from the soil solution and from the misting system are both potential sources of additional calcium ions that are difficult to eliminate or control.

There have been few studies on the implications of calcium availability on auxin uptake in cuttings. That these experiments have failed to provide any definitive conclusions on the subject should not be taken as proof that other studies are not needed. The degree of replication in these experiments may simply have been insufficient to detect small differences in root initiation rates.

Future experiments may need to concentrate on controlling calcium levels for a longer period after the auxin treatment has ceased. If calcium is important to root initiation, it may only be during the critical first few hours of induction of polarity, prior to when the root meristem is being formed. The exact timing of that event hasn't been determined in relation to auxin applied after callus formation, although it is assumed to be close to the time of auxin treatment.

4.4.6 Summary: methods of auxin application

Both the timing of auxin application and the concentration of auxin applied affect the rate of root initiation. Auxin applications are most effective if applied to cuttings four weeks after the cutting is taken from the parent plant. Optimum auxin concentration appears to be related to the duration of treatment.

Application of auxin to the apex of the cutting did not result in a significant boost to root initiation, although it is thought that a higher concentration of IBA in a lanolin paste may have a positive effect.

The effect of calcium concentration on auxin applications has not been determined conclusively. Significant differences were obtained between treating cuttings with 1 μM calcium and 100 μM. However the difference between 0 μM and 1 μM is also large, though not statistically significant. An attempt to repeat and refine this result was not conclusive.

4.4.7 Conclusions

Application of auxin by means of a basal dip is a relatively uncontrolled system. There is no control over auxin movement after it is applied, nor is there any control over how long the auxin remains in an active form within the cutting. This latter point may be of some importance in *E. nitens* cuttings.

Much research has been directed at determining the best method of applying auxin to cuttings. This has mostly concentrated on determining the relative merits of high concentration ethanolic dips and powder dips (Loach, 1988). Applying auxin at the time of cutting collection is the standard treatment, but there is no reason why this has to be the most effective time for all plants. If root initiation does not occur until a few weeks later then it should not be surprising that later applications of auxin have a greater effect. This is probably related to a higher concentration of auxin being available in the basal tissues of the cutting at the time at which it is most needed.

Application of auxin some weeks after the cuttings are first set into the mist-bed may not be a practical treatment for large scale use, particularly if it is applied as a 48 hour dip. It is possible that root initiation could be boosted by the application of auxin in the form of a soil drench or even as a foliar

application at the appropriate stage. If auxin were applied as a soil drench, at least some of the auxin would be absorbed by the part of the stem which is below the soil level. For foliar applications, there may be undesirable effects resulting from a high concentration of auxin on the foliage. If that were found to be the case, several low concentration sprays may have a similar effect. There may also be some practical difficulties that need to be addressed to allow auxin applications sufficient time to be absorbed before being diluted by the very wet conditions required for *E. nitens* cuttings.

Little, if any, attention has been directed at other factors which might influence the absorption of auxin from the treatment solution or its effectiveness, once absorbed. Studies of auxin induced polarity changes in single cells have shown that the concentration of calcium in the surrounding medium can have an effect on repolarisation. The initial experiments on this topic using cuttings have proved inconclusive and a more detailed examination is warranted.

4.5 ELECTRIC POTENTIAL DIFFERENCES

4.5.1 Introduction

Bioelectric currents have been demonstrated to be crucial in the establishment of cellular polarity in zygotes, suspension cultures and callus cultures (Harold, 1986). Harold notes that "Endogenous electric currents in aqueous systems report the flow of ions across the plasma membrane". These currents are believed to play a causal role in the growth, development and morphogenesis of cells and tissues. It follows that a change in the currents must represent an alteration to the co-ordination and physiological processes that underlie morphogenesis. Electric currents have been used to make callus and suspension cultures undergo a change in polarity or to acquire polarity where none previously existed. Applied currents have been shown to influence root initiation and organogenesis in callus *in vitro*. They have also been shown to enhance growth in whole plants. The presence of auxin has been demonstrated to play a significant role in establishing cellular polarities (Goldsworthy and Lago, 1992), as has the presence of calcium ions in the surrounding medium (Mina and Goldsworthy, 1992).

There are no instances reported where applied currents have been used to induce organogenesis from organised plant tissues. There is no obvious reason why it should not be possible to use applied currents to induce root primordia from callus at the base of a cutting. If currents of a similar magnitude to those applied to callus cultures were applied to callus on cuttings, it might be possible to induce regions of polarity. Preliminary investigations would need to concentrate on determining if and how it is possible to use currents to cause root formation. If the callus were on cuttings of varying rooting ability, it might be possible to make some deductions about the nature of the changes between cuttings of high rooting ability and cuttings of low rooting ability.

4.5.1a Overall Rationale

A series of experiments was devised to test the hypothesis that root initiation could be induced from callus that forms at the base of cuttings by exposing the cuttings, with their callus, to a lateral electric potential difference. The potential difference was applied using the apparatus described in Section 3.5.

The experiments undertaken were divided into three groups:

a) Those where exogenous auxin was present during the treatment with a potential difference;
b) Those where there was no additional auxin; and
c) Those where calcium was absent from the bathing medium.

4.5.2 Effects of an applied potential difference in the presence of IBA

4.5.2a Introduction

The general hypothesis is that root initials can be induced in callus by the action of electric currents and auxin. There are few clues to what combinations of potential difference, auxin and cutting preparation will be required to achieve this. To gain initial data, several experiments were devised to test different combinations of callused cuttings, auxin and potential difference. The cuttings were treated with IBA prior to and during their exposure to the potential difference as the greatest success in achieving repolarisation and organogenesis has been in the presence of auxin.

4.5.2b Experiment PD-1. Rationale

The first experiment was designed to investigate the effect of a potential of 3.4 Volts on cuttings with either two weeks or four weeks of callus growth. All treatments received a total of 48 hours exposure to 20 mg/l IBA. Exposure to the current occurred in the last 24 hours of the experiment to allow for uptake of the IBA prior to transferring to the treatment tank.

4.5.2c Procedures

Two experiments using the same conditions, media and cuttings were performed. One was carried out when the cuttings had been left to callus for two weeks and the other after four weeks on the mist-bed. For convenience, these have been labelled as two parts of the same experiment, although their data has been analysed separately.

A standard treatment consisting of 0.1 N Hoaglands solution was used, as outlined in the General Methods section. The medium was supplemented with 20 mg/l IBA dissolved in ethanol, giving a final ethanol concentration of 1% in the treatment solution. A potential difference of 3.4 Volts was applied during the cuttings' exposure to the current. This resulted in a current of 53 μAmpGeneral Methods to 48 μAmp flowing between the electrodes.

A single batch of node 4 cuttings was prepared and placed on the mist-bed. After either two weeks or four weeks on the mist-bed, cuttings were selected at random and transferred to the treatment solution. Prior to transferring the cuttings to the treatment solutions, they were rinsed twice with tap water.

All the cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for the last 24 or 12 hours of treatment.

4.5.2d Data and interpretation

The results for this experiment, Table 4.5a, show that the cuttings which had been callused for two weeks then exposed to the current were inhibited in their root formation. Cuttings that had been callused for four weeks have no significant differences between treatments.

Week 2 cuttings showed considerable necrosis and damage to the base of the stems from 24 hours exposure to the potential difference. 64% of cuttings were excluded from measurement as they were assessed as being dead below the ground level. Only 2.5% (1 out of 40) of cuttings in each of the other two treatments had died in the same way. There were only 3.5 % losses in the Week 4 experiment.

	Week 2	Week 4
% Root Initiation		
Control	59.0	63.2
12 hours	33.3*	47.2
24 hours	21.4*	55.0
Root Number		
Control	2.04	3.62
12 hours	2.00	2.53
24 hours	2.00	3.14
Callus Rating		
Control	2.97	3.60
12 hours	3.13	3.86
24 hours	2.86	3.50

* significantly different from control at 5% level

Table 4.5a

The effect of applied potential difference on cuttings in the presence of IBA 20 mg/l.

4.5.2e Experiment PD-2. Rationale

This experiment was designed to investigate the effects of a potential difference of 3.4 Volts on cuttings with four weeks of callus growth with different levels of IBA. The potential difference was applied to the cuttings concurrent with the last 24 hours of the IBA treatment. This allowed the cuttings to absorb some of the IBA prior to the cuttings being transferred to the treatment tank.

4.5.2f Procedures

There were three parts to this experiment using the same conditions, media and batch of cuttings. The difference in the experiments lay in the duration of the exposure to the current and in the concentration of IBA used. All treatments received a total of 48 hours exposure to IBA at concentrations of 20, 10 or 5 mg/L

A standard treatment consisting of 0.1 N Hoaglands solution was used, as outlined in the General Methods section. The medium was supplemented with IBA dissolved in ethanol, giving a final ethanol concentration of 1% in the treatment solution. A potential difference of 3.4 Volts was applied during the cuttings' exposure to the current. This resulted in a current of 58 μAmp to 48 μAmp flowing between the electrodes .

A single batch of node 4 cuttings was prepared and placed on the mist-bed. After four weeks on the mist-bed, cuttings were selected at random and transferred to the treatment solution. Prior to transferring the cuttings to the treatment solutions, they were rinsed twice with tap water.

All the cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for the last hours of auxin treatment.

4.5.2g Data and interpretation

The results obtained from treatment with a potential difference for a shorter duration showed no significant differences between the mean of the control and that of other treatments, see Table 4.5b. Treatments of 12 hours and 24 hours were intended for this experiment to allow direct comparison with experiment PD - 1, however a power failure prevented the completion of those treatments.

	% Root Initiation	Roots/Cutting	Callus Rating
Control	38.2	3.23	3.35
0.5 hours	51.5	1.53	3.03
1 hour	28.1	2.33	3.72
3 hours	42.4	1.57	3.39
6 hours	45.4	2.47	3.36

Table 4.5b

The effect of an applied potential difference on cuttings in the presence of IBA 20 mg/l for up to six hours, experiment PD - 2. There are no significnt differences between treatments.

There is a difference in rooting between the control and 0.5 hrs treatent which

suggests that a shorter treatment period might be effective but the differences are not significant.

The pattern of results obtained from treatment with 10 mg/l IBA is consistent with the results from experiment PD-1 (see Table 4.5c). The 12 hour treatment shows slight promotion of root initiation and the 24 hour treatment inhibiting root initiation. However, this may be due to a low root initiation rate being recorded for the controls. This initiation rate is lower than in the similar experiment using 5 mg/l IBA, despite the cuttings coming from the same batch of plants.

	% Root Initiation	Roots/Cutting	Callus Rating
Control	22.9	1.75	3.54
12 hours	34.2*	2.15	3.55
24 hours	13.9*	1.60	4.03

* = significant difference between treatments

Table 4.5c

The effect of applied potential difference on cuttings in the presence of 10 mg/l IBA, experiment PD - 2.

Application of a potential difference to callused cuttings in the presence of 5 mg/l IBA appeared to have no effect on root initiation (see Table 4.5d).

Overall, it seems possible that there is some promotion of root initiation in the treatments where 10 mg/l IBA was used. However, the variation in control root initiation rates and the small sample sizes in these experiments makes it difficult to be conclusive.

A further complication of these experiments was the growth of algae in the bathing medium reservoir and in the treatment tank. This was thought to be due to the presence of ethanol used to dissolve the IBA. This served as a carbon source for the algae, which would otherwise not have been able to grow in the darkened growth chamber. Subsequent experiments used K IBA which is water soluble, thus avoiding the need for ethanol.

	% Root Initiation	Roots/Cutting	Callus Rating
Control	28.6	1.80	3.29
12 hours	29.7	1.91	3.11
24 hours	25.0	2.44	3.11

Table 4.5d

The effect of applied potential difference on cuttings in the presence of IBA 5 mg/l, experiment PD - 2. There are no significant differences between treatments

4.5.2h Experiment PD-3. Rationale

This experiment was intended to re-examine the effect of a potential of approximately 3.4 Volts on cuttings with four weeks of callus growth, in the presence of 10 mg/l IBA. To prevent the growth of algae seen in the previous experiment, K-IBA dissolved in water, was used. All treatments received a total of 48 hours exposure to K-IBA, at a concentration of 12 mg/L, which is an approximately equivalent molarity to 10 mg/l IBA. Exposure to the potential difference occurred in the last 24 hours, to allow for uptake of the IBA prior to the cuttings being transferred to the treatment tank.

4.5.2i Procedures

This experiment was carried out twice, two days apart, using the same batch of cuttings and identical procedures.

A standard treatment consisting of 0.1 N Hoaglands solution was used, as outlined in the General Methods section. A potential difference of 3.3 Volts was applied during the cuttings' exposure to the current. This resulted in a current of 38 μAmp to 47 μAmp flowing between the electrodes.

A single batch of node 4 cuttings was prepared and placed on the mist-bed. After four weeks on the mist-bed, cuttings were selected at random and transferred to the treatment solution. Prior to transferring the cuttings to the treatment solutions, they were rinsed twice with tap water. All the cuttings

were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for the last 24 or 12 hours of treatment.

4.5.2j Data and interpretation

	% Root Initiation	Root Number	Callus Rating
First Run			
Control	17.0	3.44	3.72
12 hours	35.1*	2.00	3.95
24 hours	31.6	2.58	3.84
Second Run			
Control	26.3	1.73	3.10
12 hours	26.3	3.00	3.30
24 hours	32.5	2.60	3.47

* Significantly different from control at 5% level

Table 4.5e

The effect of applied potential difference on cuttings in the presence of K-IBA 12 mg/l, experiment PD- 3.

The significant difference in the first part of this experiment indicates that root initiation was promoted by exposure to the potential difference. However, the second part of the experiment fails to reproduce this effect. The variation in root initiation rate in the controls for the two parts of the experiment brings this result into question. All the cuttings were chosen at random from a single batch and should have had similar rates of root initiation for the control treatments. The significant difference could be attributed to an abnormally low root initiation rate in the control for the first run of the experiment or to a treatment effect.

As a result of removal of ethanol from the bathing medium, there was no evidence of algal growth during this experiment.

4.5.2k Experiment PD-4. Rationale

This experiment was designed to investigate the effect of a higher voltage on cuttings with either three weeks or four weeks of callus growth. All treatments received a total of 48 hours exposure to 20 mg/l IBA. Cuttings were exposed to an electric potential of approximately 10 Volts for 48, 24 or 12 hours.

4.5.2l Procedures

Two experiments using the same conditions, media and cuttings were performed. One was carried out when the cuttings had been left to callus for three weeks and the in other the cuttings were placed on the mist-bed for four weeks. For convenience these have been labelled as two parts of the same experiment, although their data has been analysed separately.

A standard treatment consisting of 0.1 N Hoaglands solution was used, as outlined in the General Methods section. The medium was supplemented with 20 mg/l IBA dissolved in ethanol, giving a final ethanol concentration of 1% in the treatment solution. A potential difference of 9.97 to 9.86 Volts was applied during the cuttings' exposure to the current. This resulted in a current of 445 μAmp to 400 μAmp flowing between the electrodes.

A single batch of node 4 cuttings was prepared and placed on the mist-bed. After either three weeks or four weeks on the mist-bed, cuttings were selected at random, rinsed twice with tap water and transferred to the treatment solution. All the cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for 48, 24 or 12 hours.

4.5.2m Data and interpretation

This experiment showed no effect from the exposure of the cuttings to currents, despite the current being approximately ten times that of the previous experiments.

	Week 3	Week 4
% Root Initiation		
Control	33.3	25.0
12 hours	29.4	29.2
24 hours	32.3	20.1
48 hours	22.9	33.3
Root Number		
Control	1.25	1.67
12 hours	1.20	1.57
24 hours	1.45	1.40
48 hours	1.50	1.25
Callus Rating		
Control	2.47	2.58
12 hours	2.56	2.79
24 hours	2.38	2.33
48 hours	2.57	2.71

Table 4.5f

The effect of applied potential difference on cuttings in the presence of IBA 20 mg/l and 10 Volts, experiment PD - 4. There were no significant diferences between treatments.

4.5.2n Discussion, effects of applied potential difference in the presence of IBA

There was substantial and significant inhibition of root initiation to cuttings with two weeks callus. This occurred after 12 hours exposure to 3.3 Volts, in the presence of 20 mg/l.IBA and caused substantial stem damage after 24 hours treatment.

Two experiments showed treatment means that were significantly different, which indicates significant promotion of root initiation. However, these results were not consistent, it is possible to interpret them as being due to chance or experimental error.

None of the experiments which used three or four week old cuttings and 20 mg/l IBA showed any difference between the controls and treatment with a potential difference, regardless of the duration of treatment or the size of the potential difference. This is surprising, as it is at this stage that the cuttings have been shown to be most responsive to the application of IBA.

It is of concern that treatment with a potential differences of approximately 3.3 Volts produced both positive and negative effects on cuttings in different situations but that a higher voltage had no measurable effects. It is difficult to propose a explanation where a potential difference of 3.3 Volts can have significant effect on tissues but one of 10 Volts has no apparent effect. If the results from experiments with lower currents are accepted as being representative of the effects of currents, then the effects of higher currents will need to be further investigated.

4.5.2o Conclusions

Overall, the results for these experiments are quite variable. However, they do point to the fact that applied potential differences can affect the development of cuttings. The positive effects of electric potential differences on root initiation need verification but are consistent with the hypothesis that led to this work.

The negative effects on two week old cuttings needs to be verified, as it indicates that there may be stages in the development of callus that are more sensitive to imposed potential differences. An underlying assumption of these experiments was that the treatments would be most likely to have an effect when there was a large amount of callus at the base of the cutting. This may need to be re-assessed.

4.5.3 Effects of an applied potential difference in the absence of IBA

4.5.3a Introduction

Some of the experiments in section 4.5.2 indicated a degree of inhibition of root initiation and even toxicity from the presence of IBA in the treatment medium. A series of experiments was designed to test the effect of 3.4 Volts on cuttings without any additional IBA being present in the treatment medium.

4.5.3b Experiment PD-5. Rationale

This experiment was designed to test the effect of approximately 3.4 Volts on cuttings in the absence of IBA. Three experiments using the same conditions, media and cuttings were performed. One was carried out on fresh cuttings, the others when the cuttings had been left to callus for two weeks and four weeks on the mist-bed. For convenience, these have been labelled as three parts of the same experiment, although their data has been analysed separately.

4.5.3c Procedures

A standard treatment consisting of 0.1 N Hoaglands solution was used, as outlined in the General Methods section. A potential difference of 3.3 Volts was applied during the cuttings exposure to the current. This resulted in a current of 56 μAmp to 47 μAmp flowing between the electrodes.

A single batch of node 4 cuttings was prepared. One third of the cuttings were treated immediately and the remainder placed on the mist-bed. After either two weeks or four weeks on the mist-bed, cuttings were selected at random and transferred to the treatment solutions. Cuttings were rinsed twice with tap water prior to treatment. Rooted cuttings were discarded.

All the cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for 48, 24 or 12 hours. All cuttings were assessed at the same time, three weeks after the final treatments and seven weeks after the first treatments were applied.

4.5.3d Data and interpretation

Root initiation is significantly promoted by the application of current to fresh cuttings for 48 hours. In addition, callus ratings for all cuttings exposed to the current were significantly different to that of the control treatment.

	Week 0	Week 2	Week 4
% Root Initiation			
Control	29.6	39.4	22.2
12 hours	37.9	29.6	18.5
24 hours	30.0	32.3	30.0
48 hours	61.3*	10.0**	23.1
Root Number			
Control	1.37	1.38	1.67
12 hours	1.73	1.12	1.40
24 hours	2.00	1.40	1.33
48 hours	1.47	1.00	1.33
Callus Rating			
Control	1.59	2.58	2.30
12 hours	2.82**	2.27	2.30
24 hours	2.53**	2.74	2.00
48 hours	2.64**	2.57	2.31

* & ** Significantly different from control at 5% and 1% levels respectively

Table 4.5g

The effect of applied potential difference on cuttings in the absence of IBA, Experiment PD-5.

There was a reduced root initiation rate for 2 week old cuttings treated with current for 48 hours. This is a similar result as was found for the same treatment combination in the presence of IBA (see Table 4.5a). In this experiment, there was not a high incidence of damage to cuttings, as found with the experiments where IBA was present.

4.5.3e Experiment PD-6. Rationale

The experiment was designed to investigate the effect of a potential difference of approximately 3.4 Volts on fresh cuttings without any additional IBA, in an attempt to replicate the promotion of root initiation that was observed in the previous experiment.

4.5.3f Procedures

To achieve a greater degree of replication, two experiments using the same conditions, media and cuttings were performed. A standard treatment consisting of 0.1 N Hoaglands solution was used, as outlined in the General Methods section. A potential difference of 3.3 Volts was applied during the cuttings' exposure to the current. This resulted in a current of 30 μAmp to 31 μAmp flowing between the electrodes. This current is slightly lower than that measured for the same applied potential difference in other, similar experiments. It is believed that an error occurred in the preparation of the Normal Hoaglands stock solution, from which the bathing solution was prepared. This resulted in the concentration of KNO_3 in the bathing medium being half its proper level and caused the medium to have a lower conductivity.

All cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for the 48, 24 or 12 hours of treatment.

The cuttings were node 4 cuttings, which were prepared from a batch of seedlings grown in the North Forest Products' nursery at Ridgley. These cuttings were from the same batch of plants as was used in the experiment described in Section 4.5.4d and whose results are contained in Table 4.5j. Root initiation in the cuttings was assessed five weeks after treatment. The results of this experiment were adversely affected by a higher than usual incidence of fungal damage, resulting in losses of 19% and 26% in the two parts of the experiment. These losses were spread evenly among the treatments. The resulting lower degree of replication makes detection of significant differences less likely.

4.5.3g Data and interpretation

There were no significant differences between treatments in the first part of this experiment, see Table 4.5h. There appears to be a trend to higher root initiation in the longer duration treatments. This is consistent with the effect found in the previous experiment, PD -5. This pattern was not repeated in the second part of the experiment. There was no trend to increased root initiation and there was one highly anomalous result, for which no satisfactory explanation can be offered.

	Part One	Part Two
% Root Initiation		
Control	23.1	26.9
12 hours	26.9	33.3
24 hours	40.0	3.6*
48 hours	40.7	20.0
Root Number		
Control	1.50	1.28
12 hours	1.43	2.00
24 hours	1.80	1.00
48 hours	1.45	1.20
Callus Rating		
Control	1.69	1.42
12 hours	1.88	1.78
24 hours	1.96	1.32
48 hours	1.89	1.52

* significantly different from control at 5% level

Table 4.5h

The effect of an applied potential difference on fresh cuttings in the absence of IBA, Experiment PD-6.

There was no appreciable trend on callus ratings. A very low callus rating was recorded in both experiments, as well as in the related Experiment PD-8, which was run at the same time, using the same batch of plants from Ridgley. The plants had a higher nutritional status and this may have affected the rate at which callus developed on the cuttings.

When the experiment was measured, it was noted that most of the roots that were visible were very short, as if they had just emerged. This suggests that the experiment should have been left on the mist-bed longer, to allow completion of root emergence. The experiment was measured five weeks after treatments were applied, which was the standard length of time to allow root emergence, but is not as long as the previous, successful experiment which was measured after seven weeks.

4.5.3h Discussion, effects of applied potential difference in the absence of IBA

There is a positive effect on root initiation from applying a current to freshly collected cuttings. However the attempt to repeat this result was not successful. This is probably due to the different source of seedlings used to provide the cuttings and other, subtle variations in experimental technique. It is difficult to determine what effect the error in the preparation of the nutrient solution had on the cuttings, although it may have had a negative effect. Such an error does not alter the potential difference to which the cuttings were exposed but it does decrease the magnitude of the current flowing around the tissues.

The inhibition of root initiation in week 2 cuttings is consistent with the results obtained for the same age cuttings in the experiment outlined in Section 4.5.2b, Experiment PD-1. This is in contrast with the results for week 4 cuttings that show no effect from applied currents.

The results of these experiments indicate that root initiation can be promoted as well as inhibited by the application of electric currents. Such promotion has not previously been reported in cuttings of any species. Substantial replication of the experiment is needed to confirm the results.

Experiments in other sections of this thesis have been carried out using distilled water as the control treatment. In this experiment, as with others in this section, the control treatment was 0.1 N Hoaglands solution. No attempt has been made to compare the root initiation rates between these two types of control treatment. While it seems unlikely that the mineral content of the treatment solution would have much effect on rooting ability, this possibility could have been excluded by the addition of an additional water control treatment. Particularly as the effect of calcium ion concentration is part of the proposal under investigation. Immersion of *E. grandis* cuttings in water for 12 hours prior to planting into soil has been reported to increased root initiation (Wilson, 1994a). However, it is not recorded whether the water used was distilled or tap water, which might contain significant amounts of calcium.

4.5.3i Conclusions

The high level of rooting promotion obtained in experiment PD-5 demonstrates that the techniques used have the potential to increase root initiation from fresh cuttings. This is despite the failure to replicate the results in Experiment PD-6. There are no reasons to reject the first result, while there are several differences in the second experiment which may have contributed to the lower root initiation.

The original hypothesis, that led to these experiments, proposed that the electric currents would induce root primordia from callus by altering cellular polarity within the callus. In fact, the experiments have demonstrated that it is difficult to induce root primordia from callus at the base of cuttings. These results indicate that cuttings are more sensitive to the effects of applied currents prior to the development of callus. They also indicate substantial variations in the sensitivity of callus to the applied current.

4.5.4 Effects of potential difference applied with calcium absent from the bathing medium

4.5.4a Rationale

As outlined in section 2.7.6, the presence of calcium ions in the bathing medium has been found to be necessary to allow the polarity of individual cells to be altered by applied electric currents (Mina and Goldsworthy, 1992). If the observed effects of currents upon cuttings are due to the establishment of new cell polarities and ion currents, then application of a potential difference to cuttings in a calcium free medium should have little or no effect on root initiation.

Two separate experiments were undertaken using calcium free medium. The first experiment used cuttings with four weeks callus growth and a medium containing K-IBA. This experiment uses a calcium free medium but in other respects, is similar to that detailed in section 4 4.1 and whose results appear in Table 4.5f. The second experiment also uses a calcium free nutrient solution but with freshly prepared cuttings and no auxin. It is similar to experiments PD-5 and PD-6, using a calcium free medium.

4.5.4b Experiment PD-7, procedures

Two identical experiments using the same conditions, media and cuttings were performed, one immediately after the other. For convenience, these have been labelled as two parts of the same experiment, although their data has been analysed separately.

A calcium free Hoaglands solution was prepared by substituting NH_4NO_3 for $Ca_2(NO_3)_2$, as outlined in Section 3.5. The medium was supplemented with 12 mg/l K^+-IBA. A potential difference of 3.3 Volts was applied during the cuttings' exposure to the current. This resulted in a current of 39 µAmp to 42 µAmp flowing between the electrodes.

The cuttings were node 4 cuttings which had been prepared and placed on the mist-bed four weeks earlier. They had developed substantial amounts of callus. Prior to the cuttings being transferred to the treatment solutions, they were

rinsed twice with distilled water, to reduce the amount of calcium contamination carried over from their rooting medium. All cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for the last 24 or 12 hours of treatment.

4.5.4c Data and interpretation

	Part One	Part Two
% Root Initiation		
Control	14.0	31.2
12 hours	18.5	13.3
24 hours	14.3	27.6
Root Number		
Control	1.37	1.95
12 hours	1.60	1.50
24 hours	1.50	1.62
Callus Rating		
Control	2.82	2.48
12 hours	2.89	2.73
24 hours	2.75	2.48

Table 4.5i

The effect of an applied potential difference applied to cuttings in the presence of K^+IBA, 12 mg/L, and in a calcium free medium, experiment PD-7.

There are no significant differences between the treatments in either part of the experiment. There is a surprising difference between the root initiation rates in the control treatments, given that all the cuttings were selected at random from within the same batch of callused cuttings. This makes interpretation of any other results difficult. There is no evidence of the promotion of root initiation by applied currents that has been observed in the other experiments, where auxin has been present.

4.5.4d Experiment PD-8, procedures

The second trial using calcium free medium was conducted using freshly. prepared cuttings, without any period of callusing on the mist-bed. A calcium free Hoaglands solution was prepared by substituting NH_4NO_3 for $Ca_2(NO_3)_2$, as outlined in the General Methods section. The medium contained no auxin. A potential difference of 3.28 Volts was applied during the exposure of the cuttings to the current. This resulted in a current of 38 μAmp to 45 μAmp flowing between the electrodes. All cuttings were held in the treatment solution for 48 hours. Cuttings exposed to the potential difference were moved to the treatment tank for the last 24 or 48 hour of treatment. The results of this experiment are presented in Table 4.5j.

The cuttings were node 4 cuttings, which were prepared from a batch of seedlings grown at Ridgley. These cuttings were from the same batch of plants as used in the experiment described in Section 4.5.3e and whose results are contained in Table 4.5h.

4.5.4e Data and interpretation

	% Root Initiation	Root Number	Callus Rating
Control	8.8	1.00	1.32
24 hours	14.7	1.40	1.37
48 hours	3.1	1.00	1.41

Table 4.5j

The effect of an applied potential difference applied to cuttings in a calcium free medium, experiment PD-8.

None of the treatment means are significantly different from that of the control. The very low root initiation rate obtained from this experiment is quite unexpected. Identical cuttings obtained from the same batch of seedlings showed a root initiation rate of 23 and 26% in control treatment of experiment PD-6, outlined in Section 4.5.3f.

This experiment was not repeated, due to a lack of suitable plants. If the results obtained in this experiment can be replicated in the future, it would indicate that the absence of calcium in the surrounding medium is of critical importance to root initiation, regardless of the application of current. This is in sharp contrast to the experiments in Section 4.4.4, where it was found that the concentration of calcium in the surrounding medium had little or no effect on root initiation The difference is that this later experiment was conducted on cuttings without basal callus and without exogenous auxin applications. Similar effects should be possible in a medium containing calcium but in the presence of a calcium channel blocker such as La^{2+}.

4.5.4f Discussion

The failure of cuttings to initiate roots in conditions that have previously caused rooting promotion is consistent with the results of Mina and Goldsworthy (1992). It does not prove that the enhanced root initiation found in the presence of calcium is due to the establishment of new polarities. It points to the likelihood that calcium channels play an important role in the establishment of cellular polarity and root meristem formation. It is also possible that the calcium concentration may be important for other ion channels involved in membrane currents, as has been suggested by Kourie (1996).

4.5.4g Conclusions

The effect of a calcium free nutrient medium on cuttings needs clarification. The results of the experiment PD-8 imply that, even in the absence of applied currents, the calcium status of the medium surrounding a freshly prepared cutting is important to rooting ability. This is the reverse of what was demonstrated for callused cuttings in the experiment PD-7 and those described in Section 4.4.4.

4.5.5 The effect of electric potential differences on root initiation

4.5.5a Consistency with previous research

The potential differences used in the experiments described in this section are broadly similar to potential differences that have been reported to affect differentiation in plant cells.

For most of the length of the treatment tank it can be assumed that the potential difference is evenly distributed and that the current is flowing uniformly through the liquid in the tank. All of the cuttings were at least 10 cm from the electrodes. The cross sectional area of the nutrient solution was 35 cm $^2 \pm 2$ cm ^{2}, resulting in a current density of between 1.1 μAmp cm^{-2} and 1.5 μAmp cm^{-2} for the experiments carried out at 3.3 Volts and currents up to 12 μAmp cm^{-2} for the 10 Volt experiment. Such currents are of a similar magnitude to those surrounding normal root meristems (Hush and Overall, 1989). This assumes that the ionic concentration of the bathing solution in the tank is uniform and the flow rate is uniform across the tank. This was found to be the case in test runs.

Somatic embryogenesis from *Medicago* protoplasts was affected by potential differences as low as 8 mVolt cm $^{-1}$ and 60 mVolt cm $^{-1}$ was lethal within a few hours (Dijak *et al*, 1986). Regeneration from tobacco callus was stimulated by the application of a 1 or 2 μAmp current, which was calculated to generate potential differences ranging from 1 to 300 mVolt cm $^{-1}$ through the callus pieces (Rathore and Goldsworthy, 1985b). These potential differences are similar to the 34 to 32 mVolt cm $^{-1}$ used in most of the experiments reported here. While in the tank, the potential difference from one side of a cutting to the other is of the order of 10 to 16 mVolt, assuming the 3.3 Volt potential difference is evenly distributed between the electrodes and that the cutting is 3 to 5 mm in diameter.

The electric fields to which the cuttings were exposed are slightly lower than those calculated to be the minimum required to cause electrophoretic migration of mobile components of cell membranes (Jaffe, 1977). A field below 0.1 mVolt per cell or 33 mVolt cm $^{-1}$ is thought to be unlikely to have any significant effect

on membrane proteins because back diffusion would be higher than the electrophoretic mobility of the molecule (Jaffe, 1977). However, the calculations by Jaffe (1977) are based on individual spherical cells, 10μm in diameter, in an uniform electric field. It has been postulated that neither of these conditions are fulfilled when complex tissues are placed in an electric field (Edmonds, 1994). In tissues, much weaker fields may be effective, especially if cells are elongated in the direction of the field or if cells are interconnected in the direction of the field.

4.5.5b Artefacts and technical considerations

It is important in experiments such as these to control artefacts such as localised pH variations and nutrient gradients (Robinson, 1985). Variations in the applied potential differences are relatively easy to control. However variations in the current flowing as a result of this potential difference are more difficult to control.

In these experiments, substantial effort was devoted to eliminate the effects of current on electrolyte accumulation in the bathing solution. If not controlled, electrophoretic migration of ions could result in substantial concentration changes in the bathing medium around electrodes, resulting in pH changes and possible growth effects.

Specific steps were taken to prevent ionic concentration occurring. The continuous flow system was designed to prevent ion accumulation around the electrode by continuously flushing the ions away from the electrode. The plant material was spatially separated from the electrode. This results in the highest concentration of electrolytes being some distance from the plants, unlike some experiments where the electrode is in close association with the plant tissues (Goldsworthy and Lago, 1992; Goldsworthy and Rathore, 1985; Rathore and Goldsworthy, 1985b).

In an experiment not reported, a failure in the power supply to the peristaltic pump occurred for three hours, while a potential difference of 3.4 Volts was maintained. Three hours exposure to these conditions did not result in any significant damage to the basal regions of those cuttings. This makes it less likely that the damage to cuttings noted from other experiments was the result of ion accumulation or pH changes. Such pH changes might be an explanation

for some of the anomalous results, but is unlikely to be the overall driving force. The difference in pH of the bathing medium, measured at the two electrodes, is quite small and would be even smaller at points closer to the bathing medium inflow, at the centre of the tank.

Although the voltage varied little over the duration of individual experiments, some variation in current was noted. The two most probable causes of these fluctuations were temperature fluctuations in the bathing medium and minor variations in the conductivity of the bathing solution. This was alleviated as much as possible by maintaining a larger reservoir of bathing solution. This resulted in less variation in temperature when fresh Hoaglands solution was added. Distilled water stocks were held in the treatment room prior to use, to allow equilibration of temperature whenever possible.

Most of the longest duration treatments were applied in the half of the tank that was closest to the cathode, but it would be better if several tanks were run together, so that there could be treatments at both poles at the same time. It is not expected that this would have any substantial effect on the cuttings, since the potential difference would be expected to be evenly distributed along the length of the tank and all cuttings would be exposed to this potential difference equally.

4.5.5c Limitations on results

The results from these experiments have shown a high degree of variability, making it difficult to obtain identical results from replicate experiments and making comparisons between related experiments difficult.

Part of this variability can be assigned to the difficulties in obtaining identical conditions for experiments. Successive experiments used cuttings from plants grown under different seasonal conditions. Temperature within the glasshouse was controlled to some extent but daylength and light intensity were not controlled. These are factors that are known to affect the rooting ability of cuttings and could be expected to affect these results. However, even in experiments conducted within a few days of each other, where the same batch of seedlings was used, there were unexpected variations in results. No satisfactory explanations for these variations have been discovered.
Slight variations in current are to be expected in an experimental set up such

as this. Minor variations in the nutrient concentration within the bathing medium, minor temperature variations and normal experimental error in preparation of nutrient supplies will all affect the conductivity. These are probably enough to explain most of the current variation that was observed. A more complete experimental set up would have direct control over the temperature of the nutrient tank, control over the temperature of the nutrient inflow and continuous monitoring of pH and conductivity of the nutrient inflow and outflows from both electrodes, as well as continuous monitoring of the applied potential difference and resultant current flowing.

All of the experiments were limited to a maximum of 40 cuttings per treatment, as this was the maximum number of cuttings that could be held in one tray in the tank in its current design. Making the tank larger, to accommodate more cuttings, presents some technical difficulties. The maximum size of the tank is largely controlled by the need to maintain efficient flushing of the nutrient solution, whilst maintaining electrical isolation by ensuring the outflow is composed of discrete drips. Increasing the size of the tank by making it longer would require a higher bathing medium flow rate, making it more difficult to maintain the dripping pattern of the outflow. A wider, shallower tank could have the same total volume and would accommodate more cuttings but would be more likely to result in the bathing medium having a non-uniform flow pattern.

To obtain a higher degree of replication would require that the tanks be duplicated. Experiments using additional tanks would need careful monitoring to ensure the results from separate tanks were comparable. Nutrient flow rates into and out of the tank would need to be equalised, as would potential differences and currents.

4.5.6 Possible modes of action

It is highly unlikely that the weak electric fields used in these experiments would penetrate the plasma membrane to exert an effect on the internal constituents of the cell. The plasma membrane itself has a high resistance and a trans-membrane potential difference that is typically of the order of 100 mVolt (Jaffe, 1986). It is unlikely that the relatively small potential differences applied in these experiments would alter this potential.

The most probable way in which such weak fields could have an effect on the orientation of growth is by causing lateral movement of membrane components by electrophoresis. Such movement has been demonstrated to occur in several instances (Robinson, 1985). The fields to which the cuttings were exposed are slightly lower than those calculated to be the minimum required to cause electrophoretic migration of mobile components within cell membranes (Jaffe, 1977). It was estimated by Jaffe (1977) that between one tenth and half of biological molecules would be polarised by a steady voltage drop of 0.8 to 4 mVolt across the diameter of a cell. For a cell of 30 μ diameter, this is equal to a potential difference of 260 to 1300 mVolt cm^{-1}. With a potential difference of this order, the time taken to equilibrate with back diffusion was calculated to be approximate-ly 3 hours. These calculations were found to be consistent with the measure-ments of Poo & Robinson (1977), who first demonstrated the electrophoretic migration of plasma membrane proteins using receptor proteins in cultured muscle cells. They demonstrated movement of the receptors in fields as low as 200 mVolt cm^{-1} applied for 24 hours but that field strengths below this produced no detectable change. Since then, responses have been reliably recorded in a number of types of animal cell cultures where cells have migrated toward the cathode at field strengths as low as 0.1 mVolt per cell diameter (Robinson, 1985).

The most effective fields in the experiments reported here were approximately 33 mVolt cm^{-1}, applied for up to 48 hours. That is, approximately one tenth the potential difference applied for ten times as long to those calculated by Jaffe (1977). There are several suggestions which could account for the apparent discrepancies. The calculations of Jaffe are based on an assumption that the fields remain uniform throughout the tissues on which they are acting. In complex tissues, such as stem segments, this is unlikely to be true. There may be intensification of the field in some regions, caused by the presence of cells of higher resistivity, or there may be a significant difference in the mobility of plasma membrane components in some plant tissues. Alternatively, lateral electrophoresis of membrane proteins may not be the mode of action of the applied potential difference in this instance.

The target molecules in the plasma membrane most likely to be affected by lateral electrophoresis would be ion channel proteins, particularly calcium channels. Calcium channels play a crucial role in the establishment of new polar currents and it seems likely that re-orientation of growth polarity would

require the involvement of calcium channels. This is supported by the evidence of Mina and Goldsworthy (1992), who found calcium involvement in the establishment of polar currents. It also appears to be supported by the results in Section 4.5.3, where an absence of calcium seems to have interfered with the normal responses to applied potential differences.

An alternative proposition is that the applied fields might have a direct effect upon the conformational changes in membrane bound macro-molecules including enzymes. A theoretical basis has been proposed for such interactions that would allow for a response to fields as low as 1 $mVolt.cm^{-1}$ (Weaver and Astumian, 1990). There is some experimental evidence to support such a proposal (Edmonds, 1994; Tsong and Gross, 1994).

A clear, causal link is yet to be established between the internally generated polar currents and the alignment of microtubules, despite their being closely associated (Hush *et al*, 1990; Hush *et al*, 1992; Hush and Overall, 1989; Hush and Overall, 1991). The currents are unlikely to be acting directly on microtubules, as microtubules have been shown to align at right angles to an applied potential difference. This is despite the tendency for isolated microtubule components, such as F-actin, to polymerise in alignment to electric fields (Hush and Overall, 1991). It is more likely that there is some interaction between currents and microtubule associated proteins that are found in the plasma membrane. One example of these proteins are the microtubule linking proteins, which form cross bridges between cortical microtubules and the plasma membrane at regular intervals along the length of the microtubule and are firmly attached to the microtubule (Giddings and Staehelin, 1991).

If ion channels are the component of the plasma membrane upon which the electric fields are having an effect, then an explanation must be found for the effects of auxin. The apparent change in sensitivity to applied electrical potential differences in the presence of auxin also requires and explanation. Such an explanation may be provided by further clarification of the effect of auxin on voltage-dependent and ATP regulated ion channels, as have been described by Barbier-Brygoo *et al,* (1996).

If the very low potential differences used in these experiments are not sufficient to exert a direct effect on membrane constituents, then the mode of action could be via an effect on some tissue factor that is external to the plasma membrane. Localised electrophoresis of ions in the extracellular medium might conceivably cause alterations in ion channel activity or affect enzyme activity.

4.5.7 Future investigations

The immediate goal of any further investigations of the effect of electric potentials on cuttings must be to verify the results obtained so far. In particular, to confirm that fresh cuttings do respond to the treatments with increased root initiation. Once that result has been confirmed, it would be desirable to discover the upper and lower limits to potential differences that can be use to affect rhizogenesis. If lower currents were found to be effective, even over longer exposure times, it may require some re-assessment of theories on electrophoretic movement within the plasma membrane.

The duration of treatments was arbitrarily set at a maximum of 48 hours. There is nothing to suggest what would result from extending the treatments but, provided the bathing medium is in homoeostasis, it is quite possible that extended treatments would result in a further increase in root initiation.

All of these experiments used 0.1 N Hoaglands solution as the bathing solution. This concentration was only chosen because it was found to result in a current that was similar to those used by other researchers (Mina and Goldsworthy, 1991). Most theories on the role of ion currents in repolarisation are based on the effects of the electric potential difference but it is possible that the effect is due to actual ion movement as part of the current generated by the potential difference. This could be tested by altering the conductivity of the bathing medium to increase or decrease the current flowing at a consant potential difference.

The experiments of Section 4.5.2 demonstrated an apparent change in sensitivity of callus to electric fields. If these experiments are found to be repeatable, it will present a method of investigating the interaction between callus and auxin at different stages of development.

The results obtained so far do not prove that the treatment effects are due to the establishment of new polarities marked by ion flows. This could really only be proved by actually measuring the ion flows around cuttings before and after exposure to currents, to demonstrate that the flow patterns for individual ions has been altered by the treatments. This may prove difficult if, as expected, the main region of rhizogenesis is in the vascular cambium. Histological studies may be useful to correlate the effects of currents to changes in the pattern of cell division that occur within the vascular cambium after treatments have been applied.

In the study by Mina and Goldsworthy (1991), re-polarisation was more pronounced in cells growing in a medium containing 2,4-D than one containing IAA. This was cited as evidence that cells growing in 2,4-D are more susceptible to repolarisation and that natural polarity is less firmly established. This suggests that treatment with low concentrations of 2,4-D might be worth investigating to see if the re-polarisation is affected by the type of auxin present in the treatment medium. It may be that the 2,4-D treatment would need to be applied over a longer period of time than the relatively short exposures to auxin that were attempted in these experiments. Investigations of possible practical applications of such treatments can really only be undertaken once there is a larger body of knowledge on the relationship between electric fields and root initiation.

4.5.8 Conclusions

The experiments reported here were based on the notion that currents applied to callused cuttings would induce polarity within the callus causing root primordia to form. This has been demonstrated to occur but only in the presence of exogenous IBA. It was not expected that fresh cuttings would respond in the same way, in the absence of callus. Callus had been thought to be essential for such treatments to be effective, as the treatments were proposed to act on undifferentiated cells.

Root initiation has previously been stimulated from callus cultures by the application of electric currents, however, the promotion of root initiation in cuttings by applied electric potential differences has not been reported.

In all of the experiments in this section of the thesis, there needs to be some reservations held over the results due to lack of replication, the small numbers of cuttings in some treatments and the uneven response to treatments. There is a clear need for greater replication and careful attention to experimental technique, to eliminate as many variables as possible. The experimental set up is generally fairly simple, in that there is no subtlety of control over the direction of the electric potential within the tank, nor over how, or where, it interacts with the cuttings. Regardless of this, these results demonstrate that such an approach could be an effective tool in future investigation of the fundamental control mechanisms of root initiation.

4.5.9 Summary of results

The experiments outlined above represent a preliminary attempt to demonstrate that endogenous currents play a causal role in root initiation. More importantly, they were intended to demonstrate that application of an external potential difference can induce additional root initiation in callus at the base of cuttings.

The results of these experiments can be summarised as follows:

- In certain situations, it is possible to induce root initiation in cuttings using applied electric potential differences;
- Roots can be induced more readily from fresh cuttings than from cuttings where callus formation has occurred;
- Callused cuttings vary in their sensitivity to applied potential differences;
- Exogenous auxin is required for the potential difference to be effective as root promoter in callused cuttings; and,
- Calcium in the bathing medium is likely to play an important role in the effect that potential differences have on cuttings.

The results from individual experiments have not been consistent or readily repeatable in all instances. However, taken overall, the experiments have provided some unique and useful information on the control of root initiation in cuttings.

4.6 VITAMIN D and RELATED COMPOUNDS

4.6.1 Introduction

The ability of vitamins D_2 and D_3 and some related sterols, to promote root initiation by acting mostly as an auxin synergist was demonstrated by Buchala and Pythoud (1988). As no specific functions have been found for these compounds within plants there is no satisfactory explanation for the effect that they have on root initiation. The efficacy of vitamin D in the propagation of eucalypts has not been tested.

4.6.2 Effect of cholecalciferol and ergocalciferol on root initiation

4.6.2a Rationale

Vitamin D_3 and vitamin D_2, cholecalciferol and ergocalciferol respectively, have been demonstrated to be the most effective sterols at increasing root initiation in a number of species through their synergistic effect on auxin applications (Buchala and Pythoud, 1988). It was proposed to investigate whether the compounds were capable of exerting a similar effect on root initiation in *E. nitens* cuttings.

4.6.2b Procedures

A series of three small experiments was undertaken, to determine the effects of vitamin D_3, and vitamin D_2 in root initiation in cuttings of *E. nitens*. The treatments were applied after the cuttings had been grown on the mist-bed for several weeks, to allow for callus formation.

The first two experiments were designed to demonstrate whether the vitamins had any effect on root initiation. Two levels of IBA treatment were compared with two levels of vitamin.

In the first experiment, node 2 cuttings were used. They were treated after two weeks on the mist-bed to allow for callus development. The treatment levels were 10 mg/l IBA and 20 mg/l vitamins. There were 24 cuttings per treatment.

In the second experiment, node 3 cuttings were used. They were treated after four weeks on the mist-bed, for callus development. The treatment levels were 20 mg/l IBA and 10 mg/l vitamins. There were 41 to 44 cuttings per treatment.

In the third experiment, calcium was included in the treatment medium, in the form of $CaCl_2$, at a concentration of 0.1 mM. This was done on the basis that the vitamins may have been acting by stimulating some calcium regulatory function, as been proposed by Buchala and Pythoud (1988). It was hypothesised that the treatment regime in the first and second experiments was a very low calcium environment and that increasing the calcium concentration could alter the response of the cuttings to vitamins. The cuttings were taken from node three. Treatments were applied after four weeks on the mist-bed, for callus development. There were 31 cuttings per treatment.

In all experiments, treatments were applied in 50 ml tubes using the method outlined in the General Methods section. Treatment solutions were prepared by dissolving IBA and the vitamins in small volumes of ethanol, which were then mixed with distilled water. The final concentration of ethanol was 1%. Control treatments consisted of distilled water containing the same concentration of ethanol.

All three experiments were assessed for root initiation three weeks after the application of treatments.

4.6.2c Data and interpretation

In the first experiment the treatment of IBA plus ergocalciferol gave a significant boost to root initiation over the root initiation from treatment with IBA alone or for untreated cuttings, see Table 4.6a. The differences between some of the other treatment means are substantial but are not significant, due to a low degree of replication in this trial.

Treatment	% Root Initiation	Roots/Cutting	Callus Rating
Control	58.3	1.71	3.83
IBA 10 mg/l	50.0	6.08*	4.54
Cholecalciferol	33.3	4.00	3.75
Ergocalciferol	37.5	1.89	3.71
Cholecalciferol + IBA	70.8	4.82*	4.87*
Ergocalciferol + IBA	83.3*	4.45*	4.33

* Significantly different from control at 5% level

Table 4.6a

The effect on root initiation of application of IBA, 10 mg/l, and cholecalciferol and ergocalciferol, 20 mg/l

Treatment with IBA alone did not have a significant effect on root initiation. This was unexpected, even though the level of IBA was lower than that normally used.

In the second experiment, see Table 4.6b, the root initiation rate was not significantly different from that obtained with IBA alone. The vitamins did significantly increase the number of roots per cutting, indicating that they were affecting root initiation in some way.

Treatment	% Root Initiation	Roots/Cutting	Callus Rating
Control	27.3	1.83	2.95
IBA 20 mg/l	75.0*	3.42	3.18
Cholecalciferol	35.7	1.93	2.78
Ergocalciferol	34.1	2.13	3.04
Cholecalciferol + IBA	65.9*	4.17*	3.25
Ergocalciferol + IBA	76.2*	3.84*	3.33

* Significantly different from control at 5% level

Table 4.6b

The effect on root initiation of application of IBA, 20 mg/l, and cholecalciferol and ergocalciferol, 10 mg/l

Treatment	% Root Initiation	Roots/Cutting	Callus Rating
Control	19.3	1.33	2.61
IBA	38.7	1.58	2.81
Ergocalciferol + IBA	35.5	2.18	2.97

Table 4.6c

The effect on root initiation of application of IBA, 20 mg/l, and ergocalciferol, 10 mg/l, with 1 mM calcium added to all treatments. There are no significant differences between treatments.

The differences between means in this experiment are not significant. However, the trends are similar to those found in the previous experiments. It is not possible to determine if the presence of calcium altered the response of cuttings.

The results from all of these experiments suggest that cholecalciferol and ergocalciferol are capable of acting as auxin synergists to boost root initiation in *E. nitens* cuttings. The vitamins had the greatest effect when applied with lower than optimal concentrations of auxin. The vitamins had no effect on root initiation when applied in the absence of auxin. These effects are both consistent with the vitamins acting as an auxin synergist.

No additional information was obtained by the addition of calcium to the treatment solution. This experiment would have been more useful with the inclusion of some calcium free treatments and with a greater degree of replication.

There has not been any extensive attempt to find the optimum treatment concentration or treatment duration for these compounds. Higher concentrations of vitamin Ds are difficult to achieve, due to their low solubility in water although use of concentrations as high as 100 mg/l have been reported (Pythoud *et al*, 1986). A water soluble form of ergocalciferol is available commercially, but the means of solubilising the compound is not clear and might affect its activity.

4.6.2d Conclusions

Ergocalciferol and cholecalciferol clearly have the ability to boost root initiation in certain situations. In all of the reports on the effects of ergocalciferol and cholecalciferol, they have been cited as boosting the number of roots formed on each cutting. Increasing the proportion of cuttings initiating roots has not previously been reported.

It should be noted that in the experiments reported by Buchala and Pythoud (1988), the vitamins were most effective in promoting root initiation if they were applied within 48 hours of the cutting being collected. In these experiments, the treatments were applied two or four weeks after the cuttings were first collected.

None of these experiments have provided any information on the mode of action of vitamin D_2 and D_3, except that they act as synergists to auxin and appear to have a slight negative effect when applied on their own. There was no evidence of a stimulatory effect in the absence of exogenous applications of auxin, as has been reported by Moncousin and Gaspar (1983).

4.6.3 Effect of stigmasterol applications

4.6.3a Rationale

In addition to the use of the secosteroids, cholecalciferol and ergocalciferol, stigmasterol has been shown to have a promotive effect on root initiation. Stigmasterol is a more attractive proposition for routine use, as it is both less toxic to humans and more resistant to oxidation. It is also readily converted to a secosteroid by photo-isomerisation.

Two experiments were devised to test the hypothesis that stigmasterol is able to act as an auxin synergist to promote root initiation in a similar manner to vitamins D_2 and D_3.

4.6.3b Procedures

Two separate experiments were used to test the effects of stigmasterol. The first compared root initiation in treatments containing 20 mg/l IBA and looked at the effects of two levels of stigmasterol in comparison with one level of ergocalciferol. The cuttings used in this experiment were node 3 cuttings that had been allowed to callus for four weeks, there were 32 cuttings per treatment.

The second experiment investigated the effect of stigmasterol applied with 40 mg/l IBA, which is close to the optimum level of IBA when used for a 48 hour treatment. Cuttings were node 4 cuttings, allowed to callus for 4 weeks prior to treatment and there were 60 cuttings per treatment.

In both experiments, treatments were applied in 50 ml tubes using the method outlined in the General Methods section. Treatment solutions were prepared by dissolving IBA and stigmasterol in small volumes of ethanol, which were then mixed with distilled water. The final concentration of ethanol was 1%. Control treatments consisted of distilled water containing the same concentration of ethanol. Tween 20 was added to all treatments at the rate of two drops per litre.

4.6.3c Data and interpretation

The results from the experiment using 20 mg/l IBA, Table 4.6d, indicate that stigmasterol does have the capacity to stimulate root initiation as an auxin synergist to a similar extent as ergocalciferol.

Treatment	% Root Initiation	Roots/Cutting	Callus Rating
IBA	32.1	8.22	2.86
Ergocalciferol 20 mg/l	67.9*	2.74*	2.39
Stigmasterol 20 mg/l	52.0	3.00*	2.84
Stigmasterol 50 mg/l	63.3*	2.68*	2.47

* Significantly different from control at 5% level

Table 4.6d

Comparison of the effect on root initiation of ergocalciferol and stigmasterol in the presence of IBA, 20 mg/l.

The results in this experiment differ from previous attempts in that in this instance the ergocalciferol + IBA result is significantly different to the IBA result. This was not recorded in those experiments of section 4.6.2 where IBA was used at a rate of 20 mg/l.

Root number per cutting appears to have been reduced substantially by the sterol treatments but this is, at least partly, a reflection of an abnormally high number of roots on a few cuttings in the IBA treatment.

An identical experiment using node 4 cuttings failed to give any usable results, due to a very low rooting rate in all treatments. Only 1 to 3 cuttings rooted in each treatment. The data for this experiment has not been presented. This extremely poor result was tentatively linked to the use of a spray of Bayleton on the plants a week prior to collection of the cuttings. This had been used to control powdery mildew and was applied at the highest recommended rate. Cuttings which had been collected from this batch of seedlings immediately prior to spraying, had much higher root initiation rate.

Treatment	% Root Initiation	Roots/Cutting	Callus Rating
Control	8.7	1.33	1.84
IBA	10.7	3.33	1.82
Stigmasterol	7.2	1.00	1.74
Stigmasterol + IBA	22.6*	1.86	1.66

* Significantly different from control at 5% level

Table 4.6e

The effect on root initiation of treatment with IBA, 40mg/l, and stigmasterol, 50mg/l.

The root initiation rate in the experiment using 40mg/l IBA, see Table 4.6e, are very poor for node 3 cuttings. The cuttings did not respond well to IBA treatment. However stigmasterol still boosted root initiation when applied with 40 mg/l IBA. The differences between the treatment means for roots per cutting are not significant because there are few degrees of freedom in each treatment.

In both of the experiments presented in this section, stigmasterol did not have

any significant effect on cuttings when it was applied without IBA. In both experiments, the root initiation rate was higher when stigmasterol was applied with IBA than when IBA was applied alone. These results provide clear evidence that stigmasterol has a significant synergistic effect on IBA induced root initiation.

The result obtained for stigmasterol is similar to that for ergocalciferol, which may indicate that they are acting in the same manner. Stigmasterol is effective at an IBA concentration found to be close to the optimum for a 48 hour treatment, whereas the ergocalciferol treatments have been found to be most effective at lower IBA concentrations.

In preparation for this experiment, it was found that treatment solutions containing stigmasterol at concentrations of 75mg/l and 100 mg/l did not remain fully dissolved. Other experimenters have compared root initiation at higher concentrations of applied sterol. It was reported that up to 100 mg/l vitamin D_3 in a 0.4% ethanolic solution was applied to cuttings (Pythoud *et al*, 1986). The authors do not detail how they managed to dissolve the sterols, and keep them in solution. It is also possible that treatment with a combination of sterols might have a greater effect.

There are some instances where the treatment with IBA and sterol failed to produce the expected boost to root initiation. This does not negate the validity of these results but it does underline the fact that there are other factors that influence rooting ability that are not affected by IBA treatment.

4.6.3d Discussion

The results from the two trials in this section indicate quite clearly that stigmasterol is also an auxin synergist, able to enhance the root initiation effects of IBA treatments. The results do not offer any particular information on a possible mode of action of the sterols, beyond it being auxin dependent.

Stigmasterol may be acting as a sterol or may be converted in the plant to the secosteroid form, as the conversion occurs readily by photo-isomerisation (Miller and Norman, 1984). Although the treatments were applied in the dark, it is conceivable that they might not become active until after the cuttings were returned to the mist-bed and were exposed to light. If such a conversion were to

occur, it would require that the sterol be transported to the upper parts of the cuttings, as the sterols were only applied to the basal 15 mm of the stem. All of this region of the cutting is below the surface of the soil when the cuttings were returned to the mist-bed. Any sterols retained in the base of the cuttings would not be subject to photo-isomerisation.

As with the experiments using vitamin D, stigmasterol is effective in promoting root initiation 4 weeks after the cuttings were collected from the plants. This is in contrast to the results of other researchers, who have only found the compounds to be effective when applied within 48 hours of preparation of the cutting (Pythoud *et al*, 1986).

4.6.4 Triazole applications

4.6.4a Rationale

This experiment was intended to check whether the application of triazole fungicide was responsible for an unexpected decrease in root initiation rates in a batch of cuttings used in section 4.6.3. One experiment in that section showed a very low level of root initiation in the control cuttings, as well as in all of the treatments. The cuttings had been collected from a batch of seedling plants which had shown quite high root initiation in cuttings collected a week earlier. The only difference that could be found in the treatment of the seedlings between the collection dates of the two batches of cuttings was that the plants had been sprayed with Bayleton to control powdery mildew. The active constituent of Bayleton is triademefon, a triazole. Triazoles are a group of chemicals that are inhibitors of isoprenoid synthesis. As the mode of action of triademefon on powdery mildew is to inhibit sterol production, it was thought possible that there could have been an effect on sterol levels within the sprayed plants. This experiment was intended to test the effect of triademefon, which is the active ingredient of Bayleton, on the rooting ability of cuttings.

4.6.4b Procedures

Cuttings were collected from seedlings that had previously been sprayed 0, 1 or 2 times with Bayleton.

A group of 300 *E. nitens* seedlings of uniform size and appearance were selected from seedlings grown for field planting at North Forest Products nursery at Ridgley. The plants were divided into 3 treatment groups of 100 plants each:

T1 - No Bayleton sprays.

T2 - A single Bayleton spray applied 4 days before the cuttings were collected.

T3 - Bayleton spray applied 11 days and 4 days before the cuttings were collected.

Bayleton was applied at a rate of 5 g/L, sprayed to run-off stage. This is the highest rate of application recommended for fungal control.

Treatments all received identical growth conditions and handling, other than the spray application. Cuttings were prepared as normal, with the lowest node at node 3, and were set into a mixture of peat and horticultural vermiculite. Treatments were applied in a randomised complete block design, with 20 cuttings per replicate. Root initiation was assessed after four weeks on a mist-bed. The number of roots for each cutting was recorded, ignoring roots emerging from leaves.

4.6.4c Data and interpretation

Application of triademefon to the seedling stock plants had no measurable effect on the percentage of cuttings initiating roots. There was a treatment effect on the mean number of roots per cutting. Although root length was not measured, it also appeared that the treated cuttings had younger, shorter roots, with less branching. This suggests that the treatments may have delayed root initiation rather than prevented it. If this is the case, it could be that the effects of the treatment were decreasing with time, allowing root initiation to proceed. A higher application rate may have produced a more distinct treatment effect.

Treatment	% Root Initiation	Mean Roots/Plant
Control	86	3.62
Single spray	79	2.87
Double spray	86	2.66 *

* Significantly different from control at 5% level

Table 4.6f

The effect of triademefon sprays on root initiation.

An alternative possibility is that the triademefon inhibited gibberellin synthesis, resulting in delayed root extension without having any effect on root initiation. Gibberellins are one of the other groups of isoprenoids whose synthesis is inhibited by triazoles. Paclobutrazol, another triazole, is often used as a gibberellin inhibitor. Inhibition of gibberellin synthesis is thought to be most likely to promote root initiation and use of paclobutrazol has, in some situations, promoted root initiation (Davis and Sankhla, 1988; George, 1993). It may not be possible to choose between the two propositions, unless exogenous gibberellins are applied to treated cuttings or the gibberellin or sterol content of tissues are measured directly.

The use of triazoles remains a potentially useful method to probe the role of sterols and applied vitamin D in root initiation, despite the lack of clear cut results from the experiment using Bayleton. Grossman (1990) cites a number of references that detail changes in sterol levels in plants that have resulted from treatment with triazoles, mostly paclobutrazol, or with tetcyclacis. In suspension cultures, these effects could be reversed by the addition of cholesterol or stigmasterol (Goad, Haughan and Lenton, 1988; Haughan *et al*, 1989; Haughan, Lenton and Goad, 1988). More recently, Sailerova and Zwiazek (1996) showed that soil applications of triadimefon altered the ratios of free sterols to sterol esters in white spruce (*Picea glauca*) seedlings.

Triazoles are most effective in fungi (Cooke and Burden, 1990), where they cause the conversion of ergosterol into a Δ^{14} sterol, resulting in changed membrane fluidity which retards or prevents growth. In plants, they are mostly seen as inhibitors of gibberellin synthesis. Paclobutrazol is the most widely used. It usually has either a promotory effect on root initiation or no effect at all (Davis and Sankhla, 1988), which is what would be expected from inhibition of gibberellin synthesis.

The mode of action of all triazoles and a number of other related fungicides is to block the action of cytochrome P-450 dependent monoxygenases (Grossman, 1990). Gibberellin synthesis is more sensitive to inhibition than the sterol synthesis pathway, although individual triazoles may have a greater or lesser effect on different branches of the pathway. Other biochemical pathways reported to be affected by the same group of growth retardants lead to the synthesis of ethylene, polyamines, abscisic acid and cytokinins (Chappell, 1995).

There are several factors in the execution of this experiment which might account for it failing to produce the expected reduction in root initiation. The cuttings were collected from plants that appeared to be considerably more vigorous than those grown at the Horticultural Research Centre. It is believed that this was mostly due to a higher level of fertiliser application in the seedling nursery and reduced leaching from less frequent overhead watering.

Another factor was that this experiment was conducted in late Spring whereas the initial experiment, from which the proposal derived was conducted in early Autumn. Cuttings from seedlings grown in these seasons have quite different rooting patterns, which may result from different sterol synthesis patterns in the parent plants

4.6.4d Conclusions

This experiment was unable to demonstrate that triazoles have a significant effect on root initiation rates. The experiment would be worth repeating at several different times of the year, using a triazole which is a better sterol inhibitor and measurement of root initiation at several stages. Application of sterols has been shown to enhance root initiation, so it would not be surprising if lowering the endogenous sterol content of the plants had a negative effect on root initiation. This might also offer an explanation for some of the seasonal

variation in rooting ability, since sterol synthesis is known to be affected by light intensity and daylength, resulting in different sterol profiles in plants at different times of the year.

4.6.5 Effect of sterols on the *in vitro* growth of *E. nitens*

4.6.5a Rationale

This experiment was based on the hypothesis that the low light levels in growth cabinets might have an effect on the amount and type of sterols being synthesised by the plants' cells. Treatments were designed to test the effect of stigmasterol, cholecalciferol and cholesterol added to proliferation and root initiation media.

If a clear effect could be shown, this would demonstrate that:

a. Sterols are important to growth as well as root initiation;
b. Root initiation and/or shoot proliferation can be improved in tissue culture.

In addition variations in growth response to different applied sterols could be used to gain information on the structural requirements for growth promotion activity.

Stigmasterol was included in this experiment on the basis of previous results, where some effect on *E. nitens* cuttings has been detected. Ergocalciferol and cholecalciferol have been reported to be effective in promoting growth *in vitro* (George, 1993; Druart, 1988). Cholesterol was selected because it is a common sterol in plants which is less difficult to use than cholecalciferol. The inclusion of stigmasterol acetate in this trial provided information on the importance of the 3β hydroxyl group to sterol functionality.

The effects of the sterols were tested in proliferation media and in root initiation media.

4.6.5b Procedures

Media preparation and experimental procedures are as described in the General Methods section.

4.6.5c Data and interpretation

In the root initiation media, one root initiated out of all of the flasks and no root initiation was recorded for the controls. No differences could be detected between treatments on visual inspection. The micro-cuttings were not measured. A root initiation rate of at least 50% had been expected for the material used.

Addition of sterols gave a clear boost to the growth of proliferation cultures in several treatments, see Table 4.6g. If the apparent increase in proliferation growth were reproduced over repeated generations of culture, it would provide a substantial boost to the productivity of a micropropagation system. The apparent decrease in growth with a higher level of stigmasterol indicates that a concentration of 50 mg/l is above the optimum level for that sterol.

Treatment	Number	Mean Weight (mg)	Std Err
Control	44	261	17.4
Stigmasterol 20 mg/l	54	352 *	21.6
Stigmasterol 50 mg/l	47	310	18.1
Cholcalciferol 20 mg/l	48	328 *	22.8
Cholesterol 20 mg/l	55	358 *	26.4
Stig'rol Acetate 20 mg/l	55	305	16.2

* Significantly different at 5% level

Table 4.6g

Effect of sterols on growth of proliferation cultures

The lower growth from the stigmasterol acetate treatment may offer clues about the structural requirements for stimulation of growth. The 3β hydroxyl group is the site of acetate bonding. This indicates that most, if not all, of the growth stimulation is prevented by bonding of the hydroxyl group on the A ring. This could be checked by the use of sterols with different hydroxyl positioning or with additional hydroxyl groups. According to Grunwald (1975), the 3

hydroxyl group must be free for phytosterols to have an effect on membranes and this allows for free interaction with membrane phospholipids. An interaction based on ion-dipole and hydrogen bonding was proposed to explain this. However, this review also claims that an additional structural requirement for interaction with phospholipids is that the molecule have a flat configuration. Based on this, Grunwald concludes that cholesterol would be the most suited and that stigmasterol would be least suited to fill this role in plasma membranes. Such an explanation does not fit well with the results of this experiment, where stigmasterol appears to be nearly as effective as cholesterol in the promotion of growth.

This hypothesis contrasts with results obtained from using paclobutrazol to inhibit *in vitro* sterol synthesis in celery suspension cultures. In this, the authors (Goad *et al*, 1988) established that sterols fulfil two roles within the plant. One is a requirement for a bulk sterol, assumed to be as a membrane constituent. The other is a requirement for a trace amount of a "trigger" sterol which is apparently needed to initiate some metabolic process essential to cell proliferation. The bulk role is best filled by any 3β-Hydroxy-Δ^5-sterol but the side chain is not important. In the trigger role, however, the requirement is best met by a sterol with a 24α ethyl group on the side chain (Goad *et al*, 1988; Haughan *et al*, 1989; Haughan *et al*, 1988).

Phytosterol synthesis is reported to be affected by light intensity, light quality and photoperiod (Grunwald, 1975; Harwood and Russell, 1984). It is possible that the growth promotion observed in this experiment is due to decreased synthesis of the required sterols *in vitro*. This could be due to the inadequacy of the light intensity or light quality in the tissue culture growth room.

The effects of sterols on root initiation remain to be discovered. The failure of root initiation media was later traced to the use of ethanol to dissolve the sterols, prior to inclusion in the medium. A small trial consisting of 50 micro-cuttings per treatment, performed by staff of North Eucalypt Technologies, showed that the effect of including 1% ethanol in root initiation media was to reduce root initiation from 70% to zero. In the same trial, 1% methanol also had a substantial inhibitory effect, reducing root initiation to 24%. If the effects of sterols on root initiation are to be investigated *in vitro*, an alternative solvent will need to be selected. There were no ethanol free controls included in either media. Given the inhibitory effect on root initiation, the effects of ethanol on *E. nitens* proliferation cultures also needs to be clarified. The

effects of ethanol on root initiation were unexpected as ethanol is commonly recommended for the preparation of tissue culture media stock solutions (George, 1993; Hartmann *et al*, 1990). Ethanol was also used as the solvent for IBA in all cuttings treatments. It is presumed that the shorter exposure time of 48 hours is the reason for ethanol not inhibiting rooting inthose situations.

There are a few instances where ethanol has been shown to inhibit growth and root initiation of *in vitro* cultures (de Klerk, ter Brugge and Marinova, 1996; Dieleman, Pierik and Kuiper, 1995; Perata, Alpi and Lo Schiavo, 1986; Perata, Lo Schiavo and Alpi, 1988). Ethanol has been also recorded as an inhibitor of root initiation in cuttings and seedling growth (Alpi, Perata and Beevers, 1985; Bhattacharya, Bhattacharya and Bhatnagar, 1985; Middleton, Jarvis and Booth, 1978), although it was not included by Wilson and van Staden (1990) in their collation of compounds known to affect root initiation. It is believed that the toxic effects of ethanol relate to its conversion within cells to acetaldehyde, which is believed to bind to proteins and inactivate enzymes (Perata and Alpi, 1991). Ethanol has also been demonstrated to have an effect on cell membrane permeability by affecting the ordering of membrane constituents (Rols *et al*, 1990): (Grunwald, 1968).

4.6.5e Conclusions

This experiment confirms that sterols boost growth in *E. nitens* in addition to having an effect on root initiation. It does not offer any particular clues as to what role phytosterols may be fulfilling in the physiological processes controlling root initiation. It cannot be determined, from these results, whether the growth promotive effect is due to alterations to plasma membrane properties, action as an auxin synergist or something different altogether. There is, of course, no reason why the applied sterols might not be having a slight stimulatory effect on more than one cellular function.

4.6.6 Extraction and measurement of ATPase activity

4.6.6a Rationale

If stigmasterol, ergocalciferol and cholecalciferol are all able to influence root initiation in cuttings, some consideration should be given to possible modes of action of these compounds. Such a mode of action should be consistent with the model of polarity induction leading to organogenesis.

One role for sterols that has been suggested fits in well with what is known about auxin action and the induction of polarity. Sterols are believed to play an important role in modulation of plasma membrane ATPase activity (Cooke *et al*, 1994; Cooke and Burden, 1990; Cooke *et al*, 1988). ATPases, particularly H^+ ATPases, play an important part in auxin reception and in initiation of ion currents, as outlined in Section 2.7.7.

ATPases are claimed to be affected by sterols in two ways

a. Bulk membrane effects, which are probably a reflection of altered membrane fluidity; and
b. Annulus effects, sometimes referred to as sparking effects, where low concentrations of specific sterols are required for activation of ATPases in conjunction with specific phospholipids.

It was proposed to undertake a simple extraction of plasma membranes from tissues at the base of cuttings. Measurement of ATPase activity in those membranes could then be used to determine if the addition of sterols to auxin treatment solutions resulted in an increase in ATPase activity.

4.6.6b Procedures

The experiment consisted of treating callused cuttings with either IBA or an IBA stigmasterol mixture for 2 days. Samples were then collected from the treated stem material, the plasma membranes were extracted and purified, and the ATPase activity of the samples measured. The cuttings to be treated were node 4 cuttings that were allowed to form callus on the mist-bed for four weeks. They were then treated for 48 hours with either a 40 mg/l solution of IBA or or a solution of IBA, 40 mg/l plus stigmasterol, 50 mg/l. The experiment was replicated two weeks later using similar cuttings and identical procedures.

In both experiments, treatments were applied in 50 ml tubes using the method outlined in the General Methods section. Treatment solutions were prepared by dissolving the IBA and the stigmasterol in small volumes of ethanol, which were mixed with distilled water. The final concentration of ethanol was 1%. Control treatments consisted of distilled water containing the same concentration of ethanol. Tween 20 was added to all treatments at the rate of two drops per litre.

At the conclusion of the 48 hour treatment, some of the cuttings were harvested for their callus and lower stem and the remainder were returned to the mist-bed. Further samples were harvested five days and seven days later. Thus samples were collected 0, 5 and 7 days after the treatments were completed.

Membrane extraction procedures, enzyme assays and protein determinations were performed as described in the General Methods section.

4.6.6c ATPase assay: data and interpretation

ATPase activity was measured as being present in all samples. However, none of the assays showed significant inhibition by the presence of 1 mM vanadate. This made it impossible to determine what proportion of the ATPase activity was due to plasma membrane H^+ ATPase and what proportion was due to other ATPases and phosphatases.

The enzyme assays were re-run using samples that had been frozen by immersion in liquid nitrogen and then stored at -80°C. The assay procedure was modified by substituting 0.2mM diethylstilbestrol (DES) for vanadate, as an inhibitor of plasma membrane ATPase. DES is a more appropriate inhibitor of plasma membrane ATPases, according to Serrano (1988). The samples that had been frozen showed no significant ATPase activity. It was concluded that freezing the samples had resulted in the loss of all ATPase activity.

ATPase activity has been measured from the extracts but it is not possible to determine if that activity is due to plasma membrane ATPase or some other ATPase or non-specific phosphatase. The inclusion of molybdate in the assay media should have prevented the activity of acid -phosphatases.

The reason for the failure of vanadate to inhibit any of the ATPase preparations is not known. Difficulties in the preparation of vanadate solutions are discussed by Gordon (1991). However, this discussion is in relation to the preparation of vanadate for use as an inhibitor of a different phosphatase and it is not clear whether the same difficulties apply to ATPases. Nor is it clear whether these would be sufficient to completely prevent the vanadate from acting as an inhibitor. It is also possible that impurities in the membrane extract could have interfered with the vanadate inhibition.

An alternative explanation is that the wrong fraction of membranes was collected from the sucrose density gradient. This could only be checked by repeating the extraction and using density beads to check the position of the 34% 45% interface or by specific staining of the plasma membranes as described by Larsson, Widell and Kjellbom, (1987).

4.6.6d Protein determination: data and interpretation

Only the results from the re-assay of protein levels using frozen samples are presented. Assays using freshly prepared extracts gave similar results, however they were less accurate, due to some of the absorbances recorded being outside the linear range of the standard curves prepared for those assays.

The protein content of the extracts varied considerably, in both replicates of the experiment but showed similar trends, see Figures 4.5i and 4.5j. In the controls, the protein content of the extracts stayed roughly constant over the three measurement dates. In the auxin treated cuttings, there was an increase in the protein levels over the sampling period. In the sterol plus auxin treatment, the protein levels also increased, probably more quickly than in the auxin treatment and probably to a higher level, although this could not be confirmed without more extensive sampling.

It is thought that the additional protein is most likely to be due to co-extraction of some other cellular protein, rather than representing some massive increase in the protein content of plasma membranes. It was apparent that the high protein extracts were a much darker colour than the extracts containing less protein. It was also noted that pure plasma membrane preparations should be white or off white (Kjellbom and Larsson, 1984). All of these preparations were coffee coloured or darker, indicating a low level of purification.

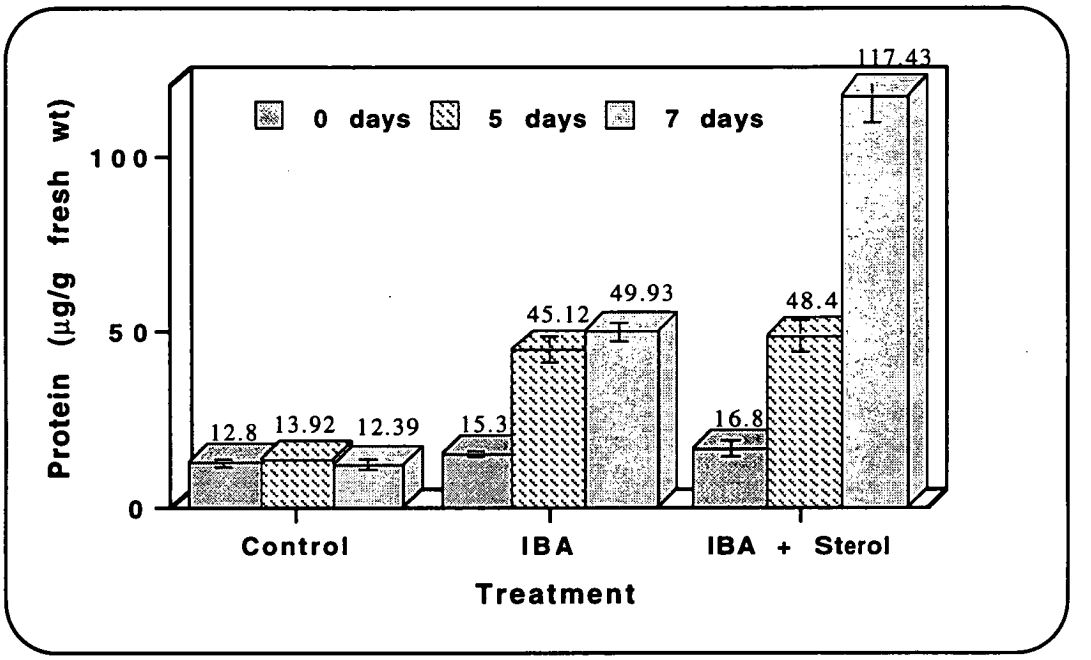


Figure 4.6h

Protein concentration, measured by micro assay, first replicate.

Bars = S.E. of means.

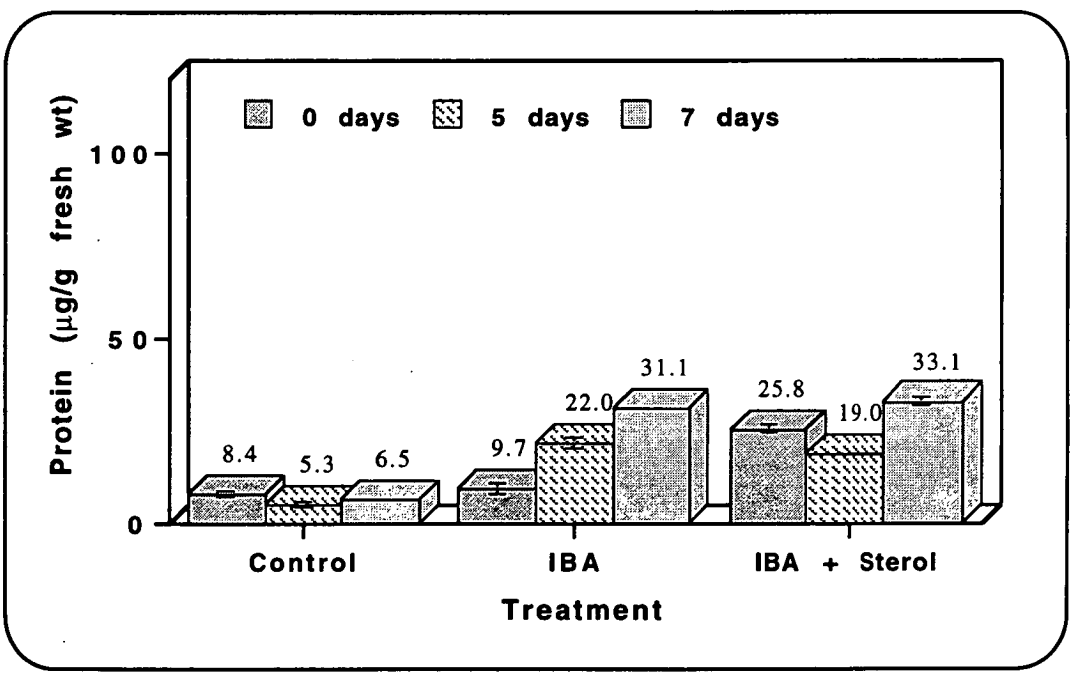


Figure 4.5i

Protein concentration, measured by micro assay, second replicate.

Bars = S.E. of means.

Substantial increases in the synthesis of soluble proteins has been noted as a feature of the early stages of root initiation (MacIsaac, Sawhney and Pohorecky, 1989). The production of such large quantities of protein within a few days of treatment makes it unlikely that the protein is directly associated with root initiation. At that stage, the proportion of cells in treated cuttings that would be directly involved in formation of root primordia would be small. It is difficult to see how such a small group of cells could effect such a substantial change in the protein content of the whole sample. It is more likely that the protein represents a general response of callus tissue to the presence of the auxin sterol combination.

4.6.6e Discussion

This experiment was intended to give some indication of ATPase levels in the presence and absence of stigmasterol. This was always likely to be difficult, especially as the technique had been adopted from use in citrus species without modification. There are many technical difficulties in obtaining pure plasma membrane extracts from samples that have never previously been extracted, so it is perhaps not surprising that the first attempt was not successful. Future attempts to extract plasma membranes from eucalypt callus should perhaps use the phase separation technique, which is less prone to contamination from other plant constituents (Larsson *et al*, 1987).

It is not possible to draw any conclusions on whether stigmasterol is stimulating ATPase activity because there has not been any positive identification of plasma membrane ATPase in the membrane preparations. This can really only be demonstrated by confirming their sensitivity to vanadate or to DES.

4.6.7 Summary of results and interpretation

There are three experiments where root initiation rates have been significantly stimulated by sterols or seco steroids (vitamin Ds) when applied with IBA. These have resulted in root initiation levels that are significantly above those obtained from the application of auxin without sterols.

That sterols are unable to promote root initiation without additional IBA points to one of two possible circumstances. Either there are insufficient endogenous

auxins in the cuttings to provide the synergistic effect or the sterols are only interacting with exogenous hormones, perhaps by enhancing absorption or by protecting them from degradation. The first explanation is more likely, as there are no reports that would indicate a role for sterols in auxin metabolism.

The limited success in treating plants with sterol synthesis inhibitors should not be taken as an indication that endogenous sterol synthesis plays no role in altering rooting ability in cuttings. Such a conclusion could only be drawn from more extensive testing of the effects of a range of triazoles.

The tissue culture experiment confirms that sterols are able to boost growth in *E. nitens*, in addition to having an effect on root initiation of cuttings. However, it does not offer any particular clues as to what role phytosterols may be fulfilling. The failure to obtain promotion of root initiation in tissue cultures is disappointing but can probably be easily rectified by repeating the experiment using a different solvent to dissolve the sterols.

The final experiment gave no indication of ATPase levels in the presence and absence of stigmasterol. It did, however, suggest that stigmasterol treatment may cause a substantial increase of some protein that co-extracts with the plasma membrane. The identity and role of that protein remains to be established.

4.6.7a Limitations of studies

All of the experiments on the effect of sterols and vitamin D on cuttings were carried out on three or four week old cuttings with extensive callus. It would be interesting to see if the sterols have the same effect on fresh cuttings, especially since other researchers have only found an effect when treatments are applied within the first 48 hours after the cuttings were collected (Buchala and Pythoud 1988).

Some caution needs to be exercised with the concentration of vitamin D being used in plant cells and applied to cuttings in these experiments which are much higher than those found in animal tissues. Studies with vitamin D_3 in chick cell nuclei demonstrated a maximal response at 26 pmol; in humans the plasma level is 10 -20 ng/ml (=2.5 x 10^{-8} M) (Miller and Norman, 1984). These concentrations are considerably lower than those being used in plants. This

may be partly due to the absence of active transport and absorption mechanisms that are present in animal cells. Rapid deactivation is unlikely in plant tissues. Buchala and Pythoud (1988) describe degradation of Vitamin D as mostly a function of microbial action rather than occurring within the plant.

4.6.8 Mode of action of sterol synergism

Although much has been done to record the effects of vitamin D and stigmasterol on root initiation and root extension in plants, there are, as yet, no definite indications of a mode of action. Other effects of sterols and steroids on plant growth and development have not been well studied and what few effects have been noted have proved difficult to reproduce (Grunwald, 1975). There are limited reports of positive effects from sterols on plant growth or proliferation *in vitro* (George, 1993).

It is possible that all or some of the effects observed for vitamin D, stigmasterol and cholesterol could be explained by reference to the role that sterols in general play in plant physiology. According to Hennessey (1992), the primary function of sterols in plants is their ability to affect membrane structure and water permeability. Sterols affect the packing of membrane bilayers. This increases the fluidity of bilayers which are below their phase transition temperature and decreases the fluidity of bilayers which are above their phase temperatures. The side chain of plant sterols is also important in determining the degree of ordering of the bilayer that occurs.

Paclobutrazol has been used to probe sterol metabolism in plants (Goad *et al*, 1988). When added to a celery cell suspension culture it caused accumulation of 14α sterols and loss of the normal sterols, campesterol, stigmasterol and sitosterol. Normal growth could be restored by adding sterols to the culture. It was established that the sterols were fulfilling two roles within the plant. One was a requirement for a bulk sterol, assumed to be as a membrane constituent. The other was a requirement for a trace amount of a "trigger" sterol, which appeared to be required to initiate some metabolic process essential to cell proliferation. The bulk role is best filled by any 3β-Hydroxy-Δ^5-sterol but the side chain was not important. In the trigger role, the requirement was best met by a sterol with a 24α ethyl group on the side chain such as stigmasterol (Goad *et al*, 1988; Haughan *et al*, 1989; Haughan *et al*, 1988).

The concept of a trace amount of sterol required for some trigger function fits in well with the proposals that sterols play a role in the modulation of ATPase activity. Results from the experiments in this thesis appear to fit the description of the bulk sterol role, particularly the results from the *in vitro* experiment, where bonding to the hydroxyl group reduced growth. Similar results have been obtained when white spruce seedlings were treated with triadimefon (Sailerova and Zwiazek , 1996),where substantial variations in water relations and plasma membrane ATPase activity were correlated with alteration in the sterol composition of the seedlings in white spruce (Picea glauca) seedlings.

It is believed that membrane lipids play a crucial role in determining the properties of the membrane proteins. Some sharp changes in enzyme activity with temperature change have been correlated with phase transition temperatures of lipids that are in close association with that enzyme. Modulation of ATPase activity by membrane factors has been studied extensively, particularly with reference to the the existence of a lipid annulus effect. This refers to the fact that some of the phospho-lipids and sterols within the plasma membrane will be in close contact with integral membrane proteins, such as ATPases (Harwood and Russell, 1984).

Phospholipids are said to bind strongly to the annulus and weakly to sterols (Cooke and Burden, 1990). Molecules bound in the annulus are far less mobile than those of the bulk membrane where there is a high degree of lateral movement of individual lipid molecules. Sterols are known to perturb phospholipid interactions and accumulate at the liquid/crystal gel interfaces, affecting the lipid domain size and stability (Stilwell, Cheng and Wassall, 1990). The rigid ring structure of the sterol provides the perturbation of the bilayer. This leads to the observation that sterols that vary only in the structure of the acyl tail should have the same effects on membrane properties.

The possibility that sterols play a role in modulation of membrane bound enzyme activity also provides a link between the role of sterols as auxin synergists and one of the known functions of auxin. Activation of ATPase is required to generate the ATPase driven transmembrane potential difference, described in Section 2.7.2, which is created by activation of auxin binding protein 1 (Barbier-Brygoo *et al*, 1992). This transmembrane potential difference may be significant in the establishment of cellular polarity.

4.6.9 Future directions

The results from the experiments in this section provide some preliminary indications of an alternative treatment to enhance root initiation. With further study, it may be possible to develop useful treatments and to obtain some additional information on the physiology of organogenesis and root initiation. One priority is to identify how the application of sterols have the effects that have been recorded in these and other experiments.

Continued attempts to demonstrate variation in ATPase activity in sterol treated tissues can be justified. Stimulation of plasma membrane ATPase activity is a mode of action that is supported by experimental evidence from other researchers and is in accordance with the model of root initiation that has been advanced in this thesis. Extraction of ATPases from treated cuttings may not be the most appropriate method of measurement. Extraction from treated callus cultures may give a higher yield of membranes and less contamination. There are also other extraction techniques which could be tested.

The ATPase extraction experiment appeared to demonstrate a substantial stimulation in the production of an unidentified protein that was co-extracted with the plasma membranes. Confirmation that the presence of sterols in root initiation treatments stimulates the production of some protein would be a significant step in identifying how auxins and sterols interact to stimulate growth and root initiation.

If sterols were demonstrated to be affecting ATPase activity it would be worthwhile investigating the effects of phospholipid manipulations on root initiation. It has been suggested that an early part of the auxin signal transduction mechanism may be via rapid changes in phospholipids within the cell to initiate a calcium mediated signal cascade (Ettlinger and Lehle, 1988). Adding phospholipids to partially purified membrane preparations has been shown to increase ATPase activity (Cocucci, 1986).

The interactions between sterols and other membrane constituents are complex (Demel and De Kruyff, 1976) and it is difficult to predict how addition of sterols will affect membrane fluidity without direct measurement. Sterol treatment may be found to significantly alter plasma membrane fluidity. Such alterations could be a significant factor in the promotion of root initiation by sterols. If so, consideration should be given to other treatments that might alter plasma

membrane fluidity. There may be other treatments which could be used to alter the lipid profile of the plasma membrane.

Regardless of how sterol synergism is achieved, these results have shown that sterols do have the potential to be used for the manipulation of rooting ability in cuttings. Further development will be required to turn these initial results into practical treatments for routine use.

It may be possible to design treatments incorporating sterols to directly enhance the root promoting activity of auxin applications. Although the sterols are not overly expensive, and are used at low concentration, vitamin D is highly toxic. Vitamin Ds and stigmasterol are light sensitive which may restrict their use. The growth promotion obtained from cholesterol *in vitro* suggests it may be possible to use use less costly and less purified forms of sterol to obtain a similar result. The method of application is a practical matter that would need to be resolved. So far, they have only been applied in dilute, 48 hour, dips. This is unlikely to be a viable method of application for large scale propagation.

Incorporation of sterols into tissue culture media holds distinct prospects for enhanced growth of proliferations. The effect of sterols on root initiation *in vitro* needs to be clarified. Given the positive effects it has on growth rates and on root initiation in cuttings it would be expected to cause a significant improvement in growth and rooting of cultures. The heat labile nature of some sterols and their low solubility in water may present some technical difficulties in preparation of media. However these difficulties can probably be overcome. There may be other sterols that are more suitable for use in tissue culture media that provide the same effect.

In vitro cultures may also provide a more suitable system for studying the effects of sterols on growth and root initiation of *E. nitens*. Microbial degradation was found to cause significant changes in the composition of sterol treatment solutions (Pythoud *et al*, 1986), making studies in a non-sterile environment open to misinterpretation. Studies into the structural requirements for growth promotion, using structural analogues of active sterols should be carried out *in vitro*.

4.6.10 Conclusions

Clear evidence has been provided of the ability of vitamins D_2 and D_3 and stigmasterol to promote root initiation. All three compounds act as synergists to auxin and, given the structural similarity between them, it seems very likely that they are acting in the same way. Growth promotion was also demonstrated by the inclusion of sterols in tissue culture media. This may also be due to the same effect, as there is exogenous auxin contained in the tissue culture medium.

The two most probable methods by which sterols could have such an effect on root initiation are by membrane fluidity or by stimulation of ATPase activity. Attempts to demonstrate the effect of sterols on ATPase activity were not successful. This failure to obtain any clear information on the status of ATPase activity in this experiment was disappointing. It was hoped to provide evidence of a mechanism by which sterols are able to stimulate root initiation, which in turn, could provide information on how root initiation is stimulated by auxin. Further attempts to measure ATPase activity in sterol treated tissues are needed to clarify their effects ATPase. Accurate and meaningful measurement of membrane fluidity may be more difficult to achieve.

4.7 THE EFFECT OF G COMPOUNDS ON ROOT INITIATION

4.7.1 Introduction

It has been proposed that G compounds are rooting inhibitors that may play a controlling role in adventitious rooting of *E. grandis* (Paton, Willing and Pryor, 1981). G compounds were applied to callused cuttings of *E. nitens* at a range of concentrations to measure any promotion or inhibition of rooting. The treatments were applied in the presence and absence of IBA, to identify any possible synergism with auxin.

4.7.2 G compounds applied without additional auxin

4.7.2a Rationale

G compounds have been shown to be rooting inhibitors but have also been claimed to be promoters of root initiation and have been proposed as a substitute for auxin (Dhawan *et al*, 1979).

A small experiment was undertaken to study the effects of several levels of G compounds on root initiation. The experiment was undertaken using well callused cuttings with a reasonable level of rooting potential, as root initiation in *E. nitens* has been demonstrated to be more readily stimulated after callus has formed. The G compounds were obtained from extractions, as detailed in the General Methods section.

4.7.2b Procedures

Treatment solutions containing G compounds were prepared in distilled water, using concentrations ranging from 10^{-4} M to 10^{-8} M. Distilled water was used as the control. To prepare treatment solutions it was assumed that the molecular weights of the mixed compounds was approximately 280 and that the sample was 90% pure. The crude extract was dissolved in ethanol. All treatment solutions had a final ethanol concentration of 1%. Tween 20 was included in the treatment solution, 4 drops per litre, to maintain the extract in solution.

Treatments were applied as basal dips, using the technique described in the General Methods section, except that 12 ml of treatment solution was used per vial, resulting in a treatment depth of approximately 20 mm.

The cuttings used for this experiment were node 2 cuttings, which have a higher rooting potential. These were selected as it was expected that there would be some inhibition of root initiation, as well as promotion. Treatments were applied three weeks after cuttings were collected to allow for callus formation. The treatments were applied to the cuttings, in the dark, for 48 hours. Root initiation was assessed after three weeks.

4.7.2c Data and interpretation

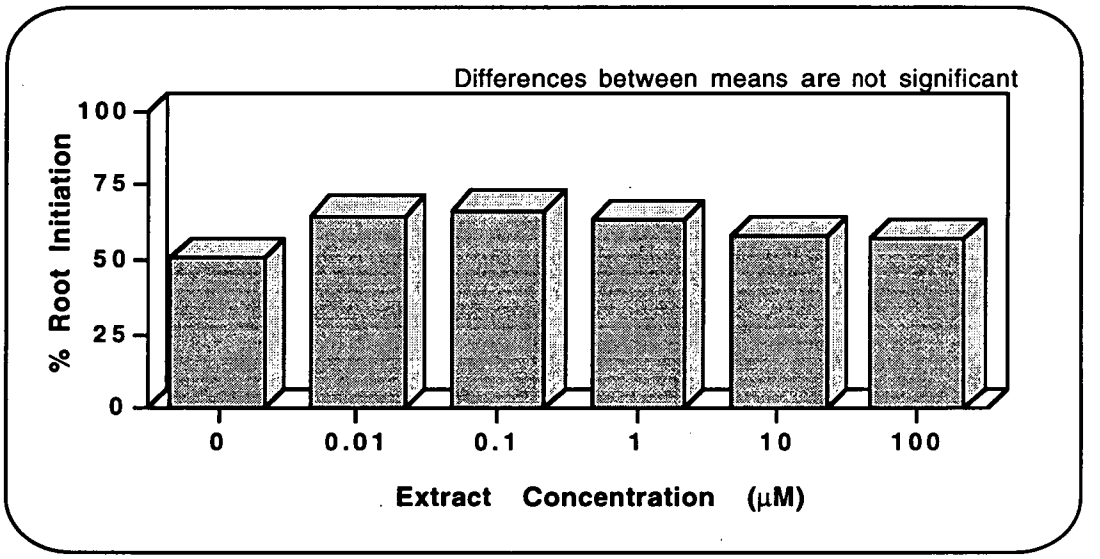


Figure 4.7a

Effect of G compound extracts on root initiation applied as a basal dip to three week old cuttings

None of the treatment means are significantly different from that of the control. The highest concentration of G compounds used in this experiment is one that has been found to be highly inhibitory to root initiation in other species of *Eucalyptus* (Paton *et al*, 1970). There is no evidence of any negative effects on root initiation in this test system, see Figure 4.7a.

There was no significant difference in the mean number of roots per cutting or in the mean callus rating for any of the treatments, see Table 4.7b. It is possible that the treatment regime used for this experiment did not supply enough G compounds for the effects to be noticed. This seems unlikely, given reports that higher levels of G compounds cause physical damage to stems (Menary, 1992). The callus at the base of the cuttings would be expected to be more delicate than whole stems, as it lacks a cuticle.

Extract concentration (μM)	100	10	1	0.1	0.01	0
% Root Initiation	57.1	57.9	63.6	66.7	64.9	50.9
Roots/Cutting	1.91	1.67	2.00	1.82	1.89	1.65
Callus Rating	2.95	2.93	3.18	2.75	3.14	3.05

No significant differences at 5% level

Table 4.7b

Effects of application of G compounds applied as a basal dip to three week old cuttings.

4.7.2d Discussion

There are three possible explanations for the failure of the treatment to affect root initiation significantly. There may have been insufficient G compounds absorbed, the duration of the treatments may not have been long enough or G compounds may not have any substantial effects on root initiation of *E. nitens* cuttings.

4.7.3 G compounds applied with IBA

4.7.3a Rationale

It has been claimed that G compounds are able to act as a synergist of auxin in the promotion of root initiation in mung beans (Menary, 1992). This supposed auxin synergism has not been tested in *E. nitens* cuttings. An experiment was undertaken, similar to the previous one, except that 40 mg/l IBA was included in the treatments. If G compounds act as an auxin synergist, at least one level of G + IBA should produce greater root initiation than IBA on its own.

4.7.3b Procedures

Treatment solutions containing G compounds were prepared as described in Section 4.7.2b. All treatments contained 40 mg/l IBA and had a final ethanol concentration of 1%. Tween 20 was included in the treatment solution, 4 drops per litre, to maintain the G compound extract in solution.

Treatments were applied as described in the General Methods section except that 12 ml of treatment solution was used per vial, resulting in a treatment depth of approximately 20 mm.

The cuttings used for this experiment were node 4 cuttings, treatments were applied four weeks after cuttings were collected to allow for callus formation. The treatments were applied to the cuttings, in the dark, for 48 hours. Root initiation was assessed after three weeks.

4.7.3c Data and interpretation

For root initiation, none of the treatment means are different from the mean of the control (see Figure 4.7c). However, there are significant differences between the treatment levels. The χ^2 test for a comparison between 0.01 µM and 10 µM means is significant at the 5% level. This may indicate a degree of inhibition of root initiation at high concentration and promotion at low concentrations. If this is a real treatment effect, then it is surprising that the 100 µM treatment, which is ten times as concentrated, had no significant effect. The concentrations of G compounds which appear to be causing promotion of root initiation are very low and are well below concentrations found to be promotory by other researchers.

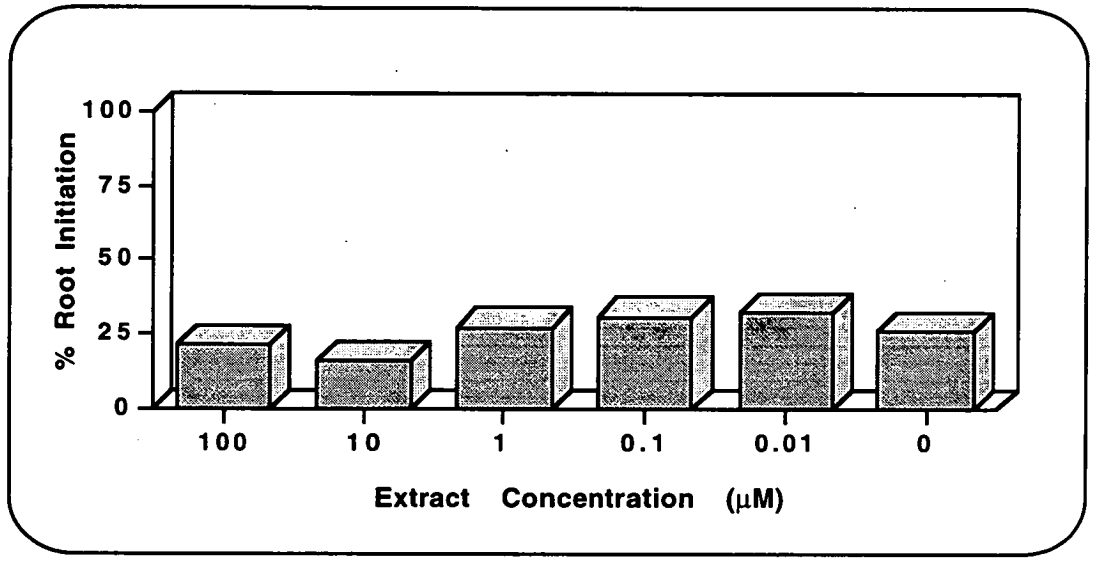


Figure 4.7c

Effects of application of G compound extracts on root initiation, in the presence of 40 mg/l IBA.

An analysis of variance for the mean number of roots per cutting and for mean callus rating show that the differences between means are not significant, see Table 4.7d.

Extract concentration (μM)	100	10	1	0.1	0.01	0
% Root Initiation	21.2	16.1	27.2	30.8	32.1	25.8
Roots/Cutting	2.14	2.60	2.50	2.05	2.76	1.94
Callus Rating	3.12	3.43	2.89	2.83	3.14	2.95

No significant differences at 5% level

Table 4.7d

Effects of application of G compounds applied as a basal dip to three week old cuttings in the presence of 40 mg/l IBA.

4.7.3d Discussion

There is some evidence for inhibition of root initiation at high concentrations of G compounds but there is no evidence that they are able to promote root initiation to a significant extent.

4.7.4 Conclusions

Application of G compounds did not increase the root initiation percentage significantly in either the presence or absence of IBA. Nor did it have any significant effect on the number of roots per cutting or on the callus rating of the cuttings. The concentrations used in these experiments are within the range used by other researchers to demonstrate both inhibition and promotion of root initiation (Dhawan *et al*, 1979; Menary, 1992; Paton *et al*, 1970).

The test system used in these experiments is one where the cuttings are only exposed to the agent for 48 hours, under the assumption that this is sufficient time for it to be absorbed into the cutting. This may not be a valid assumption, as there are no published reports that indicate how rapidly G compounds are absorbed by cuttings. Other researchers have assumed that G compounds are readily absorbed and translocated within plant tissues (Dhawan *et al*, 1979).

It was also assumed that the G compounds would be most effective if applied at the time when auxin applications are most effective on *E. nitens* cuttings. This is a valid assumption if G compounds act as an auxin synergist but may be less valid if their mode of action is independent of auxin. The results of these experiments do not clarify the mode of action of G compounds.

It is possible that the compounds may not have stayed at physiologically active concentrations for the length of time required for G compounds to exert their effects on root initiation. Although it is known that 48 hours is sufficient time for root initiation to occur, and is clearly long enough for IBA to cause stimulation of root initiation, there is no evidence that other root promoting substances work as quickly. Once removed from an exogenous source of the compounds, the endogenous levels may quickly fall below the threshold level needed to stimulate root formation.

Many of the bio-assay results obtained for G compounds could be discounted as being a result of root promotion by the imposition of sub-lethal stress or injury on the test plant, as proposed by Wilson and Van Staden (1990). However, even if all of these result are set aside, there remains a body of results which still point to G compounds being highly prevalent in tissues of *E. grandis* and having a variety of apparently significant physiological effects in that species.

However this evidence does not constitute sufficient grounds for supposing that G compounds are functioning as growth regulators, as suggested by Dhawan *et al.* (1979) nor that they are a useful adjunct in root initiation treatments in other species of *Eucalyptus*.

A variety of other researchers have studied the use of G compounds for promotion of root initiation in eucalypts (Baltas *et al*, 1992; Harrison *et al*, 1993; Menary, 1992), see also Wilson and van Staden (1990). None have published results indicating that the compounds are able to alter root initiation levels in eucalypt cuttings or tissue cultures to the extent that would make them a useful treatment. In addition, none have published any data that would indicate the mode of action by which the compounds might exert the effects that are claimed for them. At this stage, a compilation of all the data, particularly that which has not been published, on the effects of G compound applications on eucalypt cuttings would be useful. Access to all of this data may clarify whether further study of the phenomenon is likely to be of practical benefit.

5. GENERAL DISCUSSION

5.1 INTRODUCTION

In this thesis, each section of the results chapter concludes with a discussion of the results contained in that section. In this chapter, it is intended to combine and discuss the most significant results in the context of the main hypothesis that has influenced most of the research. The discussion will include some of the limitations of the results obtained so far and possible directions for future research to provide additional data to support the hypothesis. The practical implications of the hypothesis are considered and a number of proposals are made for practical treatments to enhance root initiation in *E. nitens* and in cuttings generally.

5.2 SUCCESSFUL ROOT INITIATION DEPENDS ON INDUCTION OF POLARITY

The central hypothesis towards which much of the research in this thesis has been directed is that root initiation depends on the establishment of a new axis of cellular polarity. New meristem formation, leading to root development, can only occur after a new axis of cellular polarity has been initiated within the cells or tissues. Thus, those factors which cause or control the development of cellular polarity are likely to be the most important factors in controlling root initiation.

In order to be able to optimise promotion of root initiation in cuttings, it is necessary to have a detailed understanding of how such a new axis of polarity develops. This includes an understanding of the establishment of a new polarity signal, how that polarity signal is perceived by cells or tissues and responses of the cells to the signal. This results in re-orientation of cellular constituents and co-ordination of cell development, cell division and extension.

There is good evidence that auxin flows and ion flows resulting in electric potential differences are the main method by which polarity is signalled within

and between cells. Thus, the factors which influence auxin flow or ion current establishment are likely to be important for re-polarisation of tissues and are therefore important in root initiation.

Warren Wilson and Warren Wilson (1993) have proposed that the ionic currents are a consequence of the development of polar auxin transport within tissues, developing initially by diffusion from source to sink and later strengthened by active transport of auxin. Sachs (1991), on the other hand, proposes that the development of polar auxin transport within tissues may be a later manifestation of polarity that is initially expressed by electric currents. This appears more consistent with the research on the occurrence of ionic currents in single cells and in the earliest stages of somatic embryos (Brawley *et al*, 1984; Gorst *et al*, 1987; Nuccitelli, 1986; Rathore *et al*, 1988). However, even if ionic currents are able to direct the establishment of cellular polarity and auxin flow, it is clear that auxin flows are of primary importance in the re-orientation of cellular polarity. Auxin must therefore be able to bring about re-orientation of cellular constituents, especially microtubules.

Re-polarisation implies a degree of plasticity within the cells, where it occurs there must be a re-ordering or re-orientation of cells. Since the cells are not able to physically re-orient, there must be a movement or migration of cellular components. It appears most likely that both auxin and electric currents have their major effects on the plasma membrane, where the most active receptors are situated. This is particularly true of electric fields, which are unable to penetrate the plasma membrane and are only able to exert an effect parallel to it. Auxin may be able to interact with internal receptors, on the nucleus or elsewhere, but there is no evidence presented so far, that such receptors are involved in the re-orientation of polarity or in root initiation. This implies that the physiological plasticity may be dependent on or controlled by the ease with which receptors are able to re-order or re-orient within the plasma membrane.

5.3 EXPERIMENTAL RESULTS, SUPPORT FOR THE GENERAL HYPOTHESIS

The results from all of the experiments in this thesis are consistent with the hypothesis that has been advanced. In some cases, the results provide new evidence to support the proposal that induction of polarity is a crucial step in root initiation.

5.3.1 Electrical polarity

The results obtained from the application of low voltage potential differences to cuttings are direct evidence for the importance of cellular currents in the establishment or re-orientation of cellular polarity which lead to root initiation. Although electrical potential differences have been used to enhance root initiation from callus in the past, this is the first time that the application of such a potential difference has been used to promote root initiation from organised tissues.

At present, there is no evidence as to which cells within the cutting are affected by the potential difference. There has been no attempt, yet, to determine where the root initials form under these conditions. The evidence from the studies in this thesis indicates that root initiation normally occurs either directly from the region of the vascular cambium or from callus that is well separated from the original cutting tissue. The callus also appears to arise from the vascular cambium or cells in close association with it. This leads to the conclusion that, in the case of the application of electric potentials to fresh cuttings, the potential difference is most likely to have its effect on cells in the vascular cambium.

The vascular cambium is enclosed by the cortex of the cutting and most of the cambial cells will be isolated from direct effects of currents. It is not clear how this arrangement of tissues would affect the perception of imposed electric fields within the tissues. This will be determined by the electrical properties of the cortical tissues. Not all of the cambium is isolated from direct interaction with the current. The vascular cambium was found to be disrupted for some distance from the base by the process of collecting the cuttings. The effect of this is to damage cells and disrupt connections between cells. It also provides a

continuous connection between the bathing medium and vascular tissues for some distance up from the base of the cutting. There is also a small region at the base of the cutting that is directly exposed to the tank solution and current. The effect that this disruption would have on the electrical properties of tissues within the cutting is difficult to determine.

The fact that the four week old callus on the base of cuttings did not respond to potential differences as expected does not necessarily mean that those cells are less able to repolarise in the presence of a potential difference. It may be that the potential difference and duration of treatment selected for these experiments were not optimal for callus cells at that stage of development. The extent of callus development and the distribution of actively growing cells is likely to change as a basal callus mass develops. This could result in altered cell membrane characteristics, which alter the cell's response to an applied potential difference.

In discussion of the role of ionic current on re-polarisation of tissues, it is assumed that the ionic currents are the initial polarity signal which directs early auxin flows leading to the establishment of positive feed-back and concentration of auxin flows (Sachs, 1991; Warren Wilson and Warren Wilson, 1993). If ionic currents do represent the early stages of induction of polarity, then the late stage callus may already have a strong degree of polarity signalling which prevents weaker imposed fields from having an effect. However if this is the case, it is difficult to explain why similar treatments have an effect on fresh cuttings, where the tissues have an even greater degree of determined polarity.

The interactions between applied electric fields and callused cuttings is likely to be different to that in fresh cuttings due to different electrical properties of the callus and stem tissue. Firstly, the callused cuttings either lack a cuticle in the lower regions or it is disrupted. This would result in removal of any effects that the cuticle may have on current flow. Secondly, the underlying callus is loosely packed with many intercellular spaces which may allow for the flow of current through the tissue, rather than just around it. Thirdly, the callus cells are likely to be in active growth and not under the influence of spatial and hormonal restrictions as are stem tissues in fresh cuttings. Any of these factors could explain the variation in response to similar treatments that were observed between fresh and callused cuttings.

The interpretation of the various results from application of potential differences to cuttings is complicated because the actual mechanism of re-polarisation is still obscure. The role of auxin in such imposed re-polarisation has not been determined. It has been proposed that re-polarisation occurs due to re-alignment of microtubules by movement of microtubule linking proteins embedded in the plasma membrane (Hush and Overall, 1991; Hush and Overall, 1992). However, it has not been established that such linking proteins are capable of directing microtubule orientation or if they move in response to changes in the microtubules (Giddings and Staehelin, 1991). If microtubule orientation is directed by the positioning of the linking proteins, there remains the problem of determining how auxin and electrical signals direct the positioning of the linking proteins.

It is possible that imposed potential differences cause the re-orientation of other membrane components such as ion channels, ATPases or auxin binding proteins. In this case, the imposition of the field could be seen as merely re-orienting those parts of the plasma membrane that are responsible for the maintenance of ionic currents and that microtubule re-alignment occurs as a consequence of this.

5.3.2 Sterols and the induction of polarity

The results obtained from the application of vitamin D and stigmasterol to cuttings can be interpreted as supporting a model of root initiation based on induction of polarity. Exactly how the sterols affect polarity depends on the mode of action. It is most likely that they exert their effect by alteration of ATPase activity or by modification of the composition of the plasma membrane. There is substantial evidence that sterols can modify the activity of ATPases (Cooke *et al,* 1994) but further evidence is required to demonstrate that this is the way in which sterols promote root initiation.

Alteration of ATPase activity offers a direct link to induction of polarity within tissues. Increased ATPase activity might increase the rate of polar auxin transport in line with the chemi-osmotic theory of auxin transport (Rayle and Cleland, 1992). Alternatively, sterols could alter the activity of ATPases that are stimulated by auxin and increase ionic currents of the type described by Barbier-Brygoo *et al,* (1996). These ion currents and membrane hyperpolarisation have, so far, only been observed in protoplasts and isolated

membranes. There are also discrepancies, which need clarification, between the auxin responses measured in these protoplasts and those measured in whole tissues (Hertel, 1995). Nevertheless, the similarities between these auxin responses and those required for induction of polar ion currents are substantial. The fact that ABP1 has been shown to cluster on the plasma membrane in response to auxin treatment (Diekmann *et al*, 1995) is also consistent with ABP1 having a role in the establishment of polarity.

Cholesterol has been shown to affect the activity of many other membrane bound enzymes, such as Ca^{2+} ATPases, and has an effect on Ca dependent K^+ currents in animal tissues. It is expected that phytosterols, such as stigmasterol, may have similar effects in plant cells (Hennessey, 1992) but this has not yet been demonstrated. It is possible that sterols may exert their effect by modification of bulk membrane properties. Alteration of plasma membrane fluidity has been cited as one effect from incorporation of additional sterols (Hartmann and Pierre, 1987). Increasing the proportion of sterols in a phospholipid membrane generally results in increased rigidity of the membrane (Thompson, 1984).

The way in which changes to membrane fluidity might affect the induction of cellular polarity and organogenesis is not clear. Re-polarisation by applied electric potential differences is claimed to be independent of membrane fluidity. The effect of membrane fluidity on the electrophoretic polarisation of cell membrane components have been calculated and demonstrate that membrane fluidity should have little effect on the extent of polarisation that occurs (Jaffe, 1977). This is because changes in the drag co-efficient of the membrane affect electrophoretic mobility and the rate of back diffusion equally. Thus, altering the membrane fluidity should not alter the effect of applied potential differences. These calculations may also apply to the self electrophoresis that is assumed to occur in the lead up to the establishment of polar ionic currents. However, these calculations only take into account the effect of membrane fluidity on viscosity of the membrane. Changes in fluidity have a number of other effects, such as increasing the vertical displacement of proteins within the membrane (Shinitzky, 1984). This may render the calculations less accurate.

The mobility of membrane components is not the only way by which alteration of sterol content of membranes alters the properties of the plasma membrane. Other effects recorded include changes in the permeability of the membranes to anions and changes to protein functions (Stilwell *et al*, 1990; Thompson, 1984). These effects could change the way polar currents are established or re-oriented by altering the ease with which ionic currents are maintained.

5.3.3 Morphology and the induction of polarity

All cuttings appear to form substantial amounts of basal callus without requiring the application of additional auxin. This indicates that there are tissues within the cutting that are able to undergo depolarisation and cell division. Hence, the loss of rooting ability with age in *E. nitens* is not associated with loss of ability to dedifferentiate.

The callus cells that form as a result of this dedifferentiation are also clearly influenced by a polarity signal and are able to respond to that signal. This is demonstrated by the large numbers of cells that differentiate into vascular tissues within the callus. These tissues have moved from being undifferentiated, randomly oriented cells to become polar, co-ordinated tissues. This indicates that the reason for root initiation failing to occur in basal callus is not that the cells are unable to respond to a polarity signal. It may be that the signals, in the form of auxin flows, are not of the required intensity, clarity or duration to allow root primordia to generate within the callus. The association between root primordia and vascular strands is claimed by Warren Wilson and Warren Wilson (1991) to be due to exactly this. It is their view that formation of root primordia require the auxin flows to be concentrated by channelling through vascular tissues.

In this research, the morphology of root initials found in callus is consistent with the view of Warren Wilson and Warren Wilson (1991). All root primordia were found to have a small strand of differentiating vascular tissues associated with them. Root primordia that formed in the absence of extensive callus, direct from the vascular cambium, did not fit this pattern. These roots did not show signs of vascularisation until well after the root had begun to extend. However, the formation of strands of vascular tissues is clearly not all that is required. Vascularisation of basal callus occurred extensively in callus from cuttings with a high rooting ability and in cuttings with a low rooting ability.

Several similar strands of xylem could be found in the one cutting but at most, only one or two strands had root primordia associated with them. Clearly there must be other factors which also affect the rate at which root primordia form in callus.

The intensity of the auxin signal that is perceived by the callus tissues is clearly one factor that is important. Hence, when additional auxin is applied to callused cuttings, root initiation is promoted. However, promotion of root initiation reached a maximum beyond which application of additional IBA had a negative effect. This maximum root initiation rate was well short of 100 % of cuttings. In other words, increasing the intensity of the auxin signal improved root initiation but not in all cuttings.

With regard to the duration of the auxin signal required, lateral root induction in *E. globulus* seedlings has been shown to take less than 24 hours *in vitro* (Pelosi *et al*, 1995). However there is little information to indicate how long cells require a polarity signal to be in place to cause root initiation from cuttings or from callus. It is unlikely to be less than that for lateral root primordia. IBA was applied to callused cuttings for a maximum of 48 hours in the treatments used in this thesis. Enhanced levels of IBA would be expected to persist for some time after treatments were completed. Further increases to the duration of the auxin signal may increase root initiation but this has yet not been tested.

The concept of the auxin signal requiring some measure of clarity is one that needs explanation. In fact, there is little direct evidence that any spatial or temporal restrictions being a requirement for organogenesis. However, it is difficult to envisage how a hypothetical generalised auxin flow through a large volume of cells could result in a localised, self-contained region of repolarisation occurring, initially involving just a few cells. Thus it is likely that if auxin flows are responsible for initiating organogenesis there will be some requirement for the auxin flow to be spatially restricted, as is suggested by Warren Wilson and Warren Wilson (1993). Auxin flows within tissues that do not have this spatial restriction will result in quite different patterns of development.

5.3.4 Auxin applications

The results from the section on auxin applications are largely consistent with root initiation occurring as a result of polarity induction.

The results obtained regarding the timing of auxin applications could be explained in terms of auxin being most effective after callus has developed. This is when there are greater numbers of undifferentiated callus cells which are able to respond to a polarity signal. Application of auxin treatments at this time creates an auxin gradient within the undifferentiated tissues, resulting in the formation of a new meristem. However, such reasoning would also lead to the expectation that an electric potential difference, acting as an alternative polarity signal and applied at similar times, should also result in increased root initiation. This is the reverse of what was observed, where fresh cuttings without callus responded to a potential difference of approximately 3.3 volt/metre but four week old cuttings with extensive callus did not respond to those conditions unless some IBA was present. In other words, two root initiation treatments, thought to act as polarity signals, were applied to cuttings at the same stage of development and provided different responses from those cuttings. This suggests that the two treatments must be acting at different points in the root initiation signal response chain.

The results from experiments varying the concentration and duration of auxin treatment and the method of application of auxin are also consistent with the model of root initiation that has been proposed, but do not provide any additional evidence to support the proposal.

The experiments designed to vary the concentrations of calcium in the treatment medium were expected to provide further evidence of a role for ionic currents in auxin induced root initiation. It had been supposed that the absence of calcium in the surrounding medium might block the establishment of new ionic currents, as has been demonstrated for tobacco callus cells (Mina and Goldsworthy, 1992). Demonstration of such a role for calcium would provide further evidence for the model. The experiments undertaken to test this have provided inconclusive results. The design of the experiment needs some refinement to provide some more definitive evidence. This might be achieved by improving the efficiency of the calcium treatment, increasing its duration or perhaps by the use of calcium channel blockers.

5.3.5 Loss of rooting ability with seedling age

The rapid loss of rooting ability in growing seedlings cannot be explained directly from the results obtained in this thesis. However, it is possible to speculate on changes in physiological factors which might restrict the ability of cells to respond to auxin or other root induction treatments. Similarly, the effects of G compounds on root initiation, while not demonstrated in *E. nitens,* could be related to signal transduction mechanisms.

Evidence has been presented that both auxin polarity signals and electric potential differences have their effect at the plasma membrane. Therefore, any change in the plant's physiology that reduces rooting ability may also have an effect on the plasma membrane. The list of possible changes in membrane physiology that might reduce the ability of cells to respond to a root initiation or polarity stimulus is probably very large. However, four possibilities can be discussed with reference to this thesis. They are: alteration in the density or mobility of membrane bound auxin receptors; changes in the distribution or mobility of ion channels; changes in ATPase activity; or loss of membrane fluidity which might restrict the mobility of any of the three types of membrane protein. All of these changes could result in a cell becoming unable to respond to a defined polarity signal or might reduce the sensitivity of the signal transduction mechanism. Study of factors affecting plasma membrane physiology in plants has been largely restricted to changes that occur during senescence (Thompson, 1984) but in animal cells, a wide array of factors have been identified (Shinitzky, 1984; van der Meer, 1984). Indication of possible experimental approaches might be obtained by study of this research.

Although the use of G compounds in this thesis did not result in any measurable promotion or inhibition of rooting, it is possible to speculate on a role for G compounds that is consistent with the main hypothesis. Paton (1981) noted that ease of rooting in *Eucalyptus* cuttings is inversely correlated to frost resistance. He also observed that in *E. grandis* frost resistance is well correlated with G compound accumulation and claimed that there was evidence for a similar correlation in other *Eucalyptus* species. It was claimed that the mechanism by which G compounds enhance frost resistance is via alterations to membrane properties, although no data was presented to support this. However, changes in membrane properties, especially membrane fluidity, could account for changes in rooting ability by affecting any of the membrane associated functions previously mentioned.

One difficulty in identifying what alteration in membrane properties or other factors is actually responsible for altering rooting ability is that the change need not be reflected in all tissues in the cutting. The change may only occur in some crucial target tissues. It is possible, for example, to speculate that changes in the distribution of auxin binding proteins, specific ion channels or in the activity of ATPases might occur within a small group of cells or a particular cell type within a cutting.

At this stage, there is insufficient knowledge of the distribution of auxin binding proteins in cells of different tissues or their mobility in cell membranes to indicate whether such changes in auxin binding activity exist. Similarly, study of ion channels is also not sufficiently developed to provide information on the distribution of channels within tissues or cells.

However, ATPase activity and its sensitivity to auxin stimulation has been shown to vary substantially (Masson, Szponarski and Rossignol, 1996; Michelet and Boutry, 1995; Santoni, Vansuyt and Rossignol, 1993). There exist, in plants, a variable number of isoforms of ATPase which are coded for by different genes (Sussman, 1994). It has been established that some of these isoforms are localised in particular tissues, while others are found more generally (Ewing and Bennett, 1994; Michelet *et al*, 1995). It is believed that the association of isoforms of ATPase with particular cells or tissues may be linked to specialised functions within those tissues (Michelet and Boutry, 1995; Palmgren and Christensen, 1994).

It is possible to speculate that particular isoforms of ATPase may be required for auxin stimulation of cell repolarisation. The regulation of those genes would then provide one part of a mechanism by which variation in rooting ability within plants could be explained. Such speculation points to the need to obtain a clearer understanding of the link between auxin action, ATPase activity and root initiation. It is also necessary to demonstrate that root initiation or cell repolarisation is associated with one particular or several ATPase isoforms. So far neither of these requirements has been satisfied.

5.3.6 Seasonal variation in rooting ability

Seasonal variation in rooting ability of cuttings has been recorded for *E. nitens* and *E. globulus* (Carter and Slee, 1993; de Little *et al*, 1992). Such seasonal variation also appeared in the experiments of this thesis, although this was not specifically measured. Root initiation is lowest in early winter, when parent plant growth rate is slowest (de Little *et al*, 1992). Even though the seedlings were grown in heated glasshouses, the plants were exposed to reduced daylength and light intensity during winter. The variation in rooting ability appears to correlate with growth rate, perhaps due to lower carbohydrate reserves. However, the possibility cannot be excluded that reduced light intensity, photoperiod and temperature have a more direct effect on rooting ability in eucalypts, as has been speculated for other plants (Moe and Andersen, 1988). Since exogenous sterol applications have been demonstrated to affect rooting ability, it seems likely that endogenous sterol levels would be able to affect rooting ability of cuttings in a similar way. Sterol synthesis and the proportions of different sterols found in cells is known to be affected by environmental factors, particularly light intensity and quality (Grunwald, 1975). This raises the possibility that seasonal variation in light intensity and photoperiod could alter the proportion and concentration of different phytosterols in plants. This could in turn affect the ability to initiate roots. There is no data available on the sterol composition of eucalypt tissues.

5.4 THE ROLE OF AUXIN METABOLISM IN POLARITY INDUCTION

Some of the factors that are known or thought to be implicated in root initiation could be explained in terms of the induction of polarity signals within tissues and how such signals are controlled.

Although it is generally accepted that auxin is required for root initiation and that exogenous applications of auxin result in promotion of root initiation, there is still no generally accepted model of how auxin induces roots to form within tissues. Most recent reviews on the role of auxin in root initiation are primarily concerned with regulation of auxin levels, auxin conjugation and catabolism (Blakesley, 1994; Blakesley and Chaldecott, 1993; Blakesley *et al*, 1991; Gaspar and Hofinger., 1988; Haissig, 1986; Hartmann *et al*, 1990; Jarvis, 1986; Moncousin, 1991). These issues, while undoubtedly important, have not yet provided a detailed mechanism of how root initials are formed.

A view of auxin action where its primary role in root initiation is to induce a new axis of polarity is consistent with much that is known of the effects of auxin on cuttings. Such a role is also consistent with all that is known about auxin and its effects at a cellular level. The application of exogenous auxin can be seen as resulting in stronger auxin fluxes through tissues and providing a stronger, clearer polarity signal to those tissues. These stronger signals are able to overcome existing cellular polarity, resulting in increased meristem formation.

As has been mentioned previously, at least some of the effects attributed to the presence of rooting inhibitors could also be consistent with a model of root initiation where the induction of polarity is a key step. The ability of some flavonoids to affect auxin transport has been recorded (Faulkner and Rubery, 1992). The presence of physiologically significant quantities of these compounds in tissues could result in reduced rate of auxin transport and hence an altered polarity signal. It is not clear whether the concentration of these compounds found in the cytoplasm is sufficient to have this effect. This is distinct from the concentration of phenolics that are measured in the cutting as a whole. If the major concentrations of phenolics are contained within plastids, they still may not be able to have an effect on auxin transport under normal

conditions. The damage that occurs at the time of cutting collection and consequent release of phenolics may reduce the rate of auxin transport in the base of the cutting. That reduced auxin transport occurs in the few days immediately after severance, a critical stage of root induction for most species. The significant time gap between the collection of the cutting and optimum auxin induced root initiation in *E. nitens* makes it unlikely that phenolics released during wounding play a direct role in root initiation in this species.

The concept of auxin flows and currents being responsible for the initiation of root primordia also correlates well with the hypothesis of Beakbane (1969), that physical structures such as suberin bands and resin canals might in some way interfere with root induction. Physical restrictions such as those described by Beakbane could have an effect on the transmission of auxin flux signals through tissues. They could also affect the transmission of electric currents serving as polarity signals by restricting the direction or the strength of the ion flows. Physical restrictions also have the potential to restrict organogenesis by the imposition of a pressure gradient, which can act as a polarity signal on its own. Such a signal might over-ride other auxin induced signals.

5.5 PROPOSALS FOR FUTURE RESEARCH

Additional research is required to further clarify how the results that have been obtained so far conform with the hypothesised control mechanisms of root initiation. Most of the experiments in this thesis have been designed to demonstrated the potential of new techniques or approaches to the study of root initiation. The potential for some of these techniques has been clearly demonstrated, such as the the electric potential difference experiments and in the stigmasterol and auxin timing experiments. Other experiments have given less clear cut results, most notably those looking at the role of calcium in root initiation and those using apical applications of auxin. Many of the results obtained point to the need for further study. In some cases, clarification of the effects of treatments are needed.

With respect to the effect of application of electric potential differences to cuttings, there is a clear need for further research. The first objective should be to confirm the results of the experiments which have indicated that root initiation can be promoted by the application of potential differences. Following this, it would be desirable to determine the extent to which currents are effective, by determining the minimum and maximum currents and exposure times required to produce a measurable effect. Once this data has been obtained, some studies aimed at determining the mechanism of the effects should be tried. The effects of ion substitution and ion channel blockers could be used to provide information on how the imposed potential difference affects endogenous currents. Direct measurement of ion currents using ion selective electrodes may also prove useful in this regard, but only if the induced changes are found to occur in surface cell layers, where such measurements are possible. Morphological studies are needed to determine which tissues are responding to the effect of the currents. Further study of the role of auxin, particularly auxin transport, in cellular re-polarisation could prove useful. The use of auxins not subject to polar transport and transport inhibitors may clarify this point.

The use of sterols to promote root initiation, has been known for some years but has not been the subject of detailed study. Clarification of how sterols act as auxin synergists may provide further information on how auxins induce root initiation. Detailed measurement of ATPase activity in target tissues may provide the necessary data but it is possible that there may be functional specialisation, within tissues, of ATPase isoforms, which would make the

identification of one ATPase responsible for the effect difficult. If no effect on ATPase activity can be detected, then a role for sterols in membrane fluidity changes is most likely. Direct measurement of plasma membrane fluidity is likely to be difficult to interpret due to the non-heterogeneous nature of plasma membranes (Cruzeiro-Hansson, Ipsen and Mouritsen, 1989). A more productive approach might be to determine if ion channel activities or membrane permeability were affected by sterol application.

The positive effects from sterols included in tissue culture media points to a more convenient method to study structural requirements for sterols, particularly if an alternative solvent can be found that avoids the negative effects of ethanol. The inclusion of sterol synthesis inhibitors in tissue culture media may also be helpful in elucidation of the structural requirements of sterols.

The effect of the timing of sterol applications for maximum effect has not been studied in *E. nitens*. In other species, it was found that treatments were only effective if applied within a few days of the collection of the cuttings (Buchala and Pythoud, 1988). This is clearly not the case in *E. nitens*. It is possible that the sterols may prove to be more effective if applied at some other time, rather than applied with auxin at four weeks after collection of the cutting.

It has been assumed that the factors that affect root initiation in young seedling derived cuttings are the same as those that affect the rooting ability of cuttings from clonal stock plants. Almost all of the results in this thesis have been obtained from experiments using cuttings derived from young seedlings. This technique was adopted as being a method which allows for the reliable production of large numbers of uniform cuttings when needed. In particular, it was hoped to avoid complications arising from maintaining large numbers of stock plants under controlled conditions. It was also hoped that such a strategy might reduce the seasonal variation in the rate at which cuttings were produced and in rooting ability of those cuttings. By selecting a particular node number it was also possible to obtain the rate of root initiation required for a particular experiment. In general, this method of producing cuttings was successful and convenient. However, few of the treatments described in this thesis have been tested on cuttings from clonal stock plants. Such testing may be necessary to confirm that the results obtained with seedling derived cuttings are also applicable to cuttings derived from container grown stock plants.

5.6 PRACTICAL APPLICATIONS

Most of the experiments in this thesis have been designed to obtain basic data on the processes underlying root initiation. However, the results have provided some indications of treatments and strategies that may have some practical benefits in the propagation of *E. nitens* and other difficult to root species.

Varying the timing of auxin applications is a technique that has not been adopted previously for the propagation of cuttings. The removal of cuttings from a mist-bed for the application of low concentration auxin dips is probably an impractical step for large scale propagation of all but high value plants. However, the application of auxin by other methods is worthy of exploration. Auxin application by means of a soil drench or spray could have a similar effect, provided the concentration of auxin that is used results in sufficient auxin reaching the target tissues.

The use of sterols as auxin synergists would appear to have some potential. There are, however, some practical considerations which may limit its use. Vitamin D could not be recommended for use as a practical treatment for propagation of eucalypts, due to the toxicity of the compound, its low solubility and its low stability when exposed to heat or light. Stigmasterol is less toxic but has a similar solubility. Other sterols are more soluble in water but their effect on root promotion has not yet been determined. A treatment duration of 48 hour was used in this thesis, it maybe possible to obtain a positive effect using shorter treatments. Incorporation of sterols into in vitro propagation media is certainly worthy of consideration if the measured increase in growth is maintained during continuous use.

Little is known of the mobility of sterols in plant tissues. This makes the effect of their application by other means, such as sprays, difficult to predict. A more practical approach might be to identify growth conditions that maximise natural sterol production or modify it by means of application of growth regulators.

The application of electric potentials to cuttings has not been sufficiently evaluated to be advocated for direct practical application, although if shorter treatment times were found to be effective it might become a possibility. The use of such treatments on a larger scale would present problems in maintaining a uniform potential difference and in countering the effects of electrolysis and electrophoresis of components of the treatment solution.

5.7 CONCLUSIONS

The direction of research in this thesis and the experiments that were conducted were aimed at providing some alternative perspectives on the problem of promoting root initiation in difficult-to-root woody plants. Most of the experiments were intended to trial new methods of enhancing root initiation, in the hope of finding some treatments that demonstrate a potential to enhance root initiation or to shed some light on the control of root initiation.

The results of the experiments have fulfilled that expectation, in that they have demonstrated some alternative methods of promoting root initiation in cuttings. The results have also proved to be consistent with a view of root initiation where induction of cellular polarity is the crucial first step in root initiation. Such a view of root initiation may prove useful in devising further strategies to enhance root initiation in cuttings and in understanding how existing treatments have their effect.

It will also be possible to use these experimental results to develop treatments and strategies that have a direct application to the problem of obtaining large scale clonal propagation of *Eucalyptus nitens*.

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Data for Figure4.7d: Root initiation

Summary Statistics

DF:	5	
Total Chi-Square:	5.786	p=.3276

	Control	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	Totals:
-	49	52	52	48	45	36	282
+	17	14	10	18	20	17	96
Totals:	66	66	62	66	65	53	378

	Control	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	Totals:
-	74.24%	78.79%	83.87%	72.73%	69.23%	67.92%	74.6%
+	25.76%	21.21%	16.13%	27.27%	30.77%	32.08%	25.4%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.7d: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	8.881	1.776	.875
Within groups	372	754.738	2.029	p = .4975
Total	377	763.619		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	66	.5	1.113	.137
100 µM	66	.455	1.112	.137
10 µM	62	.419	1.3	.165
1 µM	66	.682	1.986	.244
0.1 µM	65	.631	1.193	.148
0.01 µM	53	.887	1.66	.228

Data for Figure 4.7d: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	15.282	3.056	2.071
Within groups	369	544.542	1.476	p = .0683
Total	374	559.824		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	66	2.955	1.195	.147
100 µM	66	3.121	1.074	.132
10 µM	62	3.435	1.35	.172
1 µM	66	2.894	1.371	.169
0.1 µM	64	2.828	1.216	.152
0.01 µM	51	3.137	1	.14

Data from Figure 4.7b: Root inititiation

Summary Statistics

DF:	5
Total Chi-Square:	4.206 p=.5202

	Control	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	Totals:
-	28	24	24	20	19	20	135
+	29	32	33	35	38	37	204
Totals:	57	56	57	55	57	57	339

	Control	100 µM	10 µM	1 µM	0.1 µM	0.01 µM	Totals:
-	49.12%	42.86%	42.11%	36.36%	33.33%	35.09%	39.82%
+	50.88%	57.14%	57.89%	63.64%	66.67%	64.91%	60.18%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data from Figure 4.7b: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	8.11	1.622	.934
Within groups	333	578.48	1.737	p = .4592
Total	338	586.59		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	57	.842	1.099	.146
100 µM	56	1.089	1.379	.184
10 µM	57	.965	1.195	.158
1 µM	55	1.273	1.638	.221
0.1 µM	57	1.211	1.235	.164
0.01 µM	57	1.228	1.31	.173

Data for Figure 4.7b: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	6.979	1.396	.906
Within groups	333	513.021	1.541	p = .4773
Total	338	520		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	57	3.053	1.394	.185
100 µM	56	2.946	1.271	.17
10 µM	57	2.93	1.266	.168
1 µM	55	3.182	1.107	.149
0.1 µM	57	2.754	1.214	.161
0.01 µM	57	3.14	1.172	.155

Data for Figure 4.6f: Root initiation

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	177.384	88.692	1.872
Within groups	12	568.681	47.39	p = .1961
Total	14	746.065		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	5	85.917	6.785	3.035
single	5	78.75	7.126	3.187
double	5	86.167	6.734	3.012

Data for Figure 4.6f: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	2.551	1.275	4.127
Within groups	12	3.708	.309	p = .0432
Total	14	6.259		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	5	3.622	.76	.34
single	5	2.871	.211	.094
double	5	2.662	.552	.247

Data for Figure 4.6g

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	309526.503	61905.301	2.77
Within groups	297	6637700.415	22349.16	p = .0183
Total	302	6947226.917		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	44	260.75	115.453	17.405
Stigmast. 20 mg/l	54	352.333	158.601	21.583
Stigmast. 50 mg/l	47	309.681	124.041	18.093
Cholcalc. 20 mg/l	48	327.729	158.244	22.841
Cholest. 20 mg/l	55	357.909	195.434	26.352
Stig acetate 20 ...	55	305.236	120.505	16.249

Data for Figure 4.6e: Root Initiation

Summary Statistics

DF:	3	
Total Chi-Square:	8.779	p=.0324

	Control	IBA 40 mg/L	Stigma 50 ...	Stigma 50...	Totals:
-	63	50	64	48	225
+	6	6	5	14	31
Totals:	69	56	69	62	256

	Control	IBA 40 mg/L	Stigma 50 ...	Stigma 50...	Totals:
-	91.3%	89.29%	92.75%	77.42%	87.89%
+	8.7%	10.71%	7.25%	22.58%	12.11%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.6e: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	5.738	1.913	2.653
Within groups	252	181.664	.721	p = .0492
Total	255	187.402		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	69	.116	.438	.053
IBA 40 mg/L	56	.357	1.407	.188
Stigma 50 mg	69	.072	.261	.031
Stigma 50mg + IBA	62	.419	.95	.121

Data for Figure 4.6e: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	1.285	.428	.56
Within groups	252	192.652	.764	p = .6416
Total	255	193.938		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	69	1.841	.964	.116
IBA 40 mg/L	56	1.821	.897	.12
Stigma 50 mg	69	1.739	.885	.107
Stigma 50mg + IBA	62	1.661	.723	.092

Data for Figure 4.6d: Root initiation

Summary Statistics

DF:	3	
Total Chi-Square:	8.643	p=.0344

	IBA 20 mg	Ergo 20 m...	Stigma 20 ...	Stigma 50...	Totals:
-	19	9	12	11	51
+	9	19	13	19	60
Totals:	28	28	25	30	111

	IBA 20 mg	Ergo 20 m...	Stigma 20 ...	Stigma 50...	Totals:
-	67.86%	32.14%	48%	36.67%	45.95%
+	32.14%	67.86%	52%	63.33%	54.05%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.6d: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	19.359	6.453	.332
Within groups	107	2080.317	19.442	p = .8023
Total	110	2099.676		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
IBA 20 mg	28	2.643	8.01	1.514
Ergo 20 mg + IBA	28	1.857	1.938	.366
Stigma 20 mg + I...	25	1.56	2.434	.487
Stigma 50mg + IBA	30	1.7	1.896	.346

Data for Figure 4.6d: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	4.922	1.641	1.705
Within groups	107	102.934	.962	p = .1703
Total	110	107.856		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
IBA 20 mg	28	2.857	1.008	.19
Ergo 20 mg + IBA	28	2.393	1.031	.195
Stigma 20 mg + I...	25	2.84	1.028	.206
Stigma 50mg + IBA	30	2.467	.86	.157

Data for Figure 4.6c: Root initiation

Summary Statistics

DF:	2	
Total Chi-Square:	3.107	p=.2115

	Control	IBA	Ergoc + IBA	Totals:
-	25	19	20	64
+	6	12	11	29
Totals:	31	31	31	93

	Control	IBA	Ergoc + IBA	Totals:
-	80.65%	61.29%	64.52%	68.82%
+	19.35%	38.71%	35.48%	31.18%
Totals:	100%	100%	100%	100%

Data for Figure 4.6c: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	4.323	2.161	1.823
Within groups	90	106.71	1.186	p = .1675
Total	92	111.032		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	31	.258	.575	.103
IBA	31	.613	.989	.178
Ergoc + IBA	31	.774	1.499	.269

Data for Figure 4.6c: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1.957	.978	.715
Within groups	90	123.161	1.368	p = .4919
Total	92	125.118		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	31	2.613	.989	.178
IBA	31	2.806	1.302	.234
Ergoc + IBA	31	2.968	1.197	.215

Data for Figure 4.6b: Root initiation

Summary Statistics

DF:	5	
Total Chi-Square:	43.491	p=.0001

	Control	IBA	Cholcal	Ergocal	Cholc + IBA	Ergo + IBA	Totals:
-	32	11	27	29	15	10	124
+	12	33	15	15	29	32	136
Totals:	44	44	42	44	44	42	260

	Control	IBA	Cholcal	Ergocal	Cholc + IBA	Ergo + IBA	Totals:
-	72.73%	25%	64.29%	65.91%	34.09%	23.81%	47.69%
+	27.27%	75%	35.71%	34.09%	65.91%	76.19%	52.31%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure4.6b: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	292.85	58.57	8.959
Within groups	254	1660.535	6.538	p = .0001
Total	259	1953.385		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	44	.5	1.023	.154
IBA	44	2.568	3.245	.489
Cholcal	42	.69	1.239	.191
Ergocal	44	.727	1.318	.199
Cholc + IBA	44	2.75	3.724	.561
Ergo + IBA	42	2.929	3.234	.499

Data for Figure 4.6b: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	8.805	1.761	1.322
Within groups	253	336.971	1.332	p = .2551
Total	258	345.776		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	44	2.955	.987	.149
IBA	44	3.182	1.206	.182
Cholcal	41	2.78	.962	.15
Ergocal	44	3.045	1.293	.195
Cholc + IBA	44	3.25	1.081	.163
Ergo + IBA	42	3.333	1.337	.206

Data for Figure 4.6a: Root initiation

Summary Statistics

DF:	5	
Total Chi-Square:	18.112	p=.0028

	Control	IBA	Cholcal	Ergocal	Cholc + IBA	Ergo + IBA	Totals:
-	10	12	16	15	7	4	64
+	14	12	8	9	17	20	80
Totals:	24	24	24	24	24	24	144

	Control	IBA	Cholcal	Ergocal	Cholc + IBA	Ergo + IBA	Totals:
-	41.67%	50%	66.67%	62.5%	29.17%	16.67%	44.44%
+	58.33%	50%	33.33%	37.5%	70.83%	83.33%	55.56%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.6a: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	213.118	42.624	4.531
Within groups	138	1298.042	9.406	p = .0007
Total	143	1511.16		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	24	1	1.063	.217
IBA	24	3.042	4.288	.875
Cholcal	24	1.333	2.648	.541
Ergocal	24	.708	1.042	.213
Cholc + IBA	24	3.417	3.977	.812
Ergo + IBA	24	3.708	3.605	.736

Data for Figure 4.6a: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	27.951	5.59	3.232
Within groups	138	238.708	1.73	p = .0086
Total	143	266.66		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	24	3.833	1.404	.287
IBA	24	4.542	1.474	.301
Cholcal	24	3.75	1.294	.264
Ergocal	24	3.708	1.16	.237
Cholc + IBA	24	4.875	1.227	.25
Ergo + IBA	24	4.333	1.308	.267

Data for Figure 4.5i: Root intiation

Summary Statistics

DF:	2	
Total Chi-Square:	2.701	p=.2591

	Control	24 hr	48 hr	Totals:
-	31	29	31	91
+	3	5	1	9
Totals:	34	34	32	100

	Control	24 hr	48 hr	Totals:
-	91.18%	85.29%	96.88%	91%
+	8.82%	14.71%	3.12%	9%
Totals:	100%	100%	100%	100%

Data for Figure 4.5i: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	.356	.178	.889
Within groups	6	1.2	.2	p = .4591
Total	8	1.556		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	3	1	0	0
24 hr	5	1.4	.548	.245
48 hr	1	1	•	•

Data for Figure 4.5j: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	.114	.057	.168
Within groups	98	33.331	.34	p = .8457
Total	100	33.446		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	34	1.324	.475	.081
24 hr	35	1.371	.646	.109
48 hr	32	1.406	.615	.109

Data for Figure 4.5i: Root initiation, part one

Summary Statistics

DF:	2	
Total Chi-Square:	.309	p=.8568

	Control	12hr	24 hr	Totals:
-	49	22	24	95
+	8	5	4	17
Totals:	57	27	28	112

	Control	12hr	24 hr	Totals:
-	85.96%	81.48%	85.71%	84.82%
+	14.04%	18.52%	14.29%	15.18%
Totals:	100%	100%	100%	100%

Data for Figure 4.5i: Root intiation, part two

Summary Statistics

DF:	2	
Total Chi-Square:	3.455	p=.1777

	Control	12hr	24 hr	Totals:
-	44	26	21	91
+	20	4	8	32
Totals:	64	30	29	123

	Control	12hr	24 hr	Totals:
-	68.75%	86.67%	72.41%	73.98%
+	31.25%	13.33%	27.59%	26.02%
Totals:	100%	100%	100%	100%

Data for Figure 4.5i: Root number, part one

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	.16	.08	.275
Within groups	14	4.075	.291	p = .7633
Total	16	4.235		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	8	1.375	.518	.183
12hr	5	1.6	.548	.245
24 hr	4	1.5	.577	.289

Data for Figure 4.5i: Root number, part two

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1.05	.525	.425
Within groups	29	35.825	1.235	p = .6578
Total	31	36.875		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	20	1.95	1.234	.276
12hr	4	1.5	1	.5
24 hr	8	1.625	.744	.263

Data for Figure 4.5h: Callus rating, part one

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	1.018	.339	.436
Within groups	100	77.819	.778	p = .7278
Total	103	78.837		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	26	1.692	.788	.155
12hr	26	1.885	.909	.178
24 hr	25	1.96	1.06	.212
48 hr	27	1.889	.751	.145

Data for Figure 4.5h: Callus rating, part two

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	3.131	1.044	1.995
Within groups	102	53.36	.523	p = .1195
Total	105	56.491		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	26	1.423	.578	.113
12hr	27	1.778	.934	.18
24 hr	28	1.321	.612	.116
48 hr	25	1.52	.714	.143

Data for Figure 4.5i: Callus rating, part one

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	.266	.133	.127
Within groups	109	114.162	1.047	p = .8808
Total	111	114.429		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	57	2.825	.966	.128
12hr	27	2.889	1.188	.229
24 hr	28	2.75	.967	.183

Data for Figure 4.5i: Callus rating, part two

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1.412	.706	.618
Within groups	120	137.092	1.142	p = .5408
Total	122	138.504		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	64	2.484	.976	.122
12hr	30	2.733	1.048	.191
24 hr	29	2.483	1.271	.236

Data for Figure 4.5h: Root initation, part one

Summary Statistics

DF:	3	
Total Chi-Square:	2.887	p=.4093

	Control	12hr	24 hr	48 hr	Totals:
-	20	19	15	16	70
+	6	7	10	11	34
Totals:	26	26	25	27	104

	Control	12hr	24 hr	48 hr	Totals:
-	76.92%	73.08%	60%	59.26%	67.31%
+	23.08%	26.92%	40%	40.74%	32.69%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.5h: Root initiation, part two

Summary Statistics

DF:	3	
Total Chi-Square:	8.234	p=.0414

	Control	12hr	24 hr	48 hr	Totals:
-	19	18	27	20	84
+	7	9	1	5	22
Totals:	26	27	28	25	106

	Control	12hr	24 hr	48 hr	Totals:
-	73.08%	66.67%	96.43%	80%	79.25%
+	26.92%	33.33%	3.57%	20%	20.75%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.5h: Root number, part one

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.841	.28	.541
Within groups	30	15.542	.518	p = .6579
Total	33	16.382		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	6	1.5	.548	.224
12hr	7	1.429	.535	.202
24 hr	10	1.8	1.033	.327
48 hr	11	1.455	.522	.157

Data for Figure 4.5h: Root number, part two

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	3.226	1.075	1.193
Within groups	18	16.229	.902	p = .3406
Total	21	19.455		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	7	1.286	.488	.184
12hr	9	2	1.323	.441
24 hr	1	1	•	•
48 hr	5	1.2	.447	.2

Data for Figure 4.5g: Root number, week 2

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.707	.236	.846
Within groups	30	8.352	.278	p = .4794
Total	33	9.059		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	13	1.385	.65	.18
12hr	8	1.125	.354	.125
24 hr	10	1.4	.516	.163
48 hr	3	1	0	0

Data for Figure 4.5g: Root number, week 4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.479	.16	.254
Within groups	22	13.867	.63	p = .8579
Total	25	14.346		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	6	1.667	1.211	.494
12hr	5	1.4	.548	.245
24 hr	9	1.333	.5	.167
48 hr	6	1.333	.816	.333

Data for Figure 4.5g: Callus rating, week 0

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	25.259	8.42	5.557
Within groups	113	171.22	1.515	p = .0014
Total	116	196.479		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	27	1.593	.797	.153
12hr	29	2.828	1.537	.285
24 hr	30	2.533	1.306	.238
48 hr	31	2.645	1.142	.205

Data from Figure 4.5g: Callus, week2

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	3.222	1.074	.862
Within groups	116	144.478	1.246	p = .4629
Total	119	147.7		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	33	2.576	1.032	.18
12hr	26	2.269	1.002	.197
24 hr	31	2.742	1.237	.222
48 hr	30	2.567	1.165	.213

Data from Figure 4.5g: Callus, week4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	1.966	.655	.819
Within groups	106	84.798	.8	p = .4861
Total	109	86.764		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	27	2.296	1.031	.198
12hr	27	2.296	.869	.167
24 hr	30	2	.788	.144
48 hr	26	2.308	.884	.173

Data for Figure 4.5g: Root initiation, week 0

Summary Statistics

DF:	3	
Total Chi-Square:	8.353	p=.0392

	Control	12hr	24 hr	48 hr	Totals:
-	19	18	21	12	70
+	8	11	9	19	47
Totals:	27	29	30	31	117

	Control	12hr	24 hr	48 hr	Totals:
-	70.37%	62.07%	70%	38.71%	59.83%
+	29.63%	37.93%	30%	61.29%	40.17%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.5g: Root initiation, week 2

Summary Statistics

DF:	3	
Total Chi-Square:	7.245	p=.0645

	Control	12hr	24 hr	48 hr	Totals:
-	20	19	21	27	87
+	13	8	10	3	34
Totals:	33	27	31	30	121

	Control	12hr	24 hr	48 hr	Totals:
-	60.61%	70.37%	67.74%	90%	71.9%
+	39.39%	29.63%	32.26%	10%	28.1%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.5g: Root initiation, week 4

Summary Statistics

DF:	3	
Total Chi-Square:	1.099	p=.7772

	Control	12hr	24 hr	48 hr	Totals:
-	21	22	21	20	84
+	6	5	9	6	26
Totals:	27	27	30	26	110

	Control	12hr	24 hr	48 hr	Totals:
-	77.78%	81.48%	70%	76.92%	76.36%
+	22.22%	18.52%	30%	23.08%	23.64%
Totals:	100%	100%	100%	100%	100%

Data for Figure 4.5g: Root number, week 0

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	2.313	.771	1.237
Within groups	43	26.794	.623	p = .3079
Total	46	29.106		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	8	1.375	.518	.183
12hr	11	1.727	1.009	.304
24 hr	9	2	.866	.289
48 hr	19	1.474	.697	.16

Data from Figure 4.5f: Callus, week 3

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.793	.264	.207
Within groups	135	171.955	1.274	p = .8911
Total	138	172.748		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	36	2.472	1.108	.185
12 Hr	34	2.559	1.106	.19
24 Hr	34	2.382	1.181	.203
48 Hr	35	2.571	1.119	.189

Data from Figure 4.5f: Callus, week 4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	2.875	.958	.711
Within groups	92	124.083	1.349	p = .5481
Total	95	126.958		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	24	2.583	1.283	.262
12 Hr	24	2.792	1.25	.255
24 Hr	24	2.333	.761	.155
48 Hr	24	2.708	1.268	.259

Data for Figure 4.5f: Root number, Week3

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.642	.214	.351
Within groups	37	22.577	.61	p = .7888
Total	40	23.22		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	12	1.25	.622	.179
12 Hr	10	1.2	.422	.133
24 Hr	11	1.455	1.214	.366
48 Hr	8	1.5	.535	.189

Data for Figure4.5f: Root number, week 4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.714	.238	.446
Within groups	22	11.748	.534	p = .7228
Total	25	12.462		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	6	1.667	.516	.211
12 Hr	7	1.571	1.134	.429
24 Hr	5	1.4	.548	.245
48 Hr	8	1.25	.463	.164

Data from Figure 4.5f: Callus, week 3

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	.793	.264	.207
Within groups	135	171.955	1.274	p = .8911
Total	138	172.748		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	36	2.472	1.108	.185
12 Hr	34	2.559	1.106	.19
24 Hr	34	2.382	1.181	.203
48 Hr	35	2.571	1.119	.189

Data from Figure 4.5f: Callus, week 4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	2.875	.958	.711
Within groups	92	124.083	1.349	p = .5481
Total	95	126.958		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	24	2.583	1.283	.262
12 Hr	24	2.792	1.25	.255
24 Hr	24	2.333	.761	.155
48 Hr	24	2.708	1.268	.259

Data for Figure 4.5e: Callus rating, first run

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1.168	.584	.215
Within groups	125	339.699	2.718	p = .8069
Total	127	340.867		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	53	3.717	1.486	.204
12hr	37	3.946	1.632	.268
24 hr	38	3.842	1.868	.303

Data for Figure 4.5e: Callus rating, second run

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	3.241	1.62	.772
Within groups	132	277.159	2.1	p = .4643
Total	134	280.4		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	57	3.105	1.385	.183
12hr	38	3.289	1.659	.269
24 hr	40	3.475	1.32	.209

Data for Figure 4.5e: Root initiation, first run

Summary Statistics

DF:	2	
Total Chi-Square:	4.378	p=.112

	Control	12hr	24 hr	Totals:
-	44	24	26	94
+	9	13	12	34
Totals:	53	37	38	128

	Control	12hr	24 hr	Totals:
-	83.02%	64.86%	68.42%	73.44%
+	16.98%	35.14%	31.58%	26.56%
Totals:	100%	100%	100%	100%

Data for Figure 4.5e: Root initiation, second run

Summary Statistics

DF:	2	
Total Chi-Square:	.532	p=.7663

	Control	12hr	24 hr	Totals:
-	42	28	27	97
+	15	10	13	38
Totals:	57	38	40	135

	Control	12hr	24 hr	Totals:
-	73.68%	73.68%	67.5%	71.85%
+	26.32%	26.32%	32.5%	28.15%
Totals:	100%	100%	100%	100%

Data for Figure 4.5e: Root number, first run

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	11.096	5.548	.872
Within groups	31	197.139	6.359	p = .4279
Total	33	208.235		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	9	3.444	3.005	1.002
12hr	13	2	1.08	.3
24 hr	12	2.583	3.175	.917

Data for Figure 4.5e: Root number, second run

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	10.832	5.416	1.102
Within groups	35	172.01	4.915	p = .3435
Total	37	182.842		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	15	1.733	.884	.228
12hr	10	3	3.333	1.054
24 hr	13	2.615	2.256	.626

Data for Figure4.5c: Root initiation

Summary Statistics

DF:	2	
Total Chi-Square:	4.231	p=.1206

	Control	12hr	24 hr	Totals:
-	27	25	31	83
+	8	13	5	26
Totals:	35	38	36	109

	Control	12hr	24 hr	Totals:
-	77.14%	65.79%	86.11%	76.15%
+	22.86%	34.21%	13.89%	23.85%
Totals:	100%	100%	100%	100%

Data for Figure 4.5c: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1.454	.727	.332
Within groups	23	50.392	2.191	p = .721
Total	25	51.846		

Model II estimate of between component variance = -.732

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	8	1.75	1.488	.526
12hr	13	2.154	1.676	.465
24 hr	5	1.6	.548	.245

Data for Figure 4.5c: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	5.553	2.776	1.011
Within groups	106	291.053	2.746	p = .3673
Total	108	296.606		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	35	3.543	1.314	.222
12hr	38	3.553	1.796	.291
24 hr	36	4.028	1.797	.299

Data for Figure 4.5b: Root initiation

Summary Statistics

DF:	4	
Total Chi-Square:	4.098	p=.393

	6 Hr	3 Hr	1 Hr	0.5 Hr	Control	Totals:
-	18	19	23	16	21	97
+	15	14	9	17	13	68
Totals:	33	33	32	33	34	165

	6 Hr	3 Hr	1 Hr	0.5 Hr	Control	Totals:
-	54.55%	57.58%	71.88%	48.48%	61.76%	58.79%
+	45.45%	42.42%	28.12%	51.52%	38.24%	41.21%
Totals:	100%	100%	100%	100%	100%	100%

Data for Figure 4.5b: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	4	28.177	7.044	1.511
Within groups	63	293.705	4.662	p = .2097
Total	67	321.882		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
6 Hr	15	2.467	2.2	.568
3 Hr	14	1.571	1.342	.359
1 Hr	9	2.333	1.5	.5
0.5 Hr	17	1.529	.943	.229
Control	13	3.231	3.767	1.045

Data for Figure 4.5b: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	4	7.73	1.933	.738
Within groups	160	418.718	2.617	p = .5671
Total	164	426.448		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
6 Hr	33	3.364	1.517	.264
3 Hr	33	3.394	1.731	.301
1 Hr	32	3.719	1.727	.305
0.5 Hr	33	3.03	1.489	.259
Control	34	3.353	1.612	.276

Data for Figure 4.5a: Callus, week 2

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	.909	.454	.264
Within groups	89	153.048	1.72	p = .7684
Total	91	153.957		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	39	2.974	1.135	.182
12hr	39	3.128	1.321	.212
24 hr	14	2.857	1.703	.455

Data for Figure 4.5a: Callus, week 4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	2.58	1.29	.376
Within groups	111	381.385	3.436	p = .6878
Total	113	383.965		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	38	3.605	1.897	.308
12hr	36	3.861	2.031	.338
24 hr	40	3.5	1.633	.258

Data for Figure 4.5a: Root initiation, week 2

Summary Statistics

DF:	2	
Total Chi-Square:	8.221	p=.0164

	Control	12hr	24 hr	Totals:
-	16	26	11	53
+	23	13	3	39
Totals:	39	39	14	92

	Control	12hr	24 hr	Totals:
-	41.03%	66.67%	78.57%	57.61%
+	58.97%	33.33%	21.43%	42.39%
Totals:	100%	100%	100%	100%

Data for Figure 4.5a: Root initiation, week 4

Summary Statistics

DF:	2	
Total Chi-Square:	1.901	p=.3866

-	14	19	18	51
+	24	17	22	63
Totals:	38	36	40	114

	Control	12hr	24 hr	Totals:
-	36.84%	52.78%	45%	44.74%
+	63.16%	47.22%	55%	55.26%
Totals:	100%	100%	100%	100%

Data for Figure 4.5a: Root number, week 2

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	3	1.852	.617	.298
Within groups	43	88.957	2.069	p = .8263
Total	46	90.809		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	23	2.043	1.581	.33
12hr	13	2	1.472	.408
24 hr	3	2	1.732	1
Untreated	8	1.5	.535	.189

Data for Figure 4.5a: Root number, week 4

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	11.961	5.981	.99
Within groups	60	362.451	6.041	p = .3775
Total	62	374.413		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	24	3.625	3.076	.628
12hr	17	2.529	1.807	.438
24 hr	22	3.136	2.1	.448

Data for Figure 4.4j: Root initiation

Summary Statistics

DF:	5	
Total Chi-Square:	1.551	p=.9071

	500 µM	100 µM	50 µM	10 µM	1 µM	0 µM	Totals:
-	23	32	35	30	33	28	181
+	16	22	16	16	21	15	106
Totals:	39	54	51	46	54	43	287

	500 µM	100 µM	50 µM	10 µM	1 µM	0 µM	Totals:
-	58.97%	59.26%	68.63%	65.22%	61.11%	65.12%	63.07%
+	41.03%	40.74%	31.37%	34.78%	38.89%	34.88%	36.93%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.4j: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	31.361	6.272	1.209
Within groups	281	1457.266	5.186	p = .3048
Total	286	1488.627		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
500 µM	39	1.103	1.667	.267
100 µM	54	1.796	3.043	.414
50 µM	51	.961	2.289	.32
10 µM	46	1.174	2.694	.397
1 µM	54	.796	1.471	.2
0 µM	43	1.093	1.937	.295

Data for Figure 4.4j: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	8.435	1.687	2.439
Within groups	282	195.034	.692	p = .0348
Total	287	203.469		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
500 µM	39	2.026	.903	.145
100 µM	54	1.648	.781	.106
50 µM	51	1.902	.855	.12
10 µM	46	1.478	.691	.102
1 µM	54	1.759	.889	.121
0 µM	44	1.659	.861	.13

Data for Figure 4.4h: Root initiation

Summary Statistics

DF:	5	
Total Chi-Square:	6.997	p=.2209

	500 µM	100 µM	50 µM	10 µM	1 µM	0 µM	Totals:
-	24	17	25	26	34	23	149
+	27	30	29	23	22	28	159
Totals:	51	47	54	49	56	51	308

	500 µM	100 µM	50 µM	10 µM	1 µM	0 µM	Totals:
-	47.06%	36.17%	46.3%	53.06%	60.71%	45.1%	48.38%
+	52.94%	63.83%	53.7%	46.94%	39.29%	54.9%	51.62%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.4h: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	6.564	1.313	.185
Within groups	153	1082.945	7.078	p = .9677
Total	158	1089.509		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
500 µM	27	3.333	1.922	.37
100 µM	30	3.933	3.629	.663
50 µM	29	3.897	3.395	.63
10 µM	23	3.696	2.225	.464
1 µM	22	3.636	1.916	.408
0 µM	28	3.643	1.89	.357

Data for Figure 4.4h: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	11.739	2.348	.904
Within groups	303	787.006	2.597	p = .4788
Total	308	798.744		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
500 µM	52	3.731	1.634	.227
100 µM	47	3.447	1.501	.219
50 µM	54	3.815	1.638	.223
10 µM	49	3.571	1.658	.237
1 µM	56	3.25	1.455	.194
0 µM	51	3.431	1.769	.248

Data for Figure 4.4g: Root initiation

Summary Statistics

DF:	4	
Total Chi-Square:	5.295	p=.2584

	Control	Blank	IBA 2 days	IBA 4 days	IBA 6 days	Totals:
-	45	36	36	29	32	178
+	73	81	82	87	83	406
Totals:	118	117	118	116	115	584

	Control	Blank	IBA 2 days	IBA 4 days	IBA 6 days	Totals:
-	38.14%	30.77%	30.51%	25%	27.83%	30.48%
+	61.86%	69.23%	69.49%	75%	72.17%	69.52%
Totals:	100%	100%	100%	100%	100%	100%

Data for Figure 4.4g: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	4	9.118	2.28	.828
Within groups	401	1104.635	2.755	p = .5082
Total	405	1113.754		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	73	2.411	1.342	.157
Blank	81	2.531	1.566	.174
IBA 2 days	82	2.573	1.618	.179
IBA 4 days	87	2.862	1.912	.205
IBA 6 days	83	2.566	1.754	.193

Data for Figure 4.4g: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	4	5.625	1.406	.613
Within groups	576	1320.505	2.293	p = .6531
Total	580	1326.131		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	118	3.983	1.596	.147
Blank	116	3.776	1.605	.149
IBA 2 days	118	3.831	1.576	.145
IBA 4 days	114	3.868	1.392	.13
IBA 6 days	115	3.687	1.379	.129

Data for Figure 4.4f: Stem damage, 48 hour treatment

Summary Statistics

DF:	5	
Total Chi-Square:	47.005	p=.0001

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
Damaged	34	54	57	69	59	78	351
Undamaged	67	47	42	31	40	22	249
Totals:	101	101	99	100	99	100	600

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
Damaged	33.66%	53.47%	57.58%	69%	59.6%	78%	58.5%
Undamaged	66.34%	46.53%	42.42%	31%	40.4%	22%	41.5%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.4f: Stem damage, 12 hour treatment

Summary Statistics

DF:	5	
Total Chi-Square:	39.191	p=.0001

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
Damaged	35	40	37	57	59	68	296
Undamaged	66	61	64	44	40	32	307
Totals:	101	101	101	101	99	100	603

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
Damaged	34.65%	39.6%	36.63%	56.44%	59.6%	68%	49.09%
Undamaged	65.35%	60.4%	63.37%	43.56%	40.4%	32%	50.91%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for experiment 4.4.3: Callus rating - 48 hour treattment

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	8.337	1.667	1.602
Within groups	497	517.428	1.041	p = .158
Total	502	525.765		

Model II estimate of between component variance = .125

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	96	1.844	.921	.094
20 mg/L	89	2	1.234	.131
40 mg/L	85	1.976	1.069	.116
80 mg/L	83	1.952	1.092	.12
120 mg/L	77	1.922	.957	.109
160 mg/L	73	1.603	.74	.087

Data for experiment 4.4.3: Callus rating - 12 hour treattment

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	2.961	.592	.517
Within groups	515	590.037	1.146	p = .7636
Total	520	592.998		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	92	1.957	1.037	.108
20 mg/L	96	1.885	.905	.092
40 mg/L	90	2.111	1.136	.12
80 mg/L	85	2.047	1.133	.123
120 mg/L	85	2.035	1.074	.116
160 mg/L	73	1.959	1.148	.134

Data for Figure 4.4d: Root initiation, 48 hour treatment

Summary Statistics

DF:	5	
Total Chi-Square:	10.239	p=.0687

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
-	86	67	62	64	62	60	401
+	10	21	24	18	14	13	100
Totals:	96	88	86	82	76	73	501

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
-	89.58%	76.14%	72.09%	78.05%	81.58%	82.19%	80.04%
+	10.42%	23.86%	27.91%	21.95%	18.42%	17.81%	19.96%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.4d: Root initiation, 12 hour treatment

Summary Statistics

DF:	5	
Total Chi-Square:	20.741	p=.0009

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
-	83	81	77	59	57	55	412
+	8	15	14	24	26	17	104
Totals:	91	96	91	83	83	72	516

	Control	20 mg/L	40 mg/L	80 mg/L	120 mg/L	160 mg/L	Totals:
-	91.21%	84.38%	84.62%	71.08%	68.67%	76.39%	79.84%
+	8.79%	15.62%	15.38%	28.92%	31.33%	23.61%	20.16%
Totals:	100%	100%	100%	100%	100%	100%	100%

Data for Figure 4.4e: Root number - 48 hour treatment

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	70.203	14.041	4.667
Within groups	94	282.787	3.008	p = .0008
Total	99	352.99		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	10	1.2	.422	.133
20 mg/L	21	1.952	1.161	.253
40 mg/L	24	2.125	1.727	.353
80 mg/L	18	2.944	1.984	.468
120 mg/L	14	2.786	1.847	.494
160 mg/L	13	4.231	2.488	.69

Data for Figure 4.4e: Root number - 12 hour treatment

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	5	78.859	15.772	4.087
Within groups	98	378.18	3.859	p = .0021
Total	103	457.038		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	8	1	0	0
20 mg/L	15	1.333	.617	.159
40 mg/L	14	2.286	1.637	.438
80 mg/L	24	1.917	1.213	.248
120 mg/L	26	3.192	2.245	.44
160 mg/L	17	3.588	3.337	.809

Data for Figure 4.4a: Root initiation

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	7	29452.087	4207.441	4.111
Within groups	141	144301.418	1023.414	p = .0004
Total	148	173753.505		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	16	33.021	22.999	5.75
Week 0	17	28.235	30.365	7.365
Week 1	20	40.833	34.201	7.648
Week 2	18	42.963	37.081	8.74
Week 3	20	49.333	37.328	8.347
Week 4	17	66.373	25.524	6.19
Week 5	16	69.792	26.408	6.602
Untreated	25	32.333	34.554	6.911

Data for Figure 4.4b: Root number

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	7	13.123	1.875	1.561
Within groups	105	126.083	1.201	p = .1551
Total	112	139.206		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	13	1.551	.803	.223
Week 0	10	2.017	1.148	.363
Week 1	15	2.367	1.43	.369
Week 2	13	2.449	1.609	.446
Week 3	15	2.061	1.14	.294
Week 4	16	1.953	.902	.225
Week 5	16	1.88	.887	.222
Untreated	15	1.378	.572	.148

Data for Figure 4.4c: Callus rating

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	7	14.988	2.141	2.2
Within groups	141	137.233	.973	p = .0377
Total	148	152.221		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	16	2.938	.744	.186
Week 0	17	3.66	1.143	.277
Week 1	20	3.688	1.284	.287
Week 2	18	3.511	1.065	.251
Week 3	20	3.051	1.092	.244
Week 4	17	2.839	.55	.133
Week 5	16	3.059	.767	.192
Untreated	25	3.014	.939	.188

Data for experiment in section 4.4.2: Survival

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	7	8395.511	1199.359	1.622
Within groups	152	112394.419	739.437	p = .1331
Total	159	120789.931		

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	17	66.667	26.352	6.391
Week 0	17	58.824	17.793	4.315
Week 1	20	53.333	24.543	5.488
Week 2	20	43.333	30.779	6.882
Week 3	20	49.167	28.344	6.338
Week 4	20	45.833	31.933	7.141
Week 5	20	48.333	33.726	7.541
Untreated	26	44.231	20.517	4.024

Data for Figure 4.2a

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Seedling Age (A)	3	20504.901	6834.967	126.031	.0001
Hormones (B)	1	371.826	371.826	6.856	.0151
AB	3	2457.44	819.147	15.104	.0001
Error	24	1301.579	54.232		

	Hormones:	No IBA	IBA 20 mg	Totals:
Seedling Age	Node 1	4 71.675	4 85.855	8 78.765
	Node 2	4 66.935	4 43.733	8 55.334
	Node 3	4 36.26	4 57.29	8 46.775
	Node 5	4 .832	4 16.095	8 8.464
Totals:		16 43.926	16 50.743	32 47.334

Data for Figure 4.2b

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Seedling Age (A)	3	17.183	5.728	15.335	.0001
Hormones (B)	1	15.867	15.867	42.479	.0001
AB	3	4.924	1.641	4.394	.0134
Error	24	8.964	.374		

	Hormones:	No IBA	IBA 20 mg	Totals:
Seedling Age	Node 1	4 2.512	4 2.618	8 2.565
	Node 2	4 2.125	4 4.27	8 3.197
	Node 3	4 1.735	4 3.253	8 2.494
	Node 5	4 .25	4 2.115	8 1.183
Totals:		16 1.656	16 3.064	32 2.36

Data for Figure 4.2c

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Seedling Age (A)	3	.572	.191	3.426	.0332
Hormones (B)	1	4.434	4.434	79.68	.0001
AB	3	2.657	.886	15.914	.0001
Error	24	1.336	.056		

	Hormones:	No IBA	IBA 20 mg	Totals:
Seedling Age	Node 1	4 3.48	4 3.61	8 3.545
	Node 2	4 3.505	4 4.245	8 3.875
	Node 3	4 3.65	4 4.088	8 3.869
	Node 5	4 2.912	4 4.582	8 3.747
Totals:		16 3.387	16 4.131	32 3.759