THE UNIVERSITY OF CALGARY

In vitro studies of adventitious root formation in four woody species

by

Pete Steve Ronald Metivier

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

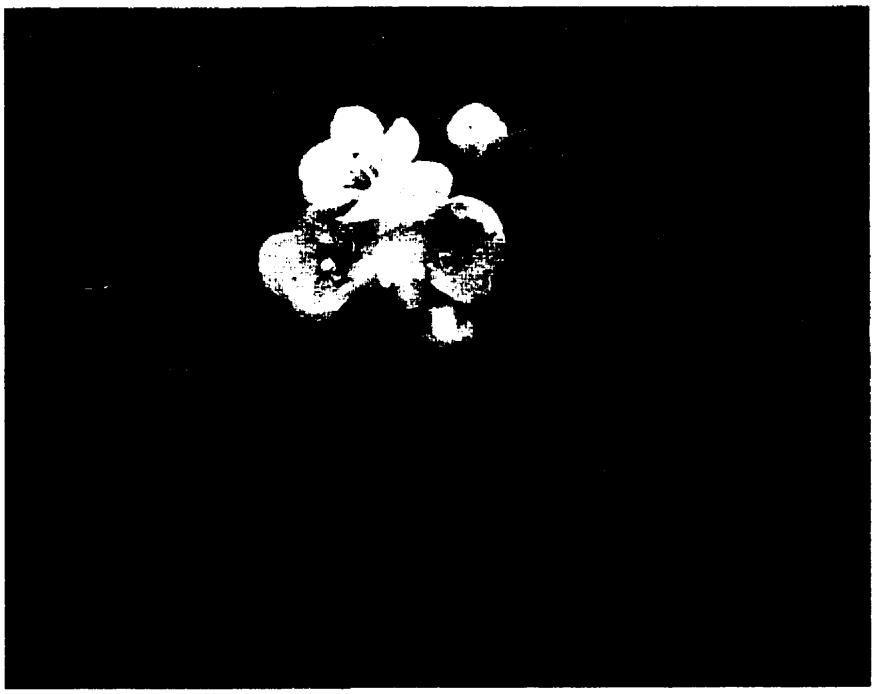
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JANUARY, 2000



***Crataegus rotundifolia* Moench**

(taken from Wild flowers of Calgary and Southern Alberta

by France Royer and Richard Dickinson)

ABSTRACT

In this research several studies were undertaken in an attempt to improve adventitious root formation and also to gain further insight into the controls and structural changes occurring during this process in micropropagated woody species. The potential use of *Agrobacteria* to promote rooting was investigated in several commercially important species, and is shown to be a specific plant-bacteria interaction in *Pinus ayacahuite*. The optimal rooting treatment using auxins was determined for two valuable ornamental species. This was 10μM IBA for *Cotinus coggygria* and 10μM IAA for *Crataegus spp.* The former species, by virtue of its potential as a model system, was used to conduct further physiological and histological studies of adventitious root formation. The length of the root inductive phase in *Cotinus coggygria* was determined to be 5 days. Two modes of adventitious root initiation and development were demonstrated in this species, the direct and indirect patterns, depending on auxin treatment.

ACKNOWLEDGEMENTS

I wish to sincerely thank Dr. T. A. Thorpe for his advice and guidance throughout this period of research. I am grateful for the opportunity to work in your lab and honored to be a fellow "Team-Thorpe" member.

I also want to thank Dr. E. C. Yeung for his encouragement and unselfish sharing of knowledge and training. Thanks are also extended to Dr. C.C. Chinnapa for assisting with film development techniques.

I gratefully acknowledge the financial assistance received as teaching and research assistantships from the Department of Biological Sciences, University of Calgary.

Finally I wish to acknowledge the help received from friends and colleagues during the course of my research who ultimately have contributed to the completion of this study.

DEDICATION

I dedicate this manuscript to my family

Sherry-Ann and Solange

for enduring this journey with me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

showing root primordium initial development.

LIST OF ABBREVIATIONS

° C - degree Celsius

µl - microliter

µM – micromolar concentration

½ AE-von Arnold and Ericksson medium (1981) at half-strength

½ GD - Gresshoff and Doy medium (1972) at half-strength

½ MCM-Bornman' medium (1983) at half-strength

2,4-D – 2,4 dichlorophenoxyacetic acid

A_{600} – absorbance at 600 nanometers

AB- AB medium

AC- Activated charcoal

AE-von Arnold and Ericksson medium (1981)

BA-N^6-benzyladenine

BFC – bud forming capacity

bp – base pair

CFB – percentage of cotyledons forming buds

DNA – deoxyribonucleic acid

GD – Gresshoff and Doy medium (1972)

HFM-Hormone-free medium

IAA – indole acetic acid

IBA- indole butyric acid

kDa – kilodalton

L - liter

M - molar concentration

MCM-Bornman's medium (1983)

mg - milligram

ml - milliliter

MS-Murashige and Skoog medium (1965)

NAA – naphthalene acetic acid

NPTII – Neomycin phosphotranferase assay

PPM – plant preservative mixture

psi – pound per square inch

T-DNA – tranferred deoxyribonucleic acid

v/v – volume to volume

w/v – weight to volume

CHAPTER 1 GENERAL INTRODUCTION

During the 1970s a lot of excitement was generated with regards to applying plant cell and tissue culture technology to improve important agricultural crop species (Conger, 1980). The impetus for this was the previous demonstration by several independent researchers of cell totipotency, regeneration of entire plants from single cells and the ability to create hybrid plants in some species by somatic cell fusions.

In the last several decades, the techniques used in plant tissue culture have emerged as powerful tools to investigate basic and applied problems in plant biology studies. While some areas have more potential practical application than others, clonal or mass propagation of selected and desirable genotypes has been the most practical application adopted. On a global scale, plant tissue culture, have found wide commercial application in the propagation of important agronomic, horticultural and forestry species. Additionally pathogen-free plants have been produced this way in vitro. Thus the potential for plant improvement and the interest in plant tissue culture techniques as a vehicle to achieve this goal remains high throughout the world.

Investigating the biology of adventitious root formation provides researchers with a forum in which to pursue fundamental research on the regulation of plant growth and development (Hess, 1994). As interest in speeding technology transfer was and remains important, the results of the research have been rapidly applied by commercial plant propagators, agronomists (Redenbaugh, 1991; Kovar and Kuchenbuch, 1994), foresters (Thorpe *et al.*, 1991; Ritchie, 1994), and horticulturists (Capellades *et al.*, 1991; Davies *et al.*, 1994). A complete understanding of the regulation of adventitious root formation is yet to be determined, so in the meantime many plant species remain difficult to root.

A review of the literature quickly reveals that adventitious root formation is paramount due to its importance in vegetative propagation of desirable genotypes. It is a prerequisite (Davies *et al.*, 1994), and a cornerstone to successful regeneration of cloned plants. In many potentially valuable horticultural and forestry crops, the inability to induce adventitious roots in mature woody species seriously limits propagation efforts (Murray *et al.*, 1994; Ritchie, 1994). If these recalcitrant species can be successfully rooted, a feat, which is currently uneconomical by traditional cuttings, then it would ultimately lead to the development of new plant products and market opportunities. Apart from improving tissue culture systems (Debergh and Zimmerman, 1991; Davies *et al.*, 1994), the use of new biotechnological techniques to address this problem opens up (Hamill and Chandler, 1994; Palme *et al.*, 1994; Riemenschneider, 1994; Tepfer *et al.*, 1994). There is a tremendous need to increase the rooting percentages, to extend the range of rooting to other genotypes and to make the root systems physiologically stronger.

The following study investigates various aspects of adventitious root formation *in vitro* by applying conventional and novel treatments to several economically and commercially important plant species. Currently, the regeneration of microshoots in these plant systems is not problematic but these species have proven to be difficult-to-root genotypes. In addition, I use some physiological and anatomical approaches are employed to further characterize the induction and development phases of adventitious root formation. The knowledge and experiences gained from these studies will undoubtedly contribute further to our fundamental understanding of adventitious root formation. With continued interaction between the fundamental and applied, the results

will be beneficial to all members of the scientific community, those involved in industry (agronomy, horticulture, forestry) and society at large.

CHAPTER 2 MICROPROPAGATION AND ADVENTITIOUS ROOTING IN PLANTS

1. LITERATURE REVIEW

1.1 Plant propagation

Our dependence on plants is evident in our food, medicines, clothing, fuel, paper, lumber, and aesthetic ornamentals. Since before recorded history, humans have continually cultivated and attempted to "improve" and propagate desirable plant genotypes for their use. Vegetative propagation of plants is an extremely old practice indeed, and has been successfully executed in ancient times in some species, as supported by the writings of Aristotle (384-322 BC), Theophrastus (371-287 BC) and Pliny the Elder (23-79 AD) which describe efforts to root cuttings (Haissig and Davis, 1994).

In this century, advances in plant propagation have involved two main issues related to adventitious root formation (Hess, 1994).

(1) the development of systems to provide a favorable environment for rooting (restricted air volumes around the cuttings, humidification systems, nozzle technology, plant tissue culture etc.), and,

(2) the search for substances that specifically promote rooting (from the elusive search for rhizocaline up to the discovery of auxins).

The latter had a major impact in root formation research and the field of plant propagation, as we know it today.

1.2 Plant cell and tissue culture

At the turn of the 20th century, Haberlandt (1902) conducted pioneering experiments to regenerate plants from single cells. Though unsuccessful, one can regard the technology as having its humble beginnings then based on his speculations on cell totipotency and the inspiration passed on to future researchers. He suggested that techniques for isolating and culturing plant cells should be developed, and further postulated that manipulation of the environment and nutritional requirements would result in recapitulation of developmental sequences of normal plant growth (Hughes, 1981). In the ensuing years following Haberlandt's paper not much progress was made with plant tissue culture.

The first success of cultured plant tissues came some 30 years later when actively growing clones of tomato roots were established (White, 1934). Subsequently, by the late 1930s other researchers reported the first successful callus cultures independently in carrot (Gautheret, 1939; and Nobécourt, 1939) and tobacco (White, 1939). Other notable successes followed (Gautheret, 1986), but the establishment of long-term plant tissue cultures provided new perspectives on improving plant propagation by employing them directly for propagation or in physiological studies of rooting (Haissig and Davies, 1994).

Plant tissue culture (or the aseptic culture of plant cells, tissue and organs) therefore had a significant impact on agriculture, in research and practical applications (Thompson

and Thorpe, 1990). Three agricultural areas identified were (1) genetic modification (2) disease-free plants and (3) clonal propagation of plants.

As a method plant tissue culture offered potentially controlled environments (both physically and chemically), so that otherwise difficult experiments could be conducted at reasonable cost (Haissig and Davies, 1994). In this same period, little progress was made in understanding the control of adventitious rooting (particularly recalcitrant rooters) in general. But plant hormone research was escalating simultaneously (Skoog and Miller, 1957; Haissig and Davies, 1994), so it became possible to address the rooting problem empirically with a host of chemicals.

1.3 Micropropagation of plant species

Since the development of plant tissue cultures under aseptic conditions, micropropagation has emerged as having the most practical application. The technology and its application to numerous plant species have developed rapidly in the last three decades. Micropropagation can be defined as the true-to-type propagation of selected genotypes using in vitro culture techniques, often associated with mass production at competitive pricing (Debergh and Read, 1991). Morel (1960) produced the first micropropagated plant (orchid), and together with a new culture medium high in mineral salts (Murashige and Skoog, 1962), provided the stimulus for applying plant cell and tissue techniques to other species.

By the late seventies, hundreds of institutions were involved in commercial micropropagation of various plants species (Murashige, 1978), and more companies

continue to come on line over the years. As early as 1974, Murashige proposed a three-stage protocol for micropropagation of plants; however much has since changed. A five-stage protocol is now generally agreed upon for developing a reliable and repeatable micropropagation scheme (Debergh and Read, 1991).

These are as follows:

1. the preparative stage (Stage 0)
2. initiation of culture (Stage 1)
3. multiplication (Stage 2)
4. elongation and root induction or development (Stage 3)
5. transfer to greenhouse conditions (Stage 4)

The regeneration may be accomplished from callus, organ, cell and protoplast cultures. Of these, callus and organ cultures have been employed with varying degrees of success in woody species over the years (Ahuja, 1993). Now organ cultures e.g. embryos, cotyledons and bud meristems are routinely used in micropropagation programs due to their relatively higher genetic stability. Protoplast and cell suspension cultures have been less successful for large scale cloning, and callus invariably exhibits genetic variability with time.

A main advantage of tissue culture in breeding programs and mass clonal production is its potential for enormous multiplication rates (Thorpe and Biondi, 1984) compared to traditional rooted cuttings. The intention is to produce large quantities of uniform plants of selected qualities (Thorpe *et al.*, 1991), and woody plants being outbreeders basically guarantee uniformity through clonal multiplication.

The potential benefits of clonal material in reforestation programs have been recognized for a long time (Harry and Thorpe, 1990), and at least a 10% increase in gain is possible when compared to selected seed families (Kleinschmit, 1974; Thorpe *et al.*, 1991). To get the maximum genetic gain, both sexual and asexual reproduction must be used in forest improvement (Hasnain and Cheliak, 1986). The sexual route prevents inbreeding by introducing new genes and genetic gain results from additive genetic effects. Asexual reproduction allows multiplication of elite full-sib families, which exhibit gain from non-additive genetic effects.

Traditional methods of vegetative propagation for woody species includes rooted cuttings, rooted needle fascicles (pine), grafting and air layering (Thorpe and Harry, 1990). When compared to herbaceous species, woody plants are more difficult to propagate asexually, partly because of the juvenility-maturation phase change they exhibit. This phase change limits regeneration of true-to-type clones in a majority of species, particularly forest trees (Thorpe and Harry, 1990). One objective is to multiply trees which have demonstrated superior traits (Thorpe *et al.*, 1991), but a decline in rooting is seen in plants exceeding 10 years old (Girouard, 1974). Juvenile material is easier to propagate and most successes involve the use of excised embryos and seedling parts, or rejuvenating donor tree parts (Bonga, 1987; Harry and Thorpe, 1994).

Other problems arising in culture includes secretion of polyphenols and tannins by woody angiosperms in response to wounding (Harry and Thorpe, 1994; Thorpe and Harry, 1990), release of volatiles into the head-space, systemic contamination, and vitrification of shoots. Overall, the most advanced micropropagation technology is still labor and cost intensive (Harry and Thorpe, 1994).

1.4 Micropropagation of ornamental and forest species

As pointed out earlier, micropropagation of ornamental species was initiated as an offshoot of efforts by Morel in France (Hughes, 1981; Zimmerman, 1986) to produce virus-free *Cymbidium* orchids from infected plants (Gautheret, 1985). Further stimulus may be attributable to the development and widespread use of the Murashige and Skoog new medium containing high concentrations of mineral salts (Hughes, 1981; Murashige and Skoog, 1962). Later, other successes with numerous herbaceous crops like tobacco, ferns, African violet, and *Syngonium* added more information to that of Morel (which was rather limited). This nevertheless fostered interest in applying the same techniques to woody plants, which were considered difficult to propagate in vitro (Zimmerman, 1986). Woody plants represent a vast array of types relative to their taxonomy or use, and include both gymnosperms and angiosperms (Thorpe and Harry, 1990). The gymnosperms are typically referred to as softwoods and the angiosperms as hardwoods.

Since the 1930's tissues of woody plants have been cultured (Gautheret, 1930; 1934), but it took another 40 years before entire plants could be regenerated in culture (Dunstan and Thorpe, 1986; Thorpe *et al.*, 1991; Ahuja, 1993). During this period, callus and organ cultures became established for woody plants, with varying degrees of organogenesis, but apparently separate development of shoots and roots. The first success of complete plantlets was reported for the angiosperm aspen, (*Populus tremuloides*) by Winton (1968), and then later for a conifer, long-leaf pine (*Pinus palustris*), by Sommer *et al.*, (1975). The triploid aspen plantlets were regenerated via organogenesis from callus and

the pine from adventitious bud development on cotyledons. By the early 1980's commercial production of woody ornamental plants had exceeded that of fruit trees (Zimmerman, 1986), with earlier successes occurring rapidly with ericaceous plants like azalea, rhododendron and laurel.

Subsequently, a number of woody species have been regenerated *in vitro* (Dunstan and Thorpe, 1986; Thorpe *et al.*, 1991; Capellades *et al.*, 1991; Ahuja, 1993; Bajaj, 1996), in addition to, woody ornamentals (Zimmerman, 1986; Capellades *et al.*, 1991; Debergh, 1994; Ma *et al.*, 1996). Regeneration was accomplished largely by enhancing axillary bud breaking, producing adventitious buds or producing somatic embryos (Harry and Thorpe, 1994).

The history and economic importance of ornamental plants have been reviewed previously (Capellades *et al.*, 1991; Debergh and Read, 1991), and one conclusion is that on a worldwide basis, more than 500 million plants are micropropagated annually with the majority being ornamentals (Debergh, 1994). In the USA (1991) export value exceeded that of all other agricultural commodities, including forestry and agronomic crops (Davies *et al.*, 1994; Anon, 1991). Wholesale value of vegetable crops was $5 billion, citrus, fruit and nut crops were $9.7 billion, and ornamentals (foliage and bedding plants, nursery crops, floriculture, greenhouse) were $8.9 billion. Consumer expenditure on ornamental crops totaled $40 billion (Davies *et al.*, 1994).

Today, micropropagation of ornamentals is the most widely used tissue culture technique (Debergh, 1994), with notable examples being *Gerbera jamesonii* (100%), *Spathiphyllum* (90%), and *Anthurium spp.* (75%) in Western Europe. Numerous types of woody ornamentals in many genera are now being produced commercially (Zimmerman,

1986), with the rose being the most economically important one in the world (Ma *et al.*, 1995). In excess of 20,000 commercial rose cultivars exist, based on only 8 of the known 200 wild species in *Rosa* (Krussmann, 1981; Roberts *et al.*, 1990).

Propagation have been almost exclusively by proliferation of axillary shoots derived from shoot or meristem tips (Zimmerman, 1986). As in other micropropagated woody plants the same stages in the process are routinely followed i.e. stages 0-4 alluded to before (Debergh and Read, 1991). Rooting of shoots using media with low salt concentration and supplemented with an auxin is preferred by many, although rooting can occur on hormone-free or charcoal-containing media (Debergh, 1994). For many herbaceous and woody ornamentals, the double layer technique (Maene and Debergh, 1985) has successfully induced elongation and/or rooting (Debergh, 1994). With this technique, rooting can be induced with minimal labor input, when the final propagation is performed in disposable containers in the culture room, and root induction medium is added automatically through the lids of the containers.

Some of the same problems of tissue cultured woody plants are encountered, chiefly the need to establish mature clones, maintain continuous explanting stock material and hampered evaluations of field performance and stability of phenotypes (Zimmerman, 1986). Major problems impeding reliability are predominantly bacterial contamination (Zimmerman, 1986; Cassells, 1988; Debergh, 1994), and somaclonal variation and physiological abnormalities (Debergh, 1994).

2. ADVENTITIOUS ROOT FORMATION

2.1 Introduction

The term "adventitious root" is widely used to designate a root arising on a site of the plant that is not itself a root e.g. shoot or leaf, or on an already lateralized root axis (Esau, 1953; Barlow, 1994). The inability to induce adventitious root formation in conventional cuttings or tissue culture is a major limiting factor when cloning plants for genetic improvement and commercial applications. Much biochemical and physiological research has thus been conducted to elucidate the control of this important developmental process (Haissig *et al.*, 1992).

The first discovered and identified plant hormone, indolyl-3-acetic acid (IAA), was shown to induce or promote adventitious rooting (Thimann and Went, 1934; Gaspar *et al.*, 1997; Kevers *et al.*, 1997). Subsequently, the idea developed that the auxin (IAA) was the main decisive factor for rooting, a notion further strengthened by the finding that other natural and synthetic auxins had the same effect. Other classes of plant hormones did not have the same effect, so the specific rooting property of auxins led to the conception of adventitious root formation as a single physiological phenomenon (Kevers *et al.*, 1997).

Today the literature refers to this concept as "traditional" because it was based on a series of wrong concepts about auxin and adventitious root formation (Gaspar *et al.*, 1997). Other misconceptions regarded auxin as the main stimulus for adventitious rooting and that exogenous supplies of auxin was required to enhance and/or maintain

high endogenous levels in the plant tissue. Evidence of the existence of inductive/adaptive enzymes that regulate exogenous auxin and cytokinin hormones, coupled with evidence in plants of altered metabolism of other hormones in response to fed hormones, do not support these notions (Gaspar *et al.*, 1997).

Much progress has been made to date and one of the major achievements is the recognition of successive interdependent physiological phases in adventitious root formation (De Klerk *et al.*, 1995; Gaspar *et al.*, 1997; Kevers *et al.*, 1997; De Klerk *et al.*, 1999). At present, a great deal of information exists providing, biological, histological, and biochemical justification for dividing the rooting process into these phases (Kevers *et al.*, 1997). The phases have different exogenous requirements, and the levels of auxin, peroxidases, polyamines, phenolic compounds, cytokinins and ethylene undergo typical changes during these phases (Kevers *et al.*, 1997; De Klerk *et al.*, 1999).

2.2 Phases of adventitious root formation

The rooting process have been divided into the following three physiological phases after exposure to an external rooting stimulus, e.g. auxin

1. Inductive phase
2. Initiation phase
3. Expression phase

The **inductive phase** refers to the period required to allow all the necessary biochemical events that precede the **initiation** of cell divisions that start formation of root

meristems and primordia organization. The period of internal growth and emergence of the root primordia from the cutting is referred to as the **expression** phase.

A prerequisite for these phases is that the target cell(s) must be competent or acquire competence to respond to the external rooting signal. Competence itself refers to a state of the cell that enables it to respond to a specific stimulus and embark on a specific developmental pathway, e.g. adventitious roots. Competent cells that are induced in this fashion, and no longer require the presence of the external signal to complete the successive developmental phases, are said to be determined, even if other environmental conditions are required (Mohnen, 1994).

The duration of the root inductive phase may vary (Gaspar *et al.*, 1997; Hausman *et al.*, 1994), being achieved in less than a day or up to several days in some species. Also, it appears that cuttings at the end of an *in vitro* multiplication cycle may already be in the inductive phase due to a gradual reversal of the auxin to cytokinin ratio in the shoots (Kevers *et al.*, 1997). This could account for previously observed discrepancies where endogenous compounds are analyzed.

2.3 Rooting of microshoots *in vitro*

The inability to induce rooting often limits cloning when using conventional cuttage or tissue culture (Haissig *et al.*, 1992), particularly true for woody species which exhibit reduced rooting capacity as they mature (Mohammed *et al.*, 1989; Thorpe and Harry, 1990; Thorpe *et al.*, 1991). Rooting in coniferous species remains problematic (Dumas

and Monteuuis, 1995; Mohammed and Vidaver, 1988) despite satisfactory results with *Pinus radiata* (Horgan and Holland, 1989).

Past research has largely involved physiological and biochemical studies that have identified factors associated with or influencing rooting but have not elucidated controls (Haissig *et al.*, 1992). The putative roles of a number of biological, physical and chemical factors affecting rhizogenesis in plants have been outlined (Gaspar and Coumans, 1987), including some of the associated metabolic changes. Typically shoots are elongated to an average length of 30mm before they are rooted and acclimatized (Harry and Thorpe, 1994). Fully formed and well-developed leaves are important at this stage as well (McCown and McCown, 1987). A major concern with rooting in vitro is to reduce the number of steps involved with the two-fold objective of reducing production costs and morphological variation (Harry and Thorpe, 1994).

Depending on the species, an auxin may or may not be used to root hardwoods, and usually it is added to agarified medium or as a liquid pulse. Auxin treatments are required for rooting in some species, such as birch and poplar (McCown and McCown, 1987), whereas other species, such as teak and eucalyptus, needed an auxin for rooting (Gupta and Mascarenhas, 1987; Mascarenhas *et al.*, 1987). Generally rootable shoots are pulsed for a short time in solutions of auxins, commonly IBA (Thorpe *et al.*, 1991). Alternatively, the auxin can be added to the substrate in low concentrations or the shoots dipped in commercial rooting powder. The mineral composition of agar medium is generally reduced to 1/4 or 1/2 strength and sucrose levels from 1-2%. Low-salt media such as WPM (woody plant medium) and GD (Greshoff and Doy, 1972) are favorable for increasing rooting percentages (Chulapa, 1987).

Rooting ability is slow and difficult in conifers compared to angiosperms, in part due to the absence of preformed root initials (Mohammed and Vidaver, 1988). Spontaneous rooting is low and ranges from 1-5%, with *P. radiata* being one exception (36% rooting) (Mohammed and Vidaver, 1988). Although most conifers have been rooted *in vitro*, a rooting treatment is required to obtain satisfactory levels. Some factors affecting rooting include shoot vigor, juvenility, reduced sucrose and minerals in the media, increases in agar concentrations and types, and the use of soil-less mixes.

Auxins are necessary for rooting of conifers, and usually NAA, IBA and IAA are used (Mohammed and Vidaver, 1988). Application can be by continuous exposure, as a rooting powder or short duration pulses. The latter have been most effective (Mohammed and Vidaver, 1988), and important considerations, as seen with *Pinus canariensis*, are the use of an effective auxin or combinations, rooting solution concentration and exposure times (Martinez Pulido *et al.*, 1990).

Usually the auxin is added to an agar substrate, which provides uniform distribution of the hormone and nutrients, as well as good shoot-substrate contact for promotion of more synchronous rooting. However root quality in agar may not always be acceptable, as is seen in white spruce where non-functional roots are problematic (Toivonen, 1985). In *Acacia*, roots in agar were lacking both fully developed vascular systems and root hairs, but were normal in liquid medium (Skolmen and Mapes, 1978). Compared to angiosperms, root primordia of conifers appear sooner (4-6 weeks) on semi-solid media and are usually thicker with fewer lateral roots (Harry and Thorpe, 1994).

Several chemicals called "auxin synergists" or "co-factors" have been found to enhance rooting response to applied auxins (Thorpe *et al.*, 1991; Mohammed and

Vidaver, 1988; Jarvis, 1986). Aromatic amino acids and simple phenolics have been demonstrated in *Pinus radiata* (Smith and Thorpe, 1977), and vitamin D in *Populus tremula* (Pythoud *et al.*, 1986).

CHAPTER 3 THE ESTABLISHMENT AND MAINTENANCE OF MICROSHOOTS AND BACTERIAL CULTURES

The following gives a detailed account of the different methods, procedures and techniques used to establish and maintain cultures of the various woody plants and bacterial species used in this investigation.

1. *Pinus ayacahuite*

Pinus ayacahuite var. *ayacahuite* Ehrenb. (Mexican white pine) is native to Central America and is found at elevations of 1800-3200m above sea level. The species is endangered due to deforestation practices, as its wood is used widely for firewood, furniture, roofing and carving purposes. Conventional propagation of *P. ayacahuite* from seeds is difficult due to a characteristic long seedling phase which is further compounded by little success in achieving genetic improvements from conventional tree breeding methods (Saborío *et al.*, 1999).

1.1 *Source of seeds*

Mature seeds of *P. ayacahuite* obtained from CAMCORE (Central America and Mexico Coniferous Resources Cooperative) were used in this study. Seeds from the provenances Los Cubes, Las Trancas and Ixtlán were selected based on the performance of their embryos (Table 3.1) demonstrated *in vitro* (Saborío *et al.*, 1999).

1.2 *Sterilization and stratification procedures*

These steps were followed as described by Saborío *et al.*, (1999). Briefly, equal quantities of these seeds were mixed and imbibed for 48 h under cold, running tap water.

Non-floating seeds were then surface sterilized for 15 min with a 30% Javex™ solution containing Tween™ (4 drops per 100 ml), followed by 3 rinses with sterile water. After 9 days of stratification at 4 °C, the seeds were surface sterilized again (same as before). The seed coats were removed and the megagametophyte given a third, and identical sterilization. The embryos were excised with care and cultured on a 1% sucrose medium solidified with 0.7% agar (DifcoBacto™) in the light.

1.3 *Bud induction and bud growth*

To induce buds, the cotyledons of 3 day old embryos cultured on sucrose (1%) and agar (0.7%) were used. Excised cotyledons were then cultured for 2 weeks on MCM medium (Bornman, 1983), containing 10 or 50 μM N^6-benzyladenine and sucrose (3%). Bud development and shoot elongation were achieved by transferring to half-strength AE medium (von Arnold and Ericksson, 1978), supplemented with AC (0.05%) and sucrose (2%) and no hormones. Shoots with a length of 3-5 cm were excised and used for rooting experiments.

Table 3.1 Comparison of the responses of three provenances of *Pinus ayacahuite* seeds to micropropagation *in vitro*. The results are based on 120 seeds from each provenance, which were hydrated for 48h under running tap water, sterilized and stratified for 7 days at 4° C. The embryos were removed and cultured on agar and sucrose medium for 3 days. Cotyledons were cultured for 15 days on bud induction medium (MCM, 3% sucrose, 50μM BA). They were then transferred to MCM plus 2% sucrose for 30 days, followed by subsequent transfers every 30 days to ½ AE plus 2% sucrose and 0.05% AC. Cotyledons were evaluated at Day 105. (Adapted from Ph.D. thesis (Saborio, 1996)).

Provenance	% Responding embryos	% CFB[1] (105 days)	Avg. no. of buds/ cotyledon (105 days) (X ± SE)	BFC[2] (X ± SE)
Los Cubes	83.33	78.89	9.09 ± 1.71	8.76 ± 1.72
Las Trancas	66.67	76.67	8.60 ± 1.33	7.75 ± 1.41
Ixtlán	63.33	74.45	7.30 ± 1.34	6.61 ± 1.40

[1] %CFB = Percentage of cotyledons forming buds
[3] BFC (Bud Forming Capacity) index = (avg. no. bud per cotyledon) x (% cotyledons forming buds)/100

2. *Picea rubens*

Picea rubens (red spruce) is commercially important for pulpwood, as well as for construction, plywood, crating and interior finishing (Hosie, 1979). It is a prominent species in the northeastern United States and the Maritime - provinces of Canada (Lu *et al.*, 1991). Recent reports indicate declining numbers at high elevations in the United States, possibly from aluminum toxicity and soil acidification. Rooting response ranges from 25-80% with an IBA or NAA pulse treatment, or a dip in rooting powder, and largely depends on shoot quality and age (Lu *et al.*, 1991).

2.1 *Source of seeds*

Red spruce shoots were regenerated by organogenesis of mature embryos. Mature seeds of *P. rubens* were obtained from the Petawawa National Forestry Institute and stored at –20 °C.

2.2 *Surface sterilization*

The seeds were imbibed in running cold tap water for 48 h and then surface sterilized for 20 min with a 30% Javex™ solution and rinsed with sterile water (Lu *et al.*, 1991). They were then treated with a 10% H_2O_2 solution for 5 min and rinsed with sterile water.

Seeds were either immediately used for embryo dissection or stored at 4 °C for future use.

2.3 *Bud induction and shoot development*

Embryos were dissected and cultured on ½ MCM medium supplemented with 3% sucrose and 10 μM BA for 2 weeks in the light. Bud development was achieved by transferring directly to ½ AE medium containing no hormones and 0.1% AC. After the buds developed into shoots they were cultured in jars and allowed to elongate on the same medium. Shoots 3-4 cm were separated and used for rooting experiments (Lu *et al.*, 1991).

3. Ornamental species

The two woody angiosperms of choice for this research were *Crataegus spp.* (Hawthorn) and *Cotinus coggygria* (Smokebush), representatives of the families *Roseaceae* and *Anarcardiceae* respectively. *In vitro* cultures of these species involved, microcuttings supplied generously by AgriForest Technologies Incorporated, a biotechnology company located in Kelowna, British Columbia (courtesy of Dr. K. Patel). Typically each microcutting comprised 2-3 nodes from which axillary buds will develop. In addition, the Hawthorn cuttings develop a large, swollen stem base and callus from which more shoots arise. The microcuttings were multiplied and maintained on full strength MS medium (Murashige and Skoog, 1962) supplemented with BA (1.0 mg/L) and 3% sucrose in clear jars. For axillary bud breaking and shoot elongation the medium

was further supplemented with 0.01% AC. The medium was solidified with Agargel™ (4 g/L). On this medium the microshoots can be regenerated every 2-3 weeks. For rooting experiments the nitrates in the medium were reduced to half-strength, and shoot lengths ranged from 2.5-3.5 cm for both ornamental species.

3.1 *Crataegus spp.*

Crataegus spp. var. Toba (Hawthorn) is considered to be difficult-to-root ornamental plant and *in vitro* rooting success is about 20% with auxins (K. Patel, personal communication). It is a large genus consisting of small trees and coarse shrubs grown as ornamentals and for woodcarving (Hosie, 1979). About 25-30 species are found across Canada, and are easily recognized by their predominant winter buds, thorns, lobed leaves, zigzag twigs, perfect clustered flowers and apple-like fruits.

When the mother explants arrived, initial attempts to establish the cultures were plagued by contamination. The source and nature of the contamination was undetermined, but it appeared to be bacterial and endogenous. As a consequence, the technique of shoot tip culture had to be employed (Nehra and Kartha, 1994). Briefly, the shoot tips (1-2 mm) which comprised the apical meristem and one or two rudimentary leaf primordia were excised and cultured on full strength MS medium in glass jars containing BA (1mg/L) and 0.01% AC (see above). These shoot tips were established fairly easily and they grew rapidly on this medium. After one week in culture the original shoot tips had proliferated greatly and frequent sub-cultures were performed every 2 weeks to the same medium until contaminant-free cultures were established. Contaminated plant materials were autoclaved before disposal. Concurrently a novel

product called PPM™ (Plant preservative mixture, Plant Cell Technology Inc.) was tested for its efficacy in controlling contaminated tissue cultures. It is a heat stable, broad spectrum preservative and biocide claimed to reduce or prevent microbial contamination in tissue cultures. It targets enzymes in the Krebs cycle and electron transport chain, being biocidal at >2ml/L and biostatic at <2ml/L. Preliminary trials showed it appeared to check the contamination, but it inhibited shoot multiplication and elongation compared to the controls (data not shown). For this reason its use was discontinued and not included in future tissue culture work.

3.2 *Cotinus coggygria*

Cotinus coggygria var. S*mokebush* Mill, is a member of the Smoke Tree genus of the cashew family (*Anacardiaceae*). The genus contains 2 species, one in North America and the other in Europe. Members are small shrubs or trees with yellowish wood. Leaves are simple, alternate, deciduous and entire along the margins. Smokebush is valued as a high priced ornamental plant in Canada. It is also considered to be difficult-to-root species *in vitro* (K. Patel, 30-40% personal communication).

4. Establishment of *Agrobacteria* cultures

4.1 *Sources of bacteria*

For this investigation two strains of *Agrobacterium tumefaciens* (A281x200 and EHA101) and several strains of *Agrobacterium rhizogenes* were used. The *A. tumefaciens*

strains were, generously supplied by Dr.Maurice Moloney (University of Calgary). Strain A281x200 carries the plasmid pCGN200 is oncogenic (armed) (Hood *et al.*, 1984; 1986), and is resistant to kanamycin. Strain EHA101 (Hood *et al.*, 1986) is disarmed and also kanamycin resistant.

The *A. rhizogenes* strains were obtained from different sources. Strain R1601, was generously contributed by Dr. Dave Ellis of BC Research Incorporated, (Vancouver, BC). Strain R1601 is both kanamycin and carbenicillin resistant. It has the chromosomal background from RA4 and carries the plasmid pTVK291, which contains the virulence region from the super-virulent Ti-plasmid pTiBo542 (Ellis *et al.*, 1989). The wild-type strain 15834 was obtained from Dr. H. Flores of the Biotechnology Institute (Pennsylvania State University, PA, USA). All strains of *Agrobacteria* were grown on solid or liquid AB-media prepared freshly from sterile stocks of 20X AB salts, 20% glucose, 1M $MgSO_4$, 0.1M $CaCl_2$ and $FeSO_4.7H_2O$ (0.25mg/ml), supplemented with the specified antibiotic (kanamycin, gentamycin, timentin or carbenicillin).

4.2 *Storage of bacteria*

To establish glycerol stocks of the bacterial strains, they were grown at 28° C on the appropriate solid medium and using the specified antibiotic (Watson *et al.*, 1975). For strains A281x200, EHA101 and R1601, it was with kanamycin (100μg/ml); and for strain 15834 it was without antibiotic but with biotin (vitamin H) at 0.2mg/ml. After 2-3 days

pure colonies were selected and inoculated into liquid media (5ml) using a sterile toothpick, along with the specified antibiotic overnight at 28° C on a shaker, to an ocular density of 0.5-0.6 (A_{600}).

Glycerol stocks, (1000 µl), were made by combining 500 µl each of a sterile 50% glycerol solution and the bacterial broth in Eppindorf tubes (1.5 ml), for a final concentration of 25%. These cultures were mixed on a vortex and subsequently stored at –80 ° C for future use. When required, sterile toothpicks were used to directly inoculate solid media in Petri dishes or liquid media in culture tubes (5 ml). The glycerol stocks were not allowed to thaw and refreeze.

5. Equipment and materials

5.1 *Tissue culture techniques*

All tissue culture procedures were conducted under aseptic conditions in a laminar flow hood (Envirolab™ Sterility Module, Envirco Incorporated, Albuquerque, New Mexico). All media were prepared from stock solutions (100X) made from chemicals supplied by Sigma® and Fisher®, and stored in the refrigerator at 4 °C. Plant hormones and other chemicals were either added directly to the media before autoclaving or after by filter sterilizing using 0.2 µm filters (Acrodisc™). All media were autoclaved at 120 °C and 15 psi for 20 min. Gelling agents included DifcoBacto™ agar (for MCM and AE media), Agargel™ agar (for MS medium) and Gelrite™ (for GD medium).

Transwells (Costar®) with a pore size of 0.1 μm were used to separate shoots from bacterial cultures in the same culture jars. Holes 0.5 cm deep were made in the solid medium with a sterile glass rod and the transwell inserted. Medium was then placed inside the transwell using sterile tips.

5.2 *Growth chamber conditions*

All cultures were maintained at 24 °C ± 1 °C under a 16 h photoperiod (8 h dark), generated by F40T12 Gro-WS lamps (Sylvania Gro-Lux™) with a photosynthetic photon flux of 80-100 μmol m^{-2} s^{-1}. All experimental units in the same experiment were kept together on the same shelves to minimize variation due to environmental conditions.

5.3 *Photography and film development*

Photographs were taken with a manually operated Nikon camera. The color film used was Kodak Gold™ 200 ISO. For black-and-white photographs, Kodak Plus-X Pan film was used, and the film was developed using TMAX developer at 20 ° C for 5.5 min. The negatives were then printed on Kodak Professional Polycontrast III RC paper in the dark room.

CHAPTER 4 *IN VITRO* ADVENTITIOUS ROOT FORMATION WITH *AGROBACTERIA*

1. The rooting of woody plants by *Agrobacteria*

In vitro micropropagation has improved the vegetative propagation of many recalcitrant woody species tremendously; however, rooting the microshoots remains far from routine. In difficult-to-root cuttings, several approaches have been adopted to promote root initiation. To enhance vegetative propagation of ornamental species, *Agrobacterium rhizogenes* have been used increasingly (Hatta *et al.*, 1996). Successful root induction using, various strains of *A. rhizogenes* have been achieved in several woody species of horticultural and forest interest. They include almond (Strobel and Nachmias, 1985), olive (Strobel *et al.*, 1988), apple (Patena *et al.*, 1988), hazelnut (Bassil *et al.*, 1991), and kiwi (Rugini *et al.*, 1991); in *Pinus monticola*, *Pinus banksiana* and *Larix laricina* (McAfee *et al.*, 1993). Recently successful rooting has also been achieved with *Ziziphus jujuba,* which is valued for its ornamental quality (Hatta *et al.*, 1996).

Other notable successes include eucalyptus, Douglas fir, *Castanea sativa*, *Malus*, larch, hybrid poplar and black locust (Burns and Schwarz, 1996). The technique is not new and had been suggested earlier by Riker (1930). Seen as a natural example of genetic engineering, the results of these studies exemplify the attention given to exploring the host range possibilities of *A. rhizogenes.*

2. The *Agrobacteria* system

The *Agrobacteria* system comprises two species, *A. rhizogenes* and *A. tumefaciens*, and exploits a natural genetic transformation ability across kingdoms. These bacteria are

capable of transferring segments of their genome into the DNA of recipient plant cells, where they are stably integrated and expressed.

Agrobacterium rhizogenes induces the pathogenesis known as hairy root at the site of infection in the plant. Hairy root disease is characterized by prolific adventitious root development. *Agrobacterium tumefaciens* causes crown gall disease, inducing tumors characterized by amorphous disorganized callus or by shoot teratomas.

In virulent strains of both of these bacteria the disease develop after transfer of the bacterial DNA into the plant cell. This transferred DNA (or T-DNA) contains genes that are responsible for the aberrant growths, and is itself part of a large extra-chromosomal bacterial plasmid (> 200 kb). The plasmid that confers the hairy root syndrome is known as the root-inducing (*Ri*) plasmid and for the tumor-inducing ability it is the *Ti*-plasmid.

Agrobacteria belong to the family *Rhizobiaceae* and are closely related to members of the genus *Rhizobium* (Bergey's manual, 1974). They are non-sporing, gram-negative rods (0.8 by 1.5-3.0 μm). The method of classification in the genus can be based on pathogenicity, type of opine synthesized, host range, and one of three biotypes based on metabolic and physiological characteristics (Nester *et al.*, 1984). Both species are soil inhabitants and in the plant they exist as intercellular parasites (Bergey's manual, 1974).

The biology of *Agrobacterium* is now better understood, and the ability to transform plant cells is correlated to the tumor-inducing (Ti) plasmid in *A. tumefaciens* or the root-inducing (Ri) plasmid in *A. rhizogenes* (Klee *et al.*, 1987). Plasmids from several strains of *Agrobacterium* have been well characterized and shown to share the same basic architecture. Two important regions of these plasmids critical for transformation by

transferred DNA are the mobile T-DNA itself and another 30kb region on the plasmid called the virulence (*vir*) region.

The T-DNA is flanked by 25 bp direct repeats which acts as a *cis* element signal for the transfer apparatus (de la Riva *et al.*, 1998). It contains oncogenic genes (*onc*) which direct the synthesis of auxin, cytokinins and opines. Opines are specific, low molecular weight, unusual amino acids produced by transformed cells, and used as a source of carbon and nitrogen by the organism (Smith and Hood, 1995). All the *onc* genes have eukaryotic regulatory sequences for expression, so that expression of these genes by host plant cells is an indicator of successful T-DNA transfer (Klee *et al.*, 1987). Constitutive expression of the hormonal genes results in hormonal imbalances and altered developmental programs in host cells leading to tumors and/or hairy roots. Virulent strains can be disarmed by removing the *onc* genes from the T-DNA segment, a practice useful in vector plasmid construction for plant transformation.

The *vir* region is a regulon containing genes essential for T-DNA transfer (*virA*, *virB*, *virD* and *virG*) or for increasing transfer efficiency (*virC* and *virE*), but are not transferred in the process (de la Riva *et al.*, 1998). The products of the virulence genes mediate the T-DNA transfer process by generating and processing a copy of the T-DNA, facilitating its movement out of the bacterium and into the plant cell. Transcription of these genes is induced by low pH or phenolic compounds (e.g. acetosyringone) produced by wounded plant cells, opines and monosaccharides (Smith and Hood, 1995). The molecular basis of *Agrobacteria* infection of host plant cells is complex and the precise mechanism of T-DNA transfer and its integration in the plant genome is still elusive.

For recent reviews on this topic see Sheng and Citovky (1996) and de la Riva *et al.*, (1998).

3. Genes isolated from *Agrobacteria* T-DNA

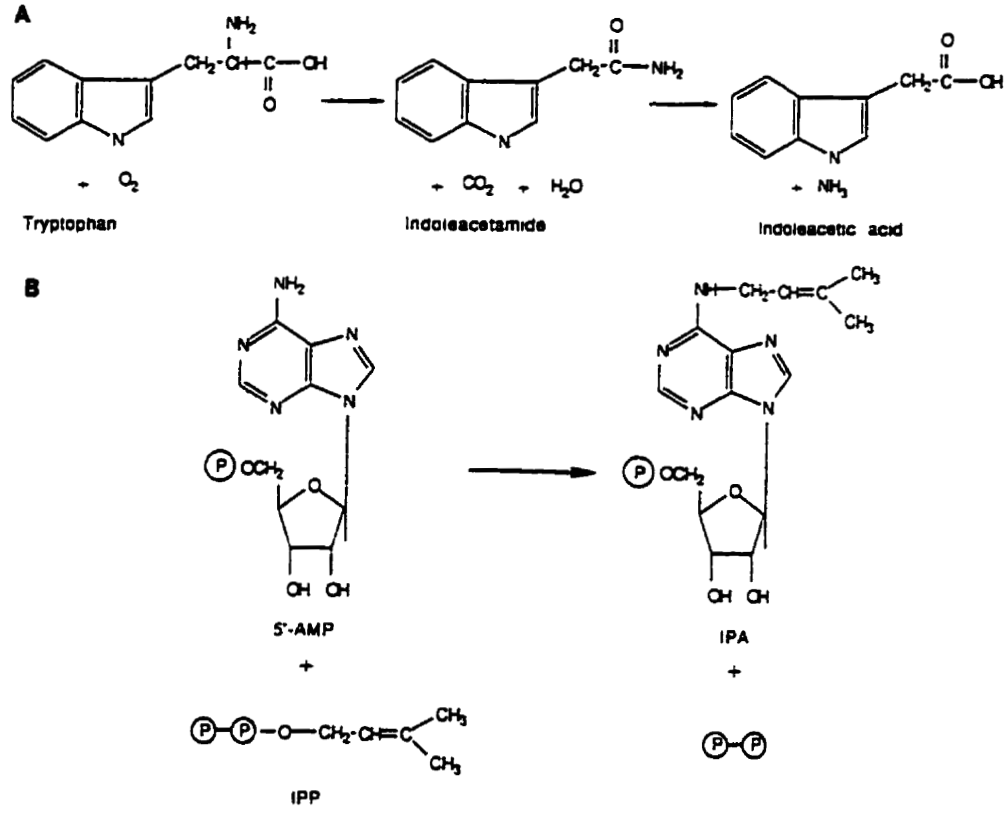
Several genes have been isolated from the T-DNA of *A. tumefaciens*, including *iaaM, iaaH, iptZ* (Figure 4.1). The *iaaM* gene codes for an enzyme tryptophan monooxygenase, which converts tryptophan to indole-3-acetamide. This is then converted to indoleacetic acid (IAA) by the product of the *iaaH* gene, indoleacetamide hydrolase. The *iptZ* gene encodes isopentenyl transferase, which synthesizes the cytokinin isopentenyl adenosine from isopentenyl pyrophosphate and adenosine monophosphate (Klee *et al.*, 1987). Another gene isolated recently is called gene 5 (Korber *et al.*, 1992; Rinallo and Mariotti, 1994), which codes for a 26 kd protein capable of transforming tryptophan into indole-3-lactate, an auxin analogue. The involvement of these genes in phytohormone biosynthesis, have been demonstrated biochemically (Klee *et al.*, 1987), and the genes are bacterial in origin, since plants do not normally utilize the biosynthetic pathway of *Agrobacterium*.

In *A. rhizogenes* four genetic loci have been identified (White *et al.*, 1985; Blakesley, 1994; Rinallo and Mariotti, 1994), *rolA*, *rolB*, *rolC* and *rolD*. *RolB* and *rolC* are important since they code for glucosidase activity that releases free auxin and cytokinin, respectively, from inactive conjugates. Although they affect the same type of hormones, the mode of action is completely different from the genes isolated from *A. tumefaciens*.

Figure 4.1 Pathways for (a) auxin and (b) cytokinin biosynthesis by the T-DNA genes of *Agrobacterium tumefaciens* (Adapted from Klee *et al.*, (1987)).

(a) The auxin pathway involves conversion of tryptophan to indoleacetamide by tryptophan monooxygenase and then to indoleacetic acid (IAA) by indoleacetamide hydrolase

(b) The cytokinin pathway involves the synthesis of isopentenyl adenosine from isopentenyl pyrophosphate and 5' – adenosine monophosphate by isopentenyl transferase.


A
Tryptophan
Indoleacetamide
Indoleacetic acid
B
5'-AMP
IPA
IPP

4. Rooting with *Agrobacterium tumefaciens*

Saborío *et al.*, (1999) recently demonstrated in our laboratory that several strains of *Agrobacterium tumefaciens* promote formation of adventitious roots in microcuttings of the tropical conifer species *Pinus ayacahuite*. This was achieved when the shoots and bacteria were in direct contact or separated by the use of Transwells™ of pore size 0.1 μm (Corstar™). The finding was significant since the rooting percentages were higher (75-100%) when compared to traditional auxin treatment (20-40%) and controls (0%). Rooting trials with auxin also yielded inconsistent results and varying degrees of spontaneous rooting was observed in some treatments. The conclusion was that the bacterial strains somehow improved the rhizosphere for adventitious root formation *in vitro*, presumably by producing some unknown but diffusable chemical substance (s). That rooting was obtained without contact between the plant cells and the bacteria supports the idea that a transformation event did not occur. If this was the case, then inclusion of bacterial culture inoculations into rooting protocols of other woody species, perhaps by pre-conditioning the rooting medium, would have practical significance in increasing overall rooting percentages in recalcitrant species.

For the above reasons, we wanted to further investigate Saborío's (1999) observations and to see if such rooting responses by co-culture with *A. tumefaciens* is specific to *P. ayacahuite*, or if the technique can be applied to other difficult to root woody species, representative of both gymnosperms and angiosperms. This becomes very important since the vast majority of economically and commercially important micropropagated woody species are difficult to root *in vitro* or *ex vitro* using conventional auxin

treatments, or they simply exhibit low rooting capacities (Mohammed and Vivader, 1988; Mohammed *et al.*, 1989; Haissig *et al.*, 1992).

For these reasons we selected two commercially valuable woody ornamental plants from different families, *Cotinus coggygria* and *Crataegus spp.* (angiosperms), and one important forest tree species, *Picea rubens* (gymnosperm) to conduct this investigation. All three of the plant systems can be regenerated via organogenesis (axillary bud breaking and bud induction on zygotic embryos respectively), but prove to have problematic and inconsistent rooting qualities (Lu *et al.*, 1991; personal communication).

Commercial production of the selected ornamentals are underway (AgriForest Industries, BC), but tremendous losses are incurred at the rooting and post-rooting stages (see Chapter 3). To alleviate this a more efficient rooting protocol needs to be developed which will yield close to 100% rooting percentages, while reducing costs and making the endeavor viable. Currently, these microshoots are given a quick dip in a rooting powder containing IBA as the active ingredient, and transferred to the greenhouse. Whatever rooting numbers are attained by this shotgun approach are accepted by the company.

The objective of this study is to conduct co-cultivation experiments with the same strains of *A. tumefaciens* used by Saborío *et al.*, (1999), to investigate the root promoting effects in vitro, and to see if the technique can be of practical use in micropropagation operations.

5. Materials and Methods

5.1 ***Shoot quality and selection:*** Shoot production for all species were maintained and proliferated as described previously in Chapter 3, and before each co-culture experiment new shoots were multiplied and elongated to the desired lengths (3-5 cm). *In vitro* shoots were obtained from cotyledons for *P. ayacahuite* and from embryos in the case of *P. rubens*. For *C. coggygria* and *Crataegus spp.*, they were obtained from axillary branching. Freshly wounded (cut) shoots were separated and inoculated immediately with *Agrobacteria* in direct co-culture trials. For experiments with Transwells™, the shoots were planted evenly around the centrally inserted unit and the latter inoculated with a sterile toothpick dipped in the *Agrobacteria* culture. Treatments (bacterial strains) were replicated twice, each comprising of 10 shoots per glass jar. For the controls, neither the shoots nor the medium were inoculated with bacteria. Rooting was evaluated after 30 and 45 days. Each experiment was repeated a minimum of three times. Cultures were maintained at 24 ± 1° C under a 16h photoperiod and photosynthetic photon flux of 80-100 μmol m^{-2} s^{-1}.

5.2 ***Rooting media and conditions*:** For the woody angiosperms the basal rooting medium was half-strength (KNO_3) MS medium (Murashige and Skoog, 1962). Neither hormones nor auxin was added to this hormone free medium (HFM), and it was solidified with Agargel™ (4g/L). In other experiments the effect of sterile vermiculite moistened with MS medium was tested. For *P. ayacahuite* shoots, the rooting medium was ½ GD

(Greshoff and Doy, 1972; half-strength macro elements and 2% sucrose). For *P. rubens* shoots, the rooting medium was ½ GD containing 2% sucrose and 0.3% Gelrite™.

5.3 ***Strains and inoculation methods:*** Several strains of *Agrobacteria* were compared for their ability to induce adventitious roots. The *A. tumefaciens* strains utilized were A281x200 and EHA101 (see Chapter 3), and the *A. rhizogenes* strains were R1601 and 15834. Shoots were inoculated with these strains by either dipping the stem bases for 1 second in a liquid broth grown overnight, or in a 2 day-old pure colony culture grown on solid media. The bacterial cultures were grown and handled under the conditions specified in Chapter 3.

5.4 ***Co-culture periods:*** Initially co-culture in direct contact with the bacteria lasted 21 days (Saborío *et al.*, 1999), but this was varied from 1, 7 14, 21 and 28 days during the course of the research. Overgrowth of bacteria was checked by frequent sub-cultures to the same medium every 3-4 days, and finally to the same medium, containing the specified antibiotic.

5.5 ***Incisions:*** The stem bases of shoots were either cut square, diagonally or further wounded by making a small vertical cut with a scalpel to determine if incision was a factor affecting the ability of the *Agrobacteria* strains to induce adventitious rooting.

5.6 ***Antibiotics:*** In all direct co-culture experiments the bacteria were killed by supplementing the respective rooting medium with the antibiotics Carbenicillin (Sigma®, di-sodium salt) at a rate of 500 mg/L or Timentin® (SmithKline Beecham) at 200 mg/L.

6. Results

6.1 Rooting with *A. tumefaciens*

When microshoots of *P. rubens*, *C. coggygria* and *Crataegus spp.* were co-cultured in direct contact with *A. tumefaciens* strains, no adventitious roots formed (Table 4.1). Instead, the cut stem bases of test shoots developed little to no callusing during co-culture. The effects of incisions, the inoculation methods, the nature of bacterial cultures and media type did not promote roots or enhanced callusing during co-culture with the bacteria. Long co-culture periods (>7 days) reduced survival of the shoots and in some cases caused death, particularly with the ornamental shoots.

When *P. ayacahuite* shoots were inoculated with *A. tumefaciens* and co-cultured for 21 days, rooting was observed, but only after 60 days in culture (Table 4.1 and Figure 4.2 A, B and C). The percent rooting was 20-30% compared to controls and previously published results. When the experiment was repeated no rooting was observed.

No adventitious rooting in the test species, (including *P. ayacahuite*), occurred when the shoots and the bacteria were separated by the Transwell™ membrane (0.1 μm) in the same culture jars (Table 4.1). Some of the *C. coggygria* shoots developed a tumor-like structure at the site of inoculation (Figure 4.3, A and B).

Table 4.1 The responses of various woody species *in vitro*, co-cultivated with two strains of *Agrobacterium tumefaciens*. The shoots were either in direct contact with the bacteria or separated by a Transwell™ unit with a pore size of 0.1 μm.

				Direct Contact
		Microshoots		
Agrobacteria	*P. ayacahuite*	*P. rubens*	*C. coggygria*	*Crataegus spp.*
A281x200	roots	no roots	no roots	no roots
EHA101	roots	no roots	no roots	no roots
				Transwells™
		Microshoots		
Agrobacteria	*P. ayacahuite*	*P. rubens*	*C. coggygria*	*Crataegus spp.*
A281x200	no roots	no roots	no roots	no roots
EHA101	no roots	no roots	no roots	no roots

Figure 4.2 Shoots of *Pinus ayacahuite* rooted *in vitro* by *Agrobacteria* strains.

A and B: Shoots produced roots when co-cultured with *Agrobacterium tumefaciens*, strain A281x200.

C. A shoot rooted by *A. tumefaciens*, strain EHA101.

D, E and F: Shoots produced roots when co-cultured with *A. rhizogenes*, wild type strain 15834.

6.2 Rooting with *A. rhizogenes*

In similar co-culture experiments with several strains of *A. rhizogenes*, the bacteria did not infect the host plants and incite the hairy root syndrome. Strains R1000, 13333 and ROLD produced a lot of bacterial overgrowth in the cultures and could not be controlled with available antibiotics. The shoots eventually succumbed to the presence of these antibiotics and died. In one experiment with *P. ayacahuite* microshoots, adventitious roots developed but repeated trials did not yield the same results (Table 4.2 and Figure 4.2, D, E and F). In the other microshoots tested, the cut stem bases did not respond to the treatment (no callus or visible cell division activity) and became black in color. Shoots of *P. rubens* were not included in these trials.

When stem cuttings (3-5mm) of *C. coggygria* were co-cultured with *A. rhizogenes* for 1 day and then transferred to antibiotic containing medium, heavy callusing occurred at the cut ends, but no hairy roots developed from *in situ* cells or the callus. This was seen in 100% of the stem cuttings when co-cultured with both strains 15834 and R1601, but not in the controls (Table 4.3 and Figure 4.3 D, E and F).

Table 4.2 The responses of various woody species *in vitro* co-cultivated with two strains of *Agrobacterium rhizogenes*. The shoots were in direct contact with the bacteria. (na = not assessed)

		Direct Contact		
		Microshoots		
Agrobacteria	*P. ayacahuite*	*P. rubens*	*C. coggygria*	*Crataegus spp.*
15834	roots	no roots	no roots	no roots
R1601	no roots	no roots	no roots	no roots
13333	na	na	no roots	no roots
ROLD	na	na	no roots	no roots
R1000	na	na	no roots	no roots

Table 4.3 The effect of *A. rhizogenes* strains *in vitro* on stem cuttings and microshoots of *C. coggygria*.

	Response (roots/callus)		
C. coggygria	Control	R1601	15834
Shoot	none	none	none
Stem cutting	none	callus	callus

Figure 4.3 Microshoots and stem cuttings of *C. coggygria* showing different responses to *Agrobacteria.*

A. Microshoots showing tumor-like structures at the cut stem bases when planted directly into the agar after inoculation with strain A281x200.

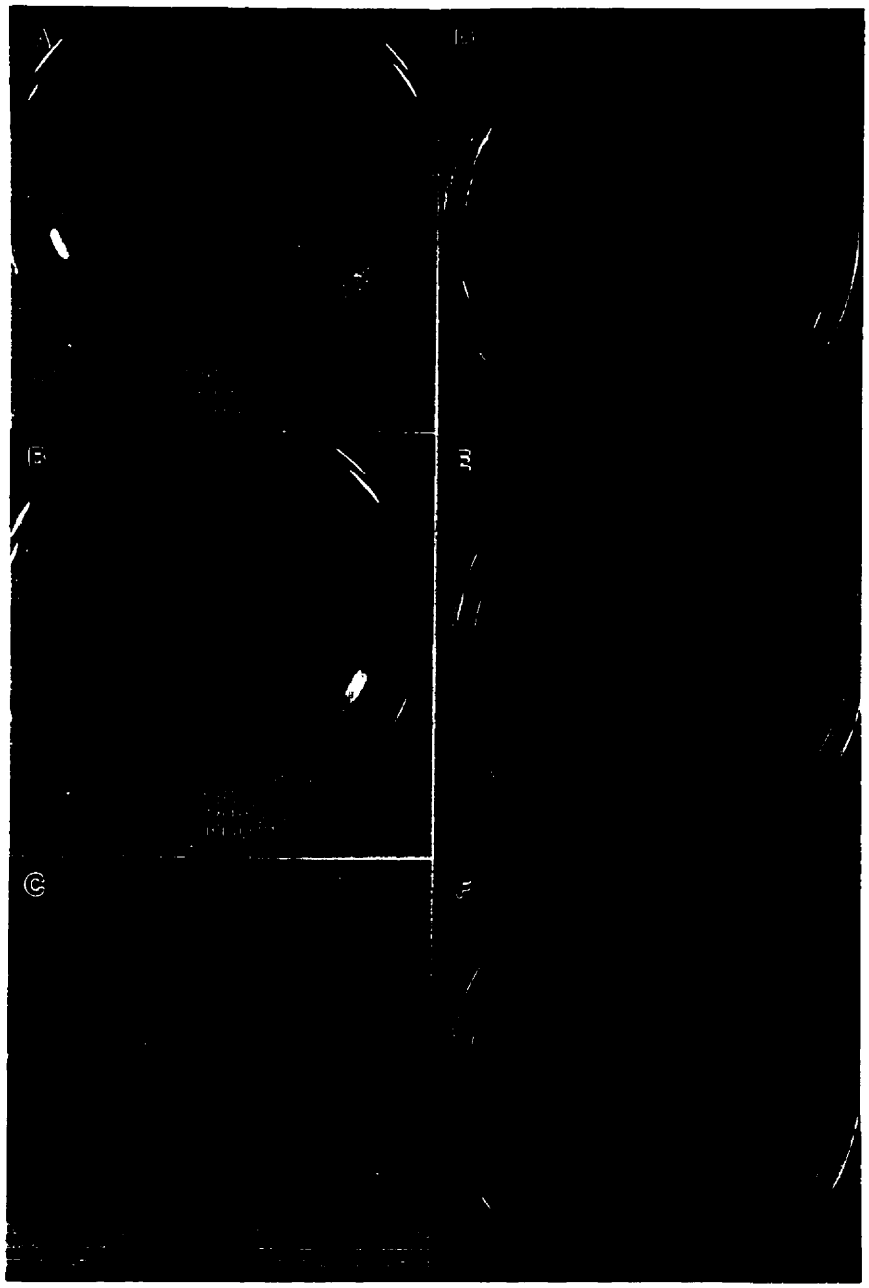
B. The shoots produced fewer tumor-like structures when they were gently placed on the agar for 1-2 days after infection with A281x200.

C. Microshoots in culture tubes after inoculation with *A. rhizogenes* (strain 15834).

D. Control stem cuttings on MS medium.

E. Callus formed in response to *A. rhizogenes* co-culture with strain 15834 for 2 days. The cuttings were later transferred to antibiotic medium (500mg/L Carbenicillin)

F. Similar response seen when co-cultured with *A. rhizogenes* strain R1601.



7. Discussion

The initial objective of the foregoing research was to assess whether *Agrobacterium tumefaciens* promoted the formation of adventitious roots *in vitro* in several economically important woody species. Up to eleven strains of this bacteria were shown to promote rooting in *Pinus ayacahuite* (Saborio *et al.*, 1999). The rooting response was reported in situations where the *in vitro* regenerated shoots were co-cultured in direct contact with the bacteria in the same culture vessel, and also when they were separated by Transwell™ units with a pore size of 0.1 μM, which restricts the migration of the bacteria. The strains shown to be most effective by Saborio *et al.*, (1999) were A281x200 (oncogenic) and EHA101 (disarmed).

The occurrence of a transformation event was ruled out since collectively, the rooted shoots did not produce galls or tumors, the EHA101 strain is non-oncogenic and the Transwell™ units occluded physical contact between the wounded plant cells and the bacteria (Saborio *et al.*, 1999). *A. tumefaciens* is the soil pathogen that incites the crown gall disease in susceptible host plants (Nester *et al.*, 1984), and is not expected to produce adventitious roots in infected plants. It was concluded that the bacteria produced a root promoting substance or combination of chemicals that was diffusable in nature and capable of improving the immediate rhizosphere where they are grown.

Similar conclusions were drawn in other co-culture studies (McAfee *et al.*, 1993) whereby increased rooting could not be ascribed to DNA transfer and the possibility of low copy transformation may be involved. Although the nature of this interaction

between microshoots of *P. ayacahuite* and *A. tumefaciens* remains elusive, the increased rooting percentages attained by their co-cultivation, is certainly an attractive one. Additionally, it was not determined whether this observation was specific to *P. ayacahuite* shoots or can be demonstrated in other plant systems.

In this study, I tested the efficacy of adventitious root induction by *A. tumefaciens* for microshoots of *Crataegus spp., C. coggygria* and *Picea rubens* (in addition to *P. ayacahuite* shoots) under *in vitro* conditions. Microshoots of these species did not produce adventitious roots in either direct contact with the bacteria, or when separated by Transwell™ units (Table 4.1). Various factors such as incisions, inoculation methods, co-cultivation period etc. were also evaluated during the course of the study, but these did not influence rooting. All shoots produced little to no callus at the cut stem bases in both treatment and control conditions, suggesting that such responses are not attributable to the *Agrobacteria.* Also shoot growth and vigor deteriorated rapidly as the co-culture periods were increased, particularly when in direct contact with the bacteria. Attempts to validate the response seen with *P. ayacahuite* under similar conditions resulted in a limited rooting response (Figure 4.1, A, B and C), and further attempts were not successful. The physiological state of the shoots, the decline in quality of seeds and the source of the seeds (provenance) may have possibly contributed to these results.

Only some shoots of *C. coggygria* produced tumor-like growths when co-cultured in direct contact with strain A281x200 (Figure 4.2, A, and B). Although the response was not 100%, it is consistent with the mode of action typical of *A. tumefaciens* infection of susceptible host plants. These findings suggest that *A. tumefaciens* are not capable of inducing adventitious roots in other woody species and confirms that the interaction

between *P. ayacahuite* and this bacterium may be a specific one. It also demonstrates that at least one species (*C. coggygria*) is susceptible to the virulent A281x200 bacterium.

It is well established that *A. tumefaciens* is capable of expressing the transferred genes *iaaM*, *iaaH* and *iptZ* genes in host plant cells and brings about the crown gall phenotype (Nester *et al.*, 1984). These genes are bacterial in origin but contains eukaryotic regulatory sequences and codes for key enzymes in auxin and cytokinin biosynthesis respectively in the tumorous cells. The auxin biosynthetic pathway utilized by *Agrobacterium* is not normally used by plants (Klee *et al.*, 1987). Several reports substantiate the fact that some strains of *A. tumefaciens* produce significant levels of IAA and cytokinins when grown in culture (Nester *et al.*, 1984). Weiler and Spanier (1981) demonstrated the production of a range of compounds with plant regulatory properties produced by *A. tumefaciens* and released into the culture medium. Using the virulent strain B6 and a non-virulent strain B6-37, they followed the kinetics of the formation of these compounds and quantified several growth regulatory substances. Among these were IAA, ABA, $GA_{3/7}$, trans-zeatin and isopentenyladenine-type cytokinins which were mainly produced during the mid to late log phase. Under the tested conditions, the virulent strain was shown to produce slightly more IAA than the avirulent strain. These results however confirmed previous reports of IAA production by *A. tumefaciens* (Liu and Kado, 1979). In both these studies, the bacteria were either grown in a rich medium or included tyrosine or tryptophan.

When Saborio *et al.*, (1999) evaluated bacterial filtrates of *A. tumefaciens*, no roots were induced in *P. ayacahuite* and the bacteria were grown on a minimal AB medium. The observation that strain B6 releases growth regulatory substances might support the

view that these can improve the rhizosphere and aid in inducing roots or tumors in wounded plant cells during co-culture.

Other types of root stimulating bacteria (RSB) have been shown to have a root stimulating effect during co-culture *in vitro* with slash pine (Burns and Schwarz, 1996). The bacterium was isolated from the explant tissue (*Pinus elliottii* Engelm.) and remains unidentified; however it promoted up to 90% rooting in seedling-derived hypocotyls. The researchers are currently exploring the direct application of RSB to other important pines and conifers, and determining the mechanism of its morphogenetic activity. Co-culture with mycorrhiza have also stimulated adventitious root formation in *Pinus pinaster* (David *et al.*, 1983), and affected root morphology in *Prunus cerasifera* (Berta *et al.*, 1995).

The poor rooting capacity of many micropropagated shoots has resulted in increased use of *Agrobacterium rhizogenes* in many studies for improving rooting numbers and quality (Kevers *et al.*, 1997). In experiments where the shoots were co-cultured with *A. rhizogenes* for 1-3 days (Table 4.2), none of the microshoots rooted, except for several *P. ayacahuite* shoot in one trial. Figure 4.1 (A, B and C) shows the phenotype of the shoot and roots produced with strain 15834 (WT), and they do not display the hairy root phenotype. Rooting of conifer shoots by *A. rhizogenes* have been demonstrated previously (McAfee *et al.*, 1993). Attempts to repeat the experiment gave negative results. The other woody angiosperms did not form roots and generally showed no apparent response (e.g. callus) to the treatments. Instead the stems developed a black color in the rooting zone and growth of the shoots appeared to be arrested. In another experiment (Table 4.3), *C. coggygria* stem segments developed huge calluses at the cut

ends when co-cultured with strains 15834 and R1601 compared to the controls (Figure 4.2, A, B and C). No roots were observed from the stem tissue or callus. This was not observed when whole microshoots were co-cultured with the same strains. The reason for this is unknown and these strains are not expected to produce prolific callus.

The soil bacterium *A. rhizogenes* is responsible for the hairy root syndrome in dicotyledonous plants, producing numerous adventitious roots at the infection site. This was demonstrated in tobacco leaf discs (data not shown) to validate the virulence of strain 15834. The neoplastic roots are transformed by fragments of T-DNA from *Ri*-plasmids (Kevers *et al.*, 1997). In agropine strains such as 15834, the T-DNA consists of a left region (TL) and a right region (TR) which contains the agropine and auxin biosynthetic genes. During hairy root induction, the auxin genes have been shown to play an accessory role by providing auxin required to trigger differentiation of cells transformed by the T-DNA (Cardarelli *et al.*, 1987; Kevers *et al.*, 1997), when endogenous plant levels is insufficient. The TL region contains the genes involved in hairy root formation, referred to as *rol* genes A, B, C and D, and corresponds to four of 18 open reading frames (ORF) in this region. Among these, *rolB* is the most crucial and has putative function as a glucosidase capable of hydrolyzing inactive IAA-conjugates or as a component of the auxin (IAA) receptorial complex in transformed cells. The other *rol* genes are involved in accessory functions.

The literature reports on many plant species that are highly recalcitrant to *Agrobacterium* infections (Rinallo and Mariotti, 1994), and assumes the strains possess different virulence levels or they are sometimes inhibited by antimicrobial compounds in plant exudates immediately after wounding (e.g. separation from the mother explant).

Several *A. tumefaciens* strains have been identified that show a wide host range (Smith and Hood, 1995), each strain infecting a large variety of plants with overlapping ranges. Despite this, the strains are all different. Variation in the infection response are sometimes observed e.g. *A. rhizogenes* occasionally produces proliferating callus instead of adventitious roots (Rinallo and Mariotti, 1994). This can be explained in terms of the interaction between the T-DNA and the endogenous level of hormones in the plant tissue. It is not clear if this response seen in *C. coggygria* stem cuttings reflect these arguments.

CHAPTER 5 ADVENTITIOUS ROOTING OF *COTINUS COGGYGRIA* AND *CRATAEGUS SPP.* WITH AUXINS *IN VITRO*

1. Choice of auxin for rooting ornamental *in vitro* shoots

1.1 Introduction

The woody ornamental species used in this research are usually rooted *in vitro* on a mixed auxin regime comprising 0.1 mg/L NAA, 1.0 mg/L IAA and 0.1 mg/L IBA. However rooting success is only 20-30% (K. Patel, personal communication). When the microshoots are rooted under greenhouse conditions after a quick dip in rooting powder (IBA is the active ingredient), rooting success is 40-50%. To compound this, a further 50% loss is encountered while in the greenhouse, and before they get to market, presumably due to root functionality problems. The microshoots of these woody ornamentals can be regenerated with relative ease from axillary buds, but to date no precise *in vitro* rooting protocols have been developed for *C. coggygria* and *Crataegus spp.* respectively.

From preliminary rooting experiments that I performed *in vitro* with *C. coggygria* shoots on this mixed auxin regime, I got slightly higher rooting percentages (50-80%). Although multiple roots (6-7) were produced, the quality of the rooted shoot was poor (Figure 5.1, A, B and C). Heavy callusing and swelling of the shoot bases developed and roots were separated very easily from the shoot base when gently tugged. This is indicative of problems that may be encountered later with the functioning of the root and root-shoot junction; and also, the loss of roots during handling and transfers from the culture room to the greenhouse. A further concern is the exposure to a higher incidence of diseases and infections. These conditions, coupled with the excessive callusing, makes such micropropagated plants very undesirable, and, contributes to accruing of significant

costs in the long run. A treatment with an auxin that produces intact and fully functional roots and root-shoot junctions with little or no callusing would be desirable for commercial markets. For this reason it is important to determine the best choice of auxin or combination of auxins, and the optimal concentration(s) thereof, to produce viable micropropagated plantlets. My preliminary experiments also demonstrated that a small and inconsistent amount of spontaneous rooting occurs in *C. coggygria*. When this happens the microshoots produce an average of 1-2 roots compared to those rooted on the mixed auxins. The increased number of roots in response to the auxins is certainly desirable and can confer greater survival throughout the culture period, if they are intact and fully functional.

As was discussed in Chapter 2, normally some kind of auxin treatment is required to root the micropropagated shoots. For *ex vitro* rooting, cuttings are usually given a quick dip in rooting powder or a concentrated solution of auxin. The result is only a brief exposure to the auxin, with IBA being the auxin of choice (De Klerk *et al.*, 1998). Microcuttings can be rooted in exactly the same fashion, but the preferred way is to root them *in vitro*. Using this approach the shoots may also be pulsed for short periods in solutions of concentrated auxin(s), but most often are exposed to the auxin for lengthy periods. Because of the long duration of exposure to auxin in the media, other auxins or combination of auxins may be optimal for rooting (Gaspar *et al.*, 1997; De Klerk *et al.*, 1998). So for any viable and successful micropropagation scheme, one needs to determine the best choice of auxin(s) for the species in question.

In this study the performance of three auxins commonly used for rooting (NAA, IBA, IAA), and 2,4-D, were tested in *C. coggygria* and *Crataegus spp.* microshoots. The

objective is to determine the best or most suitable rooting treatment for the two ornamental species.

1.2. Determination of the length of the root inductive phase in *C. coggygria*.

1.2.1 ***Exogenous requirements of auxin***: Most woody species such as *C. coggygria* requires treatment with an exogenous source of auxin for adventitious root formation *in vitro*. Researchers have shown that application of auxin is not necessary throughout the entire rooting process, but can be limited to the root inductive phase (Hausman *et al.*, 1995). Continuous exposure to the auxin in some species can result in inhibitory effects, that hinders further root initiation and development.

1.2.2 ***Root inductive phase***: This phase of adventitious root formation have been defined as the time required for all the necessary biochemical events preceding cell divisions that ultimately lead to formation of root primordia. From a practical standpoint, its duration is defined as the minimum time required for the presence of the external rooting stimulus for competent cells (Jarvis, 1986; Moncousin, 1991; Hand, 1994; Gaspar *et al.*, 1997). In previous studies, two approaches have been demonstrated in poplar microshoots to determine the length of the root inductive phase (Hausman *et al.*, 1994). The first approach was to cultivate the shoots on auxin for various times and then transfer to non-auxinic medium. The second approach consisted of measuring peroxidase activity in the basal part of shoots during the rooting process. The length of this induction period can last from hours to days.

In this study I determine the length of the root inductive phase of *C. coggygria* microcutting physiologically by treatment with IBA. This period corresponds to the minimum time required on IBA to yield 100% rooting. *C. coggygria* microshoots were cultured on auxinic medium for various periods as indicated, and then transferred to non-auxinic media. The percentage of shoots that rooted for each exposure time indicated was determined after 12 days.

2. Material and Methods

2.1 *Rooting medium*: The basal rooting medium was a modified MS medium with half-strength nitrates (Murashige and Skoog, 1962). To this medium, increasing concentrations of the auxins NAA, IBA, IAA and 2,4-D were added prior to autoclaving (0.1, 1, 5, 8, 10 and 100 μM). The pH was adjusted to 5.8 and the media solidified with Agargel™ (4g/L) in glass jars (100 ml). For the controls the rooting medium was not supplemented with auxin or any other hormone.

2.2 *Selection of microshoots*: Shoot production of both *C. coggygria* and *Crataegus spp.* species were maintained as described previously (Chapter 3). Prior to rooting trials, microshoots were multiplied and elongated on MS media (Murashige and Skoog, 1962) supplemented with BA and activated charcoal at 24 ° C ± 1 in the growth chamber. Shoots 2-3cm long were selected and cut midway in the internodal regions and planted immediately in the rooting medium, and cultured in the light. For each treatment level in each rooting experiment, 10-15 microshoots per treatment were used. Each treatment level was replicated twice and the experiment at least two times. Rooting was evaluated after 14 days in culture with respect to the percentage of shoots that rooted and the mean number of roots per rooted shoot.

2.3 Determination of root inductive phase

Microshoots of *C. coggygria* shoots were cultivated on MS medium supplemented with 10 μM IBA in jars (Murashige and Skoog, 1962). Shoots (2-3cm) were exposed to

IBA for 0, 1, 2, 3, 4, 5 and 6 days and then transferred to the same medium without IBA. A total of 20 shoots were transferred at the times indicated (i.e. 2 replicates comprising 10 shoots per jar). Previous experiments have indicated that roots emerge from the shoots after 5 days on IBA, but most produce roots closer to Day 10 while on the auxin medium. Control shoots (Day 0) were cultured on non-auxinic medium. After 12 days in culture the number of rooted shoots were counted and the percent rooting was determined. This experiment was repeated twice in the light.

3. Results

3.1 Effect of NAA, IAA, IBA and 2,4-D on rooting in *C. coggygria.*

The microshoots were cultured with increasing concentrations of IAA, NAA, IBA and 2,4-D (Figure 5.2). IBA outperformed all the auxins tested by producing the highest percentage of rooted shoots and over a wide range (1-100μM). Complete rooting occurred at 10μM (100%) and 100μM (100%), and both levels gave maximal root numbers (Table 5.1). NAA also produced a high number of rooted shoots at the1μM level (86%), but it produced fewer roots (Table 5.1). IAA gave lower rooting percentages except where the initial concentration was very high (94%). The number of roots per shoot was also highest for the levels tested at the higher concentrations of IAA (Figure 5.1). 2,4-D was inhibitory at all concentrations tested and did not induce adventitious roots in *C. coggygria.*

The quality of the rooted plantlets from IBA treated shoots were also superior compared to the other auxins treatments. The roots emerged earlier (between Day 5 and

10) and subsequently were able to elongate faster in the culture jars (Figure 5.1, D). This growth spurt was observed shortly after emergence of root primordia. The stems were thicker and the leaves large and greener in color compared to other treatments. The degree of callusing was also less when IBA was used at the 10μM level (Table 5.2). At the 100μM level, all the auxins induced excessive callusing in the shoot bases. IAA and NAA also produced undesirable callus in the shoots at the 10μM level, despite producing many roots. At the 1μM level, IAA and NAA produced acceptable amounts of callus (Table 5.2), but fewer roots. Generally the medium turned purple whenever roots were produced, indicating the release of phenolics into the medium. Also control shoots produced little or no callus during the root induction period.

3.2 Length of the root inductive phase

To determine the length of the root inductive phase in *C. coggygria*, microshoots were cultured on IBA medium for different periods of time, before transferring to the same medium without IBA. The number of rooted shoots was scored on Day 12. Figure 5.3 shows the percentage of rooted shoots for the various exposures to IBA. The percent rooting increased with increasing time spent on the auxin. Shoots transferred to non-auxinic medium by Day 5 and 6 rooted to 100%. This suggests a period of 5 days is sufficient to induce 100% rooting in *C. coggygria* microshoots and coincides with the end of the root inductive phase. The control shoots did not root during these experiments.

Figure 5.1 *C. coggygria* and *Crataegus spp.* microshoots rooted on different auxin regimes *in vitro*.

A. *C. coggygria* shoots treated with mixed auxins (IAA, NAA and IBA) responded to the treatment by producing a callus and numerous nodular structures in the rooting zone (arrowheads).

B. *C. coggygria* shoots eventually produced adventitious roots from the swollen stem bases 2-3 weeks after treatment with mixed auxins.

C. Comparison of a control shoot (left) of *C. coggygria*, and a shoot rooted with the mixed auxins (right)

D. *C. coggygria* microshoots rooted at various concentration of IBA. Note the spontaneous rooting in some shoots (arrow).

E. *Crataegus spp.* microshoots rooted at various concentrations of IAA. Control shoots did not root. Note the heavy callus formation at 100μM IAA.

A

B

C


D
CTRL
1µM
10µM
100µM
E
CTRL
1µM
10µM
100µM

Figure 5.2 Rooting percentages obtained with *C. coggygria* microshoots *in vitro* to a range of concentrations of various auxins (IAA, NAA, IBA), and 2,4-D.

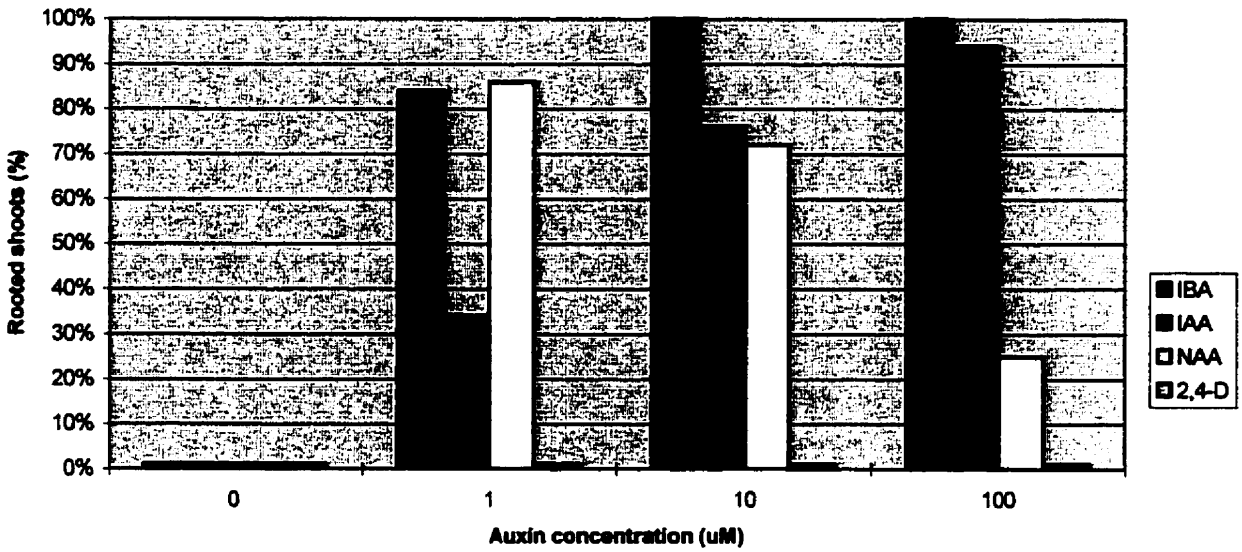

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rooted shoots (%)
0
1
10
100
Auxin concentration (uM)
IBA
IAA
NAA
2,4-D

Table 5.1 The number of adventitious roots formed by *C. coggygria* microshoots in response to a range of concentrations of different auxins *in vitro*. (Means ± SE).

	Concentration (μM)			
Auxin	0	1	10	100
IAA	0 ± 0	3.72 ± 0.60	7.03 ± 0.11	8.13 ± 0.11
IBA	0 ± 0	5.23 ± 0.42	8.35 ± 0.40	9.0 ± 0.40
NAA	0 ± 0	2.78 ± 0.25	5.04 ± 0.32	3.43 ± 0.32
2,4-D	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 5.2 Callus formation in the stem bases of *C. coggygria* microshoots in response to a range of concentrations of different auxins *in vitro*.

	Callus Formation			
Auxin	0	1	10	100
IAA	+	++	+++	+++
IBA	+	+	++	+++
NAA	+	++	+++	+++

+ = little or no callus
++ = acceptable callus
+++ = excessive callus

3.3 Effect of NAA, IAA, IBA and 2,4-D on rooting in *Crataegus spp.*

Crataegus spp. microshoots did not respond to auxins, as strongly as with *C. coggygria* and this species remains very difficult to root. IAA gave the best rooting response in microshoots of *Crataegus spp.* (Table 5.3). Roots were visible after 8-10 days, but very few roots developed (Figure 5.1, E). A similar scoring system was used to determine the degree of callusing as in the *C. coggygria* shoots (Table 5.3). Callusing increased as the concentration of IAA increased from 1 to 100 μM.

IAA at the 10μM level gave 71% rooting in the shoots but produced fewer roots and callused within acceptable levels (Figure 5.1, E). Although the number of rooted shoots and number of roots per shoot were higher at 100μM IAA (86%) there was excessive callusing in the rooting zone.

When IBA was tested, the shoots responded by producing varying degrees of callusing. At 1μM, numerous root primordia were visible in the callus by Day 10, however these failed to elongate during the next ten days. Higher concentrations (10 and 100μM), IBA induced a mass of callus, (1-1.5 cm in diameter), and roots were barely visible. These also failed to elongate and develop further.

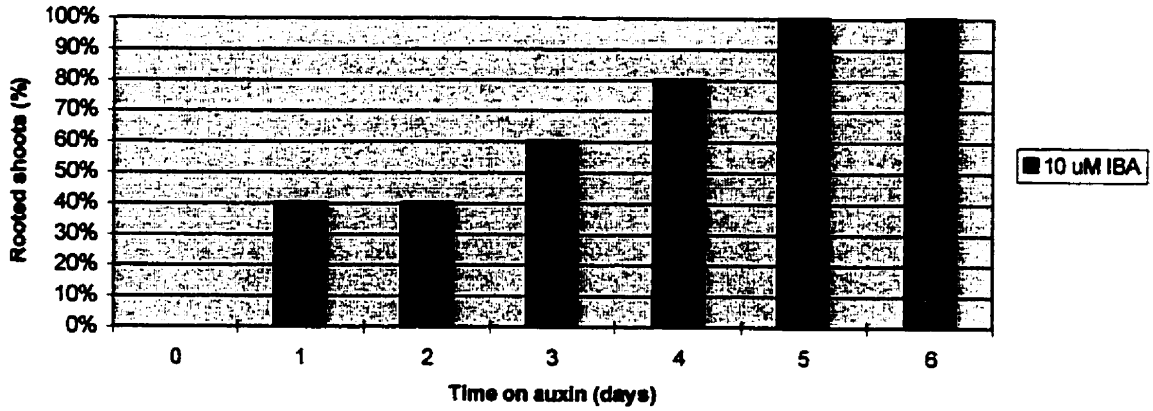
In the presence of NAA, the microshoots responded by producing heavy callusing in the stem base, and no adventitious roots were produced. Similarly, 2,4-D in the rooting medium produced heavy callus and no roots in the stem base, and shoots eventually died.

Table 5.3 The performance of *Crataegus spp.* microshoots when rooted on a range of concentrations of IAA *in vitro*. (Means ± SE).

	Concentration of IAA (μM)			
	0	1	10	100
Percent rooted shoots	0 ± 0.00	28.5% ± 0.28	71% ± 0.10	86% ± 0.14
Number of roots per shoot	0 ± 0.00	1.5 ± 0.00	2.13 ± 0.60	4.13 ± 0.70
Root lengths	0 ± 0.00	2.94 ± 2.90	2.25 ± 0.00	1.93 ± 0.40
Callus formation	+	+	+ +	+ + +

+ = little or no callus
+ + = acceptable callus
+ + + = excessive callus

Figure 5.3 The rooting percentage of *in vitro* cultured *C. coggygria* microshoots on non-auxinic medium after transfer from auxin medium (10μM IBA) at the times indicated.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rooted shoots (%)
0
1
2
3
4
5
6
Time on auxin (days)
10 uM IBA

4. Discussion

The micropropagation of desirable genotypes in large quantities for commercial markets is crucial in horticulture, forestry and genetic improvement programs. The inability to induce adventitious roots is often the limiting factor in conventional cuttings and tissue culture (Haissig *et al.*, 1992). The objective of this study was to determine the best auxin treatment for rooting microcuttings of *C. coggygria* and *Crataegus spp. in vitro*. The shoots were exposed to a range of concentrations of various auxins, namely IAA, IBA, NAA and 2,4-D. The results reveal that two different auxins were effective in the induction of adventitious roots in these systems. For *C. coggygria* microcuttings, the best performance was achieved with 10μM IBA i.e. 100% rooting, whereas for *Crataegus spp.*, up to 71% rooting was attained with 10μM IAA. IAA, IBA and NAA were all effective in promoting multiple roots in *C. coggygria*, whereas in Crateagus spp., a high degree of callusing and reduced adventitious rooting was observed. In both systems 2,4-D at the concentrations tested was not capable of inducing rooting. Several criteria were taken into consideration in determining the best rooting treatments, such as the maximal percentage of rooted shoots, the maximal root numbers, the timing of emergence of roots, shoot quality and growth (e.g. leaf, stem and apex development) and the degree of callusing observed in the shoot bases. All these characteristics are essential for rapid resumption of growth after transfer to greenhouse and soil.

The efficacy of the different auxins generally depends on the concentration of the active auxin at the site of action, and on tissue sensitivity (Van der Krieken *et al.*, 1997).

The active auxin concentration in turn depends on uptake of the auxin by the tissue, transport to the site of action and on metabolic inactivation. The induction of adventitious roots has been achieved by wounding (Zobel, 1992; Blazková *et al.*, 1996), and this wound response is known to involve production of phenolic compounds, stimulation of peroxidases and expression of wound-specific genes. Although many factors can affect adventitious rooting, in most species the application of auxin is required to achieve adventitious rooting. IAA was the first plant hormone to be used for rooting (Cooper, 1935; Epstein and Müller, 1993). Soon after the synthetic auxins IBA (now shown to be naturally occurring in plants) and NAA were discovered and demonstrated to also promote rooting.

Auxins occur in plants naturally as the free acid and in conjugated forms (covalently bound to other molecules) (Blakesley, 1994; Bandurski *et al.*, 1995). The free acid is considered to be the active form, whereas the conjugated form may function as a supply of free auxin without *de novo* synthesis. The auxin (IAA) is covalently bonded by an amide or ester linkage to an amino acid, (e.g. IAA-Asp) or sugar/inositol (e.g. indole-3-acetylglucose).

Auxin can be applied to the microcutting in low concentration (micromolar range) for days to weeks, or at high concentrations (millimolar range) for brief periods e.g. seconds to minutes. The latter is practiced in horticulture by dipping cuttings in a concentrated auxin solution or in rooting powder (DeKlerk *et al.*, 1998). The applied auxin in the rooting medium enters the microcuttings through the cut surface, and is taken up by the cells by pH trapping and influx carriers (Rubery and Sheldrake, 1973; Delbarre *et al.*, 1996; DeKlerk *et al.*, 1998). Once taken up by the cells, the auxin may be inactivated by

oxidation or by conjugation. Plant tissues have been shown to be capable of non-decarboxylative IAA degradation and decarboxylative IAA oxidation. IAA is inactivated irreversibly by the oxidative pathway and its catabolism resembles that of enzymes involved in the wound reaction (Blakesley, 1994; DeKlerk *et al.*, 1998). This suggests that IAA oxidation is catalyzed by non-specific peroxidases related to wounding. Several pathways of IAA degradation have been identified (Blakesley, 1994), which lead to major products such as indole-3-aldehyde, 3-methyleneoxindole and indole-3-methanol (an intermediate compound). Additionally IAA in the light is decomposed rapidly by photo-oxidation (Nissen and Sutter, 1990; Epstein and Müller, 1993; DeKlerk *et al.*, 1998). IBA is inactivated by oxidation to a lesser extent and NAA is not oxidized. Conjugation of the auxin (IAA, NAA and IBA) after uptake by the cells is a reversible inactivation and the free auxin may be released from the conjugates. Because of conjugation, only about 1% of the total auxin taken up by the tissue occurs in the free form (DeKlerk *et al.*, 1998).

IBA have been demonstrated as a very effective auxin in promoting rooting of a wide variety of plants, and is used commercially to root many plant species worldwide (Hartmann, 1990; Epstein and Müller, 1993). However IBA was not effective in inducing adventitious roots in *Crataegus spp.* as seen in this study. It is generally assumed that IBA has a greater ability to induce adventitious roots than IAA because of its relatively higher stability. The concentration of IAA and IBA was shown previously to be reduced by 40% and 20% in autoclaved medium, respectively, (Nissen and Sutter, 1990). In liquid medium IAA was more sensitive to non-biological degradation than IBA. When applied to the medium, both are taken up the cells and shown to be conjugated with amino acids

and sugars in comparable amounts (Nordström *et al.*, 1991; Van der Krieken *et al.*, 1997). Though not physiologically active, the IAA and IBA conjugates form a potential source of free auxin. It has been demonstrated that IBA can be converted to IAA (by ß-oxidation), implying IBA may not be active itself but acts as a naturally occurring slow release source of IAA (Van der Krieken *et al.*, 1997). Several possible hypotheses can be put forward to explain the better rooting seen with IBA (Epstein and Müller, 1993; Van der Krieken *et al.*, 1997). First, both IBA and IAA are physiologically active in root induction, but the rate of conjugation may be slower for IBA than IAA. Second, the free acid of IBA might be more active than that of IAA, and third, the IBA might not be active itself but exerts its action by conversion to IAA. There is still not enough evidence available to support these hypotheses and more work needs to be done to elucidate the role of IBA and IAA as rooting factors in plants.

The length of the root induction period for *C. coggygria* microshoots was determined to be equivalent to 5 days. After this time on IBA and subsequent transfer to the same media without auxin, 100% rooting was achieved. This suggests that during this period, the auxin must be present to ensure all the necessary biochemical events preceding the initial cell divisions, and that the signal is required during the formation of root primordium initials and root primordia. Prolonged exposure to the auxin does not inhibit root elongation, root formation or development of additional root primordia.

CHAPTER 6 DEVELOPMENTAL ANATOMY OF ADVENTITIOUS ROOTS IN *COTINUS COGGYGRIA*.

1. Introduction

A structural analysis of plant developmental processes is an important first step in the study of plant morphogenesis, and in many areas of research, different histological techniques are widely utilized to accomplish this (Wetmore and Wardlaw, 1951; Yeung, 1999). As pointed out in Chapter 2, histological examinations have identified successive but interdependent developmental phases during adventitious rooting (Hausman *et al.*, 1997; Kevers *et al.*, 1997). Several steps in the rooting process have been distinguished based on histological observations by earlier researchers (Favre, 1970; Favre, 1973; Mitsuhashi-Kato *et al.*, 1978; Hicks, 1987; Moncousin *et al.*, 1988; Bollmark *et al.*, 1988). These include early cytological events such as nucleus swelling, followed by unorganized clusters of cell divisions leading to formation of organized root primordia. Later events include internal growth of the root primordia and its eventual emergence from the plant tissue.

As pointed out by Altamura (1996), histological studies on adventitious rooting have been conducted to determine one or all of the following:

1. To identify the cells or tissues that give rise to the root i.e. the target for auxin or other root-inducing factors
2. To establish the presence or absence of pre-formed root primordia
3. To determine whether anatomical features account for low rooting capacity
4. To relate biochemical and physiological data to anatomical stages of root development

Adventitious root formation is viewed as a very complex multicellular process requiring cells or a group of cells to embark on a new developmental program. All too often the rooting competence of some cells remain unexpressed in planta, but may be elicited *in vitro* by the action of a specific inducer, e.g. auxin. Thus *in vitro* culture of tissue and organ systems can be instrumental in identifying and characterizing cytohistological components of the rhizogenic process.

1.1 Patterns of adventitious root formation

Typically, two patterns of adventitious root formation are observed in microcuttings of both herbaceous and woody plant species (Altamura, 1996). These two rhizogenic patterns reflect how competence is attained by the explant *in vitro*.

1. **Indirect pattern**: competence is achieved during culture and root primordia develop from a previously formed callus.
2. **Direct pattern**: competence is already present at the onset of culture and root primordia develop from cells in close association with the vasculature.

Competence for rooting is defined here as the ability of specific cells in the explant to respond to a specific root inducing stimuli, e.g. auxin or wound response. The molecular basis for competence is not known, but may involve specific receptor sites (Mohnen, 1994). Competent cells that no longer require the rooting stimulus are said to be determined for rooting, even if other environmental conditions are needed to complete the remaining stages of root development (Mohnen, 1994; Gaspar *et al.*, 1997).

Both direct and indirect pathways to adventitious root formation can be activated in the same explant either woody or herbaceous, e.g. in tobacco leaf macroexplants cultured

on 0.6 μM IAA (Altamura, 1996). The two patterns may arise from differentiated, differentiating or meristematic cells. Some of the cell types involved in the direct pattern of rooting of woody stem explants include cells of the vascular cambium, phloem parenchyma and phloem ray, medullary ray, pericycle, bud and leaf gaps, outgrowths of the lenticels, margins of differentiating resin ducts and secretory cavities (Lovell and White, 1986; Altamura, 1996). An early cellular event is dedifferentiation for the reactivation of the cell cycle for both differentiated and differentiating cells. For meristematic cells, changes occur during the culture (e.g. already dividing cambial cells change the orientation of cell division planes).

1.2 Formation of root primordium initials

The formation of root primordium initials is a key step in adventitious root formation and is independent of the pattern of root rhizogenesis (i.e. direct or indirect). It is defined as a clump of cells containing chromophil nuclei and nucleoli, and lacks starch (Altamura, 1996). Also it is characterized by synchronous mitotic cell divisions whose plane of division is tangential to the center of the cell clump. In the case of direct rhizogenesis, root primordium arises from localized cell divisions in the primary explant, and is produced *de novo* in the callus during the indirect process. In both cases the root primordia initials eventually give rise to root primordia.

The relationship between root primordium initials and determination is not well characterized. Some evidence suggests that determination is achieved before formation of root primordium initials e.g. in *Convolvulus*, English Ivy and tobacco (Christianson and Warnick, 1985; Geneve *et al.*, 1988; Atfield and Evans, 1991; Warnick, 1992; Mohnen,

1994; Altamura, 1996). Other researchers describe root primordium initials as undetermined structures and developmentally plastic (Bonnett and Torrey, 1966; Thorpe, 1978, 1980; De Klerk *et al.*, 1995), which become determined at some time after initiation or during formation.

According to Altamura (1996) the conflicting opinions in the literature are due to a lack of histological studies on competence and determination. This is coupled to the difficulty of recognizing the root primordium initials in different tissue types at the time of their initiation. The overall process of adventitious root formation can therefore be illustrated as follows:

Competence > Determination > Root primordium initials > Root Primordia

1.3 Objectives

In this study, the main objective is to further characterize the rhizogenic process in *Cotinus coggygria*, and to compare the findings to the literature of other difficult-to-root woody species. The ontogeny of the rooting process is examined after induction by the auxin IBA. The sequence of events occurring in the rooting zone associated with root meristem formation, organization and development is investigated in this system. Shoots rooted on a mixed auxin regime are also examined histologically and compared to controls and other treatments. This approach remains a powerful investigative tool to determine and document early structural and cytological events occurring immediately after auxin stimulation (i.e. root induction, during root meristem and primordia development).

2. METHODS AND MATERIALS

2.1 Shoot regeneration

Microshoots of *Cotinus coggygria* (Smokebush) were regenerated in culture as reported previously in Chapter 2. Briefly, stem cuttings were cultured on MSF medium supplemented with BA (benzyladenine) at a concentration of 1.0 mg/L, 0.1% AC (activated charcoal; Sigma®) and solidified with 4g/L Agargel™, to generate and elongate axillary shoots for the rooting treatment.

2.2 Auxin treatment

For auxin treatments, the elongated shoots were excised with a #10 blade and cut at a 90° angle to the main axis. They were placed on the same medium containing half-strength nitrates, no AC, no BA and supplemented with 10μM IBA (indole-butyric acid) i.e. rooting medium. Once on the auxinic medium, 10 shoots were harvested every day up until the time of root primordia emergence. Extended exposure to this concentration of IBA was previously shown not to inhibit additional primordia formation or development (Chapter 5). Furthermore previous rooting trials have consistently demonstrated the emergence of adventitious roots as early as 5-10 days on IBA medium (Chapter 5). Control shoots were harvested at Day 0, 5 and 10. Shoots cultured on mixed auxins [IAA (1.0mg/L), IBA (0.1mg/L) and NAA (0.1mg/L)] were harvested and fixed at the end of the rooting period.

2.3 Histological procedures

2.3.1 ***Fixative and dehydration***: For each harvest date the basal 4-6mm from each shoot was removed with a razor blade and fixed in formalin-acetic acid-alcohol (FAA) in vials at room temperature (Jensen, 1962). A total of 5 explants each were destined for transverse and longitudinal sectioning respectively. The fixed stem material was then subjected to a tertiary-butyl alcohol (TBA) dehydration series, 70%, 85%, 95% and 100% (Jensen, 1962), the latter (100%) containing safranin for specimen identification. The samples were in each series for a minimum of 12h. The end of the dehydration step comprised 2 changes of absolute TBA at 40° C on a slide warmer, again for a minimum time of 12h.

2.3.2 ***Paraffin and Vacuum infiltration:*** The tissues were infiltrated with molten paraffin during a course of 3 changes (min. 12 h) in the oven at 56° C (Fisher Isotemp® 100 series, Model 116G). Later they were vacuum-infiltrated for 10 min (Welch Duo-Seal®, Model 1400, Sargent-Welch Scientific Co.) at 60° C in a heated vacuum oven (National Appliance Co.).

2.3.3 ***Embedding:*** The processed stem bases were then embedded in paraffin wax (Paraplast™), as detailed by Jensen (1962). Briefly, the explants were separated in paper

boats and covered with fresh molten paraffin. After solidification, they were isolated with a heated knife and mounted onto mounting blocks in preparation for sectioning.

2.3.4 ***Sectioning***: Longitudinal, and transverse serial sections 7 μm thick, were obtained using a rotary microtome (820 Spencer Microtome, American Optical Corporation). The ribbons were cut and separated into approximately 2.5 cm long pieces, and expanded on water on glass slides, and later dried overnight on the slide warmer (Precision Scientific Company, Chicago, USA).

2.3.5 ***Staining and light microscopy***: All sections were treated with xylene to remove the wax and rehydrated with an ethanol down-series. They were then stained in a solution containing safranin O, basic fuschin and crystal violet, then counter-stained with fast green (Yeung and Peterson, 1972). Cover slips were put on the stained slides after adding 2-3 drops of slide mounting medium (Cytoseal™, Stephens Scientific). All light microscopic observations on these slides were done using the Leitz Aristoplan microscope (Leica, Canada).

2.3.6 ***Photography***: Photographs of stained sections were taken using a Leitz Aristoplan™ microscope and Wild Leitz camera accessory (Western Opti-Tech), using Kodak Ektachrome ASA160 tungsten color slide film and Kodak Color ASA 200 for print development.

3. Results

3.1 Treatment with 10 µM IBA

As was seen in previous rooting trials (Chapter 5), fully developed and elongating adventitious roots were visible at about Day 10 when *C. coggygria* shoots were cultured on 10 µM IBA (Figure 6.1, A). When the shoots were harvested, the arrangement of the tissues was typical of stems of dicotyledons. The epidermis comprised a single layer of cells surrounding a narrow cortex of 6-7 layers of parenchyma cells. Typical collateral vascular bundles (7-8 in number) were arranged in a circular fashion around the stem enclosing the central pith (Figure 6.1, B). Associated with each vascular bundle is a secretory duct, which is typical of the family *Anarcardiaceae*. These secretory ducts were located exteriorly to the phloem tissue in the cortex (Figure 6.1, B). The phloem tissue is separated from xylem tissue by the procambium and consists of 2-3 layers of cells. The amount of phloem tissue is small compared to the amount of xylem tissue present. The medullary ray between bundles are very narrow (Figure 6.1, B). No obvious mitotic cell division activity was apparent at this time. Also other specialized cell types such as collenchyma and sclerenchyma were absent from the stem tissues. After 24 h in culture, no obvious structural changes were seen in the tissues close to the cut stem bases. The cells remained lightly stained and nuclei were visible in only a few cells.

Secondary growth occurred 72 h after culture with the formation of the vascular cambium (Figure 6.1, C). The cells in the interfasicular region also became activated and mitotic cell divisions could be seen in this region. Recently divided cells and their

derivatives were identified by their prominent nuclei and a denser cytoplasm compared to earlier sections (Figure 6.1, C).

The derivatives, especially those derived from the interfasicular cambium, assumed an isodiametric shape and continued to divide to produce files of cells leading to the formation of root primordium initials adjacent to the cambium (Figure 6.2, A). The root primordium initials were located between pairs of secretory ducts (Figure 6.2, B). As development progressed, continuous divisions within the root primordium initial lead to the formation of a root primordium (Figure 6.2, C). Some cells located at the proximal end of the root primordium differentiated into tracheids and established vascular continuity with the stem (Figure 6.2, C).

After five days in culture, one or several of the root primordium initials had developed further to produce well-organized root primordium (Figure 6.3, A). These root primordia began to differentiate internally and elongate at a right angle to the main axis toward the epidermis. By Day 10 several of these root primordia had grown through the cortex and were visible in the culture vessels (Figure 6.3, B). Elongation was rapid at this time and by the next 2 days the roots had an average length of 1.0cm. The organization of the tissues in the emerged root contained the typical arrangement seen during primary root growth.

As observed in Figure 6.3, A, the development of adventitious roots was not synchronous, as root primordia in different stages of development can be seen in the same shoot base cross section. Newly formed root primordia were also observed near the shoot apical end of the stems, whereas older or completely developed root primordia formed closer to the base of the stem. This observation also suggests that prolonged

exposure to auxin did not inhibit the initiation and development of new roots. Additionally, adventitious roots were not observed to arise from any other location or tissue when treated with 10 μM IBA and not all the root primordia initials developed into fully formed adventitious roots by Day 10.

At the end of the rooting period (10 days), the control shoots had not produced any adventitious roots (Figure 6.4, A). A similar degree of secondary growth was observed whereby the vascular cambium was formed (2-3 layers thick), separating the phloem from the xylem (Figure 6.4, B). Cells in the interfasicular region, were also activated and gave rise to an interfasicular cambium (Figure 6.4, C). Derivatives of the interfasicular cambium were clearly visible but no meristematic activity was detected because the cells stained lightly and prominent nuclei and/or newly divided cells were not visible (Figure 6.4, C). The cut stem bases also did not develop any considerable amount of callus (Figure 6.4, A and D).

Figure 6.1 Developmental stages of adventitious root formation in *C. coggygria in vitro*.

A. Adventitious root formation at Day 10 in *C. coggygria* microshoots rooted on 10μM IBA.

B. Light micrograph of a cross-section of the shoot base of *C. coggygria* at Day 0, showing the arrangement of tissues.

C. Light micrograph of a cross-section after 72 h of culture. The vascular cambium has formed and mitotic cell divisions can be seen in the interfasicular region (arrows). Note the prominent nuclei and dense cytoplasm.

Abbreviations: co, cortex; se, secretory duct; me, medullary ray; ph, phloem; pr, procambium; xy, xylem; pi, pith; ep, epidermis.

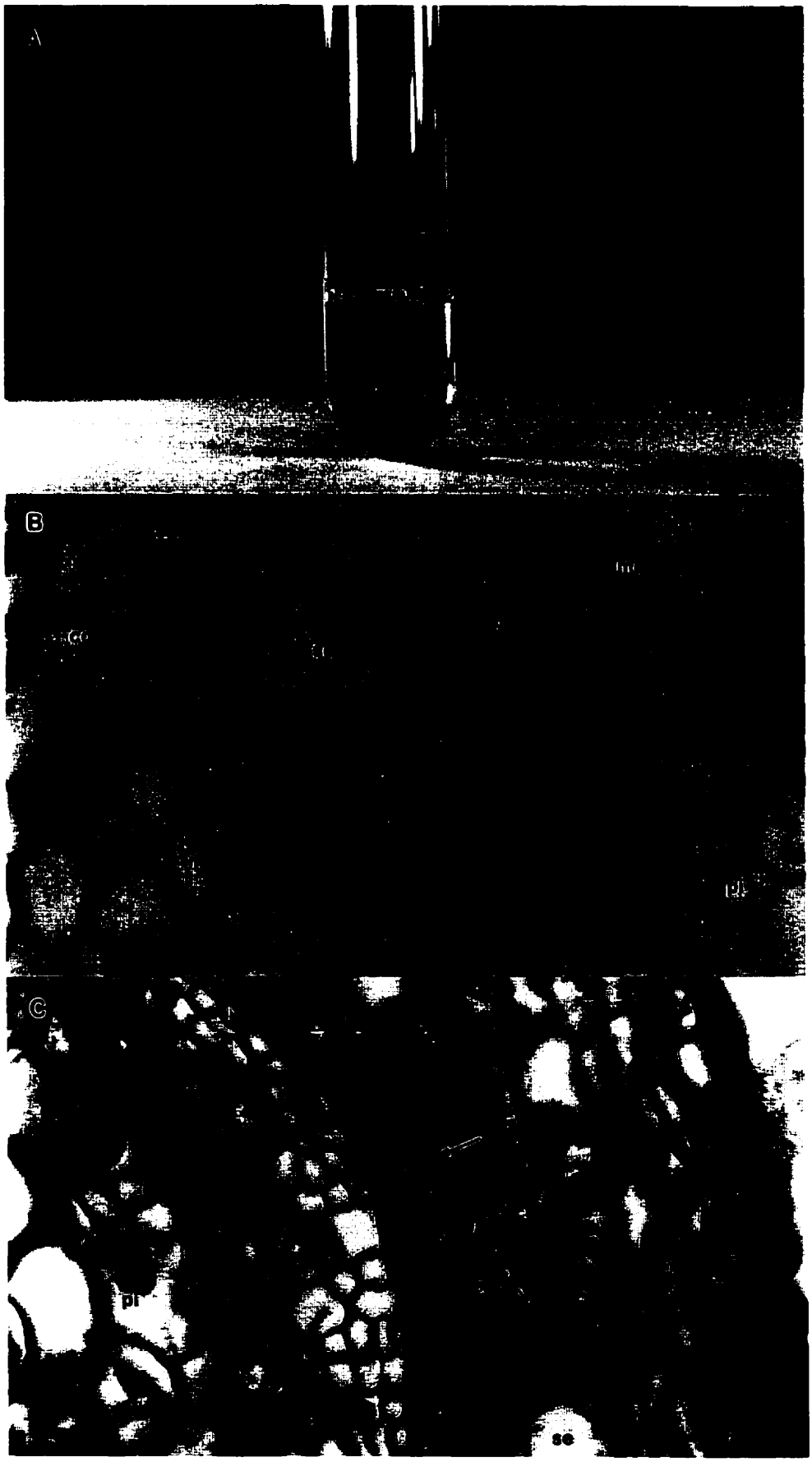

A
B
C

Figure 6.2 Light micrographs of cross-sections of *C. coggygria* stem after several days of culture on 10μM IBA.

A. Cell divisions in the interfasicular region at Day 3, leading up to the formation of root primordium initials. Note the files of cells cut off by periclinal divisions from derivatives of the cambium (arrow).

B. Cross-section showing a single root primordium initial between two secretory ducts and developing from derivatives of the interfasicular cambium. Note the dense staining of the root primordium initial (arrow).

C. Cross-section of the shoot base showing a developing root primordium in the viscinity of the interfasicular cambium. Note the organized outline and the differentiating tracheids close to the shoot vasculature (arrowheads).

Abbreviations: ifc, interfasicular cambium; fi, files of cells; se, secretory duct; rpi, root primordium initial; pi, pith; vc, vascular cambium; rp, root primordium.

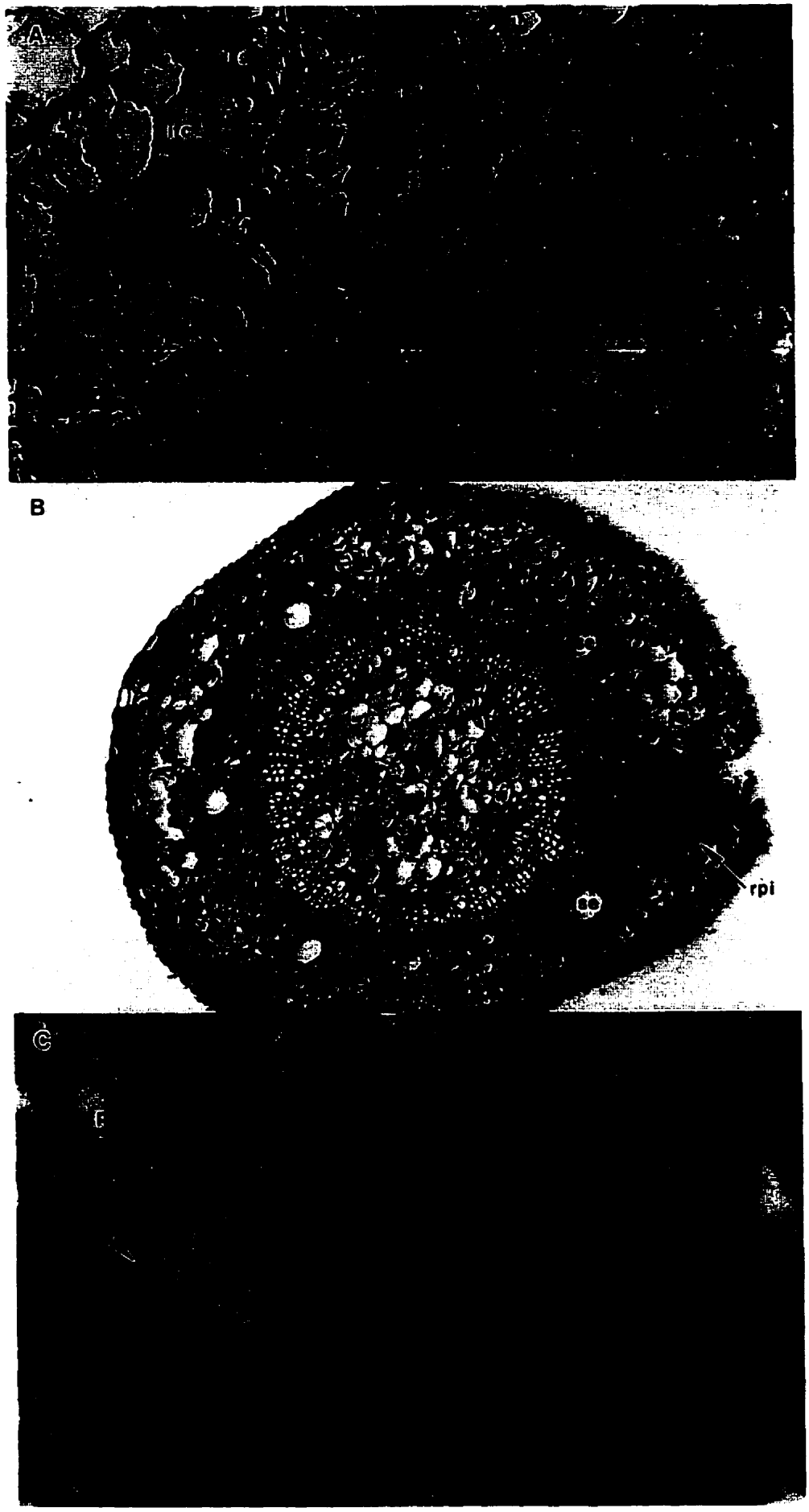

A
B
rpi
C

Figure 6.3 A. Cross-section of *C. coggygria* shoot at Day 5. Several root primordium initials have developed simultaneously in this shoot (arrowheads).

B. Longitudinal section of a root primordium soon after emergence from the cortex tissue at Day 10.

Abbreviations: rp, root primordium; rcp, root cap.

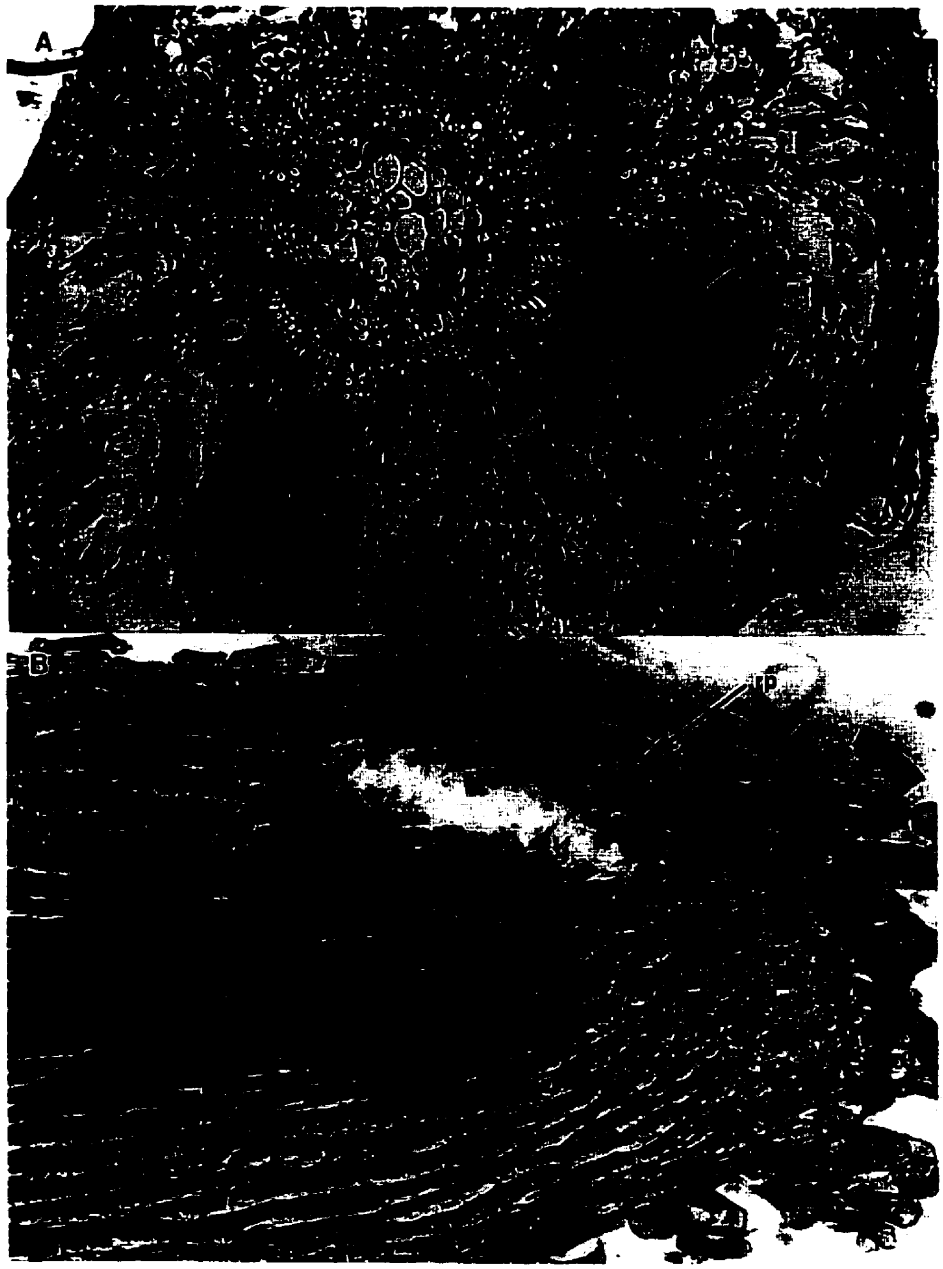

A
B
P

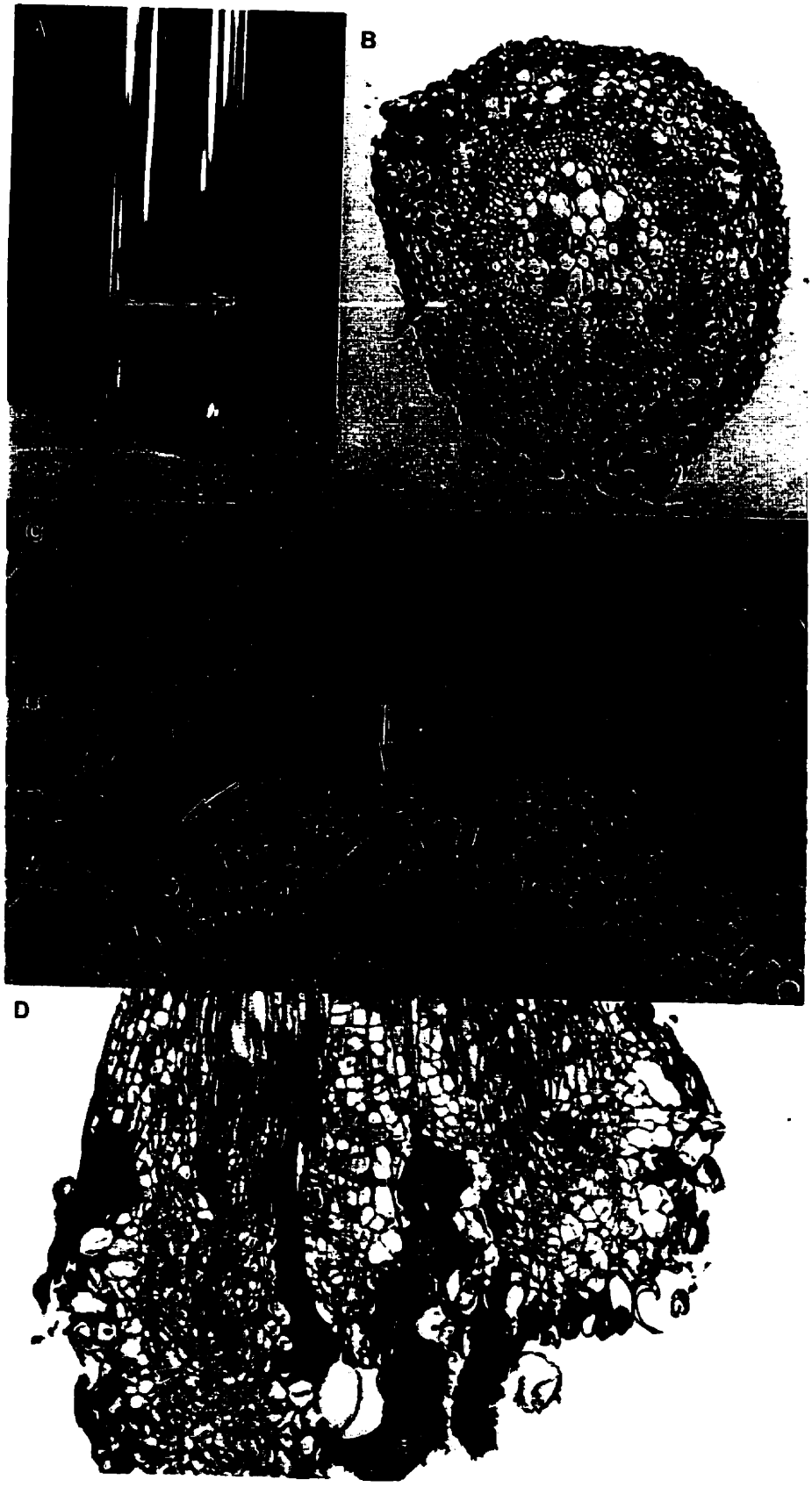
Figure 6.4 A. A control shoot of *C. coggygria* in a culture tube at the end of the rooting period. Note that very little callus has developed at the cut stem base and no roots were formed.

B. Cross-section of a control shoot showing secondary growth and the arrangement of the tissues at the end of the rooting period.

C. Cross-section of a control shoot at higher magnification. Note the activated vascular cambium and interfasicular cambium. No meristematic activity is seen in the interfasicular region (arrow).

D. Longitudinal section of a control shoot illustrating the limited development of callus in the shoot base.

Abbreviations: se, secretory duct; ifc, interfasicular cambium; ca, vascular cambium; ph, phloem; xy, xylem; pi, pith.


B
D

3.2 Treatment with mixed auxins

Adventitious roots initiated differently when the shoots were induced on a mixed auxin regime IAA (1.0mg/L), IBA (0.1mg/L) and NAA (0.1mg/L). After seven days of culture the stem bases became heavily swollen, with no adventitious rooting. By Day 14, the majority of shoots had produced adventitious roots. When viewed under a dissecting microscope, these shoots had characteristic bumps along the stem bases in contact with the auxin medium (see Figure 5.1 A, Chapter 5). Longitudinal sections of the rooted and non-rooted shoots revealed that the bumps and distorted growth corresponded to large masses of proliferating and undifferentiated cells of the cortex (Figure 6.5, A). In both situations these proliferated areas remained largely as internal callus, and in some instances disrupted the epidermis.

In other rooted and non-rooted shoots, centers of meristematic activity were observed within the callus (Figure 6.5, B and C). These cells had characteristically heavily stained cytoplasm and large prominent nuclei. They were located at a distance from the vasculature and cambium, and dividing cells could not be traced to these tissues. Cell division in several different planes increased the volume and number of cells in these centers and resulted in a circular root nodule (Figure 6.5, B and C). Other cells in the vicinity of the heavily stained central cells differentiated into tracheids and also divided and contributed to the overall size of the nodule (Figure 6.6, A). Figure 6.6, B, illustrates that vascular continuity is eventually established and that the root-shoot junction is continuous in the rooted shoots. Also both the interfasicular and fasicular cambium were activated, resulting in numerous regular files of cells, consisting of several layers thick

(Figure 6.6, B). Although they seem to contribute to both the phloem and xylem tissues respectively, neither meristematic cells nor adventitious root primordia developed from these derivatives. The adventitious roots also appear to develop and establish vascular continuity in the interfasicular region of the stem. Control shoots had similar structural changes as observed previously when they were cultured in the absence of auxin in the rooting medium.

Figure 6.5 A. Longitudinal section of *C. coggygria* microshoot rooted on mixed auxins, showing the proliferation of callus in the cortex and shoot base. Root primordia are visible in this callus and are connected to the vasculature.

B. Longitudinal section of a stem base treated with mixed auxins and showing nodular structure. Within the structure is a mass of proliferating cortical cells. A zone of meristematic activity is visible in the callus (arrowhead) distant from the vasculature. Note the prominent nuclei and dense staining of the cytoplasm.

C. Cross-section of the shoot showing a similar meristematic structure developing in the callus at a distance from the vasculature (arrowhead).

Abbreviations: rp, root primordium; pi, pith; v, vasculature; ic, internal callus; de, derivatives;


A
B
C

Figure 6.6 Light micrographs of *C. coggygria* shoot bases rooted on mixed auxins.

A. Actively dividing cells in the internal callus of the cortex. Note the cell divisions in different orientations, the prominent nuclei and dense cytoplasmic staining. Some cells are differentiating into tracheids (arrow).

B. Cross-section through a stem showing the extensive secondary growth arising from the cambium and interfasicular cambium. Note the files of cells cut off to the outside but do not show meristematic activity (arrow).

Abbreviations: ic, internal callus; rp, root primordium; pi, pith; se, secretory duct; ifc, interfasicular cambium; ca, vascular cambium.


Pl

4. Discussion

The auxin IBA at a concentration of 10 μM promotes the formation of adventitious roots in *C. coggygria* microshoots during the final days of a 10-day culture period. The first indication of root formation is the formation of root primordium initials in the stem (Hartmann *et al.*, 1990) and the origin of the initials vary with the species (Altamura, 1996). These initials are identified as small meristematic cells possessing large nuclei and densely stained cytoplasm. The root primordia may be formed directly from stem cells *in situ* (root primordium initials) or indirectly from callus (Hartmann *et al.*, 1990; Altamura, 1996). In the first instance cells are competent to form root initials prior to the auxin stimulus, while in the latter case, competence is achieved during the culture period in the callus.

Shoots of *C. coggygria* displayed the direct pattern of rhizogenesis when cultured on IBA alone. The root initials were identifiable 72 h into culture and originated in the interfasicular region of the stem. They appeared to be direct derivatives of cells from a recently activated interfasicular cambium (between 48-72 h) after the auxin treatment. The derivatives became meristematic soon after they were cut off from the cambium by periclinal cell divisions. These meristematic cells continued to divide in many different planes to eventually form root primordia initials and organized root primordia by Day 5. These results are comparable with those described by other researchers for the direct pattern of rooting in other species with woody stems (Hicks, 1987; Zhou *et al.*, 1992; De Klerk *et al.*, 1995; Altamura, 1996). Root elongation and emergence also followed the

typical pattern seen in endogenous root formation. Vascular differentiation and continuity was established very early and before the emergence of the elongating root primordia.

This histological analysis in *C. coggygria* reveals two recognizable developmental stages during adventitious root formation. The first is induction (72 h), which coincides with the first cell divisions leading to the formation of root primordium initials. This was followed by cell proliferation within the root primordium initials, which led to the formation of root primordia.

Shoots were rooted on a mixed auxin regime first responded by producing an internal callus in the cortex. Within this proliferating mass of cells, meristematic activity was established at a distance from the vasculature and eventually developed into nodular-like structures. With continued cell divisions in different orientations, these structures became organized into root primordium initials, complete with differentiating tracheids. This differentiation appears to progress towards the vasculature of the shoot until connectivity is established. It is not clear when this occurs relative to primordium emergence, because several of the roots separated easily when tugged gently. This raises questions concerning root functionality when they are induced on a mixed auxin regime. This mode of root initiation is typical of the indirect pattern of root formation (Hicks, 1987; Hartmann *et al.*, 1990; Altamura, 1996), where competence to root is attained during culture.

Both types of rooting pattern can be seen in the same woody species (e.g. olive trees: Altamura, 1996). In olive both patterns are active and some shoots exhibit direct genesis from cambial cells, whereas others produce an internal callus from the cambium, and meristemoids are formed from the first derivatives at a point distant from the cambium. *C. coggygria*. shoots also developed an internal callus in the cortex, but no meristematic

activity or derivatives could be traced to the active cambial zone. The difference in development of adventitious roots between the two treatments suggests that different initial processes may be occurring, because no internal callus formed when IBA alone was the signal. The uptake and targeting of auxin apparently differs in the two scenarios. IBA alone appears to target competent cells in the interfasicular region, whereas the other auxin(s) target cells in the cortex, initiating an internal callus from which competent cells develop.

Cross sections of the rooted shoots on the mixed auxin regime also showed extensive periclinal divisions of the cambium, producing in several layers of cells arranged in regular files. This was also observed in *Malus domestica* (Zhou *et al.*, 1992) where root initials and root primordium initials were produced from meristematic cells distant from the cambium. However, this was not seen in *C. coggygria* stem sections and the files of cells appeared to be dormant. When compared to shoots rooted on IBA alone or the controls, this extensive arrangement of files of cells is not evident in *C. coggygria.*

Hence, depending on the treatment *C. coggygria* can exhibit both patterns of adventitious root formation seen in woody plants (direct and indirect). Differences in the mode of initiation exist which cannot be explained by histology alone. IBA at the optimum concentration brings about earlier rooting and produces a more desirable phenotype (no callusing) compared to mixed auxin treatments. However both pathways led to vascular continuity in the root-shoot junctions, and the location of the adventitious roots were similar. This histological examination also established that stems of *C. coggygria* are devoid of anatomical structures that may be barriers to rooting (e.g. fibers), and that no preformed roots existed in the tissues. Also roots developed *in situ* from cells

close to the cambium and in callus, but not from any other tissue or location such as from cells lining the secretory ducts.

CHAPTER 7 SUMMARY

The formation of adventitious roots in important micropropagated species is still a limiting factor in the regeneration of plantlets in many commercial endeavors. This study attempted to investigate and exploit the root promoting ability of *Agrobacterium tumefaciens* among several commercially important forest and ornamental species. This potential rooting treatment was demonstrated in *Pinus ayacahuite* previously in our laboratory (Saborio *et al.*, 1999). The results of the experiments suggest that this promoting effect of *A. tumefaciens* may be specific to this conifer species, and is not transferable to other woody conifers (*Picea rubens*) and woody ornamentals (*C. coggygria and Crataegus spp.*). The study showed on a limited basis, the induction of adventitious roots in *P. ayacahuite* with both strains of *A. tumefaciens* and *Agrobacterium rhizogenes*. In microshoots of *C. coggygria*, *A. rhizogenes* was not capable of inducing adventitious root formation, but instead produced callus on stem cuttings. Since optimal rooting treatments with auxin was not developed at the time for the ornamental species, the next set of experiments were performed to determine the best choice and concentration of auxin for *C. coggygria* and *Crataegus spp.* respectively. The best choice of auxin for *C. coggygria* was IBA (10 μM) and for *Crataegus spp.* was IAA (10 μM).

The performance of *C. coggygria* microshoots on 10μM IBA suggested that it could be used as a potential model for further studies of adventitious root formation in woody plant species. The shoots root uniformly and 100% in 9-10 days, with some roots emerging as early as 5 days. In the absence of auxin, spontaneous roots sometimes arise but these are usually single and infrequent (<10%). However shoots rooted on auxin develop multiple roots compared to controls (0%) or spontaneous rooting therefore they

are easy to distinguish and indicate the induction of many root primordium initials within the rooting zone.

The duration of the root induction period was 5 days on 10μM IBA. This is the minimum time that the auxin signal must be available to realize 100% rooting of the microshoots. Periods less than this do not yield 100% rooting.

A histological study was then performed to look at the structural changes occurring in the rooting zone of *C. coggygria* during adventitious root ontogenesis. Apparently IBA activates the procambium resulting in development of the vascular and interfasicular cambium. Derivatives cut off by periclinal divisions of the interfasicular cambium (towards the cortex) appear to be targeted by the auxin, and through successive cell divisions give rise to the root primordium initials. These continue to develop in the interfasicular region and eventually emerge as functional root primordia after 9-10 days. The root primordium initials are visible in the rooting zone as early as 3 days after auxin treatment, and organized root primordia formed as early as 5 days. This mode of adventitious root initiation and development (direct rhizogenesis) is typically found in a variety of woody species.

When the same shoots were treated using a combination of auxins they developed severe callusing initially (Day 7), and from this callus adventitious roots emerged subsequently. Vascular continuity was physically tested, by pulling gently on the roots, and in some instances they were separated easily. The dissecting microscope revealed combination of nodular structures and developed roots on the majority of shoots. In other shoots only the calluses and nodular structures were obvious. Histological examinations of both types of shoots revealed a presumably different mode of adventitious root

formation with mixed auxins in this species. Although the ontogeny of root formation was not followed with this treatment, it appears that the auxins target cells of the cortex and incites the development of an internal callus. Within this proliferating mass of cells, competence is achieved and a root primordium initial develops at a point distant from the vasculature. This was observed both in shoots that produced roots at the end of the rooting period and in those that did not. Cells within the initials differentiated into tracheids, so it seems probable that as they continue to develop in the callus, vascular connection is established at a later time. This mode of adventitious root initiation and development corresponds to the indirect pathway of rhizogenesis in woody shoots.

REFERENCES

Ahuja MR (1993) Micropropagation á la carte. In: Micropropagation of woody plants. Ahuja MR (Ed). Kluwer Academic Publishers, Dordrecht. pp:3-9.

Altamura MM (1996) Root histogenesis in herbaceous and woody explants cultured in vitro. A critical review. Agronomie: plant genetics and breeding. 16:589-602.

Anon (1991) National Agriculture Statistics Service, USDA/ARS, Washington.

Attfield EM and Evans PK (1991) Developmental pattern of root and shoot organogenesis in cultured leaf explants of *Nicotiana tabacum* cv Xanthi nc. J. Exp. Bot. 42:51-57.

Bajaj YPS (1996) Biotechnology in Agriculture and Forestry 35 (Trees IV). Bajaj YPS (Ed) Springer-Verlag Berlin Heidelberg New York. pp:3-420

Bandurski RS, Cohen JD, Slovin JP and Reineke M (1995) Hormone biosynthesis and metabolism. In: Plant Hormones. Physiology, Biochemistry and Molecular Biology. Davies PJ (Ed.). Kluwer Academic Publishers Dordrecht, The Netherlands. pp: 39-65.

Barlow PW (1994) The origin, diversity and biology of shoot-borne roots. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 1-23.

Bassil NV, Proebsting WM, Moore LW and Lightfoot DA (1991) Propagation of hazelnut stem cuttings using *Agrobacterium rhizogenes*. HortSci. 26:1058-1060.

Bergeys Manual of Determinative Bacteriology (8th edition) (1974). The William and Wilkens Company, Baltimore, USA. pp:265-267.

Berta G, Trotta A, Fusconi A, Hooker JE, Munro M, Atkinson D, Giovnnetti M, Morini S, Fortuna P, Tisserant B, Gianinazzi-Pearson V and Gianinazzi S (1995) Arbuscular

mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera*. Tree Physiol. 15:281-293.

Blakesley D (1994) Auxin metabolism and adventitious root formation. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 143-154.

Blazková A, Sotta B, Tranvan H, Maldiney R, Bonnet M, Einhorn J, Kerhoas L and Miginiac E (1997) Auxin metabolism and rooting in young and mature clones of *Sequoia sempervirens*. Physiol. Plant. 99:73-80.

Bollmark M, Kubat B and Eliasson L (1988) Variations in endogenous cytokinin content during adventitious root formation in pea cuttings. J. Plant Physiol. 132:262-265.

Bonga JM (1987) Clonal propagation of mature trees: problems and possible solutions. In: Cell and tissue culture in forestry. Bonga JM and Durzan DJ (Eds). Martinus Nijhoff Publishers, Dordrecht. pp:249-271.

Bonnett HT and Torrey JG (1966) Comparative anatomy of endogenous bud and lateral root formation in *Convolvulus arvensis* roots cultured *in vitro*. Am. J. Bot. 53:496-507

Bornman CH (1983) Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro*. Physiol. Plant. 57: 5-16.

Burns JA and Schwarz OJ (1996) Bacterial stimulation od adventitious rooting on *in vitro* cultured slash pine (*Pinus elliottii* Engelm.) seedling explants. Plant Cell Rep. 15: 405-408.

Capellades M, Beruto M, Vanderschaeghe A and Debergh PC (1991). Ornamentals. In: Micropropagation: Technology and Application. Debergh PC and Zimmerman RH (Eds.) Kluwer Academic Publishers, Dordrecht. pp. 215-229.

Cardarelli M, Spano L, Mariotti D, Mauro ML and Contantino P (1987) The role of auxin in hairy root induction. Mol. Gen. Genet. 208:457-463.

Cassells AC (1988) Symposium on bacterial and bacterial-like contaminants of plant tissue cultures. Acta Hort. 225: ISHS The Netherlands.

Christianson ML and Warnick DA (1985) Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. Dev. Biol. 112:494-497.

Chulapa V (1987) European Hardwoods. In: Cell and Tissue Culture in Forestry. Vol 3. Bonga JM and Durzan DJ (Eds). Martinus Nijhoff Publishers, Dordrecht. pp:224-246.

Conger BV (1980) Cloning agricultural plants via in vitro techniques. CRC Press, Boca Raton, Florida.

Cooper WC (1935) Hormones in relation to root formation on stem cuttings. Plant Physiol. 10:789-794.

David A, Faye M and Rancillac M (1983) Influence of auxin and mycorrhizal fungi on the *in vitro* formation and growth of *Pinus pinaster* roots. Plant and Soil. 71:501-505.

Davies FT, Davis TD and Kester DE (1994) Commercial importance of adventitious rooting to horticulture. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 53-59.

De la Riva GA, Gonzalez-Cabrera J, Vazquez-Padron R and Ayra-Pardo C (1998) Agrobacterium tumefaciens: a natural tool for plant transformation. EJB. (www.ejb.org).

Debergh P (1994) *In vitro* culture of ornamentals. In: Plant Cell and Tissue Culture. Vasil IK and Thorpe TA (Eds). Kluwer Academic Publishers, Dordrecht. pp: 561-573.

Debergh PC and Read PE (1991) Micropropagation. In: Micropropagation-technology and application. Debergh PC and Zimmerman RH (Eds). Kluwer Academic Publishers, Dordrecht. pp:1-13

Debergh PC and Zimmerman RH (1991) Micropropagation: Technology and application. Kluwer Academic Publishers, Dordrecht.

Delbarre A, Muller P, Imhoff V and Guern J (1996) Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198:532-541.

De Klerk GJ, Keppel M, Ter Brugge J and Meekes H (1995) Timing of the phases in adventitious root formation in apple microcuttings. J. Exp. Bot. 46:965-972.

De Klerk GJ, Van Der Krieken WM and de Jong JC (1999) The formation of adventitious roots: new concepts, new possibilities. In Vitro Cell. Dev. Biol.- Plant 35:189-199.

Dumas E and Monteuuis O (1995) *In vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants: influence of activated charcoal. Plant Cell Tiss. Org. Cult. 40:231-235.

Dunstan DI and Thorpe TA (1986) Regeneration in forest trees. In: Cell culture and somatic cell genetics in plants, Vol 3. Vasil IK (Ed) pp: 223-241 Academic Press, NY.

Ellis D, Roberts D, Sutton B, Lazaroff W, Webb D and Flinn B (1989) Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens*. Plant Cell Reports 8:16-20.

Epstein E and Müller J (1993) Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport. Physiol. Plant. 88:382-389.

Esau K (1953) Plant Anatomy. J. Wiley and Sons Inc, New York.

Favre JM (1970) Influences de facteurs internes sur la cinétique d'enracinement de la Vigne cultivée in vitro. Rev. Gén. Bot. 77:519-522.

Favre JM (1973) Effets corrélatifs de facteurs internes et externes sur la rhizogenèse d'un clone de vigne (*Vitis riparia* x *Vitis rupestris*) cultivé in vitro. Rev. Gén. Bot. 80:279-361.

Gaspar T and Coumans M (1987) Root formation. In: Cell and tissue culture in forestry, Vol 2. Bonga JM and Durzan DJ (Eds) Martinus Nijhoff Publishers, Dordrecht. pp:202-217.

Gaspar T, Kevers C and Hausman JF (1997) Indissociable chief factors in the inductive phase of adventitious rooting. In: Biology of Root Formation and Development. Altman A and Waisel Y (Eds.) Plenum Press, New York. pp:55-63

Gautheret RJ (1934) Cultur du tissu cambial. CR Acad Sci (Paris) 198:2195-2196.

Gautheret RJ (1939) Sur la possibilité de réalizer la culture indéfinie des tissus de tubercules de carrote. C. R. Acad. Sci. 208:118.

Gautheret RJ (1940) Nouvelles recherches sur le bourgeonnement di tissu cambial d'*Ulmus campestris* Cultive *in vitro*. C.R. Acad. Sci. (Paris) 210:744-746.

Gautheret RJ (1985) History of plant tissue and cell culture: a personal account. In: Cell Culture and Somatic Cell Genetics in Plants. Vasil IK (Ed.). Vol. 2. Academic Press, Orlando, Florida. pp: 1-59.

Geneve RL, Hackett WP and Swanson BT (1988) Adventitious root initiation in de-bladed petioles from the juvenile and mature phases of English Ivy. J. Am. Hortic. Sci. 113:630-636.

Girouard RM (1974) Propagation of spruce by stem cuttings. NZ J. For. Sci. 4:140-149.

Gupta PK and Mascarenhas AF (1987) Eucalyptus. In: Cell and Tissue Culture in Forestry, Vol 3. Bonga JM and Durzan DJ (Eds). Martinus Nijhoff Publishers, Dordrecht. pp:385-399.

Gresshoff PM and Doy CH (1972) Development and differentiation of haploid *Lycopersicum esculentum* (tomato). Planta 107:161-170.

Haberlandt G (1902) Kulturversuche mit isolierten Pflanzenzellen, Sitzungsber Math-Naturwiss. KI. Kais. Akad. Wiss. Wien. 11:69.

Haissig BE, Davis TD and Riemenschneider DE (1992) Researching the controls of adventitious rooting. Physiol. Planta. 84: 310-317.

Haissig BE and Davis TD (1994) A historical evaluation of adventitious rooting research to 1993. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 275-331.

Hamill JD and Chandler SF (1994) Use of transformed roots for root development and metabolism studies and progress in characterizing root-specific gene expression. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 163-179.

Hand P (1994) Biochemical and molecular markers of cellular competence for adventitious rooting. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 111-121.

Harry IS and Thorpe TA (1991) Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. Bot. Gaz. 152:446-452.

Harry IS and Thorpe TA (1994) *In vitro* culture of forest trees. In: Plant Cell and Tissue Culture. Vasil IK and Thorpe TA (Eds) Kluwer Academic Publishers, Dordrecht. pp:539-599.

Hartmann HT, Kester DE and Davies JT (1990) Plant propagation, principles and practices. 5th ed. Prentice Hall, Englewood Cliffs, NJ.

Hasnain S and Cheliak W (1986) Tissue culture in forestry: economic and genetic potential. For. Chron. 82: 219-225.

Hatta M, Beyl CA, Garton S and Diner AM (1996) Induction of roots on jujube softwood using *Agrobacterium rhizogenes*. J. Hort. Sci. 71:881-886.

Hausman JF, Kevers C and Gaspar T (1994) Involvement of putrescine in the inductive rooting phase of poplar shoots raised *in vitro*. Physiol. Plant. 92:201-206.

Hausman JF, Evers D, Kevers C and Gaspar T (1997) Internal controls of root induction of poplar shoots raised *in vitro*. J. Applied Bot. 71:104-107.

Hess CE (1994) Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York.

Hicks GS (1987) Adventitious rooting of apple microcuttings in vitro: an anatomical study. Can. J. Bot. 65:1913-1920.

Hood EE, Jen G, Kayes L, Kramer J, Fraley RT and Chiltons MD (1984) Restriction endonuclease map of Ptio542, a potential Ti plasmid vector for genetic engineering of plants. Bio/Tech. 2:702-709.

Hood EE, Helmer GL, Fraley RT and Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTio542 outside of T-DNA. J. Bact. 168:1291-1301.

Hood EE, Clapham DH, Ekberg I and Johannson T (1990) T-DNA presence and opine production in tumors of *Picea abies* (L.) Karst induced by *Agrobacterium tumefaciens* A281. Plant Mol. Biol. 14:111-117.

Horgan K and Holland L (1989) Rooting micropropagated shoots from mature radiata pine. Can. J. For. Res. 19: 1309-1315

Hosie RC (1979) Native trees of Canada. Fitzhenry and Whiteside, Don Mills, Ontario

Hughes KW (1981) Ornamental species. In: Cloning agricultural plants via in vitro techniques. Conger BV (Ed.). CRC Press, Boca Raton, Florida. pp:5-50.

Jarvis BC (1986) Endogenous control of adventitious rooting in non-woody cuttings. In: New Root Formation in Plants and Cuttings. Jackson MB (Ed). Martinus Nijhoff Publishers, Boston. pp:191-222.

Jensen WA (1962) Botanical Histochemistry. Principles and practice. Freeman WA, San Francisco. pp:408

Kevers C, Hausman JF, Faivre-Rampant O, Evers D and Gaspar T (1997) Hormonal Control of Adventitious Rooting: Progress and Questions. Angewandte Botanik. 71:71-79.

Klee H, Horsch R and Rogers S (1987) *Agrobacterium*-mediated plant transformation and its further applications to plant biology. Ann. Rev. Plant Physiol. 38: 467-486.

Kleinschmidt J (1974) A program for large scale cutting propagation of Norway spruce. New Zealand J. For. Sci. 4:359-366.

Korber H, Strizhov N, Staiger D, Feldwisch J, Olsson O, Sandberg G, Palme K, Schell J and Konez S (1992) T-DNA gene 5 of *Agrobactrium* modulated auxin response by autoregulated synthesis of a growth hormone antagonist in plants. EMBO Journal. 12:3983-3991.

Kovar JL and Kuchenbuch (1994) Commercial importance of adventitious rooting to agronomy. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 25-35.

Krussmann G (1981) The complete book of roses. Timber Press, Portland, OR.

Lovell PH and White J (1986) Anatomical changes during adventitious root formation. In: New Root Formation in Plants and Cuttings. Jackson MB (Ed.) Martinus Nijhoff Publishers, Dordrecht, The Netherlands. pp: 111-140.

Liu ST and Kado CI (1979) Indoleacetic acid production: a plasmid function of *Agrobacterium tumefaciens* C58. Biochem. Biophys. Res. Commun. 90:171-178.

Lu CY, Harry IS, Thompson MR and Thorpe TA (1991) Plantlet regeneration from cultured embryos and seedling parts of red spruce (*Picea rubens* Sarg.) Bot. Gaz. 152:42-50.

Ma Y, Byrne DH and Chen J (1996) Propagation of rose species in vitro. In Vitro Cell. Dev. Biol. 32: 103-108

Maene LJ and Debergh PC (1985) Liquid medium additions to established tissue cultures to improve elongation and/or rooting in vitro. Plant Cell Tissue Org. Cult. 5:23-33.

Martinez-Pulido C, Harry IS and Thorpe TA (1990) *In vitro* regeneration of plantlets of Canary Island pine (*Pinus cararíensis*). Can. J. For. Res. 20: 1200-1211.

Mascarenhas AF, Kendurkar SV, Gupta PK, Khuspe SS and Agrawal DC (1987) Teak. In: Cell and Tissue Culture in Forestry, Vol 3. Bonga JM and Durzan DJ (Eds). Martinus Nijhoff Publishers, Dordrecht. pp:300-315.

McAfee BJ, White EE, Pelcher LE and Lapp MS (1993) Root induction in pine (*Pinus*) and larch (*Larix*) spp. using *Agrobacterium rhizogenes.* Plant Cell Tiss. Org. Cult. 34:53-62.

McCown DD and McCown BH (1987) North American Hardwoods. In: Cell and Tissue Culture in Forestry, Vol 3. Martinus Nijhoff Publishers, Dordrecht. pp:247-260.

Mitsuhashi-Kato M, Shiboaka H and Shimokoriyama RJ (1978) The nature of the dual effect of auxin on root formation in *Azukia* cuttings. Plant Cell Physiol. 19:1535-1542.

Mohammed GH and Vivader WE (1988) Root production and plantlet development in tissue-cultured conifers. Plant Cell Tiss. Org. Cult. 14: 137-160.

Mohammed GH, Patel KR and Vidaver WE (1989) The control of adventitious root production in tissue cultured Douglas-fir. Can. J. For. Res. 19:1322-1329.

Mohnen D (1994) Novel experimental systems for determining cellular competence and determination. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.) Plenum Press, New York. pp:87-98.

Moncousin C, Favre JM and Gaspar T (1988) Changes in peroxidase activity and endogenous IAA levels during adventitious rooting in vine cuttings. In: Physiology and Biochemistry of Auxins in Plants. Kutacek M, Bandurski RS and Krekule J (Eds.) Academia Praha. pp: 331-337.

Moncousin C (1991) Rooting of in vitro cuttings. In: Biotechnology in Agriculture and forestry. Vol 17. High-tech and micropropagation I. Bajaj YPS (Ed.). Springer-Verlag, Berlin.

Morel G (1960) Producing virus-free Cymbidiums. Amer. J. Bot. 29:495-497.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497

Murashige T (1974a) Plant propagation through tissue cultures. Annu. Rev. Plant Physiol. 25:35-166.

Murashige T (1974b) Propagation through tissue cultures. HortScience 9:2-3.

Murashige T (1978) The impact of plant tissue culture on agriculture. In: Frontiers of Plant Tissue culture 1978. Thorpe TA (Ed.). University of Calgary Printing Services, Canada.

Murray JR, Concepcion Sanchez M, Smith AG and Hackett WP (1994) Differential competence for adventitious root formation in histologically similar cell types. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 99-109.

Nehra NS and Kartha KK (1994) Meristem and shoot tip culture: requirements and applications. In: Plant Cell and Tissue Culture. Vasil IK and Thorpe TA (Eds.) Dordrecht, Kluwer Academic Publishers, Netherlands pp: 37-70.

Nester EW, Gordon MP, Amasino RM and Yanofsky (1984) Crown gall: a molecular and physiological analysis. Ann. Rev. Plant Physiol. 35: 387-413.

Nissen SJ and Sutter EG (1990) Stability of IAA and IBA in nutrient medium of several tissue culture procedures. HortScience 25:800-802.

Nobécourt P (1939) Sur la perennite de l'augmentation de volume des cultures de tissus vegetaux. C. R. Soc. Biol. 130:1270.

Nordström AC, Jacobs FA and Eliason L (1991) Effect of exogenous indole-3-acetic acid and indole-3-butyric acid on internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cuttings. Plant Physiol. 96:856-861.

Palme K, Hesse T, Garbers C, Simmons C and Söll D (1994) The *ERabp* gene family: structural and physiological analyses. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 155-161.

Patena L, Sutter EG and Dandekar AM (1988) Root induction by *Agrobacterium rhizogenes* in a difficult-to-root woody species. Acta Hort. 227:324-329.

Pythoud F, Buchala AJ and Schimd A (1986) Adventitious root formation in green cuttings of *Populus tremula* : characterization of the effect of vitamin D and indolebutyric acid. Physiol. Plant. 68:93-99.

Redenbaugh K (1991) Applications of micropropagation for agronomic crops. In: Micropropagation. Debergh PC and Zimmerman RH (Eds.). Kluwer Academic Publishers, Netherlands. pp: 285-310.

Richie GA (1994) Commercial application of adventitious rooting to forestry. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 37-52.

Riemenschneider DE (1994) Genomic manipulation of plant materials for adventitious rooting research. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 61-76.

Riker AJ (1930) Studies on infectious hairy root of nursery apple trees. J. Agr. Res. 4:507-540.

Rinallo C and Mariotti D (1994) *Agrobacterium* as a tool for gene transfer in woody species: the state of the art and practical perspectives. A review. Giorn. Bot. Ital. 128:729-740.

Roberts AV, Horan I and Matthews D (1990) Protoplast technology and somatic embryogenesis in *Rosa.* In: Integration of *in vitro* techniques in ornamental plant breeding. de Jong J (Ed) Proc. Eucarpia Symp. pp:110-115.

Rubery PH and Sheldrake AR (1973) Effect of pH and surface charge on cell uptake of auxin. Nature New Biol. 244:285-288.

Rugini E, Pellegrineschi A, Mencuccini M and Mariotti D (1991) Increase of rooting ability in the woody species kiwi (*Actinidia deliciosa* A. Chev.) by transformation with *Agrobacterium rhizogenes rol* genes. Plant Cell Rep. 10: 291-295.

Saborio FJ, Moloney MM, Tung P and Thorpe TA (1999) Root induction in *Pinus ayacahuite* by co-culture with *Agrobacterium tumefaciens* strains. Tree Physiology 19:383-389.

Salisbury FB and Ross CW (1985) Hormones and growth regulators. In: Plant Physiology (3rd Ed.) Wadsworth Publishing Company Inc. Belmont California USA pp:309-348.

Sheng J and Citovsky V (1996) *Agrobacterium* – plant cell DNA transport: have virulence proteins, will travel. Plant Cell 8:1699-1710.

Skolmen RG and Mapes MO (1978) Aftercare procedures required for field survival of tissue cultured propagated *Acacia koa*. Comb. Proc. Intern. Plant. Prop. Soc. 28:156-1642.

Skoog F and Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. In: Biological Action of Growth Substanes 11th Symp. Soc. Exp. Biol. Cambridge Univ. Press. Cambridge.pp: 118.

Smith DR and Thorpe TA (1977) Root initiation in cuttings of *Pinus radiata* seedlings: Effects of aromatic amino acids and simple phenylpropanoids. Bot. Gaz. 138:434-437.

Smith RH and Hood EE (1995) *Agrobacterium tumefaciens* transformation of monocotyledons: review and interpretation. Crop Sci. 35:301-309.

Sommer HE, Brown CL and Kormanik PD (1975) Differentiation of plantlets in longleaf pine (*Pinus palutris* Mill.) tissue cultured in vitro. Bot. Gaz. 136:196-200.

Strobel GA and Nachmias A (1985). *Agrobacterium rhizogenes* promotes the initial growth of bare root stock almond. J. Gen. Microbiol. 131:1245-1249.

Strobel GA, Nachmias A and Hess WM (1988). Improvements in the growth and yield of olive trees by transformation with Ri plasmid of *Agrobacterium rhizogenes*. Can. J. Bot. 66: 2581-2585

Tepfer D, Damon JP, Ben-Hayyim G, Pellegrineschi A, Burtin D and Martin-Tanguy J (1994) Control of root system achitecture through chemical and genetic alterations of polyamine metabolism. In: Biology of Adventitious Root Formation. Davis TD and Haissig BE (Eds.). Plenum Press, New York. pp: 181-189.

Thimann KV and Went FW (1934) On the chemical nature of the root forming hormone. Proc. Kon. Akad. Wetensch. 37:456-459.

Thompson MR and Thorpe TA (1990) Biochemical perspectives in tissue culture for crop improvement. In: Biochemical Aspects of Crop Improvement. Khanna KR (Ed) CRC Press Boca Raton Ann Arbor Boston.

Thorpe TA (1978) Physiological and biochemical aspects of organogenesis in vitro. In: Frontiers of Plant Tissue Culture. Proc. 4th Int. Congr. Plant Tissue and Cell Culture. Thorpe TA (Ed.) University of Calgary Press, Calgary, Canada. pp:49-58.

Thorpe TA (1980) Organogenesis in vitro: structural, physiological and biochemical aspects. In: International Review of Cytology. Suppl. 11A. Vasil IK (Ed.) Academic Press, New York. pp: 71-111.

Thorpe TA and Biondi S (1984) Conifers. In: Handbook of plant cell culture, Vol 2. Sharp WR, Evans DA, Ammirato PV & Yamada Y (Eds) pp: 435-470 Macmillan, NY.

Thorpe TA and Hasnain S (1988). Micropropagation of conifers: methods, opportunities and costs. *In* Tree Improvement-Progressing Together. Proceedings of the 21st Meeting of the Canadian Tree Improvement Association, 1987,Truro, N.S. Morgenstern EK and Boyle JB (Eds.) Canadian Forestry Service, Chalk River. pp. 68-84.

Thorpe TA and Harry IS (1990) Special problems and prospects in the propagation of woody species. Plant Aging: Basic and Applied Approaches. Rodriguez *et al.* (Eds). Plenum Press, NY. pp:67-74.

Thorpe TA, Harry IS and Kumar PP (1991) Application of micropropagation to forestry. In: Micropropagation. Debergh PC & Zimmerman RH (Eds). Kluwer Academic Publishers, Dordrecht. pp: 311-336.

Toivonen PMA (1985) The development of a photophysiological assessment system for white spruce (*Picea gluaca* (Moench) Voss) seedlings and micropropagated plantlets. Ph.D. Thesis, Simon Fraser University, Burnaby.

Van der Krieken WM, Kodde J, Visser MHM, Tsardakas D, Blaakmeer A, de Groot K and Leegstra L (1997) Increased induction of adventitious rooting by slow release auxins and elicitors. In: Biology of Root Formation and Development. Altman A and Waisel Y (Eds.) Plenum Press, New York. pp:95-104.

von Arnold S and Eriksson T (1978) Induction of adventitious buds on embryos of Norway spruce grown *in vitro*. Physiol. Plant. 44:283-287.

von Arnold S and Eriksson T (1981) In vitro studies of adventitious shoot formation in *Pinus contorta*. Can. J. Bot. 59:870-874.

Warnick DA (1992) Developmental biology of rhizogenesis in vitro in *Convolvulus arvensis*. MS Thesis, San Jose State Univ., Dept. Biol. Sci. San Jose, CA, USA.

Watson B, Currier TC, Gordon MP, Chilton MD and Nester EW (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bateriol. 123:255-264.

Weiler EW and Spanier K (1981) Phytohormones in the formation of crown gall tumors. Planta 153:326-337.

Wetmore RH and Wardlaw CW (1951) Experimental morphogenesis in vascular plants. Annu. Rev. Plant Physiol. 2:269-292.

White PR (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9:585

White PR (1939) Potentially unlimited growth of excised plant callus in an artificial nutrient. Amer. J. Bot. 26:59

White FF, Taylor BH, Huffman GA, Gordon MP and Nester EW (1985) Molecular and genetic analysis of the tranferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J. Bacteriol. 164:33-45.

Winton LL (1968) Plantlet formation in aspen tissue culture. Science 160:1234-1235.

Yeung EC and Peterson RL (1972) Studies on the rosette plant *Hieracium floribundum*. I. Observations related to flowering and axillary bud development. Can. J. Bot. 50:73-78.

Yeung EC (1999) The use of histology in the study of plant tissue culture systems-some practical comments. In Vitro Cell. Dev. Biol. Plant. 35:137-143.

Zhou J, Wu H and Collet GF (1992) Histological study of initiation and development in vitro of adventitious roots in minicuttings of apple rootstocks of M26 and EMLA9. Physiol. Plant. 84:433-440.

Zimmerman RH (1986). Regeneration in Woody Ornamentals and Fruit Trees. In: Cell Culture and Somatic Cell Genetics of Plants. Vol. 3. Vasil IK (Ed) Academic Press, NY. pp:243-258.

Zobel B (1992) Vegetative propagation in production forestry: Rooted cutting use has been very successful for some species. J. For. 90:29-33.