ADVENTITIOUS ROOTING IN STEM CUTTINGS OF *EUCALYPTUS GRANDIS* HILL EX MAID.

by

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FRONTISPIECE

A stand of the study species, *Eucalyptus grandis* Hill ex Maid.
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PREFACE

This thesis has not been submitted, in part or whole, to another university. Except where the work of others is acknowledged it is the product of my own work.

Philip John Wilson

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ABSTRACT

Adventitious rooting in stem cuttings of *Eucalyptus grandis* Hill ex Maid. was thought to be influenced by a putative inhibitor. In previous studies it has been usual to infer the presence of putative rooting inhibitors and promoters from the mung bean bioassay, but the possibility was raised that treatment responses in this assay could be mediated more by the concentration of the treatment solution than by the chemical identity of the solute.

This appeared to be so: several solutes, including hydrochloric acid and common salt, were found to promote the rooting of mung bean cuttings when present in the treatment solution at an apparently injurous concentration. The concept of promoters and inhibitors of adventitious rooting, as constituted at present, was considered to be an unfavourable approach for further studies.

Stem cuttings must contain a morphogen, broadly defined, which operates the 'switch' from stem to adventitious root. The leaves and buds of *E.grandis* stem cuttings did not appear to be sole sources of a morphogen (as is often assumed), but nevertheless the activity of the leaves and buds was good for rooting. This activity was reflected in the pattern of root emergence. A slight preponderance emerged from the leaf trace sectors of the stem, suggesting that the leaves and buds cause a morphogen (of unknown origin) to circulate in the cutting.

The existence of a vascular morphogen was confirmed and it proved to be very mobile in the stem, suggesting that it is well distributed circumferentially at the base of the cutting rather than confined to the leaf trace sectors. It appeared to be super-abundant at the base of easy-to-root cuttings, but it was not possible to tell to what extent the morphogen was rendered accessible to the sites where roots initiate.

In general, the rate of efflux from the transporting tissues, the rate of attenuation of the morphogen after efflux, and the number of potential sites for root initiation must interact on a small scale to determine rooting ability. The relative prominence of these groups of factors would be expected to vary with circumstances, for example at different locations within a single stem cutting, so the traditional concept of a limiting morphogen ('rhizocaline') is unhelpful in its simplest form.

Nevertheless, the rhizocaline concept provides a starting point towards a more comprehensive view of adventitious rooting, which is required in order to predict and improve rooting ability. This view remains a remote objective because many of the factors which could be important have recieved very little attention and will be difficult to elucidate.

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CHAPTER ONE

GENERAL INTRODUCTION AND OBJECTIVE

1.1 INTRODUCTION

Tree improvement by breeding is slow because there is a long interval between generations, and because trees are very heterozygous (LIBBY, STETTLER & SEITZ, 1969). These constraints can be circumvented in the shorter term by selecting superior trees (which may be hybrids) and propagating them vegetatively. Non-additive genetic gain is transferred in full in vegetative propagation, which is particularly useful in rapidly improving those attributes which are poorly heritable, such as volume growth and cellulose yield (ZOBEL & IKEMORI, 1983).

The cheapest and easiest technique for propagating forest trees vegetatively is by stem cuttings, but traditionally only a few very easy-to-root genera such as *Populus* L. have been propagated routinely in this way. Recently, however, various attempts have been made to afforest on a commercial scale with *Eucalyptus* L'Her. stem cuttings. The outstanding example is the Brazilian company Aracruz Florestal SA, which began afforesting with rooted cuttings in 1979. By 1983 the company's annual planting of 12.5 million trees was almost exclusively of rooted cuttings (ZOBEL & IKEMORI, 1983), and average increases in yield had been estimated, over the pre-existing seedling stands, of 112% in volume growth (from 33 to 70 m^3/ha/yr), and of 135% in pulp yield/ha/yr (BRANDAO, 1984).

The rooting ability of stem cuttings of *Eucalyptus* (and many other woody genera) varies markedly between species, between clones within a species, and within clones. The objective of the work in this thesis was to contribute to an understanding of adventitious rooting in stem cuttings of *E.grandis* Hill ex Maid. so that, ultimately, rooting ability could be predicted or improved in practice.

Adventitious roots have been defined more or less broadly. According to ESAU (1977) they are those roots which have not originated in normal acropetal sequence from the radicle, including roots which arise on aerial plant parts, underground stems and relatively old roots. BARLOW (1986) confined the term to those roots which arise from the shoot.

In many studies, reviewed in the following Chapter, the rooting ability of stem cuttings has been seen to be determined by various putative promotors and inhibitors. These

include a group of rooting inhibitors isolated from *E.grandis*. The amount of inhibitory activity in *E.grandis* cuttings (inferred from bioassays) increased as rooting ability decreased, suggesting a direct quantitative association between inhibitor content and rooting ability (PATON, WILLING, NICHOLLS & PRYOR, 1970). This relationship was emphasized by PATON, WILLING & PRYOR (1981) and PATON (1983).

CHAPTER TWO

RHIZOCALINE, ROOTING CO-FACTORS AND THE CONCEPT OF PROMOTERS AND INHIBITORS OF ADVENTITIOUS ROOTING

2.1 INTRODUCTION

According to HARTMANN & KESTER (1983), DUHAMEL DU MONCEAU (1758) suggested that the formation of adventitious roots on stems was due to the downward movement of sap, and SACHS (1880,1882) assumed the existence of an active substance formed in the leaves and buds which accumulated at the base of stem cuttings. The hypothetical root-forming hormone (rhizocaline) was sought in many later studies and, by extension, various other hypothetical constituents of plants, which were also supposed to be predominantly important in regulating rooting, have been invoked. These include co-factors (auxin synergists), and promoters and inhibitors of adventitious rooting with no known modes of action.

Broadly speaking, the concept of promoters and inhibitors of adventitious rooting could be said to encompass all of those studies in which known and unknown compounds have been found to promote or inhibit rooting when applied to stem cuttings. However, this review is confined to those for which no well established mode of action has been put forward. These compounds have been considered together because it is suggested that they have a common mode of action, and because they are related through the original concept of rhizocaline. The effects of the nutrients, hormones, *etc.* have been reviewed by HAISSIG (1986) and JARVIS (1986).

2.2 RHIZOCALINE

The old idea that the flow of sap in plants can inhibit or promote the development of shoots and roots was developed by LOEB (1915, 1917). A portion of stem left on leaf cuttings of *Bryophyllum calycinum* Salisb. inhibited the development of leaf buds whereas the leaf promoted adventitious rooting in the stem, suggesting that the stem receives a regeneration stimulus from the leaves (LOEB, 1915). The presence of leaves stimulated rooting on horizontal stem lengths of *B.calycinum* especially when at the apical end of the stem, suggesting a preferential basipetal movement of the hypothetical regeneration stimulus (LOEB, 1917).

WENT (1929) observed that the leaves and buds on stem cuttings of *Acalypha wilkesiana* Muell. Arg. also promoted rooting, and assumed that they were the source of the hypothetical 'root-forming phytohormone' (rhizocaline). WENT (1934) then sought to make his studies of rhizocaline quantitative by establishing a standard bioassay procedure using etiolated pea epicotyls. He proposed the 'rhizocaline unit', each of which would produce one root above the control value when tested under the conditions specified. This technique was the forerunner of the mung bean bioassay, which became popular for inferring the presence of promoters and inhibitors of adventitious rooting in plant extracts.

After the discovery of the chemical identity of the naturally occurring auxin indoleacetic acid (IAA), its ability to promote adventitious rooting was soon established (COOPER, 1935; THIMANN & KOEPFLI, 1935). However, later work, in which the response of cuttings to applied auxins was variable, led to the suggestion that hormones other than auxin are required for root formation (COOPER, 1938; WENT, 1938; THIMANN & DELISLE, 1939).

VAN OVERBEEK & GREGORY (1945) suggested that the leaf factors other than auxin which induce rooting could be, at least in part, nutritional. The promotory effects of leaves on the initiation of roots in *Hibiscus rosa-sinensis* L. cuttings were replaced by a mixture of auxin, sucrose and nitrogenous compounds (VAN OVERBEEK, GORDON & GREGORY, 1946).

SPEIGEL (1954) extended the view of substances active in rooting to include inhibitory substances. Leachate from the bases of the difficult-to-root *Vitis* L. '41B' inhibited rooting, when supplied to cuttings of the easy-to-root *Vitis vinifera* L. The inhibitor content in extracts from various vines was then inferred using the *Lepidium sativum* L. root growth bioassay and the *Avena* L. coleoptile growth bioassay. The difficult-to-root species and hybrids tended to have a relatively high inhibitor content.

BOUILLENNE & BOUILLENNE-WALRAND (1955) proposed that rhizocaline consists of the product of a reaction (facilitated by an oxidase enzyme) between auxin and an ortho-diphenolic. In many studies, reviewed in section 2.3.4, phenolics have been shown to promote rooting when applied to stem cuttings, tending to support the proposal that phenolics are a component of rhizocaline. However, several other possible modes of action have been proposed for phenolics, which were reviewed by HAISSIG (1974a). Phenolics may a) protect auxin from oxidation; b) participate in auxin synthesis; c) act in the first of two hypothetical stages of root formation, conserving auxin for the auxin-specific second stage or d) act directly in association with auxin. HAISSIG (1974a) concluded by favouring the hypothesis that ortho-diphenolics react directly with auxin

to form one or more auxin-phenolic conjugates, which then create the predisposition to root. This is similar to the scheme put forward by BOUILLENNE & BOUILLENNE-WALRAND (1955).

2.3 COFACTORS, PROMOTERS AND INHIBITORS

Co-factors are promoters of adventitious rooting which are synergistic with auxin (HESS, 1964a). However, there is no satisfactory distinction between co-factors and other promoters. For example, when applied phenolics alone have promoted rooting in the absence of applied auxin (GORTER, 1962; FERNQVIST, 1966; KAWASE, 1971; TOGNONI & LORENZI, 1972; BORJARCZUK, 1978; JAMES & THURBON, 1979; RAVIV, REUVENI & GOLDSCHMIT, 1986; Chapter 3), it is possible to speculate that the promotory effect depended on the presence of endogenous auxin (JAMES & THURBON, 1979).

The presence of promoters and inhibitors of adventitious rooting in plant extracts has often been inferred from the *Vigna radiata* (L.) Wilczek (mung bean) bioassay, developed by HESS (1964a). HESS (1964a) separated extracts of *Hedera helix* L. in the juvenile and adult growth phases, and extracts of easy- and difficult-to-root *Hibiscus* L. cultivars. The methanolic extracts were separated by paper chromatography and the various Rf zones were eluted in water, to give test solutions in which mung bean cuttings were placed.

The number of roots per cutting proved to vary markedly with treatment, allowing areas of promotion and inhibition to be identified relative to a control value. Difficult-to-root material seemed to have smaller areas of promotion than easy-to-root material, leading HESS (1964a) to infer the existence of promotory substances which would be present at relatively high concentrations in easy-to-root material. HESS (1964a) called these hypothetical substances 'co-factors' since they were thought to be most promotory in the presence of auxin.

In many studies, the bioassay approach pioneered by WENT (1934) and developed by HESS (1964a) has been used to infer the presence of unknown promoters or inhibitors in plant extracts. In most, promotory or inhibitory activity was related to the rooting ability of the plants extracted.

2.3.1 UNKNOWN ENDOGENOUS PROMOTERS AND INHIBITORS

A positive association between promotory activity and rooting ability was reported for two *Malus* Mill. (apple) rootstocks, one made easy-to-root by high temperature winter storage (CHALLENGER, LACEY & HOWARD, 1965). Promoters accumulated above a girdle in an easy-to-root but not a difficult-to-root cultivar of *Hibiscus* (STOLZ & HESS, 1966). Easy-to-root juvenile *Hedera helix* generally contained more promotory activity than the difficult-to-root adult form (GIROUARD, 1969). Promotory activity was correlated with rooting ability in three cultivars of *Rhododendron* L. (LEE, McGUIRE & KITCHIN, 1969). A correlation was found between promotory activity and seasonal variation in the rooting ability of *Salix viminalis* L. cuttings (GESTO, VAZQUEZ & VIEITEZ, 1977); and promotory activity was correlated with rooting ability in ten clones of *Persea americana* Mill. (avocado) (RAVIV & REUVENI, 1984).

Inhibitory activity was implicated in the rooting ability of *Carya illinoensis* (Wang) K.Koch cuttings (TAYLOR & ODOM, 1970). Seasonal variation in the rooting ability of cuttings of the apple cultivars 'MM106', 'MM109' and 'EM1X' was correlated with inhibitory activity (LIPECKI & DENNIS, 1972). More inhibitory activity was present in extracts and xylem exudate of a difficult-to-root cultivar of *Dahlia variabilis* Desf. than in an easy-to-root cultivar (BIRAN & HALEVY, 1973b). And in *Phoenix dactylifera* L., a difficult-to-root clone contained higher inhibitory activity compared to an easy-to-root clone (REUVENI & ADATO, 1974).

In *Pyrus* L. (pear), an easy-to-root cultivar had high promotory activity whereas a difficult-to-root clone contained inhibitors (FADL & HARTMANN, 1967a); and more promotory activity and less inhibitory activity was associated with easy- as opposed to difficult-to-root cultivars of *Camellia* L. (RICHARDS, 1964), apple (ASHIRU & CARLSON, 1968) and *Syringa vulgaris* L. (BOJARCZUK, 1978).

Several unexpected results have been reported. Promotory activity of *Juniperus horizontalis* Moench. 'Plumosa', *Taxus cuspidata* Sieb. and Zucc. (LAMPHEAR & MEAHL, 1963) and *Castanea sativa* Mill. (GESTO, VAZQUEZ & VIEITEZ, 1977) extracts was similar throughout the year, despite a marked seasonal variation in rooting ability. Promotory activity was as great in flowering trees of *Pinus taeda* L. and *P.elliotii* Engelm. as in relatively easy-to-root seedlings of the same species (ZIMMERMAN, 1963). No consistent differences in promotory or inhibitory activity were evident between juvenile and adult apple '5' extracts (QUAMME & NELSON, 1965). An easy-to-root cultivar of *Chrysanthemum morifolium* Ramat. contained more promotory activity in the stem, but a difficult-to-root cultivar contained more in the leaves (STOLZ, 1968). The

levels of three promoters in mung bean were not related to a decrease in rooting ability with age (HEUSER & HESS, 1972a). More promotory activity was observed in the difficult-to-root *Picea glauca* (Moench.) Voss. var. *albertiana* (S.Brown) Sargent 'Conica' compared to the easy-to-root *Chamaecyparis lawsoniana* var. *fletcheri* Hornibrook (TOGNONI & LORENZI, 1972); and no satisfactory correlation could be found between promotory or inhibitory activity and the rooting ability of juvenile and mature *Pistacia vera* L. or of easy- and difficult-to-root cultivars of *Prunus* L. (cherry) (AL BARAZI & SCHWABE, 1985).

Various suggestions have been made to account for inconsistent results obtained in mung bean bioassay studies. Inconsistencies could be due to the common use for assay of alcoholic extracts. Aqueous extracts (TOGNONI, KAWASE & ALPI, 1977), centrifugate (KAWASE, 1970) or vacuum extracts of sap (BASSUK & HOWARD, 1981), appeared to give better correlations. Inconsistencies could also be due to variations in the extent to which co-factors are mobilized to the base of cuttings (LAMPHEAR & MEAHL, 1963). Finally, a rooting bioassay may lack sensitivity when the assay species is different from the species assayed. For example, more satisfactory results were obtained when separated extracts of *Pistacia vera* and cherry were assayed by cuttings of the same species than when assayed by cuttings of mung bean (AL BARAZI & SCHWABE, 1985).

The bioassay of plant extracts also ignores some potentially important sources of variation. The concentration and composition of the treatment solution are unknown, and many constituents of plant extracts would be expected to have nutritive value to cuttings. Treatment differences in many bioassay studies were not substantiated statistically and are therefore difficult to interpret.

2.3.2 KNOWN ENDOGENOUS PROMOTERS AND INHIBITORS

Rooting promoters (inferred as such in the mung bean bioassay) from separated plant extracts have been more or less positively identified in several studies. These include an aminophenol from juvenile *Hedera helix* and *Hibiscus rosa-sinensis* (HESS, 1964b); iso-chlorogenic acid (HESS, 1965) and a mixture of oxygenated terpenoids (HESS, 1966) from juvenile *Hedera helix*; a tentatively identified phenolic compound from *Pelargonium* L'Her. (JACKSON & HARNEY, 1970); lipid-like compounds from juvenile *Hedera helix* (HEUSER & HESS, 1972b); sesquiterpene lactones from *Helianthus tuberosus* L. (SHIBOAKA, MITSUHASHI & SHIMOKORIYAMA, 1967) and *Chrysanthemum moriflorum* (OSAWA, SUZUKI, TAMURA, OHASHI & SASADA, 1973); a bicyclic

terpene with a perhydroazulene nucleus from *Portulaca grandiflora* Hook. (MITSUHASHI, SHIBAOKA & SHIMOKORIYAMA, 1969); putative auxin-phenolic conjugates from pear (FADL & HARTMANN, 1967b) and *Hedera helix* (GIROUARD, 1969); a dihydrocholesterol from *Picea glauca* 'Conica' (TOGNONI & LORENZI, 1983); four compounds, the most active of which was 1,2,4 trihydroxy-n-heptadeca-16-yn, from avocado (RAVIV, BECKER & SAHALI, 1986); the wound metabolite glyceollin from *Glycine max* Merr. (YOSHIKAWA, GEMMA, SOBAJIMA & MASAGO, 1986); and a diterpene with a conjugated diketone function from *Coleus scutellarioides* Benth. and *C.blumei* Benth.(DEVRIESE, BUFFEL & GEUNS, 1988). Vitamins K, H' (HEMBERG, 1953) and D (BUCHALA & SCHMID, 1979) have also promoted rooting in bioassays.

Rooting inhibitors from *Eucalyptus grandis* have been identified as three closely related compounds which possess a fused bicyclic structure with a peroxide linkage (NICHOLLS, CROW & PATON, 1970), and as a 13C or 14C aromatic structure with an isobutyryl side chain (CROW, OSAWA, PATON & WILLING, 1977). In *Castanea sativa*, mature cuttings were found to contain two ellagic acid derivatives, which were absent from juvenile and etiolated cuttings, one of which significantly inhibited rooting in a *Phaseolus vulgaris* L. 'Contender' bioassay (VIEITEZ, KINGSTON, BALLESTER & VIEITEZ, 1987).

It would be surprising if these diverse plant constituents all had direct or specific roles in adventitious rooting, particularly as many of them have not been mentioned as being physiologically active in other contexts. This raises the possibility that they may have unspecific modes of action. This possibility tends to be supported by considering the yet greater diversity of non-endogenous compounds which have been found to promote rooting in mung bean (and other) cuttings.

2.3.3 NON-ENDOGENOUS PROMOTERS

This class of compounds includes potassium permanganate (CURTIS, 1918), hydrogen peroxide (WINKLER, 1927), acids (STOUTEMYER, 1938), naphthol sulphonate and potassium anthroquinone sulphonate (WENT, 1939), dimethylsulphoxide and tobacco smoke extract (STOLZ, 1951), sulphuric acid, potassium hydroxide and mercuric chloride (SOEJARKO, 1965), dinitrophenol (KRUL, 1968), sodium metabisulphite (GURUMURTI, CHIBBAR & NANDA, 1974), dihydroasparagusic acid (KUHNLE, CORSE & CHAN, 1975), cortisol (LOEYS & GEUNS, 1978), various antibiotics (SHANMUGASUNDARAM, JANARDHANAN & LAKSHMANAN, 1983), and a

fungal glycoprotein (MITSUHASHI, MAEDA, & FUJII, 1985).

Inhibitors of protein, RNA and DNA synthesis have also often promoted rooting, even though it is well established that new synthesis of protein, RNA and DNA are required during root initiation. This aspect was reviewed by HAISSIG (1986). The inhibitors may have indirect effects, for example by causing chemical injury (HAISSIG, 1986), or by delaying the development of the first formed primordia so that more of the later-forming primordia are allowed to develop (ANZAI, SHIBAOKA & SHIMOKORIYAMA, 1971; JARVIS, SHANNON & YASMIN, 1983).

2.3.4 PHENOLICS

According to the proposals of BOUILLENNE & BOUILLENNE-WALRAND (1955) and HAISSIG (1974a), ortho-diphenolics react with auxin to form 'rhizocaline'. After tentatively identifying a rooting co-factor in juvenile *Hedera helix* and *Hibiscus rosa-sinensis* as an aminophenol, HESS (1964b) supplied various known substances to mung bean cuttings and concluded that the promotory activity of the aminophenol could be attributed to a diphenolic moiety.

Applied phenolics are now well known as promoters of adventitious rooting in stem cuttings. Of the many phenolics tested on mung bean cuttings, 31 of 32 (BASSUK, HUNTER & HOWARD, 1981), all 13 tested (FERNQVIST, 1966), and 7 of 13 (HESS,1964b) promoted rooting. Four phenolics were promotory when applied to *Eranthemum tricolor* Nichols cuttings (BASU, BOSE, ROY & MUKHOPADHYAY, 1969). Three were mostly promotory to cuttings of five fruit tree species (MITRA, 1986). Four gave variable results when applied to cuttings of *Pinus radiata* D.Don. (SMITH & THORPE, 1977). One of four (2,5-dihydrobenzoic acid) promoted rooting in *Tilia americana* L. cuttings (MORSINK & SMITH, 1975). Coumarin promoted rooting in cuttings of *Impatiens balsamina* L. (DHAWAN & NANDA, 1982). Umbelliferone promoted rooting in *Phaseolus vulgaris* cuttings (VAZQUEZ, 1973). And rutin promoted rooting in cuttings of *Euonymus alatus* Sieb. 'Compactus' (LEE & TUKEY, 1971).

In vitro, phloretic acid promoted the rooting of apple 'M7' shoots (JONES & HATFIELD, 1976). Phloroglucinol promoted rooting in shoots of apple 'M7' (JONES & HATFIELD, 1976), apple 'M9' (JAMES & THURBON, 1981b; JAMES, 1983) and several other apple cultivars (ZIMMERMAN, 1984), *Prunus* L. (plum) 'Pixy' and cherry 'F12/1' (JONES & HOPGOOD, 1979), and *Theobroma cacao* L. 'Amelonado' (PASSEY

& JONES, 1983). Chlorogenic acid promoted rooting in shoots of plum 'Myrobalan' (HAMMERSCHLAG, 1982); and catechol promoted rooting in shoots of *Hedera helix* (HACKETT, 1970).

Thirteen of seventeen phenolics promoted rooting in cuttings of *Phaseolus vulgaris* (POAPST & DURKEE, 1967), but in the mildly alkaline test solutions used the phenolics were more or less oxidized to a mixture of quinones and complex substances. The more easily oxidized substances were most promotory, suggesting that the various oxidation products, rather than the original phenolics, were most active (POAPST, DURKEE & JOHNSTON, 1970). BASSUK & HOWARD (1981) found that the level or activity of a rooting promoter in the xylem sap of apple 'M26' shoots increased with a seasonal increase in rooting ability, and later showed that the promoter was similar to an oxidation product of phloridzin, an abundant phenolic in apple (BASSUK, HUNTER & HOWARD, 1981). POAPST & DURKEE (1967) suggested that one of the *in vivo* functions of the oxidized phenolics produced at a wound might be to improve rooting ability.

The anthocyanins are a common group of phenolics, easily recognized as the red pigments of some leaves, petals and young stems. There is some circumstantial evidence that anthocyanin metabolism can affect rooting ability (HAISSIG, 1986). For example, a high leaf anthocyanin content was associated with good rooting ability in stem cuttings of *Acer rubrum* L. (BACHELARD & STOWE, 1963) and *Hedera canariensis* Willd. (STOUTEMYER, BRITT & GOODWIN, 1961).

The anthocyanins themselves have not been shown to be promotory in a rooting bioassay but the possible precursors of anthocyanin biosynthesis, sucrose and riboflavin, improved rooting ability when supplied to *Eucalyptus camaldulensis* Dehnh. cuttings (BACHELARD & STOWE, 1962). The anthocyanins in relation to adventitious rooting in *Eucalyptus grandis* cuttings are discussed in Chapter 7.

2.4 CRITIQUE OF RHIZOCALINE

BOUILLENNE & BOUILLENNE-WALRAND (1955) proposed that rhizocaline is the

product of a reaction (facilitated by an oxidase enzyme) between auxin and an orthodiphenolic. Their only evidence to support this proposal was a reference to the work of NOEL (1951) and SIRONVAL (1947), whose studies: "...have shown that the initiation of adventitious roots is linked to the previous appearance of orthodiphenolics."[1] These studies were not identified by BOUILLENNE & BOUILLENNE-WALRAND (1955) and therefore cannot be cited. The requirement for the third component of rhizocaline, the oxygen-requiring enzyme, was simply inferred by BOUILLENNE & BOUILLENNE-WALRAND (1955) (assuming that a reaction occurs between auxin and another substance) since: "...studies on adventitious root initiation and growth have all shown that these processes require oxygen."[2]

All stem cuttings are wounded, which releases formerly compartmented phenolics, and IAA is thought to be ubiquitous in higher plants. Obviously the existence of rhizocaline depends on whether these two classes of substances react together *in vivo*. The work referred to by BOUILLENNE & BOUILLENNE-WALRAND (1955) evidently does not demonstrate this.

FADL & HARTMANN (1967b) and GIROUARD (1969) gave evidence for auxin-phenolic conjugates in plant extracts (in the latter case tentatively identified later by HAISSIG (1974a)). However, in a review of the many bound (conjugated) forms of auxin which occur naturally, COHEN & BANDURSKI (1982) did not mention auxin-phenolic conjugates.

Although they may not occur naturally, auxin-phenolic conjugates can be produced *in vitro*: IAA formed addition products spontaneously with the quinones produced by the action of phenolase on various phenolics (LEOPOLD & PLUMMER, 1961).

The only evidence that putative auxin-phenolic conjugates are active in root initiation was given by FADL & HARTMANN (1967b), who found that the putative conjugate promoted rooting in mung bean cuttings. Since such a large diversity of compounds have promotory activity in the mung bean bioassay, the physiological significance of this observation is uncertain.

1 "ont montre que la neoformation des racines adventives est liee a l'apparition prealable de radicaux orthodiphenoliques."

2 "...les etudes sur la cinetique de la rhizogenese (neoformation et croissance) ont toutes montre que ces phenomenes exigent la presence d'oxygene..."

Both applied auxins and applied phenolics (especially oxidized forms) generally promote adventitious rooting in stem cuttings. Where both have been applied, phenolics generally have been able to evoke a response in addition to that evoked by auxin. This has led very many workers to describe phenolics as synergists or 'co-factors' of auxin.

However, there is no justification for assuming that auxins and phenolics work somehow in concert. No association between the modes of action of phenolics and auxin has been demonstrated. That applied phenolics and applied auxin are synergistic when applied together is not satisfactory evidence for a direct association. To postulate an *in vivo* reaction between auxin and phenolics is quite unnecessary. Moreover, there is no strong evidence that auxin-phenolic conjugates occur naturally in plants, or (if they do) that they have any role in adventitious rooting.

2.5 OTHER MODES OF ACTION SUGGESTED FOR PHENOLICS

Dissatisfaction with the rhizocaline concept was implicit in the discussion of JAMES & THRURBON (1981a) and BASSUK, HUNTER & HOWARD (1981). Both sought a more general explanation for the promotory activity of phenolics.

2.5.1 REDOX BALANCE

JAMES & THURBON (1981a) revived the idea, proposed by STONIER, HUDEK, VANDE-STOUWE & YANG (1970), that some phenolics might: (i) protect IAA by inhibiting peroxidase ('IAA oxidase'), and (ii) inhibit oxidation reactions in general, maintaining the cell in a reduced state and perhaps allowing cells to divide.

Monophenols are supposed to enhance, whereas polyphenols are supposed to inhibit, IAA oxidation *in vitro*, and hence the latter 'protect' auxin (HARE, 1964). However, the *in vivo* significance of such observations has not been well established (WAREING & PHILLIPS, 1981). The auxin protection hypothesis was reviewed (but not favoured) by HAISSIG (1974a).

The apparent inhibition of IAA oxidase by polyphenolics *in vitro* could be an artefact since these phenolics and phenolase can react at alkaline pH to give quinones, which could then oxidize tryptophan to IAA (GORDON & PALEG, 1961). The net rate of IAA destruction would therefore be diminished, but not necessarily the actual rate. This pathway is unlikely to be followed *in vivo* because phenolics and phenolase are probably

effectively separated in the intact cell.

Peroxidase activity increases in an early phase of root formation and decreases in a later phase, and it is often assumed that this fluctuation has an effect on root initiation *via* IAA metabolism (HAISSIG, 1986; JARVIS, 1986). However, peroxidase may also participate in respiration, cell wall synthesis, lignin formation, senescence and defence against pathogens, and is also associated with the evolution of ethylene and various stresses, including salt stress and hypoxia (GASPAR, PENEL, THORPE & GREPPIN, 1982). In view of its numerous forms and versatile catalytic abilities, peroxidase is a confusing enzyme (STAFFORD, 1974), and its role in adventitious rooting need not be an effect on auxin metabolism.

Some early work sought to establish an association between redox state and mitosis. Reduced-state hydroquinones were characteristic of dividing cells, whereas mature cells contained the quinones themselves (VAN FLEET, 1954). The reducing agent cysteine hydrochloride promoted mitosis, perhaps due to its ability to maintain phenolics in the reduced state (REED, 1949); and cell division was associated with a thiol-disulphide equilibrium, the reduced thiols being characteristic of metabolic activity (SIEGEL & PORTO, 1961).

This approach no longer appears to attract attention. No unifying hypothesis for the control of cell growth and division has emerged, and the biochemical basis for variations in the length of the cell cycle, thought to reside in the G1 phase (the period between mitosis and DNA synthesis), remains obscure (LLOYD, POOLE & EDWARDS, 1982).

In summary, any *in vivo* role for phenolics in IAA metabolism (*via* an effect on peroxidase), or on rooting ability *via* an effect on the capacity for mitosis, has not been well substantiated.

2.5.2 THE WOUND RESPONSE

BASSUK, HUNTER & HOWARD (1981), following POAPST & DURKEE (1967), drew attention to the role of phenolics and their derivatives in the wound response.

Wounding causes conspicuous changes in the phenolic metabolism of affected tissues (RHODES & WOOLTORTON, 1978). Cells which are damaged may suffer a breakdown in compartmentation, which results in the oxidation of pre-existing phenolics by phenolase. The phenolics may then polymerize to brown or black tannin-like substances which, like tannins, can precipitate proteins and impede the progress of

infecting organisms. Since the reactions beyond phenolase are thought to proceed non-enzymatically it seems likely that the highly reactive o-quinones could react with other potential reactants in the cell, apart from each other, such as those with amino, amine or thiol groups (PIERPOINT, 1970).

Wound responses can also be evoked in undisrupted cells in the vicinity of a wound. Such cells frequently show an increase in phenylpropanoid metabolism, which results in the new synthesis of phenolics (RHODES & WOOLTORTON, 1978), regardless of whether the cells are responding to a mechanical or chemical wound or to infection by microorganisms (KOSUGE, 1969). The role of these accumulating phenolics (especially cinnamic acid derivatives) is unclear, although quinone products and other phenolics may inhibit microbial growth, and phenolics participate in the formation of lignin and suberin which may act as a physical barrier at the surface of a wound (McCLURE, 1960; SALIN & BRIDGES, 1981). A local accumulation of phenolics close to a wound may be supplemented by the basipetal translocation of soluble phenolics, as occurs in *Pinus banksiana* Lamb. seedling cuttings (MONTAIN, HAISSIG & CURTIS, 1983).

However, wounds evoke many other responses apart from their effects on phenolic metabolism. Wound signals, which may be essentially chemical (GUSTAFSON & RYAN, 1976) or electrical (VAN SAMBEEK & PICKARD, 1976), may be propagated throughout the plant. A systemic wound signal appeared to weaken the cell membrane, inferred from a lowered protoplast yield (WALKER-SIMMONS, HOLLANDER-CZYTKO, ANDERSEN & RYAN, 1984). Wound signals have also increased cell membrane permeability, causing an ion influx (ZOCCHI & HANSON, 1982) or increasing the capacity for protein synthesis (DAVIES & SCHUSTER, 1981). In mung bean seedlings, severing stems from their roots to make stem cuttings stimulated RNA and DNA synthesis (inferred from the incorporation of radioactive uridine and thymidine) locally throughout the hypocotyl, whereas roots developed subsequently only at the base (TRIPEPI, HEUSER & SHANNON, 1983).

Membranes within cells affected by wounding may break down and be subjected to peroxidation (THEOLOGIS & LATIES, 1981; THOMPSON, LEGGE & BARBER, 1987). Membranes may be destroyed either by the dissociation of their constituents or by autophagy (MORRE, 1975). Autophagy, accomplished by lysosomes, can be induced or increased by many sub-lethal treatments (PITT, 1975). Membrane breakdown has been associated with an accumulation of lipids, thought to be membrane breakdown products (LATIES, 1978). This lipid accumulation may lead to the release of free fatty acids, impairing respiration and temporarily inducing the cyanide insensitive pathway (LATIES, 1982).

Ethylene is evolved, as a consequence of wounding and various other stresses (ABELES, 1973), in proportion to the number of cells which are perturbed without being killed (ELSTNER & KONZE, 1976). Ethylene can induce peroxidase activity (GASPAR, PENEL, THORPE & GREPPIN, 1982) as well as the enzymes concerned with phenylpropanoid metabolism (CHALUTZ, 1973; RHODES, HILL & WOOLTORTON, 1976).

Any membrane breakdown is followed by new membrane synthesis, which may result in changes in compartmentation and membrane composition (BENEVISTE, 1978). For example, the inner membranes of mitochondria from wounded tissue have properties which differ from those from fresh tissue (ASAHI, 1978).

Terpenoids (STOESSL, STOTHERS & WARD, 1976; KUC & LISKER, 1978) and ascorbic acid (JOHNSON & SCHAAL, 1957) can also increase in response to wounding, and free fatty acids can be translocated from an unwounded to a wounded part of the plant (BREDEMEIJER & HEINEN, 1968).

Other wound responses could be expected at the base of the stem cutting by virtue of its position in the rooting medium, which is dark, wet and probably hypoxic in relation to the aerial environment. Ethylene may accumulate, either through being unable to escape (KAWASE, 1976) or through new synthesis (CLEMENS & PEARSON, 1977). Flooding-intolerant species produce ethanol when waterlogged (McMANMON & CRAWFORD, 1971) but other less toxic products are associated with flooding tolerance. These include shikimic acid (TYLER & CRAWFORD, 1970), lactic acid (HOOK, BROWN & WETMORE, 1972), malic acid (CRAWFORD, 1967), succinic acid (CRAWFORD & TYLER, 1969), an amino butyric acid (FULTON, ERICKSON & TOLBERT, 1964) and glycerol (CRAWFORD, 1971). Anaerobiosis caused by submergence can also cause changes in protein synthesis (MOCQUAT, PRAT, MOUCHES & PRADET, 1981).

Most of these various wound responses have not received attention in relation to adventitious rooting, and caution is obviously required before assigning any consequence of the wound response to phenolics alone. It is probably more realistic, if not more illuminating, to accept that the entire metabolism of affected cells changes in response to wounding.

There is no well substantiated role for phenolics in adventitious rooting: their mode (or modes) of action as rooting promoters, when supplied to stem cuttings, is unknown. Very many other promoters have unknown modes of action, and it would be satisfying if they all acted in the same way. Since such a large diversity of promoters have been identified,

they could have a common mode of action only if promotory activity was largely unrelated to chemical identity. One way in which this could happen can be envisaged: stress or injury caused by a high concentration of a treatment solution could promote rooting.

2.6 PROMOTION OF ROOTING DUE TO STRESS OR INJURY (ADDITIONAL TO THE WOUND RESPONSE)

After finding that naphthol sulphonate and potassium anthroquinone sulphonate promoted the rooting of *Pisum sativum* L. cuttings, WENT (1939) suggested that there are two stages in root formation: a first stage, which had been satisfied by the (presumably) non-specific chemicals, and a second stage which was auxin-specific. The concept of a two-stage process was supported by MITSUHASHI, SHIBAOKA & SHIMOKORIYAMA (1969), who showed that portulal, a bicyclic terpene, could promote rooting when applied at an early stage in several assay species.

An apparently unspecific early stage could be related to stress or injury. SOEKARJO (1965) promoted rooting in *Coleus scutellarioides* cuttings by supplying just sub-lethal concentrations of IAA or the unspecific chemicals H_2SO_4, KOH and $HgCl_2$. Indole and naphthol were also more synergistic than various phenolics when supplied to cuttings of *Phaseolus vulgaris* (GORTER, 1962; 1969). This was an unexpected result in relation to the auxin protection hypothesis, according to which the ortho-diphenolics should have shown the greatest synergism. SOEKARJO (1965) expressed the view, endorsed by GORTER (1969), that the various treatments had allowed affected cells to become more susceptible to the influence of endogenous auxins. JARVIS (1986) pointed out that this could be equated with the first of WENT's (1939) two phases of root formation.

Treatment of *Coleus* Lour. internodes with H_2SO_4 and KOH caused inhibitors of IAA oxidase to be liberated, suggesting a possible way in which these chemicals could promote rooting (SOEKARJO & JANSSEN, 1969). Alternatively, since 10^{-3}M IAA had begun to destroy the cytoplasm of leaf epidermal cells of *Rhoeo discolor* Hance within 20 minutes of application (VON GUTTENBERG & MEINL, 1952), SOEKARJO (1965) considered that injurous concentrations of a treatment solution would result in a loss of organization or differentiation within the cell. This would then render the cells more able to differentiate in a new direction, making root initiation more likely.

This view has received support from later work, including that already mentioned in relation to the diversity of the wound response. Wounding can initiate a degenerative phase in the metabolism of affected cells which causes the loss of some of the structural

and biochemical attributes of differentiation. However, while this loss can be defined as dedifferentiation (ESAU,1977), its relationship to developmental plasticity is obscure because so little is known about 'determination', or the commitment to a particular developmental pathway (WAREING, 1982).

BONGA (1982) has speculated that morphogenic ability is inversely related to the quantity of organellar DNA in the cell. For example, the cells lining the resin ducts of conifers have high morphogenic ability *in vitro* (BONGA, 1981), and also have few plastids of simple structure relative to the surrounding parenchyma (WOODING & NORTHCOTE, 1965). Similarly, cells entering meiosis, which achieve the most plastic state possible, suffer partial lysis of their contents (DICKINSON & HESLOP-HARRISON, 1977; SHEFFIELD & BELL, 1979; LUCK & JORDON, 1980), although in this case lysis also occurs within the nucleus (SHEFFIELD, CAWOOD, BELL & DICKINSON, 1979). In general, in the course of re-differentiation, old biochemical or structural differentiation would be expected to be lost to some extent.

So many diverse substances could promote rooting when applied to stem cuttings simply by increasing the magnitude of certain wound responses, which would cause differentiation to be weakened or lost. Presumably, as in SOEKARJO's (1965) study, rooting would be promoted most by a sub-lethal concentration of the treatment solute, and its chemical identity would be relatively unimportant.

2.6.1 THE POSSIBLE *IN VIVO* OCCURRENCE OF PROMOTORY STRESS OR INJURY (ADDITIONAL TO THE WOUND RESPONSE)

At first sight it seems improbable that an endogenous substance could stress or injure the tissue which produced it. However, the concept of stress or injury at the sub-cellular level can be easily oversimplified. Cell constituents turn over quite rapidly in healthy cells. For example, membrane proteins have exponential decay curves, and the membranes of the endoplasmic reticulum have half lives of 2-4 days (MORRE, 1975). WHATLEY (1978) proposed a cyclical pattern of development for plastids implying that 'stress', which reduces plastid organization in prokaryotes (WHATLEY, 1978) and eukaryotes (NIR, KLEIN & POLJAKOFF-MAYBER, 1969; WHATLEY, 1971; HSIAO, 1973), is part of the 'normal' life cycle (WHATLEY, 1978). TREWAVAS & JENNINGS (1986) pointed out that higher plants, being sessile, rely on plastic developmental changes to adapt to environmental variation, making it particularly difficult to define normality.

Endogenous phenolics may reach effectively sub-lethal concentrations in some circumstances, such as in the novel environment at the base of a cutting. For example, newly explanted cultures *in vitro* sometimes suffer a progressive and fatal 'browning reaction', which is assumed to be due to an unregulated wound response. Applied phenolics generally promote rooting at about 10^{-3}M (JAMES & THURBON, 1981a), a high concentration which could be injurous depending on the toxicity of the phenolics concerned.

Phenolics are toxic to plants. They are generally glycosylated or otherwise conjugated in the plant (GLASS & BOHM, 1971; HARBORNE, 1979), to increase their solubility and to reduce their toxicity (PRIDHAM, 1959). Nevertheless, phosphate uptake by barley roots was inhibited by concentrations of phenolic acids as low as 5×10^{-5}M (GLASS, 1973). The relatively high activity of oxidized as opposed to reduced phenolics in promoting rooting (POAPST & DURKEE, 1967) could be due simply to their higher toxicity (reactivity). An easy-to-root clone of *Rhododendron* contained more phenolase than a difficult-to-root clone (FOONG & BARNES, 1981), which would perhaps result in more oxidized phenolics at the site of a wound.

Several observations suggest that endogenous phenolics may be associated with rooting ability. ROY, ROYCHOUDHURY, BOSE & BASU (1972) found a correlation between the kind of phenolics characteristic of a species and rooting ability. Phloridzin (an abundant phenolic in apple) increased in the xylem sap of apple 'M26' shoots prior to a seasonal increase in rooting ability (BASSUK, HUNTER & HOWARD, 1981). Apple shoot cultures which had been grown on a medium containing phloroglucinol subsequently rooted better than those which had been grown in its absence (JAMES & THURBON, 1981b). And applied phenolics increased the rooting ability of shoot cultures of the difficult-to-root apple clone 'M9' (JAMES & THURBON, 1981a), but had no promotive effect on the easy-to-root clone 'M26' (JAMES & THURBON, 1981a).

It is conceivable that other constituents of the plant could also reach locally high concentrations at the base of the cutting, perhaps sufficient to cause de-differentiation.

2.7 CONCLUSION

The concept of promoters and inhibitors of adventitious rooting (including rhizocaline and rooting co-factors) represents the original and traditional approach to the problem of adventitious rooting in stem cuttings. The concept is founded on the bioassay principle,

in which plant extracts or known compounds are supplied to stem cuttings and found to promote or inhibit rooting.

It is conventional to assume that activity in the bioassay reflects *in vivo* activity and hence has physiological significance. However, this assumption is not necessarily warranted because, although a large diversity of known and unknown compounds has been found to inhibit or promote rooting in rooting bioassays, no well substantiated mode of action for them has been put forward.

One mode of action for rooting promoters is suggested, which would operate if the active substance were present in a treatment solution at very high (sub-lethal) concentrations. Under some circumstances this could be envisaged to occur *in vivo*, but it could equally occur in suitable bioassay conditions.

In either case the action would be unspecific, analagous to the first of WENT's (1939) two phases of root formation as developed by SOEKARJO (1965). Various observations suggest that a physical wound propagates a signal (which may be systemic), which induces changes in the metabolism of affected cells. The wound-induced changes would be likely to be both degenerative and regenerative, and they could be initiated, exacerbated or prolonged by chemical injury additional to the physical wound. The perturbed cells could become relatively open to new influences (generated by setting the cutting), and thus more likely to initiate roots.

This mode of action would have several consequences. If a solute could be injurous at one concentration it could also be lethal at a higher concentration. Thus, a solute could either promote or inhibit rooting depending on its concentration in the treatment solution, and therefore could not be classified as a 'promoter' or 'inhibitor' on the basis of bioassay evidence alone. The most promotory concentrations of a treatment solution would be adjacent to inhibitory concentrations, and the range of promotory concentrations would probably not be great. Also, known 'promoters' and 'inhibitors', as well as solutes with no *in vivo* function, should all have comparable promotory activity. These possibilities are considered in the following chapter through work with the mung bean bioassay.

CHAPTER THREE

PROMOTION AND INHIBITION OF ADVENTITIOUS ROOTING IN THE MUNG BEAN BIOASSAY

3.1 INTRODUCTION

In many studies, eluates of separated plant extracts and various known endogenous and synthetic (non-endogenous) substances have promoted or inhibited rooting when applied to stem cuttings of mung bean. These were reviewed in the previous chapter and a common mode of action for them was proposed, according to which rooting could be promoted by injurous concentrations of a treatment solute.

SOEKARJO (1965) suggested that injury caused by high concentrations of a treatment solute could promote rooting, and this view was endorsed by GORTER (1969). MITSUHASHI MAEDA & FUJII (1985) also suggested that promotion of rooting by metabolic inhibitors might be closely related to cell damage, and interpreted the promotory effect of a fungal glycoprotein on rooting of mung bean cuttings as perhaps due to a hypersensitive reaction in the cutting.

SOEKARJO & JANSSEN (1969) suggested that IAA might be conserved by sub-lethal treatments, since applied acid or base liberated inhibitors of IAA oxidase in *Coleus* internodes. LEE, PAUL & HACKETT (1977) confirmed that pretreatment with acid or base promoted rooting in cuttings of several species, but considered that the acid treatment worked best for calciphilous species while treatment with base was best for calcifuges. Similarly, KHOSH-KHUI & TAFAZOLI (1979) found that the rooting of cuttings of *Rosa damascena* Mill., which grows well on alkaline soils, was promoted by acid whereas base had no effect.

The mode of action put forward by SOEKARJO (1965) would have several consequences: a solute could either promote or inhibit rooting, depending on its concentration in the treatment solution; the most promotory concentrations of a treatment solution would be adjacent to inhibitory concentrations; the range of promotory concentrations may not be great; and known 'promoters' and 'inhibitors', as well as solutes with no *in vivo* function, should all have comparable promotory activity. These predictions are tested in this chapter, through concentration series experiments using the mung bean bioassay.

The mung beans used for the experimental work were bought locally and are assumed to belong to *Vigna radiata* (L.) Wilczek. RACHIE & ROBERTS (1974) recognize mung beans and urd beans as subspecies of *V.radiata*: var. *aureus* and var. mungo respectively, but FERY (1980) preferred to recognize two species, *V.radiata* (L.) Wilczek and *V.mungo* (L.) Hepper, since the U.S. Department of Agriculture does so. Mung and urd beans are distinguished from each other by pod characteristics (RACHIE & ROBERTS, 1974), which are not observed in bioassay work, and given the recent changes in taxonomic status and the ability of both beans to hybridize with several taxa, including each other (FERY, 1980), it is not possible to be categorical about the botanical identity of the test plants used in the assay.

3.2 MATERIALS AND METHODS

Mung bean seeds were surface sterilized in 3% hypochlorite solution for 10 to 20 minutes then rinsed and soaked in tap water for 4 to 8 hours. The seeds were placed with forceps in seed trays containing vermiculite at a spacing of 1.8 × 1.6 cm (3 cm^2 per seed), and covered lightly with vermiculite. They were grown in a cabinet with 20 hours of 250 μE. P.A.R. and 4 hours dark, a constant temperature of 25°C and unknown humidity. The beans were harvested once the cotyledons had begun to abscise and could be dislodged without injury to the stem. This was done so that the cotyledons would not drop into and contaminate the test solutions. The cuttings were prepared by severing the bean plants close to the medium level and placing them, within a few seconds, in a water-filled tray so that their bases were immersed. After up to an hour they were put in vials filled to 4 cm depth with tap water (the control treatments), or solutions of known solutes prepared with tap water. The cuttings were then replaced in the cabinet, under the same environmental conditions as which they had been grown.

Each treatment within an experiment consisted of three vials arranged in randomized complete blocks. The number of cuttings per vial varied between experiments from six to eight, with the exception of the experiment shown in Figure 3.3a, in which the number of cuttings per vial was three. The vials stood in trays at a spacing of 4.4 × 4.4 cm. After 24 hours the beans were rinsed and transferred to clean tap water if appropriate. Tap water gave a higher number of roots per cutting than once-distilled glass-distilled water, perhaps due to the presence of promoters such as boron (MIDDLETON, JARVIS & BOOTH, 1978). Vials were topped up (every 24 hours) with distilled water because the rate of water uptake varied between treatments. Adventitious roots began to emerge after 3 to 4 days and were counted after 7 days.

Eight solutes at various concentrations were supplied: phenol and catechol, which should promote rooting according to the view that they resemble endogenous 'co-factors'; indole-3-butyric acid (IBA), the well known synthetic auxin and promoter of adventitious rooting; 'G' (short for *grandis*), the putative rooting inhibitor isolated from *Eucalyptus grandis* (NICHOLLS, CROW & PATON, 1970); an acid (HCl), a base (NaOH), a salt (NaCl) and polyethelene glycol (mol. wt. *c.* 200). The phenol, catechol, IBA and G experiments were repeated once.

The solutes were directly soluble in water except for IBA and G. These latter were dissolved in a few drops of ethanol then added drop-wise to warm water. The water was kept at close to 80°C for a few minutes to drive off the ethanol, cooled and made up to the volume required to give the highest desired molarity. As with the other solutes the lower molarities were then obtained by dilution.

The G was obtained from Dr. D.M. Paton (Australian National University, Canberra, A.C.T., Australia). It consisted of a mixture of three closely related compounds, G1, G2 and G3, and probably contained traces of grandinol (PATON, D.M., 1986; personal communication).

Data from several experiments were combined to determine whether the mean of mung bean root counts is related to the standard deviation. Mung bean root counts have not been transformed before analysis in previous studies, except by JACKSON & HARNEY (1970). They used the log transformation, implying a linear relationship between the mean and standard deviation.

Mung bean root count data were transformed $X = \log(X + 1)$ before being analyzed by analysis of variance. A large number of zeros in the data probably render this transformation unsatisfactory (see results), so markedly inhibitory treatments were excluded from the analyses. This procedure was justifiable because all of the solutes tested were able to completely inhibit rooting at high concentrations by killing the cuttings.

Means presented graphically (Figures 3.2a and b, 3.3a and b, 3.4 and 3.5) are the detransformed (derived) values: $X = (\text{antilog } X)-1$. However, the numerical summary of the analyses (Table 3.1) is of the transformed values since this is more appropriate for the presentation of least significant differences (L.S.D.s).

3.3 RESULTS

The means and standard deviations of mung bean root counts are linearly related, indicating that the log transformation X = log (X) would be appropriate, or X = log (X + 1) if the data contain zeros (Figure 3.1).

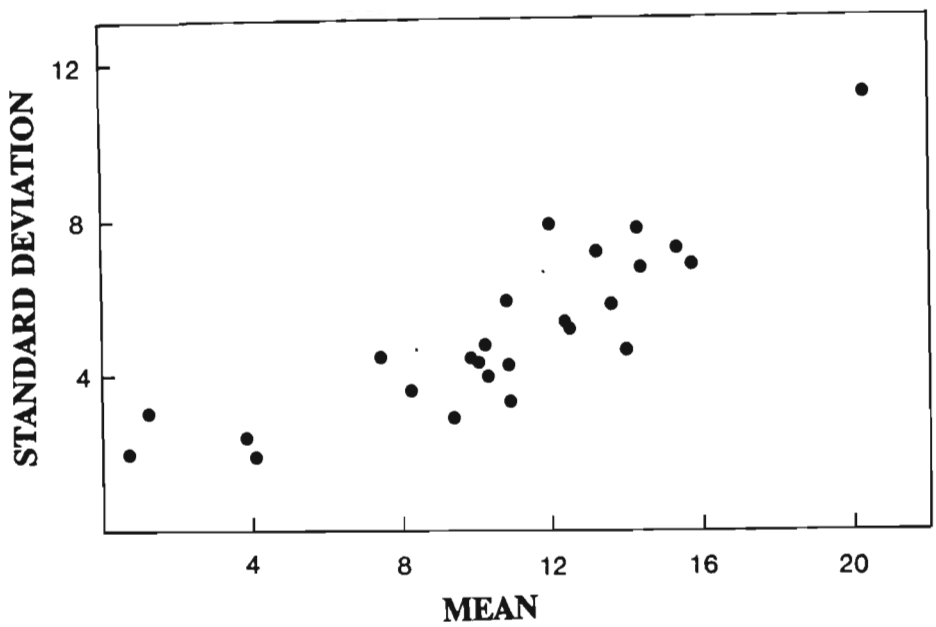

Figure 3.1. Relationship between the mean and standard deviation of mung bean root counts.

The two points with the lowest means in Figure 3.1 may represent a real deviation from the linear relationship. These treatments were almost lethal to cuttings. Generally, many of the cuttings in such treatments were killed whereas those which survived often had moderate or high numbers of roots, increasing skewness and variability. This suggests that the transformation X = log (X + 1) may not be satisfactory for data which contain many zeros.

Figures 3.2a and b, 3.3a and b, 3.4 and 3.5 show that all eight solutes were able to kill mung bean cuttings and inhibit rooting at high concentrations, and all promoted rooting at lower concentrations. This promotion was confirmed as being statistically significant at the 95% confidence level in most cases (Table 3.1), although the error variances were high. The promotion was invariably significant at the 90% confidence level. The phenol, catechol, IBA and G experiments were repeated once (Figure 3.2a and b, 3.3a and b), and show that the variation between occasions is also high. Nevertheless the trends are consistent.

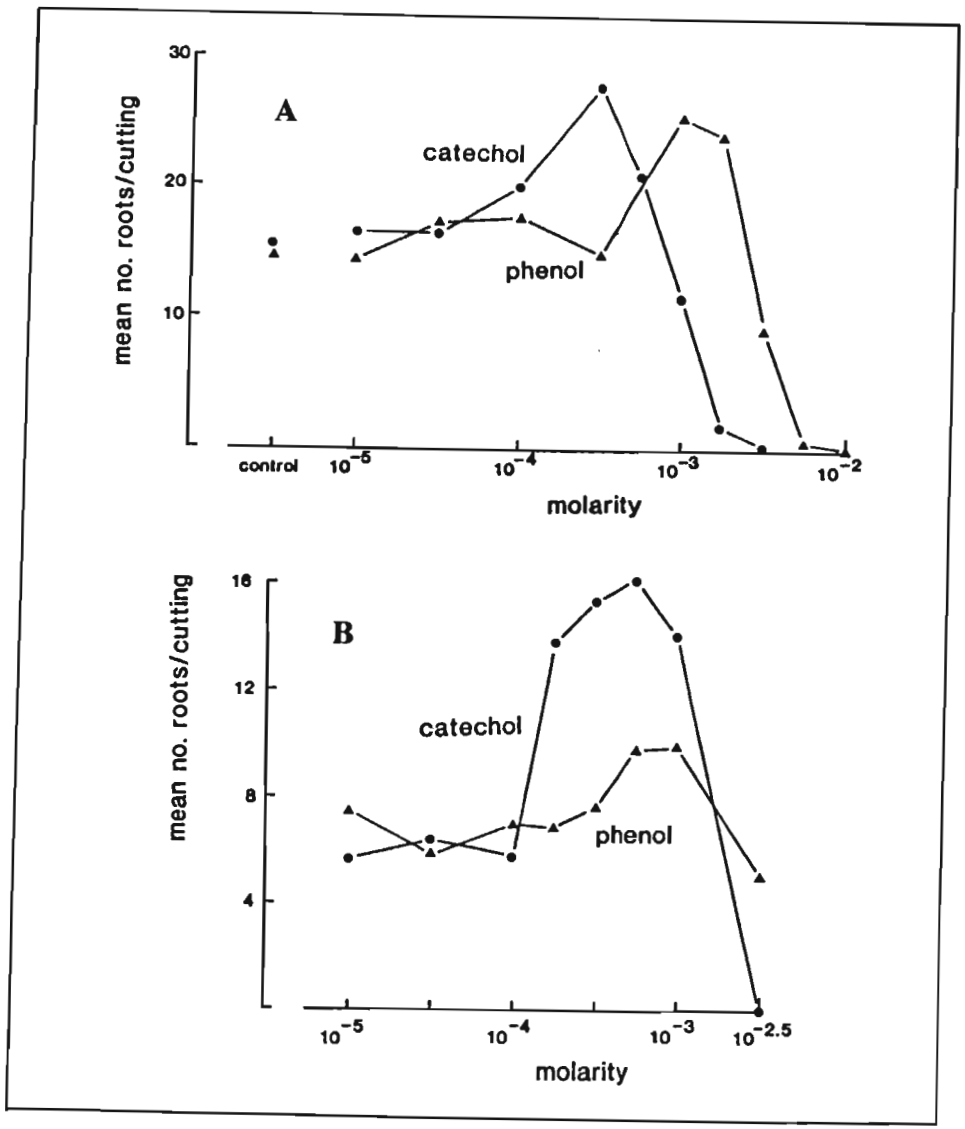

Figure 3.2a and b. The effect of the molarity of phenol and catechol on the de-transformed mean number of roots per mung bean cutting.

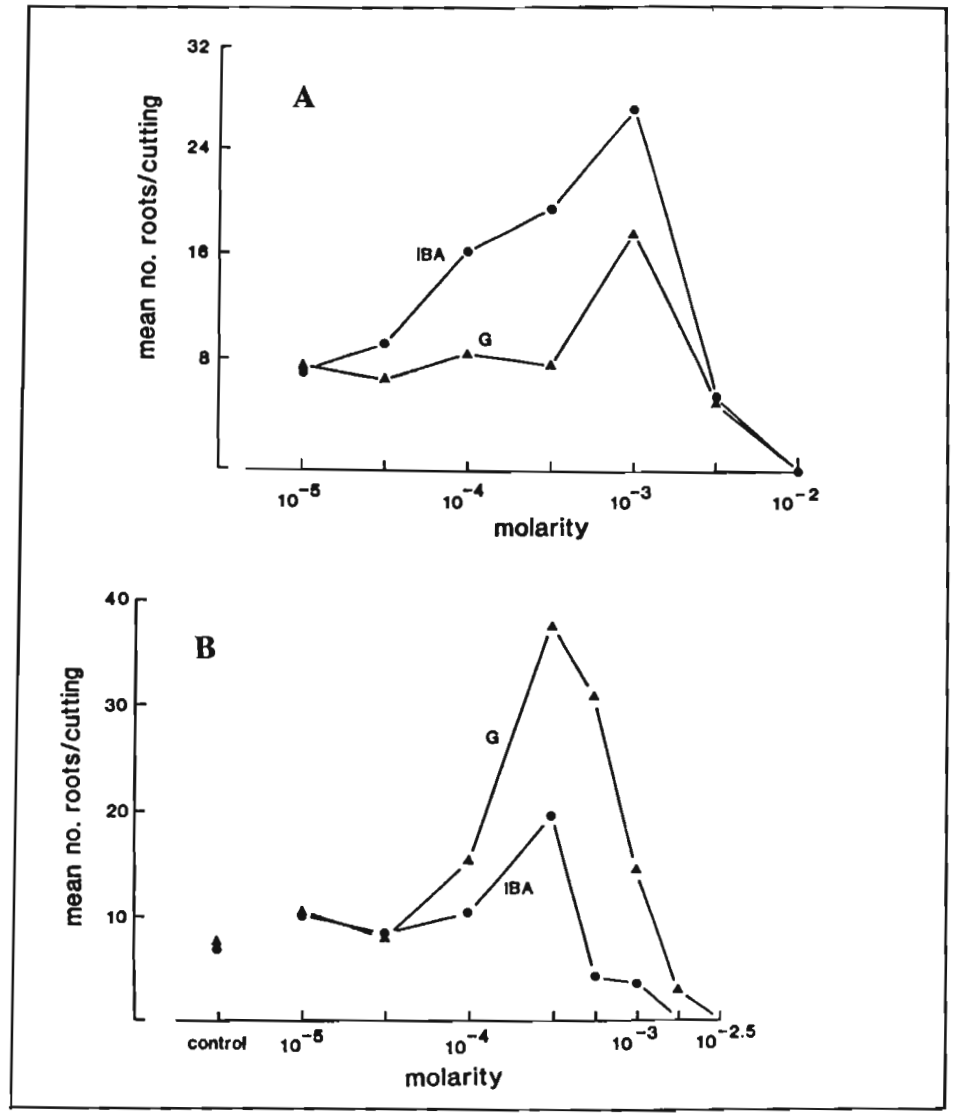

Figure 3.3a and b. The effect of the molarity of IBA and G, the putative rooting inhibitor from E. grandis, on the de-transformed mean number of roots per mung bean cutting.

Table 3.1. The effect of the molarity of various solutes on the transformed mean number of roots per mung bean cutting. Lines connect means which are not significantly different at the 95% confidence level. Asterisks denote treatments which were lethal or sub-lethal and which were excluded from the analyses.

		MOLARITY																
		CONTROL	10^{-5}	-4.5	-4	-3.75	-3.5	-3.25	-3	-2.75	-2.5	-2.25	-2	-1.75	-1.5	-1.25	-1	-0.5	L.S.D.
FIGURE	SOLUTE																		
3.2a	PHENOL	2.737	2.730	2.895	2.917		2.752		3.269	3.212	2.314	*	*						0.344
3.2a	CATECHOL	2.821	2.857	2.849	3.041		3.355	3.085	2.523	*	*								0.327
3.2b	PHENOL		2.133	1.916	2.077	2.062	2.149	2.377	2.385		*								0.319
3.2b	CATECHOL		1.889	1.993	1.904	2.687	2.789	2.839	2.705		*								0.219
3.3a	IBA		2.102	2.308	2.842		3.022		3.346		*		*						0.858
3.3a	G		2.104	2.007	2.233		2.142		2.812		*		*						0.917
3.3b	IBA	2.119	2.405	2.240	2.443		3.029	*	*	*									0.307
3.3b	G	2.134	2.441	2.196	2.796		3.657	3.463	2.751	*	*								0.448
3.4	HCl	2.364							2.152		2.608		2.805	*	*				0.453
3.4	NaOH	2.364							2.371		2.333		2.428	2.627	*	*			0.289
3.5	NaCl	2.328							2.304		2.412		2.529	2.660	2.375	2.050	1.550		0.240
3.5	P.E.G.	2.328							2.441		2.523		2.703	2.740	2.580	2.537	2.347	1.455	0.364

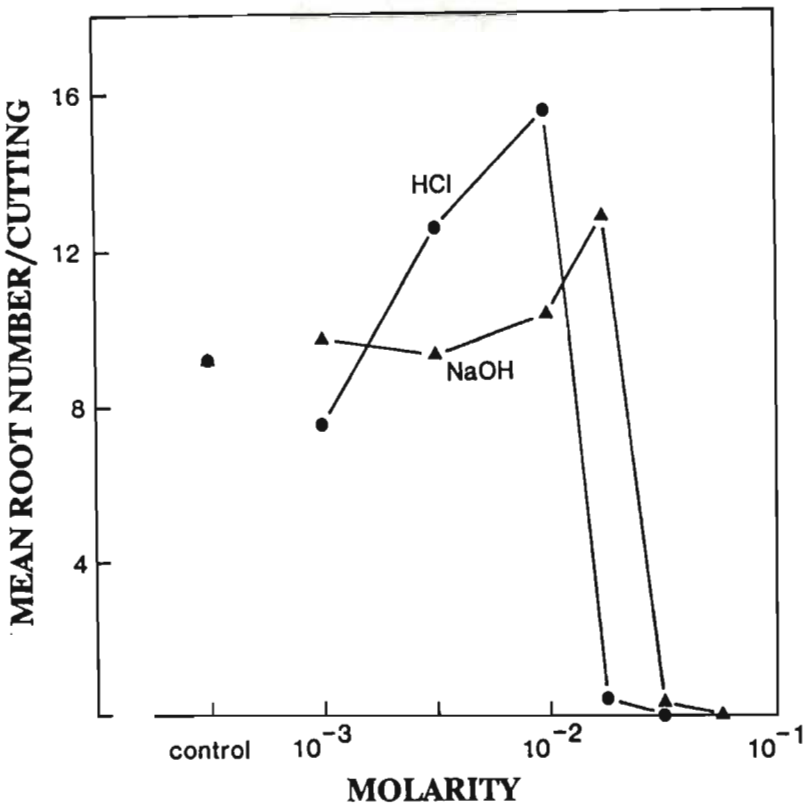

Figure 3.4. The effect of the molarity of HCl and NaOH on the de-transformed mean number of roots per mung bean cutting.

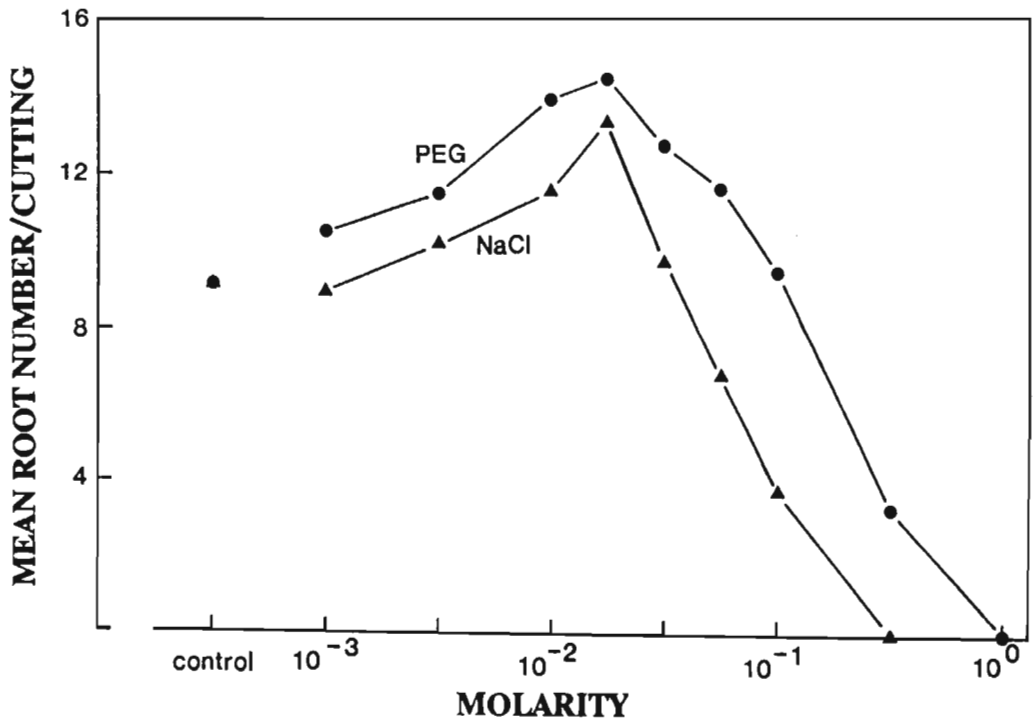

Figure 3.5. The effect of the molarity of common salt and polyethelene glycol on the de-transformed mean number of roots per mung bean cutting.

3.4 DISCUSSION

The results indicate that a non-specific effect promotes rooting in mung bean cuttings, and largely bear out the four predictions of the previous chapter. The eight solutes tested were able to both promote and inhibit rooting, depending on their concentration in the treatment solution. The promotory concentrations were 'adjacent' to inhibitory ones. The range of promotory concentrations was generally not great. And 'promoters', 'inhibitors' and compounds with no *in vivo* function were all able to promote rooting to some extent. Any additional promotory activity which might have been expected from auxin or phenolic 'co-factors', or any additional inhibitory activity which might have been expected from 'G', the *Eucalyptus* inhibitor, was not apparent. Although there was a lot of variation within and between experiments, the trends were consistent: the treatment effects evidently owed more to the concentration of the various solutes than to their chemical identity.

A few incidental observations in previous studies also suggest that most promotory activity in the mung bean bioassay is elicited at close to inhibitory concentrations. High rooting in assay plants was associated with flaccidity in the lowest part of the stem (CHALLENGER, LACEY & HOWARD, 1965; TOGNONI & LORENZI, 1972). Experience suggests that the lower stems become flaccid and die when the treatment solution is at a sub-lethal concentration. Treatments with IBA and ethephon, which promoted rooting in mung bean cuttings, resulted in a vertical re-distribution of roots away from the base of the cuttings (ROBBINS, KAYS & DIRR, 1983), perhaps for the same reason. In a concentration series experiment in which a putative promoter from avocado was supplied to mung bean cuttings, the first inhibitory treatment was distinctly injurous at the base of the cuttings (judged from the photographs), and the next more dilute treatment was the most promotory (RAVIV, REUVENI & GOLDSCHMIT, 1986). A concentration series of IAA gave similar results: the most promotory concentrations were adjacent to the superoptimal (inhibitory) ones (JACKSON & HARNEY, 1970).

The mode of action of applied phenolics, which are well known to promote rooting, may also be related to injury since they generally elicit a response at about 10^{-3}M, far in excess of their normal *in vivo* concentrations (JAMES & THURBON, 1981a). Similarly, auxin applied at just below a toxic concentration is considered to be most favourable for root initiation (HARTMANN & KESTER, 1983).

In mung bean biossay studies the association between injury and high rooting ability is easily observable and it is surprising that it has not been explicitly remarked upon before,

except by SOEKARJO (1965) for *Coleus*. It is probably easy to assume, when expecting promotory activity, that inhibitory concentrations are superoptimal and could be disregarded. For example, KRUL (1968) found that pinto bean (*Phaseolus vulgaris* 'Pinto') cuttings treated with the most promotory concentration of dinitrophenol sometimes developed 'necrotic lesions', but these cuttings were excluded from his analyses.

In one case where inhibitory activity was expected, 'suboptimal' (promotory) concentrations of the 'inhibitor' were assumed to demonstrate auxin-like activity (DHAWAN, PATON & WILLING, 1979). In support of this interpretation, DHAWAN, PATON & WILLING (1979) drew attention to the similarity between the endogenous concentration of G in easy- and difficult-to-root juvenile and adult *E.grandis* leaves, and concentrations of applied G that promoted and inhibited rooting in their mung bean bioassay (10^{-5}M and at least 5×10^{-4}M respectively). However, in two experiments from the present study, G was promotory in the mung bean bioassay at 10^{-3}M and $10^{-3.5}$M, and was markedly inhibitory at $10^{-2.5}$M and $10^{-2.75}$M. The easiest explanation for the large discrepancy between the two studies is that the local conditions of the bioassay affect the concentrations at which promotion and inhibition is elicited. Even when experiments were repeated under close to identical conditions (cf. Figures 3.2a and b; Figures 3.3a and b) these concentrations differed appreciably.

Although G was originally described as a rooting inhibitor (PATON, WILLING, NICHOLLS & PRYOR, 1970), other functions have been proposed: G could act as a fungicide, insecticide or germination inhibitor in litter (NICHOLLS, CROW & PATON, 1970); it may confer frost resistance since its levels are high in resistant tissue (PATON, 1981); applied G has an antitranspirant effect, which may be an *in vivo* function (PATON, DHAWAN & WILLING, 1980), and G can also reduce the rate of photosynthesis (SHARKEY, STEVENSON & PATON, 1982).

The observed effect on rooting is non-specific, but whether it is mediated through stress or injury to the potential sites at which roots initiate, according to the speculation of the previous chapter, is open to question. Other conceivable explanations are that the cell walls are loosened, increasing the uptake of water or facilitating the emergence of root initials (LEE, PAUL & HACKETT, 1977).

However, it did appear that promotory concentrations of a treatment solution were generally injurous to mung bean cuttings: they often died back from the base and tended to look sickly. The leaves were also adversely affected, judged from their appearance, suggesting that the action of a treatment solute need not be confined to the base of the cutting.

Whatever the explanation, it is clear that the activity of putative promoters and inhibitors in the mung bean bioassay need not reflect *in vivo* activity. The interpretation of the existing body of work is therefore open to question and a more critical approach is required.

CHAPTER FOUR

EVIDENCE FOR A ROOTING MORPHOGEN IN WOODY STEM CUTTINGS

4.1 INTRODUCTION

The foregoing chapters have established that the concept of promoters and inhibitors of adventitious rooting (defined in Chapter 2) is not a favourable approach for future studies. In particular, the evidence that 'G' (the putative rooting inhibitor in *Eucalyptus grandis*) limits rooting is based on bioassay evidence, the interpretation of which is open to question.

Attention has been drawn to a non-specific effect which promotes rooting in mung bean cuttings. This effect has also been observed in *E.grandis* cuttings (data not shown) but was not evoked strongly by the treatments tested. While there is probably scope for improving rooting ability by developing similar but more effective treatments, this approach would tend to be empirical since the nature of the non-specific effect, and any association it has with a hypothetical morphogen, is purely speculative.

For this reason a broader approach is adopted. In this chapter the evidence for a rooting morphogen in woody stem cuttings is reviewed.

4.2 SCHEMES FOR ADVENTITIOUS ROOTING IN WOODY STEM CUTTINGS

Generally, knowledge is required of the sub-systems of a system before a conceptual scheme can be synthesized (THORNLEY, 1980). For adventitious rooting in woody stem cuttings this knowledge is lacking. Several possible schemes are discussed, which are illustrated diagrammatically in Figure 4.1. No attempt is made to estimate the probability of each; the objective is to discuss what is conceivable.

The factors affecting adventitious rooting have been divided into 'exogenous' and 'endogenous' factors. Potential root initials are defined as cells which are capable of becoming root initials in the presence of a suitable concentration of a rooting morphogen (exogenous agent 2; Figure 4.1). Root initial cells may then either proceed directly to the first divisions of root initiation or else require the intervention of a

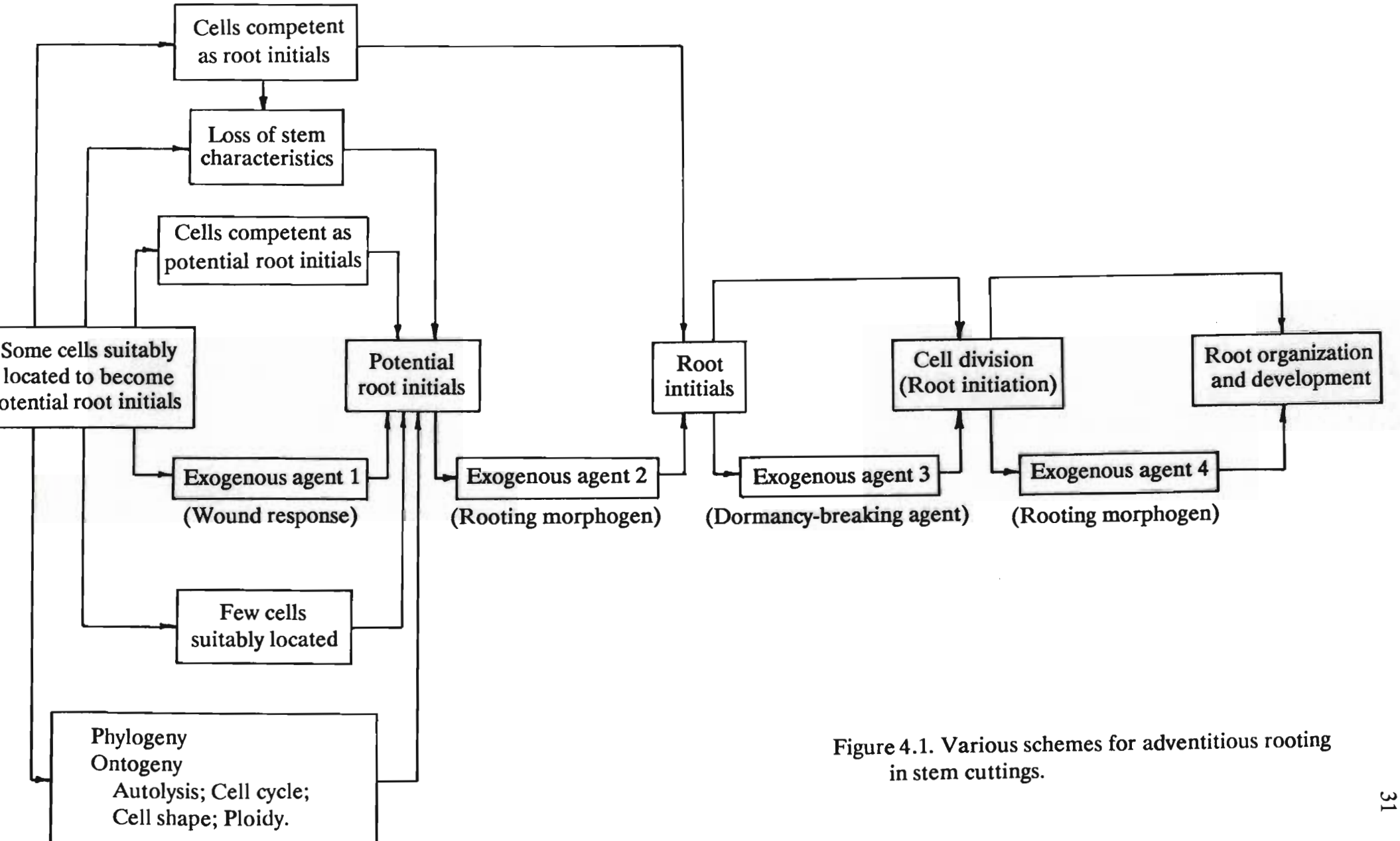

Figure 4.1. Various schemes for adventitious rooting in stem cuttings.

dormancy-breaking agent (exogenous agent 3). The subsequent development and organization of the primordium may be regulated endogenously or may require another exogenous agent (exogenous agent 4). This would be a rooting morphogen if the primordium became determined as a root only after beginning development.

Exogenous agent 1 is a consequence of the wound response (see chapter 2), which may create either potential root initials or root initials. In the latter case it would include the function of exogenous agent 2, the rooting morphogen.

4.2.1 STEM CELLS SUITABLY LOCATED TO BECOME POTENTIAL ROOT INITIALS

The simplest sequence of anatomical events is assumed, in which adventitious roots arise directly from pre-existing stem cells rather than from sites created after the cutting is set. Other possible sequences are described by LOVELL & WHITE (1986).

4.2.2 SOME CELLS COMPETENT AS ROOT INITIALS

Competence implies a commitment to a particular pathway of development in advance of its expression (WAREING, 1982). Since adventitious organs other than roots rarely if ever arise on stem cuttings, all or a proportion of stem parenchyma cells could be competent as root initials. However, this seems unlikely as stem and root parenchyma are differentiated both biochemically and structurally.

In tobacco there are 25000 to 30000 mRNA sequences per organ, of which only about 8000 are common to all (GOLDBERG, 1980). Evidence for organ-specific proteins has also been given by PATE (1966) and RAFF, HUTCHINSON, KNOX & CLARKE (1979). Chloroplasts in light can synthesize up to about 80 polypeptides (GRIERSON & COVEY, 1984) whereas root plastids are not exposed to light.

The plastids also represent the most conspicuous structural difference between stem and root parenchyma. In light-grown leaves and stems they are usually green, whereas those of roots are colourless. When illuminated, roots of *Convolvulus arvensis* L. (HELTNE & BONNET, 1970) and *Lupinus albus* L. (MESQUITA, 1971) turn green, but root plastids often remain colourless in the light (SCHNEPF, 1980), or else roots may appear reddish as in eucalypts. On the other hand, etiolated (white) stems which are exposed to light invariably turn green. Thus, in many species, even if the plastids at the base of a stem

cutting attained their least differentiated state (perhaps owing to the absence of light in the rooting medium), their developmental 'program' would still differ from that of root cells.

If variations in the differentiation of stem parenchyma were such that a proportion of cells lacked significant stem characteristics, it seems more likely that they would consist of potential root initials (on which a rooting morphogen would need to act) rather than root initials.

Root initials do not develop in the intact stem of most woody species: they are activated only after severance. If there were any root initials in the intact stem, either a limiting dormancy-breaking agent or an inhibitor (characteristic of the stem) would be required. The cells concerned are usually located in the cambium or those tissues external to the cambium, in which dormancy is broken periodically in the intact stem to allow for radial and tangential growth. Thus, any root initials would be more likely to be prevented from developing by an inhibitor characteristic of the stem.

4.2.3 THE LOSS OF STEM CHARACTERISTICS

Cells which lose previously developed characteristics can be said to dedifferentiate (ESAU, 1977). A morphogen might need to increase to effect dedifferentiation in stem cuttings (HAISSIG, 1974a), or substances characteristic of the stem might need to be lost. Shoots import various substances from the roots and severance of the stem cutting would prevent any further import, perhaps allowing their levels to diminish. Substances in this category include cytokinins, which are well known as promoters of shoot morphogenesis, and various root-synthesized amino acids (PATE, 1966). The stem may also be the source of specific inhibitors of adventitious rooting (for example 'G', the putative rooting inhibitor in *Eucalyptus grandis*; PATON, WILLING, NICHOLLS & PRYOR, 1970) (see Chapter 2).

Alternatively, the process of dedifferentiation could be essentially degenerative. For example, autolysis could significantly disrupt biochemical and/or structural differentiation. Cells might undergo some autolysis at certain points in their ontogeny (see section 4.2.6. below), or in response to wounding (exogenous agent 1). The wound response could be a consequence of severance or of stresses imposed when the base of the cutting is set in the dark, wet and probably hypoxic rooting medium (see Chapter 2).

4.2.4 SOME CELLS COMPETENT AS POTENTIAL ROOT INITIALS

If the number of potential root initials were non-limiting, the exogenous agents would be limiting both in the intact stem and in the stem cutting. Although up to four have been conceived, only one may exist or be limiting, or one substance could fill more than one role. For example auxin, if applied at a high concentration, could evoke a wound response, but it could also be the rooting morphogen and could act to break dormancy. This is the traditional view of adventitious rooting.

4.2.5 FEW CELLS SUITABLY LOCATED TO BE POTENTIAL ROOT INITIALS

The number of potential root initials could be limited by the number of suitable locations. This might occur if the concentration of a rooting morphogen varied significantly in different localities of the rooting zone. For example, a phloem-mobile morphogen might exceed the concentration necessary to initiate roots only in those cells with a distinctive relationship to the most actively unloading sieve elements.

4.2.6 VARIATION BETWEEN CELLS

If only a proportion of suitably located cells are, or become, competent as potential root initials, these cells might be expected to be in some way distinctive. Parenchyma cells can be specialized in various ways for photosynthesis, storage, deposition (ESAU, 1977) and perhaps transport (MURMANIS & EVERT, 1967) so it is possible that only a small proportion are sufficiently unspecialized to be potential root initials.

Differences between cells of one cell type must also exist, created endogenously by variations in phylogeny (lineage) or ontogeny. Relevant ontogenetic processes could include pre-programmed autolysis, the cell cycle, cell shape or ploidy.

Phylogeny

In the water fern *Ceratopteris thalictroides* Brongn., the precursor of the lateral root is always a particular cell in the lineage forming the endodermis (CHIANG & GIFFORD, 1971). Similarly, 2 to 4 cells in the apical meristem of the corn plant are solely responsible for tassle formation (COE & NEUFFER, 1978). It is not inconceivable that lateral root formation is determined by lineage in angiosperms (BARLOW, 1982), so the frequency

of root initial cells in stems could be determined in the same way.

Autolytic processes

Cells autolyze during lysiginous air space formation and during lignification. Isolated cell senescence and death can also occur within otherwise vigorous tissue, for example in embryogenesis (SAUNDERS, 1966), in the development of the pollen tube (JENSEN, 1969), in young regions of the cress root (BERJAK & LAWTON, 1973), and in floral apices (HICKS, 1973).

If autolysis were to contribute to the formation of root initials or potential root initials, the process would obviously have to be reversible since subsequent development depends on synthetic processes (HAISSIG, 1986). This may be possible since senescence, which involves autolysis, can be reversed, at least experimentally. Soybean cotyledons were found to regreen, following decapitation of the seedlings, even though 90% of their nucleic acids and 80% of their protein had been lost (KRUL, 1974).

The cell cycle

In growing stems a proportion of cells in the extra-cambial tissues are dividing at any one time to allow for tangential growth, and in woody stem cuttings relatively good rooting ability is often associated with vigorous growth of the parent shoot, when cell divisions would be numerous. This is so in eucalypts (DURAND-CRESSWELL, BOULAY & FRANCLET, 1982) as well as in other species (HARTMANN & LORETI, 1965; HARE & LAND, 1982). The parenchyma of the pith in *E.grandis* cuttings is adjacent to an internal phloem, but adventitious roots effectively never arise in the pith, whose cells are quiescent in the intact growing stem irrespective of the rate of growth.

The metabolic state of the cell, which could be regarded as biochemical differentiation, varies as cells progress through the cell cycle (YEOMAN & AITCHISON, 1973). The RNA complement of the cell increases stepwise, immediately after telophase, during DNA synthesis (the S phase) and immediately before prophase (YEOMAN & AITCHISON, 1973). Cytoplasmic protein also accumulates, and the cytoplasm grows and vacuolates in preparation for division (DYER, 1976a). The resting phase of cells is associated with arrest in the G1 phase, between mitosis (M) and S (BROWN, 1976), and both the G1 phase and the G2 phase (between S and M) are of variable duration (DYER, 1976a). The pericycle cells of radish seedling roots from which lateral roots initiated had a 4C DNA complement, indicating that they had synthesized DNA and were in the G2

phase (BLAKELY & EVANS, 1979). This may have allowed the cells to proceed rapidly to mitosis following auxin treatment (BLAKELY & EVANS, 1979).

During cell division the nuclear membrane breaks down and molecules from the cytoplasm, perhaps including those conveying new positional information, could then affect the nuclear genome directly (MACLEAN, 1977). Alternatively, the cell could be particularly responsive to an outside influence during the S phase when the chromosomes become unravelled (DYER, 1976a), perhaps rendering their genes more accessible. It is conceivable that the accessibility of different parts of the genome might also vary with gene activity, since this causes a local unravelling of the chromosome (MILLER & BEATTY, 1969), or with the local turnover of 'metabolic' DNA (ANKER, STROUN, GREPPIN & FREDJ, 1971; HURST & GAHAN, 1975).

Hypoxia caused mitoses to be slowed in pea root tips (AMOORE, 1961). Hypoxia could be experienced by the base of stem cuttings and could therefore prolong a potentially responsive period during the cell cycle. Hypoxia could also stimulate air-space development (ERDMANN, HOFFMANN & WIEDENROTH, 1986).

Cell shape

One of the earliest visible events in lateral root initiation is a change in polarity, which may occur in the absence of cell division (FOARD, HABER & FISHMAN, 1965; BAYER, FOY, MALLORY & CUTTER, 1967; CHARLTON, 1977). Isolated cells lose their polarity, suggesting that (in multi-cellular systems) the adjacent cells, the tissue or the plant body as a whole contribute to polarity (SCHNEPF, 1986). The signals which convey the necessary positional information are largely unknown, but include the flux of auxin (SCHNEPF, 1986).

The ease with which a cell changes polarity might depend on the shape of the cell: the less equidimensional the cell, the more difficult the change might be. Axial stem parenchyma cells (as opposed to ray parenchyma) are more or less elongate (ESAU, 1977), so it is conceivable that the ability to change polarity is confined to the least elongate cells.

Ploidy

An increase in ploidy is common in differentiating (LIST, 1963) and ageing plant cells (ANKER, STROUN, GREPPIN & FREDJ, 1971), and cells of the cortex and stele may

be predominantly polyploid (DYER, 1976b). Polyploidy results from nuclear division which is not followed by cell division (ESAU, 1977). It is well known as a conservative mechanism in evolution, but the genus *Eucalyptus* appears to lack any of these mechanisms, including polyploidy, perhaps because the eucalypts are generally long-lived (RYE, 1979). Cells which become polyploid appear not to divide subsequently (BUTTERFASS, 1980), and polyploidy may also impair organogenesis *in vitro*: in cell cultures of *Daucus* L. organogenesis was restricted to diploid cells (MITRA, MAPES & STEWARD, 1960).

4.2.7 CONCLUSION

The schemes discussed are arbitrary to some extent and may not include factors which are important or limiting in practice. They could all contribute to rooting ability, which may be influenced by a large number of small effects rather than by one or a small number of large ones.

It has been established, at least in principle, that either an exogenous agent, acting on the sites where roots initiate, or an endogenous process, taking place within the sites themselves, may be the more important in affecting rooting ability. The evidence for and against these two possibilities is considered below.

4.3 EVIDENCE FOR AN EXOGENOUS INFLUENCE (A MORPHOGEN) IN WOODY STEM CUTTINGS

4.3.1 INTRODUCTION

The stem cells of a cutting may either retain their identity or give rise to adventitious roots. Intermediate stages in metabolism (HAISSIG, 1986) and anatomy (LOVELL & WHITE, 1986) have been described, but these do not appear to represent alternative pathways of development. Thus the cells concerned are bistable and switch from one state to another. An autonomous system cannot exhibit true switch behaviour, since an agent must operate the switch (THORNLEY, 1972). This indicates that those stem cells which represent potential sites for root initiation are acted on by an outside influence, generally assumed to be a rooting morphogen.

If regulation in the cell resided largely in the nuclear genome then everything but this

could be considered to be exogenous. Similarly the nucleus, the whole cell or a group of cells could be considered as the most suitable entity which must be acted on by an exogenous influence. Single cells can differentiate (WADA & O'BRIEN, 1975) but large numbers of cells acting in concert could also perceive stimuli (O'BRIEN, 1982). An exogenous influence could therefore originate in the cytoplasm of affected cells, from neighbouring cells, from more or less remote regions of the cutting such as the leaves or buds, from the wound responses, or from outside the cutting.

The point at which stem cells become determined as root initials has not been defined. Shoot primordia originating in the pericycle of roots of *Euphorbia esula* L. are at first indistinguishable from root primordia (BAKSHI & COUPLAND, 1959). It is only when the primordium begins to show the organization characteristic of a root (when it consists of about 1500 cells in *Agathis australis* (D.Don) Lindl.; WHITE & LOVELL, 1984) that it can be said with confidence to be determined (LOVELL & WHITE, 1986). On the other hand, there is no evidence that other organs apart from roots arise adventitiously from stem cuttings.

While the development of stem-borne adventitious roots (HAISSIG, 1974b) and lateral roots (ALLEN & ALLEN, 1949) may halt at some intermediate stage in intact plants, in stem cuttings of apple (MACKENZIE, HOWARD & HARRISON-MURRAY, 1986), *Pseudotsuga menziesii* (Mirb.) Franco (HEAMAN & OWENS, 1972) and *Pinus radiata* (CAMERON & THOMPSON, 1969), there is no evidence that root primordia fail to develop once they have formed. Thus, the earliest events are likely to be limiting. In *Pinus radiata* seedling cuttings, in which root primordia initiate directly from the stem rather than from callus, the earliest visible events preceded any cell division (SMITH & THORPE, 1975). This is consistent with the sequence of events observed in lateral root initiation (see section 4.2.6.).

The evidence suggests that any rooting morphogen acts before the development and organization of the primordium, possibly before any cell division.

4.3.2 THE IDENTITY OF A MORPHOGEN

Outside influences which establish differences between cells include electrical fields (WAREING, 1982), physical stress (SACHS, 1986) and various kinds of informational molecules. Informational molecules may act over shorter or longer distances. By analogy with animal physiology, the short range molecules might be expected to have higher specificity and therefore would carry more information than the long range molecules,

such as the known plant hormones (HESLOP-HARRISON & LINSKENS, 1984). On the other hand, while short range molecules are known to be involved in cell recognition interactions in plants, there is as yet no information that such interactions are important in cell differentiation (WAREING, 1982).

The sites at which adventitious roots (CHANDRA, GREGORY & WORLEY, 1971; LOVELL & WHITE, 1986) and lateral roots (MALLORY, CHIANG, CUTTER & GIFFORD, 1970; CHARLTON, 1975) normally initiate are close to pre-existing or induced vascular tissue, suggesting that long range molecules of vascular origin are important. These include: sugars, sugar alcohols, amino acids, amides, organic acids, vitamins and growth substances (ZIEGLER, 1975), as well as various phenolics (AUCLAIR, 1963; MACLEOD & PRIDHAM, 1966; MONTAIN, HAISSIG & CURTIS, 1983).

In *Eucalyptus grandis* cuttings, the majority of adventitious roots emerge directly from the stem (rather than from the callus at the base of the cutting; see Chapter 7), and the site of initiation of these roots also appears to be in, or close to, the external phloem.

The rooting zone of many *E.grandis* and *E.camaldulensis* seedling cuttings was sectioned at the onset of rooting. Roots in a very early stage of development were not found (tending to confirm that the earliest events are limiting; see section 4.3.1), but the pattern of later development was similar in the two species. The earliest stage of development was found in an *E.camaldulensis* cutting, and is illustrated in Plate 4.1.

4.3.3 POLARITY

The polarity, or apparent polarity, of root emergence is one of the lines of evidence implicating auxin in root initiation (BATTEN & GOODWIN, 1978; JARVIS, 1986). The conventional view is that auxin is produced mainly in the shoot apices (WAREING & PHILLIPS, 1981), from which it moves strictly basipetally in shoots (WAREING, 1982). This creates the impression that auxin accumulates specifically at the base of stem cuttings.

Adventitious roots generally emerge at the base of the cutting. This could be due to polarity or to orientation, depending on the kind of cutting, as can be demonstrated by inverting cuttings. For example, inverted cuttings of *Rosa indica* Lindl. (NIELSON-JONES, 1925), *Salix* L. (WAREING & PHILLIPS, 1981) and *Vitis* (HARTMANN & KESTER, 1983) developed roots as usual at the proximal end (now the top of the cutting), demonstrating polarity independent of orientation. On the other

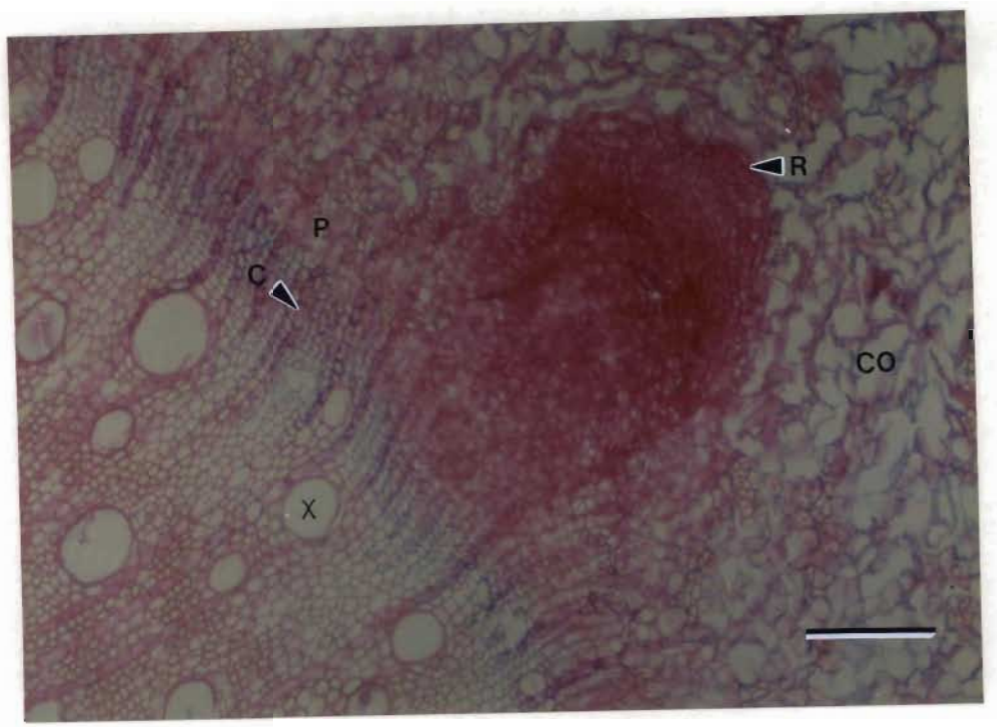

Plate 4.1 A transverse section through the base of a *Eucalyptus camaldulensis* seedling cutting, 7 days after being set, showing a developing adventitious root. The base of the cutting has swelled radially, disrupting the cortex but leaving the underlying tissues relatively unaffected. The root appears to be bounded tangentially by two vascular rays, and to have initiated in or close to the external phloem. The section was embedded in wax and stained with safranin and fast green (see section 6.2). X xylem; C cambium; P external phloem; R adventitious root; CO cortex. Scale bar = 50 μm.

hand, any polarity may be overcome by inversion, when roots emerge from the distal end (now the base of the cutting). Species like this include *Aloe frutescens* Salm-Dyck (NEILSON-JONES, 1925), *Tagetes* L. (WENT, 1941), *Salix viminalis* (HOAD, HILLMAN & WAREING, 1971), *Nicotiana tabacum* L., *Lycopersicon esculentum* L. (SHELDRAKE, 1974), *Carya illinoensis* (SMITH, 1972) and *Ribes sativum* Syme. (HARTMANN & KESTER, 1983).

Auxin moves polarly (basipetally) through the parenchyma associated with the vascular tissue (WANGERMANN, 1974) and is an active process, being dependent on respiration (NIEDERGANG-KAMIEN & LEOPOLD, 1957). WENT (1941) suggested that the polarity of auxin movement may be reversed in inverted cuttings, accounting for the reversed polarity of root and shoot emergence in *Tagetes*, but SHELDRAKE (1974)

could find no evidence for this except in those cuttings whose pith had degenerated, allowing passive bidirectional transport. Since the polarity of root and shoot emergence was reversed, auxin would have tended to accumulate *via* the polar transport system at the proximal (top) end of the cutting, where shoots rather than roots developed.

Auxin arriving at the proximal end of a stem cutting *via* the polar transport system is probably redistributed acropetally. Auxin applied to one end of stem cuttings distributed itself throughout the cutting (STRYDOM & HARTMANN, 1960; McGUIRE, ALBERT & SHUTAK, 1969), and in mung bean cuttings the rate of basipetal auxin transport from the leaves probably reflects the amount being imported (JARVIS & SHAHEED, 1986).

Apart from the polar pathway, four other pathways for the movement of auxin have been mentioned. Applied auxin can move non-polarly in the phloem (HOAD, HILLMAN & WAREING, 1971; MORRIS & KADIR, 1972; GOLDSMITH, CATALDO, KARN, BRENNEMAN & TRIP, 1974; ALLEN & BAKER, 1980; ABO HAMED, COLLIN & HARDWICK, 1984), including the internal phloem (ZAMSKI & TSIVION, 1977). Transport in this pathway has the characteristics of normal transport in the phloem, and may therefore proceed in either direction depending on source-sink relationships.

Auxin flux induces xylem differentiation (SACHS & COHEN, 1982; ALONI & ZIMMERMANN, 1983), suggesting that auxin can move in this tissue. This movement could also be in either direction since xylem may differentiate acropetally as well as basipetally (ESAU, 1965; LARSON, 1983).

Auxin has been found in the xylem sap (HALL & MEDLOW, 1974), but there is a possibility that its presence in this pathway is an artifact caused by microbial contamination (ALLEN, GREENWAY & BAKER, 1979).

Finally, on account of its high membrane permeability (RAVEN & RUBERY, 1982), auxin can also move radially between pathways (SKOOG, 1938; ZAMSKI & WAREING, 1974).

Auxin appears to be to able to circulate in stem cuttings, assuming any vascular flux in the stem. Efflux would be likely at a wound which interrupts the transporting tissues, but the effects of efflux from the various pathways, including the phloem, would be indistinguishable. Thus, the composition of a morphogen of vascular origin would be unknown.

Polarity or orientation effects make it clear that, irrespective of which end of the cutting the roots emerge, the morphogen has limited accessibility to the potential sites for root

initiation.

4.3.4 AUXIN

Of the known plant hormones, it is generally accepted that auxin has a central role in adventitious rooting, whereas it is unclear whether the other hormones play a major role under natural conditions (JARVIS, 1986).

One line of evidence suggests that auxin has an ancient and fundamental role in promoting rooting. The earliest land plants of the Psilophyta had horizontal rhizomes connecting upwardly growing stems which, in the case of representatives of the Rhyniales, bore unicellular rhizoids (BARLOW, 1986). These rhizoids presumably evolved into roots. Rhizoids can be induced to develop on some modern-day liverworts by environmental auxin in the soil (SHELDRAKE, 1971), which is produced by soil microorganisms (ROBERTS & ROBERTS, 1939; BROWN, 1972). In modern higher plants root growth and root hair development can also be stimulated by environmental auxin (SHELDRAKE, 1973; TIEN, GASKINS & HUBBELL, 1979; LOPER & SCHROTH, 1986). Proteoid roots (unusually prolific lateral roots) are induced by non-infecting rhizosphere microorganisms (LAMONT & McCOMB, 1974; MALAJCZUK & BOWEN, 1974), perhaps by the same means.

Thus, originally, IAA might have been a messenger of microbial activity (hence fertility) in the substratum (SHELDRAKE, 1971) and the plant would have gained adaptive advantage by allowing IAA to evoke appropriate responses. These could have involved the breaking of dormancy and/or morphogenesis.

The widespread efficacy with which supplied auxin promotes root formation may be taken as evidence that it is often the sole factor limiting root initiation in non-woody cuttings (JARVIS, 1986), although it has been pointed out that this and other evidence implicating auxin is circumstantial (BATTEN & GOODWIN, 1978).

Auxin may also limit lateral root initiation. Decapitation inhibited lateral root initiation in *Acer saccharinum* L. (RICHARDSON, 1958) and pea (McDAVID, SAGAR & MARSHALL, 1972), but the rate of initiation could be restored by supplying auxin to the cut stumps.

Applied auxin is well known as a promoter of adventitious rooting in woody stem cuttings. However, in woody cuttings, while IAA induces root initiation in predisposed cells, it cannot itself create the necessary predisposition (HAISSIG, 1970). Thus, auxin has little

or no promotory effect when applied to difficult-to-root cuttings. For example, GAUTAM & CHAUHAN (1986), HUCKENPAHLER (1955), KADMAN & BEN-YAACOV (1965) and MEREDITH, JOINER & BIGGS (1970) failed to improve rooting ability by applying auxin to cuttings of *Juglans regia* L., *Liriodendron tulipifera* L., avocado and two clones of *Feijoa sellowiana* Berg. respectively.

These and similar results could be taken to indicate that applied auxin is either not taken up or is catabolized before the target cells are capable of responding. However, STEPONKUS & HOGAN (1967), STOLTZ (1968), GREENWOOD, ATKINSON & YAWNEY (1976) and BIRAN & HALEVY (1973b) found no correlation between the endogenous auxin content and rooting ability in *Abelia grandiflora* Rehd. 'Prostrata', *Chrysanthemum morifolium*, *Acer saccharum* Marsh. and *Dahlia variabilis* Desf. cuttings respectively. Similarly, there was no correlation between the levels of endogenous auxin and the number of root primordia initiated in either intact or decapitated root segments of *Zea mays* L. cultured *in vitro* (GOLAZ & PILET, 1987).

A rooting morphogen would have to act in a direct and fundamental way in order to satisfy the definition used in this chapter. This has not been demonstrated for auxin, the response to which appears to depend on the 'competence' of the responding cell or tissue, the molecular basis for which remains unknown (WAREING, 1982). Moreover, auxins have many functions in stem cuttings, any of which could affect rooting ability.

Auxin applied to the base of cuttings may lead to a more rapid accumulation there of phloem-mobile compounds, which could promote rooting if limiting. This could be achieved in four ways. 1. The rate at which sugar is loaded into the phloem from storage parenchyma may be increased (LEPP & PEEL, 1971; HAYES & PATRICK, 1986). 2. Unloading from the phloem may be stimulated (PATRICK, 1979; HAYES & PATRICK, 1986). 3. Phloem transport itself may be promoted (LEPP & PEEL, 1971; ALTMAN & WAREING, 1975; PATRICK, 1979). Or 4. The strength of the sink may be increased (PATRICK, 1979), through an increase (for example) in respiration or callus development at the base of the cutting (STRYDOM, 1960).

The first two effects should increase the concentration of phloem-mobile substances at the base of the cutting, and the third and fourth may also do so depending on the inter-relationships between the rates of efflux, influx and sink activity. The relevant phloem-mobile substances may be dormancy-breaking agents such as the nutrients, but may also include other exogenous agents including auxin itself.

Applied auxin can promote rooting by stimulating callus production at the base of the cutting. This effect is particularly apparent in stem cuttings of those species, such as apple,

in which many or all adventitious roots arise in callus (MACKENZIE, HOWARD & HARRISON-MURRAY, 1986). In eucalypt stem cuttings adventitious roots usually develop directly from the stem rather than from callus (see Chapter 7), and the promotion of callus growth by applied auxin would therefore have indirect effects (if any) on rooting ability.

The generally promotive effects of applied auxin need not be related to its roles as an endogenous substance. In one of the earliest studies on the effects of synthetic auxin-like compounds on rooting ability, ZIMMERMAN & WILCOXON (1935) identified eight promotors, bringing the total of synthetic auxin-like promoters identified at that time to 16. ZIMMERMAN & WILCOXON (1935) felt that this large number militated against the idea of specificity of action. HARTMANN & KESTER (1983) state that a concentration of applied auxin just below the toxic concentration is considered to be the most favourable for root promotion, which also suggests a non-specific effect perhaps due to injury. And in Chapter 3, a sub-lethal concentration of applied auxin was found to be most promotory in mung bean cuttings.

Rather than being a rooting morphogen, auxin could be a dormancy-breaking agent. In mung bean seedlings, severance induced DNA synthesis locally throughout the hypocotyl, but the subsequent development of these sites into root primordia was dependent on applied auxin (TRIPEPI, HEUSER & SHANNON, 1983).

4.3.5 OTHER POSSIBLE MORPHOGENS

Wound metabolites, induced by the severance wound at the base of the cutting or by the dark, wet and probably hypoxic environment close to the base (if the cutting is set in rooting medium), could participate in adventitious rooting (see Chapter 2). Hypoxia at the base of the cutting could also be inhibitory to rooting, in which case oxygen would be an important component of the morphogen.

A morphogen could consist of more than one substance. Regulation of the patterns of regenerating cambia could be accounted for by the ratio of two diffusible morphogens, suggested to be auxin and sucrose (WARREN-WILSON, 1978; WARREN-WILSON & WARREN-WILSON, 1981). Similarly, SKOOG & MILLER (1957) showed that shoot *versus* root development could be regulated *in vitro* by the ratio of auxins and cytokinins. This latter ratio is also important in the development of the crown gall tumour: whether shoots or roots are formed depends on the kind of mutation in the tumour-inducing plasmid (GARFINKEL & NESTER, 1980). The root-inducing plasmid causes proteins

to be produced which participate directly in auxin synthesis (NESTER, GORDON, AMASINO & YANOFSKI, 1984), modifying the auxin/cytokinin balance of the tissue accordingly (AKIYOSHI, MORRIS, HIMZ, MISCHKE, KOSUGE, GARFINKEL, GORDON & NESTER, 1983).

An indefinite number of other compounds could participate in morphogenesis. These include: nitrogen compounds, which appear to have a direct effect in promoting lateral rooting in roots (HACKETT, 1972; DREW, SAKER & ASHLEY, 1973); oligopeptides similar in structure to IAA (KLAMBT, 1983); non-indolic auxins such as phenylacetic acid (SCHNEIDER & WIGHTMAN, 1974); and carbohydrates (THORPE, 1980; TRAN THANH VAN, TOUBART, COUSSON, DARVILL, GOLLIN, CHELF & ALBERSHEIM, 1985) which can affect morphogenesis *in vitro*.

The composition and concentration of a morphogen could be less relevant than other criteria. For example, by analogy to the differentiation of wound-induced vascular tissue (SACHS & COHEN, 1982; ALONI & ZIMMERMANN, 1983), the rate of flux (kg/s^{-1}) or the flux density ($kg/m^2/s^{-1}$) of the morphogen could be the most important variable. So could flux direction, given that a change of polarity is required when roots are initiated. Any criterion of quantity may also be cumulative, in which case it would interact with time. This is possible since roots may emerge from woody cuttings, such as those of *E.grandis*, over a period of several weeks.

Metabolic oscillations are characteristic of living things, because reaction kinetics are often non-linear (owing to cyclic interactions such as feedback) and because the system is maintained far from thermodynamic equilibrium (LLOYD, POOLE & EDWARDS, 1982). For example, the rate of basipetal efflux of IAA from stem segments of *Pinus sylvestris* L. showed short term oscillations (WODZICKI, ABE, WODZICKI, PHARIS & COHEN, 1987), and the pool sizes of the intermediates of glycolysis have been shown to oscillate in the range 10^{-5}M to 10^{-3}M within periods of the order of minutes (HESS, 1979). Thus, a maximum value could be very short-lived, or if the morphogen consists of more than one oscillating component, the correspondence of oscillations could be a rare event.

4.4 EVIDENCE FOR ENDOGENOUS INFLUENCES AFFECTING THE NUMBER OF SITES FOR ROOT INITIATION IN WOODY STEM CUTTINGS

No previous study has explicitly addressed the possibility that the frequency of sites in

stem cuttings may determine rooting ability. However, the following observations may be relevant.

HALMA (1931) made grafts between different *Citrus* L. species of known rooting ability. The grafts were set as stem cuttings so that the scion provided the upper (leafy) part of the cutting, while the stock provided the base where rooting subsequently took place. HALMA (1931) found that the speed of rooting was governed by the identity of the stock, provided that the leaves of the scion were healthy.

VAN OVERBEEK & GREGORY (1945) conducted similar experiments but arrived at the opposite conclusion: that rooting ability was determined by the identity of the scion. In their study, scions of the easy-to-root *Hibiscus rosa-sinensis* were grafted on to stocks of the difficult-to-root *Hibiscus* cultivar 'Ruth Wilcox'. When set as cuttings the rooting ability of the stock was promoted, relative to that of entire 'Ruth Wilcox' cuttings, but this promotion was negated by girdling the scion. VAN OVERBEEK & GREGORY (1945) concluded that the easy-to-root scion was the source of promoters which moved downwards in the phloem to improve the rooting ability of the stock.

However, RYAN, FROLICH & KINSELLA (1958) pointed out that there was evidence in the paper of VAN OVERBEEK & GREGORY (1945) that the difficult-to-root cultivar had suffered relatively rapid leaf abscission. They expressed the view that, so long as healthy leaves are present on the cutting, the rooting ability of the stock should not be influenced by the characteristics of the cultivar furnishing the leaves. An easy-to-root scion would improve the rooting ability of a difficult-to-root stock only if the propagation conditions rendered the leaves of the difficult-to-root cultivar relatively unhealthy. RYAN, FROLICH & KINSELLA (1958) made a large number of reciprocal grafts between easy- and difficult-to-root clones of *Citrus*, avocado, *Camellia*, *Hibiscus* and *Macadamia ternifolia* F.Muell., and demonstrated convincingly that the rooting ability of grafted cuttings was determined largely by the identity of the stock.

These results, together with those of HALMA (1931), could be interpreted to mean that the wound responses of easy-to-root stocks are in some way more able to promote rooting than those of difficult-to-root stocks. Alternatively, the easy-to-root stocks could have contained significantly more reserve materials. However, in a variation of the technique, MES (1951) made reciprocal bark grafts between the easy-to-root citron (*Citrus medica* L.) and the difficult-to-root sour orange (*Citrus aurantium* L.). Cuttings were prepared so that the bark grafts were located close to the base of the cuttings. Bark of citron on sour orange rooted whereas sour orange alone did not root; in the reciprocal graft, bark of sour orange on citron rooted sparingly while citron alone rooted prolifically. In this study, any differences between species in the nature of the wound response at the base

of the cutting, or in the resources of the stem within the cambium, failed to markedly affect the rooting ability of the grafted tissue, supporting the conclusion that there was a higher frequency of potential sites for root initiation in the easy-to-root tissue.

4.5 SUMMARY

Several schemes for adventitious rooting in woody stem cuttings have been conceived, all of which require the intervention of an exogenous agent or agents. The minimum requirement would be for an inhibitor characteristic of the stem to be lost, or for a dormancy-breaking agent to accumulate, but in most schemes a rooting morphogen is also required. It could not be established, however, that the presence or absence of the required exogenous influence would be limiting in the rooting zone.

Exogenous influences could originate from the leaves, buds, or stem, from the wound responses of the cutting, or from outside the cutting. The vascular system is a prominent source of exogenous influence at the base of the cutting, but the importance and identity of the influence, at least in woody stem cuttings, has not been established. Auxin is well known as a promoter of adventitious rooting, but if it is the rooting morphogen in woody stem cuttings it often appears to be non-limiting. Other substances, or a combination of substances possibly including auxin, or some other kind of influence, could equally well constitute the morphogen.

A morphogen could be non-limiting in concentration or composition, but inaccessible. This appears to be so over much of the length of the stem. A variable such as flux could also be a more relevant criterion of quantity than concentration or composition.

The number of potential sites for root initiation in the rooting zone could be an important source of variability. Only a small proportion of apparently suitably located cells ever initiate roots, even when rooting ability is 'good', so these cells might be expected to be in some way distinctive. Several sources of variation between cells were considered, which could affect the responsiveness of cells to a morphogen, but there was little direct evidence for or against their participation in adventitious rooting. However, a small number of indirect observations suggest that the number of sites in the stem may vary sufficiently to have an important effect on rooting ability.

CHAPTER FIVE

THE CONTRIBUTION OF THE LEAVES AND BUDS TO THE ROOTING ABILITY OF *EUCALYPTUS GRANDIS* STEM CUTTINGS

5.1 INTRODUCTION

The leaves and buds of stem cuttings are commonly assumed to be the source of a morphogen which limits rooting ability. In this chapter, the contribution of the leaves and buds to the rooting ability of *Eucalyptus grandis* cuttings is investigated. A second possibility, that the wound responses of the cutting generate a limiting morphogen, is considered briefly in Chapter 7.

Stem cuttings without leaves rarely root unless new leaves develop during the propagation period (LEAKEY, 1985). This observation, that the absence of leaves prevents rooting, is often interpreted to mean that the presence of leaves causes rooting, for example because they are the source of an 'endogenous rooting stimulus' (HAISSIG, 1982). This stimulus has been variously assumed to consist of auxin, nutritional factors or other 'co-factors' (JARVIS, 1986).

Active leaves, rather than actually producing auxin, could simply enhance the uptake of supplied auxin, or may load supplied auxin into the appropriate tissue for downward transport to the sites of root initiation (JARVIS, 1986). For example, JARVIS & SHAHEED (1986) found that the rooting ability of mung bean cuttings was related to the quantity of auxin moving downward in the hypocotyl (a small fraction of the total), rather than to the total quantity of auxin taken up by the cuttings. Similar results were obtained by BATTEN & GOODWIN (1981). Mung bean cuttings were floated on IBA solutions so that uptake was not dependent on transport, but the auxin transport inhibitor TIBA nevertheless inhibited rooting.

The leaves could also load endogenous auxin, or any other endogenous substances active in rooting, in the same way, and would also be responsible for any recirculation. WAREING (1985) emphasized that the only relevant quantity of a morphogen is that prevailing at the site of action, and in the case of adventitious rooting this quantity could be affected by the rate of flux generated by the leaves.

The leaf can affect the development of the bud in its axil. Reducing leaf area promoted bud development and improved rooting ability in stem cuttings of *Eucalyptus*

camaldulensis (GEARY & HARDING, 1984).

Like mature leaves, developing buds (*ie.* young leaves and shoot apices) on stem cuttings are often assumed to be the source of a limiting morphogen. Removal of the shoot apices and buds from pea cuttings within four days of setting caused a marked reduction in subsequent rooting ability (ERIKSEN, 1973), but this could be restored by applying IAA to the stumps (ERIKSEN & MOHAMMED, 1974). On the other hand, while cuttings of *Populus* x robusta Schneid. with active apices rooted better than cuttings with dormant apices, the auxin content of the two kinds of cuttings was similar (SMITH & WAREING, 1972).

Buds on cuttings do not always promote rooting. They may have little or no effect (KATSUMI, CHIBA & FUKUYAMA, 1969; MIDDLETON, JARVIS & BOOTH, 1980), or they may be inhibitory if carbohydrates are limiting, since developing buds may be a significant sink under these circumstances (FISCHER & HANSEN, 1977). Developing flower buds inhibited rooting more than developing vegetative buds in cuttings of *Dahlia variabilis*, perhaps because they were a stronger sink (BIRAN & HALEVY, 1973a). Dormant buds, as opposed to active ones, may also inhibit rooting by producing inhibitors (FADL & HARTMANN, 1967c; ROBERTS, 1969).

Promotion or inhibition of rooting through the loss of buds in disbudding experiments may be artifactual. For example, a 'promotive' effect of buds was observed because sufficient water was lost through disbudding wounds to adversely affect the cuttings (HOWARD, 1980). An 'inhibitory' effect of buds was due to the release, in disbudded cuttings, of a wound-induced stimulus (HOWARD, 1968).

5.2 MATERIALS AND METHODS

Eucalyptus grandis cuttings were prepared from coppice shoots 0.6 to 1.0 m long grown from the stumps of one-year-old trees at the University Farm, Ukulinga (Plate 5.1).

All the nodes of shoots were converted to cuttings with the exception of the soft apical nodes, the smallest nodes and those nodes whose leaves had abscised, were senescent or were in otherwise poor condition. Unless otherwise stated, the cuttings consisted of one pair of leaves severed across the lamina to give an approximate area per leaf of 4 cm^3, their petioles and axillary meristems, and the internode below. The internodes varied in length from 3 cm to 6 cm, and in diameter from 1 mm to 5 mm (Plate 5.2).

The cuttings were soaked in 2 g l^{-1} of the systemic fungicide Benlate (active ingredient

Plate 5.1. Coppice shoots arising from one-year-old stumps of *Eucalyptus grandis*. These shoots, when 0.6 to 1.0 m high, were harvested for stem cuttings.

benomyl (benzimidazole) 50 g kg^{-1}) for up to one hour. They were then allocated to plots non-randomly on the basis of size and appearance, so that like cuttings were roughly equally represented in each plot.

After various treatments (see below), the base of each cutting was dipped in talc containing 8000 mg l^{-1} IBA (unless otherwise stated). The cuttings were set to a depth of 2 to 3 cm in vermiculite, each in a polystyrene cavity of area 4.4 × 4.4 cm.

The cuttings were kept well wetted with intermittent mist spray in a greenhouse, the regime varying with the weather. (On average about 15 seconds mist every 15 minutes during the day, and every 2 hours at night). The temperature in the greenhouse also varied with ambient conditions, from about 35°C (the hottest day) to about 10°C (the coolest night).

Plate 5.2. The range of variability in the stem cuttings of *Eucalyptus grandis* used for propagation experiments. Internode length varies from 3 cm to 6 cm.

Where the proportion of cuttings rooted per plot was the variable analyzed, the data were transformed using the angular (arcsine) transformation before analysis of variance.

Each treatment within an experiment consisted of at least two plots of 32 cuttings each. Where there were more than two treatments these were arranged in randomized complete blocks, with the restriction that like treatments should not be adjacent. In experiments consisting of two treatments only this meant that plots were arranged alternately. Plot values within a treatment were combined only if the results were consistent between blocks. The χ^2 values to compare two proportions were calculated using the formula:

$$\chi^2 = \frac{\begin{vmatrix} a & b \\ c & d \end{vmatrix} \begin{matrix} a+b \\ c+d \end{matrix}}{a+c \quad b+d \quad n} = \frac{(|ad - bc| - n/2)^2 \times n}{(a+b) \times (c+d) \times (a+c) \times (b+d)}$$

This formula incorporates Yates' correction for continuity (QUENOUILLE, 1969). The

test assumes that the proportions are normally distributed, and the correction improves the normal approximation when the total sample size is less than about 200 (SOKAL & ROLFE, 1969).

5.2.1 THE CONTRIBUTION OF THE LEAVES

Cuttings of one clone were prepared with a leaf area per leaf of about 4 cm^2. Before setting the cuttings any axillary shoots were removed without injuring the axil. After being allocated to plots: a) the lower leaf was removed, b) the upper leaf was removed, or c) both leaves were retained. Each treatment consisted of 2 plots of 32 cuttings each. The cuttings were not treated with IBA. After 69 days the number of roots per plot and the proportion of leaves abscised from the surviving cuttings was recorded. Before recording abscission, leaves were subjected to a gentle downward pressure, causing all leaves with well developed abscission zones to drop. The axillary buds which had developed during the propagation period were removed, oven-dried at 50°C for 48 hours and weighed.

In analyses of variance of root number and leaf abscission, using the randomized complete blocks design, the effect due to blocks was not significant. To conserve degrees of freedom (SNEDECOR & COCHRANE, 1980) the data were re-analyzed using the unpaired t test which, when there are only two treatments, is equivalent to the analysis of variance of a completely randomized design (FREESE, 1967).

At the end of six propagation experiments involving three clones and various treatments, the leaves of surviving (two-leaf) cuttings were subjected to a gentle downward pressure, causing all leaves with well developed abscission zones to drop. When one leaf only was retained, its position (upper or lower) was recorded if the vertical distance between the insertion of the petioles was greater than 5 mm. The expected proportion of upper and lower leaves abscised, assuming no difference in the rate of abscission, was 0.5. A goodness-of-fit test was used to compare the observed and expected proportions (FREESE, 1967).

One-leaf cuttings of one clone were prepared with leaves unreduced in area. Any axillary shoots were removed without injuring the axil. The leaf area was then reduced to approximately 0, 1.5, 4.5 and 13.5 cm^2/leaf (a logarithmic series) before the cuttings were set. This was achieved by removing the excess leaf area with one cut perpendicular to the midrib. The approximately correct position of the cut was established by placing the leaf against a suitable template depending on the original size of the leaf. Each leaf area treatment consisted of 2 plots of 32 cuttings each. The number of roots per plot was

recorded after 54 days. The axillary buds which had developed into small shoots during the propagation period were removed, oven-dried at 50°C for 48 hours and weighed.

At the end of the experiments a sample of leaves was removed from each treatment, and their actual leaf areas were measured individually using a leaf area meter. The mean actual leaf area (and associated confidence limits) was then calculated for each treatment.

5.2.2 THE CONTRIBUTION OF THE BUDS

Two experiments were performed in which the axillary buds were removed from cuttings immediately before being set. In a third experiment, stock plants were disbudded three weeks before their harvest for cuttings. Three clones were used in each experiment.

1. The axillary buds and their associated meristems were excised with a V-shaped notch of tissue before the cuttings were set. The notch was about 1 mm across and extended to the wood less than 1 mm below the surface of the axil. In the control cuttings a notch of tissue of similar size was removed from the stem 1 mm to 2 mm above the axil. Each of the two treatments consisted of 6 plots of 32 cuttings each (two plots of each of three clones). The proportion of cuttings rooted and the proportion of leaves abscised were recorded after 44 and 23 days respectively.

2. Any developing axillary buds were carefully removed with a scalpel flush with the surface of the axil. The axils themselves were undamaged, so the bud meristems tended to begin new development. Developing buds were removed when the cuttings were set, and thereafter weekly throughout the propagation period, so that they never exceeded a few millimetres in length. The buds of the control cuttings were allowed to develop normally. Each of the two treatments consisted of 6 plots of 32 cuttings each (two plots of each of three clones). The proportion of cuttings rooted and the proportion of leaves abscised were recorded after 41 and 27 days respectively.

3. Six rooted cuttings (two of each of three clones) were open-grown in pots to height 0.8 m to 1.0 m. Twenty one days before they themselves were harvested for stem cuttings, one plant of each clone was disbudded by removing the apices of all shoots at the internode at which the proximal leaf had reached full size. Any developing axillary buds were also removed with a scalpel. Fourteen and seven days before harvest any newly developing axillary buds were again removed with a scalpel, causing the subsequent bud outgrowth to be progressively more vigorous. Twelve days after the cuttings were set, mean bud length per cutting was recorded, and the proportion of cuttings rooted and the proportion of leaves abscised (from the unrooted survivors) was recorded after 53 days.

5.3 RESULTS

5.3.1 THE CONTRIBUTION OF THE LEAVES

Cuttings from one clone were prepared with leaf areas close to 4 cm^2. Three treatments were compared: control (two leaves), upper leaf removed and lower leaf removed. The results, combining plot values, are summarized in Table 5.1.

Table 5.1. Survival (%), root number and leaf abscission (%) of two-leaf cuttings and one-leaf cuttings of *E.grandis* (with either the lower or the upper leaf retained). Each treatment consisted of 64 cuttings.

	CONTROL (2 LEAVES)	LOWER LEAF RETAINED	UPPER LEAF RETAINED
SURVIVAL (%)	95.3	90.6	92.2
ROOT NUMBER	102	122	156
LEAF ABSCISSION (%) OF SURVIVORS (DAY 69)	25.4	14.8	6.8

The one-leaf cuttings had significantly higher root numbers ($t = 3.89$; $t_{0.05,3\ df} = 2.78$), and significantly less leaf abscission ($t = 4.95$; data angular-transformed), than two-leaf cuttings. Survival was similar between treatments.

Total bud development per cutting was similar between treatments, but the distribution of bud growth between axils differed (Figure 5.1). The upper bud developed more rapidly than the lower bud in two-leaf cuttings. In one-leaf cuttings the presence of a leaf tended to promote the development of the bud in its axil, either overcoming the greater vigour of the upper bud (lower leaf retained) or making it more pronounced (upper leaf retained).

There was an indication that, in one-leaf cuttings with the upper leaf retained, leaf abscission was slower and bud development in the axil of the retained leaf was more rapid, than in one-leaf cuttings with the lower leaf retained (Figure 5.1).

In general, during the propagation period, the lower leaf abscised more rapidly than the

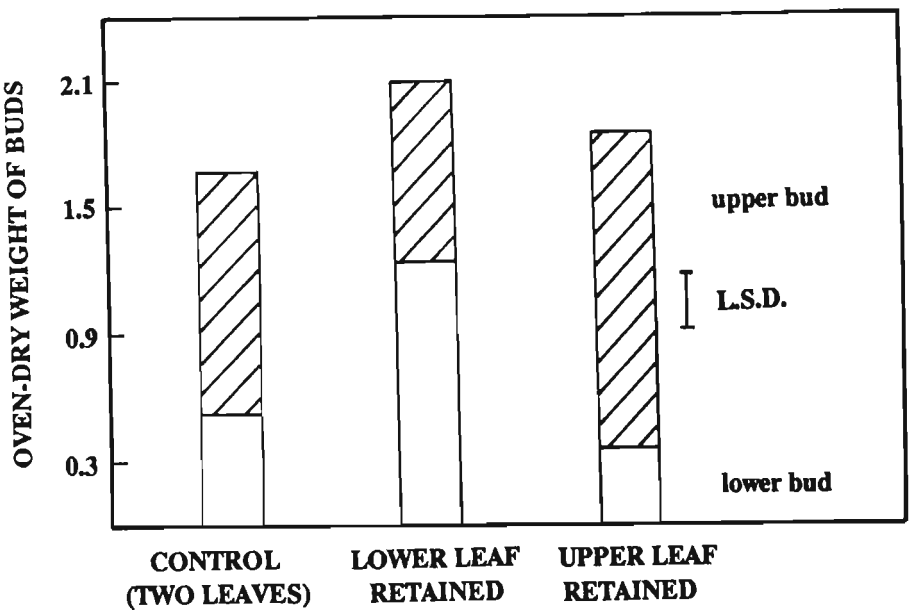

Figure 5.1. Axillary bud development during the propagation period in two-leaf and one-leaf cuttings of *E.grandis* (lower or upper leaf retained).

upper leaf from two-leaf cuttings. Of a total of 2404 cuttings set in six experiments, 429 had retained one leaf. The observed number of upper and lower leaves retained was compared to the expected number (assuming no difference in the rate of abscission, *ie.* p = 0.5). Table 5.2 shows that upper leaves tended to be retained longer than lower ones.

Table 5.2. The observed number of upper or lower leaves abscised from two-leaf cuttings retaining one leaf at the end of the propagation period, compared to the expected number assuming no difference in the rate of abscission.

	UPPER LEAF ABSCISED	LOWER LEAF ABSCISED	TOTAL	
OBSERVED	152	277	429	
EXPECTED	214.5	214.5	429	$\chi^2 = 36.4$

Of the cuttings which had lost one leaf, 64.6% retained the upper one while 35.4% retained the lower one. This is an overall value for three clones treated in various ways.

One-leaf cuttings were prepared with intended leaf areas of 0, 0.5, 1.5, 4.5 and 13.5 cm^2

(a logarithmic series). The actual areas were: 0, 0.6 ± 0.03, 2.0 ± 0.07, 5.3 ± 0.05, and 12.2 ± 0.12. These are described below as leaf areas 1, 2, 3 and 4 respectively.

Cuttings with zero leaf area failed to root and died rapidly. This was observed both when the cuttings were prepared in this way and after the leaf of the leafy cuttings had abscised during the propagation period. All cuttings surviving at the end of the propagation period had retained their leaf, whereas the remainder had died after their leaves had abscised. Survival was therefore an index of the rate of leaf abscission. These variables were largely unaffected by leaf area except at the smallest area, at which the rates of mortality and abscission were markedly accelerated (Figure 5.2).

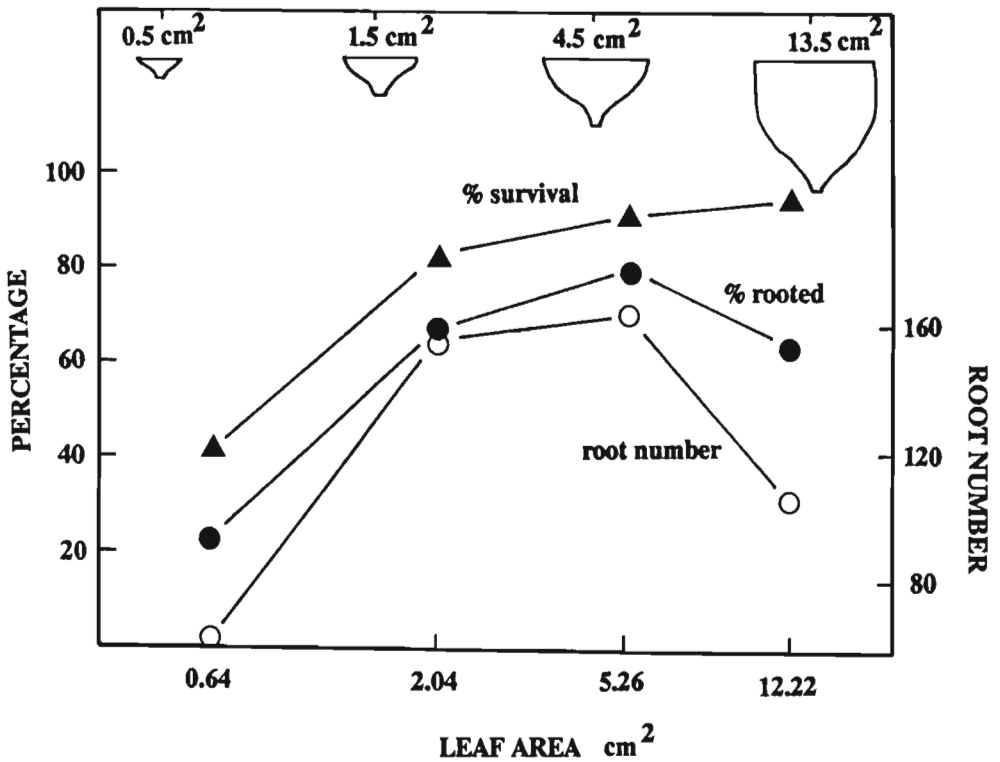

Figure 5.2. The relationship between leaf area, survival and rooting ability in one-leaf stem cuttings of *E.grandis*. Survival is an index of the rate of leaf abscission.

Although the highest leaf area did not promote leaf abscission it was nevertheless inhibitory to rooting ability (especially root number). The highest leaf area also inhibited bud development (Figure 5.3).

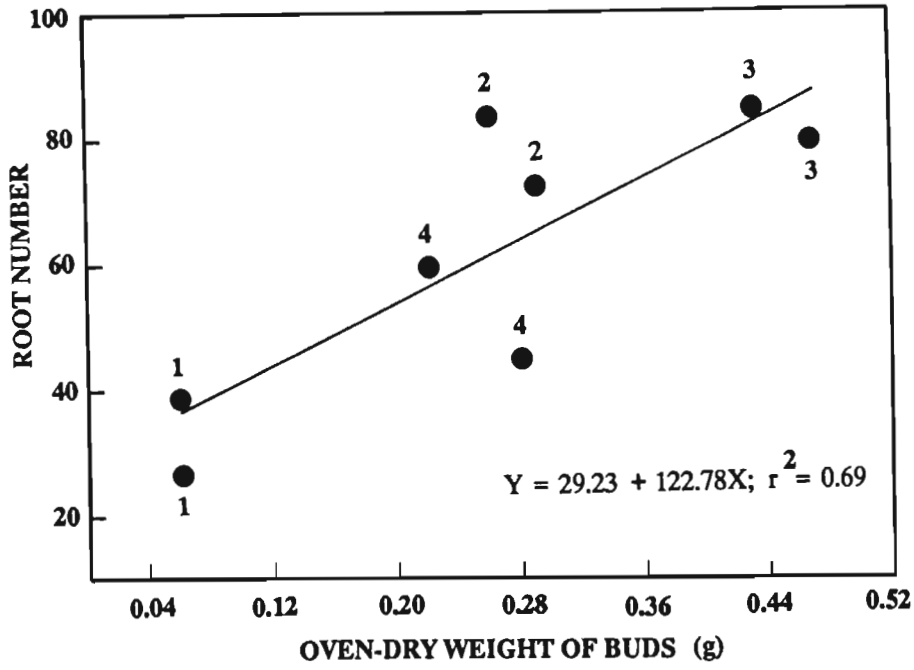

Figure 5.3. The relationship between rooting ability and bud development in one-leaf cuttings of *E.grandis* with various leaf areas. The points marked '1' had the smallest leaf area and the points marked '4' the largest.

Figure 5.3 emphasizes the linearity of the relationship between bud development and root number, but also demonstrates the importance of leaf area. There was a large difference in bud development between leaf areas 2 and 3, but no difference in root number. Bud development was similar at leaf areas 2 and 4 but root number was different.

5.3.2 THE CONTRIBUTION OF THE BUDS

1. Removing both the axillary buds and the bud meristems from cuttings before setting them reduced rooting ability, and also increased the rate of leaf abscission (Table 5.3). Rooting ability was reduced by about a third in all three clones and, on average, leaf abscission was accelerated by a comparable proportion. However, in clone 3, bud excision had no detectable effect on the rate of leaf abscission.

2. A second experiment was performed, in which the buds developing during the propagation period were removed with as little disturbance to the axil as possible. The bud meristems remained intact in this treatment, so that they tended to begin new development. These newly developing buds were removed at weekly intervals

Table 5.3. The effect of bud and bud meristem excision on rooting ability and the rate of leaf abscission in stem cuttings of three clones of *E.grandis* (each unit value is based on 64 cuttings).

	% CUTTINGS ROOTED DAY 44		% LEAF ABSCISSION DAY 23	
CLONE	CONTROL	BUDS EXCISED	CONTROL	BUDS EXCISED
1	65.6	45.9	24.2	37.1
2	64.0	43.7	25.8	41.4
3	75.0	41.9	11.7	13.0
MEANS	68.2	43.8	20.6	30.5

throughout the propagation period. Thus the treated cuttings had active bud meristems and, on average, small axillary buds not exceeding a few millimetres in length. The results are shown in Table 5.4.

Table 5.4. The effect of removing developing buds from stem cuttings, on rooting ability and the rate of leaf abscission in stem cuttings of three clones of *E.grandis* (each unit value is based on 64 cuttings).

	% CUTTINGS ROOTED DAY 41		% LEAF ABSCISSION DAY 27	
CLONE	CONTROL	BUDS REMOVED	CONTROL	BUDS REMOVED
1	48.4	18.7	25.8	57.0
2	34.4	15.6	3.9	17.2
3	68.7	51.6	3.1	3.9
MEANS	50.5	28.6	10.9	26.0

The results are comparable to those of the bud and bud meristem excision experiment. Clone 3 appears to be relatively unaffected by bud removal, possibly because leaf abscission was accelerated only during the latter part of the propagation period in this clone. At the end of the propagation period (day 41), leaf abscission in the control and

buds-removed treatments was 23.4% and 39.1% respectively.

3. Disbudding stock plants 21 days before harvesting them for stem cuttings accelerated subsequent bud development on the cuttings and improved rooting ability, but the effect on leaf abscission was inconsistent (Table 5.5).

Table 5.5. The effect of disbudding stock plants of *E.grandis*, 21 days before harvesting them for stem cuttings, on mean bud length per cutting after 12 days, and % rooting and % leaf abscission after 53 days.

CLONE	N	% ROOTED	BUD LENGTH	% ABSCISSION
		CONTROL		
1	80	11.2	1.2	69.2
2	76	7.9	1.8	21.8
3	68	45.6	13.2	17.6
MEANS	74.7	21.6	5.4	36.2
		STOCK PLANTS DISBUDDED		
1	81	23.5	5.5	39.3
2	97	14.4	3.0	31.7
3	51	64.7	21.4	13.2
MEANS	76.3	34.2	10.0	28.1

The inconsistent effect of disbudding on leaf abscission may be explained by the variation in the original number of cuttings set, which reflects the original size of the stock plants. Since pot volume was constant the smaller plants may have been growing more rapidly (or been in otherwise better condition) than the larger plants at harvest, although this was not apparent from the appearance of the plants.

In clone 1, the control and disbudded plants were similar in size (80 and 81 cuttings harvested respectively), and cuttings from the disbudded plant had a significantly slower rate of leaf abscission (see Table 5.6).

Table 5.6. The effect, on leaf abscission from *E.grandis* stem cuttings, of disbudding the stock plant 21 days before the harvest of the cuttings.

	LEAVES LOST	LEAVES RETAINED	TOTAL	
CONTROL	36	16	52	
DISBUDDED	33	51	84	$x^2 = 10.36$

5.4 DISCUSSION

It is unlikely that the leaves and buds of *E.grandis* cuttings wholly produce a rooting morphogen. Cuttings with the small leaf area of about 2 cm^2 had close to the maximum rooting ability, indicating that any 'fixed' resource related to leaf area is unlikely to limit rooting. Similarly, the loss of buds caused only a modest reduction in rooting ability which could be attributed, at least in part, to an increase in the rate of leaf abscission.

The larger the bud in the axil of the subtending leaf at the end of the propagation period the slower the rate of leaf abscission and, in general, the better the rooting ability of the cutting. This relationship was observed between axils within a cutting (the upper bud tended to develop quicker than the lower bud), when bud development was accelerated, and when the buds were removed. The presence of the bud meristems and small buds (removed weekly throughout the propagation period) had no ameliorating effect compared to the complete absence of buds and meristems.

The developing bud may inhibit the abscission of its subtending leaf, in *E.grandis* cuttings, by creating sink activity. Rather than being inhibitory, by depleting the 'fixed' resources of the cutting, sink activity could be beneficial to the function of the leaf. The rate of photosynthesis is sink-dependent (THORNE & KOLLER, 1974; GEIGER, 1976; HEROLD, 1980), and sink activity is probably severely diminished when the cutting is excised. The larger the bud the stronger its expected sink activity, which could therefore increase the metabolic activity and health of the leaf.

Leaf abscission can be seen as the consquence of leaf dysfunction, in which case it would be affected by the transit of many factors through the petiole, as well as their rate of transit (TREWAVAS, SEXTON & KELLY, 1984). The transit rate would have been diminished in leaves reduced to 0.6 cm^2, which suffered markedly more rapid leaf abscission than the larger leaf areas. However, leaves with the super-optimal leaf area of 12.2 cm^2, which were relatively quiescent (as judged from the rate of development of

their axillary buds and the rooting ability of the cuttings), did not abscise quicker than leaves with lesser leaf areas.

This could be explained if the rate of flow into the leaf, as well as the rate of flow out of the leaf, affects abscission. Leaves with super-optimal leaf areas probably have a high demand for water relative to the supply, which would result in water stress. Their rate of import in the transpiration stream could nevertheless be relatively high, since stressed leaves of eucalypts may continue to transpire, either through the stomata (PRYOR, 1976) or after the stomata have closed (PEREIRA & KOZLOWSKI, 1976). Water stress would, however, tend to inhibit basipetal translocation (PLAUT & REINHOLD, 1965; BREVEDAN & HODGES, 1973), perhaps inhibiting rooting ability. Cuttings with the largest leaf area rooted less well than expected, according to their rate of bud development (*cf.* leaf areas 2 and 4, Figure 5.3), perhaps for this reason.

The development of roots on stem cuttings could affect bud development and leaf function, rather than *vice versa*. In leaf squares of *Peperomia sandersii* A.D.C., buds developed after roots, and each arose close to the origin of a root (HARRIS & HART, 1964). Similarly, the continued growth of buds on tobacco explants depended on the presence of a vascular connection with a root (SKOOG & MILLER, 1948). The development of roots on the petioles of detached runner-bean leaves prevented the rapid leaf senescence which otherwise followed excision (CHIBNALL, 1954).

Roots begin to emerge from *E.grandis* cuttings after 7 to 10 days, so that interactions between the roots, and the leaves and buds, would be likely by the end of the propagation period (over 40 days). Other sources/sinks in the cutting are possible, the activity of which could depend on activity elsewhere in the cutting. For example, bud break in *Betula pendula* Roth. is associated with a change in the partitioning of carbohydrates in the stem (SAUTER & AMBROSIUS, 1986). Clearly, the source/sink interactions within a cutting could be complicated. Nevertheless, it seems likely that most source/sink activity, at least initially, is located in the leaves and buds.

The buds must demand from, and supply to, the extra-cambial tissues (where roots initiate), since the bud meristem is located external to the wood. The leaves, on the other hand, have the option of utilizing the internal phloem which is present in eucalypts (METCALFE & CHALK, 1950). The buds, having developed in the propagation environment, may also be relatively well adapted to the prevailing conditions. Nevertheless, it is leaf activity which appears to be critical to rooting ability. Bud activity would be relatively low during the earlier part of the propagation period, when many roots initiate, and in the optimum range of leaf area (2 cm^2 to 5 cm^2), rooting ability was insensitive to variation in the rate of bud development (*cf.* leaf areas 2 and 3, Figure 5.3).

The rooting ability of *E.grandis* cuttings cannot be attributed to direct effects of the leaves and buds, such as might be expected if the leaves and buds were sole sources of a limiting morphogen. However, the activity of the leaves and buds is clearly favourable for root initiation. Mature leaves are sinks as well as sources (CANNY, 1973), and the buds are known to import auxin as well as to export it (see Chapter 4). It is therefore possible that the leaves and buds import the precursors of the morphogen, or that they simply cause the morphogen to circulate. In either case the activity of the leaves and buds would be more important than their *de novo* supply of the morphogen, which would originate from the stem, the wound responses, or from outside the cutting.

Both mature leaves (ESCHRICH, 1975) and buds (see Chapter 4) export basipetally. If the activity of the leaves and buds determines the rate of export of the rooting morphogen, this activity should be reflected in the tangential distribution of roots: a higher frequency of roots would be expected in the leaf trace sectors of the stem. This possibility is considered in the next chapter.

CHAPTER SIX

THE TANGENTIAL DISTRIBUTION OF ADVENTITIOUS ROOTS ON STEM CUTTINGS OF *EUCALYPTUS GRANDIS*

6.1 INTRODUCTION

The tangential distribution of adventitious roots emerging from woody stem cuttings has not received attention. However, the stem of conventional-sized *Eucalyptus grandis* cuttings is asymmetrical in several respects in the radial tangential plane. These asymmetries, illustrated in Plates 6.1 and 6.2, may or may not be reflected in the frequency of root emergence.

The terminal shoots of *E.grandis* coppice grow more or less vertically, whereas the lateral shoots grow obliquely. In practice a high proportion of cuttings often originate from the lateral shoots, the stems of which are characteristically red above and green (with no admixture of red) below (Plate 6.1).

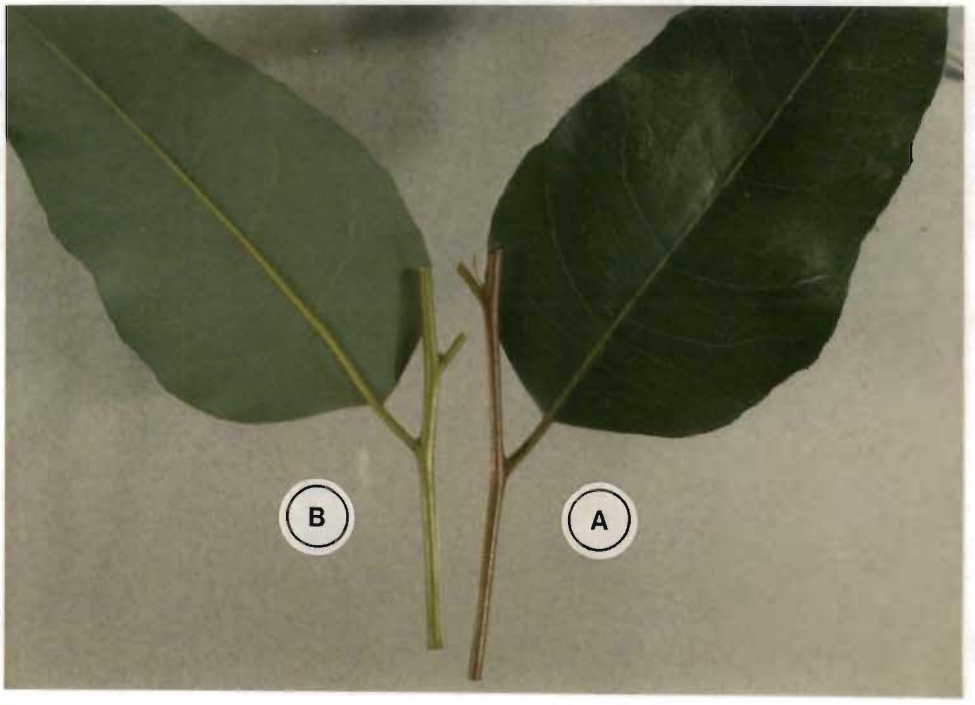

Plate 6.1. The (a) upper and (b) lower surface of a lateral shoot from *E.grandis* coppice. The shoots are characteristically red above and green below. The stems are 4 cm to 5 cm long.

The red upper surface contains anthocyanins, a common group of phenolics well known as red pigments in the young leaves and bark of first-year eucalypt twigs (SHARMA & CROWDEN, 1974). Anthocyanins (or the metabolism associated with them) have been implicated in adventitious rooting in several studies.

Leaves of *Hedera canariensis* with juvenile morphology were found to contain more anthocyanins than leaves with adult morphology, and juvenile cuttings also rooted better than adult ones (STOUTEMYER, BRITT & GOODWIN, 1961). BACHELARD & STOWE (1963) observed a positive correlation between leaf anthocyanin content and rooting ability in *Acer rubrum* cuttings, and the possible precursors of anthocyanin biosynthesis, sucrose and riboflavin, improved rooting ability when applied to *Eucalyptus camaldulensis* cuttings (BACHELARD & STOWE, 1962). Thus, according to the hypothesis that anthocyanins (or the metabolism associated with them) promote rooting, more roots would be expected to emerge from the red than from the green face of the stem in *E.grandis* stem cuttings.

The cross-section of the stem approximates to an oblong with rounded corners (Plate 6.2).

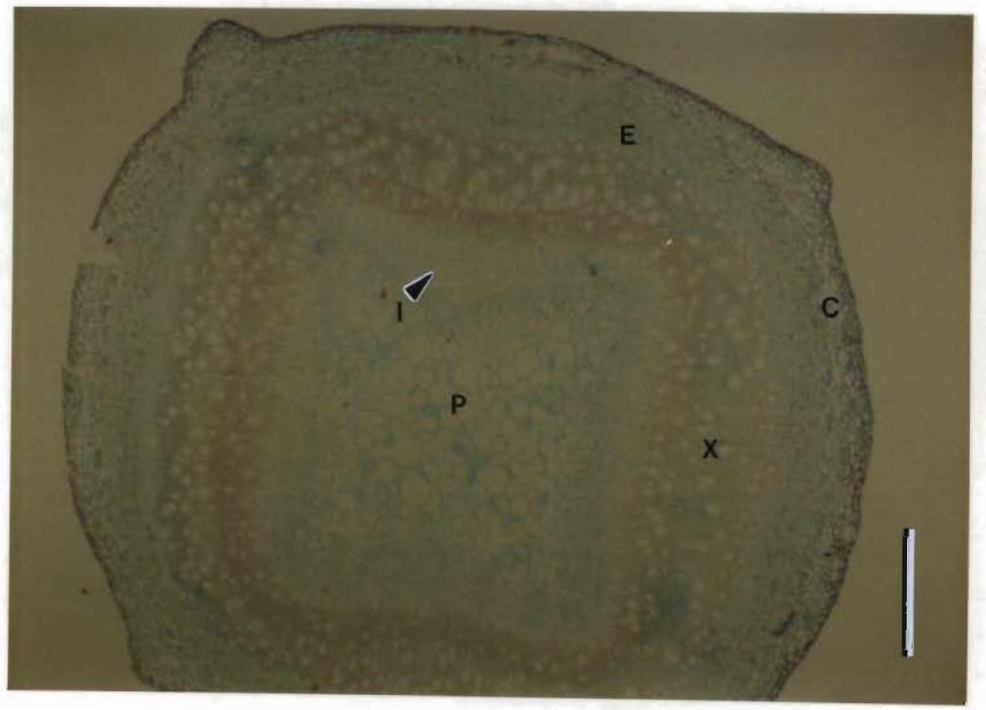

Plate 6.2. Transverse section through the stem of an *E.grandis* seedling. The stem is squarish, and the internal phloem appears to be relatively poorly developed in the corner sectors. The section was embedded in wax and stained with safranin and fast green (see section 6.2). P = pith; I = internal phloem; X = xylem; E = external phloem; C = cortex. Scale bar = 0.5 mm.

The physical stresses set up by radial growth or bending could easily be different at the corners compared to the flatter faces of the stem. When a root bends round an obstacle, lateral roots tend to develop on the convex side (WOODROOF & WOODROOF, 1934; MCQUILKIN, 1935), and these laterals may be large relative to other laterals (HORSLEY & WILSON, 1971). This suggests that tensile stresses may be promotory to root initiation.

Eucalyptus species have an internal phloem which is distributed asymmetrically, being most extensive at the mid-points of the flatter faces of the stem and least extensive at the corners (METCALFE & CHALK, 1950; Plate 6.2).

The leaves have discrete vascular traces, which should remain well defined over the vertical distance from the insertion of the petioles to the base of the *E.grandis* cutting (3 to 6 cm). Vascular elements can deviate from the vertical, but the deviation is typically less than 1% in phloem (ZIMMERMANN, 1983).

It is assumed that actual or potential sites occur (at one height) in a ring of constant thickness a constant distance from the epidermis. Thus, all other factors being equal, the number of sites (at one height) should be directly proportional to the length of the circumference of the stem. By calculating the length of the circumference in different sectors of the stem, the numbers of emerged roots could be compared to the predicted numbers of sites available. Discrepancies could be taken as evidence for an exogenous influence which, as it increases (for example), would either create sites or would increase the proportion of potential sites which actually initiate roots. On the other hand, rooting ability would appear to be influenced more by the number of sites available if a constant number of emerged roots, relative to the predicted number of sites, was observed.

6.2 MATERIALS AND METHODS

The specimens from which the sections shown in Plate 6.2 and Plate 4.1 were prepared were fixed in F.A.A., dehydrated in a concentration series of ethanol and tertiary butyl alcohol, impregnated with paraffin wax and embedded. They were sectioned on a sliding microtome (section thickness 10 μm), dewaxed, stained with safranin and fast green and mounted, using a conventional procedure.

Cuttings of *E.grandis* were treated and cultured as described in section 5.2, unless otherwise stated.

The cross-section of the stem of coppice shoots approximates to an oblong with rounded

corners. The leaf traces from the node above are easy to identify, even when the stem is twisted, because they occupy the two shorter sides of the rectangle.

A circle was drawn with eight 45° sectors radiating from its centre. That portion of a stem bearing roots was severed and positioned vertically at the centre of the circle, with the estimated mid-point of the leaf traces (marked with mounting needles) occupying the mid-points of two opposing sectors. Since the pith is more rectangular in cross section than the surface of the stem (see Plate 6.2), reference to this assisted in locating the mid-point of the faces of the stem containing the leaf traces. The numbers of roots emerging in the eight sectors were then recorded.

Experiments were performed in order to compare the frequency of roots emerging from: (i) The upper (red) and lower (green) surfaces of the stem in two-leaf cuttings. (ii) The sides (leaf traces), top and bottom, and corners of the stem in two-leaf cuttings, and (iii) The two leaf traces in one-leaf cuttings. Any roots which appeared to emerge from basal callus, rather than directly from the stem itself, were excluded from the analyses. In experiments (i) and (iii) 'borderline' roots were excluded. In experiment (ii) all roots were made to fall into one sector or another.

6.2.1 COMPARISON OF THE FREQUENCY OF ROOT EMERGENCE FROM THE UPPER (RED) AND LOWER (GREEN) SURFACES OF THE STEM

The numbers of emerged roots in the two sectors were recorded in a total of 663 cuttings (four batches) of one clone, 51 to 64 days after the cuttings were set. The batches of cuttings rooted with 38.3% to 87.4% success, 0.87 to 3.32 roots per cutting. The results were consistent between batches and were combined. The upper surface of the stem varied from very deeply red to lightly tinged with red. The lower surface was uniformly green with no admixture of red. The two surfaces were assumed to be of equal length, so that the expected proportion of roots emerging from each (assuming no difference in frequency) was 0.5. A goodness-of-fit test was used to compare the observed and expected proportions (FREESE, 1967).

6.2.2 COMPARISON OF THE FREQUENCY OF ROOT EMERGENCE FROM THE SIDES, TOP AND BOTTOM, AND CORNERS OF THE STEM

A sample of 128 cuttings was harvested from the coppice growing from a single stump of *Eucalyptus grandis*. To ensure high rooting ability, the coppice was shorter than usual

when harvested (0.3m to 0.5m in length) and a high proportion of cuttings came from terminal rather than from lateral shoots. At this stage little or no red pigmentation was present in the upper surface of laterals. The cross-section of the stem was assumed to be (on average) quadlaterally symmetrical. Thus the eight sectors could be combined, on the basis of length of sector, into three non-equivalent categories: the sides of the stem (leaf traces); the surfaces perpendicular to the leaf traces; and the corners.

The thickest and thinnest cuttings were excluded, so that stem diameter varied in the range 2cm to 3 cm. The tangential distribution of the emerged roots was recorded after 38 days.

Before the cuttings were set a formal random subsample of 28 cuttings was selected and 5 mm of shoot from the base of each was cut into formalin: glacial acetic acid: 95% ethanol: water (2:1:10:7). After 10 days, transverse hand sections were cut from these lengths of shoot and photographed using a dissecting photomicroscope, the magnification chosen so that the size of the image from top to bottom of the section was constant (on average × 25). The negatives were magnified by photocopying so that this dimension was close to 6 cm.

As with the rooted cuttings, 45^{o} sectors were superimposed on the outline of these reproduced stem sections, orientated so that the mid-points of two opposing sectors corresponded to the estimated mid-points of the leaf traces. The length of the circumference in each sector was then estimated (see section 6.3 below). The total length of circumference (with confidence limits) for all 128 cuttings was also estimated from the sample values, for the three categories of sector recognized, using the formulae for linear functions given by FREESE (1967). The predicted numbers of sites (*ie.* the length of circumference) in each category of sector was compared with the numbers of emerged roots.

6.2.3 COMPARISON OF THE FREQUENCY OF ROOT EMERGENCE FROM THE TWO LEAF TRACES OF ONE-LEAF CUTTINGS

The numbers of emerged roots in the two leaf trace sectors were recorded in two samples of cuttings of one clone: a) 192 cuttings, 52 days after the cuttings were set and b) 128 cuttings 69 days after the cuttings were set. The results were consistent between samples and were combined. The two leaf trace sectors were assumed to be of equal length so that the expected proportion of roots emerging from each (assuming no difference in frequency) was 0.5.

6.3 RESULTS

6.3.1 COMPARISON OF THE FREQUENCY OF ROOT EMERGENCE FROM THE UPPER (RED) AND LOWER (GREEN) SURFACES OF THE STEM

Significantly more roots emerged from the lower (green) surface of the stem than from the upper (red) surface (Table 6.1).

Table 6.1. The observed numbers of roots emerging from the upper (red) and lower (green) surfaces of the stem, compared to the expected numbers assuming that roots emerge with equal frequency from both surfaces.

	RED	GREEN	TOTAL	
OBSERVED	65	110	175	
EXPECTED	87.5	87.5	175	$\chi^2 = 11.57$

6.3.2 COMPARISON OF THE FREQUENCY OF ROOT EMERGENCE FROM THE SIDES, TOP AND BOTTOM, AND CORNERS OF THE STEM

Construction of an average cross-section for the stem

All measurements were made on the reproduced stem sections and were doubled for the calculations (this allowed a check with a convenient graphical scale). Trigonometric formulae were given by DURELL, (1937). The symbol '<' denotes an angle.

Initially, the circumference in each 45° sector of the stem was assumed to represent the arc of a circle of unknown diameter. The arc length (of each sector in each of 28 sample cross-sections) was calculated from measurements of the chord length (VW) and the greatest perpendicular distance (YZ) from the chord to the circumference (Figure 6.1).

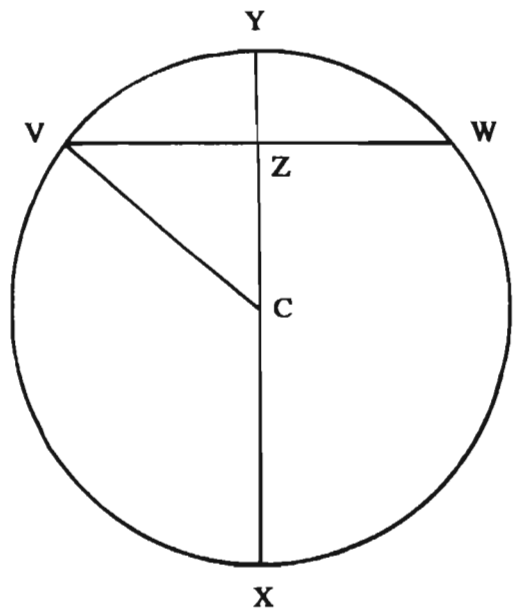

Figure 6.1. The calculation of the length of the arc VW (an estimator of circumference) in each 45° sector of the stem. Note that the circle is an extrapolation of the arc and does not represent the cross section of the stem.

To find the diameter XY of the arc VW

If XY is the diameter of a circle and the perpendicular bisector of the chord VW, then:

$VZ^2 = YZ \times XZ$.

YZ and VZ (= 0.5 VW) are known, so XZ can be calculated.

XY = XZ + YZ = diameter.

The radii calculated for the three sectors were: leaf traces 87.18: top and bottom 86.31: corners 42.76

To find the length of the arc VW

The radius (CV = 0.5 XY) is known.

$< VCY = (VZ \div CV) \sin^{-1}$

$< VCW = 2 \times < VCY$

Length of arc = $(< VCW \div 360) \times (\pi \times XY)$.

The interior dimensions of the sector boundaries (aa, bb, cc and dd: Figure 6.2) were

measured and the pooled means for aa and bb (113.00), and cc and dd (123.06), were calculated.

The procedure to calculate the length of an arc, described above, was thought to be satisfactory for estimating the length of the circumference in the flatter sectors of the stem, *ie*.the sides (leaf traces) and upper and lower surfaces, but not for the corners. The stem proved on average to approximate to an oblong, the larger dimension being from leaf trace to leaf trace (sector 1 in Figure 6.2). This asymmetry caused the arc calculated for the corners to 'balloon' slightly (shown in the upper left corner of Figure 6.2). In addition, during measurement, it was found that the maximum dimension from the chord to the circumference tended not to bisect the chord, so that one of the assumptions required for calculating the length of the circumference was not met.

It appeared that a better approximation to the true shape of the corners would be obtained by extrapolating the arcs from the flatter faces of the stem (shown in the upper right corner of Figure 6.2), if the point of intersection could be rounded off. This was achieved in the following calculations, and is illustrated in the lower left and right corners of Figure 6.2.

It was necessary to assume that the radii of the two extrapolated arcs were the same, whereas in fact they differed by approximately 1% (86.31 *versus* 87.18). The calculations shown assume that the common radius is 86.31. The calculations were then repeated assuming a common radius of 87.18, and the mean was taken as the best estimate of the length of the circumference of the corners.

The calculations can be divided into the following stages: 1) The calculation of the lengths of the arcs AD and AH (Figure 6.2). 2) The location of an imaginary point M, defining the chord DM of which the line DH is a part (Figure 6.3). 3) Moving clockwise from D, the location of the point P at which a perpendicular from the line DH to the arc AD has the observed (measured) value (Figure 6.4). 4) The arc AD was assumed to stop at this point and, similarly, the arc AH was assumed to stop at a point T an equal distance from A. The corner was then rounded off with the construction of an arc of smaller diameter such that i) the arcs met at points P and T, and ii) the arcs had common tangents at these points (Figure 6.5). 5) The calculation of the length of the circumference, consisting of the arc AP, the arc HT, and the arc PT connecting them.

To find the lengths of the arcs AD and AH (Figure 6.2)

$< EFD = < GFH = 22.5$; $FD = 56.20$; $FH = 61.53$; $AC = CD = 86.31$

Assume $AB = BH = 86.31$.

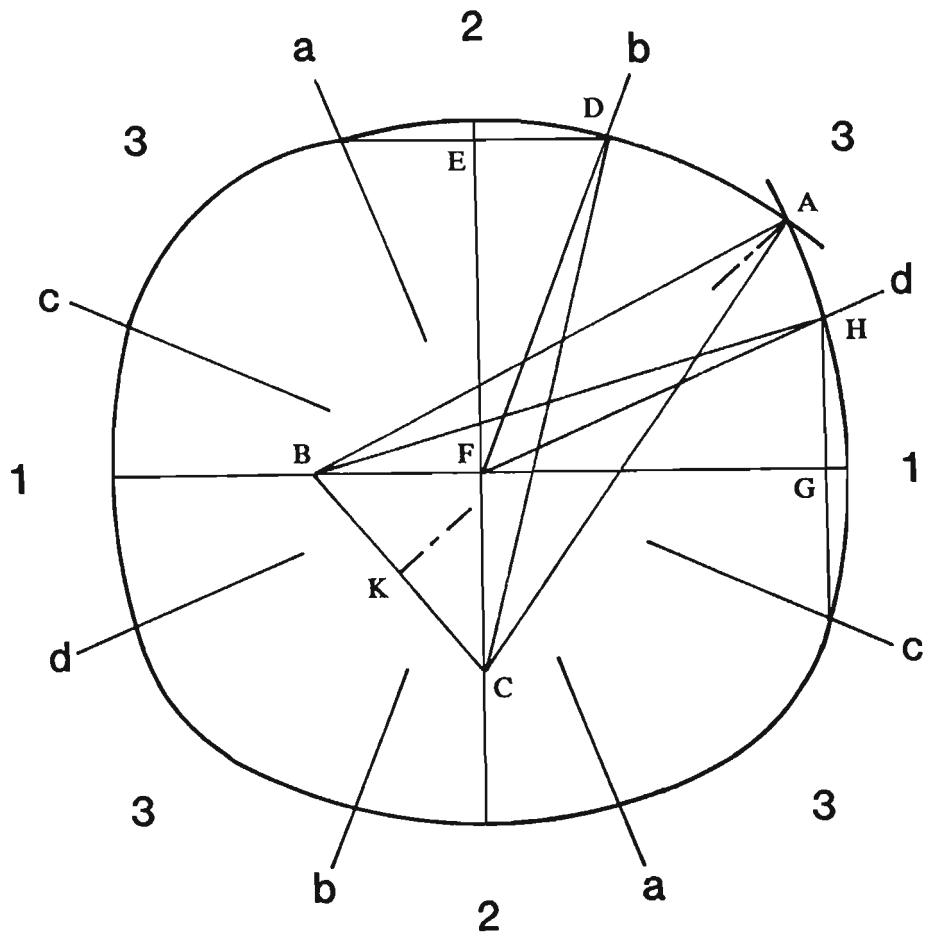

Figure 6.2. The mean cross section through the stems of 28 *E.grandis* stem cuttings. Three versions of the corners are shown (see text). The preferred version is shown in the lower left and lower right corners.

To find CF:

$ED = \sin 22.5^{o} \times FD = 21.62$

$EF = \cos 22.5^{o} \times FD = 52.20$

$< ECD = (ED \div CD) \sin^{-1} = 14.51^{o}$

$CE = \cos < ECD \times CD = 83.56$

$CF = CE - EF = 31.36.$

To find BF:

$GH = \sin 22.5 \times FH = 23.55$

$FG = \cos 22.5 \times FH = 56.85$

$< GBH = (GH \div BH) \sin^{-1} = 15.83^{o}$

$BG = \cos < HBG \times BH = 83.04$

$BF = BG - FG = 26.19.$

To find BC:

$BC^2 = CF^2 + BF^2$; $BC = 40.86.$

To find $< ABC$:

AK is perpendicular to BC; AB = AC

$BK = CK = 0.5\ BC = 20.43.$

$< ABC = (BK \div AB) \cos^{-1} = 76.31^{o}.$

To find ACD:

$< BCF = (CF \div BC) \cos^{-1} = 39.87^{o}$

$< ACB = < ABC = 76.31^{o}$ (since AB = AC)

$< ACD = < ACB - < ECD - < BCF = 21.93^{o}$

Arc $AD = (< ACD \div 360) \times (\pi \times \text{diameter}) = 33.04.$

To find $< ABH$:

$< CBF = (CF \div BF) \tan^{-1} = 50.13$

$< ABH = < ABC - < GBH - < CBF = 10.35$

Arc $AH = (< ABH \div 360) \times (\pi \times \text{diameter}) = 15.59$

Length of circumference of corner (assuming no rounding):

length = arc AD + arc AH = 48.63.

To locate the imaginary point M (Figure 6.3)

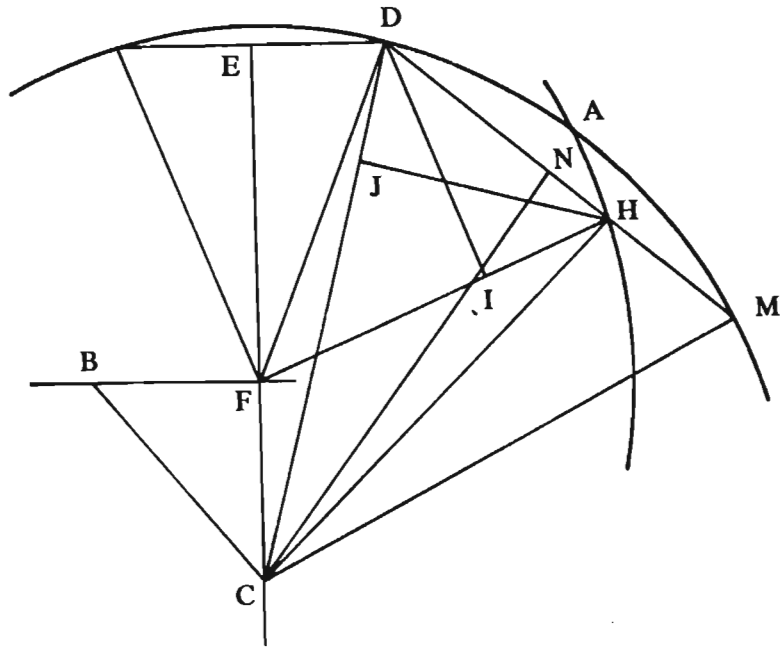

Figure 6.3. A detail of the mean cross section of the stem (shown in Figure 6.2), illustrating the location of the imaginary point M.

$CD = CM = 86.31$; $DF = 56.5$; $FH = 61.53$; $< DFH = 45^o$; $< EFD = 22.5^o$.

To find $< CDH$:

$< DFI = 45^o$; $< FDI = 45^o$; $DI = FI$.

$DF = 56.5$; $DI = \sin 45^o \times 56.5$; $DI = 39.95$; $FI = 39.95$

$FH = 61.53$; $IH = FH - FI = 21.58$

$DH^2 = DI^2 + IH^2$; $DH = 45.41$

$< IDH = (IH \div DH) \sin^{-1} = 28.37^o$

$CD = 86.31$; $ED = \sin 22.5^o \times 56.5 = 21.62$

$< DCE = (ED \div CD) \sin^{-1} = 14.51$

$< EFD = 22.5^o$; $< DFC = 157.5^o$; $< DCF = 14.51^o$

$< CDF = 180^o - < DFC - < DCF = 7.99^o$

$< CDH = < IDH + < FDI - < CDF = 65.38^o$

To find CH:

$HJ = \sin < CDH \times DH = 41.28$

$DJ = \cos < CDH \times DH = 18.92$

$CJ = CD - DJ; CD = 86.31; CJ = 67.39$

$CH^2 = CJ^2 + HJ^2; CH = 79.03.$

To find DM:

$< CHJ = (CJ \div HJ) \tan^{-1} = 58.51^o$

$< DHJ = (DJ \div DH) \sin^{-1} = 24.62^o$

$< CHM = 180^o - < CHJ - < DHJ = 96.87^o$

$< CHN = 180^o - < CHM = 83.13^o$

$HN = \cos < CHN \times CH = 9.45$

$CN = \sin < CHN \times CH = 78.46$

$CM = 86.31; CM^2 = CN^2 + MN^2; MN = 35.96$

$DM = DH + MN - HN = 71.92.$

To locate the point P, at which a perpendicular from the line DH to the arc AD has the observed value (Figure 6.4).

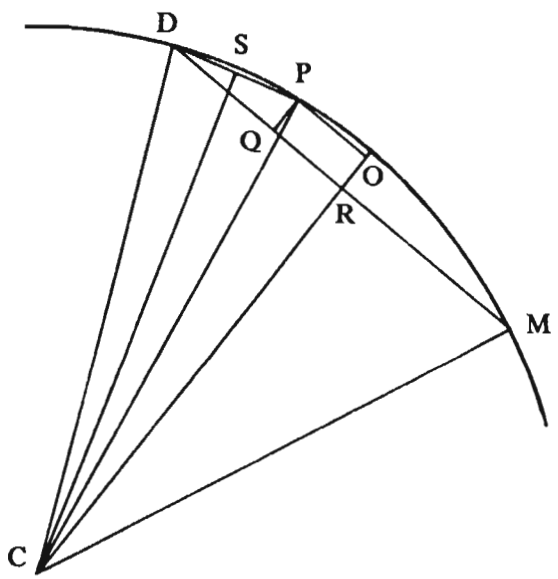

Figure 6.4. A detail of the mean cross section of the stem (shown in Figure 6.2), illustrating the location of the point P.

To find the length of the arc DP:

CD = CM = CP = 86.31; PQ = OR = 6.516; DM = 71.92; DR = 0.5 DM = 35.96.

$CD^2 = DR^2 + CR^2$; CR = 78.46

CO = CR + RO = 84.98

$CP^2 = CO^2 + OP^2$; OP = QR = 15.09

DQ = DR - QR = 20.87

$DP^2 = DQ^2 + PQ^2$; DP = 21.86

DS = 0.5 DP = 10.93

$< DCS = (DS \div CD) \sin^{-1} = 7.28^o$

$< DCP = 2 \times < DCS = 14.56^o$

length of arc DP = $(< DCP \div 360) \times (\pi \times \text{diameter}) = 21.93$.

To find the length of the arc PT of radius UP (= UT) (Figure 6.5)

$< ECD = 14.51^o$; $< DCP = 14.56^o$; $< BCF = 39.87^o$; CK = 20.43; AB = AC.

$< ECP = < ECD + < DCP = 29.07$

$< BCP = < BCU = < BCF + < ECP = 68.94$

$< BCU = < CBU$

$< PUT = < BUC = 180^0 - (< BCU + < CBU) = 42.12^o$

$CU = (CK \div \cos < BCP) = 56.85$

UP = CP - CU = 29.46 = radius

arc PT = $(< PUT \div 360) \times (\pi \times \text{diameter}) = 21.66$.

To find the total length of circumference of the rounded corner

(i) Assuming (as in the above calculations) that AC = CD = AB = BH = 86.31:

Arc AD = 33.04; arc DP = 21.93.

Arc AT = arc AP = arc AD - arc DP = 11.11

total length = (arc AD + arc AH) - arc AP - arc AT + arc PT = 48.07.

(ii) Total length recalculated assuming AC = CD = AB = BH = 87.18:

total length = 47.93

This estimate of length is less because, although the radial distances are greater, the tangential distances are less (the arcs are flatter). The difference between the two estimates is approximately 0.3% and the mean = 48.00.

This value could be a slight overestimate since the method for calculating the third arc caused the observed maximum perpendicular distance from the chord to the circumference of the sector (PQ, Figure 6.4) to be slightly exceeded in the model. However, the three versions of the shape of the corners assuming that they consist of one

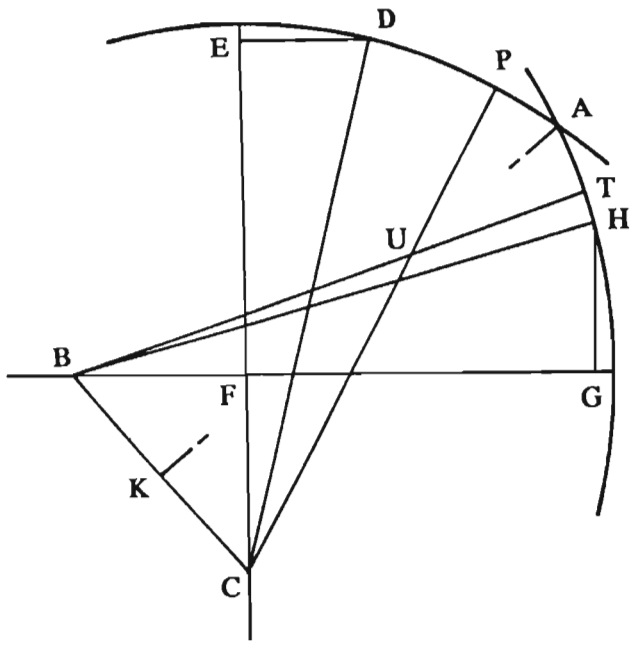

Figure 6.5 A detail of the mean cross-section of the stem (shown in Figure 6.2), illustrating the construction of the arc PT.

arc, two intersecting arcs or three arcs gave very similar lengths of circumference, indicating that any errors were minor.

The comparison of the predicted numbers of emerged roots with the actual numbers

The length of the circumference was estimated in a sample of 224 sectors (28 cuttings, 8 sectors/cutting), and the means and standard errors were calculated for the three sectors recognized: sector 1 (leaf traces, n = 56); sector 2 (upper and lower surfaces, n = 56); and sector 3 (corners, n = 112). This allowed the total lengths (128 cuttings) with confidence limits to be calculated:

Sector 1

Total length:

$$NX = 256 \times 48.161 = 12329.2.$$

Where N = total number of sectors in population of 128 cuttings.

X = mean length of sector in sample of 28 cuttings.

Standard error of total:

$$NS_{\bar{X}} = 256 \times 0.4572 = 117.0432.$$

Where $S_{\bar{X}}$ = sample standard error (n = 56), incorporating the finite population correction (1 n ÷N).

Total length with 95% confidence limits:

$$NX \pm NS_{\bar{X}} \times t_{0.05,\ 55\ df} = 12329.2 \pm 234.7.$$

Sector 2

Total length = 11157.3 ± 142.8.

Sector 3

Total length = 24549.2 ± 320.8.

In this calculation the corners were assumed to consist of one arc. The total length of the corners assuming that they consist of three arcs is 48.00 × 512 = 24576, a difference of approximately 0.1%.

No sampling error is associated with numbers of emerged roots since the whole population of 128 cuttings was enumerated. The total numbers of emerged roots is compared with the total lengths of circumference in Table 6.2.

Table 6.2. The numbers of roots emerging from 1. The leaf traces 2. The upper and lower surfaces, and 3. The corners of the stem, compared to the total length of circumference in each category.

SECTOR	NUMBER OF ROOTS	PROPORTION OF TOTAL	LENGTH OF CIRCUMFERENCE	PROPORTION OF TOTAL
1	234	0.2445	12329.2 ± 234.7	0.2565 ± 0.0049
2	224	0.2341	11157.3 ± 142.8	0.2321 ± 0.0030
3	499	0.5214	24576.0 ± 320.8	0.5113 ± 0.0067

The frequency of root emergence was about 5% greater than expected from the leaf trace sectors and 2% less than expected from the corner sectors of the stem. The frequency from the top and bottom sectors of the stem was within the confidence limits calculated for the expected value.

6.3.3 COMPARISON OF THE FREQUENCY OF ROOT EMERGENCE FROM THE TWO LEAF TRACES OF ONE-LEAF CUTTINGS

The results are shown in Table 6.3. Significantly more roots emerged from the trace below the leaf than from the trace on the opposite side of the stem (below the absent leaf).

Table 6.3. The observed numbers of roots emerging from the two leaf traces of one-leaf cuttings, compared to the expected numbers assuming that roots emerge with equal frequency from both.

	BELOW LEAF	BELOW ABSENT LEAF	TOTAL	
OBSERVED	78	51	129	
EXPECTED	64.5	64.5	129	$x^2 = 5.65$

6.4 DISCUSSION

According to the suggestion that anthocyanins, or the metabolism associated with them, are implicated in the rooting ability of woody stem cuttings (BACHELARD & STOWE, 1963), more roots might be expected to emerge from the upper red surface of the *E.grandis* stem than from the opposing green surface. However, significantly more roots emerged from the green surface. This could be interpreted to mean that anthocyanins, or aspects of anthocyanin metabolism, effectively inhibited rooting.

This interpretation would be supported by the suggestion made by HAISSIG (1986), that anthocyanins are associated with maturity and that the precursors, which may be essential to rooting, would be available in those tissues lacking anthocyanins. However, it is not known whether significantly different levels of anthocyanin precursors could be maintained in the opposing faces of the stem of *E.grandis* cuttings during the propagation period.

Anthocyanins, or aspects of their metabolism, may have no direct role in adventitious rooting. They could have various other functions. They may: confer frost resistance (WILCOX, 1982), represent an adaption to low light (LEE, LOWRY & STONE, 1979; WILCOX, 1982), or participate in wound responses in leaves (BOPP, 1959). As is well known, they also participate in leaf senescence in temperate hardwoods, and may be

formed when carbohydrates are super-abundant. Unbalanced growth in *Lemna minor* L. led to an accumulation of both carbohydrates and anthocyanins (WHITE, 1937), and sun leaves of plum contained more anthocyanins than shade leaves (SWAIN, 1959). Anthocyanins often accumulate only in the epidermis and sub-epidermis (McCLURE, 1979), which is remote from the usual sites of adventitious root initiation (close to the vascular system).

The upper (red) surface of the obliquely orientated *E.grandis* stem receives a much higher intensity of light than the lower surface. As well as affecting carbohydrate levels, high irradiance might affect such variables as the frequency of lenticels or cell wall characteristics. The base of the *E.grandis* cutting often swells slightly, and this almost invariably proceeds more rapidly on the green (lower) than on the red (upper) surface of two-leaf cuttings. Whether this could affect rooting is not known.

In easy-to-root two-leaf cuttings, a small but significant role for the leaves and/or buds was apparent, since 5% more roots than expected emerged from the leaf trace sectors of the stem. This role was confirmed in subsequent experiments, in which the frequency of roots emerging from the sector below the leaf of one-leaf cuttings was about 20% higher than the expected (mean) value, whereas the frequency from the opposing sector (below the absent leaf) was about 20% less than expected.

Leaf/bud activity could only be communicated to the base of the cutting *via* a flux in the transporting tissues of the stem, and suggests that efflux/influx at the base of the cutting promotes rooting. The efflux of a promotory morphogen would be consistent with the observation of JARVIS & SHAHEED (1986), that the rooting ability of mung bean cuttings was related to the quantity of auxin moving basipetally in the stem. There is no convincing evidence to support the alternative explanation, that leaf/bud activity accelerates the influx of mobile rooting inhibitors at the base of the cutting.

The frequency of root emergence from the upper and lower surfaces of the stem combined was not significantly different from the expected, but the frequency from the corners was (significantly) about 2% less than expected. The internal phloem is least developed in the corner sectors of the stem (METCALFE & CHALK, 1950), and this could account for the result if the internal phloem participates in transport in *E.grandis* stem cuttings.

In some circumstances roots tend to emerge from stem cuttings, such as those of plum, directly below the buds (HARTMANN & KESTER, 1983). In easy-to-root two-leaf *E.grandis* cuttings, however, the observed distribution of roots was remarkably close to the predicted distribution, notwithstanding various asymmetries in the stem. This raises

the possibility that the potential sites for root initiation are relatively insensitive to variations in the level of the morphogen. If the morphogen were unloaded in the leaf trace sectors of the stem marked variations in its tangential distribution might be expected. However, a uniform distribution of the morphogen at the base of the cutting could be achieved in two ways.

1. The morphogen could arrive asymmetrically (in the leaf traces for example), be unloaded and then diffuse outwards, reaching a close-to-constant concentration in all sectors of the stem. The rate of efflux would need to be low in relation to the rate of tangential migration to prevent appreciable concentration gradients from developing.

2. The morphogen could migrate tangentially within the transporting tissues, without becoming accessible to potential sites for root intitiation, before arriving at the base of the cutting. In this case, any concentration gradients would not affect the tangential distribution of roots. The gradients would be on too small a scale, perhaps (for example) at the level of individual sieve elements.

The results of this chapter do not allow these possibilities to be distinguished. They are considered again in the following chapter, in which the vertical distribution of roots, from the base of the cutting upwards, is investigated.

Chapter 5.

Cuttings in experiments 1 and 2 had simple transverse severance cuts at the base.

Experiment 3. Cuttings of one clone were prepared with either: a) a simple transverse cut at the base, or b) an additional radial longitudinal cut extending upwards from the base of the cutting for 5 to 6 mm. The treatments consisted of two plots of 32 cuttings each, arranged alternately.

The cuttings in experiments 2 and 3 were not treated with IBA. The three experiments were enumerated after 50, 69 and 38 days respectively.

Experiment 4. Cuttings of one clone were prepared with an additional radial longitudinal cut extending upwards from the base of the cutting for 13.7 ± 1.1 mm. The cut was orientated perpendicular to the leaf traces. The cuttings were either: a) left like this, or b) one portion of the split base was removed, with a close-to-horizontal cut at the upper extremity of the additional wound, after springing the chosen portion away from the longitudinal (Figure 7.1).

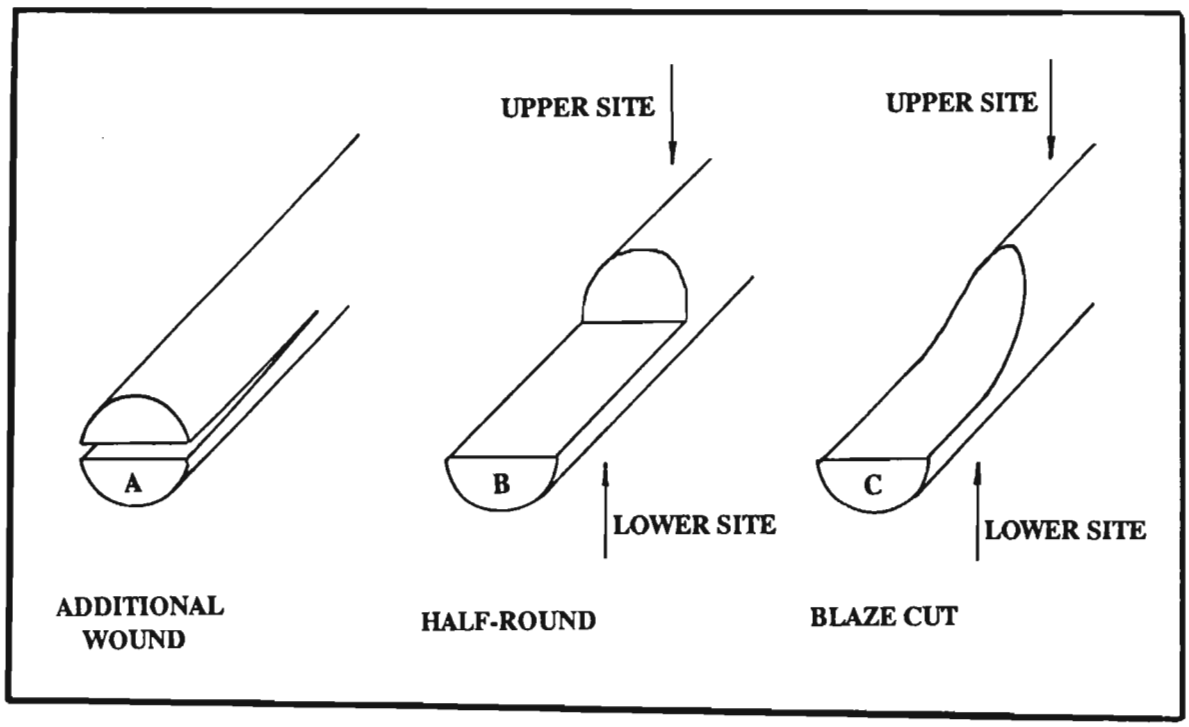

Figure 7.1. Illustration of wounds applied to cuttings in experiments 4 and 5 (see text). A: Control treatment, both experiments. B: Base of cutting reduced to one half with one vertical cut and one close-to-horizontal cut (experiment 4). C: Base of cutting reduced to up to one half with a single blaze cut (experiment 5). In B and C, roots emerged from either the 'upper' or 'lower' positions indicated.

The portion to be removed was chosen at random, so that the best unbiassed estimate of the proportion of the stem remaining was one half. Each treatment consisted of four plots, two of 32 cuttings each and two of 24 cuttings each. The same-sized plots were arranged in blocks and the treatments within blocks were arranged alternately.

Experiment 5. Cuttings of one clone were prepared either: a) with an additional radial longitudinal cut at the base, as in the control treatment of experiment 4 above, or b) with a blaze cut of approximately the same length, which removed up to half the base of the stem (Figure 7.1). The treatments consisted of two plots of 32 cuttings each, arranged alternately.

In experiments 4 and 5, cuttings were not treated with IBA and were set in the medium to a depth of 2.5 cm to 3.0 cm. The numbers of roots per plot emerging from the lowermost (half-round) portion of the stem, and from above the upper extremity of the wound on the opposite side of the stem (Figure 7.1), were recorded after 64 and 38 days respectively.

7.3 RESULTS

Figures 7.2, 7.3 and 7.4a show the vertical distribution of emerged roots in three experiments, in which the cuttings were severed by a simple transverse cut. The frequency distributions have similar shapes irrespective of rooting ability, which was poor (0.24 to 0.86 roots per cutting; Figure 7.2), intermediate (1.59 to 2.17 roots per cutting; Figure 7.3) and good (7.38 roots per cutting; Figure 7.4a).

An additional radial longitudinal wound extending upwards from the base of the cutting for 5 mm to 6 mm (Figure 7.4b), compared to a simple transverse wound (Figure 7.4a), had little effect on the overall vertical distribution of emerged roots. However, on a larger and relative scale (Figure 7.5), it can be seen that the additional wound inhibited rooting in its immediate vicinity (*ie.* for up to 5 mm to 6 mm from the base of the cutting), but promoted rooting at greater distances than this from the base. Overall, the additional wound promoted rooting (Table 7.1).

The data on the vertical distribution of roots emerging from simply-wounded cuttings, shown graphically in Figures 7.2, 7.3 and 7.4a, were re-analyzed. The number of roots emerging 0 mm to 2 mm from the base of the cutting were calculated as proportions of the total number of emerged roots in each of the five groups of cuttings. These proportions were then related to cuttings rooted (%) and mean number of roots per cutting (Figure 7.6).

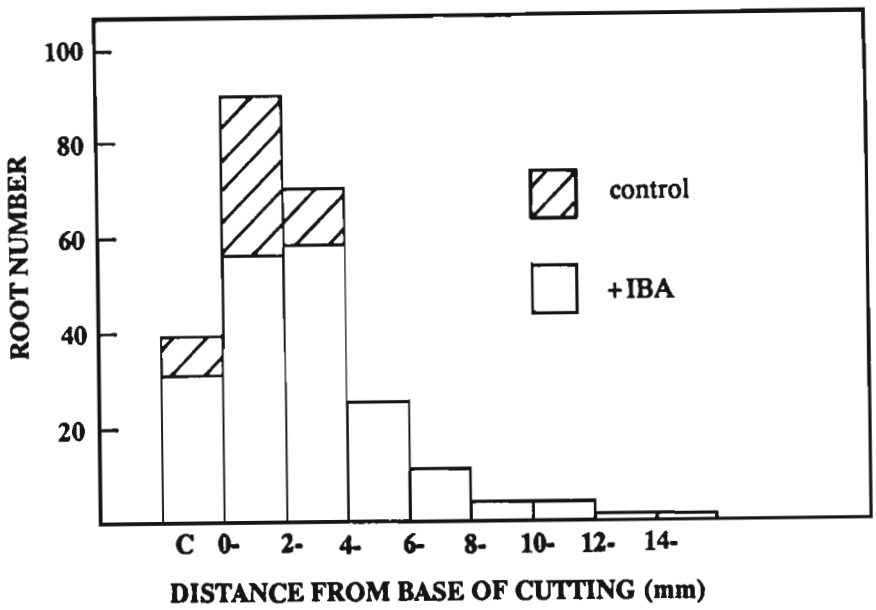

Figure 7.2. The vertical distribution of emerged roots in a batch of cuttings with poor rooting ability. The cuttings were either treated with IBA or left untreated.

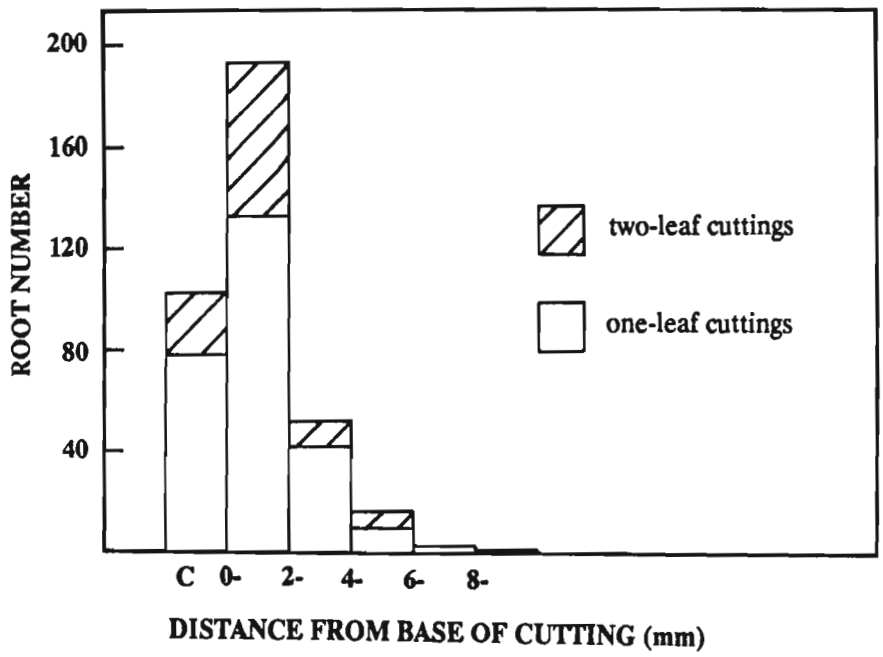

Figure 7.3. The vertical distribution of emerged roots in a batch of cuttings with intermediate rooting ability. The cuttings were prepared with either one leaf or two leaves per cutting.

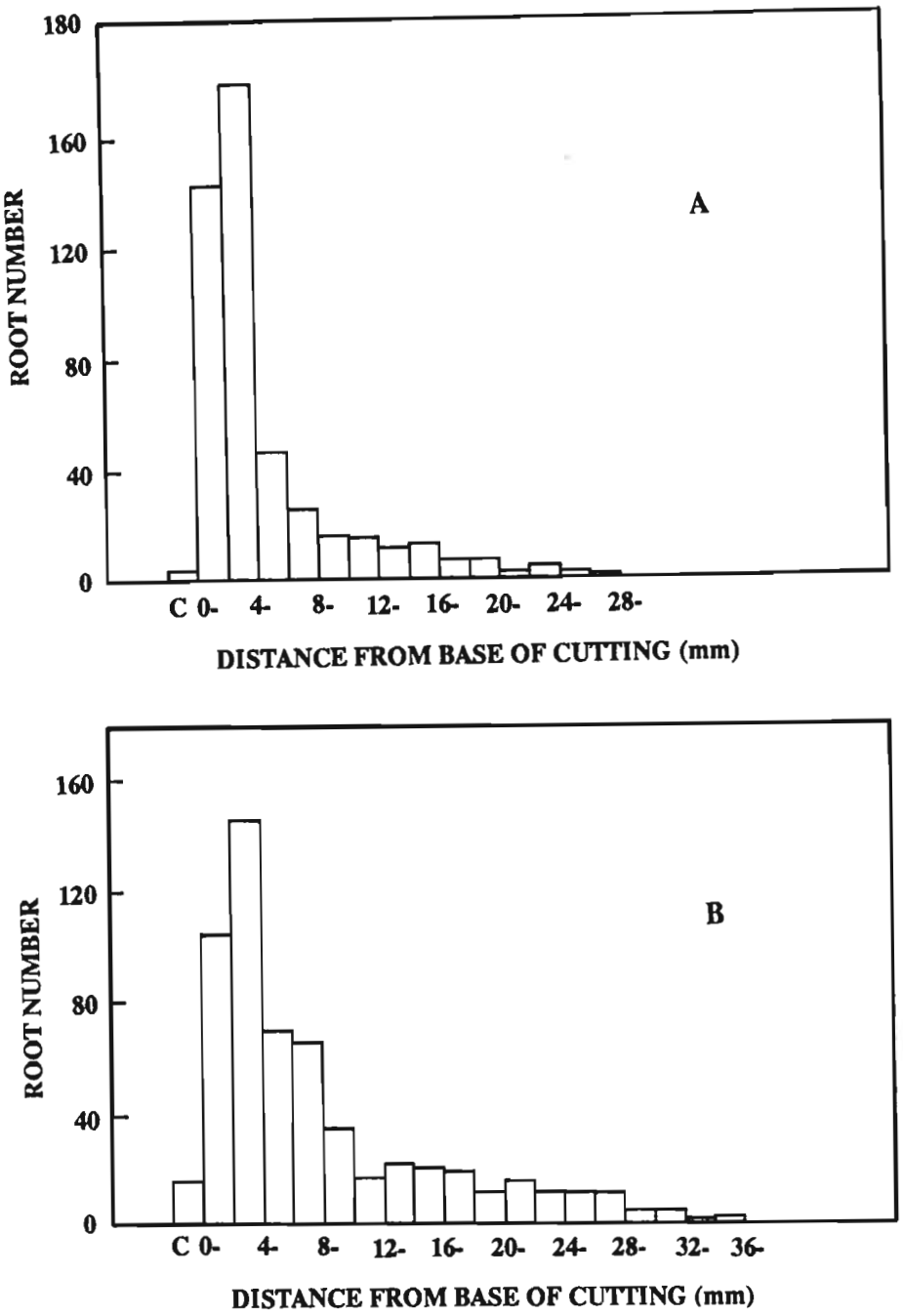

Figure 7.4 a and b. The vertical distribution of emerged roots in a batch of cuttings with good rooting ability. A. Cuttings with a simple transverse cut at the base. B. Cuttings with an additional radial longitudinal wound extending upwards for 5mm to 6 mm.

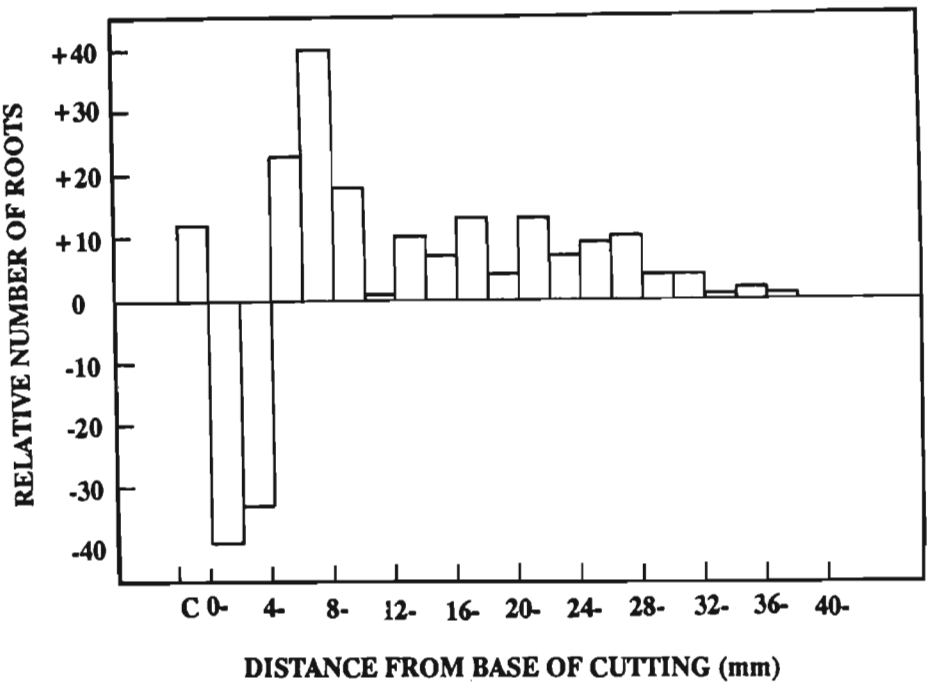

Figure 7.5 The vertical distribution of roots emerging from cuttings with an additional wound at the base (extending upwards for 5-6 mm) relative to cuttings with a simple transverse cut at the base.

Table 7.1. Observed numbers of roots emerging from cuttings with either a simple transverse cut at the base or an additional radial longitudinal wound, compared to the expected number assuming no difference between treatments.

	SIMPLE WOUND	ADDITIONAL WOUND	TOTAL	
OBSERVED	472	579	1051	
EXPECTED	525.5	525.5	1051	$\chi^2 = 10.89$

The proportion of roots emerging 0 mm to 2 mm from the base of the cutting decreased linearly as rooting ability increased, with the apparent exception of the IBA-treated cuttings. In this group the proportion was much lower than expected, signifying that the IBA treatment had resulted in an upward re-distribution of roots. This proportion was assumed to be significantly different from the remainder and was excluded from the regression calculations. The two criteria of rooting ability (cuttings rooted (%) and mean root number per cutting) gave very similar results (Figure 7.6).

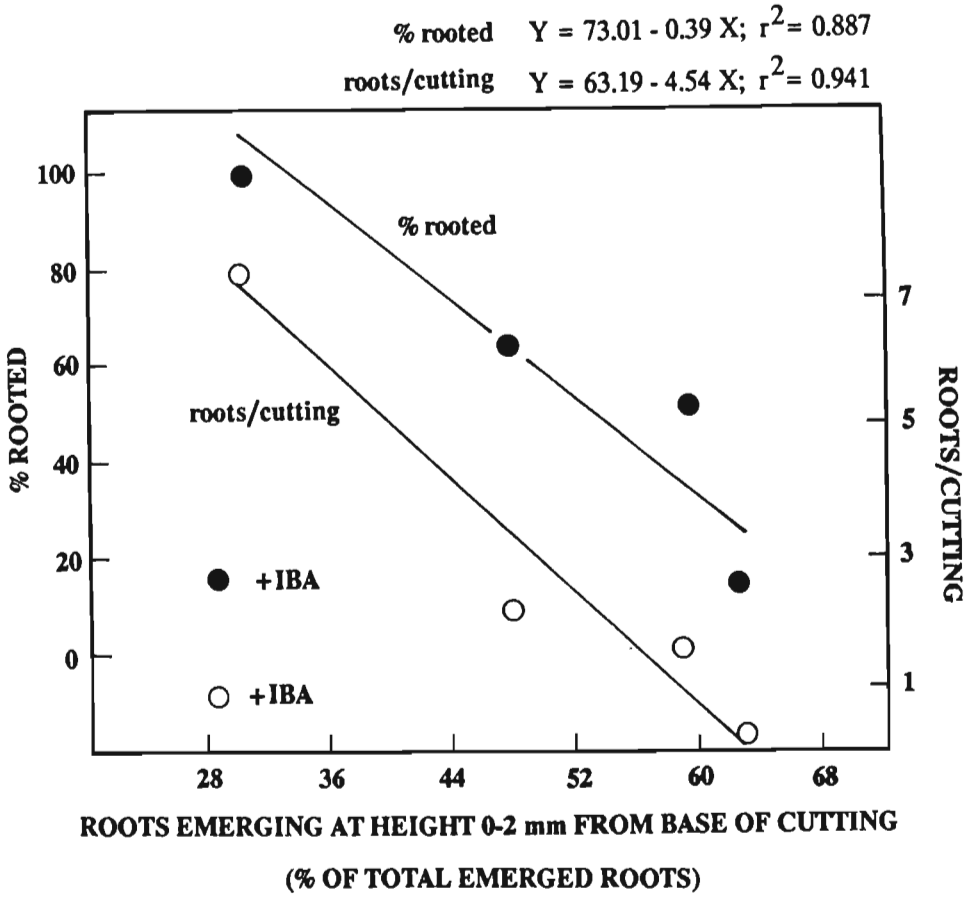

Figure 7.6. The relationship between rooting ability and the proportion of roots emerging 0 mm to 2 mm from the base of the cutting. The value for the IBA treatment was excluded from the regressions.

Cuttings were additionally wounded (as above, except that the mean length of the additional wound was 13.7 mm), then either reduced to a half-round cross-section at the base or not reduced.

Removing half of the base of the cutting reduced the wound area by approximately half. Since the additional wound increased rooting by 22.6% (579 / 472; Table 7.1), reduced cuttings might be expected to root 11.3% less well than the unreduced cuttings, by virtue of the wound effect alone. (This is an approximation since it assumes a linear relationship between the wound area at the base of the cutting and rooting ability).

The reduced cuttings rooted less well than the unreduced cuttings (Table 7.2). After allowing for the wound effect by subtracting 11.3% from the numbers of roots in the 'not reduced' treatment, the reduced cuttings still rooted less well: the value of t in the paired t test was 9.02 ($t_{0.05,\ 5\ df} = 2.57$).

Table 7.2. The total number of roots per plot emerging from cuttings either reduced or not reduced in cross-section at the base. The upper wound surface of the reduced cuttings was either transverse (plots 1 to 4) or oblique (plots 5 and 6).

PAIRED PLOTS	1	2	3	4	5	6
CUTTINGS REDUCED	77	49	134	152	210	153
CUTTINGS NOT REDUCED	114	78	165	134	273	215

Reducing the base of the cutting by both the half-round and blaze-cut treatments caused roots to emerge from: (i) the lowermost, reduced, portion of the stem (from the extra-cambial region only, as usual), or (ii) immediately above the upper extremity of the wound on the opposite side of the stem (see Figure 7.1). Owing to their vertical separation the two sites of emergence could be clearly distinguished.

The higher the number of roots per cutting in the half-round treated cuttings, the higher the proportion which emerged from the upper as opposed to the lower site of root emergence. The proportion varied from about 6% to about 26%. The proportion of roots emerging at the upper site in blaze-wounded cuttings was less: close to 13% in both plots compared to a mean value of about 22% predicted from the regression of the half-round treated cuttings (Figure 7.7).

The frequency of root emergence at the lower site was similar in both wounding treatments, and was assumed to be the same for the purpose of calculating the regression shown in Figure 7.8.

The regression predicts that, in the range of rooting ability encountered, 66% to 67% of the roots which emerged from the unreduced cuttings emerged from the lower site of emergence in the reduced cuttings. Allowing for the wound effect, this percentage becomes 74% to 76%. Thus, although relatively few roots emerged from the upper site in reduced cuttings, this was compensated for by relatively many roots emerging from the lower site.

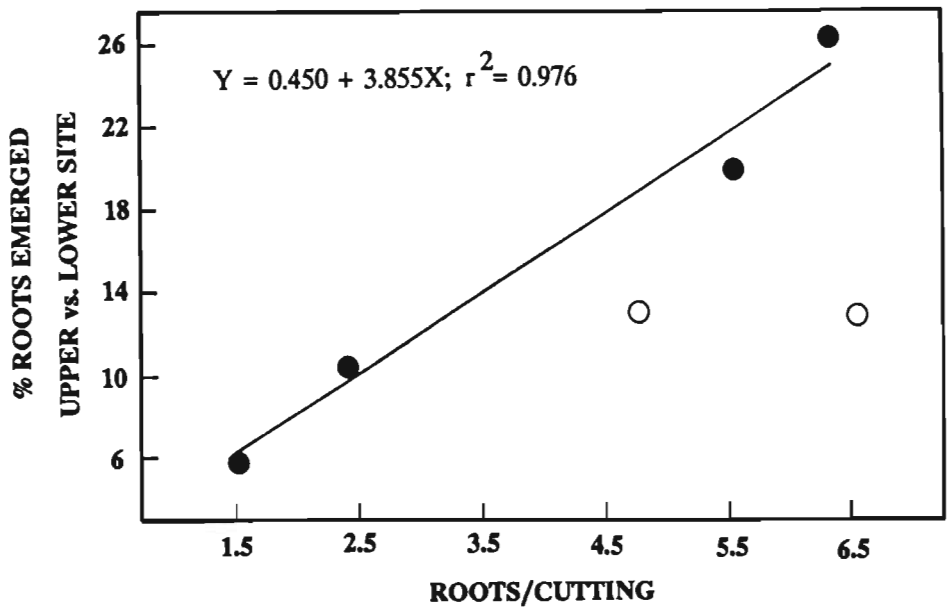

Figure 7.7. The relationship between rooting ability and the proportion of emerged roots at the upper site of root emergence, in cuttings reduced to a half-round cross section by two wounding treatments: upper wound surface transverse (●); upper wound surface oblique (○).

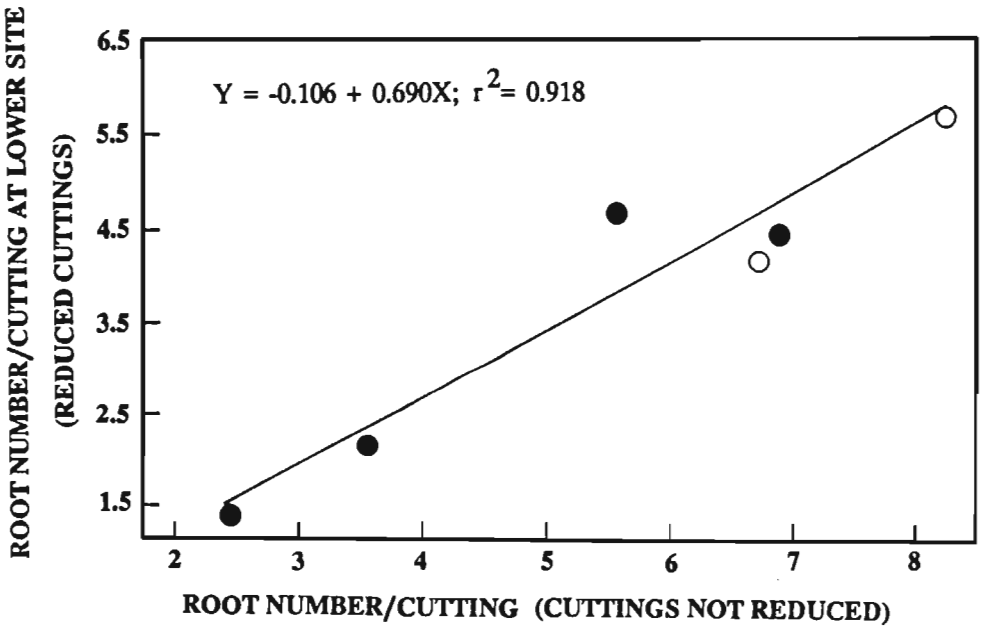

Figure 7.8. The relationship between mean number of roots per plot at the lower site of root emergence, in cuttings reduced to a half-round cross-section by two kinds of wound, and root number per paired plot in unreduced cuttings. Upper wound surface transverse (●); upper wound surface oblique (○).

7.4 DISCUSSION

When cuttings were additionally-wounded at the base with a radial longitudinal cut 5mm to 6 mm long, rooting was inhibited in the region of the stem containing the additional wound but was promoted overall. Direct effects due to the wound seem unlikely. There was no evidence that rooting had been inhibited at the base of simply wounded cuttings, as would be expected if the wound was directly inhibitory, and any directly promotory effect should have been manifested most strongly adjacent to the wound.

A wound morphogen could be loaded into the transporting tissues of the stem before being able to act. If it remained unmetabolized and was able to circulate in the cutting, its action would be indistinguishable from that of other components of a morphogen mobile in the transporting tissues. While there is no evidence for or against this possibility it seems more likely that the overall promotory effect of the additional wound is due to less direct factors.

The additional wound passed through the centre of the cutting, destroying the internal phloem. If the internal phloem participates in transport in the *E.grandis* stem cutting, its destruction could have resulted in a greater efflux of the morphogen, in the region of the external phloem at the base of the cutting, or efflux over a greater vertical distance. The pattern of efflux (inferred from the vertical distribution of roots) was consistent with this possibility, the highest frequency of roots emerging in the vicinity of the upper extremity the additional wound.

A roughly exponential decrease of root emergence with distance from the base of the cutting was observed. This distribution may directly reflect the distribution of the morphogen released from the transporting tissues, or the morphogen may be released at or close to the base of the cutting and then migrate upwards by diffusion or mass flow.

As rooting ability increased, the proportion of the total number of roots emerging at height 0 mm to 2 mm from the base of the cutting decreased: they emerged, on average, higher up the stem. There are several possible reasons for this. The rate of catabolism of the morphogen unloaded from the transporting tissues could vary with rooting ability, but the effect on the vertical distribution of roots (if any) would depend on unknown factors such as the kinetics of the reactions involved. Leaching from the base of the cutting might inhibit rooting in the adjacent region of the stem, but a rapid rate of leaching seems unlikely to be associated with good rooting ability. Callus developing at the base of the cutting could also be an appreciable sink, inhibiting rooting at the very base of the cutting, but the easiest-to-root cuttings had relatively poor callus development (judged from the proportion of roots developing from callus).

A higher concentration of the morphogen, or more rapid efflux at the base of the cutting, would both tend to increase rooting ability. They would result in a lower proportion of roots at the base of the cutting if the morphogen was super-abundant over a longer vertical distance in easy- as opposed to difficult-to-root cuttings. (The highest levels could even be inhibitory). The morphogen could be unloaded over a longer vertical distance in easy-to-root cuttings, or the efflux could be confined to the base of the cutting, after which upward migration would be required.

When cuttings were reduced at their base to a half-round cross-section roots emerged from an upper site, above the upper extremity of the wound, or from a lower site in the opposing half of the stem. The distribution of emerged roots was markedly asymmetrical. Only a small proportion emerged from above the upper extremity of the wound, although this site occupied half of the circumference of the stem. At the same time rooting was more profuse at the lower site, compared to an equivalent location in unreduced cuttings.

This is additional evidence for a morphogen mobile in the stem, and indicates that both the pattern of efflux and the quantity of the morphogen are likely to be important in affecting rooting ability: some of the morphogen which apparently escaped efflux at the upper site evoked an additional rooting response at the lower site. However, overall, rooting was less good in reduced than in unreduced cuttings. Either the additional morphogen at the lower site was rendered accessible but did not evoke a proportionate rooting response (through being super-abundant), or part remained inaccessible owing to a changed pattern of efflux there.

As rooting ability increased the proportion of roots emerging from the upper site increased (when the upper extremity of the reduction wound was a close-to-transverse cut). Again, the morphogen may have become progressively less able to evoke a proportionate rooting response at the lower site, or efflux at the upper site may have increased more rapidly than a capacity for 're-direction', as rooting ability increased.

The pattern of efflux at the upper site was affected by the orientation of the cut at the upper extremity of the reduction wound, so 're-direction' can occur. An oblique cut caused a low proportion of roots to emerge at the upper site (relative to a close-to-transverse cut), and the proportion appeared to be relatively insensitive to variation in rooting ability.

The high radial/tangential mobility of the morphogen suggests that its distribution at the base of simply wounded two-leaf cuttings is close-to-uniform, rather than confined to certain sectors of the stem. However, this does not necessarily mean that the morphogen is equally accessible to all of the sites at which roots initiate. The pattern of efflux can

evidently vary, and nothing is known of the steepness of the gradients set up after efflux.

CHAPTER EIGHT

GENERAL DISCUSSION

In stem cuttings of *E.grandis* the rooting morphogen, or an important component of it, is mobile in the stem. The activity of the leaves and buds induces a flux of the morphogen, in tissues which render it inaccessible to the sites where roots initiate. Wounds which interrupt these tissues, such as the severance cut at the base of the cutting, induce efflux to some extent, when the morphogen becomes potentially available to induce root initiation. Figure 8.1 summarizes this view of adventitious rooting.

This scheme essentially represents the original rhizocaline concept, since rhizocaline was conceived to be a vascular morphogen (see Chapter 2). However, the scheme makes it clear that aspects of adventitious rooting which have received very little attention in previous studies could have an important effect on rooting ability.

Almost nothing is known about the factors which render the morphogen accessible to the sites where roots initiate. Efflux at the base of the cutting must depend on sink activity there, but even in the intact plant the regulation of sink activity is poorly understood (WYSE, 1986). Like phloem loading (REINHOLD, 1975), phloem unloading is an active process (WEATHERLEY, 1975), and the 'pull', exerted by the sink is thought to be at least as important as the 'push' exerted by the source in driving translocation in the intact plant (GEIGER, 1975). Translocation itself is widely recognized as being non-limiting in source/sink relationships (GIFFORD & EVANS, 1981).

Since stem cuttings must have much diminished sink activity relative to the intact plant, the activity of the sink at the base of the cutting might be expected to limit the rate of downward translocation, which in turn may be so slow as to be detrimental to leaf and bud function. Thus, high sink activity could promote rooting.

Several factors attenuate the morphogen after efflux (callus growth, any other regeneration at the wound, catabolism, leaching, diffusion, mass flow, or influx back into the transporting tissues), all of which could contribute to sink activity. These have received almost no attention in previous studies, with the exception of IAA catabolism. Generally, despite a popular assumption to the contrary, high 'IAA oxidase' (peroxidase) activity at the base of the cutting seems to be associated with good rooting ability (HAISSIG, 1986).

However, rather than reflecting high sink activity, high 'IAA oxidase' activity could

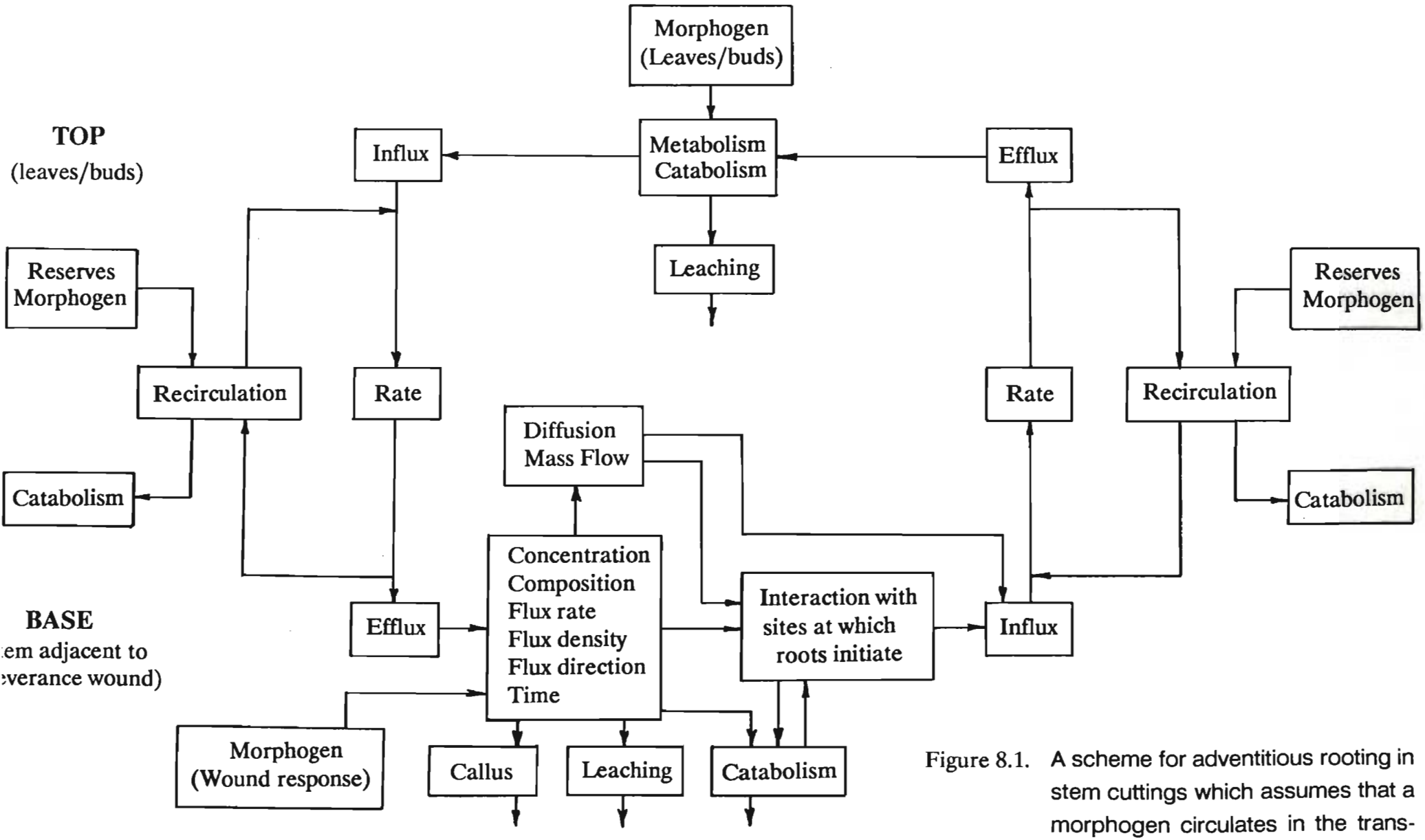

Figure 8.1. A scheme for adventitious rooting in stem cuttings which assumes that a morphogen circulates in the transporting tissues of the cutting.

simply reflect the quantity of IAA available for catabolism. The cortex has a high capacity for IAA catabolism relative to the stele in *Zea mays* L. roots (NONHEBEL, HILLMAN, CROZIER & WILKINS, 1985), and IAA induces 'IAA oxidase' activity (JENSEN, 1955; LEE, 1971). Alternatively, high 'IAA oxidase' activity could simply reflect the capacity for any metabolic activity, given that the responding cells are probably more or less dormant.

Most sink activity would be expected to be created *in situ*, but this could not account for the asymmetry of root emergence in reduced cuttings. If sink activity is indeed limiting it must be created (at least in part) by a component of the vascular morphogen, which appears to move actively basipetally. Auxin is the obvious candidate for this role (see Chapter 4).

After efflux the morphogen, whatever its composition, may or may not be accessible to the sites where roots initiate. This would depend on the distance apart of the points of efflux, and the steepness of the gradients set up as a consequence of efflux. The morphogen has high radial and tangential mobility in the stem, suggesting that (in simply-wounded two-leaf cuttings) its distribution at the base of the cutting is close-to-uniform. However, this does not necessarily mean that there are very many points of efflux. For example, the sieve tubes make up about two-thirds of the cross-section of the phloem, excluding rays and sclerenchyma, (CANNY, 1973), but not all need be equally active. In the intact plant the activity of individual sieve tubes varies markedly (ESCHRICH, 1967).

Nothing is known about the steepness of the gradients set up by efflux so, even if the distance between points of efflux were known, it would not be possible to estimate the proportion of sites which remain inaccessible to the morphogen.

It is conceivable that the morphogen is so uniformly distributed at the base of the cutting that any accessibility constraints are insignificant. Rooting ability, at least at the base of easy-to-root cuttings, would then be limited by the number of potential sites for root initiation, whose relevant attributes would be rare. In the region of the stem 0 mm to 2 mm above the base of easy-to-root cuttings (7.4 roots per cutting in total), an average of 2.8 roots per cutting emerged, compared to the hundreds or thousands of cells in this region of the stem which appear to be suitably located as potential root initials.

On the other hand, accessibility constraints could be severe if the points of efflux are widely separated, or if the gradients set up by efflux are steep. On efflux at the base of the cutting an important (but completely neglected) attenuating factor could easily be the re-absorption of the morphogen into a pathway active in the acropetal direction. For

example, the ability of auxin to re-circulate in cuttings is well established (see Chapter 4). If the acropetal pathway was close to the basipetal pathway, the accessibility of the morphogen could be severely limited, depending on the rate of influx relative to efflux.

It is conceivable that components of the morphogen arrive in different pathways. For example, auxin could move in the polar pathway (axial parenchyma), whereas other components of the morphogen could be mobile in the sieve tubes or rays. This would obviously complicate the question of accessibility.

According to TORREY (1956), the factor limiting lateral root initiation is expected to change with experimental conditions. This is probably true also of rooting ability in stem cuttings. In *E.grandis* cuttings, the relative importance of the factors involved probably varies within a single cutting, as well as between batches of cuttings with different rooting abilities. The single limiting factor is therefore illusory, and a broader approach is required.

The original concept that 'rhizocaline', the hypothetical rooting hormone, induces rooting is unhelpful in its simplest form and is also unfashionable. This is partly because it is historically associated with bioassay studies (see Chapters 2 and 3), but also because it led directly, very early on, to studies on the metabolism and biochemistry of the morphogen. Generally, such studies have been preponderant in plant physiology (PASSIOURA, 1979), but in the case of adventitious rooting they have yielded few insights (HAISSIG, 1986). In particular, conventional biochemical studies give averages for the rooting zone, which are unhelpful regarding conditions in individual or small groups of cells (HAISSIG, 1986).

At least in *E.grandis* cuttings, the interactions between the pattern of efflux of the morphogen, the factors which then attenuate the efflux, and the potential sites for root initiation are thought to occur on a small scale. Thus the gradients set up between small groups of cells are likely to be all-important.

Nevertheless the rhizocaline concept provides a starting point towards a more comprehensive view of adventitious rooting. This view is a remote objective because many of the factors which could be important in affecting rooting ability have not recieved attention, and will be difficult to elucidate.

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