

1-25-110
MICHIGAN STATE UNIVERSITY
EAST LANSING, MICH. 48824

This is to certify that the
thesis entitled

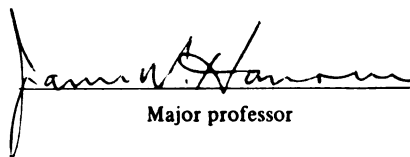
STUDIES ON ADVENTITIOUS ROOT FORMATION IN SPRUCE

presented by

Robert Charles Freyman

has been accepted towards fulfillment
of the requirements for

M.S. degree in Forestry


Major professor

Date 1-12-84



RETURNING MATERIALS:

Place in book drop to
remove this checkout from
your record. FINES will
be charged if book is
returned after the date
stamped below.

DO NOT STAMP

BOOK USE ONLY

STUDIES ON ADVENTITIOUS ROOT FORMATION IN SPRUCE

By

Robert Charles Freyman

A THESIS

Submitted to

Michigan State University
in partial fulfillment of the requirements
for the degree of
Master of Science

Department of Forestry

1984

ABSTRACT

STUDIES ON ADVENTITIOUS ROOT FORMATION IN SPRUCE

By

Robert Charles Freyman

Three studies investigating adventitious root formation in spruce were carried out in newly designed and constructed mist chambers. Chapter 1 details the design and construction of the mist chambers. Chapter 2 describes an anatomical investigation of adventitious root formation and development in Picea pungens, Picea glauca, and Picea engelmannii. Adventitious root formation and development was investigated with light microscopy in hardwood cuttings of one year old blue spruce, and three year old Engelmann and white spruce. Chapter 3 covers an experiment which compared the rooting response in young and old blue spruce (Picea pungens) to eight auxin conjugates. Old spruce (15 years) did not root. The glycine-indole-3-butyric acid produced greatest percent rooting and mean roots per cutting for the young spruce (2 years greenhouse grown). The final chapter discusses an experiment involving the use of Agrobacterium rhizogenes to induce adventitious root formation in seven tree species.

ACKNOWLEDGEMENTS

I would like to thank Dr. James Hanover for his support throughout the length of this study. I gratefully acknowledge Dr. Arthur Cameron and Dr. Daniel Keathley for reviewing this thesis and being on my committee. I would also like to thank Mr. John Heckman for his assistance and the sharing of his knowledge in microtechnique. I would like to thank Dr. Bruce Haissig for the two phenyl-auxin conjugates and Mr. David Boyles for critical directions and suggestions needed for the successful synthesis of the two conjugates. I am indebted to Mr. Roger Hangarter for the amino acid-auxin conjugates. Finally, I would like to thank Dr. Dennis Fullbright for the Agrobacterium rhizogenes and suggestions on culture techniques.

TABLE OF CONTENTS

	<u>Page</u>
List of Tables	iv
List of Figures	v
 Design of a Mist Propagation System	 1
Introduction	1
Materials and Methods	1
Discussion	8
 Anatomical Investigation of Adventitious Root Initiation and Development in <u>Picea pungens</u> , <u>Picea glauca</u> , and <u>Picea engelmannii</u>	 9
Introduction	9
Materials and Methods	12
Results	14
Discussion	20
 Comparison of Rooting Response in Young and Old Blue Spruce (<u>Picea pungens</u>) to Eight Auxin Conjugates	 61
Introduction	61
Materials and Methods	62
Results and Discussion	65
 Use of <u>Agrobacterium rhizogenes</u> to Induce Adventitious Root Formation in Seven Tree Species	 70
Introduction	70
Materials and Methods	71
Results and Discussion	72
 References	 76

LIST OF TABLES

<u>Table</u>	<u>Page</u>
Percent Rooting	66
Mean Number of Roots per Cutting	66
Mean Length of Longest Root per Cutting	67
Mean Mist	67
Analysis of Covariance	68

LIST OF FIGURES

<u>Figures</u>	<u>Page</u>
Mist Chamber Diagram	2
Materials List	4
Timer Board Layout	6
Misting Sequence	7
Normal anatomy - white spruce	29
Normal anatomy - blue spruce	29
Normal anatomy - Engelmann spruce	31
Cutting base	31
Normal anatomy	33
Mid-section of rooting zone	33
Lower rooting zone section	35
Trachied column	35
Needle trace - normal anatomy	37
Needle trace - rooting zone	37
Needle trace - rooting zone	39
Needle trace primordium	41
Needle trace meristematic spheres	41
Needle trace mature root	43
Root-trace connection	43
Cortical resin duct-root connection	45
Root tip emergency	45
Phelloderm/phellogen proliferation	47
Meristematic sphere in phelloderm/phellogen	47
Root from phelloderm/phellogen	49
Upper rooting zone section	49
Mid-rooting zone section	51
Lower rooting zone section	51
Needle gap proliferation	53
Meristematic sphere	53
Early primordium	55
Primordium	55
Early adventitious root	57
Early adventitious root	57
Primordium	59
Auxin designation and structure	64

Chapter 1

Design of a Mist Propagation System

INTRODUCTION

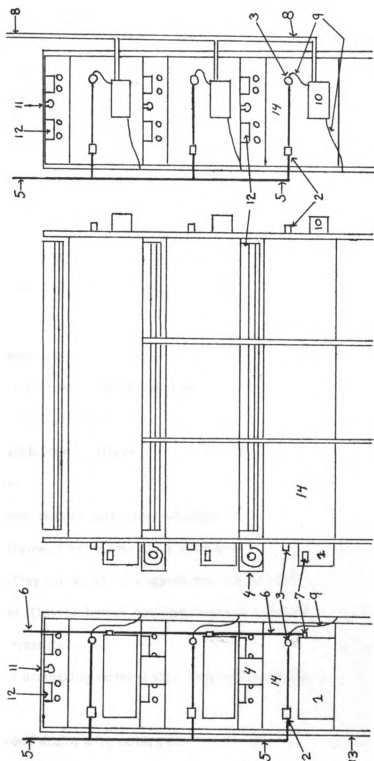
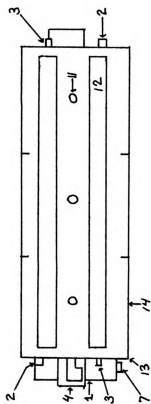
Greenhouse propagation facilities often prove unsatisfactory for research studies. A system is needed which affords good control over relative humidity, ambient air temperature, and photoperiod. These goals were met by erecting a plant growth frame located with mist capabilities in a building room. The room contained a whole room air conditioning system, floor drains, distilled water lines, and compressed air lines. The growth frames were constructed of 1-1/2" x 1/4" iron angle and consisted of three levels with each level being approximately 3' x 3' x 8'.

MATERIALS AND METHODS

A diagram of the mist chambers is presented in Figure 1 and a materials list is presented in Figure 2. The shell of the chambers is 3/8" exterior grade plywood painted with a penetrating, oil base, wood stain. The exterior stain offers advantages over paint in future maintenance and by having a fungus inhibitor. Each chamber measures 3' x 2' x 8' (w x h x l). The chambers are separated from the lights by corrugated fiberglass. A squirrel cage blower forces air through the light compartment to prevent heating of the upper chamber. The light system consists of four, eight-foot fluorescent tubes and three incandescent bulbs. In each chamber mist is produced by two 1/4" JH pneumatic atomizing nozzles. These nozzles produce an extremely fine mist. Compressed air atomizing mist heads can produce a finer mist than hydraulic type mist heads. Distilled water is used in the system to avoid nozzle clogging from salt

1. Timer box
2. Solenoid valve
3. Mist head
4. Fan and fan box
5. Compressed air line
6. Power (in 1/2" rigid conduit)
7. Junction box
8. Distilled water line
9. 1/4" flexible plastic water tubing
10. Water box with float valve
11. Incandescent fixture
12. Fluorescent fixture
13. 1-1/2" x 1/4" iron angle
14. 3/8" plywood (exterior)

Figure 1. Mist Chamber Diagram

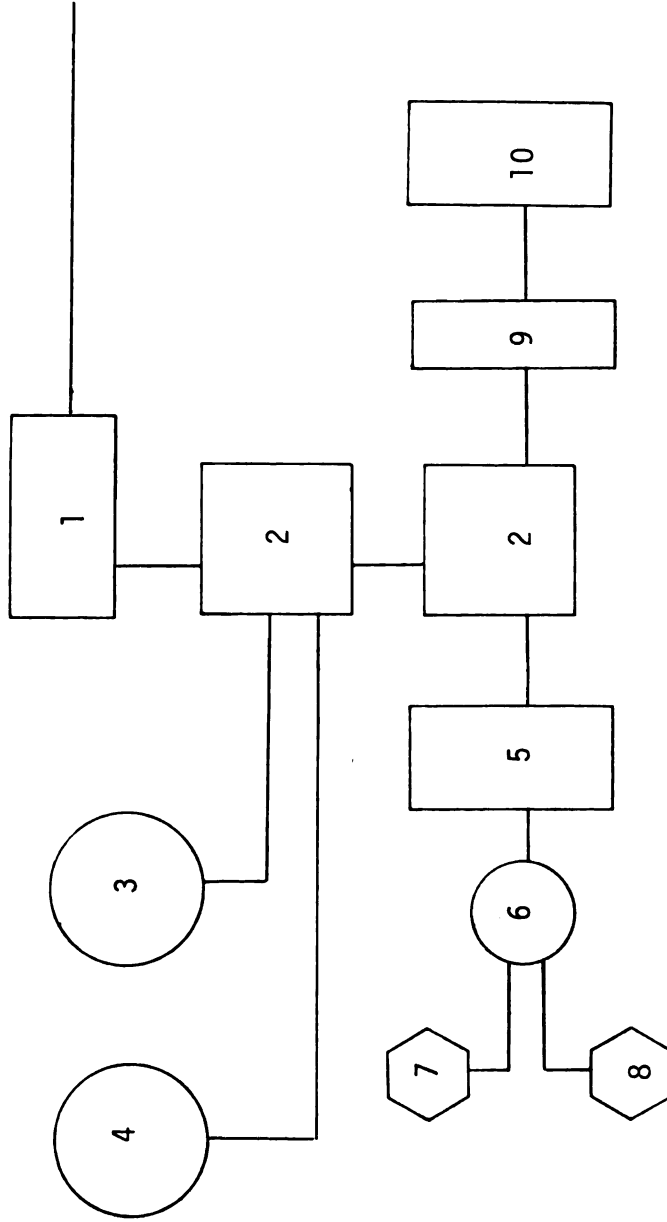


1. 1-1/2" x 1/4" iron angle
2. 6 sheets exterior 3/8" plywood
3. 3 3' x 8' sections corrugated fiberglass
4. Flexible 1/4" ID air hose
5. Air hose end fittings and elbows
6. 1/2" rigid conduit
7. #12 awg wire
8. 1/4" flexible plastic water tubing
9. Plastic hose fitting - tube adapters (United States Plastic Corp.,
1390 Neubrecht Road, Lima, Ohio 45801)
10. 3 water boxes constructed of 1/8" pvc sheet plastic
11. 3 float valves
12. 3 duplex outlets, boxes, and covers
13. 6 single throw, single pole switches, boxes, and covers
14. 6 8 foot, 2 tube fluorescent fixtures
15. 9 surface mount incandescent fixtures
16. 2 shaded pole blowers
17. 6 24 hour single throw, double pole time switches
18. 3 30 minute single throw, double pole time switches
19. 3 time delay relays (Dayton brand, Grainger's stock # 6X154)
20. 3 interval time relays (Dayton brand, Grainger's stock #5X829)
21. 3 remote probe thermostats
22. 6 1/4" JH pneumatic atomizing nozzles (Spraying Systems Co.,
Wheaton, Illinois)
23. Various fasteners, wood stain, wire nuts, etc.

Figure 2. Materials List

build-up. Bottom heat is supplied by heating cables; however, any system which plugs into a duplex outlet (120v) could be used.

A diagram of the timer board is shown in Figure 3. The incandescent and fluorescent lights are each switched with a 24 hour timer. The fan may be connected to either time clock. Bottom heat is controlled by a remote-probe thermostat. Mist is controlled by a 30 minute cycle time switch and by two relays. Figure 4 presents the mist cycle sequence. The 30 minute time switch is a single throw, double pole switch. Two different relays are used - a time delay relay and an interval time relay. Each relay controls one of the two mist heads. A cycle begins with the 30 minute time switch in the normally closed position (in between trippers). The time delay relay is energized and the delay period begins. At the end of the delay the relay energizes a solenoid valve which allows a mist head to fire (mist head "A"). Mist head "A" continues to mist until a tripper on the 30 minute timer throws the switch from the normally closed position to the normally open pole. At this point mist head "A" stops misting and mist head "B" fires (which is controlled by the interval time relay) and mists for the amount of time set on the interval time relay. For example: if the time between trippers is 1 minute, and each tripper lasts 15 seconds (or a total cycle could last a maximum 1 minute, 15 seconds), one may get a 4 second mist period (each mist head mists for 2 seconds) by setting the time delay relay to 58 seconds and the interval time relay to 2 seconds. When the switch is at the normally closed position there will be a 58 second delay allowing a 2 second mist before the switch in the 30 minute timer throws to the normally open position for a 2 second mist by the other mist head. When the switch is thrown back to the normally closed position (after the 15 second tripper time) the cycle begins again with the 58 second delay.



1. Board switch
2. Junction box
3. Fluorescent 24 hour time switch
4. Incandescent 24 hour time switch
5. Mist (on/off) switch
6. 30 minute time switch
7. Time delay relay
8. Interval time relay
9. Remote probe thermostat
10. Duplex outlet

Figure 3. Timer Board Layout

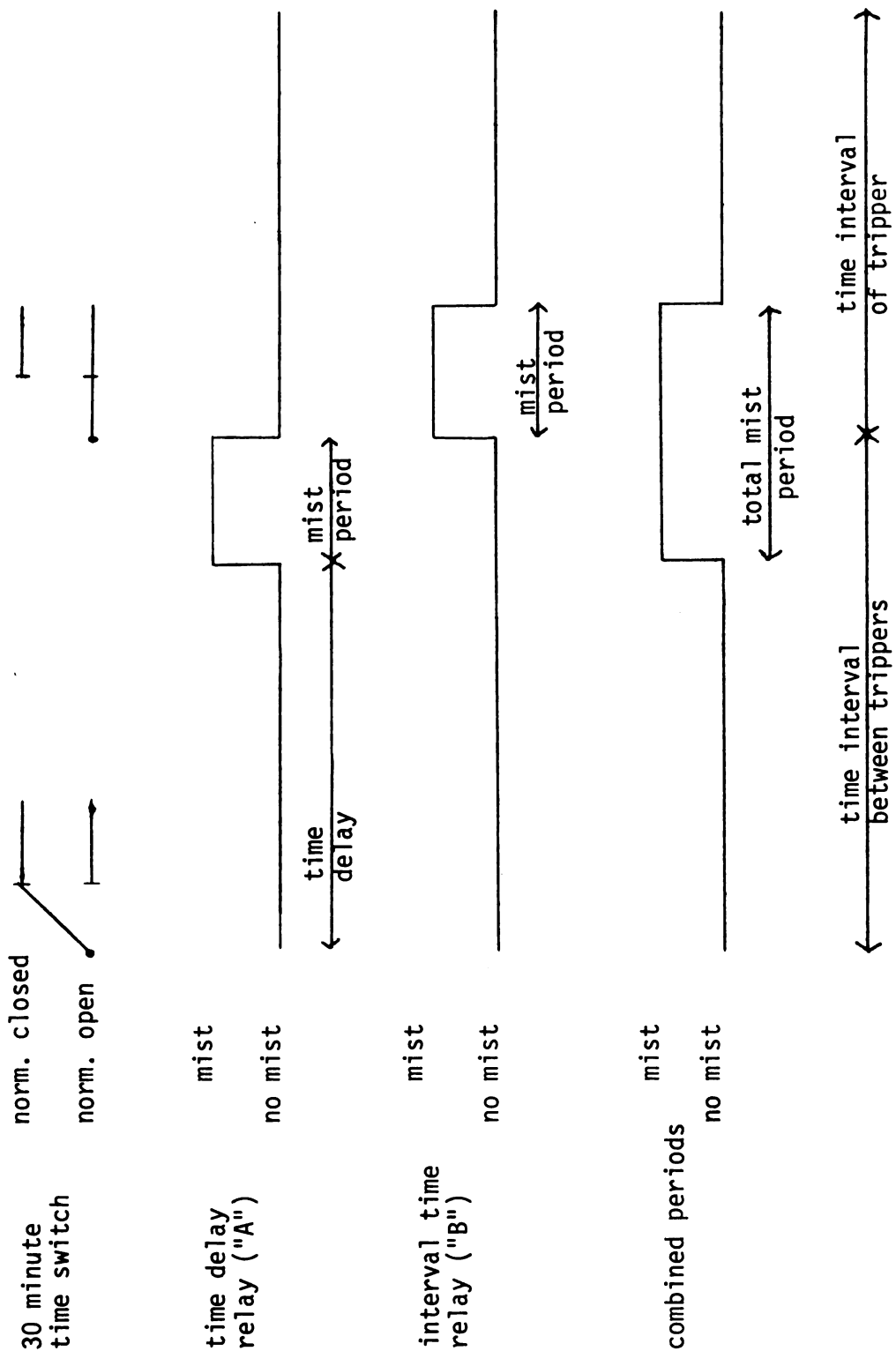


Figure 4. Misting sequence

DISCUSSION

A number of advantages are offered by the system and its location. The air conditioned room gives good control over the actual level and range of temperatures. This is a big improvement over a greenhouse. Total light energy received by the cuttings and photoperiod are also easily controlled. The timing, position, and type of mist head may be an improvement over standard mist apparatus. The mist period for each individual mist head may be separately adjusted allowing adjustment for differences in mist output by each mist head. The relays give good flexibility for choosing the proper mist period. One is not constrained by the 30 minute time clock tripper time. The mist heads at diagonal corners give a swirling action in the chamber, but dry corners are minimized because the mist heads fire in series. Because the mist is so fine, a high relative humidity is possible without heavy wetting of the plant material. This should reduce disease problems and nutrient leaching from the plant material. Drainage problems are also reduced so the use of a heavier rooting medium may be possible. All fixtures, timers, blowers, solenoids, and mist heads are outside of the chamber to aid maintenance. (Mist head nozzles project into the chamber through a rubber septum as does the cord to the heating cable and probe to the thermostat.) Any defective component can be quickly replaced.

By stacking the chambers, a large number of cuttings can be rooted in a small area. Use of bands with a 1-1/2" by 1-1/2" cell allows 1134 cuttings per chamber.

CHAPTER 2

Anatomical Investigation of Adventitious Root Initiation and Development in Picea pungens, Picea glauca, and Picea engelmannii

INTRODUCTION

Rooting stem cuttings as a method of vegetative propagation may become more important for the commercial production of forest trees. However, most important forest tree species, especially the conifers, are difficult to root. Understanding the anatomy of adventitious root initiation and formation may indicate the reasons for the difficulty in rooting. Hartmann and Kester (1975) divide the process of adventitious root formation in stem cuttings into three phases: 1) cellular dedifferentiation then redifferentiation to meristematic cells, 2) differentiation of meristematic cell groups into recognizable root primordia and 3) the growth and emergence of new roots. Anatomical investigations may show if problems in rooting are specifically related to one of these three general stages. For example, some investigators (as reviewed by Girouard, 1967) believe that some species are difficult to root because a sheath of sclereids and fibers prevents root emergence even though primordia may form. In fact, a test for amount and distribution of phloem fibers as a method for gauging juvenility and ease of rooting in apple rootstocks has been suggested (Nelson, 1978). However, inhibited root emergence has not been suggested as a reason for low rootability in forest tree species. It appears problems are greater in the processes involved with primordia initiation.

Adventitious roots may arise from preformed primordia or from induced primordia (Haissig, 1974a). There has been no report of adventitious roots arising from preformed primordia in conifer cuttings (Dalgas, 1973). Satoo (1956) investigated rooting in many conifer species and concluded that root primordia may

arise in the following five tissues: 1) Cambial and phloem portions of ray tissues, 2) leaf and branch traces, 3) bud meristem and traces, 4) irregularly arranged parenchymatous tissue, and 5) callus tissue. In general it appears that callus formation is more important for primordia formation in species that are difficult to root (Satoo, 1956).

Esau (1977) defines callus as parenchymatous tissue resulting from the proliferation of various cells near the surface of a wound. But, as emphasized by Montain, et al. (1983b), several tissues (other than root primordia) differentiate within the callus, such as vascular cambium, secondary xylem and phloem, periderm, resin canals with some parenchymatous cells remaining. Therefore, callus which develops at the base of a cutting is not a mass of parenchyma, but a complex of many tissues (Montain, et al., 1983a).

Difficulty in rooting is generally considered in the context of species comparisons. However, rooting difficulty also varies within a species--most notably in relation to phase change with juvenile cuttings being much easier to root than mature material. Similarly, differences are observed in the origin of adventitious roots in very young and older material. For example, Cameron and Thompson (1969) determined that root initiation in Pinus radiata (material from 5 year old trees) occurred mainly in callus tissue while Smith and Thorpe (1975a) found root initiation in hypocotyl cuttings of Pinus radiata (about 10 days after emergence) on the margins of differentiating resin ducts or in parenchyma tissues external to the ducts but always within the limits of the inner cortical tissue. A similar contrast is seen in Pinus banksiana where primordia in cuttings from approximately 3 month old material arise at the periphery of the callus complex produced at the base of the cutting (Montain, et al., 1983a) while primordia in 20 day old hypocotyl cuttings arise just outside axial resin canals (Montain, 1983b). Age differences are also mentioned by Delisle (1942) who observed the

cortex of young white pine cuttings becomes meristematic whereas that the cortex in cuttings from older trees remains unchanged or degenerates.

The conditions under which adventitious roots arise in stems may affect the location of primordia initiation. Picea and Abies are difficult to root with callusing considered essential to the rooting process in cuttings, but in layered branches adventitious roots arose from dormant axillary buds, not callus (Bannan, 1942).

The source of callus tissue in conifers has been identified to be the vascular cambium, phloem and xylem parenchyma, the cortex, and pith (Heaman and Owens, 1971; Bhella and Roberts, 1975b; Cameron and Thompson, 1969; Reines and McAlpine, 1959). Callus development is heaviest at the cutting base and decreases acropetally. Within callus, primordia may form. Primordia often arise in callus in conjunction with differentiating vascular tissue (Girouard, 1967; Haissig, 1974a). In Douglas fir, hybrid larch, and slash pine the primordia formed in callus are associated with tracheid nests which are composed of vascular tissue roughly spirally oriented (Bhella and Roberts, 1975b; Heaman and Owens, 1971; John, 1978; Reines and McAlpine, 1959). In Pinus radiata root primordia developed in association with tracheids produced in callus arranged more or less parallel to the main vascular system (Cameron and Thompson, 1969).

However, the need for callus production in difficult to root species has not always been emphasized by investigators. It was reported by Dalgas (1973) that primordia in Norway spruce cuttings arose in proximity to vascular elements and therefore near the cambium and sometimes in leaf traces. In white pine, root primordia apparently arose from meristematic cells produced by the cambium. These initiation sites were often associated with rays or leaf traces (Delisle, 1942). This is in some contrast to the above evidence which emphasized the need for callus production.

Because the changes that occur in the cutting base are very complex, the descriptions offered by various authors are sometimes difficult to interpret. The overall impression from the literature is that at the base of conifer cuttings a callus complex develops. The origin of the callus has been determined to be practically every tissue originally present in the cutting base (i.e., pith, xylem parenchyma, cambium, phloem parenchyma, and cortex). Within callus a number of tissues may differentiate. Those most emphasized by investigators are tracheids and meristematic zones. Previous investigators often pinpoint the origin of root primordia to be in relation to differentiating vascular tissue or to meristematic zones. However, the differentiating vascular tissues often appear to be derived from these callus meristematic zones. Therefore, by suggesting one tissue as the point of primordia initiation (for instance, differentiating tracheids), one should consider the other as being important (the meristematic zones).

Because blue, white, and Engelmann spruce cutting material was available from an initial trial of a newly constructed mist chamber, this study was undertaken to evaluate the rooting anatomy of blue, white, and Engelmann spruce. Cuttings were examined to attain a general view of the changes that occur in the cutting base and to pinpoint a specific location or tissue at which primordia commonly arise. An effort was also made to identify the earliest stages of adventitious root primordia formation. This study attempts to lay groundwork for further experimentation where treatments may be better evaluated with respect to all stages of adventitious root formation and development.

MATERIALS AND METHODS

Plant material and rooting conditions

Blue, white, and Engelmann spruce hardwood cuttings were taken 1/24/82. Cuttings which were kept in a cooler in the field were placed in the mist chambers

the day of removal from the ortet. Before sticking, the needles were stripped from the cutting base and the bottom retrimmed. Cuttings were four to seven centimeters long. All cuttings were dipped in a powder mixture of Hormodin 3 : Captan (1:1) as suggested by Hartmann and Kester (1977). Blue spruce hardwood cuttings were from one year old greenhouse grown seedlings. Engelmann and white spruce hardwood cuttings were from three year old potted trees. Cuttings were placed in mist chambers equipped with two humidifying 1/4 JH pneumatic atomizing nozzles (Spraying Systems Co., Wheaton, Illinois). Rooting medium was vermiculite:coarse perlite (1:1). Bottom heat was supplied with heating cables thermostatically controlled at 24° C. A sixteen hour photoperiod was supplied by four eight foot fluorescent tubes (warm white 1500 mv) and three incandescent bulbs (75 w.) giving an average $70 \mu\text{E m}^{-2} \text{sec}^{-1}$ at cutting level. Temperature was 22° C with lights on and 20° C with lights off. Cuttings were removed after 9 weeks and the bottom centimeter of selected cuttings was removed for sectioning. A total of 62 blue, 41 white and 48 Engelmann spruce samples taken.

Sample processing and examination

Sample preparation followed methods of Berlyn and Miksche (1976). Samples were fixed in a formalin-acetic acid-alcohol solution under vacuum for three days. Samples were dehydrated in a tertiary butyl alcohol series and embedded in paraffin. Twelve micrometer serial sections were made of each sample on an AO Spencer 820 rotary microtome. Sections were stained in safranin and fast green or hemalum and safranin. Slides were viewed on a Leitz microscope.

RESULTS

The rooting percentage of the three species was blue-24%, white-4%, and Engelmann-12%. The anatomical examination showed that blue, white, and Engelmann spruce of the ages examined are extremely similar with respect to the anatomy of adventitious root development. Therefore, the three species will be treated as a group in the following discussion and differences will be pointed out only where they are meaningful. In many cuttings various stages of callus and adventitious root development existed and there were large differences between individual cuttings in development.

Normal anatomy

Normal anatomy of blue, white, and Engelmann spruce stems is shown in Figures 5-7. All species are similar anatomically. The white and Engelmann spruce contain large piths. The secondary xylem produced while in the rooting chamber contain larger lumens than the xylem produced the prior year in the pots. Blue spruce produced tracheids of about the same lumen size in the rooting chamber as in the greenhouse. The secondary phloem is very regular in alignment and distribution about the central xylem cylinder. The cortex is composed of large, irregularly shaped parenchyma. The distribution of cell sizes is large (as compared to callus parenchyma). Many cells are uniformly filled with dark staining substances. Two layers of cells surround a resin duct, the inner layer being less densely staining and the outer layer composed of very dense staining cells. The periderm is composed of a layer or two of phelloderm, a phellogen, and a few layers of phellum. Exterior to the periderm is a cushion layer which is covered with an epidermis.

Rooting zone transformations

Figures 8-12 show various features of the anatomy typical of the rooting zone. Moving basipitally through the rooting zone one of the most noticeable changes occurs in the appearance of the secondary xylem and phloem. The secondary xylem produced in the rooting zone is irregularly arranged. Tracheids possess larger lumens with greater variability in lumen size among the tracheids produced. The tracheids are shorter and blockier. Phloem is produced in scattered columns. The magnitude of this transformation increases down the rooting zone. At the cutting base the xylem produced is highly irregular with tracheids elongating in various directions and differentiation occurring discontinuously in the cellular mass. Little, if any, phloem is produced. It should also be noticed that those tracheids produced first are the most irregular and those produced later are more normal. The earliest tracheids are shorter and wider while those produced later are more elongated with narrow lumens. Lignification is often incomplete and radial alignment is severely disrupted in the early tracheids.

It is difficult to demarcate the parenchyma of the original cortex and parenchyma of the callus mass that exists outside the tracheid region. In general, however, callus parenchyma are smaller and more uniform in size than cortical parenchyma. Callus parenchyma are lighter staining and often exhibit a prominent nuclei. Callus parenchyma appear to mature and differentiate into (among other cell types) cortical type parenchyma. A well-developed periderm covers the basal cellular mass. In the example the callus mass has covered the cut base of the cutting. This is not always observed. Some cuttings show no covering of the basal cut surface and others are intermediate in basal callus production.

In some cuttings (usually blue or Engelmann spruce), columns of tracheids form within the cortical or callus parenchyma (Figure 12). These appear to arise spontaneously. No consistent association with any other tissue could be

observed. These columns are longitudinally oriented, sometimes connecting with the central xylem cylinder at needle (leaf) gaps and sometimes being completely isolated in the cortex or callus parenchyma produced from resin ducts or phellogen/phelloderm. The tracheids produced are very similar in appearance to those produced by needle traces in the rooting zone (see below).

Needle traces

Figures 13-19 show needle traces and the formations associated with them. Needle traces are associated with root formations in two ways. One manner is where roots arise directly from cells which proliferate from some portion of the trace in the cortex, and the second is from the leaf gap area where the needle trace enters the central xylem cylinder. The greater proportion of needle trace primordium arise directly from some location along the trace.

The strong radial orientation of xylem and phloem within the trace bundle indicates a cambial like layer (trace cambium) being the source of the initial tracheid and sieve cell formation. That is, the vast majority of the tracheids in the trace appear not to differentiate from procambium derived from the apex, but were produced by a cambial layer. In addition to the cambial layer situated between the trace xylem and phloem, there are meristematic cells on the lateral portions of the trace forming a lateral trace cambium. Though no secondary growth should occur in a trace (Esau, 1977), it is observed that many traces in the rooting zone do undergo secondary growth. It appears the trace and lateral cambiums become activated and begin producing cells, some of which go on to differentiate into tracheids. These secondary trace tracheids may completely encircle the trace. Lumen size delimits these secondary trace tracheids from those comprising the original trace. The secondary tracheids are very short and wide, similar to those produced by the vascular cambium in the rooting zone.

Not all of the cells produced by the trace and lateral cambiums differentiate into tracheids but maintain a meristematic appearance and go on to divide further, both horizontally and longitudinally, forming proliferations off the needle trace.

The second way traces are associated with primordia is at the leaf or needle gap. At this area cells proliferate and protrude out from the gap into the cortex (Figure 28). Some roots, especially in blue spruce, are observed to connect with the central xylem cylinder at a needle (leaf) gap and presumably originated from a needle (leaf) gap proliferation.

Cortical Resin ducts

Figures 20 and 21 show resin duct formations. The epithelial cells of the cortical resin ducts produce callus parenchyma in two ways. One manner is through a proliferation off the side of the resin duct. The second is at the bottom of the resin duct where the duct fills in with cells.

A consistent observation about the cortical resin ducts in the rooting zone is that the epithelial cells take on a "rejuvenated" appearance as compared to ducts above the rooting zone. The outer of the two layers of epithelial surrounding resin ducts above the rooting zone are darkly staining with the inner layer appearing more active. In the cutting base the outer ring of cells lose their densely staining contents with cells of both layers acquiring more prominent nuclei. Callus parenchyma that forms off the side of the resin ducts appears to arise directly from the epithelial cells, or a sphere of callus parenchyma may form at the base of the cortical resin duct.

Cortical resin duct formations were observed only in blue and Engelmann spruce and not in white spruce.

Phelloderm/Phellogen

Phelloderm/phellogen formations are shown in Figures 22-24. It is not certain if the phellogen or phelloderm is responsible for the cell proliferations observed, but the production may be very extensive in some portions of the cutting bases. Large amounts of phellum are not observed in any of the cuttings. Tracheid columns may form within these phelloderm/phellogen masses, sometimes becoming very wide and giving rise to cambial type regions. These columns do not connect with leaf gaps but begin and end within the cell mass itself. Primordia were never observed originating from these tracheid columns.

All three species showed extensive phelloderm/phellogen production. Differentiation in these cells was observed in only the blue and Engelmann spruce and not in the white spruce.

Cortex

Cortical formations are shown in Figures 25-27. Often no clear demarcation between callus parenchyma and the cortex could be observed. The cortical cells would be indistinguishable from callus parenchyma derived from the cambium with no noticeable crushing delineating the interface. If tracheids differentiated in the callus from the vascular cambium, crushing between the cortex and callus would occur. Wound tracheids and tracheid columns differentiate in cortical parenchyma. Rarely would spheres of small meristematic type parenchyma be observed.

Vascular cambium - callus cambium

The vascular cambium appears to be very important in the dynamics that take place in the rooting zone. As discussed above, the cambium produces unusual tracheids in the rooting zone with anomalous xylem increasing basipetally and

centripetally. It appears the processes of division and tracheid differentiation interact to give this increasing variability and the observed increase in cell number at the base. The vascular cambium cuts off cells toward the pith, which in the upper rooting zone differentiate into tracheids immediately, thereby preserving radial alignment (though radial enlargement of the individual tracheids increases before lignification occurs as compared to normal tracheids) while in the lower rooting zone further divisions of the tracheid mother cells occur before differentiation and lignification (if lignification occurs), thus disrupting the radial alignment of the secondary xylem. Division before differentiation is more frequent lower in the rooting zone. This would give rise to the large tracheid mass produced at the cutting base shown in Figure 8. The continued divisions of cambial derivatives would, perhaps, displace the cambial layer and produce the callus mass that encloses some cutting bases. A cambial layer appears to be responsible for the tracheids that are produced at and over the cutting base. This cambial layer (callus cambium) is continuous with the normal vascular cambium.

As stated above a trace cambium is also activated in some traces in the rooting zone. However, not all traces in the rooting zone show secondary growth.

The trace, vascular, and callus cambia appear to be responsible for the overwhelming majority of primordia and roots. It is difficult to state whether primordia arise directly from the cambiums, or arise from cells that are "close" derivatives of the cambiums.

Primordia

The apparent sequence of primordia development is similar from all tissues and in all species. These stages are shown in Figures 29-32. The first identifiable stage is a group of small parenchymatous cells (as compared to surrounding

parenchyma). These cells continue to divide producing more and smaller cells thereby taking on the appearance of a meristem. A layer of larger cells containing ergastic materials develops on the perimeter of the meristematic ball, sometimes forming a complete cover (except at the point of contact with the trace or cambial area) or only toward the outer and lower portions of the ball and not the top. Polarity then begins to occur with formation of a slight elongation zone forming and a more defined initial area. Cells surrounding the primordium ball may become suberized. As elongation continues definable regions appear in the root/primordium. Suberization increases forming a root cap. Vascular differentiation occurs toward the xylem cylinder or trace. A definite xylem connection may or may not be observed in emerging roots.

DISCUSSION

In species without preformed initials, adventitious roots must develop from tissue that are newly formed or that have dedifferentiated then redifferentiated into initials. Literature reviewed in the introduction indicates that primordia develop in newly produced tissue. Much of this new cell production at the cutting base would be designated callus. But, to call the basal tissue callus is somewhat misleading. As referenced previously, Esua (1977) defines callus to be a parenchymatous tissue formed at cut surfaces, yet the "callus mass" at the cutting base is not a proliferation of parenchyma, but a complex of many tissues. Some of these tissues may be anatomically unusual, but the overall arrangement of these tissues, as shown in Figure 8, is exactly that of a normal stem (see also Figure 16 in Montain, et al., 1983b). That is, the basal callus consists of a centrally located xylem mass, a cambium, phloem (which is sometimes difficult to identify), cortical type tissue, and a periderm. In fact, it would be difficult to draw a line separating the callus tissue from the non-callus tissue of the stem. The appearance

of the secondary xylem (that xylem produced in the rooting chamber) changes gradually down the rooting zone, as does the amount of phloem produced and the appearance of cortical resin duct epithelial cells. The point to be made is that the extent of transformation of the rooting zone occurs as a gradient down the stem with the greatest changes occurring at the cutting base. Therefore, the process of callus formation of the cutting base is not just the accumulation of "callus tissue" at the cut surface, but more of a description of the gradient of change that is present in the rooting zone.

When cuttings are placed in the rooting chamber and released from imposed dormancy the vascular cambium initiates cell division along the entire length of the cutting. Also, cell division occurs at the cut surface. Though the site of initial cell production at the cutting base could not be identified (i.e., that tissue(s) which may be considered to have produced the first callus cells) it appears certain that a cambium which is continuous with the vascular cambium of the upper rooting zone is quickly established. This new cambium (or "callus cambium") appears to be responsible for the xylem proliferation that is produced at the cutting base which may cover, to varying degrees, the cut surface of the central xylem cylinder. In addition to the activity of the vascular and callus cambiums in the rooting zone, there is the activity of the trace cambiums of some needle traces. Though no prior description of a trace cambium could be found in the literature, the existence of a trace cambium is strongly indicated by the evidence shown in Figures 13 and 14. It appears the majority of trace xylem and phloem is of trace cambial and not procambial origin. While not every trace in the rooting zone shows cambial activation and secondary trace growth, it is only the rooting zone trace cambiums which became active; no secondary trace growth was observed in the upper portions of the cuttings. The vascular, callus, and trace cambia are responsible for much of the change that occurs in the

rooting zone, i.e., the irregular secondary (X2) xylem produced by the vascular cambium, the basal xylem proliferation produced by the callus cambium, and the secondary trace tissue produced by the trace cambium.

Adventitious root primordia and roots were consistently associated with one of the three cambiums. The strong radial alignment of meristematic cells seen in Figures 29 and 30 suggests a cambial origin. Regions of apparent meristematic potential did occur in other tissues of blue and Engelmann spruce, such as the cortex, periderm or phellogen, and cortical resin ducts. However, while it was not unusual to observe callus type parenchyma and differentiated wound tracheids produced by these tissues, only two roots and two or three meristematic balls could be confidently associated with one of these tissues.

The above discussion opens the question of whether or not "callusing" is necessary for adventitious root production. As reviewed in the introduction, many investigators believe callus is essential for adventitious root formation in the difficult-to-root species. The observations made in this study indicate this may not be true. Part of the problem is in deciding what actually is callus, or callusing. Previous investigations state that primordia arise within callus, as if callus is an identifiable region of tissue. As stated previously, it appears the process of callus formation in the species and ages examined in this study is more of a description of a gradient of transformation down the rooting zone, with some accumulation of tissue at the very base, some of which may cover the cut surface. It is evident that most adventitious roots originate at the base of the cutting where the transformation is greatest; but this is not obligatory. As shown in Figure 33, adventitious roots may form at regions in the rooting zone that do not show the large amounts of unusual tissue production that is usually present in cutting bases. The observations made in this study indicate that callusing and adventitious root formation may be parallel responses to the

same set of stimuli, and that these stimuli appear to center their effects in the cambiums present in the rooting zone. Cambiums appear to play the largest role in the rooting zone transformations called callusing, and in adventitious root production.

Haissig (1974a) suggests that the stem cambium may not constitute a favorable site for primordia initiation. He reasons that cambial cells show a high degree of differentiation and whatever factors maintain that degree of differentiation may preclude the type of dedifferentiation necessary to produce primordia initials. Primordia initials are usually formed from isodiametric cells, not fusiform shaped cells and factors that maintain the integrity of the cambial layer must be overcome. Haissig (1974a) also hypothesizes that cambial cells are probably subject to precise regulation of auxin supply by a system that insures normal development of secondary xylem and phloem.

However, all observations point to some alteration of the mechanism which regulates cambial activity and development of cambial derivatives, and that the magnitude of the change increases down through the rooting zone. Moving basipitally through the rooting zone one observes tracheids produced by the vascular and callus cambiums becoming shorter and wider, even blocky near the cutting base. Isodiametrically shaped cells are also produced in secondary trace growth. As shown in Figure 15, small vigorous cells may be produced through horizontal divisions of cells produced either by the trace cambial initials or by division of the cambial initials themselves. The regulatory mechanism of the cambium and its derivatives which apparently changes moving down the cutting also appears to change over the time course of cambial activity. As shown in Figures 8, 10, 17, and 34, the most irregular cambial derivatives are produced when the cambium is first activated. As the number of derivatives increase the more normal they become in arrangement and dimension. Even the

latest derivatives produced by the callus cambium are more elongated, slimmer, and radially aligned than those produced earliest. In general, normal cambial regulation is weakest at the cutting base and increases both acropetally and as the cambium ages.

As stated at the end of the introduction, cambiums and the differentiating derivatives should be considered as a group in relation to primordia initiation. The literature indicates that factors which effect cambial regulation and differentiation of derivatives also appear to effect adventitious root formation. Reviews by Girourd (1967) and Haissig (1974a) have emphasized the relationship between differentiating xylem and primordium initiation.

Auxin is important in secondary tissue production and differentiation (Shininger, 1979). Auxin is also believed to be crucial for primordia initiation (Haissig, 1970; Smith and Thorpe, 1975a). Sheldrake (1973) suggested autolysing cells (such as differentiating xylem) may control localized IAA production through increased availability of tryptophan. This would help explain the observed auxin gradient found across differentiating xylem, cambium, and differentiating phloem (Sheldrake, 1971). It might also be suggested that differentiating xylem may provide conditions more suitable for primordia initiation by increasing localized IAA production through increased tryptophan availability. However, evidence presented by Maloney and Elliot (1982) does not support the theory of auxin production control by substrate availability. These authors suggest enzymatic regulation. If this is the case, then the association of differentiating xylem and primordium may be differing responses to an identical stimulus.

More than auxin is needed for vascular differentiation and likewise it appears non-auxin factors (or cofactors) are also involved in primordia initiation and development. In addition to the auxin gradient, there exists a sugar gradient across xylem and phloem. Shininger (1979) reviewed evidence indicating the

importance of the auxin:sugar ratio in vascular development. Evidence has also been presented indicating that such ratios may be important in adventitious root formation (Eliasson, 1978; Hansen, et al., 1978; Nanda, et al., 1971; Vkierskov, 1976), but firm conclusions are difficult to draw (Haissig, 1982).

Phenolic substances may be involved in the rooting process by effecting IAA metabolism or action in such a way as to increase IAA effectiveness in producing root primordia (Haissig, 1974b; James and Thurbon, 1979). Phenylpropane units such as caffeic acid are also important in lignin biosynthesis. Savedge and Wareing (1981) have presented evidence of a tracheid differentiating factor in mature needles of lodgepole pine. In their experiments IAA and an intact needle pair in light was needed for vascular cambium cell division and tracheid differentiation. (Applied IAA and sucrose had no effect.) Similarly Hyun (1967) presented evidence that needles of juvenile pitch pine contained a growth/rooting promoter which consisted of IAA and polyphenols including chlorogenic and caffeic acid. It may be that Savedge and Wareing's tracheid differentiating factor is composed of poly phenolic substance(s) and the interaction of auxin with these substances may be involved in tracheid differentiation or primordia formation. A tracheid/primordia differentiating factor in needles may explain in part the primordia arising from needle traces as seen in this study and mentioned by other investigators (Dalgas, 1973; Delsie, 1942). Cameron and Thompson (1969) also make the observation that callus xylem formed most profusely where the cut surface of the cortex was 1-2 mm from a trace leading to a fascicle shoot.

While cambiums and their differentiating derivatives appeared to be the major site of primordia initiation, meristematic cell groups and sometimes roots were observed originating from other tissue. The most notable of these are cortical resin ducts. Cortical resin duct epithelial cells appeared to become

rejuvenated in the rooting zone. The outer of the two cell layers which usually contained ergastic substances, losses these substances and cells of both layers often acquire more prominent nuclei. Cell proliferations occurred off the sides of the ducts or at the bases of the ducts which filled in with parenchyma. Various cell types were observed in these proliferations were observed, such as small meristematic cells, callus parenchyma, and wound tracheids. This evidence indicates that cortical resin duct epithelial cells have the potential to renew cell division and perhaps the potential to initiate primordia. Cortical resin ducts have been observed to be major sites of root initiation in pine hypocotyl cuttings (Montain, 1983a; Smith and Thorpe, 1975a), but this is not reported for older material.

Often the cell proliferations observed on the cortical resin ducts (and in other tissues) exhibit a zone of densely staining cells at the interface with cortical parenchyma (see Figures 8, 23, 26, and 27). However, it is not certain that these proliferations would continue to develop into primordia. It is difficult to determine if all meristematic cell groups which exhibit the zone of densely staining cells should be considered to be early primordia. It may be that the accumulation of the dark staining cells may be only a response to the expanding cellular ball and should not be considered as a landmark to identify primordia. Once polarity appears the cell groups may be considered to be primordia. However, polarity is only an overt anatomical indication. The question may be posed as to when is a primordia determined in species without preformed root initials? Smith and Thorpe (1975) were able to identify in Pinus radiata hypocotyl cuttings a single cell as a meristematic locus from and around which a primordia develops, but a single cell has not been identified in other species or older material. As mentioned previously, auxin appears to be most crucial for primordia formation (as opposed to development) (Haissig, 1970; Smith and Thorpe, 1975b). Haissig's

results (from studying preformed initials in brittle willow) indicated that "competent" cells were needed before auxin was effective, that is auxin was not involved with the creation of the initials but was needed for the development of the initials into primordia. Haissig found that without auxin the initials differentiated into parenchyma. The cell proliferations observed in this study may be the "competent" cells which did not receive the needed stimulus to differentiate into primordia. It appears that conditions which are suitable for producing groups of small meristematic cells may be different from conditions needed for the development of the groups into primordia. Likewise, it has been shown that substances which stimulate adventitious root formation at one point may have no effect or are inhibitory at other stages (James, 1983; Smith and Thorpe, 1975b, 1977).

The above discussion attempts to show relationships between the anatomical observations made in this study and by others with physiological/biochemical observations made by some investigators. Most rooting studies would benefit from a concomitant anatomical investigation. In this way treatment differences may be better understood. For instance, it is well known that cutting rootability declines drastically in cuttings taken during true dormancy. Bhella and Roberts (1975) showed this decrease was not due to an inactive cambium. Haissig (1970) also used anatomical information in conjunction with his treatments to pinpoint probable sources of endogenous auxin and stage of primordia development at which auxin is most important. It may be of interest to determine if treatments effective in promoting rooting in species without preformed primordia enhance processes involved with formation of meristematic cell groups or the differentiation of these groups into primordia. Treatment effects may also be noticed in cambial response.

From this study only general conclusions can be drawn. These conclusions are:

1. Blue, white, and Engelmann spruce (of the ages examined) are very similar with respect to the anatomy of rooting zone transformations, and adventitious root formation and development.
2. A gradient of transformation occurs in the rooting zone with the greatest change occurring at the cutting base and in the earliest produced cambial derivatives.
3. Cambiums in the rooting zone (vascular, callus, and trace) appear to play the central role in producing the rooting zone transformations.
4. Adventitious root primordia and roots are consistently associated with a cambium in the rooting zone.

There are two major flaws of this study. One is that samples were not taken over time. Though great variation between and within cuttings with respect to anatomical changes in the rooting zone were observed, certain questions may have been more fully answered, such as: 1) From where and in what manner are the first cells in the cutting base produced?, 2) How does the callus cambium form?, 3) Are there distinguishable "landmarks" as to where and what cells will initiate primordium? The second flaw is that no cuttings were given a no auxin treatment. The changes which occur may be attributed to a number of factors such as the auxin application, wounding or etiolation. The last two are common to all cuttings. It would be valuable to detect any effect due to auxin application.

chamber.
phloem, X1 = xylem produced previous year, X2 = xylem produced in cooling
needle (leaf) gap, NT = needle trace, P = pith, PR = periderm, SP = secondary
cortex, CL = cushion layer, CRD = cortical resin duct, EP = epidermis, NG =

Figure 7.

entering the central xylem cylinder in Figure 6 and the trace in the cortex in
layer and a lighter staining inner epidermal layer. Note also the needle trace.
The cortical resin ducts are very asymmetrical with a dark staining outer epidermal
often filled with erastic substances and usually no noticeable nuclei is present.
greenhouse and propagation chamber, etc. (Figure 6). Cortical parenchyma are
seedlings in pots. The tracheids produced by the blue spruce (Figure 6) in the
and white (Figure 7) spruce contain larger lumens than that produced by the
secondary xylem produced in the propagation chamber by the Engelmann (Figure 7)
secondary xylem and phloem with little variation in tracheid lumen size. The
Note in Figures 6, 7 and 7 (following page) the strong radial alignment of

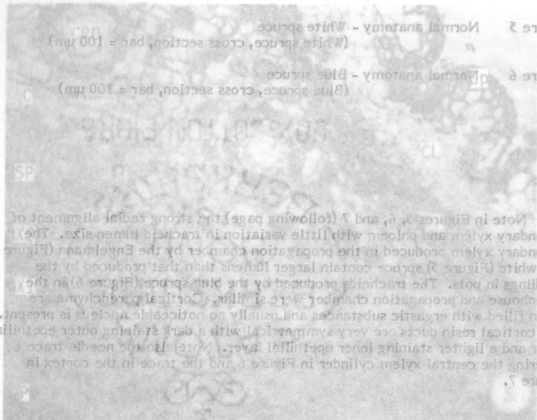


Figure 6
Normal anatomy - Blue spruce
(Blue spruce, cross section, bar = 100 μ m)

Figure 7
Normal anatomy - White spruce
(White spruce, cross section, bar = 100 μ m)

Figure 5 Normal anatomy - White spruce
(White spruce, cross section, bar = 100 μm)

Figure 6 Normal anatomy - Blue spruce
(Blue spruce, cross section, bar = 100 μm)

Note in Figures 5, 6, and 7 (following page) the strong radial alignment of secondary xylem and phloem with little variation in tracheid lumen size. The secondary xylem produced in the propagation chamber by the Engelmann (Figure 7) and white (Figure 5) spruce contain larger lumens than that produced by the seedlings in pots. The tracheids produced by the blue spruce (Figure 6) in the greenhouse and propagation chamber were similar. Cortical parenchyma are often filled with ergastic substances and usually no noticeable nucleus is present. The cortical resin ducts are very symmetrical with a dark staining outer epithelial layer and a lighter staining inner epithelial layer. Note also the needle trace entering the central xylem cylinder in Figure 6 and the trace in the cortex in Figure 7.

C = cortex, CL = cushion layer, CRD = cortical resin duct, EP = epidermis, NG = needle (leaf) gap, NT = needle trace, P = pith, PR = periderm, SP = secondary phloem, X1 = xylem produced previous year, X2 = xylem produced in rooting chamber.

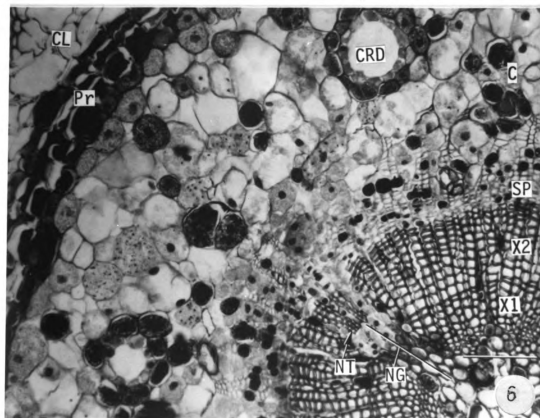
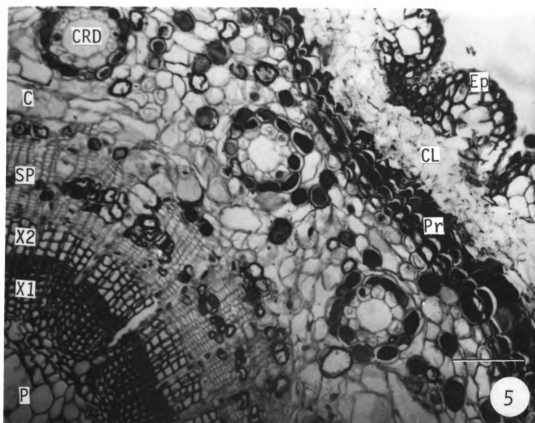


Figure 7 Normal anatomy - Engelmann spruce
(Engelmann spruce, cross section, bar = 100 μm)
(see comments on page 29)

Figure 8 Cutting base (Engelmann spruce, radial long. section, bar = 100 μm)

Note the differences between X1 xylem and X2 xylem. Tracheids in X2 are shorter and wider, becoming blocky at the base. (See also Figures 15, 33, and 34.) Those tracheids produced earliest are most abnormal. Moving out from the center, the tracheids become more normal, i.e., slimmer and longer. A large xylem proliferation exists at the cutting base in what may be considered the callus mass. The alignment of the tracheid production in the callus indicates a cambium, though the exact callus cambial layer is difficult to identify, as is the origin of the callus cambium. No clear demarcation exists between parenchyma of the cortex and parenchyma of the callus mass. Note the proliferation at the cortical resin duct base with small parenchyma containing noticeable nuclei in the proliferation interior and the more cortical like parenchyma toward the exterior of the proliferation. Wound tracheids have differentiated in the center of the proliferation. The callus mass covers the base of the cuttings with a well developed periderm covering the callus. (Compare to cutting base in Figure 34.)

C = cortex, CL = cushion layer, CRD = cortical resin duct, NT = needle (leaf) trace, P = pith, PR = periderm, X1 = xylem produced previous year, X2 = xylem produced in rooting chamber.

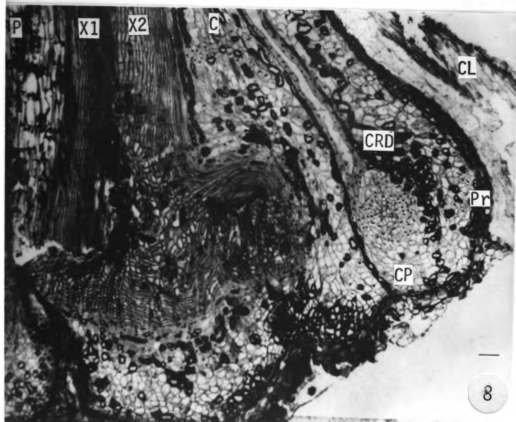
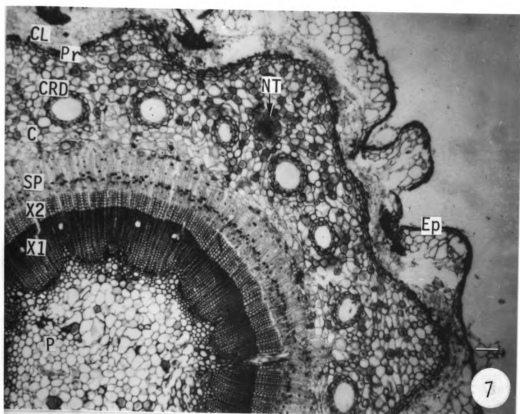


Figure 9 Normal anatomy (Engelmann spruce, cross section, bar = 100 μm)

Figure 10 Mid-section of rooting zone (Engelmann spruce, cross section, bar = 100 μm)

Figures 9, 10, and 11 (following page) are cross sections at various levels in the same cutting. Note the changes that occur in the X2 xylem and secondary phloem from the section in the upper, normal area of the cutting (Figure 9), to the mid-section of the rooting zone (Figure 10) to the base of the rooting zone (Figure 11). Secondary xylem becomes more irregular. Lignification may not occur or occurs after further division of the cambial derivatives and/or after unusual radial enlargement of the lumen. Note the great variation in lumen size and disruption of radial alignment. The greatest irregularity occurs in the earliest produced tracheids while those produced last seem less abnormal (see also Figure 9). Secondary phloem is sparser in Figure 10 and almost non-existent in Figure 11.

C = cortex, CRD = cortical resin duct, SP = secondary phloem, X1 = xylem produced previous year, X2 = xylem produced in rooting chamber.

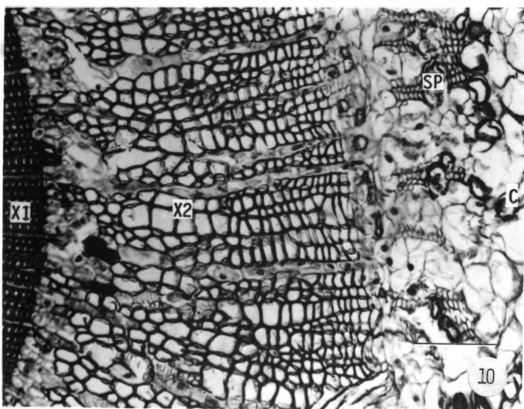
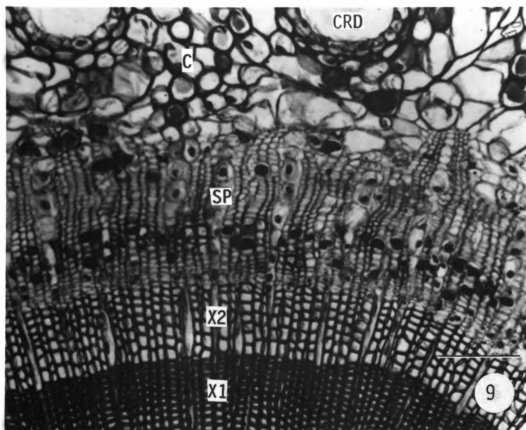


Figure 11 Lower rooting zone section (Engelmann spruce, cross section,
bar = 100 μm)
(see comments on page 33)

Figure 12 Trachied column (Blue spruce, cross section, bar = 50 μm)

Note the parenchyma in the center of the column surrounded by radially aligned rows of tracheids. Not all cells in the rows have differentiated into tracheids, but have remained parenchymatous with noticeable nuclei. (Compare the secondary trace tracheids in Figure 14.)

C = cortex, SP = secondary phloem, X1 = xylem produced previous year, X2 = xylem produced in rooting chamber.

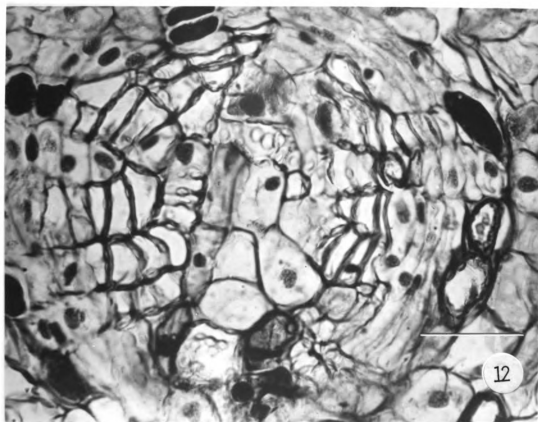
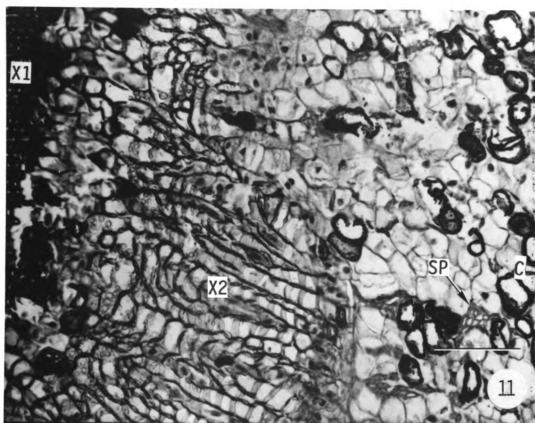


Figure 13 Needle trace, normal anatomy (Engelmann spruce, cross section, bar = 50 μm)

Note the thick walled tracheids with small lumens that make up the initial trace xylem. (Compare X1 in Figure 7.) The tracheids and sieve cells are radially aligned with cells of an apparent cambial layer (trace cambium). Note the small size and large nuclei of the cambial initials. (Compare with surrounding cortical parenchyma.) These trace cambial initials are also found on the lateral portions of the trace xylem forming a lateral trace cambium.

Figure 14 Needle trace, rooting zone (Engelmann spruce, cross section, bar = 100 μm)

This section is from the same cutting from which section in Figure 13 was taken. Note the lumen size difference between tracheids of the initial trace xylem and those of the secondary trace xylem. Not all derivatives of the trace cambium have lignified but some have retained a vigorous appearance. Compare size and appearance of these parenchyma with surrounding cortical parenchyma. Compare the secondary trace tracheids and parenchyma to cells of the tracheid column in Figure 12.

C = cortex, ITP = initial trace phloem, ITX = initial trace xylem, LTC = lateral trace cambium, STP = secondary trace phloem, STX = secondary trace xylem, TC = trace cambium.

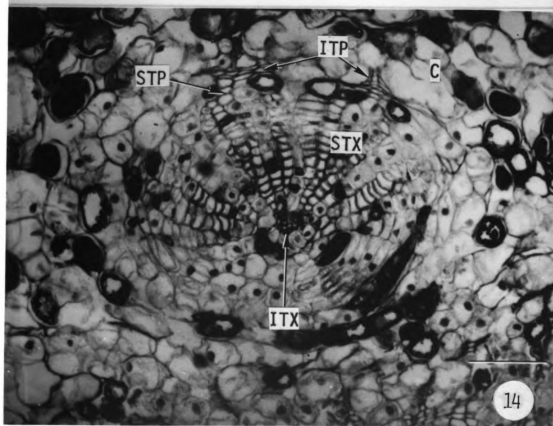
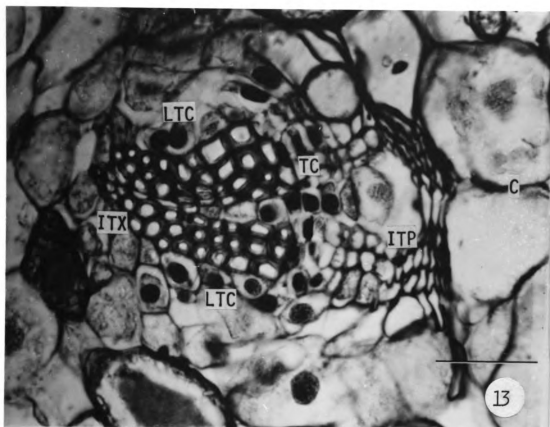


Figure 15 Needle trace, rooting zone (Engelmann spruce, long. section,
bar = 100 μm)

Note the short wide secondary trace tracheids. (Compare to X2 in Figure 8.)
Small, vigorous, isodiametrically shaped parenchyma with large nuclei, are produced
through horizontal and longitudinal divisions (unmarked arrow).

C = cortex, ITX = initial trace xylem, STX = secondary trace xylem, X2 = xylem
produced in rooting chamber.

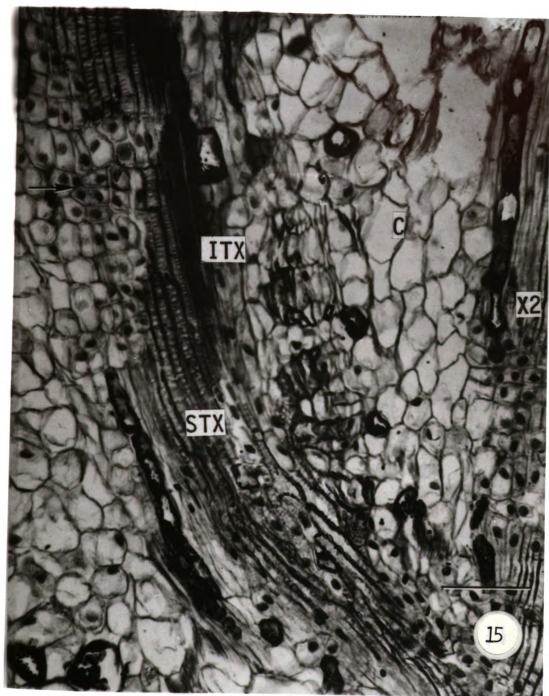


Figure 16 Needle trace primordium (Engelmann spruce, cross section,
bar = 100 μm)

Note the thick walled, small lumened, initial trace tracheids and secondary trace tracheids with much larger lumens. A more distinct connection between the primordium and trace can be seen in sections above the one shown in Figure 16 (these sections not shown).

Figure 17 Needle trace meristematic spheres (Engelmann spruce, cross section,
bar = 100 μm)

Two balls of meristematic cells associated with a trace entering the central xylem cylinder are shown. The lower (in the figure) meristematic sphere has wound tracheids differentiating adjacent to the trace xylem. Note also the tracheids of the secondary xylem (X2) with their large irregular lumens. See also Figures 25, 26, and 27 for needle trace formations.

C = cortex, CRD = cortical resin duct, ITX = initial trace xylem, NT = needle (leaf) trace, STX = secondary trace xylem, X2 = xylem produced in rooting chamber.

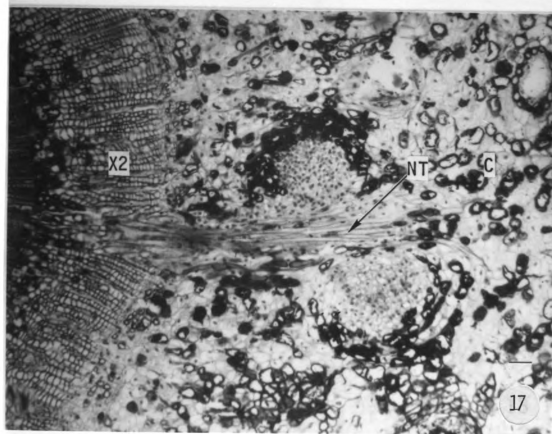
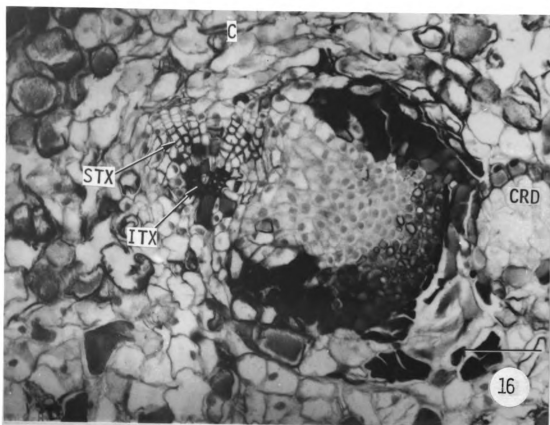
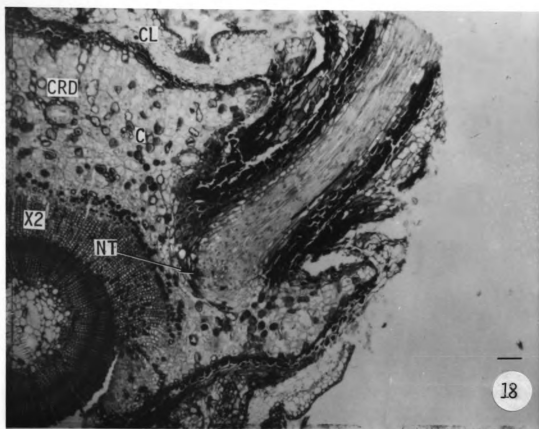


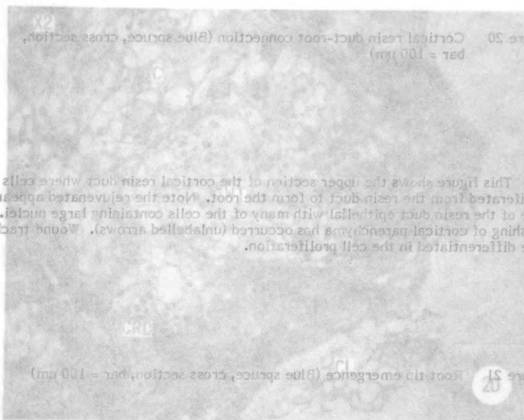
Figure 18 Needle trace mature root (White spruce, cross section,
bar = 100 μm)

Figure 19 Root-trace connection (White spruce, cross section, bar = 100 μm)

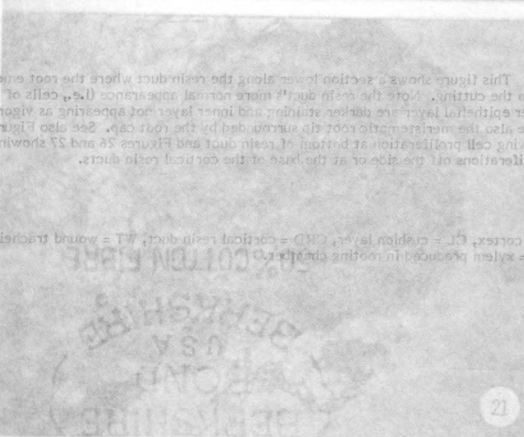
Note no connection between root and central xylem cylinder. Secondary xylem (X2) adjacent to the root trace connection is fairly normal.

C = cortex, CL = cushion layer, CRD = cortical resin duct, NT = needle (leaf) trace, X2 = xylem produced in rooting chamber.





This figure shows the upper section of the cortical resin duct where cells proliferated from the resin duct to form the root. Note the rejuvenated appearance of the resin duct epithelial with many of the cells containing large nuclei. Crushing of cortical parenchyma was occurred (unlabelled arrows). Wound tracheids have differentiated in the cell proliferation.



This figure shows a section lower along the resin duct where the root emerges from the cutting. Note the resin duct's more normal appearance (i.e., cells of outer epithelial layer are darker staining and inner layer not appearing as vigorous). Note also the intermediate root tip surrounded by the root cap. See also Figure 8 showing cell proliferation at bottom of resin duct and Figures 26 and 27 showing proliferations on the side of the cortical resin ducts.

C = cortex, CL = clasp layer, CRD = cortical resin duct, WT = wound tracheids, X2 = xylem produced in rooting stage.

Figure 20 Cortical resin duct-root connection (Blue spruce, cross section, bar = 100 μm)

This figure shows the upper section of the cortical resin duct where cells proliferated from the resin duct to form the root. Note the rejuvenated appearance of the resin duct epithelial with many of the cells containing large nuclei. Crushing of cortical parenchyma has occurred (unlabelled arrows). Wound tracheids have differentiated in the cell proliferation.

Figure 21 Root tip emergence (Blue spruce, cross section, bar = 100 μm)

This figure shows a section lower along the resin duct where the root emerges from the cutting. Note the resin duct's more normal appearance (i.e., cells of outer epithelial layer are darker staining and inner layer not appearing as vigorous). Note also the meristematic root tip surrounded by the root cap. See also Figure 8 showing cell proliferation at bottom of resin duct and Figures 26 and 27 showing proliferations off the side or at the base of the cortical resin ducts.

C = cortex, CL = cushion layer, CRD = cortical resin duct, WT = wound tracheids, X2 = xylem produced in rooting chamber.

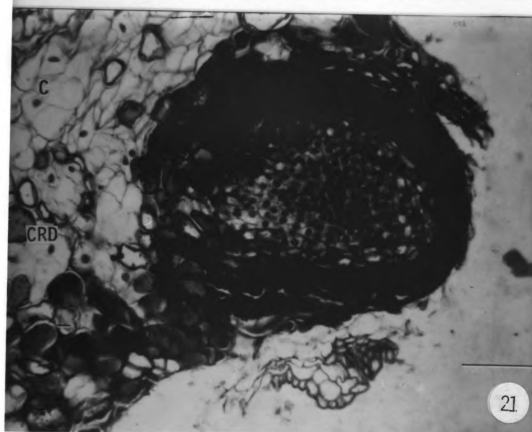
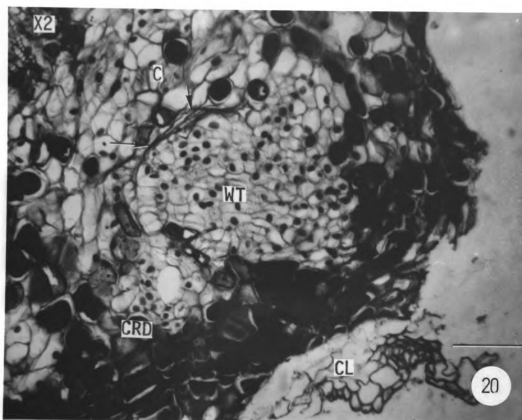


Figure 22 Phelloderm/phellogen proliferation (Blue spruce, cross section, bar = 100 μm)

This figure shows a proliferation of vigorous parenchyma apparently originating from phelloderm or phellogen as indicated by radial alignment of cells and enlargement of the proliferation by anticlinal divisions (unlabelled arrows).

Figure 23 Meristematic sphere in phelloderm/phellogen (Blue spruce, cross section, bar = 100 μm)

This figure shows a sphere of small meristematic type cells. The sphere is covered by densely staining cells (similar to those of an early root cap) on all sides except for the top (these sections not shown).

C = cortex

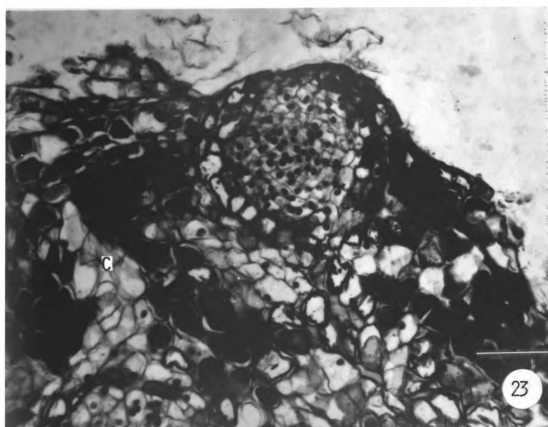
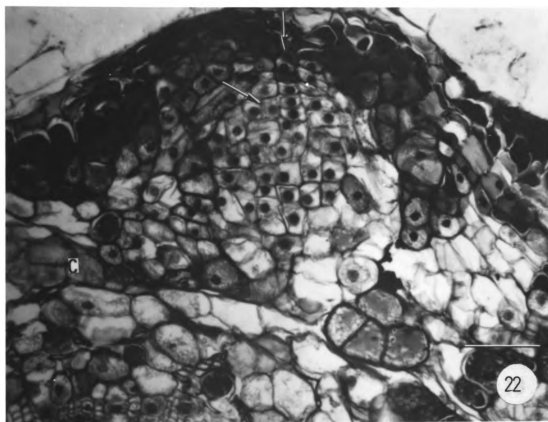


Figure 24 Root from phelloderm/prellogen (Blue spruce, cross section, bar = 100 μ m)

This figure shows a root apparently originating from the phelloderm or prellogen layer. Note resin duct, but no clear connection with the root is seen. The pictured section shows the closest association. The epithelial cells of the resin duct have taken on a rejuvenated appearance. Wound tracheids have differentiated within the root.

Figure 25 Upper rooting zone section (Engelmann spruce, cross section, bar = 100 μ m)

Figures 25, 26, and 27 show sections at various levels in the rooting zone of the same cutting. Note the secondary xylem (X2) in Figure 25 is becoming irregular with some tracheids having developed larger lumens and radial alignment disrupted in places. The labelled needle trace shows secondary trace xylem production. Four cortical resin ducts are labelled. Note some of the outer epithelial cells loosing the dense staining substances. (Compare resin ducts shown in Figure 6.)

CRD = cortical resin duct, NT = needle trace, WT = wound tracheids, X2 = xylem produced in rooting chamber.

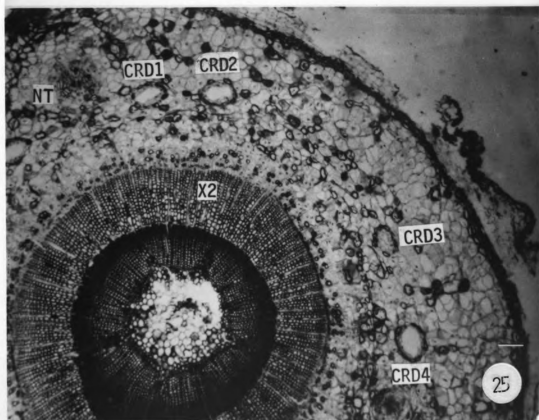
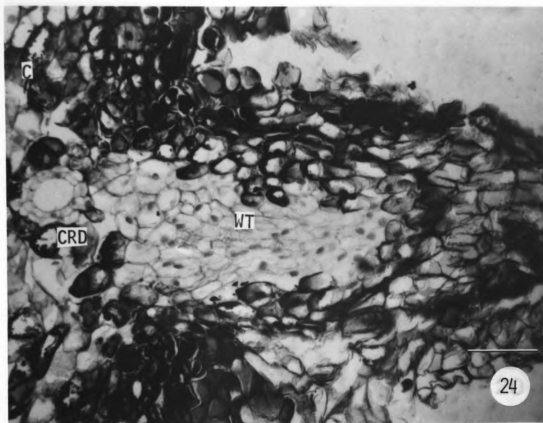


Figure 26 Mid-rooting zone section (Engelmann spruce, cross section,
bar = 100 μm)

Note the increased amount of secondary trace tracheid production around the needle trace. The secondary xylem (X2) is very irregular with blocky tracheids being produced or elongating at various orientations. Cortical resin ducts show cellular activity with a proliferation of meristematic type cells off the side of CRD2, and CRD3 filling in with small parenchyma. Resin duct epithelial shows rejuvenated appearance. A proliferation of meristematic cells has been produced in the cortex. Also note the zone of densely staining cells at the interface of the cell proliferations and cortex.

Figure 27 Lower rooting zone section (Engelmann spruce, cross section,
bar = 100 μm)

Note the secondary xylem (X2) is extremely irregular. A proliferation of meristematic type cells was produced from the needle trace. Three of four resin ducts have filled in with cells.

CPR = cortical proliferation, CRD = cortical resin duct, NT = needle trace, X2 = xylem produced in rooting chamber.

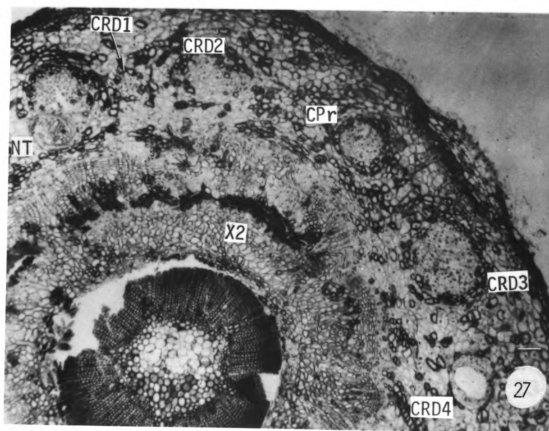
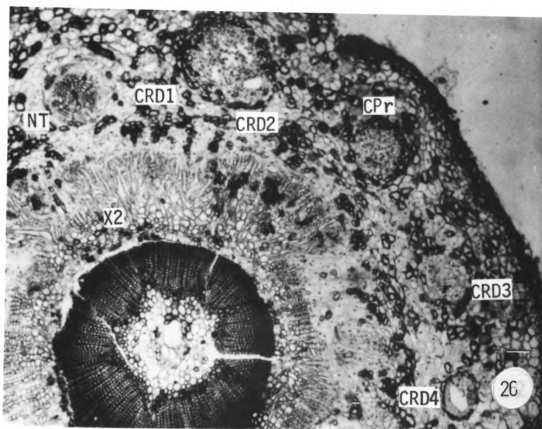


Figure 28 Needle gap proliferation (Blue spruce, cross section, bar = 100 μm)

Note the large proliferation from the needle gap extending out into the cortex. Notice the cortex that has been cut off by a periderm and the amount of cells produced by the phellogen toward the central xylem cylinder (as indicated by unlabelled arrow).

Figure 29 Meristematic sphere (White spruce, cross section, bar = 100 μm)

This figure shows a meristematic sphere being a homogenous group of small vigorous cells with large nuclei. The sphere is contiguous with the central xylem cylinder. No distinct zone of dark staining cells covers the ball.

NG = needle (leaf) gap, SP = secondary phloem, X2 = xylem produced in rooting chamber.

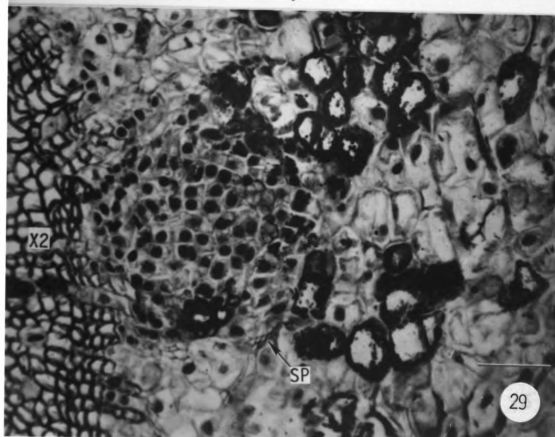
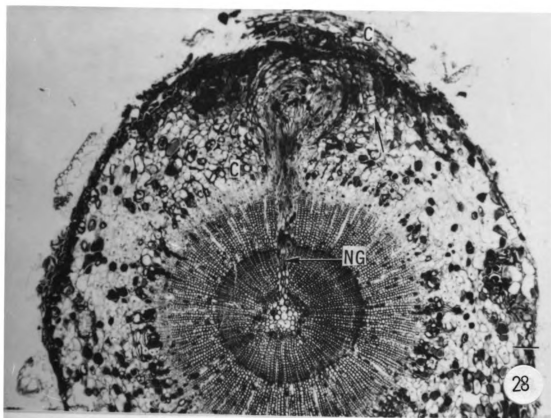


Figure 30 Early primordium (Engelmann spruce, cross section, bar = 100 μm)

Cells at primordium-cortex interface have acquired dark staining substances. (Note this feature on other proliferations of cells or primordia such as the proliferation at the resin duct base in Figure 8, and the meristematic cell groups in Figures 17, 23, 26, 27, and 34.) Note rows of radially aligned cells (unlabelled arrows). (This radial alignment is more noticeable in this figure, but rows can also be identified in Figure 29.)

Figure 31 Primordium (Engelmann spruce, cross section, bar = 100 μm)

Dark staining suberized cells surround the primordium forming an early rootcap. (Also compare primordium root caps in Figures 16 and 21.) Polarity is beginning to appear with cells adjacent to the central xylem cylinder elongating and those in the "apex" of the primordium forming a meristematic region.

EZ = elongation zone, MZ = meristematic zone, RC = root cap, X1 = xylem produced previous year, X2 = xylem produced in rooting chamber.

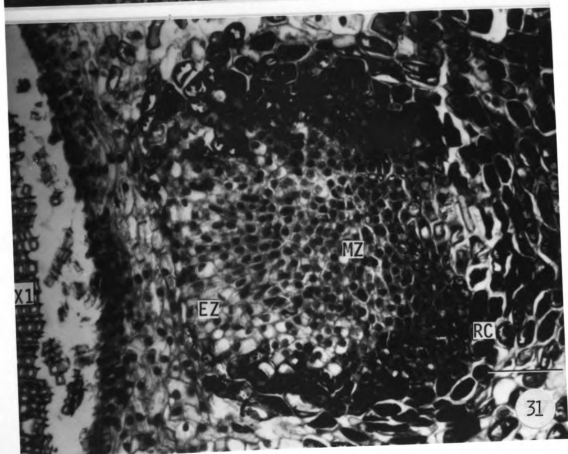
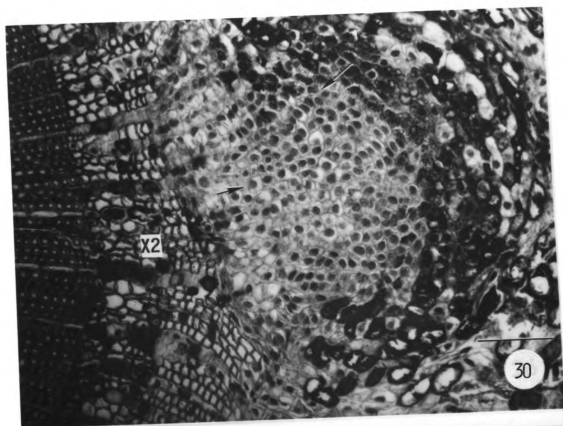


Figure 32 Early adventitious root (Engelmann spruce, cross section,
bar = 100 μm)

Note distinct root cap and meristematic zone. Cells behind the meristematic region are in various stages of elongation and differentiation.

Figure 33 Early adventitious root (Engelmann spruce, long. section,
bar = 100 μm)

This figure shows two primordia developing into roots with the lower primordium having initiated earlier than the upper. Both show developing root caps with a meristematic apex and elongated cells differentiated into tracheids. Also note the difference in appearance between X1 and X2 xylem. These two adventitious roots developed above the "callus mass" present at the base.

MZ = meristematic zone, RC = root cap, X1 = xylem produced previous year,
X2 = xylem produced in rooting chamber.

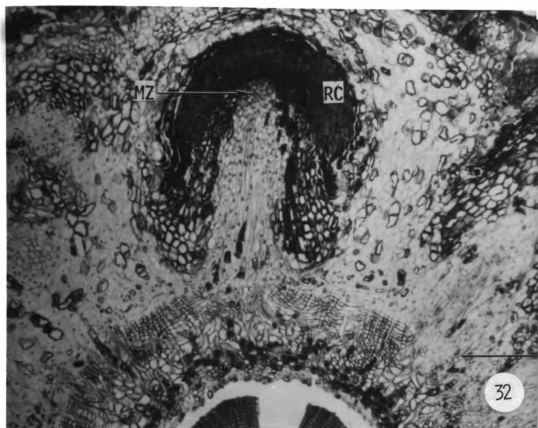


Figure 34 Primordium (Engelmann spruce, long. section, bar = 100 μ m)

This figure shows a primordium developing adjacent to a xylem proliferation at the cutting base. (Compare also the xylem proliferation in Figure 8.) Note the small, isodiametric cells with large nuclei forming a meristematic region in the primordium apex and elongating cells adjacent to the xylem proliferation. Note also the radial alignment of the tracheids in the xylem proliferation indicating a cambial origin for the xylem in the proliferation.

X1 = xylem produced previous year, X2 = xylem produced in rooting chamber.



CHAPTER 3

Comparison of Rooting Response in Young and Old Blue Spruce (Picea pungens) to Eight Auxin Conjugates

INTRODUCTION

Some evidence indicates that other substances play an important role in the metabolism and action of auxins with respect to enhancement of adventitious root formation. It has been proposed that these substances may act independently of the auxin molecule or as a conjugate of the auxin.

Much research emphasis has been placed on the role of phenolics in auxin physiology. Some phenols may enhance the enzymatic oxidation of IAA, specifically simple monophenols, while phenols with two or three adjacent hydroxyl groups (i.e., caffeic acid and chlorogenic acid) enhance the action of IAA (Letham, 1978). Some have suggested that certain phenolics may effect IAA activity by controlling IAA oxidation (thereby regulating the effective IAA concentration) (Letham, 1978).

Haissig (1974b) reviewed evidence in conflict with the above theory of phenolic controlled IAA oxidation. He proposes an auxin-phenolic conjugate as the active form of the auxin and in adventitious root formation the auxin conjugate is needed to initiate cellular dedifferentiation and root primordia initiation.

Recently synthesized aryl conjugates of indolebutyric acid (Haissig, et al., 1978; Boyles, et al., 1982a, 1982b) have shown promise as better stimulators of adventitious root formation, compared to conventional auxins, in cuttings of difficult to root species (Haissig, 1978, 1983a). Structurally similar aryl ethers do not give comparable results indicating the uniqueness of the auxin conjugate activity (Haissig, 1983b).

In addition to the conjugates tested by Haissig, auxin-amino acid conjugates have been tested in tissue culture systems (Hangarter, et al., 1980; Hangarter and Good, 1981). The investigators suggest these conjugates may be "slow release" forms of auxin through gradual hydrolysis of the covalent bond between the auxin and the conjugating moiety (Hangarter and Good, 1981). In addition these investigators suggest some of the conjugates may also have unique functions other than being slow release forms (Hangarter, et al., 1980).

There has been no previous experiment testing both amino acid and phenyl auxin conjugates for enhancement of adventitious root formation. This experiment is an attempt to evaluate the relative ability of two phenyl-auxin conjugates and six amino acid-auxin conjugates to enhance adventitious root formation in stem cuttings of blue spruce (Picea pungens) of two ages.

MATERIALS AND METHODS

Two age groups of blue spruce were used. Old blue spruce were 15 years old and young blue spruce were two years greenhouse grown. Three ortets were used for the old blue spruce. Two of the ortets were of the same full sib family and the third was from an open pollinated family. Tree position was considered more important than individual genotype of the ortet so cuttings from the trees were kept in three groups (blocks) according to tree position. The positions were: 1) lower 2/3 north side of tree, 2) lower 2/3 south side of tree, 3) upper 1/3 both sides of tree. Many seedlings were used as donors for young blue spruce. Only two or three cuttings per seedling were taken. Cuttings were kept in a cooler in the field and stuck the day of removal (2/10/83). Old cuttings were trimmed before treatment to 10 cm. Needles were stripped from the base of old cuttings. Young cuttings were 5-8 cm. The base was retrimmed before treatment. Needles were not stripped. The bottom two cm. of all cuttings was

given a two second quick-dip in a 10 mmol concentration of the auxin treatment dissolved in 100% ethanol. After drying the base was dipped in a captan:talc (1:2) mixture and stuck in the rooting medium. Nine auxins and conjugates and a control (100% ethanol only) were used. Figure 35 presents the auxin structure and designation.

Cuttings were rooted in a 3' x 2' x 8' (w x h x l) mist chamber equipped with two humidifying 1/4 JH pneumatic atomizing nozzles (Spraying Systems Co., Wheaton, Illinois). Rooting medium was vermiculite:coarse perlite (1:1). Bottom heat was supplied with heating cables thermostatically controlled at 24°C. A sixteen hour photoperiod was supplied by four eight foot fluorescent tubes (warm white, 1500 mv) and three incandescent bulbs (75w) giving an average $72\mu \text{ Em}^{-2} \text{ sec}^{-1}$ at cutting level. Air temperature was usually 22°C with lights on and 20°C with lights off. The chamber was divided into three regions (blocks, which also corresponded to tree position) according to light intensity and expected mist distribution.

Cuttings were removed 5/4/83. The following data were taken on each cutting: 1) whether the cutting rooted or not, 2) number of roots per cutting, 3) length of longest root.

The experiment consisted of a randomized complete block design with two factors, ortet age (two treatments) and auxin (ten treatments). The twenty experimental units were randomized in each of three blocks (blocking on tree position of old spruce and chamber location). Eighteen cuttings were used in each experimental unit.

During the course of the experiment a strong rooting response to a moisture gradient was observed. To get a relative measure of this gradient, Whatmann #3 filter paper 7 cm circles were stuck on toothpicks to make "sails". These were placed in a regular manner (9 rows x 21 columns) throughout the chamber.

<u>Designation</u>	<u>Structure</u>
IBA	$\text{HO}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{indole}$
P-IBA	$\text{indole}-\text{O}-\overset{\text{O}}{\parallel}{\text{C}}-$
NP-IBA	$\text{indole}-\text{NH}-\overset{\text{O}}{\parallel}{\text{C}}-$
Gly-IBA	$\text{HOOC}-\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}{\text{C}}-$
Phe-IBA	$\text{indole}-\text{CH}_2-\overset{\text{COOH}}{\underset{ }{\text{CH}}}-\overset{\text{O}}{\parallel}{\text{C}}-$
Ala-IBA	$\text{H}_3\text{C}-\overset{\text{COOH}}{\underset{ }{\text{CH}}}-\text{NH}-\overset{\text{O}}{\parallel}{\text{C}}-$
Gly-NAA	$\text{HOOC}-\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{naphthalene}$
Phe-NAA	$\text{indole}-\text{CH}-\overset{\text{COOH}}{\underset{ }{\text{CH}}}-\text{NH}-\overset{\text{O}}{\parallel}{\text{C}}-$
Gly-IAA	$\text{HOOC}-\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{indole}$

Figure 35. Auxin designation and structure

The amount of moisture held by each disk was measured. These data were used as a covariate in the analysis.

RESULTS AND DISCUSSION

Old blue spruce gave very low rooting (<2%) and so was not analyzed. All data and analysis presented are for the young blue spruce. Observation showed that the blocks in the chamber were ineffective, so analysis was done by regression on a per cutting basis. This increased the number of experimental units from sixty to five hundred forty.

Percent rooting (adjusted for the covariate) is presented in Table 1. Gly-IBA gave the greatest percent rooting as well as number of roots per cutting (Table 2) and longest root (Table 3). The control gave the next highest rooting percent, number of roots, and root length.

Covariate analysis is shown in Table 5. The large mist effect on percent rooting, number of roots, and root length is obvious. The covariate, on the average, represents about 29% of the total variation while the treatments represented about 5% of the total variation. Treatments still represent a significant portion of variation in each case. According to Tukey's W range test (Steele and Torrie, 1980, p. 185), each auxin is significantly different from the other for percent rooting. Though the differences are statistically significant most are not practically significant. The range test does not give any clear breaks for number of roots or length of root.

Ranking the data presented in Tables 1, 2, and 3 loosely defines three groups for percent rooting and number of roots. Gly-IBA stands out as the best treatment. The second group (above the overall average) contains the control, P-IBA and Phe-NAA. When ranking the conjugates by mean number of roots (rooted cuttings only), Gly-NAA has a very high average. Phe-NAA and Phe-

Table 1. Percent Rooting*

	<u>Gly IBA</u>	<u>Control</u>	<u>P IBA</u>	<u>Phe NAA</u>	<u>NP IBA</u>	<u>Gly IAA</u>	<u>Gly NAA</u>	<u>IBA</u>	<u>Phe IBA</u>	<u>Ala IBA</u>	<u>Overall</u>
Adjusted	77.8	70.5	64.8	61.0	57.3	51.8	46.2	42.9	42.6	37.0	55.2

Table 2. Mean Number of Roots Per Cutting*

	<u>Gly IBA</u>	<u>Control</u>	<u>Gly NAA</u>	<u>P IBA</u>	<u>Phe NAA</u>	<u>Gly IAA</u>	<u>Ala IBA</u>	<u>Phe IBA</u>	<u>NP IBA</u>	<u>IBA</u>	<u>Overall</u>
Adjusted, transformed**	1.69	1.46	1.46	1.39	1.39	1.30	1.22	1.21	1.20	1.20	1.35
Unadjusted (rooted cuttings only)***	3.2	2.2	6.6	3.0	4.0	2.6	2.6	3.9	2.9	3.2	3.3
	n=42	n=38	n=25	n=35	n=33	n=28	n=20	n=23	n=31	n=23	n=298

* average of 54 cuttings except where specifically specified

** $Y'_\lambda = \sqrt{Y_\lambda + .05}$

*** n=number of rooted cuttings

Table 3. Mean Length of Longest Root Per Cutting (in millimeters)*

	<u>Gly IBA</u>	<u>Control</u>	<u>P IBA</u>	<u>Phe NAA</u>	<u>Gly IAA</u>	<u>NP IBA</u>	<u>Gly NAA</u>	<u>Ala IBA</u>	<u>IBA</u>	<u>Phe IBA</u>	<u>Overall</u>
Adjusted, transformed**	3.34	2.90	2.64	2.24	2.09	1.98	1.78	1.70	1.69	1.64	2.20
Unadjusted (rooted cuttings only)***	75	38	74	78	64	69	91	56	65	68	70
	n=42	n=38	n=35	n=33	n=28	n=31	n=25	n=20	n=23	n=23	n=298

Table 4. Mean Mist ($\times 10^{-4}$ gm per 7 cm disk Whatmann #3 filter paper)*

<u>Ala IBA</u>	<u>Control</u>	<u>Gly IBA</u>	<u>Gly IAA</u>	<u>P IBA</u>	<u>Phe IBA</u>	<u>Gly NAA</u>	<u>NP IBA</u>	<u>Phe NAA</u>	<u>Overall</u>
3389	3339	3194	3049	3048	2950	2903	2676	2624	2991

* average of 54 cuttings except where specifically specified

** $Y'_1 = \log_e (Y_1 + 1)$

*** n = number of rooted cuttings

Table 5. Analysis of Covariance

Percent Rooting:

<u>Due to</u>	<u>df</u>	<u>ss</u>	<u>ms</u>	<u>F</u>
Total	539	133.5		
Treatment	9	7.4	0.82	4.81
Mist+(Mist) ²	2	34.7	17.34	101.54
Residual	528	90.2	0.17	

effective standard error = 0.057

*Gly		P	Phe	NP	Gly	Gly		Phe	Ala
<u>IBA</u>	<u>Control</u>	<u>IBA</u>	<u>NAA</u>	<u>IBA</u>	<u>IAA</u>	<u>NAA</u>	<u>IBA</u>	<u>IBA</u>	<u>IBA</u>

Number of roots per cutting**

<u>Due to</u>	<u>df</u>	<u>ss</u>	<u>ms</u>	<u>F</u>
Total	539	285.9		
Treatment	9	12.5	1.39	4.06
Mist	1	89.4	89.46	261.29
Residual	529	181.2	.34	

effective standard error = 0.082

*Gly		Gly	P	Phe	Gly	Ala	Phe	NP	
<u>IBA</u>	<u>Control</u>	<u>NAA</u>	<u>IBA</u>	<u>NAA</u>	<u>IAA</u>	<u>IBA</u>	<u>IBA</u>	<u>IBA</u>	<u>IBA</u>

Length of longest root***

<u>Due to</u>	<u>df</u>	<u>ss</u>	<u>ms</u>	<u>F</u>
Total	539	2351.8		
Treatment	9	126.9	14.11	4.77
Mist+(Mist) ²	2	641.0	320.53	108.51
Residual	528	1559.9	2.95	

effective standard error = .238

*Gly		P	Phe	Gly	NP	Gly	Ala		Phe
<u>IBA</u>	<u>Control</u>	<u>IBA</u>	<u>NAA</u>	<u>IAA</u>	<u>IBA</u>	<u>NAA</u>	<u>IBA</u>	<u>IBA</u>	<u>IBA</u>

* Tukey's w range test ($\alpha = 0.5$)** Data transformed $Y_{ij}' = \sqrt{Y_{ij} + .05}$ *** Data transformed $Y_{ij}' = \log_e (Y_{ij} + 1.0)$

IBA are also above the overall average. Though these three conjugates experienced "drier" conditions (average mist is below the overall average, Table 4), there may have been some treatment effect as the aryl conjugates were also "drier" but did not produce the above average number of roots.

Because moisture was such a controlling factor, no firm conclusions with respect to auxin application can be drawn. Further experimentation is needed, perhaps with more emphasis on Gly-NAA, Gly-IBA, Phe-NAA, Phe-IBA and P, NP-IBA.

CHAPTER 4

Use of Agrobacterium rhizogenes to Induce Adventitious Root Formation in Seven Tree Species

INTRODUCTION

Agrobacterium tumefaciens induces crown gall tumors in a wide range of plant species belonging to at least 142 genera distributed among 61 families of dicotyledonous angiosperms and certain gymnosperms. Monocotyledonous plants are not reported to be susceptible to the disease (Braun, 1978). Virulent strains of A. tumefaciens contain a large extra chromosomal DNA plasmid (the tumor inducing or Ti plasmid) which is responsible for the oncogenic properties of the bacterium. Transformation of the host plant involves the transfer and integration of a small segment of the plasmid into the host plant's nuclear DNA causing tumorous cell growth which may be maintained on media without cytokinins or auxins and free from the bacteria. The transformed cells also produce amino acid derivatives classed as opines which may be used as a carbon and nitrogen source by the bacteria (Schell, et al., 1979).

Similarly, Agrobacterium rhizogenes transforms host plant cells by integrating a portion of plasmid DNA into the host plant nuclear DNA. However, instead of producing tumorous cell growth, a proliferation of adventitious roots are produced at the wound site (Chilton, et al., 1982; Moore, et al., 1979; White, et al., 1982).

Because A. rhizogenes produces adventitious root formation at an infected wound and because cuttings possess a wounded base, an experiment was conducted to test the possibility of using A. rhizogenes to induce adventitious root formation in stem cuttings of woody plants.

MATERIALS AND METHODS

Seven tree species were tested in the experiment. Apple (Malus var. "polleret") was used as a control because of its known susceptibility to A. rhizogenes (Riker, 1930). DeCleene and DeLey (1976) list blue spruce (Picea pungens) as susceptible and eastern white pine (Pinus strobus) as resistant to A. tumefaciens. These species were included to give a possible indication of parallel host susceptibility or resistance to A. rhizogenes. Other species included were hybrid pine (Pinus densiflora x Pinus nigra), hybrid spruce (Picea glauca x P. pungens), and white spruce (P. glauca). Ages of the ortets were: Apple - 23 years, blue spruce - 2 years greenhouse grown, Douglas fir - 15 years, white pine - 12 years, hybrid pine -23 years, hybrid spruce -15 years, and white spruce - 15 years.

Cuttings, which were stored in a cooler in the field, were stuck the day of collection (2/26,27/83). The cuttings were treated with either a solution of A. rhizogenes, indolebutyric acid, or 0.85% NaCl.

Bacteria from isolated single colonies were grown in 50 ml of nutrient broth (Difco Laboratories, Detroit, Michigan) for 24 hours on a shaker. Colonies were centrifuged and washed twice in 0.85% NaCl solution and then stored, refrigerated in a 0.85% NaCl solution which contained 10^9 bacteria/ml. Carrot disks inoculated with the solution showed hairy root syndrome after two weeks indicating virulence of the bacteria.

On day 1 of the experiment (the day of collection) the cuttings were retrimmed and the basal 2-3 cm of the cutting soaked for 1 minute in the bacteria solution. After cuttings were stuck in the rooting medium, 1 ml of the bacteria solution was injected at the cutting base with a pasteur type pipet. On day 3 another 1 ml was injected at the cutting base. Control cuttings were treated exactly the same with a 0.85% NaCl solution. Auxin treated cuttings were retrimmed and

given a 2 second quick dip in a 25 mmol IBA solution in 100% ethanol. Fresh solutions from bulk stock were used for each replication.

Cuttings were rooted in conditions as described in the previous chapters.

The experiment was a randomized complete block design (blocks being chamber location) with two factors (species - seven treatments and solutions - three treatments). The twenty-one experimental units were randomized in each of six blocks with eighteen cuttings per experimental unit.

RESULTS AND DISCUSSION

Due to a disease problem caused by excess moisture, no meaningful data were attained but comments on further experimentation are in order.

The idea to use an infectious agent to enhance adventitious root formation in cuttings is not new. McDonald and Hoff (1970) investigated the effects of Cronartium ribicola on rooting needle fascicles of Pinus monticola. The investigators noted previous work where characteristic symptoms of blister rust were produced by the exogenous application of an indoleacetic acid and gibberellic acid mixture. They reasoned that C. ribicola may produce the blister rust effects by the same mechanism and perhaps infection by the organism, by altering hormonal ratios, may enhance root initiation. Unfortunately, in this case, rooting was reduced.

However, with Agrobacterium rhizogenes it is known that infection causes adventitious root production. The questions to be answered are: 1) What species can be infected by what strains? 2) What is the most efficient and successful infection technique? 3) How do A. rhizogenes induced roots compare with normal adventitious roots in both the physiological and anatomical events during the rooting process? 4) How is growth of the cutting effected by A. rhizogenes?

Some specific experimental areas that may help answer the above questions should include the following: 1) It would be beneficial to run an infection trial on potted seedlings (of suitable size and caliper) by wounding and infecting the root collars of various species to determine species susceptibility to A. rhizogenes. Comparisons could be made between A. rhizogenes and A. tumefaciens. If a given species cannot be infected by a given strain of A. rhizogenes, other strains should be tested. There is some host specificity among the various strains so there is a good possibility a virulent strain could be found for many species (Nester and Kosuge, 1981). Braun (1958) mentions that certain plant species (such as sunflower or Paris daisy) may exhibit secondary tumors that arise at locations on the plant distant from the original point of infection by A. tumefaciens and these secondary tumors are free of the bacteria. It would be very interesting if uninoculated cuttings taken from an A. rhizogenes infected stock plant would show hairy root at the basal wound. 2) Stage of stock plant development (age and period of growth) may effect susceptibility. 3) The timing of the infection after wounding may be important. Braun (1978) reviews evidence which shows that the stage of development of the wounding response effects infectivity and type of tumor produced by A. tumefaciens. It appears that infection during the period just before the first cell divisions appear in the wound area produces the most vigorous tumors. Braun (1978) gives maximum susceptibility occurring between the 48th and 72nd hours after the wound is made. Therefore, it may be necessary to allow the cuttings to incubate a few days before treatment with bacteria (hardwood cuttings probably longer than softwood cuttings). 4) During the early phases of crown gall infection a temperature sensitive step occurs (Braun, 1978). It may be necessary to determine an optimum temperature for a soaking solution or bottom heat of the rooting medium. 5) It may be possible to enhance the infection process of A. rhizogenes. Substance(s) have been isolated that appear

to enhance crown gall tumor initiation (Bouekaert-Urban and Vendrig, 1982; Bouekaert-Urban, et al., 1982). These same substances may also enhance A. rhizogenes infection or the procedure presented by the investigators may be used to identify substances specific for A. rhizogenes. Perhaps cuttings could be soaked in these substances as a pre-treatment before infection at the optimum time period. 6) There are strains of A. tumefaciens which have been altered with insertions at various locations on the Ti plasmid. Depending on the location, different tumor morphologies arise (i.e., the rooty, shooty, and large) (Akiyoshi, et al., 1983; Doms, et al., 1981, and references therein). An altered strain of A. tumefaciens may be used with, or in place of, A. rhizogenes. 7) If initial experimentation indicates that A. rhizogenes induces adventitious roots in stem cuttings, a simple assay procedure for the appropriate opine used by the particular strain of bacteria should be developed (see Otten and Schilperoort, 1978) since the detection of the opine will confirm infection and transformation.

Most interest in Agrobacterium is centered on its use as a vector for genetic engineering of plants and to investigate gene regulation. Agrobacterium may also be used to investigate the physiological processes involved in the production of various tumor or callus types. If, as has been argued, the transformation process either activates the already present biosynthetic systems to produce the observed cell production and differentiation or fixes the systems which are activated by the act of wounding (Braun, 1978), then those results derived from an Agrobacterium system should reveal the actual processes involved, for instance, in adventitious root formation. The conclusions drawn from the Agrobacterium system should apply to similar situations without the bacteria. For example, the general rule of thumb for callus cultures is that high cytokinin/auxin ratios favor shoot production and a low ratio favors root production. Evidence has been presented which supports this theory in which the different tumor morphologies attained depending

on the point of insertion in the Ti plasmid of A. tumefaciens is due to different ratios of endogenously produced cytokinins and auxins (Akiyoshi, et al., 1983, Doms, et al., 1981). However, throughout the rooting literature there exists a persistent theme of some non-auxin component being important in producing adventitious roots. This has been variously referred to as rhizocalin, cofactors, non-auxin endogenous rooting substance, or some conjugating moiety. An Agrobacterium system may allow determination of such a substance. Using Agrobacterium may allow greater control over the plant material since a specific tumor or callus mass morphology can be expected with, perhaps, less variability. A problem with analyzing sequential physiological changes during the course of adventitious root formation is the great variability among individual cuttings. Perhaps A. rhizogenes induced root formation would reduce this variability.

Another advantage of exploring an Agrobacterium system will be the availability of an increasing pool of current information. Because of its promise as system for the genetic engineering of higher plants, the amount of research on Agrobacterium should only increase. This may be the tool needed to look at the molecular biological foundation of adventitious root formation.

REFERENCES

- Akiyoshi, D.E., R.O. Morris, R. Hinz, B.S. Mischke, T. Kosuge, D.J. Garfinkel, M.P. Gordon, and E.W. Nester. 1983. Cytokinin/auxin balance in crown-gall tumors is regulated by specific loci in the T-DNA. *Proc. Natl. Acad. Sci.* 80: 407-411.
- Bannan, M.W. 1942. Notes on the origin of adventitious roots in the native Ontario conifers. *Am. J. Bot.* 29(8): 593-598.
- Berlyn, G.P. and J.P. Miksche. 1976. *Botanical Microtechnique and Cytochemistry*. The Iowa State University Press, Ames, Iowa.
- Bhella, H.S. and A.N. Roberts. 1975a. Bud and cambial activity in douglas-fir as related to stem cutting rootability. *Forest Sci.* 21(3): 269-275.
- Bhella, H.S. and A.N. Roberts. 1975b. Seasonal changes in origin and rate of development of root initials in douglas-fir stem cuttings. *J. Amer. Soc. Hort. Sci.* 100(6): 643-646.
- Bouekaert-Urban, A.M. and J.C. Vendrig. 1982. Evidence for the existence of a universal crown-gall tumor initiation enhancer. *Planta*. 156: 359-363.
- Bouekaert-Urban, A.M., G. Brouwers, L. Theolen and J.C. Vendrig. 1982. Influence of a crown-gall tumor initiation enhancer on bacterial attachment to the host plant cell wall. *Planta*. 156: 364-368.
- Boyles, D.A., B.E. Haissig, and J.R. Gaines. 1982a. Novel synthesis of aryl esters and thioesters of indole-3-acetic and indole-3-butyric acids and their use as auxin growth regulators. U.S. Patent Application, Serial No. 367,638, Filed April 12, 1982. U.S. Dept. of Commerce, Commissioner of Patents and Trademarks, Washington, DC 20231.
- Boyles, D.A., B.E. Haissig, and J.R. Gaines. 1982b. Novel synthesis of N-phenyl and N-substituted phenyl indole-3-acetamide and indole-3-butyramide and related esters and thioesters and their use as auxin growth regulators. U.S. Patent Application, Serial No. 367,639, Filed April 12, 1982. U.S. Dept. of Commerce, Commissioner of Patents and Trademarks, Washington, DC 20231.
- Braun, A.C. 1958. A physiological basis for autonomous growth of the crown-gall tumor cell. *Proc. Natl. Acad. Sci.* 44: 344-349.
- Braun, A.C. 1978. Plant tumors. *Biochem. Biophys. Acta*. 516: 167-191.
- Cameron, R.J. and G.V. Thomson. 1969. The vegetative propagation of Pinus radiata: Root initiation in cuttings. *Bot. Gaz.* 130(4): 242-251.
- Chilton, M.D., D.A. Tepfer, A. Petit, C. David, F. Casse-Delbart, and J. Tempe. 1982. Agrobacterium rhizogenes inserts T-DNA into the genomes of the host plant root cells. *Nature*. 295: 432-434.

- Dalgas, F. 1973. Anatomical studies on cuttings of Norway spruce undergoing the rooting process. *Forest Tree Improvement* 5: 4-19.
- DeCleene, M. and J. DeLey. 1976. The host range of crown gall. *Bot. Rev.* 42(4): 389-466.
- Delisle, A.L. 1942. Histological and anatomical changes induced by indoleacetic acid in rooting cuttings of Pinus strobus L. *The Va. Jor. of Sci.* 3(5): 118-123.
- Eliason, L. 1978. Effects of nutrients and light on growth and root formation in Pisum sativum cuttings. *Physiol. Plant.* 43: 13-18.
- Esau, K. 1977. *Anatomy of seed plants.* John Wiley and Sons, New York.
- Girouard, R.M. 1967. Anatomy of adventitious root formation in stem cuttings. *Proc. Int. Pl. Prop. Soc.* 17: 289-302.
- Haissig, B.E. 1970. Influence of indole-3-acetic acid on adventitious root primordia of brittle willow. *Planta* 95: 27-35.
- Haissig, B.E. 1974a. Origins of adventitious roots. *N. Z. J. For. Sci.* 4(2): 299-310.
- Haissig, B.E. 1974b. Influences of auxins and auxin synergists on adventitious root primordium initiation and development. *N. Z. J. For. Sci.* 4(2): 311-323.
- Haissig, B.E. 1979. Influence of aryl esters of indole-3-acetic and indole-3-butyric acids on adventitious root primordium initiation and development. *Physiol. Plant.* 47: 29-33.
- Haissig, B.E., J.R. Gaines, and G. Giacoletto. 1981. Tree rooting using synthetic auxins. U.S. Patent 4,297,127. Issued October 27, 1981. U.S. Dept. of Commerce, Commissioner of Patents and Trademarks, Washington, DC 20231.
- Haissig, B.E. 1982. Carbohydrate and amino acid concentrations during adventitious root primordium development in Pinus banksiana Lamb. cuttings. *Forest Sci.* 28(4): 813-821.
- Haissig, B.E. 1983a. N-phenyl indolyl-3-butyramide and phenyl indole-3-thiolobutyrate enhance adventitious root primordium development. *Physiol. Plant.* 57: 435-440.
- Haissig, B.E. 1983b. Influence of phenyl tryptophyl ether on adventitious root development in bean cuttings. *Can. J. Bot.* 61: 1548-1549.
- Hangarter, R.P., M.D. Peterson, and N.E. Good. 1980. Biological activities of indoleacetyl amino acids and their use as auxins in tissue culture. *Plant Physiol.* 65: 761-767.

- Hangarter, R.P. and N.E. Good. 1981. Evidence that IAA conjugates are slow release sources of free IAA in plant tissues. *Plant Physiol.* 68: 1424-1427.
- Hansen, J., L.H. Stromquist, and A. Ericsson. 1978. Influence of the irradiance on carbohydrate content and rooting of cuttings of pine seedlings (*Pinus sylvestris* L.). *Plant. Physiol.* 61: 975-979.
- Hartmann, T.H. and D.E. Kester. 1975. *Plant propagation--principles and practices*. Englewood Cliffs, New Jersey: Prentice-Hall, Inc.
- Heaman, J.C. and J.N. Owens. 1971. Callus formation and root initiation in stem cuttings of douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco). *Can. J. For. Res.* 2: 121-134.
- Hyun, S.K. 1967. Physiological differences among trees with respect to rooting. XIV IUFRO Congress, pp. 168-190.
- James, D.J. and I.J. Thurban. 1979. Rapid in vitro rooting of apple rootstock M.9. *H. Hort. Sci.* 54(4):309-311.
- James, J.J. 1983. Adventitious root formation "in vitro" in apple rootstocks (*Malus pumila*). I. Factors affecting the length of the auxin-sensitive phase in m.9. *Physiol. Plant.* 57: 149-153.
- John, A. 1978. An anatomical study of root initiation in stem cuttings of hybrid larch. *New Phytol.* 81: 111-116.
- Letham, D.S. 1978. Natural-occurring plant growth regulators other than the principal hormones of higher plants. pp. 349-465. In D.S. Letham, P.B. Goodwin, and T.J.V. Higgins (eds.), *The biochemistry of phytohormones and related compounds: a comprehensive treatise*. Vol. I. Elsevier/North Holland, Amsterdam.
- Mitsubishi-Kato, M., H. Shibaoka, and M. Shimokoriyama. 1978. The nature of the dual effect of auxin on root formation in *Azukia* cuttings. *Plant and Cell Physiol.* 19(8): 1535-1542.
- Maloney, M.M. and M.C. Elliott. 1982. Tryptophan and indole-3-acetic acid accumulation in *Acer* cell cultures and its relationship with cell autolysis. *Planta.* 156: 326-331.
- McDonald, G.I. And R.J. Hoff. 1970. Effects of *Cronartium ribicola* infection on rooting potential of detached *Pinus monticola* needle bundles. *Can. J. Bot.* 48: 1943-1945.
- Montain, C.R., B.E. Haissig, and J.D. Curtis. 1983a. Initiation of adventitious root primordia in very young *Pinus banksiana* seedling cuttings. *Can. J. For. Res.* 13: 191-195.
- Montain, C.R., B.E. Haissig, and J.D. Curtis. 1983b. Differentiation of adventitious root primordia in callus of *Pinus banksiana* seedling cuttings. *Can. J. For. Res.* 13: 195-200.

- Moore, L., G. Warren, and G. Strobel. 1979. Involvement of a plasmid in the hairy root disease of plants caused by Agrobacterium rhizogenes. *Plasmid*. 2: 617-626.
- Nanda, K.K., M.K. Jain, and S. Malhotra. 1971. Effects of glucose and auxin in rooting etiolated stem segments of Populus nigra. *Physiol. Plant*. 24: 387-391.
- Nelson, S.H. 1978. A test for juvenility as an index of rootability in clonal apple rootstocks. *Can. J. Plant Sci.* 58: 605-609.
- Nester, E.W. and T. Kosuge. 1981. Plasmids specifying plant hyperplasias. *Ann. Rev. Microbiol.* 35: 531-565.
- Ooms, G., P.J.J. Hooykaas, G. Moolenaar, and R.A. Schilperroot. 1981. Crown gall plant tumors of abnormal morphology, induced by Agrobacterium tumefaciens carrying mutated octopine Ti plasmids; analysis of T-DNA functions. *Gene*. 14: 33-50.
- Otten, L.A.B.M. and R.A. Schilperroot. 1978. A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta*. 527: 497-500.
- Reines, M. and R.G. McAlpine. 1959. The morphology of normal, callused, and rooted dwarf shoots of slash pine. *Bot. Gaz.* 130: 242-251.
- Riker, A.J. 1930. Studies on infectious hairy root of nursery apple trees. *Jour. Ag. Res.* 41(7): 507-539.
- Satto, S. 1956. Anatomical studies on the rooting of cuttings in coniferous species. *Bull. Tokyo Univ. For.* 51: 109-157.
- Savidge, R.A. and P.F. Wareing. 1981. A trachied-differentiation factor from pine needles. *Planta*. 153: 395-404.
- Schell, J., M. Van Montagu, M. DeBeuckeleer, M. DeBlock, A. Depicker, M. DeWilde, G. Engler, C. Genetello, J.P. Hernalsteens, M. Holsters, J. Seurinck, B. Silva, F. VanVliet, and R. Villarroel. 1979. Interactions and DNA transfer between Agrobacterium tumefaciens, the Ti-plasmid and the plant host. *Proc. Roy. Soc. London Ser. B*. 204: 251-266.
- Sheldrake, A.R. 1971. Auxin in the cambium and its differentiating derivatives. *J. Exp. Bot.* 22(72): 735-740.
- Sheldrake, A.R. 1973. The production of hormones in plants. *Biol. Rev.* 48: 509-559.
- Shininger, T.L. 1979. The control of vascular development. *Ann. Rev. Plant Physiol.* 30: 313-337.
- Smith, D.R. and T.A. Thorpe. 1975a. Root initiation in cuttings of Pinus radiata seedlings. I. Developmental sequence. *J. Exp. Bot.* 26(91): 184-192.

- Smith, D.R. and T.A. Thorpe. 1975b. Root initiation in cuttings of Pinus radiata seedlings. II. Growth regulator interactions. J. Exp. Bot. 26(91): 193-202.
- Smith, D.R. and T.A. Thorpe. 1977. Root initiation in cuttings of Pinus radiata seedlings: effects of aromatic amino acids and simple phenylpropanoids. Bot. Gaz. 138(4): 434-437.
- Steel, R.G.D. and J.H. Torrie. 1980. Principles and procedures of statistics, A biometrical approach. McGraw-Hill Book Company, New York.
- Veierskov, B., J. Hansen, and A.S. Anderson. 1976. Influence of cotyledon excision and sucrose on root formation in pea cuttings. Physiol. Plant. 36: 105-109.
- White, F.F., G. Ghidossi, M.P. Gordon, and E.W. Nester. 1982. Tumor induction by Agrobacterium rhizogenes involves the transfer of plasmid DNA to the plant genome. Proc. Natl. Acad. Sci. 79: 3193-3197.

MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 03056 6909