AN ABSTRACT OF THE THESIS OF

Ching-Te Chien for the degree of Master of Science in Forest Science presented on August 17, 1988.

Title: Plagiogravitropic Growth in Rooted Stem Cuttings of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) Related to Indole-3-Acetic Acid and Cytokinins

Abstract approved: Signature redacted for privacy.

Dr. Joe B. Zaerr

Endogenous concentrations of indole-3-acetic acid (IAA) and cytokinins were compared to the tendency for plagiogravitropic growth in rooted stem cuttings of Douglas-fir. A chromatographic method was developed for the purification and quantitation of trace levels of IAA. Two elutions with reverse-phase high performance liquid chromatography (HPLC) using a fluorescence detector resulted in a recovery of about 50%. The IAA peak was confirmed by gas chromatography/mass-spectrometry. Affinity chromatography using monoclonal antibodies raised against zeatin riboside and isopentenyladenosine, combined with HPLC and radioimmunoassay were used here for quantitation of cytokinins.

Buds, apical needles and stems of branch terminals of eight year-old clones (clone 12-plagiogravitropic growth
and clone 15-orthogravitropic growth), collections for quantitation of IAA and cytokinins were made in September, December, January, and February. The highest concentration of IAA in both clones was found in buds collected in December. Differences in IAA concentration between clones from samples collected in January and February were not significant. Significant differences were found in September and December. A high concentration of a cytokinin conjugate which was determined to be a hexose conjugate of zeatin riboside (ZX) but not zeatin ribose-O-glucoside, was found in the fall and winter buds. Some significant differences in cytokinin concentrations were found between these two clones. The interaction of ZX and ZR in buds on the four collection dates could indicate a role for ZX in the plagiogravitropic mechanism.

Clonal differences in IAA concentrations between the morphological upper and lower halves of stem sections of rooted cuttings were not significant. The highest concentration of IAA was detected in the section of stem sampled immediately above the stem area exhibiting the most plagiogravitropic curvature.

The failure to find differences of IAA concentrations between morphological upper and lower halves of curvature did not support the related Cholodny-Went hypothesis that higher concentration of IAA was on the upper half. However, there is some evidence to suggest that lateral distribution of IAA could be occurring in rooted cuttings.
and that compression wood might be associated with this asymmetrical distribution of IAA. The curvature of rooted cuttings may also be directly affected by root growth potential. Higher concentration of endogenous IAA in December bud may be linked to high mortality of seedlings lifted and outplanted before December. The high levels of ZX in fall buds may be an inactive, storage form of biologically active zeatin riboside.
Plagiogravitropic Growth in Rooted Stem Cuttings
of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco)
Related to Indole-3-Acetic Acid and Cytokinins

by

Ching-Te Chien

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Plant Materials</td>
<td>13</td>
</tr>
<tr>
<td>Extraction and Analysis of IAA</td>
<td>14</td>
</tr>
<tr>
<td>Extraction and Purification of Cytokinins</td>
<td>24</td>
</tr>
<tr>
<td>Separation of Cytokinins</td>
<td>25</td>
</tr>
<tr>
<td>Radioimmunoassay of Cytokinins</td>
<td>27</td>
</tr>
<tr>
<td>Gas Chromatography–Mass Spectrometry</td>
<td>27</td>
</tr>
<tr>
<td>RESULTS</td>
<td>30</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>43</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>54</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>56</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Rooted stem cuttings of Douglas-fir from clone 12 and clone 15 five months after placement in rooting beds. Cuttings from clone 12 exhibited higher plagiogravitropic habit than those from clone 15.</td>
</tr>
<tr>
<td>2.</td>
<td>Flow diagram of method used to measure IAA.</td>
</tr>
<tr>
<td>3.</td>
<td>Diagram of the apparatus for the production of diazomethane in diethyl ether</td>
</tr>
<tr>
<td>4.</td>
<td>HPLC chromatogram.</td>
</tr>
<tr>
<td>5.</td>
<td>Mass spectrum of MeIAA.</td>
</tr>
<tr>
<td>6.</td>
<td>Flow diagram of method used to measure cytokinins.</td>
</tr>
<tr>
<td>7.</td>
<td>Chromatogram of cytokinins.</td>
</tr>
<tr>
<td>8.</td>
<td>Levels of endogenous IAA in buds, needles and stems of clone 12 and clone 15 on four collection dates.</td>
</tr>
</tbody>
</table>
   (Cytokinins in clone 12)

10. Cytokinins levels in vegetative buds, needles and stems of Douglas-fir on four collection dates.
    (Cytokinins in clone 15)


12. Endogenous IAA levels in sections from intact rooted stem cuttings of Douglas-fir after 2 seasons of growth.
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Concentration of endogenous IAA in vegetative buds, needles and stems of Douglas-fir collected on four dates.</td>
<td>34</td>
</tr>
<tr>
<td>2. Levels of endogenous cytokinins in Douglas-fir vegetative buds, needles and stems from clone 12 and clone 15 on four collection dates.</td>
<td>38</td>
</tr>
<tr>
<td>3. Endogenous IAA in morphological upper and lower halves of curvature, apical shoots, and roots from current rooted stem cuttings of Douglas-fir clone 12 and clone 15.</td>
<td>40</td>
</tr>
<tr>
<td>4. Endogenous IAA levels in apex, shoot section, and roots of a plagiogravitropic Douglas-fir clone.</td>
<td>41</td>
</tr>
</tbody>
</table>
Plagiogravitropic Growth in Rooted Stem Cuttings of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) Related to Indole-3-Acetic Acid and Cytokinins

INTRODUCTION AND LITERATURE REVIEW

Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) is one of the most important timber species in North America. It is also the primary Christmas tree species propagated in the Pacific Northwest. Vegetative propagation may be an important tool in future tree improvement programs since all of the genetic potential of the parent tree is transferred to the progeny by this method. Vegetative propagation of Douglas-fir by stem cuttings has been successful (Copes 1983; Proebsting 1984), but large-scale utilization of rooted cuttings of this species by cuttings has several practical limitations: growth rate of cuttings from older ortets is slower than that of cuttings taken from seedlings (Copes 1976; Sweet and Wells 1974), rootability of ortets from mature trees is poorer than from seedlings, and the plagiogravitropic growth (tendency to grow in a direction other than vertical) for several years is common in rooted stem cuttings (Black 1973; Copes 1980; Starbuck 1979). For vegetative propagation to be successful on a large scale, we must be able to produce orthogravitropic rooted cuttings.
which are capable of rapid growth after out-planting.

The phenomenon of plagiogravitropic growth is ubiquitous in nature. Most roots, lateral shoots and leaves, for example, grow at an angle to gravity. Plagiogravitropic growth in lateral stem cutting from some species, e.g., *Auracaria excelsa*, *Picea abies*, *Abies alba*, and *Pseudotsuga menziesii*, has been reported to maintain branch-like angles for several years or longer before growing upright (Black 1973). Such growth is a serious obstacle to the general use of rooted cuttings of these species for reforestation or in tree improvement projects.

Two hypotheses to explain gravitropism were proposed earlier in this century. The starch-statolith hypothesis of gravity perception was set forth independently in 1900 by Haberlandt and Nemec. The primary principle of this hypothesis is that statoliths in statocytes (gravity-perceiving cells) migrate by sedimentation to the lowermost side of the cell and have the capacity to cause the tissue to respond to gravity. The statoliths in fact were starch grains. Starch grains never occur free in a cell, but are found bound together in groups of 2 to 8 within a membrane called the amyloplast. The other hypothesis involving the lateral transport and asymmetric distribution of auxin as the cause of stem curvature was proposed by Cholodny and Went in 1926.

There is little evidence to indicate that the starch-statolith theory is related to plagiogravitropism in
stem cuttings. A great deal of evidence indicates that sedimentable amyloplast movement in graviperception by plants possibly plays an important role, but this evidence is limited to sensitive organs such as the root cap, the coleoptile, and the bundle sheath in higher plants. In their reviews, Audus (1979), Volkmann and Sievers (1979), and Wilkins (1984) pointed out the close correlation between the presence of sedimentable amyloplasts in organs and their ability to respond to gravity. Several convincing lines of evidence from experiments supporting the starch-statolith hypothesis are: A) Destarching of amyloplast in organs has shown no response to gravity (Iversen 1969, 1974; Pickard and Thimann 1966). B) Removal of the root cap with statocytes supported the statolith theory (Barlow 1974; Hillman and Wilkins 1982; Juniper et al. 1966). C) There was an interaction between amyloplasts and the endoplasmic reticulum (Juniper and French 1973; Ray 1977; Sievers and Volkmann 1972, 1977; Volkmann 1974). D) Rotation experiments of roots on a horizontal clinostat showed effects of gravity on the polarity and the integrity of the statocytes (Hensel and Sievers 1980, 1981; Sievers and Heyder-Caspers 1983). E) Genetic mutants proved to be a valuable tool for studying gravitropism (Filner et al. 1970; Hertel et al. 1969; Miles 1981; Olsen and Iversen 1980 a, b). In experiments conducted on Flight 61-C of the space shuttle Columbia, Moore et al. (1986) found that the volume of amyloplasts and relative volume of starch in
Amyloplast of flight-grown seedlings are significantly less than that of earth-grown seedlings but that low gravity does not significantly alter the volume of cells, the average number of amyloplast per cell, or the number of starch grains per amyloplast. Although the correlations are convincing, they must be tempered by the report (Caspar and Somerville 1985) that roots of an Arabidopsis mutant are gravireponsive despite the fact that their columella cells lack amyloplasts.

The role of protons and calcium in gravitropism.

Asymmetrical H⁺ efflux and Ca²⁺ movements in roots and shoots of higher plants have been proposed to be transduction steps in the stimulus-response sequence of gravitropism (Cleland 1977, Lee et al. 1983a, 1983b, 1984, Mulkey and Evans 1983, Slocum and Roux 1983, Wright and Rayle 1983). It appears that such ion gradients occurring in the apoplast are probably an early and direct expression of IAA asymmetry and are related to growth induced by IAA.

Hasenstein and Evans (1986) recently indicated that calcium may act as a second messenger of auxin action to establish a Ca²⁺ gradient without a gradient in auxin. Such a mechanism could account for the inability of some workers to detect an auxin gradient.

Taken together, these reports indicated that a close coupling between auxin and Ca²⁺ movements could exist. Two
models to explain this relationship were proposed:

Model 1) When IAA is redistributed to the lower side of a shoot after gravistimulation, H⁺ secretion is stimulated and the apoplastic calcium will rise. Additionally the secreted H⁺ tends to make the lower tissue electropositive with respect to the upper tissue, causing Ca²⁺ to migrate down its apoplastic electrochemical gradient from the IAA-enriched (lower) to the IAA-impoverished (upper) side of the shoot.

Model 2) In roots the increased level of calcium in the lower side of the root cap may lead to enhanced acropetal movement of auxin into the elongation zone on the lower side of the root. The increased level of auxin in this elongation zone inhibits growth, resulting in downward curvature (Lee et al. 1983b).

Unfortunately, measurements of endogenous IAA to test these two models have not been reported by the authors. One reason for this may be the lack of suitable techniques for measuring very low quantities of this hormone.

The role of plant growth substances in plagiogravitropic growth.

Little information is available about the mechanism of plagiogravitropism in stem cuttings, especially in coniferous species. Starbuck (1979) found that ¹⁴C-IAA applied to decapitated current rooted cuttings of
Douglas-fir moved down the stem, but not evenly. After 24 hours, nearly twice as much $^{14}$C was detected in extracts from the adaxial (morphologically upper sides) than from the abaxial sides of stem cuttings. Moreover, an examination of compression wood on the upper and lower sides of each growth ring showed significantly greater compression wood on the morphologically upper than on the lower side in the basal section (1.5 to 2.0 cm from the base) of rooted cuttings. On the other hand, compression wood in the distal section (end of the stem at the time of cutting) was greater on the upper side the first season (year of rooting) but was greater on the lower side the second season. This result suggested that the formation of compression wood the second season tended to compensate for plagiogravitropic growth, at least in the (original) distal region of the stem. Formation of compression wood on the upper side of stem cuttings was indirect evidence for the involvement of growth substances in plagiogravitropic growth.

Copes (1980) found that the intensity and severity of plagiogravitropic habit in Douglas-fir grafts appear to be inversely related to the rootstock vigor or growth-promoting potential. It is still unknown whether or not this phenomenon is related to plant growth substances and/or other causes.

In lateral branches, apical control seems to play an important role in controlling branch angle (Blake et al. 1980; Pharis and Kuo 1977). When the apical control of
branch angle is abolished by decapitation of the main stem, one or more of the remaining branches assumes the vertical position as a result of enhanced formation of compression wood (in conifers) along the lower side of the branch (Wareing 1964, Westing 1965, Wilson 1977). Wilson (1973) found that a vertical branch formed by decapitating the apical stem induced compression wood on the under side and subsequently formed compression wood on the upper side to establish an "equilibrium position". There is considerable evidence indicating that IAA is involved in the process of compression wood formation (Blake et al. 1980, Little 1970, Yamaguchi et al. 1980). These studies have shown that upward movement of conifer branches in response to stem decapitation can be prevented or retarded by application of IAA to the cut stump. In general, more endogenous auxin is found on the lower side of leaning stems, which is the site of compression wood formation, than on the upper side. White pine branches on a clinostat grew more and formed compression wood on the upper side opposite the site of previous compression wood formation (Wilson 1973).

Gibberellins (GAs) appear to counteract the effect of the apex or applied auxin in the control of branch angle. Thus, exogenous application of GAs may induce branches to turn up in many species (Pharish and Kuo 1977). Pharish et al. (1981) reported asymmetrical concentration of \(^3\)H-GA applied to gravistimulated leaf-sheath and internodal pulvini of oat plants. Increases in \(^3\)H-GA glucosyl
conjugate-like substances and metabolites occurred in the upper halves, whereas free $^3$H-GA and its GA metabolites, as well as endogenous GA-like substances accumulated in the lower halves. Using $^3$HGA$_{20}$, which is a native GA of highly specific radioactivity and has high mobility and is converted to the probable 'effector' GA in maize shoots, to measure the difference of metabolites in the lower and upper halves of maize shoots after gravistimulation Rood et al. (1987) found that there was a significantly higher proportion of total free radioactive metabolites of $^3$HGA$_{20}$, $^3$HGA$_1$ plus $^3$HGA$_{20}$, in the bottom halves, and $^3$HGA glucosyl conjugates were lower in the bottom halves relative to upper halves. But Law and Hamilton (1984) showed that spraying GA on two-week-old dwarf pea plants caused an increase in levels of IAA by as much as 8-fold and decreased the level of indole-3-acetyl aspartic acid (conjugated form of IAA). A possible interpretation of these results is that GA promotes hydrolysis of conjugated IAA, or reduces activity of IAA oxidase.

Gravity-induced ethylene could affect bud outgrowth according to a recent review of Hillman (1984). Prasad and Cline (1987) found that bending down a shoot induced release of apical dominance in Pharbitis nil but that this release is inhibited by a horizontal clinostat. The release of apical dominance in a bending shoot may be accounted for by the hypothesis of gravity stress and ethylene-induced retardation of the elongation of the bending shoot. The
interaction of auxin and ethylene in leaf petioles, grass nodes and branches is documented for many species (Abeles 1973, Hayes et al. 1976, Hayes 1981, Osborne 1974, Wright et al. 1978). The study by Blake et al. (1980) on Cupressus arizonica seedling indicates that ethylene may play a role in the apical control of branch angle. Decapitation, application of GAs, high light intensity, and combinations of these treatments increased ethylene evolution and branch upturning. IAA applied to the cut stems of decapitated seedlings at first promoted and then retarded both ethylene production and branch hyponasty. But a recent study by Kaufman et al. (1985) concluded that the increase in production of ethylene in stimulated leaf-sheath bases of Avena sativa has no major effect or role in the induction of upward bending, and that the release of ethylene may occur as a result of the stress of gravistimulation rather than cause a response to gravity. The elevated level of free IAA that occurs on the lower sides as a response of gravity discussed above could be responsible for inducing ethylene production.

Cytokinins have not been related directly to the gravitropic response in woody stems. However, there is some evidence indicating that cytokinins play an important role in apical dominance. Sachs and Thimann (1967) found that axillary buds of peas treated with kinetin and IAA were released more than those treated with kinetin alone. Cytokinins could suppress the conversion of IAA into IAA
conjugates, and the higher free IAA could be responsible for higher ethylene production which was found in test of *Phaseolus mungo* L, and calcium tested synergistically with cytokinins also had the same function as IAA with cytokinins for stimulating ethylene production. (Lau and Yang 1973, 1976, Lau et al. 1977).

A number of studies have concentrated on the lateral transport of growth regulators in gravitropically stimulated primary tissues of higher plants other than woody plants. These studies have generally supported the Cholodny-Went theory which was first proposed in 1926 (see reviews by Wilkins 1984, Pickard 1985). However, in a study by Mertens and Weiler (1983) using immunoassays on seedlings of dicotyledons, sunflower (*Helianthus annuus*) and broadbean (*Vicia faba*), no significant changes were found in the amounts of IAA, GA, and ABA in top and bottom halves of gravistimulated shoots before, at the time of bending, or after bending was well underway. Thus they challenged the Cholodny-Went model as it may apply to dicotyledonous seedlings. Another study strongly arguing against a role for ABA as a inhibitor in root gravitropism comes from recent work of Moore and Smith (1985). Using carotenoid-deficient mutants of maize that lack detectable ABA, these authors showed that gravitropic curving could still occur. Weyers (1985), using a tomato mutant which was ABA-deficient, also found that ABA was not involved in gravitropism.
Although there is no direct evidence to link the statolith theory to plagiogravitropism, a proposed mechanism linking amyloplast settling to the downward movement of calcium ions and auxin in the rootcap was suggested by Evans et al. (1986). Those authors proposed that amyloplasts could act as a signal to the endoplasmic reticulum which in turn could release calcium and/or auxin to cause curvature of the root. An auxin-wave pattern rather than auxin gradients found in the cambial region of stem of conifers may be another type of auxin polar transport. (Zajaczkowski 1983, Wodzicki 1987). Additional research and new approaches are necessary to improve our understanding of gravitropism. Advances can be made by the following:

1) Improving techniques for purifying, detecting, and measuring plant growth substances.

2) Establishing how and when hormone asymmetry occurs during gravitropism in both roots and shoots.

3) Understanding which PGRs are primary and which are secondary in initiating gravitropic curvature.

4) Locating receptor sites for PGRs in gravitropically sensitive organs.

5) Increasing the use of PGR-deficient mutant plants.

Observations on Douglas-fir

The first signs of plagiogravitropism in stem cuttings of Douglas-fir are often observed in the rooting bench.
After the first year, the terminal shoot of rooted cuttings not only continues to grow obliquely, but the curvature at the base of the cutting also continues to accentuate the oblique habit. This curvature suggests a growth asymmetry favoring the morphologically upper side of the stem by responding to a higher concentration of IAA on that side. The experiments of Starbuck (1979) indicate that such may be the case although that author did not measure endogenous IAA.

Objectives

The objectives of this study were:

(1) To determine the level of endogenous IAA and cytokinins in buds, needles and stems of two clones of Douglas-fir at various times during the fall and winter. One clone exhibits plagiogravitropic growth habit, whereas the other clone exhibits an orthogravitropic growth habit.

(2) To determine the endogenous IAA concentration in the morphologically upper and lower sides of curvature, in the shoot apex, and in the roots in newly rooted cuttings of the two clones.

(3) To determine the endogenous IAA concentration in the shoot apex, the morphologically upper and lower sides of various sections of the shoot, and in the roots of Douglas-fir stem cuttings which have grown for one year after rooting.
MATERIALS AND METHODS

Plant Materials

Branch terminals 10 cm long were excised from two clones (12 and 15) of Douglas-fir. These clones had been established from rooted cuttings in a nursery near Corvallis 8 years previously. Clone 12 required about 10 years to regain an upright growth habit while clone 15 grew upright within two years of rooting (Black, 1973). The branch terminals from 3 ramets of each clone were collected on September 9, and December 23, 1985, and January 21, and February 24, 1986. Vegetative buds were collected from each tree on each of the four dates, immediately frozen in liquid nitrogen and stored at -80 °C. Two-cm long apical shoot of the debudded branch terminals were also collected and stored for IAA and cytokinin determination.

Propagation of the stem cuttings commenced in early February, 1986. Cuttings from clones 12 and 15 were trimmed to 15 cm and the needles on the basal 5 cm were removed. Cuttings were treated by dipping the base of each cutting into a dry powder of Rootone F (Union Carbide Company), and Hormex no.45 (Brooker Chemical Company), or by soaking in an aqueous solution of 7.4 mM naphthalene acetic acid (NAA) for 15 seconds (Proebsting, 1984). The cuttings were then placed in a propagation bench consisting of small gravel and
peat (3:1, v/v) or coarse quartz sand. An intermittent spray system controlled by an electronic timer applied mist for 10 seconds every 10 minutes. Day and night temperature in the greenhouse was maintained at approximately 25 °C. Evaporative coolers were used to moderate daytime temperatures. After three months the mist interval was increased to 20 minutes.

Three rooted cuttings of each clone were taken on July 13th, 1986. By this date cuttings from clone 12 exhibited more plagiogravitropic habit than cuttings from clone 15 (Fig.1). Curvature parts on the shoot were divided into an upper and lower halves, and these parts with the 2 cm long apical shoot and roots of each cutting were frozen and stored at -80 °C.

Cuttings from a local Douglas-fir tree were rooted in January of 1985 and grown in the greenhouse as described above, then transplanted into one liter containers in the late spring, 1985. These plants exhibited a strong plagiogravitropic growth habit (Fig.12). Three of these rooted cuttings were harvested on September 3, 1986, divided into several portions as shown in figure 12, frozen, and stored at -80 °C.

Extraction and Analysis of IAA

1) Preparation of DEAE-cellulose (Whatman, DE 32) column.

DEAE-cellulose was first suspended in 95% ethanol for 30
Fig. 1. Rooted stem cuttings of Douglas-fir from clone 12 and clone 15 five months after placement in rooting bed. Cuttings from clone 12 exhibited higher plagio- or gravitropic habit than those from clone 15.
min, filtered, then washed consecutively with 0.5 M NaOH (1 hour), distilled water, 0.5 M HCl (30 min), distilled water, 0.5 M NaOH (30 min), and distilled water until the pH decreased to 9.0. The washed DEAE-cellulose was suspended in 5 X buffer (0.1M ammonium acetate (AAc) buffer, pH 6.5), and stirred gently for 30 minutes. The pH was adjusted back to 6.5 periodically with acetic acid during this time. It was then washed with distilled water and resuspended in 2 X buffer (20 mM AAc buffer pH 6.5). The pH was again adjusted back to 6.5 if necessary with acetic acid. The prepared DEAE-cellulose buffer was then stored in a refrigerator with 0.1% sodium azide, an anti-microbial agent.

The DEAE-cellulose buffer was poured into a 20 ml syringe barrel fitted with a double filter paper at the bottom and a vacuum was applied at the bottom of the syringe to remove liquid solution. Additional DEAE-cellulose was added until a bed volume of 10 ml was achieved. The column was then washed with AAc buffer (20 mM pH 6.5) and was ready for use.

2) Preparation of Polyvinylpyrrolidone (PVP) Column.

PVP was suspended in methanol (MeOH) for several minutes. This suspension was poured into a 20 ml syringe barrel fitted with a double filter paper at the bottom and a vacuum applied at the bottom of the syringe. PVP was added until a bed volume of 10 ml was achieved. The column was then washed with 10 ml AAc buffer (20 mM pH 6.5) and was ready for use.
3) Preparation of the Sepralyte C18-silica Column

C18-silica (Analytichem International) was suspended in MeOH and packed into a 2 ml syringe barrel as described above. It was then eluted with approximately 10 ml of 1 M acetic acid which had been adjusted to pH 3.1 with NH4OH and maintained in this solution until used.

4) Extraction and Purification

Plant tissue (0.5-1.0 g) was suspended in the following materials: methanol 5 ml, AAc buffer 2ml, sodium diethylthiocarbamate (DIECA) 30 mg, 2,6-di-tert-butyl-4-methylphenol (BHT) 20 ul (20mg/ml in MeOH), Ascorbate 100 ul (20mg/ml in MeOH), 2-mercaptoethanol (MCE) 100 ul, and 3H-IAA (internal standard, about 20,000 cpm). The mixture was kept cold in an ice bath and homogenized with a polytron (Brinkman instruments) at full speed for 30 seconds, then stored in the dark at 5 °C for 30 minutes. The chilled mixture was centrifuged at 13,500 X g for 5 min. in a bench-top centrifuge and the supernatant decanted into a silanized tube. The pellet was resuspended in 5 ml of AAc buffer, stirred, centrifuged, and the supernatant combined with the previous supernatant.

The PVP column was attached to the DEAE-cellulose column as shown in figure 2 and washed with 100 ml AAc buffer (20 mM pH 6.5). Prepurified nitrogen pressure was used to maintain a flow rate of 1 ml/min.

The supernatant from the extract described above was diluted to 50 ml with AAc buffer containing DIECA (200 ug/l)
Fig. 2. Flow diagram of method used to measure IAA
and MCE (200 ul/l) as anti-oxidants, then passed through the washed PVP and DEAE-cellulose columns in tandem as shown in figure 2. Prepurified nitrogen pressure was used to control the flow rate at 1 ml/min. Both columns were washed with 20 ml AAc buffer containing DIECA and MCE. The IAA remained on the DEAE-cellulose. The DEAE-cellulose column was removed from the PVP column and a 2 ml C_{18}-silica column was attached in tandem below the DEAE-cellulose column, which was eluted with 50 ml of 1 M acetic acid (pH 3.1) containing MCE (200 ul/l). The IAA remained on the C_{18}-silica column.

The C_{18}-silica column was removed, washed with 10 ml of distilled water containing MCE (200 ul/l), and the IAA was eluted (1 ml/min) from it with 4 ml of MeOH. A sample of 50 ul of the eluate was taken and the ^3H-IAA measured in a scintillation counter (Beckman LS-250) to calculate recovery at this point in the analysis. The eluate was dried in a Speed Vac Concentrator (Savant) and stored at -20 °C. This procedure usually resulted in recovery of about 75% of the IAA.

5) Analysis by High Performance Liquid Chromatography (HPLC).

The analysis for IAA was achieved by two HPLC steps using a Varian Model 5020 single pump system fitted with a C_{18} reverse phase column (Altex Ultrasphere-ODS, 250x4.6 mm) and coupled with a Perkin-Elmer Model 650-10S Fluorescence Spectrophotometer detector set at Ex285 / Em345nm. The solvents used were Baker HPLC-grade MeOH and triethylamine
ammonium acetate buffer (TEAAc, 40 mM acetic acid was adjusted with distilled triethylamine to pH 3.6). The buffer of TEAAc was filtered on a C₁₈ Millipore System.

For the first HPLC step, the dry sample was dissolved in 50 ul of 50% MeOH/TEAAc, and a 20 ul aliquot was injected into the chromatograph. The mobile phase was 45% MeOH in TEAAc and the flow rate was 1 ml/min. The IAA eluted at 8 minutes and was collected. A 100 ul sample was taken for measurement of ³H-IAA with a scintillation counter to calculate the final recovery. Then the collected sample was dried by Speed Vac.

For the second HPLC step, the dried IAA was methylated by adding a few drops of freshly generated diazomethane in ether (Fig.3) and shaken frequently for 15 min. at room temperature. The ether in the sample was then evaporated under a stream of nitrogen and the sample resuspended in 50 ul of 50% MeOH/water. The total sample was injected into the HPLC. The mobile phase was 50% MeOH/water and the flow rate was 1 ml/min. The methylated IAA (MeIAA) eluted at 13 minutes. The MeIAA peak obtained in this second HPLC step was resolved to the baseline (Fig.4). An authentic standard of MeIAA was used to construct a standard curve to calculate (along with the final recovery above) the concentration of IAA in the original plant sample. The MeIAA peak was confirmed by gas chromatography/mass spectrometry (GC-MS), showing a parent ion peak at m/z 189, and a base peak at m/z 130 (Fig.5).
Fig. 3. Diagram of the apparatus for the production of diazomethane in diethyl ether. All work should be carried out in a hood. Reagents and products are toxic and explosive.
(Revised from Cohen 1984)
Fig. 4. HPLC chromatogram of PVP-DEAE-C18 semipurified extract before and after methylation. Column: 250x4.6 mm ODS; Solvent: A & B) 45% MeOH in 40 mM TEAAc buffer pH 3.6, 1 ml/min. C) 50% MeOH in clean water, 1 ml/min; Detector: fluorescence spectrophotometer at Ex 285/Em 345 nm.
Fig. 5. Mass spectra of methylated authentic IAA and of extract from Douglas-fir needles and stems. Parent ion peak at m/z 189, and base peak at m/z 130.
Extraction and Purification of Cytokinins

Stored plant material (1-2 g) for each extraction was homogenized for 30 seconds with a polytron in 10 ml of 80% MeOH/AAc buffer (20 mM pH 6.5) which contained 250 ul DIECA (20 mg/ml in MeOH), 250 ul MCE, 250 ul BHT (50 mg/ml in MeOH), 250 ul ascorbate (20 mg/ml in MeOH) and about 20,000 cpm $^3$H-isopentenyladenosine (iPA) dialcohol as an internal standard. The homogenate was stirred for 30 min at 4 °C, centrifuged (15 min at 13,500 X g), and the supernatant decanted into a silanized tube. The homogenate was reextracted as before and the two supernatants were combined and reduced in volume under a nitrogen stream to remove most of the methanol. The extract was diluted to 50 ml with AAc buffer (20 mM, pH 6.5) containing DIECA and MCE (200 ul/l) as anti-oxidants.

The diluted sample (50 ml) was purified on two columns in tandem. The first (upper) contained a 20 ml bed volume of DEAE-cellulose (Whatman DE 32) prepared as described above for analysis of IAA. The second (lower) column was a 1 ml bed volume of Cytokinin-Specific Immunoaffinity-cellulose (IgG) material described by MacDonald and Morris (1985). The sample was eluted through the two columns at a flow rate of 0.8 ml/min, then washed with 40 ml of AAc buffer (20 mM, pH 6.5). Cytokinins remain on the IgG column. The DEAE-cellulose column was removed
and discarded and the IgG column was washed first with a 5 ml mixture of AAc buffer (20 mM, pH 6.5)/0.5 M NaCl/2% dimethylsulphoxide (DMSO), then with 5 ml AAc buffer (20 mM, pH 6.5). The IgG column was eluted with 3 ml of MeOH (0.8 ml/min). A 50 ul aliquot of the eluate was taken and the $^3$H measured in a scintillation counter to determine recovery. The sample was evaporated in a Speed Vac and stored at -20°C. The extraction procedure was shown in figure 6.

Separation of Cytokinins.

The separation of cytokinins was achieved by HPLC using a Beckman model 420 system fitted with a C$_{18}$ reverse phase column (Altex Ultrasphere-ODS, 250 x 4.6 mm) and a Beckman model 160 uv detector monitoring at 260nm. The HPLC solvents were triethylamine ammonium acetate buffer (TEAAc buffer, 40 mM acetic acid was adjusted with distilled triethylamine to pH 3.4) which was filtered on a C$_{18}$ Millipore system, and Baker HPLC-grade acetonitrile. The column was eluted at 1 ml/min with the following gradient: initial conditions 6% acetonitrile:94% buffer, 6% to 11% acetonitrile over 15 min, 11% to 35% over 15 min. The injection sample was in 50 ul of methanol and triethylammonium acetate buffer (1:1 v/v). Fractions were collected every 0.5 min, and were divided into two replicates for radioimmunoassay (RIA) or combined for GC-MS. Fractions were dried in a Speed Vac and stored at -20°C.
A) Extraction and Purification

Polytron (Brinkmann) → Evaporate MeOH with prepurified Na gas → DEAE cellulose

Tissue (1-2 g)
MeOH (8 ml) + AAC buffer (2 ml)
250 μl DIECA (20 mg/ml in MeOH)
250 μl 2-mercaptoethanol
250 μl BHT (50 mg/ml in MeOH)
250 μl Ascorbate (20 mg/ml in MeOH)
3H-1PA dialcohol (20,000 cpm)

B) Quantitation

RIA

○ Antigen
⊙ Radioactive antigen
△ Antibody

Mix and incubate → Precipitation with (NH₄)₂SO₄ → Radioactive measurement

Prepurified Na
20 mM AAC buffer (pH 6.5)
Cytokinins directly pass through the column.

Wash the column with
1) 5 ml AAC buffer/0.5M NaCl/2% DMSO.
2) 5 ml AAC buffer (pH 6.5). Elute with 3 ml MeOH.
Dry by Speed Vac.

HPLC
UV DETECTOR 260nm
FRACTION COLLECTOR

Fig. 6. Flow diagram of method used to measure cytokinins.
Recovery from the extraction and purification steps as calculated using the internal standard was around 50%.

Radioimmunoassay of Cytokinins

Appropriate fractions collected during HPLC were assayed with anti-zeatin riboside IgG using $^3$H-ZR dialcohol; or with anti-iPA IgG using $^3$H-iPA dialcohol as described previously by MacDonald and Morris (1985). Samples collected in 1.2 ml Eppendorf centrifuge tubes were separated into two equal sets and dried for RIA. Phosphate buffered saline (PBS) buffer was then added to each tube along with radioactive ZR or iPA, and the antibody serum, mixed, and set aside for 20 minutes. 600 ul of 90% saturated ammonium sulfate (pH 7.0) was added for 10 minutes to precipitate the protein. The tube was then centrifuged for 30 seconds and the liquid aspirated. In the last step, 100 ul of MeOH was added to the tube and mixed, followed by 1 ml of scintillation flour. The $^3$H was measured in a scintillation counter (Beckman LS-250). A representative chromatogram and corresponding results from the RIA are shown in figure 7.

Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) was accomplished on a Finnigan Automated Gas Chromatograph EI-CI
Mass Spectrometer System M-400. Cytokinins were chromatographed as permethylated and deuteromethylated derivatives (Morris 1977).
Fig. 7. A) Chromatogram of cytokinin standards by reverse-phase HPLC. A mixture of approximately 10 ng each of tZ (trans-zeatin), DHZ (dihydrozeatin), cZ (cis-zeatin), tZR (trans-zeatin-riboside), DHZR (dihydrozeatin riboside), K (kinetin), iPA (iso-pentenyladenosine) and iP (iso-pentenyladenine) was applied to a 250x4.6 mm, ODS column equilibrated with TEAAc buffer 40 mM pH 3.4 containing 6% acetonitrile. The concentration of acetonitrile was increased to 11% over 15 min, then to 35% over 15 min, and finally to 100% over 1 min. Flow rate: 1 ml/min.

B) HPLC chromatogram of immunoaffinity purified cytokinins from Douglas-fir vegetative buds. 

C) Histogram of radioimmunoassay of HPLC fractions of immunoaffinity purified cytokinins from Douglas-fir vegetative buds.
RESULTS

Development of procedures for extracting and quantitating IAA

The procedure developed by Thompson et al. (1981) for measuring IAA by HPLC and fluorescence detection has been in use for several years, but that procedure sometimes results in poor recovery. Furthermore, an unknown substance has sometimes interfered with the determination. The method developed for the present study improved upon Thompson's technique and resulted in higher recoveries of IAA. By following these precautions, recovery of about 75% was common. The modified procedure incorporated the following precautions:

1. The combined DEAE and PVP columns were washed with 150 ml or more of 20 mM AAc buffer (pH 6.5) before starting the sample extraction.
2. Less than 0.5 mg of BHT was added to each sample. More than 0.5 mg of BHT reduced the recovery.
3. The flow rate for all elution steps was 1 ml/min or less.
4. The IAA-like material from the first HPLC separation was evaporated in the Speed Vac to a minimum volume but not dried completely.
5. Diethyl ether was evaporated with a stream of nitrogen rather than with the Speed Vac.

Rooting of stem cuttings.

Naphthaleneacetic acid (NAA) and indolebutyric acid (IBA) are frequently used in rooting cuttings of woody plants. In the present experiment, 7.4 mM NAA, the optimum concentration suggested by Proebsting (1984), resulted in 46% rooting percentage. Rootone F, a commercial preparation including 1-naphthaleneacetamide (0.067%), 2-methyl-1-naphthaleneacetic acid (0.033%), 2-methyl-1-naphthaleneacetamide (0.013%), indole-3-butyric acid (0.057%) and inert ingredient (99.83%), produced 31% rooting, and Hormex 45, another commercial product including indole-3-butyric acid (4.5%) and inert ingredient (95.5%), produced 50% rooting. Only 7% of the untreated cuttings rooted.

Estimation of endogenous IAA and cytokinins in different tissues and seasons of two Douglas-fir clones.

In general, a low concentration of IAA was found in buds, stems, and needles on four collection dates of September, December, January, and February. Although IAA levels were the highest in vegetative buds in December, the levels found in the stems and needles at that time were low
There were significant differences between the two clones in September's buds, needles and stems, and between buds in December, whereas differences on the other dates were not significantly different (Table 1).

A cytokinin conjugate (ZX), which was determined to be a hexose conjugate but not zeatin ribose-0-glucoside, was found in the vegetative buds of Douglas-fir (J.W. Morris, personal communications). In the present study, high concentrations of ZX were also found in vegetative buds of Douglas-fir, whereas low concentrations of ZX were found in needles and stems. The ZX compound extracted from vegetative buds was confirmed by GC-MS (Fig.11).

Some significant differences in concentration of cytokinins appeared in buds, needles and stems between clone 12 and clone 15, especially in ZX, ZR, and iPA (Table 2). There is a tendency of ZX and ZR interaction between clones that higher concentrations of ZX in clone 12 were measured than clone 15 on four collection dates whereas higher concentrations of ZR in clone 15 measured on the same dates. There also appears to be a tendency for higher levels of ZX and ZR to occur in September, then ZX gradually decreased from September to February. ZR, however, remained about constant, and iPA gradually increased to February (Figure 9 and 10).

Quantitation of IAA in upper and lower halves of sections from 5-month old rooted cuttings
Fig. 8. Levels of endogenous IAA in buds, needles and stems of clone 12 and clone 15 on four collection dates. (○---○ clone 12 bud; ●---● clone 15 bud; □---□ clone 12 N+S; ■---■ clone 15 N+S)
Table 1. Concentration of endogenous IAA in vegetative buds, needles and stems of Douglas-fir collected on four dates. Values are ng/g fresh wt. ± S.D. (n=3). ** indicates significant difference between clones (P<0.01) for that collecting date.

<table>
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</thead>
<tbody>
<tr>
<td></td>
<td>clone 12</td>
<td>clone 15</td>
<td>clone 12</td>
<td>clone 15</td>
</tr>
<tr>
<td>Bud</td>
<td>23.53+0.67</td>
<td>13.94+1.07</td>
<td>279.43+29.60</td>
<td>204.74+6.84</td>
</tr>
<tr>
<td>Needle + Stem</td>
<td>46.57+4.21</td>
<td>31.84+0.97</td>
<td>16.77+2.40</td>
<td>17.98+2.24</td>
</tr>
</tbody>
</table>
Fig. 9. Cytokinin levels in vegetative buds, needles and stems of Douglas-fir on four collection dates. * significantly different at $P<0.05$; ** significantly different at $P<0.01$. 
Fig. 10. Cytokinin levels in vegetative buds, needles, and stems of Douglas-fir on four collection dates. * significantly different at $P=0.05$; ** significantly different at $P=0.01$. 

CYTOKININS IN CLONE 15
Fig. 11. Mass spectrum of permethylated ZX from vegetative buds of Douglas-fir.
Table 2. Levels of endogenous cytokinins (ng/g fresh wt.) in Douglas-fir vegetative buds, needles and stems from clone 12 (plagiogravitropic growth) and clone 15 (orthogravitropic growth) on four collection dates. Each value is the average of 3 measurements.

<table>
<thead>
<tr>
<th></th>
<th>September</th>
<th>December</th>
<th>January</th>
<th>February</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>clone 12</td>
<td>clone 15</td>
<td>clone 12</td>
<td>clone 15</td>
</tr>
<tr>
<td><strong>tZ</strong></td>
<td>1.6</td>
<td>1.3</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>ZX</strong></td>
<td>79.2**</td>
<td>57.1**</td>
<td>35.3*</td>
<td>23.6*</td>
</tr>
<tr>
<td><strong>ZR</strong></td>
<td>32.7*</td>
<td>56.1*</td>
<td>17.4</td>
<td>24.8</td>
</tr>
<tr>
<td><strong>iPA</strong></td>
<td>11.9</td>
<td>9.8</td>
<td>4.7</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>IP</strong></td>
<td>4.3**</td>
<td>2.2**</td>
<td>0.2**</td>
<td>1.4**</td>
</tr>
<tr>
<td><strong>tZ</strong></td>
<td>0.3</td>
<td>0.2</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>ZX</strong></td>
<td>3.3**</td>
<td>7.5**</td>
<td>1.8</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>ZR</strong></td>
<td>36.8*</td>
<td>51.4*</td>
<td>7.8**</td>
<td>20.5**</td>
</tr>
<tr>
<td><strong>iPA</strong></td>
<td>7.6</td>
<td>5.5</td>
<td>10.3</td>
<td>15.1</td>
</tr>
<tr>
<td><strong>IP</strong></td>
<td>0.7</td>
<td>1.0</td>
<td>0.8</td>
<td>1.1</td>
</tr>
</tbody>
</table>

** = significant at P=0.01 between clone 12 and clone 15 in each collection date.

* = significant at P=0.05 between clone 12 and clone 15 in each collection date.
Three current rooted cuttings of each clone (12 and 15) which exhibited the plagiogravitropic and orthogravitropic growth habit were taken on July 13, 1986 and divided along the longitudinal axis into a morphological upper and lower half. Those cuttings had formed a small terminal bud. There were no significant differences in IAA content between the upper and lower halves of these plants in either clone (Table 3). Likewise, there were no differences in IAA content between clone 12 and clone 15. But the data suggest the greater concentration of IAA in the upper half of plagiogravitropic clone, and concentration of IAA is higher in the roots than the shoots.

IAA levels in strongly plagiogravitropic rooted cuttings

Another set of two-year old rooted cuttings were harvested on September 3, 1986. These cuttings were not from clone 12 or 15 and their shoots were still elongating when collected. Each cutting was divided as shown in figure 12 and each component was analyzed for IAA. There did not appear to be a difference in IAA concentration between the morphological upper and lower halves of the stem (Table 4). But the upper half of all sections had on the average greater IAA concentrations. The highest levels of IAA appeared to be in section 2, which was the first growth
Table 3. Endogenous IAA (ng/g fresh wt.) in the morphological upper and lower halves of curvature, apical shoots, and roots from current rooted stem cuttings of Douglas-fir clone 12 (plagiogravitropic growth) and clone 15 (orthogravitropic growth) collected on July 13, 1986. Values represent the mean ± S.D. (n=3). Only one measurement of three combined apical shoots were made for each clone. There are no significant differences in IAA content between the upper half and the lower half of curvature in either clone.

<table>
<thead>
<tr>
<th></th>
<th>apical shoots</th>
<th>upper half of curvature</th>
<th>lower half of curvature</th>
<th>roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>clone 12</td>
<td>31.89</td>
<td>34.16±3.27</td>
<td>30.22±5.67</td>
<td>45.47±1.72</td>
</tr>
<tr>
<td>clone 15</td>
<td>33.45</td>
<td>32.31±5.19</td>
<td>36.75±4.47</td>
<td>44.86±4.44</td>
</tr>
</tbody>
</table>
Table 4. Endogenous IAA levels in apex, shoot section, and roots of a plagiogravitropic Douglas-fir clone (see Fig. 12). Values represent the mean (ng/g fresh wt.) ± S.D. (n=3). Only one measurement was made in section 4. There are no significant differences in IAA content between morphological upper and lower halves of the stem sections.

<table>
<thead>
<tr>
<th>section</th>
<th>apical shoots</th>
<th>roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>upper half</td>
<td>11.95±1.10</td>
<td>26.22±4.81</td>
</tr>
<tr>
<td>lower half</td>
<td>11.54±4.73</td>
<td>24.43±2.11</td>
</tr>
</tbody>
</table>
flush of the second year rooted cutting. The section 2 was just above the portion of stem exhibiting the strongest plagiogravitropic curvature which was the section including the first year leader increment. IAA concentration decreased in both directions from section 2, but was higher in the roots than in the shoot apex.
DISCUSSION

IAA quantitation

After purification by PVP-DEAE-C_{18}, the extracts were subjected to two HPLC separations. The purpose of the second HPLC step was to obtain a more accurate measurement by separating MeIAA from the last trace of contaminants. Recovery of the radiolabelled internal standard was about 50% after the first HPLC step. A high concentration of diazomethane in ether is essential for complete methylation of IAA. Diazomethane is a useful agent for methylation, but because of its toxicity and instability, all work with diazomethane should be carried out in a hood and precautions taken to avoid explosions and contact with the chemical. Figure 3 is a diagram of the apparatus used for producing a high concentration of diazomethane. Detection by fluorescence was less sensitive after methylation, but good resolution and quantitation are still obtained.

The distribution of endogenous IAA in young rooted cuttings

In the studies on distribution of endogenous IAA levels in the two year-old rooted stem cuttings (Fig.12), there were no significant differences between the upper and lower halves in each section, although there appeared to be a
Fig. 12. Endogenous IAA levels in sections from intact rooted stem cuttings of Douglas-fir after 2 seasons of growth. The rooted cuttings were still growing (no bud formation) at the time of collection (September 3, 1986). Note shadow of rooted cutting.
tendency for the upper half to contain more IAA than the lower half (Table 4). Table 3 also shows no significant differences in IAA levels between the upper and lower halves of 5-month-old rooted stem cuttings collected in July.

Results from the present study do not support the basic premise of the Cholodny-Went model with respect to an asymmetrical distribution of IAA. The results are similar to a paper from Mertens and Weiler (1983), who used sensitive and selective immunoassays, and indicated that no significant difference of lateral asymmetry of growth substances (IAA, ABA, and GA₇) was detected in hypocotyls of Helianthus annuus and root tips of Zea mays and Vicia faba. Hasenstein and Evans (1986), investigating the interaction of Ca²⁺ and auxin on root elongation in seedlings of Zea mays, indicated that Ca²⁺ possibly acts as a second messenger of auxin action to explain the failure of gravity-induced IAA redistribution by some workers. In the present study we have no information on the levels of Ca²⁺ in plagiogravitropism.

There were no significant differences between the upper half and lower half of vertical branches in the studies by Starbuck (1979) on distribution of activity from ¹⁴C-IAA in the current season's growth of horizontal and vertical (tied vertically for 2 weeks) branch terminals on Douglas-fir. But significantly more activity was detected in the upper than in the lower side of horizontal branch terminals. Why ¹⁴C-IAA levels in upper and lower sides of vertical branch
were not different when the horizontal branches and intact rooted stem cuttings described previously have significantly more $^{14}$C-IAA in the upper than in the lower side has not been explained. If the original compression wood formation on the lower side of branch terminals or cuttings from ramets is the result of a transverse gradient in auxin content, the original compression wood formation on the lower side may limit the capacity of IAA to be transported resulting in the higher IAA levels on the upper side. The horizontal branch terminals after being tied vertically resulted in the compression wood formation on the upper half for establishing an "equilibrium position" might be the result of no difference of $^{14}$C-IAA on both halves. This hypothesis is in need of testing.

Seasonal variation in IAA levels frequently has been described as the primary physiological factor regulating cambial activity (Digby and Wareing 1966, Little and Wareing 1981, Wareing 1958). It is possible that a small increase in IAA concentration may trigger a relatively large increase in cambial activity. In the present study, excised branches from ramets which have original compression wood formation in the morphologically lower side as a response to gravity were placed vertically in a propagation bench. After bud-burst and rooting, asymmetrical IAA transport in rooted cuttings may result in a large increase in cambial activity on the upper side. Starbuck (1979) found that the bending near the base of Douglas-fir stem cuttings was brought
about by the formation of compression wood on the morphological upper sides. We also found on the propagation bench that curvature direction of the new shoot of rooted cuttings was always in the same direction as the original branch. These results could be explained if plagiogravitropic growth were caused by asymmetric IAA distribution and compression wood formation.

Plagiogravitropic growth on Douglas-fir stem cuttings from branch terminals was first observed from April to June but occurred mostly from October to December. This growth habit persists for several years or more. The mechanism of curvature in these plants is apparently different from that observed in tissues of coleoptiles, hypocotyles, and roots of other plants in which the curvature occurs within a period of several minutes to hours after gravistimulation. But Starbuck (1979) found that ¹⁴C-IAA applied to excised segments of a branch terminal, migrated unequally to the upper and lower sides. This result could mean that the apex of a tree regulates branch angle and compression wood formation, and IAA is related to this regulation system. Once a branch was detached the regulation system would be destroyed, causing no apparent difference of IAA migration. IAA lateral distribution could occur again after rooting. But the lateral distribution of IAA wasn't found in the present study. It is possible that minute difference of IAA levels on upper and lower halves that presently can not measured may cause the curvature (See table 4). The
unsuitable collection time and/or disparity of collected tissues from ramets in our studies could also account for the result of no difference of IAA concentration between upper and lower halves.

As shown in figure 12, the highest IAA levels were measured in section 2, which was the first flush growth of the second year and contained the most needles. This highest IAA levels of section 2 near the region of strong curvature may be accounted for by the accumulation of IAA produced in the apical meristem and transported to the area of curvature. Higher concentration of IAA was also measured in the roots than apical shoot. It is often found that roots develop unevenly at the base of cuttings. More roots develop on the side opposite the direction of curvature growth (personal observation). Copes (1980) found that grafting on 4 year-old field-grown rootstocks of Douglas-fir produced vigorously orthogravitropic leaders whereas grafting on container-grown stocks grew significantly shorter branches and more plagiogravitropic growth. He proposed that rootstock vigor might influence both leader and branch-like growth habit. We also observed that two-year old rooted cuttings in one-liter containers were very plagiogravitropic.

No attempt was made to measure conjugated IAA such as IAA-amino acid conjugates or glycosyl IAA conjugates. Several prior studies have provided direct and indirect evidence that IAA conjugates may be related to the ability

Variation of IAA and cytokinins levels in buds, needles and stems of 10-year-old rooted cuttings

Seasonal changes in the endogenous IAA levels in buds, shoots and cambial zones have been reported for several coniferous species. Commonly, low levels of IAA were found in dormant periods (Alden 1971, Little et al 1978, Little and Wareing 1981, Savidge and Wareing 1984) although Savidge and Wareing (1982) detected higher IAA levels in cambial tissue of lodgepole pine throughout the winter. The higher levels of IAA detected in December's buds of Douglas-fir than those of other collection dates may be indicative of active buds at this time. Why IAA levels in buds are still very high during deep dormancy (December) cannot be explained with available data although the relatively warm winter temperatures of Western Oregon allow for much biological activity to proceed in conifers.

The survival of outplanted seedlings in reforestation is associated with the time of lifting, cold storage, and outplanting sequence in nursery management. Lavender (1964) noted that seedlings lifted prior to December, or in April after buds swell in the spring, were affected adversely by outplanting and by cold storage. No mitotic activity was found in the dormant apex of Douglas-fir from December
through February (Owens and Molder 1973). Based on published data and numerous operational observations, Lavender (1985) demonstrated that Douglas-fir seedlings are most resistant to the stresses in nursery harvest, storage, and outplanting from December through February. On the other hand, buds of Douglas-fir seedlings are still active during the fall and may account for the increase in mortality after outplanting when seedlings are lifted from the nursery in early fall. It is possible that the failure of new plantings is correlated with the higher endogenous levels of IAA in the fall, a time when vegetative buds are still differentiating. But the lower concentrations of cytokinins measured in December could explained the interaction of two hormones for maintaining bud dormancy.

The lowest concentrations of IAA in January's buds are shown in the figure 8, and increased in the end of February. The gradually increased IAA levels and ZR of cytokinins in February could imply the possibility of early physiological changes in buds prior to April budburst.

It is well known that cytokinins are an essential component for culture of most species and that an appropriate ratio of cytokinins to auxins often leads to organogenesis. However, only a few studies on cytokinins in leaves and buds of conifers have been reported (Lorenzi et al. 1975; Taylor et al. 1984). Those authors found that high levels of glucosyl conjugates of cytokinins, such as zeatin riboside-0-glucoside, zeatin-7-glucoside, and
zeatin-9-glucoside, were detected in winter, and more active zeatin riboside was detected in initiating shoots. Some authors suggested that cytokinins could be associated with the flowering process (Doumas et al. 1986, Tompsett 1977, Zaerr and Bonnet-Masimbert 1985). In the present study, endogenous ZX and ZR levels in buds and ZR in needles and stems of Douglas-fir implies that ZX and ZR may play a role in the winter and maintain a balance with IAA for bud differentiating and dormancy. This ZX may be the same compound that was found in Radiata pine by Taylor et al 1984. Another iP A-type compound also found in needles and stems and gradually increased from September to February. High levels of iP A with low levels of zeatin-type cytokinins were found in pollen-cone buds (J.W. Morris, personal communication). The physiological function of iP A is still unknown.

ZX levels gradually decrease from September through February whereas ZR maintained near-constant levels throughout the study period. ZX may be an inactive or a modulator form of the biologically active ZR. The higher ZX levels in buds than in needles and stems could reflect its site of storage. The interaction tendency of ZX and ZR levels in buds between clone 12 and clone 15 on different collection dates might indicate that the orthogravitropic growth of clone 15 could relate to this higher levels of active ZR in the period although we didn’t look at the consecutive data to bud break.
Suggestions for future work

The experiments and results described above are still left with many unanswered questions. The mechanisms of plagiogravitropic growth of rooted cuttings is still not clear. Some experiments which might lead to a better understanding of plagiogravitropic growth in shoots might include the following:

1. Force horizontal branch terminals on the parent plant into the vertical position for some period of time prior to rooting them; then compare their tendency to grow plagiogravitropically with normal branch cuttings from the same tree. This experiment might offer clues as to how cuttings "remember" their previous orientation.

2. Correlate the amount and location of compression wood in cuttings with their tendency to grow plagiogravitropically. Compression wood might be the initial stimulus for asymmetrical distribution of IAA, which could lead to the formation of additional compression wood.

3. Compare the pattern of the root distribution around the base of cuttings with their tendency or direction of plagiogravitropic growth. Also examine the nutrient conditions which might bring about differences in root growth. Asymmetrical development of roots could cause asymmetrical formation of compression wood in the shoot and vice versa. Rooting cuttings in a hydroponic medium might
be an approach to this experiment.

4. Apply transport inhibitors such as morphactin, naphthyllphthalamic acid, and 2,3,5,-triiodobenzoic acid to the upper or lower side of rooted cuttings to see if differences in IAA transport could influence plagiogravitropic growth.

5. Apply calcium to the apex of shoot or roots of rooted cuttings to see if exogenously applied calcium could modify plagiogravitropic growth, or measure the endogenous IAA contents on upper and lower sides to see if calcium distribution is related to IAA lateral transport.

6. Examine the differences of free or conjugated cytokinin and gibberellin concentrations in the upper and lower sides of stem sections to see if lateral gradients in cytokinins and/or gibberellins occur in plagiogravitropic plants.
CONCLUSIONS

The concentrations of IAA in buds, apical needles and stems of branch terminals varied from September to February. The significant differences in the IAA content between two clones were significant in September and December, but the overall content of IAA in the shoot is not consistent enough to explain the tendency for plagiogravitropism. The highest concentration of IAA with lower concentration of cytokinins in buds measured in December may explain the balance of the two plant growth substances for differentiating and dormancy. The increased IAA and ZR levels, but decreased ZX levels in buds from January to February could be accounted for by the preparation of budbreak.

There were differences between the two clones in the concentration of certain cytokinins (including an unidentified hexose conjugate of zeatin riboside, ZX) on some collection dates, and the interaction of ZX and ZR in buds between two clones on four collection dates could suggest that some cytokinins content was related to the tendency for plagiogravitropism of rooted cuttings.

There was no significant differences in concentrations of IAA between the morphologically upper and the lower halves of plagiogravitropic rooted cuttings from shoot apex
to the bottom, but the mean of the measurements showed more IAA in the upper half of the stem. The highest concentration of IAA in the stem was found in the section immediately above (toward the shoot apex) the portion of stem with the strongest curvature. There was more IAA in roots than in shoot apices.

Results from these experiments suggest that IAA and/or cytokinins are not simply or solely involved in control of plagiogravitropic growth of rooted cuttings. Minor internal gradients of either IAA or cytokinins may still be in partial control of the growth mechanism. Such hormone gradients could be the promoter of compression wood, which in turn, brings about curvature of the stem.
BIBLIOGRAPHY


Bialek, K., W.J. Meudt and J.D. Cohen. Indole-3-acetic acid (IAA) and IAA conjugates applied to bean stem sections: IAA content and the growth response. Plant Physiol. 73:130-134.


Cohen, J.D. 1984. Convenient apparatus for the generation


auxin-wave in the cambial region of pine stems: validation of IAA as the auxin component by the Avena coleoptile curvature assay and by gas chromatography-mass spectrometry-selected ion monitoring. Plant Physiol. 84:135-143.


