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Rooting of flowering Dogwood (Cornus florida) microshoots

Anjana R. Sharma

To the Graduate Council:

I am submitting herewith a thesis written by Anjana R. Sharma entitled "Rooting of flowering Dogwood (Cornus florida) microshoots." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Landscape Architecture.

Robert N. Trigiano, Major Professor

We have read this thesis and recommend its acceptance:

Willard T. Witte, Otto J. Schwarz

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Rooting of flowering dogwood (*Cornus florida*) microshoots

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Anjana R. Sharma

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DEDICATION

This thesis is dedicated to my parents

Ranjana and Rajen Sharma

for their love, support and encouragement.

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ABSTRACT

Before flowering dogwood (*Cornus florida* L.) can be commercially propagated by tissue culture methods, a reliable and efficient method of production must be developed. *Cornus florida* has been micropropagated successfully but with a low rooting efficiency of about 50 %. Rooting has been a major problem in tissue culture of many other plants as well. This study was conducted to increase rooting efficiency of flowering dogwood microshoots over that previously achieved. Five to six week old microshoots originating from acclimatized axillary and nodal bud stock cultures were excised and used in the rooting experiments. *Cornus florida* microshoots were treated with various root promoting substances and bacteria. Microshoots were dipped for 24 h in aqueous diffusates that were prepared by leaching stem cuttings of either black locust (*Robinia pseudoacacia* L.) or contorted willow (*Salix* x *erythroflexuosa* Rag.). Explants were then transferred to Woody Plant Medium (WPM) with and without indole-3-butyric acid (IBA). Various concentrations of salicylic acid and acetylsalicylic acid as root stimulating moeities were tested as a continuous exposure and a 24 h pulse treatments. A recently discovered root stimulating bacterium (RSB) promoted rooting in *Pinus* seedlings was also used to study rooting of flowering dogwood microshoots. Microshoots were treated with RSB cells as well as extracts obtained at pH 3 and pH 7 from the spent medium in which RSB was grown. Rooting efficiencies of microshoots grown on WPM and treated with these root promoting substances, bacterium and

bacterial culture extracts were compared to that of microshoots grown on WPM with 4.9 μM IBA. Microshoots formed roots in all the experiments. Locust and willow diffusate slightly inhibited root formation on microshoots. Continuous exposure to 100 μM salicylic acid and 50 and 100 μM acetylsalicylic acid promoted root generation but not significantly different from that achieved with IBA. The highest mean number of roots/explant were produced when pulsed with 10 μM salicylic acid, 25 and 50 μM acetylsalicylic acid. Significant rooting was not observed on direct exposure of microshoots to live RSB. However, high rooting percentages of 70 % and 90 % were observed when microshoots were transferred to WPM amended with 2.5 ml and 0.5 ml of RSB extract obtained at pH 7 respectively. Of all the treatments tested, treatment of 5 to 6 wk old microshoots with 4.9 μM IBA stimulated the best and most consistent rooting efficiency of 70 to 100 %. Histological studies of root formation and leaf growth were also conducted. Root formation from microshoots started within 10 days of transfer to rooting medium. Microshoots grown on WPM amended with 4.9 μM IBA and leaf samples taken from various stages of micropropagation were sectioned and stained using Flemings triple stain. Leaf sections from plants in the greenhouse were found to be fully acclimatized as the anatomy and arrangement of tissues strongly resembled those from mature dogwood trees.

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LIST OF ABBREVIATIONS

ASA - acetylsalicylic acid

BA - 6-benzylaminopurine

BE - bacterial extract

BE3 - bacterial extract obtained at pH 3

BE7 - bacterial extract obtained at pH 7.14

IBA - indole-3-butyric acid

LD - black locust diffusate

PGR - plant growth regulator

RSB - root stimulating bacterium

SA - salicylic acid

WD - willow diffusate

WPM - woody plant medium

CHAPTER 1

INTRODUCTION

Flowering dogwood (*Cornus florida* L.) is a very common tree found in the eastern United states (Brinkman 1974; Coartney et al. 1991; Pirone 1980; Santamour and McArdle 1985). It is a valuable tree for the nursery and landscape industry, especially in Tennessee where it provides significant income for nursery producers (Badenhop et al. 1985; Witte 1995; Southards 1995). Powdery mildew (Hagan et al. 1995; Klein et al. 1998; McRitchie 1994; Ranney et al. 1994; Windham 1996; Windham and Witte 1998) and dogwood anthracnose (Daughtrey et al. 1996; Hibben and Daughtrey1988; Redlin 1991) are the two diseases that have, in the last few years, caused much damage to *C. florida* trees in both landscape and natural environment.

Generic flowering dogwoods are commercially propagated from seeds. Named cultivars are propagated by vegetative methods such as grafting or rooting cuttings (Badenhop et al. 1985; Dirr and Heuser 1987; Witte 1995). Both these vegetative production methods depend upon the season and success can be unpredictable. If a reliable method of producing flowering dogwoods through tissue culture is developed, it can be used to rapidly produce many copies of an important cultivar (genotype) that may have extraordinary horticultural attributes or resistance to either dogwood anthracnose, powdery mildew or both. To date, commercial propagation of flowering dogwood by tissue culture has not been feasible due to lack of a reliable protocol.

Many studies involving in vitro culture of *C. florida* have been done. Of the various media and plant growth regulators (PGRs) used for shoot proliferation of *C. florida* axillary bud cultures, WPM (Lloyd and McCown 1980) supplemented with 6-benzylaminopurine (BA) stimulated the most microshoots per culture (Declerck and Korban 1994; Kaveriappa et al. 1997). Greatest number of microshoots was produced on WPM amended with 4.4 μM BA (Kaveriappa et al. 1997). Although microshoot production is easily accomplished with seedling cultures, the microshoots have been difficult to root in vitro. Maximum root formation in *C. florida* microshoots derived from axillary and nodal cultures was achieved when placed on WPM augmented with 4.9 μM IBA (Kaveriappa et al. 1997). This was the first complete micropropagation of *C. florida* and had a rooting efficiency of about 50 %.

Since the percentage of dogwood microshoots rooting in vitro is relatively low, alternate treatments (other than IBA) may be necessary to stimulate adventitious rooting and improve the whole plant regeneration efficiency of the propagation system. Alternatives to auxins have been used to stimulate rooting in microshoots of various species. Aqueous diffusates of black locust (*Robinia pseudoacacia* L.) and contorted willow (*Salix erythroflexuosa* Rag.) influenced rooting of stem cuttings of Chinese fringetree and white fringetree (Arena 1997). Salicylic acid (SA), which has been classified as a PGR (Raskin 1992), and acetylsalicylic acid (ASA) have also been reported to have some effect on root formation (Kling and Meyer 1983; Mensuali-Sodi et al. 1995). Microorganisms have also been described to influence adventitious root formation in culture (Baker and Dyer 1996; Bassil et al. 1991; Mihaljevic et al. 1996;

Patena et al. 1988; Strobel and Nachmias 1988). A recently discovered root stimulating bacterium (RSB) induced root formation in cultured pine seedlings (Burns and Schwarz 1996).

This study was conducted to improve the rooting efficiency of microshoots of flowering dogwood in culture. The thesis consisted of three experiments where the effect of locust diffusate, willow diffusate, SA, ASA, RSB and its extracts on rooting microshoots was observed.

The following were the objectives of this research:

1. to improve rooting of flowering dogwood microshoots;
2. to investigate black locust and contorted willow diffusates as a pretreatment for rooting *C. florida* microshoots;
3. to study the effect of salicylic acid and acetylsalicylic acid on rooting *C. florida* microshoots;
4. to study the effect of the Root Stimulating Bacterium and it's extracts obtained at pH 3 and pH 7 on rooting *C. florida* microshoots.

CHAPTER 2

LITERATURE REVIEW

2.1 Distribution of Dogwoods

Dogwoods belong to the family Cornaceae and genus *Cornus* L. that consists of approximately 65 distinct species (Eyde 1988). They are mainly distributed in the temperate regions of the North America, Europe and Asia, although some are located in Africa and Peru (Brinkman 1974; Elias 1980). Three large-bracted dogwood species commonly grown in the US are *C. florida* L., *C. nuttallii* Aud. and *C. kousa* Hance. Within the United States, *C. florida* or flowering dogwood is mainly found in the eastern US (Brinkman 1974; Santamour and McArdle 1985). The natural range of *C. florida* extends from Maine to Florida and from Michigan to Alabama. West of the Mississippi, the range extends to Missouri, Arkansas, Oklahoma, Texas and Louisiana. *Cornus nuttallii* or Pacific dogwood is mainly found on the western US coast from southern California to British Columbia. A small disjunct population is located in central Idaho. *Cornus kousa,* originally found in Japan, Korea and China, is another widely cultivated species in US. *Cornus capitata* Wallich from the Himalayas and South-central China has been grown for some time in California (Santamour and McArdle 1985). Some other dogwood species found in the US. are *C. sericea* L., *C. alternifolia* L., *C. amomum* Mill., *C. canadensis* L. and *C. racemosa* Lam. (Dirr 1998).

2.2 Dogwood Morphology

Most dogwood species are small trees or shrubs, but two exhibit a herbaceous habit. Mature flowering dogwood trees can reach a height of 40 ft and spread equal to or considerably greater than the height (Dirr 1998). They can be broadly classified into two categories based on their bract size and fruit color (Eyde 1988). The first group has small inconspicuous bracts when present and blue or white fruits. The other group has large basal bracts and/or bright red fruits when mature. This group consists of the dwarf cornels, cornelian cherries and large-bracted dogwoods (Eyde 1988). The leaves in most dogwood species are opposite but sometimes alternate as in *C. alternifolia* and *C. nuttallii*. The flowers are small, perfect, 4-merous and in cymose or head-like clusters (Brinkman 1974). Flower petals may be pale white, yellow or green in color, but bracts are the most conspicuous feature of the plant. They extend well beyond the clusters of flowers and may be either red, pink or white. The flowering season for dogwoods is between March and July. Fruits are globular or ovoid and ripen in late summer or fall. They have a single two celled and two seeded bony stone. In many cases only one seed per fruit develops fully (Brinkman 1974).

2.3 Significance of Dogwoods

Dogwoods in the US are mainly cultivated for their ornamental beauty and landscape value. Annually they account for 16% of the woody ornamental production in Tennessee and have a wholesale value of about 48 million dollars (Witte 1995). Seventy-five percent of all dogwoods produced in the United States are from seedlings, rooted cuttings or grafted plants grown in Tennessee (Southards 1995). In addition to this, Dogwood Festivals are a significant source of revenue for many communities; these celebrations are held in many states including Tennessee, Georgia and Washington to name a few. The Dogwood Festival in Knoxville, Tennessee brings in 10-13 million dollars annually (Southards 1995).

Dogwoods also have a few other minor uses. *Cornus officinalis* Sieb. and Zucc. is used in Chinese medicine as an astringent, tonic and hemostatic agent (Yazaki and Okuda 1989). It is a sour, astringent, diuretic herb that acts mainly as an energy tonic for the liver and kidney and for nervous exhaustion and tension headaches. The tonic stops bleeding, lowers blood pressure and controls bacterial and fungal infections. The redosier dogwood, *C. sericea* L., is used to cure indigestion, diarrhea and vomiting.

The origin of the generic name *Cornus* is from the word "cornu", or horn, which described the strength and heaviness of the close-grained dogwoods (Keeler 1912). The wood from flowering dogwood trees is hard and used in handicrafts, in turnery and for making charcoal (Brinkman 1974) and also has a few industrial applications such as for

shuttle blocks in textile weaving (Hall 1949). The wood has also been used for hubs of small wheels (Keeler 1912), handles of tools, mallets (Keeler 1912), barrel hoops and engravers' blocks (Elias 1980). Some dogwood species produce edible fruits (Anonymous 1950; Anonymous and May 1951). The fruits of *C. mas* are used to make jelly (Trigiano et al. 1992). A compound extracted from the bark of some dogwoods has been used as a substitute for quinine as a remedy for fevers (Brinkman 1974). Dogwoods are also important ecologically as they provide food and habitat for many birds and wildlife (Boring et al. 1981; Chellemi et al. 1992; Eyde 1988; Jaynes et al. 1993; Pirone 1980). The fruits are eaten by many animals such as the squirrels, raccoons, bobwhite quail, grouse, songbirds and wild turkey while the leaves and twigs are browsed by whitetail deer (Elias 1980).

2.4 Dogwood Pests and Diseases

There are a few insect pests that cause minor damage to dogwoods. But when the plant is under stress this damage can be significant (Bailey and Brown 1991). Some of the insect pests are dogwood twig borer and clubgall midge that cause tip dieback (Bir et al. 1988). Dogwood borer is another common pest that enters the tree through the injured areas of bark. The larvae feed on the cambial layer and may girdle and kill branches or trees. Seedcorn maggot flies can also cause death of twigs (Bir et al. 1988).

Greater damage is caused by fungal pathogens. Some of the common diseases of dogwoods are spot anthracnose, leaf spots, botrytis petal blight, trunk canker, damping off and root rot.

Flowering dogwoods are affected by two major fungal diseases, powdery mildew and dogwood anthracnose. Powdery mildew, caused by *Microsphaera pulchra* Cooke and Peck, infects *C. florida* in both landscape and natural environments (Klein et al. 1998). This disease was rarely reported before 1994 but has become a major problem in production of *C. florida* in the eastern US (Hagan et al. 1995; Klein et al. 1998; McRitchie 1994; Ranney et al. 1994; Windham 1996; Windham and Witte 1998). The disease first appears on young leaves as small areas covered with powdery white fungus (McRitchie 1994). It results in twisted and distorted leaves, stunted seedlings, slow growth and development of flowers and trees (McRitchie 1994). There is also an increase in red pigmentation of leaves (Santamour and McArdle1985; Windham and Witte 1998). Heavily infected plants may die or show twig blight. The leaf surfaces are covered with mycelia bearing conidia and ascocarps (Klein et al. 1998).

Dogwood anthracnose is caused by the fungus *Discula destructiva* Redlin (Redlin 1991) and *Discula* sp. that belong to the group of imperfect fungi (Salogga 1982). Plants affected with anthracnose can be identified by the water soaked necrotic lesions on leaves, cankers, lower branch dieback, production of epicormic shoots and dead leaves clinging to trees, even through winter (Daughtrey et al. 1996; Hibben and Daughtrey 1988; Smith 1991). These symptoms were first observed in the late 1970s in the New

York City area among *C. florida* trees (Daughtrey et al.1996). Similar symptoms were also first observed on pacific dogwoods in western Washington in 1976 (Byther and Davidson 1979). The sudden appearance and rapid spread of the disease on both the east and the west coast of the United States suggest that the causal organism is an introduced pathogen (Hibben and Daughtrey 1988; Redlin 1991). DNA amplification fingerprinting of the two *Discula* species provided evidence that *D. destructiva* is an introduced pathogen (Trigiano et al. 1995).

2.5 Dogwood Culture

Generic native white flowering dogwood is commercially propagated from seeds. Propagation of various cultivars is usually carried out by vegetative methods. They are produced by grafting buds onto *C. florida* seedling or by rooting of softwood cuttings (Badenhop et al. 1985; Dirr and Heuser 1987; Witte 1995). Of the two production methods, budding is more common whereas less than 5% production is by rooting cuttings. Scientists are attempting to increase production by rooting cuttings as it has several advantages over budding. Trees produced by rooted cuttings have straighter trunks and are less susceptible to diseases and pests since wounds are not created during their production (Badenhop et al. 1985). Budding may also result in undesirable sprout production from the understock and requires skilled and experienced labor. If carried out on a large scale by big nurseries with good field irrigation and greenhouse facilities, it is

suggested that rooting cuttings may be economically beneficial (Badenhop et al. 1985). However both these methods depend upon the season and success can be unpredictable. Tissue culture methods can be used to rapidly produce multiple copies of an important cultivar (genotype), that may have extraordinary horticultural attributes or have certain disease resistance genes, from a small stock.

2.6 Micropropagation Stages

The process of micropropagation can be divided into five stages that represent points at which there is a change in the cultural environment (Miller and Murashige 1976). These stages have been explained well by Kane (1996).

Stage 0 is the selection of donor plant and preparation of explants. This is an important stage because the responsiveness of plants in vitro depends greatly on the condition of the donor plant (Debergh and Maene 1981). During this stage, plants are maintained in a clean, disease free environment. Lower humidity, use of drip irrigation and antibiotic sprays are suitable as they help to reduce the risk of contamination. Efforts to alter the physiological state of the plant may also be made to increase responsiveness of the explant.

Stage 1 is the initiation and establishment of contaminant free explants. The plant parts to be cultured are excised and surface disinfested. They are maintained on medium that may or may not contain PGRs. The media and the types and quantities of PGRs to

be used vary from species to species. Growth media and PGRs are selected in a way to maintain shoot growth but reduce callus and adventitious root formation. Multiple subcultures on this medium are required in order to obtain consistent growth and shoot multiplication (Kane 1996).

Stage 2 involves axillary shoot proliferation. During this stage, plants are maintained on a medium that allows the formation of several axillary shoots. Usually a higher cytokinin to auxin ratio is used to supplement the medium used for this stage. The type and concentration of cytokinin to be used varies between different cultures and must be experimentally determined. These cultures can also be subdivided into smaller clusters to increase the culture population. Individual shoots can be excised and used for rooting and acclimatization.

During stage 3, plants are prepared for transfer to soil. Elongated microshoots are excised and treated with auxins to promote root formation. This treatment can be carried out by dipping shoots in an auxin solution for a short period of time or by growing them on a medium supplemented with auxin. The type of auxin and the concentration to be used varies greatly between species and might not be required for certain herbaceous plants that root spontaneously.

Acclimatization or stage 4 is the final stage that determines the success of the micropropagation method. Plants cultured in vitro have low photosynthetic activity as they have been cultured in the presence of sucrose and under low light and gas exchange conditions. They also have a poor control of water loss due to reduced epicuticular wax,

abnormal stomata, poor vascular connection between roots and shoot and poorly developed mesophyll. During this stage, efforts to convert plants from a heterotrophic to a photoautotrophic state are made. This is done by transferring plants to a well drained medium and gradually exposing the plants to increasing light intensities and low humidity conditions.

2.7 Dogwood Studies

2.7.1 Micropropagation of Dogwoods

Pennel (1983) conducted micropropagation studies of dogwood with *C. canadensis* but details were not provided. The Pacific dogwood *C. nuttallii* was propagated by Edson et.al (1994) using WPM. They rooted the plants ex vitro with an IBA talc dip. *Cornus kousa* has also been cultured (reported in Trigiano et al. 1992).

2.7.2 Micropropagation of *Cornus florida*

The earliest reference to *in vitro* culture of *C. florida* was that of Coker (1982). She cultured *C. florida* in vitro by micrografting dormant and non-dormant buds onto seedlings which were cultured on Knop's medium. However, any shoots that proliferated could not be rooted successfully. Somatic embryogenesis was achieved in *C. florida* from zygotic embryos (Trigiano et al. 1989). Many of these embryos germinated to produce roots, but only about 12% regenerated entire plants. Axillary bud cultures from

seedlings were found to be easier to establish than those from mature trees (Trigiano et al. 1992). Declerck and Korban (1994) studied the effects of various media and hormones on shoot proliferation of *C. florida* axillary bud cultures. They obtained maximum shoot proliferation with WPM macronutrients supplemented with 6-benzylaminopurine (BA). However they did not try to root these cultures. The first successful and complete micropropagation of *C. florida* was accomplished using nodal and apical meristems of seedlings (Kaveriappa et al. 1997). The greatest mean number of microshoots was produced on WPM augmented with 4.4 μM BA. The mean number of microshoots per culture was also influenced by the source of the seed, indicating that there may be a genetic component to the ability of some plants to produce shoots in culture. Although some rooting was obtained with most treatments, WPM supplemented with 4.9 μM IBA produced the greatest number of rooted shoots.

2.7.3 Miscellaneous

Some of work with dogwood species includes studies of ultrastructural changes in cells of calluses exposed to chilling temperatures (Niki et al. 1978; 1979), alternate respiratory pathway due to low temperature stress (Yoshida and Tagawa 1979) and the production of secondary metabolites by cell cultures (Yazaki and Okuda 1989). Studies to determine the identity of powdery mildew fungi on *C. florida* and *C. amomum* have been conducted (Klein et al. 1998). This was done using histological techniques to

observe ingress and colonization by the powdery mildew pathogens *M. pulchra* and *Phyllactinia guttata* (Wallr.:Fr.) Lév.

2.8 Root Stimulating Compounds

There are many chemicals and microorganisms besides PGRs that affect adventitious root formation in plants. Also, many plant tissues have been found to contain substances that promote root formation alone or when combined with other auxins (Davis et al. 1988; Davis and Haissig 1994; Hartmann et al. 1990).

Kawase (1970) identified root promoting substances in the weeping willow *Salix alba* and used them to root mung bean. The diffusate also showed a synergistic effect with IAA on rooting of mung bean cuttings (Kawase 1964; 1971). Cuttings of other species treated with the willow extracts also formed more roots than non-treated ones (LeClerc and Chong 1982). However, the extract failed to stimulate rooting in Rhododendrons (Proebsting 1983). Diffusates from the willow tree improved rooting in other moderate and difficult to root plants such as chinese fringetree and white fringetree (Arena 1997).

Salicylic acid (SA) or orthohydroxy benzoic acid has been recognized as an endogenous regulator in plants (Yalpani et al. 1991). SA is a plant phenolic compound and consists of an aromatic ring with a hydroxyl group at the ortho position. SA occurs in the form of esters in several plants, one of which is the willow tree (*Salix* sp.). Study

on SA levels in thermogenic and non-thermogenic plants suggest that it is ubiquitously distributed (Raskin et al. 1990). Acetylsalicylic acid (ASA) is hydrolyzed to SA under aqueous conditions and has similar effects as SA in many plants (Leslie and Romani 1988). ASA is commonly known as aspirin, which is the trade name given to it by the Bayer company in 1898 (Raskin 1992).

SA and ASA affect many biological processes, such as flower formation (Cleland and Ajami 1974; Khurana and Maheshwari 1983), vegetative bud formation (Fries 1984), disease resistance to pathogens and production of pathogenesis-related proteins (Antoniw and White 1980; Hooft van Huijsduijnen 1986; Pennazio et al. 1987; van Loon and Antoniw 1982; White 1979; Yalpani et al. 1991). SA and ASA also affect adventitious root formation (Kling and Meyer 1983; Mensuali-Sodi et al. 1995; Still et al. 1976) and somatic embryogenesis (Meijer and Brown 1988). SA stimulated adventitious root formation in mung bean cuttings (Kling and Meyer 1983) whereas it showed a negative effect on root formation in lavandin microcuttings by inhibiting ethylene reduction (Mensuali-Sodi et al. 1995).

Co-culturing plants and explants with certain bacteria such as *Agrobacterium rhizogenes* promoted adventitious rooting (Baker and Dyer 1996; Bassil et al. 1991; Mihaljevic et al. 1996; Patena et al. 1988; Strobel and Nachmias 1988). Recently, a non-pathogenic root stimulating bacterium (RSB) was isolated (Burns and Schwarz 1996). RSB is a rod shaped Gram negative bacterium that grows well at 30C to form thick bright-yellow to mustard-yellow colonies (Burns and Schwarz 1996). RSB stimulated

adventitious root formation of in vitro cultured *Pinus elliottii* Engelm. RSB-induced roots resemble roots formed by seedlings instead of those induced by hormonal treatments (Burns 1990).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Explants

Fruits from seven wild type flowering dogwood trees on the University of Tennessee Agricultural campus and 'Cherokee Chief', a red-bracted cultivar located at a private residence were collected on September 19, 1997. Fruits were soaked in tap water for two days, depulped by hand and allowed to dry overnight. Seeds were placed in a mixture of sand and peat moss (ratio 1:1) that was moistened with warm water to its holding capacity. Seeds in the moistened medium were placed in small 'zip lock' plastic bags and stratified in a refrigerator at about 4C for 4 months. Another set of seeds was collected from seven trees on the University of Tennessee Agricultural Campus, Knoxville on October 8, 1997 and stratified in the same way. At the end of the 4 month cold stratification period some seeds had germinated. Seeds were transferred to trays containing a mixture of composted pine bark and promix BX (Premium Peat Co.) in the ratio of 3.5:1 and grown in the greenhouse. They were fertilized weekly with 200 ppm N using Peters 21-7-7 acid special (Grace-Sierra Co., Milpitas, California, USA).

After about 8 weeks in the greenhouse, the seedlings had 2-3 sets of true leaves. Nodal segments were obtained by cutting the stems above and below the node and by removing the leaf lamina leaving a small portion of the petiole attached to the stem. Segments containing the apical bud were obtained by cutting the stem about 1 cm below

the apical bud. Nodal segments and apical buds were surface disinfested by soaking them in a 20% bleach solution (Clorox) amended with a few drops of Triton X-100 and constant stirring for 15 min. They were then rinsed three times in sterile distilled water. About 2 mm of the cut ends that were exposed to the bleach were removed and the explants transferred to petri dishes (polystyrene, disposable 60 x 20 mm)containing woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with 30g/L sucrose, 0.1 g/L myo-inositol, 1mg/L thiamine, 8g/L phytagar (Gibco) and 4.4 μM 6-benzylaminopurine (BA). The pH of the medium was adjusted to 5.7 prior to autoclaving. Petri dishes were sealed with parafilm® (American National Can™, Chicago, IL). Cultures were maintained in incubators at 23C under 25 $\mu mol.m^{-2}.sec^{-1}$ light provided by fluorescent tubes for 16/8 h light-dark photoperiod. They were transferred every 5 weeks to fresh medium with similar composition except for the additional vitamins present that consisted of 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 0.1 mg/L thiamine. Explants and any subsequent growth were acclimatized to in vitro conditions for at least 4-5 culture cycles before the microshoots were used for rooting experiments.

3.2 Preparation of Willow Diffusate (WD) and Black Locust Diffusate (LD)

Aqueous diffusates were prepared from black locust (*Robinia pseudoacacia* L.) and willow (*Salix* x *erythroflexuosa* Rag.) (Arena 1997). On the morning of July 22, 1998, 1.25 kg of fresh terminal shoots of willow was collected from the trial gardens at

the University of Tennessee Agriculture Campus, Knoxville. Leaves were removed and stems were cut into about 4 cm long segments. Stem segments were placed in a galvanized steel bucket containing 8 L of tap water and steeped for about 24 h at room temperature. Large pieces of stems and other debris were removed and diffusate filtered through several layers of cheesecloth three times. The volume of WD was divided into 400 ml aliquots and stored in plastic bottles at -20C. LD was prepared on 23 June 1998 from 0.9 kg of black locust shoots, collected from the trial gardens at the University of Tennessee Agricultural Campus, Knoxville, soaked in 5 L of tap water. The diffusate was filtered and stored as 400 ml aliquots in plastic bottles at -20C.

The diffusates were thawed and first filtered through Whatman number 1 filter paper just before use in the experiments. Diffusates were filter sterilized subsequently using 0.22 μm filters.

3.3 Preparation of Root Stimulating Bacterial Extract

3.3.1 Bacterial Culture Preparation

The root stimulating bacterium (RSB) was cultured on modified WPM as described by Burns and Schwarz (1996; Appendix I). The medium was adjusted to pH 5.5 before autoclaving and supplemented with 10 ml/L of GD (Gresshoff and Doy 1972) iron stock, 20 g/L sucrose, 2g/L casein hydrolysate and 15 g/L Bacto™ Agar. Bacterial cultures were obtained from Dr. O. J. Schwarz, Department of Botany, University of Tennessee. The T-streak method (Claus 1988) was used to subculture these bacteria and

obtain single cell derived colonies. Bacteria were cultured in 100x15 mm petri dishes and maintained at 27 C in the dark. Dense bacterial colonies were observed after 5-6 days.

3.3.2 Bacterial Extract Preparation

RSB was grown on modified WPM (Burns and Schwarz 1996; Appendix 1). A loopfull of bacterial colony was inoculated into liquid modified WPM of the same composition except without agar. Liquid bacterial cultures were grown on a shaker incubated at 28C and after 5-6 days, about 450 ml of liquid containing RSB were poured into two bottles and centrifuged at 6000 rpm (~5860xg) for 10 min at 5C. Bacterial cells were collected at the bottom of the tubes and were discarded, leaving about 400 ml of supernatant. The pH of the supernatant was adjusted to pH 7.14 with 1 N KOH and 200 ml of ethyl acetate added. The mixture was subjected to constant agitation via a stir bar and stir plate for 45 min. The liquid was poured into a separatory funnel and the water fraction (lower layer) discarded. The ethyl acetate fraction was poured into a flask and dried using a rotary evaporator. Another fraction was obtained by adjusting the original ethyl acetate fraction to pH 3 with 1 N HCl and extracting in the same way. The residue was collected by adding 50 ml of 70% ethanol that turned slightly yellow. The residue that was collected from the pH3 fraction had a strong odor. Bacterial extracts obtained at pH 7.14 (BE7) and pH 3 (BE3) were stored in separate plastic bottles at 4C in the dark. Both extracts were filter sterilized using a 0.22 μm filter just before use in experiment.

3.4 Preparation of Salicylic acid and Acetylsalicylic acid Solutions

A 0.1 M solution of salicylic acid (MW = 138.12) was prepared and dilutions were made to obtain 10, 25, 50, 100 and 200 μM salicylic acid. Similarly, a 0.1 M solution of acetylsalicylic acid (MW = 180.15) was prepared and dilutions were made to obtain 10, 25, 50, 100 and 200 μM acetylsalicylic acid. Solutions were sterilized by passing through a 0.22 μm filter. Solutions were added to cooled medium for continuous exposure of microshoots. For the 24 h pulse exposure, the solutions were poured into sterile magenta boxes.

3.5 Preparation of Samples for Histological Studies

Leaf and root samples for histological studies were collected in April-May 1999. The following samples were used to study anatomy: leaves from a mature dogwood tree on the Agriculture campus, leaves from acclimatized tissue cultured plants in the greenhouse, leaves from plants in peat pellets from the incubator and from stock cultures on WPM + BA. Stem cuttings were collected from microshoots placed on WPM + 4.9 μM IBA after 10, 12 and 15 days. Cuttings from adventitious roots were collected 1 and 3 weeks after erupting from the surface of the microshoots.

Samples were rinsed in water, cut into pieces not more than seven mm long and transferred to vials containing Histochoice®(Amresco, Solon, Ohio) a non-aldehyde fixative + 20% ethanol. Samples (except the older root samples) were vacuum aspirated

for 1 h in the fixative and the solution replaced with fresh fixative of the same composition. Samples were stored at room temperature until processed for histology.

Samples were dehydrated using a graded series (30, 50, 70, 95 + Erythrosin B, and 100 %) of isopropyl alcohol, each for 30 min. Samples were rinsed in 100% isopropyl alcohol 2 more times for 15 min each. Samples were gradually infiltrated with Paraplast +® (Oxford ® Labware, St. Louis, Missouri) by adding a few chips of Paraplast+ to the vials in a 60C oven. A few chips were added every 30 min. After this was repeated several times, sufficient Paraplast+ was added to cover the tissue. The vials were left in the oven overnight to evaporate all of the isopropyl alcohol. The Paraplast+ was discarded and replaced with fresh melted Paraplast+. Samples were cast into blocks by placing them in 8 x 8mm truncated peel away embedding molds (Polysciences Inc., Warrington, Pennsylvania) containing Paraplast+.

3.6 Design of Experiment

A randomized incomplete block design was used for all microshoot rooting experiments. This design consists of blocks that do not contain all treatments and is especially suitable for experiments with many treatments or for those where a limited source of homogeneous explants are available (Kuklin et al. 1993). Also, blocking by explant source (i.e. culture) can be done with this design. This design conserves experimental materials and reduces labor and yet is equally powerful and efficient as the other designs (Kuklin et al. 1993).

3.7 Rooting with Willow Diffusate and Locust Diffusate

3.7.1 Experimental Design

The experimental design used was a randomized incomplete block design with 6 treatments. There were 10 replications for each treatment and 30 blocks with 2 treatments per block. The experimental unit consisted of one microshoot per test tube (disposable polystyrene 17 x 100 mm with snap caps) and the 2 microshoots within in the same block were from the same 'mother' culture. The experiment was physically set-up in labeled test tube racks and incubated at 26 C under 25 $\mu mol.m^{-2}.sec^{-1}$ for a 16/8 h light-dark photoperiod. The treatments were the following: sterile, distilled water, WD, 4.9 μM IBA + WD, LD, 4.9 μM IBA + LD and water + 4.9 μM IBA.

3.7.2 Experimental Procedure

Supports to hold the microshoots were made from Whatman number 1 filter paper, which had 2 mm holes punched in it with a teasing needle. The supports were placed in Magenta GA-7 vessels and autoclaved (25 min at 121 C). Dogwood microshoots that were at least 1 cm long were excised and inserted into filter paper supports and pretreated for 24 h with either filter sterilized WD, LD or sterilized water. The cut end of the microshoots were placed 2 mm into the diffusates (Figure 1). After 24 h, microshoots were transferred to test tubes containing about 10 ml of WPM or WPM

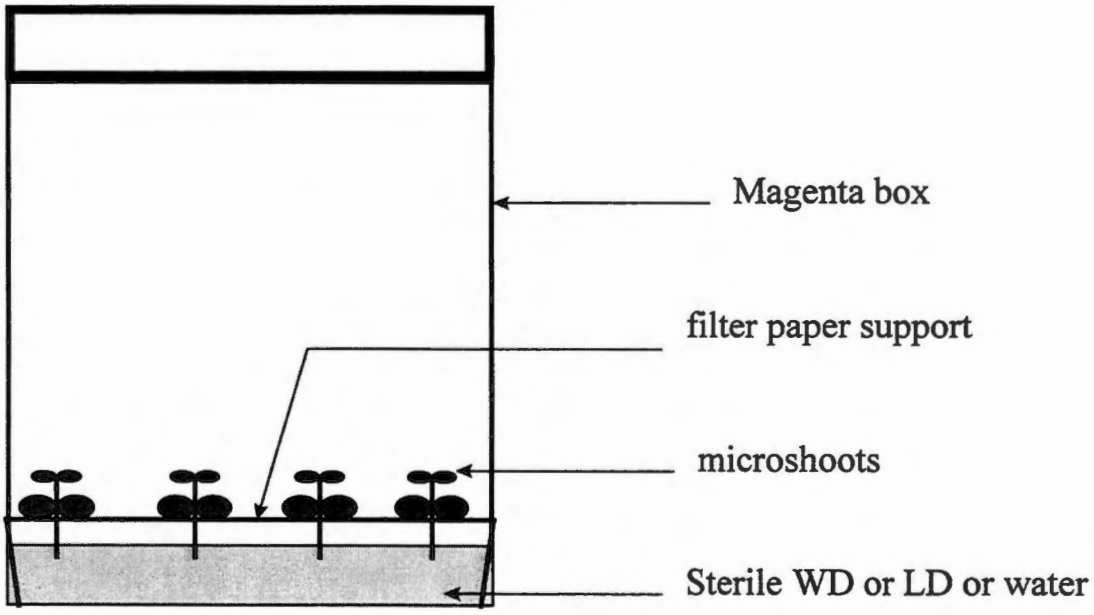


Figure 1. Diagrammatic representation of the treatment method used to dip microshoots in treatment solutions

+ IBA. The tubes were placed in test tube racks and incubated at 26C for 5 weeks under 25 $\mu mol.m^{-2}.sec^{-1}$ with a 16/8 h light-dark photoperiod. The number of roots regenerated from each microshoot was counted after 5 weeks. After rooting, the microshoots were transferred to WPM with 4 g/L phytagar (Gibco) and without any IBA for a week and then to WPM with 10 g/L sucrose and 4 g/L phytagar for one additional week. Finally, they were placed in WPM without sucrose and 6 g/L phytagar for 2 to 3 weeks.

Agar on the roots was washed off with tap water before placing rooted microshoots in Jiffy peat pellets (Jiffy products (N. B.) Ltd. Shippagan, Canada), which were presoaked in warm water until they expanded and were soft. Rooted microshoots were placed in the peat pellets very carefully to avoid breaking the roots that were brittle and weak. Peat pellets with entire plants were placed in Magenta GA-7 vessels and incubated at 17C with about 40 $\mu mol.m^{-2}.s^{-1}$ light provided by fluorescent tubes for 16/8 h light-dark photoperiod. They were initially watered with 50% WPM basal salt solution without any sucrose and thereafter with 10% WPM basal salt solution when needed. The lids of the vessels were securely closed for the first week and then opened each day for an increasing amount of time starting with one hour until they were acclimatized to the growth chamber conditions after about 6 weeks.

When the experiment was repeated the peat pellets were maintained in trays with wells to hold the peat pellets. The lids for the trays were made of clear plastic and remained closed for about one week. Lids with increasing size of holes (2.5 cm diameter to 3 x 4 cm) were prepared and used to cover the trays during the next few weeks after which no lids were placed.

Plants were transferred to 15 cm square pots containing dogwood mix (Appendix 2) after the leaves had fully expanded and placed in the greenhouse. They were maintained in trays under 50% shade cloth (250 $\mu mol.m^{-2}.s^{-1}$ light). For winter culture, incandescent lighting (three, 300 watt bulbs) were positioned 1 meter over the pots to increase day length to 18 h. Osmocote 14-14-14 (5 gms), a slow release fertilizer that becomes activated about 2 weeks after distribution, was sprinkled over the surface soil around the plant. For the first two weeks plants were watered with 150 ppm Peters 20-20-20 soluble fertilizer. Plants were watered with regular tap water and maintained in the greenhouse.

3.8 Rooting with Root Stimulating Bacterium and Bacterial Extracts

Preliminary experiments were performed by dipping microshoots in RSB colonies growing on modified WPM (Appendix 1) with and without casein hydrolysate. Microshoots were then transferred to test tubes containing about 10 ml of the same medium. Extensive bacterial growth was observed in the test tubes after 2 weeks and no significant rooting was seen. Further experiments were designed to use RSB extracts obtained at pH3 and pH7.

3.8.1 Extracts at pH 3 and pH 7

3.8.1.1 Experimental Design

The design used for this experiment was a randomized incomplete block with 7 treatments. There were 15 replications for each treatment and 35 blocks with 3 treatments per block. The experimental unit consisted of one microshoot per test tube.

In the first experiment, different volumes of BE obtained at pH 3 and pH 7 were added to WPM. The treatments were the following:

WPM + 12.5 ml BE3,

WPM + 2.5 ml BE3,

WPM + 0.5 ml BE3,

WPM + 12.5 ml BE7,

WPM + 2.5 ml BE7,

WPM + 0.5 ml BE7 and

WPM + 4.9 μM IBA.

3.8.1.2 Experimental Procedure

Microshoots at least 1 cm long were excised from dogwood cultures maintained on WPM + 4.4 μM BA and transferred to test tubes containing about 10 ml of one of the above listed media. The cultures were placed in test tube racks in the incubator at 26C for 5 weeks under 25 $\mu mol.m^{-2}.sec^{-1}$ with a 16/8 h light-dark photoperiod. The number of roots regenerated from each microshoot was counted after 5 weeks. After rooting,

microshoots were transferred to WPM with 4g/L phytagar (Gibco) without IBA for a week and then to WPM with lesser concentration of sucrose (10 g/L) and 4g/L phytagar for one more week. Finally, they were placed in WPM without sucrose containing 6 g/L phytagar for 2 weeks. Rooted microshoots were acclimatized as described under section 3.6.2.

3.8.2 Extracts at pH 7

None of the plants from the BE3 treatment survived. The second experiment was designed to have treatments only containing different volumes of BE7.

3.8.2.1 Experimental Design

The experimental design used was a randomized incomplete block with 6 treatments. There were 10 replications for each treatment and 30 blocks with 2 treatments per block. The experimental unit consisted of one microshoot per test tube. In the second experiment, different volumes of BE obtained at pH 7 were added to WPM, resulting in the following treatments:

WPM + Water (No rooting agent),

WPM + 4.9 μM IBA,

WPM + 0.25 ml BE7,

WPM + 0.5 ml BE7,

WPM + 1.0 ml BE7 and

WPM + 5.0 ml BE7.

3.8.2.2 Experimental Procedure

The experimental procedure and data collection method used for this experiment was the same as that used for the experiment with BE3 and BE7 (Section 3.7.1.2).

3.9 Rooting with Salicylic acid and Acetylsalicylic acid

Two methods of exposing microshoots to rooting agent (SA or ASA) were compared. In one experiment, microshoots were constantly exposed to rooting agent, whereas another experiment used a pulse method. The same design was used for both these experiments.

3.9.1 Continuous Treatment

Microshoots were continuously exposed to rooting agent (SA or ASA) by incorporating the rooting agent in WPM amended with 4.9 μM IBA.

3.9.1.1 Experimental Design

The experimental design used was an incomplete block with 7 treatments. There were 12 replications for each treatment and 28 blocks with 3 treatments per block. The experimental unit consisted of one microshoot per test tube. In the first experiment different amounts of SA and ASA were added to WPM supplemented with 4.9 μM IBA. The treatments were the following:

WPM + 4.9 μM IBA (No additional rooting agent),

WPM + 4.9 μM IBA + 50 μM SA,

WPM + 4.9 μM IBA + 100 μM SA,

WPM + 4.9 μM IBA + 200 μM SA,

WPM + 4.9 μM IBA + 50 μM ASA,

WPM + 4.9 μM IBA + 100 μM ASA and

WPM + 4.9 μM IBA + 200 μM ASA.

3.9.1.2 Experimental Procedure

The experimental procedure and data collection method used for this experiment was the same as that used for the experiment with BE3 and BE7 (Section 3.7.1.2).

3.9.2 Pulse Treatment

Pulse treatment consisted of exposing the microshoots to the rooting agent (SA or ASA) for a 24 h period. The microshoots were exposed to SA and ASA in the same way as for the WD and LD experiment (Section 3.7.2; Figure 1) and then transferred to WPM amended with 4.9 μM IBA.

3.9.2.1 Experimental Design

The experimental design used in this experiment was the same as that for the continuous treatment. The treatments were the following:

WPM + 4.9 μM IBA + 10 μM SA,

WPM + 4.9 μM IBA + 25 μM SA,

WPM + 4.9 μM IBA + 50 μM SA,

WPM + 4.9 μM IBA + 10 μM ASA,

WPM + 4.9 μM IBA + 25 μM ASA,

WPM + 4.9 μM IBA + 50 μM ASA and

WPM + 4.9 μM IBA + water (autoclaved).

3.9.2.2 Experimental Procedure

In the second experiment, 24 h pulse treatments of SA and ASA were used. Microshoots were excised from stock cultures and maintained on filter paper supports in sterilized Magenta GA-7 boxes containing different concentrations of SA and ASA. These were treated in the same way as those in the experiment with WD and LD (Section 3.6.2). After 24 h the microshoots were transferred to test tubes containing about 10 ml of WPM + 4.9 μM IBA. Whole plants were then dealt with and data collected in the same way as for the BE3 and BE7 experiment.

3.10 Histological Studies

Embedded samples were mounted on a rotary microtome (Reichert-Jung 820 Histocut) and 12 μm thick sections were cut. Sections were mounted on clean Superfrost glass slides and allowed to dry for 24 h before staining. Modified Flemings Triple stain (Johansen 1940, Appendix 3) was used. Coverslips were secured to slides with Permount ® (Fisher Scientific, Fairlawn, New Jersey) as the mounting medium. Slides were

viewed with a Nikon microscope and photographs taken using a Nikon FX-35WA camera and Techpan film (Eastman Kodak Co., Rochester, New York). Black and white photographs were developed (Appendix 7) and printed on Kodabrome II RC Paper (Eastman Kodak Co., Rochester, New York).

3.11 Statistics and Data Analysis

Data collection consisted of counting the number of roots that formed on each microshoot. Since many microshoots in some of the treatments failed to produce roots, transformation of the counts was done to normalize the data. This conversion was done as follows:

Transformed number = $\sqrt{}$(number of roots + 1). Normality of the data was tested. A mixed model analysis of variance was performed (SAS Institute Inc., 1997). Mean separation was done on the transformed data using the method of least significant differences (LSD). Percentage rooting, least square (LS) mean number of roots/treatment and LS mean number of roots/rooted microshoot were calculated on the transformed data. Least square mean number of roots/treatment was calculated by taking into consideration all the replications for the treatment irrespective of whether the microshoots rooted or not. The LS mean number of roots/rooted microshoot was calculated considering only those microshoots that actually rooted for each treatment.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Rooting Microshoots with Willow and Locust Diffusates

This experiment was repeated three times. Data was analyzed separately for each experiment and also after combining results from all three trials.

Trial 1:

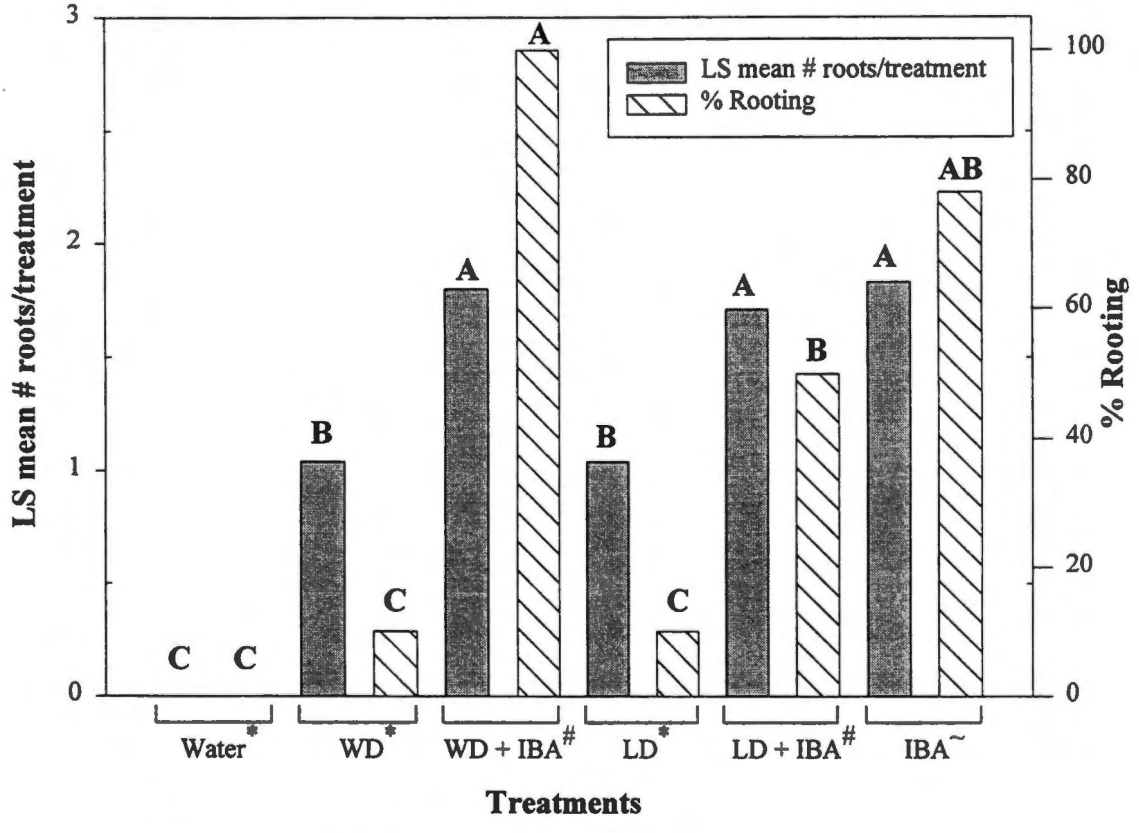
Significant differences ($P \leq 0.05$) between the mean number of roots generated per microshoot among various treatments were observed. Microshoots pretreated with water for 24 h before transfer to WPM did not produce roots (Table 1; Figure 2). Microshoots exposed to WD and LD for 24 h before transfer to WPM formed a mean (least square [LS]) of one root (Table 1; Figure 2). Microshoots transferred to WPM + 4.9 μM IBA generated more roots compared to those placed in WPM alone. There was a slight, but not significant, reduction in the number of roots formed per microshoot when pretreated with WD and LD before transfer to medium with IBA compared to those placed directly in IBA.

All microshoots generated adventitious roots when the cut surfaces were placed in WD for 24 h before transfer to WPM supplemented with 4.9 μM IBA. In contrast, only 78 percent of the explants formed roots with the 4.9 μM IBA treatment. Ten percent or fewer microshoots rooted when exposed to LD, WD or water (Table 1; Figure 2). Among

Table 1. Percentage rooting, least square mean number of roots/treatment and least square mean number of roots/rooted microshoot for the various willow and locust diffusate treatments for rooting *Cornus florida* microshoots.

Treatments	Least square mean number of roots/treatment				Least square mean number of roots/rooted microshoot				Percentage rooting			
	Trial 1	Trial 2	Trial 3	Mean	Trial 1	Trial 2	Trial 3	Mean	Trial 1	Trial 2	Trial 3	Mean
Water*	0^{AB}	1.4^{B}	1.3^{B}	1.2^{C}	-	1.4^{B}	1.6^{AB}	-	0^{C}	68^{AB}	50^{BC}	40^{C}
WD*	1.0^{B}	1.3^{B}	1.2^{B}	1.1^{C}	1.4^{A}	1.3^{B}	1.6^{AB}	1.5^{BC}	10^{C}	45^{BC}	39^{C}	30^{C}
WD + IBA#	1.8^{A}	1.5^{B}	2.0^{A}	1.7^{AB}	1.8^{A}	1.5^{AB}	2.0^{A}	1.9^{AB}	100^{A}	55^{BC}	92^{A}	83^{AB}
LD*	1.0^{B}	1.1^{B}	1.2^{B}	1.1^{C}	1.4^{A}	1.1^{B}	1.4^{B}	1.4^{C}	10^{C}	27^{C}	50^{BC}	27^{C}
LD + IBA#	1.7^{A}	1.5^{B}	1.9^{A}	1.6^{B}	1.7^{A}	1.5^{AB}	2.1^{A}	1.9^{AB}	50^{B}	64^{AB}	89^{AB}	67^{B}
IBA~	1.8^{A}	2.2^{A}	1.8^{A}	1.9^{A}	1.9^{A}	2.2^{A}	1.9^{AB}	2.0^{A}	78^{AB}	91^{A}	89^{AB}	89^{A}

* Microshoots transferred to WPM
# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA
- No roots formed
Numeric values followed by the same letter are not significantly different at the 0.05 level of significance. Mean separation was performed on the transformed data.



* Microshoots transferred to WPM
\# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 2. Effect of willow diffusate and locust diffusate on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots (Trial 1). Bars having different letters are significantly different at $P \leq 0.05$.

the microshoots that rooted, no significant differences ($P \leq 0.05$) were observed for the number of roots stimulated by various treatments (Table 1; Figure 3).

Trial 2:

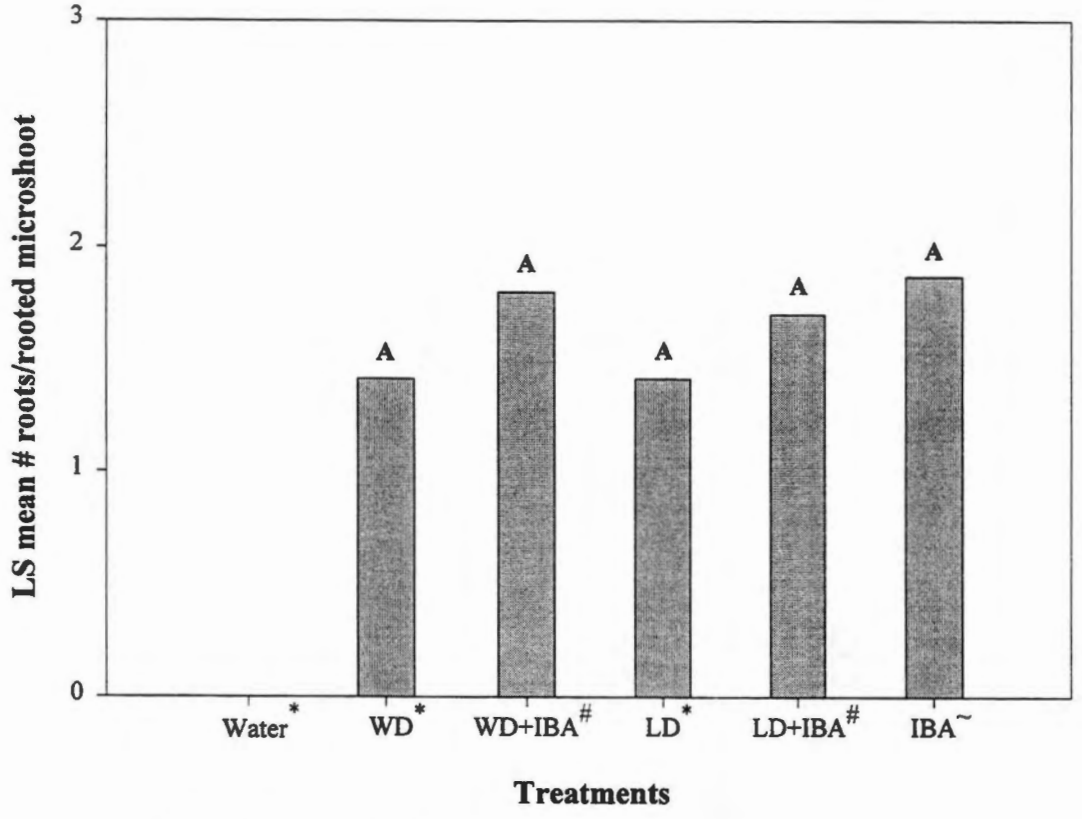
The results of this trial (Table 1; Figure 4) showed similar results to trial 1 for the number of roots stimulated by each treatment except that 70% of the microshoots treated with water rooted compared to 0% in Trial 1. However, significant differences for rooting percentages among various treatments were observed. Best rooting efficiency of > 90% was observed for microshoots treated with 4.9 μM IBA (Table 1; Figure 4).

Microshoots placed directly in medium containing 4.9 μM IBA also formed the most number of roots followed by those pretreated with WD or LD and transferred to WPM amended with IBA. Microshoots treated with 4.9 μM IBA formed a LS mean of 2.2 roots/treatment (Table 1; Figure 4). Among the microshoots that rooted, those transferred to medium amended with IBA formed significantly greater LS mean number of roots than those placed in WPM alone (Table 1; Figure 5).

Trial 3:

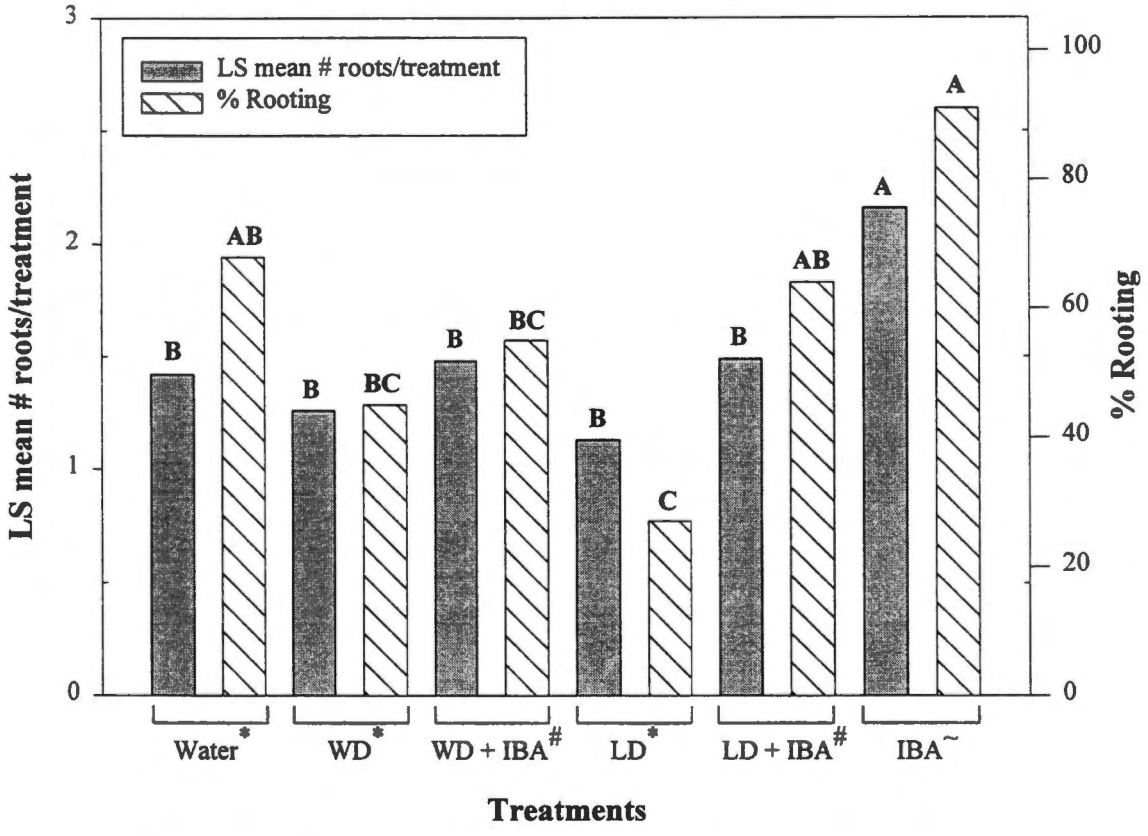
Microshoots treated with WD + IBA and LD + IBA formed a greater, although statistically similar ($P \leq 0.05$), LS mean number of roots than those treated with IBA alone (Table 1; Figure 6). Microshoots treated with water, WD or LD alone were similar to each other but produced fewer mean number of roots/treatment than other treatments.

Rooting percentages were higher for microshoots in the WD + IBA, LD + IBA



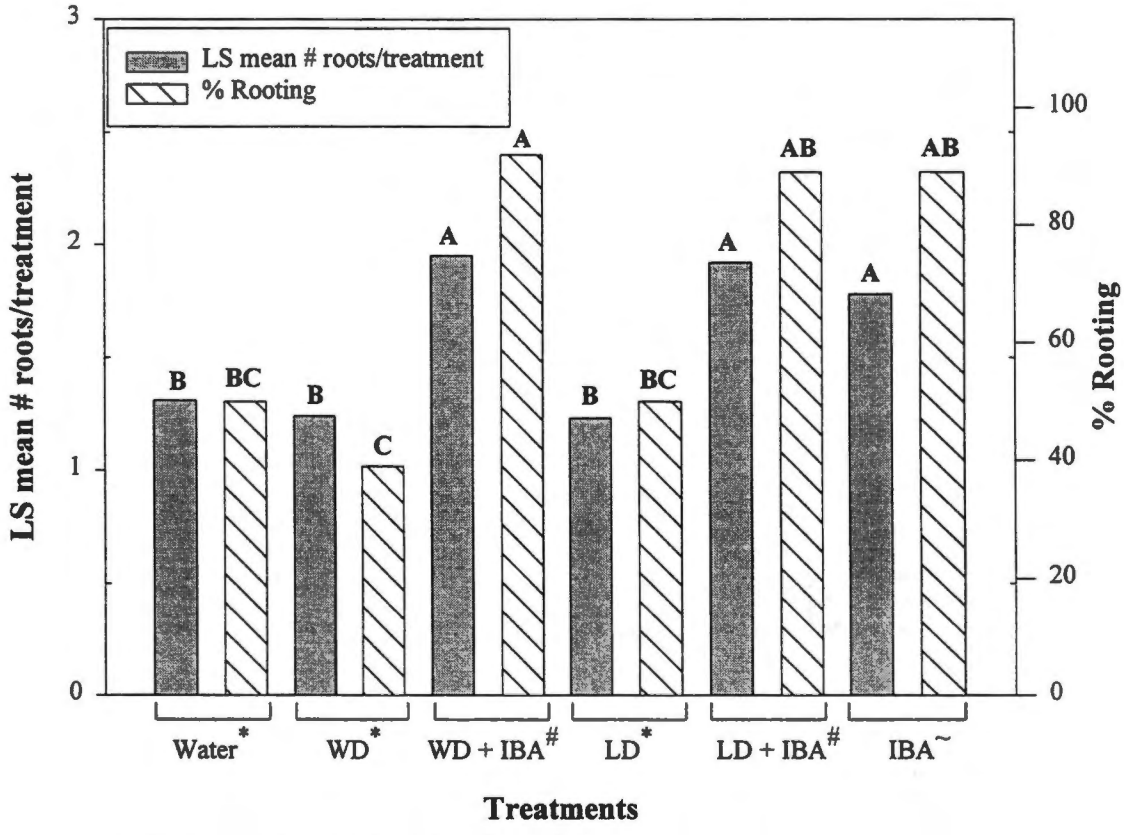
* Microshoots transferred to WPM
# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 3. Effect of willow diffusate and locust diffusate on least square mean number of roots/rooted microshootof *Cornus florida* (Trial 1). Bars having different letters are significantly different at $P \leq 0.05$.



* Microshoots transferred to WPM
\# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 4. Effect of willow diffusate and locust diffusate on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots (Trial 2). Bars having different letters are significantly different at $P \leq 0.05$.



* Microshoots transferred to WPM
\# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 6. Effect of willow diffusate and locust diffusate on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots (Trial 3). Bars having different letters are significantly different at $P \leq 0.05$.

and IBA treatments than for the water, WD or LD treatments. Treatment of explants with water, WD or LD resulted in fewer (%) rooted microshoots.

Among microshoots that rooted, those treated with LD alone formed fewer roots than all other treatments (Table 1; Figure 7).

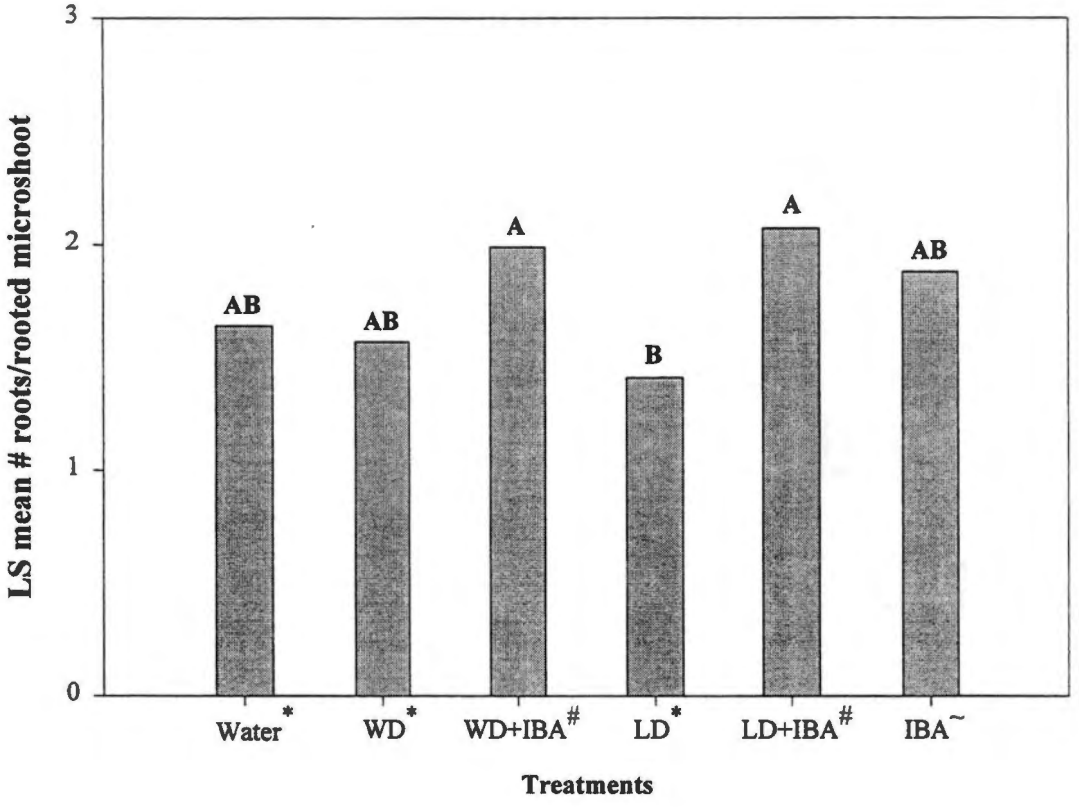
Mean of Trials 1, 2 and 3:

Results (Table 1; Figure 8) from the three similar experiments repeated at different times were pooled together and analyzed statistically. Microshoots pretreated with water, WD and LD alone formed significantly fewer number of roots/treatment than the other treatments (Table 1; Figure 8). Microshoots that received the LD + IBA treatment generated significantly fewer roots/treatment than those in the IBA treatment. The WD + IBA treatment was statistically similar ($P \leq 0.05$) to the IBA treatment for the number of roots formed per treatment.

The water, WD and LD pretreatments alone resulted in significantly fewer microshoots that rooted (Table 1; Figure 9). Rooting of greater than 80% were observed for the IBA and WD + IBA treatments. The LD + IBA treatment stimulated roots to form on 64% of the microshoots.

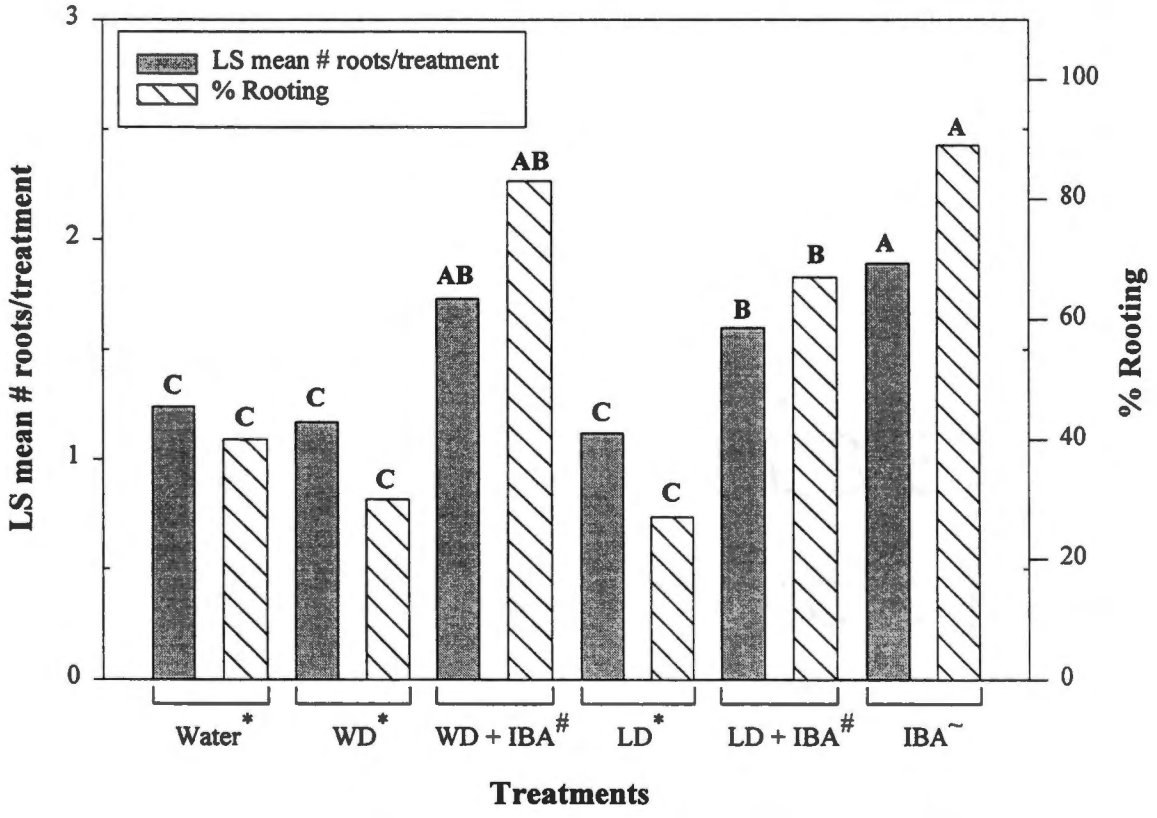
Among the microshoots that rooted, those treated with WD + IBA, LD + IBA or IBA alone formed significantly greater LS mean number of roots than the water, WD and LD treatments alone (Table 1; Figure 9).

Least square mean number of roots formed per treatment and percent rooting of microshoots was used to evaluate rooting efficiency. In summary, highest rooting



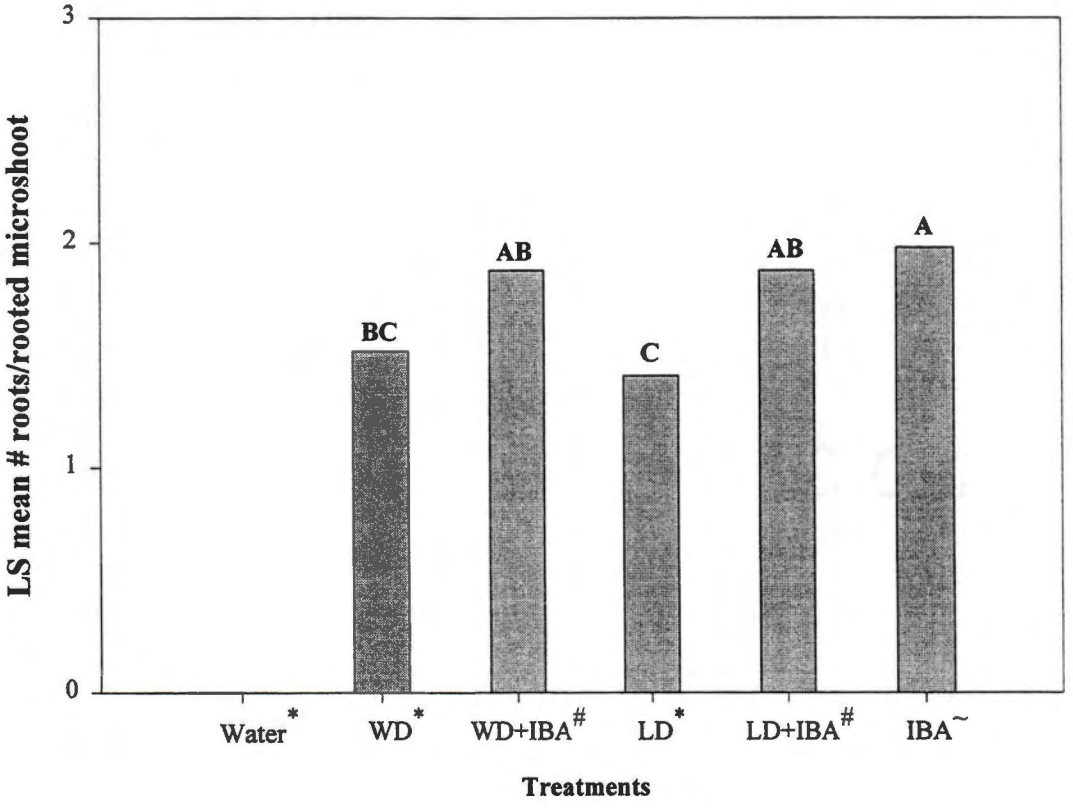
* Microshoots transferred to WPM
\# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 7. Effect of willow diffusate and locust diffusate on least square mean number of roots/rooted microshoot of *Cornus florida* (Trial 3). Bars having different letters are significantly different at $P \leq 0.05$.



* Microshoots transferred to WPM
\# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 8. Effect of willow diffusate and locust diffusate on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots (Mean of trials 1, 2 and 3). Bars having different letters are significantly different at $P \leq 0.05$.



* Microshoots transferred to WPM
# Microshoots transferred to WPM + 4.9 μM IBA
~ Microshoots placed directly in WPM + 4.9 μM IBA

Figure 9. Effect of willow diffusate and locust diffusate on least square mean number of roots/rooted microshoot of *Cornus florida* (Mean of trials 1, 2 and 3). Bars having different letters are significantly different at $P \leq 0.05$.

efficiency was produced when microshoots were placed directly in WPM containing 4.9 μM IBA. None of the pre-treatments stimulated better rooting than 4.9 μM IBA. WD + IBA, although similar to IBA alone, does not show significantly better rooting. Pre-treatment with WD or LD does not seem to improve rooting of *C. florida* microshoots.

WD and LD slightly inhibited root induction in Trials 1 and 2, which agrees with the results of Proebsting (1983) who observed that willow water inhibited rooting in Rhododendron cuttings. However, results of Trial 3 suggests that the use of WD and LD in combination with IBA enhances rooting in plants, as suggested by Hess (1964), Kawase (1970; 1971) and LeClerc and Chong (1983). Perhaps WD and LD contain certain cofactors that induce rooting (Hess 1961; 1963). WD and LD seem to have an additive effect with IBA in root formation on *C. florida* microshoots.

The average of the three trials showed that the WD pretreatment, although statistically ($P \leq 0.05$) similar to IBA, had a lower rooting percentage. Significantly lower rooting percentage as well as mean number of roots/treatment than the IBA treatment resulted from the LD pretreatment. Reduced rooting efficiency may be due to the inhibitory activity of certain compounds present in the diffusates. WD and LD may contain substances that stimulate rooting of *C. florida* microshoots or may not enhance the activity of IBA as suggested by Hess (1964), Kawase (1970; 1971) and LeClerc and Chong (1983). However, the results of the experiment agree with the results of Proebsting (1983) that the extracts fail to stimulate rooting. Addition of 4.9 μM IBA to WPM enhanced the root forming capacity of the microshoots compared to the IBA treatment with water pretreatment (control). This agrees with the findings of Kaveriappa

et al. (1997) that IBA can be used to stimulate rooting of *C. florida* microshoots. Rooting percentages ranged between 70 and 100 when microshoots were treated with IBA alone.

Higher rooting efficiencies were observed in these experiments compared to those obtained earlier by Kaveriappa et al. (1997). Microshoots grown on WPM amended with BA were green in color for the first few weeks. After about 4 weeks, the stem color started turning reddish-brown. Microshoots that were excised and used for rooting after they had started turning reddish-brown (presumably more mature) rooted more easily than those that were green. The microshoots used in the experiments described here were about 5-6 weeks old whereas the microshoots used in the experiments by Kaveriappa et al. (1997) were 4 weeks old. Based on these observations and results obtained from the WD and LD experiments it seems that the age of the microshoots might also have a role in rooting efficiency. This could be the reason why higher rooting efficiencies than those by Kaveriappa et al (1997) were obtained when the same concentration of IBA was used in both experiments.

Higher auxin to cytokinin ratios stimulate root formation in plants (Sutter 1996). A possible reason for the apparent age related ability to form roots is that there could be a change in the auxin:cytokinin ratio at the site of root initiation. In these experiments, microshoots were obtained from cultures grown in WPM supplemented with 4.4 μM BA. After 4-5 weeks, most of the nutrients and PGRs (cytokinin; BA) may have been depleted from the medium and the stem tissues. Perhaps this results in better rooting of microshoots when placed in medium containing auxin IBA. An interesting experiment

would be to determine the levels of BA in the microshoots and correlate it with the ability to generate roots.

4.2 Rooting with Root Stimulating Bacterium and Bacterial Extracts

Preliminary experiments were performed by dipping microshoots in RSB colonies growing on modified WPM (Appendix 1) with and without casein hydrolysate. Extensive bacterial growth was observed 2 weeks after transfer of microshoots to 10 ml of the same medium but no significant rooting occurred even after 8 weeks. Further experiments were designed instead to use RSB extracts obtained at pH3 (BE3) and pH7 (BE7).

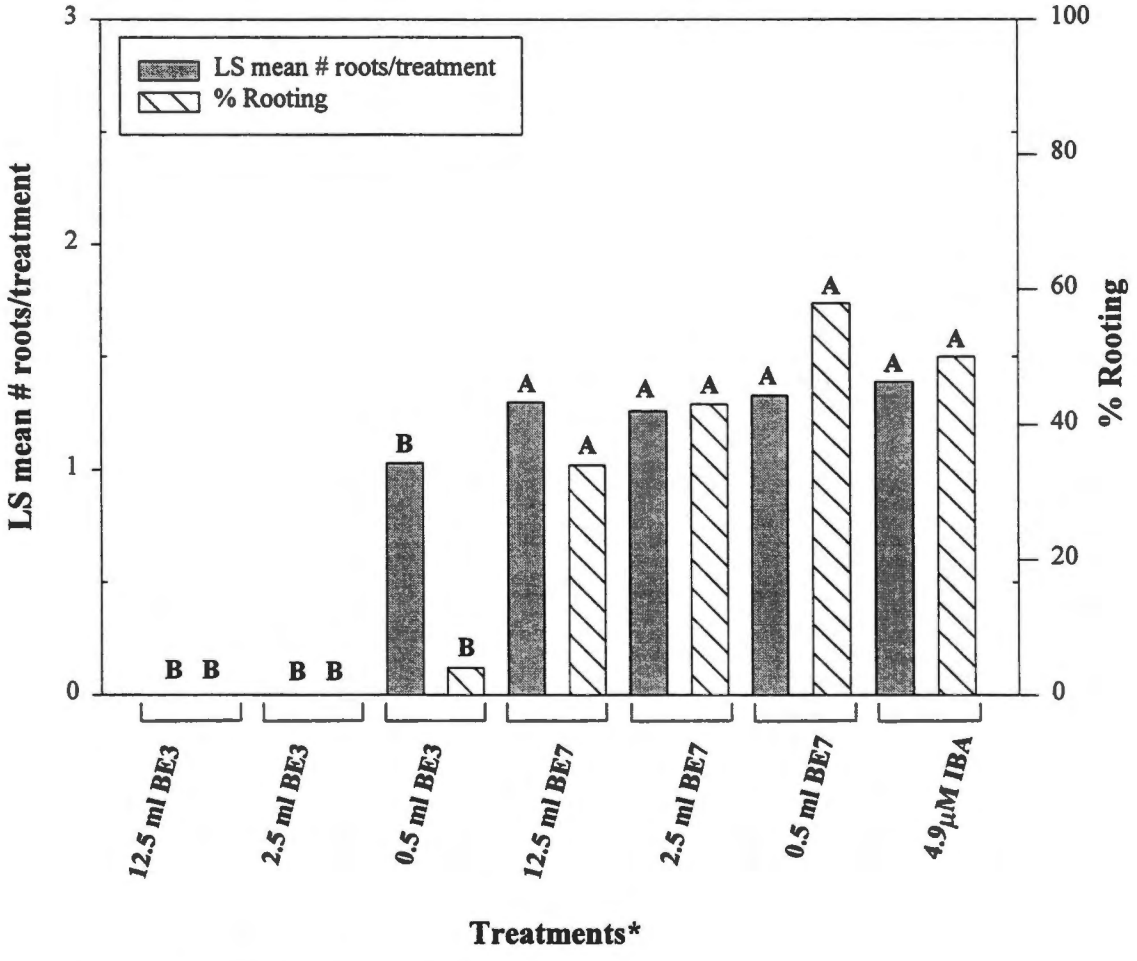
4.2.1 Extracts at pH3 and pH7

Clear differences were evident between the ability of BE3 and BE7 to stimulate rooting of *C. florida* microshoots (Table 2; Figure 10). Leaves of microshoots turned yellow 2-3 weeks after transfer to WPM containing any amount of BE3. None of the microshoots from any of the three BE3 treatments tested survived (Table 2, Figure 10). In the three BE7 treatments tested, the LS mean number of roots formed was statistically ($P \leq 0.05$) similar to the number of roots/treatment formed with 4.9 μM IBA. Although not statistically significant, some differences in rooting percentages of microshoots were observed among the various BE7 treatments. A higher percentage of microshoots rooted

Table 2. Percentage rooting, least square mean number of roots/treatment and least square mean number of roots/rooted microshoot for the various BE3 and BE7 treatments for rooting *Cornus florida* microshoots.

Treatments*	Least square mean number of roots/treatment	Least square mean number of roots/rooted microshoot	Percentage rooting
12.5 ml BE3	0^{B}	-	0^{B}
2.5 ml BE3	0^{B}	-	0^{B}
0.5 ml BE3	1.0^{B}	-	4^{B}
12.5 ml BE7	1.3^{A}	1.9^{A}	34^{A}
2.5 ml BE7	1.3^{A}	1.6^{A}	43^{A}
0.5 ml BE7	1.3^{A}	1.6^{A}	58^{A}
4.9 μM IBA	1.4^{A}	1.8^{A}	50^{A}

* Volumes added per liter of WPM
- No roots formed
Numeric values followed by the same letter are not significantly different at the 0.05 level of significance. Mean separation was performed on the transformed data.



* Volumes added per liter of WPM

Figure 10. Effect of different volumes of BE3 and BE7 on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots. Microshoots were cultured on WPM. Bars having different letters are significantly different at $P \leq 0.05$.

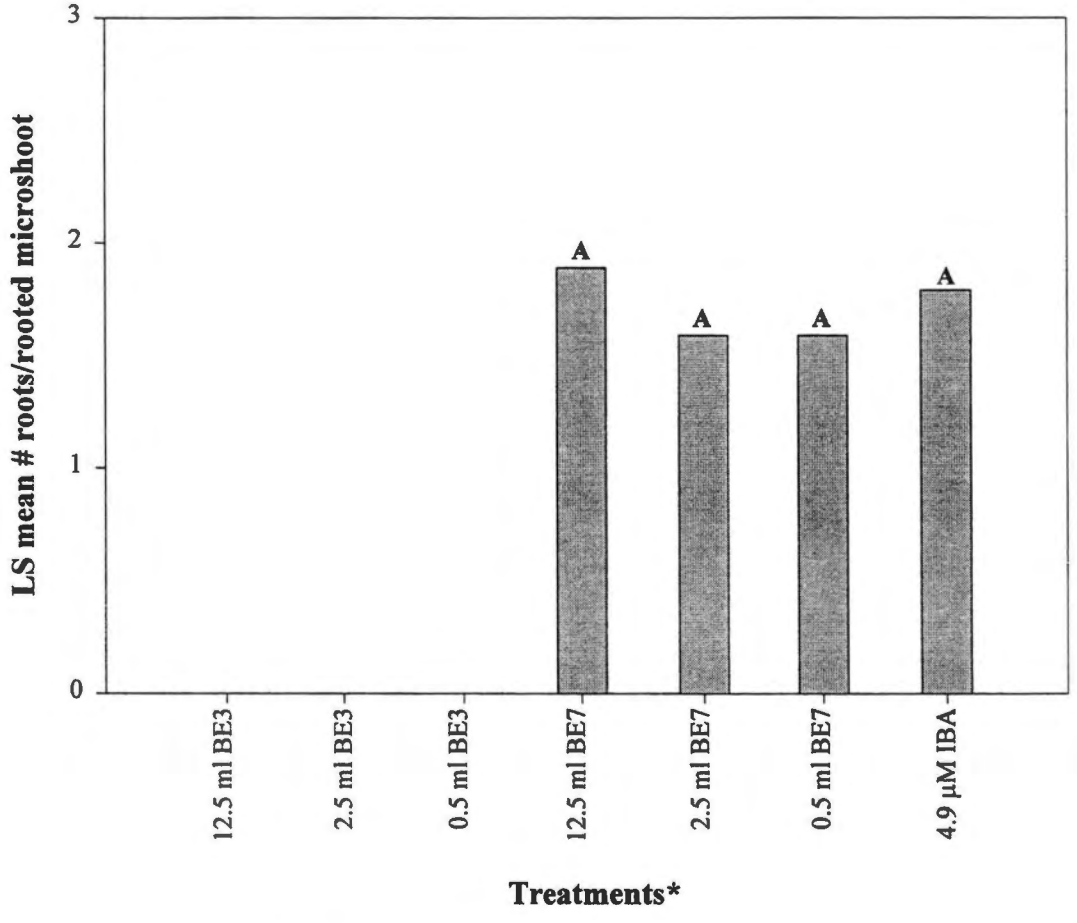
with 0.5 ml BE7 than those treated with 4.9 μM IBA. Rooting percentages increased with decreasing BE7 volume.

A linear increase in percent rooting seemed to be inversely related to the volume of BE added to the medium. However, the concentration required to induce maximum rooting was not established as volumes below 0.5 ml were not tested. Among the microshoots that rooted, no significant differences ($P \leq 0.05$) were observed between different treatments (Table 2, Figure 11). Different volumes of BE7 apparently did not significantly affect the number of roots formed per microshoot.

None of the microshoots from the various BE3 treatments survived. The reason for this observation could not be established. Since the pH of the medium was adjusted to 5.7 after addition of different volumes of BE, acidic conditions could not have been the cause. It might be that the BE3 extract contains substances that might be toxic or severely affect some physiological processes that are vital for growth of the microshoots.

4.2.2 Extracts at pH 7

The experiment was repeated using different volumes of bacterial extract at pH7 (BE7). Of the various BE7 volumes tested, roots were formed only on microshoots treated with 5 ml of BE7/L of WPM (Table 3; Figure 12). Microshoots on WPM with 5.0 ml of BE7/L formed a mean of about 2 roots/treatment, but had a very low rooting percentage of 10. The 4.9 μM IBA treatment resulted in a LS mean of 2 roots/culture and 100 % of the microshoots rooted. With regard to number of roots/rooted microshoot, no



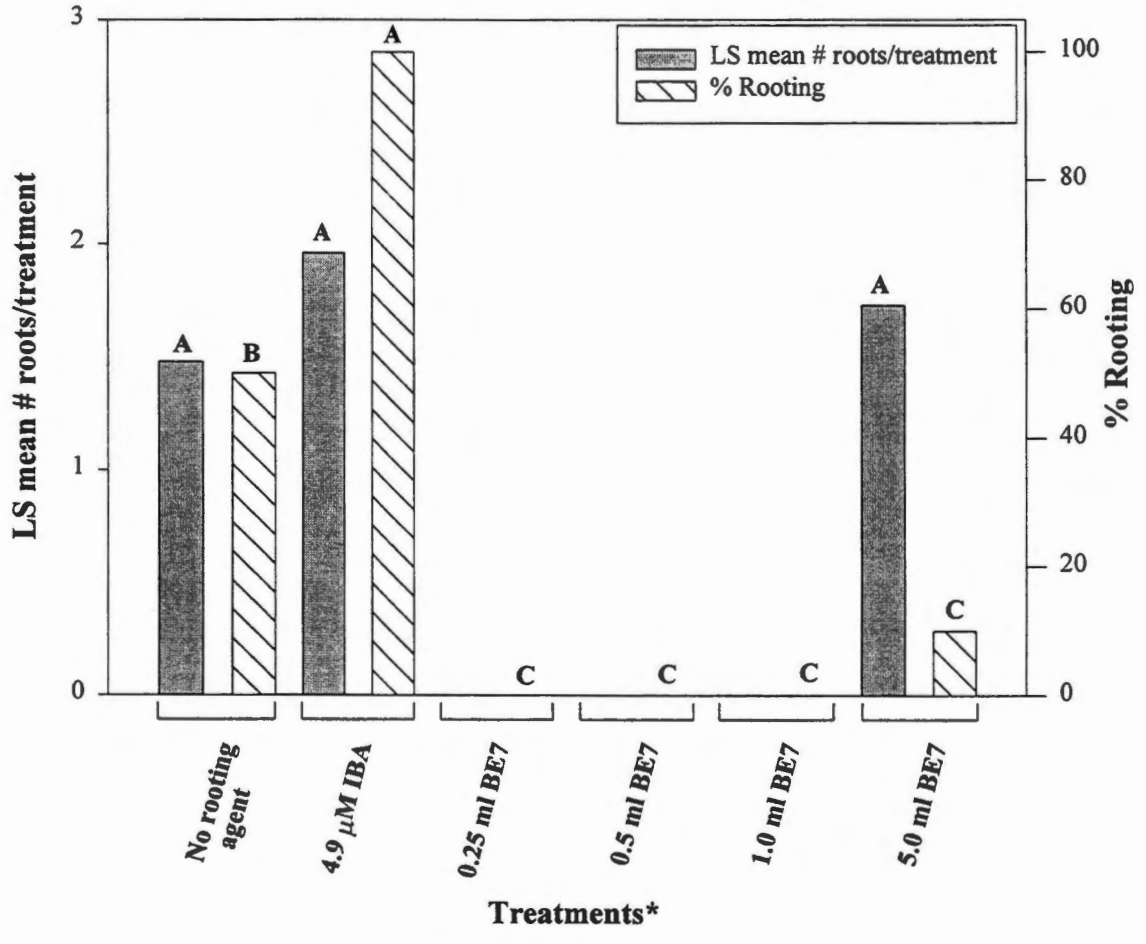
* Volumes added per liter of WPM

Figure 11. Effect of different volumes of BE3 and BE7 on least square mean number of roots/rooted microshoot of *C. florida*. Microshoots were cultured on WPM containing 4.9 µM IBA. Bars having different letters are significantly different at $P \leq 0.05$.

Table 3. Percentage rooting, least square mean number of roots/treatment and least square mean number of roots/rooted microshoot for the various BE7 treatments for rooting *Cornus florida* microshoots.

Treatments*	Least square mean number of roots/treatment	Least square mean number of roots/rooted microshoot	Percentage rooting
No rooting agent	1.5^{A}	1.5^{A}	50^{B}
4.9 µM IBA	2.0^{A}	2.0^{A}	100^{A}
0.25 ml BE7	-	-	0^{C}
0.5 ml BE7	-	-	0^{C}
1.0 ml BE7	-	-	0^{C}
5.0 ml BE7	1.7^{A}	1.7^{A}	10^{C}

* Volumes added per liter of WPM.
- No roots formed
Numeric values followed by the same letter are not significantly different at the 0.05 level of significance. Mean separation was performed on the transformed data.



* Volumes added per liter of WPM

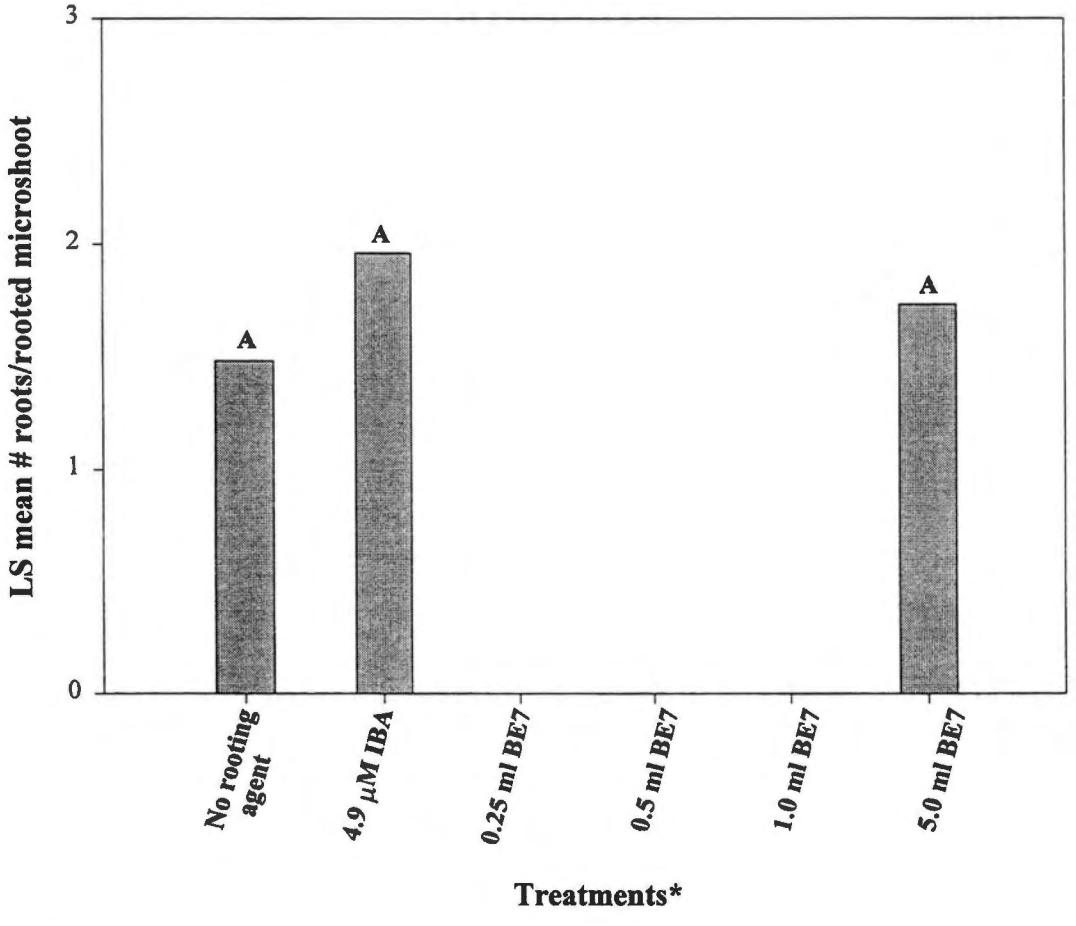
Figure 12. Effect of different volumes of BE7 on least square mean number of roots/treatment and percent rooting of *Cornus florida* microshoots. Microshoots were cultured on WPM. Bars having different letters are significantly different at $P \leq 0.05$.

significant differences ($P \leq 0.05$) were observed between different treatments (Table 3; Figure 13).

Since these results conflict with those in the previous experiment, not much could be said about the effect of BE7 in rooting dogwood microshoots. The highest volume of BE7 tested (5 ml/L) induced 10 % rooting and stimulated the formation of about 2 roots/microshoot. Addition of low volumes of BE7 to WPM did not induce rooting of microshoots. Volumes greater than 5.0 ml could be more effective in rooting *C. florida* microshoots, but this needs to be further tested. BE7 seems to contain some root promoting substances and therefore concentrating the extract before adding to growth medium may be beneficial.

In summary, the number of roots formed per treatment on addition of the bacterial extracts seems to increase with increasing volume of BE7 whereas the rooting percentage increases with decreasing volume of BE7.

At low pH, acidic components in a solution are known to become protonated and become less polar. Thus, the ethyl acetate fraction would contain the acidic components when extracted at pH 3. At neutral pH, the acidic components are not protonated and remain negatively charged and would not be extracted in the ethyl acetate fraction, but only in the water fraction. Since the extract at neutral pH (BE7) showed some root promoting properties compared to none in the acidic fraction, root stimulating compounds that might be present in the bacterial extract are compounds that do not contain acidic groups.



* Volumes added per liter of WPM

Figure 13. Effect of different volumes of BE7 on least square mean number of roots/rooted microshoot of *C. florida*. Microshoots were cultured on WPM containing 4.9 μM IBA. Bars having different letters are significantly different at $P \leq 0.05$.

4.3 Rooting with Salicylic acid (SA) and Acetylsalicylic acid (ASA)

4.3.1 Continuous Treatment

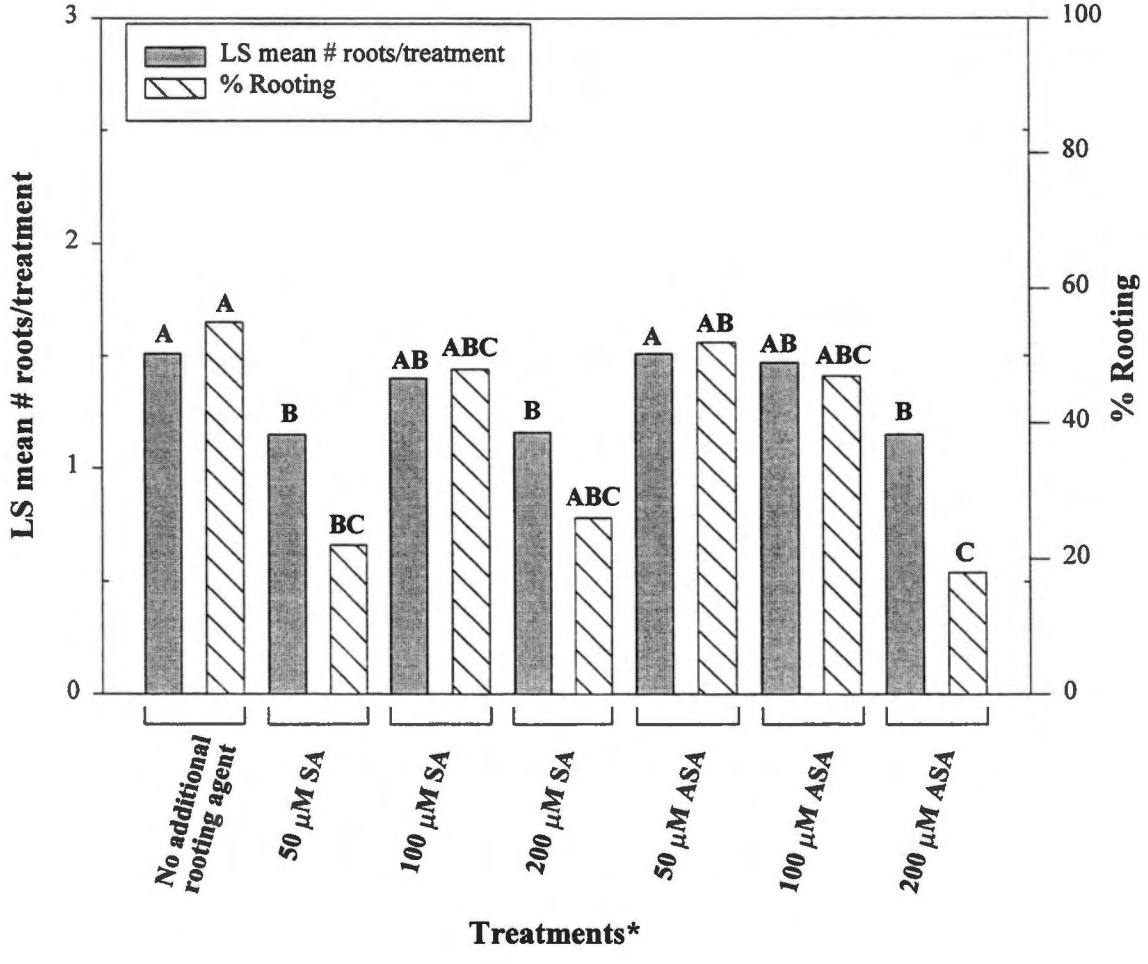
Continuous treatment of SA and ASA caused significant differences ($P \leq 0.05$) in rooting flowering dogwood microshoots. Microshoots pretreated with 100 μM SA, 50 μM ASA and 100 μM ASA generated LS mean number of roots that were statistically similar to the treatment where microshoots were placed directly in WPM amended with 4.9 μM IBA (Table 4, Figure 14). Microshoots that received these treatments also had a higher rooting percentage of about 50 compared to the other treatments (Table 4; Figure 14). The 50 and 200 μM SA treatments stimulated fewer explants to root compared to the other treatments.

Treatment of microshoots with higher (200 μM) ASA concentration and lower (50 μM) SA concentration resulted in lower rooting percentages. The 100 μM SA treatment stimulated rooting similar to the IBA treatment. This contradicts the results of Mensuali-Sodi et al. (1995), which indicate that 100 μM SA severely inhibited rooting of lavandin microcuttings. Among the various concentrations of ASA tested, the lower concentrations of 50 and 100 μM stimulated higher rooting efficiency than those stimulated by high concentration of 200 μM ASA. Inhibitory rooting activity was observed when microshoots were treated with 50 and 100 μM SA and 200 μM ASA (Table 4, figure 14). Among the microshoots that rooted, no significant differences ($P \leq 0.05$) were observed for the number of roots stimulated by various treatments (Table 4; Figure 15).

Table 4. Percentage rooting least square mean number of roots/treatment and least square mean number of roots/rooted microshoot for the various salicylic acid and acetylsalicylic acid continuous treatments for rooting *Cornus florida* microshoots.

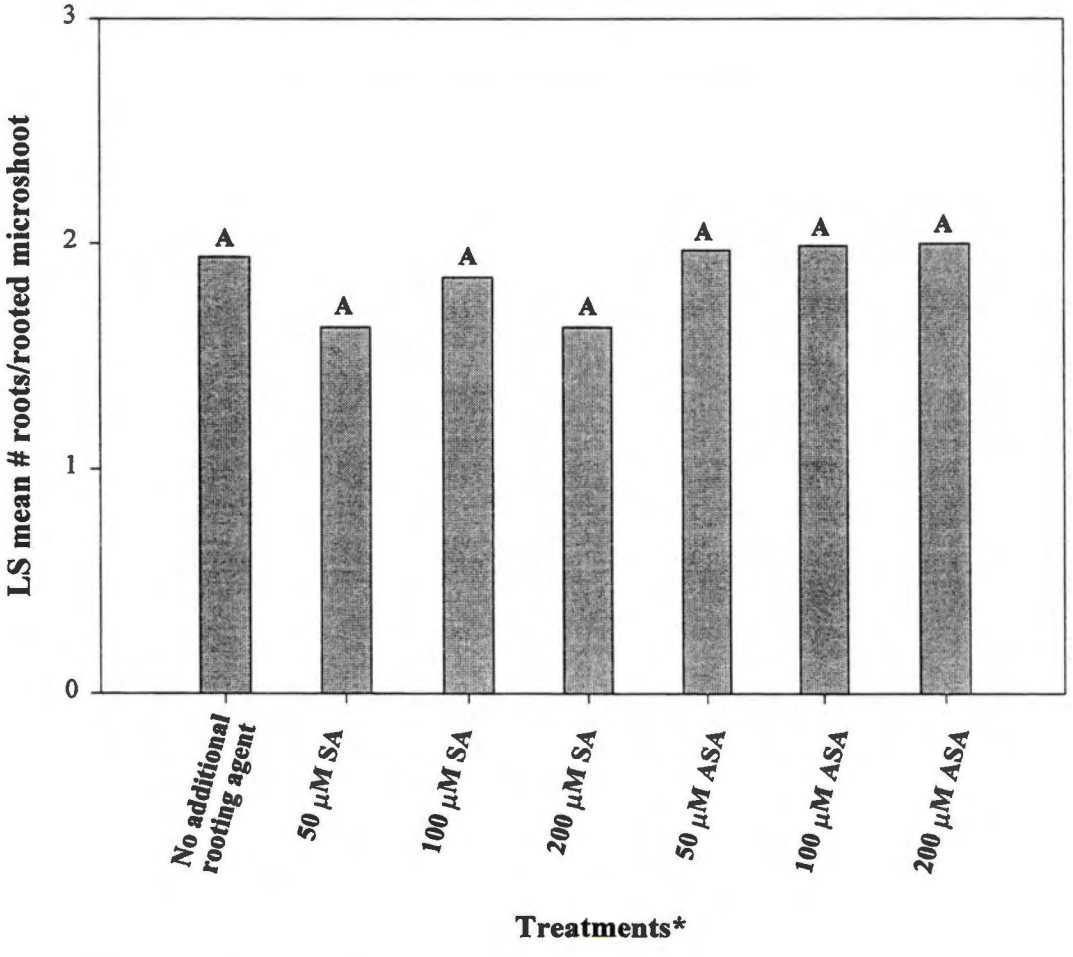
Treatments*	Least square mean number of roots/treatment	Least square mean number of roots/rooted microshoot	Percentage rooting
No additional rooting agent	1.5^{A}	1.9^{A}	55^{A}
50 µM SA	1.2^{B}	1.6^{A}	22^{BC}
100 µM SA	1.4^{AB}	1.9^{A}	48^{ABC}
200 µM SA	1.2^{B}	1.6^{A}	26^{ABC}
50 µM ASA	1.5^{A}	2.0^{A}	52^{AB}
100 µM ASA	1.4^{AB}	2.0^{A}	47^{ABC}
200 µM ASA	1.2^{B}	2.0^{A}	18^{C}

* Volumes added per liter of WPM + 4.9 µM IBA
Numeric values followed by the same letter are not significantly different at the 0.05 level of significance. Mean separation was performed on the transformed data.



* Volumes added per liter of WPM + 4.9 μM IBA

Figure 14. Effect of different concentrations of salicylic acid and acetylsalicylic acid continuous treatment on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots. Microshoots were cultured on WPM containing 4.9 μM IBA. Bars having different letters are significantly different at $P \leq 0.05$.



* Volumes added per liter of WPM + 4.9 μM IBA

Figure 15. Effect of different concentrations of salicylic acid and acetylsalicylic acid continuous treatment on least square mean number of roots/rooted microshoot of *C. florida*. Microshoots were cultured on WPM containing 4.9 μM IBA. Bars having different letters are significantly different at $P \leq 0.05$.

Very high concentrations of PGRs can inhibit root forming activity, whereas extremely low levels may not stimulate rooting at all (Arteca 1995). The activity of the PGRs can be shown to follow a bell shaped curve with low activities at low and high concentrations and optimum concentrations somewhere in between (Arteca, 1995). The results from the SA treatments seem to form a bell shape curve as the lower and higher concentrations have lower rooting activity. Similarly, the ASA treatments at high concentrations of 200 μM caused lower rooting efficiency. Concentrations greater than 200 μM and less than 50 μM need to be tested to establish a dose-response curve.

All microshoots treated with SA and ASA formed fewer than or significantly similar number of roots/treatment to IBA control. Thus, SA or ASA did not stimulate better rooting than IBA. The 50 and 100 μM SA and 200 μM ASA treatments inhibited rooting activity. This contradicts the suggestion of Kling and Meyer (1983) that SA stimulates rooting in mung bean. However, the negative effect on rooting supports the findings of Mensuali-Sodi et al. (1995) who found that SA has an inhibitory effect on lavandin microcuttings.

Salicylic acid (100 μM) inhibited root formation in lavandin microcuttings (Mensuali-Sodi et al. 1995), whereas the same concentration stimulated rooting in *C. florida* microshoots, but to a lesser degree than the IBA treatment. SA (100 μM and 1 μM) reportedly stimulated root production in mung bean cuttings (Kling and Meyer 1983). Still et al. (1976) also reported stimulation of root production in mung bean when treated with 1000 μM SA. Microshoots treated with various continuous SA treatments

formed fewer or similar number of roots/treatment than those treated with IBA alone. It is possible that the concentration and continuous exposure to SA may be inhibitory to adventitious root formation.

4.3.2 Pulse Treatment

Since relatively high concentrations and continuous exposure to SA inhibited rooting, another experiment was performed using lower concentrations and pulse treatments of SA and ASA. The LS mean numbers of roots generated by microshoots exposed to 10 μM SA and 25 and 50 μM ASA were statistically similar to those formed by the water pretreatment (Table 5; Figure 16).

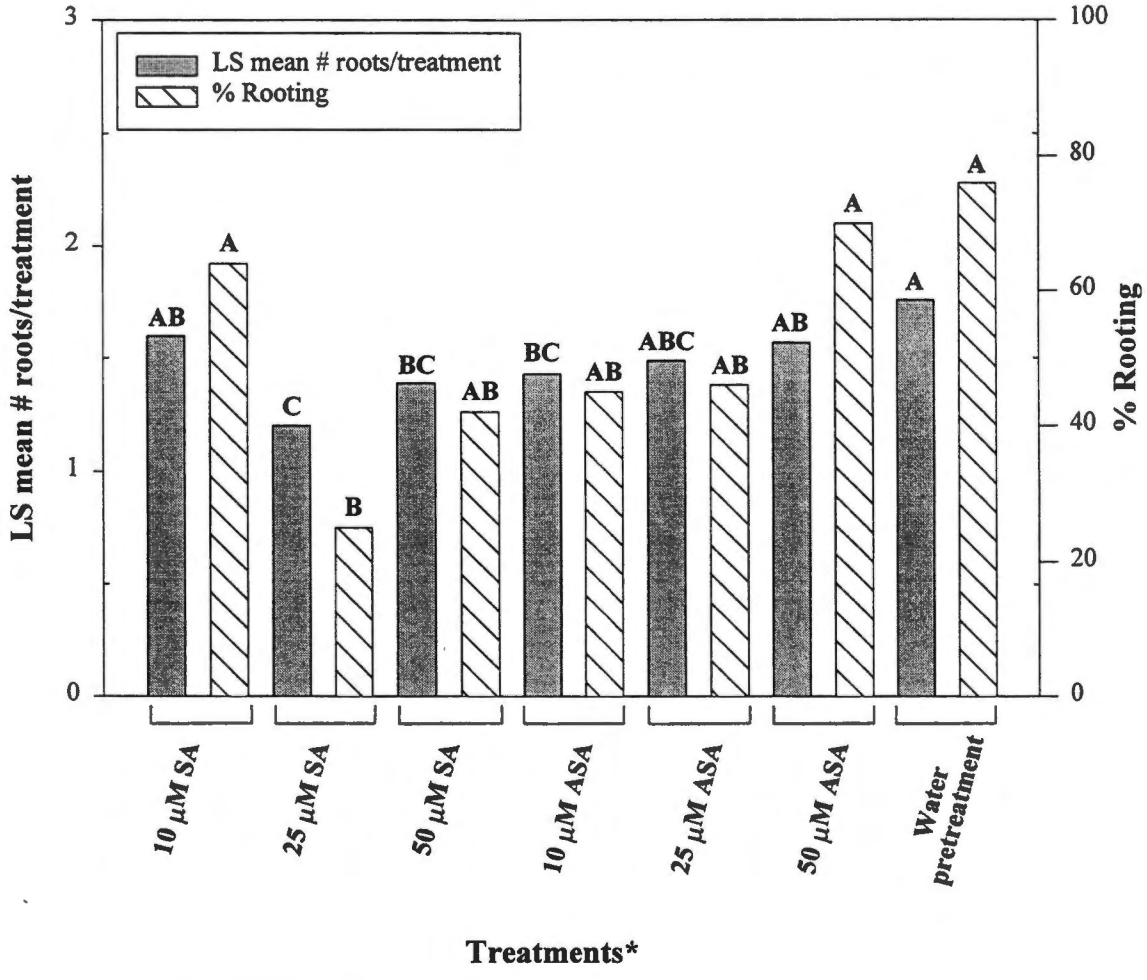
Higher concentrations of SA inhibited root forming activity that supports the findings of Arteca (1995). The ability to form roots is partially dependent on the concentration of the rooting compounds or PGRs found in the tissues (Arteca 1995). There is an optimum concentration with declining activity at higher and lower levels of PGRs. The optimum concentration was not determined in these experiments, but probably is below 10 μM. Lower concentrations of ASA (10 μM) slightly inhibited rooting of *C. florida* microshoots (Table 5; Figure 16). Among the microshoots that rooted, no significant differences ($P \leq 0.05$) were observed for the number of roots stimulated by various treatments (Table 5; Figure 17).

SA and ASA have been reported to influence the activity of other PGRs such as ethylene. Certain levels of ethylene are optimum for root formation whereas extreme

Table 5. Percentage rooting, least square mean number of roots/treatment and least square mean number of roots/rooted microshoot for the various salicylic acid and acetylsalicylic acid pulse treatments for rooting *Cornus florida* microshoots.

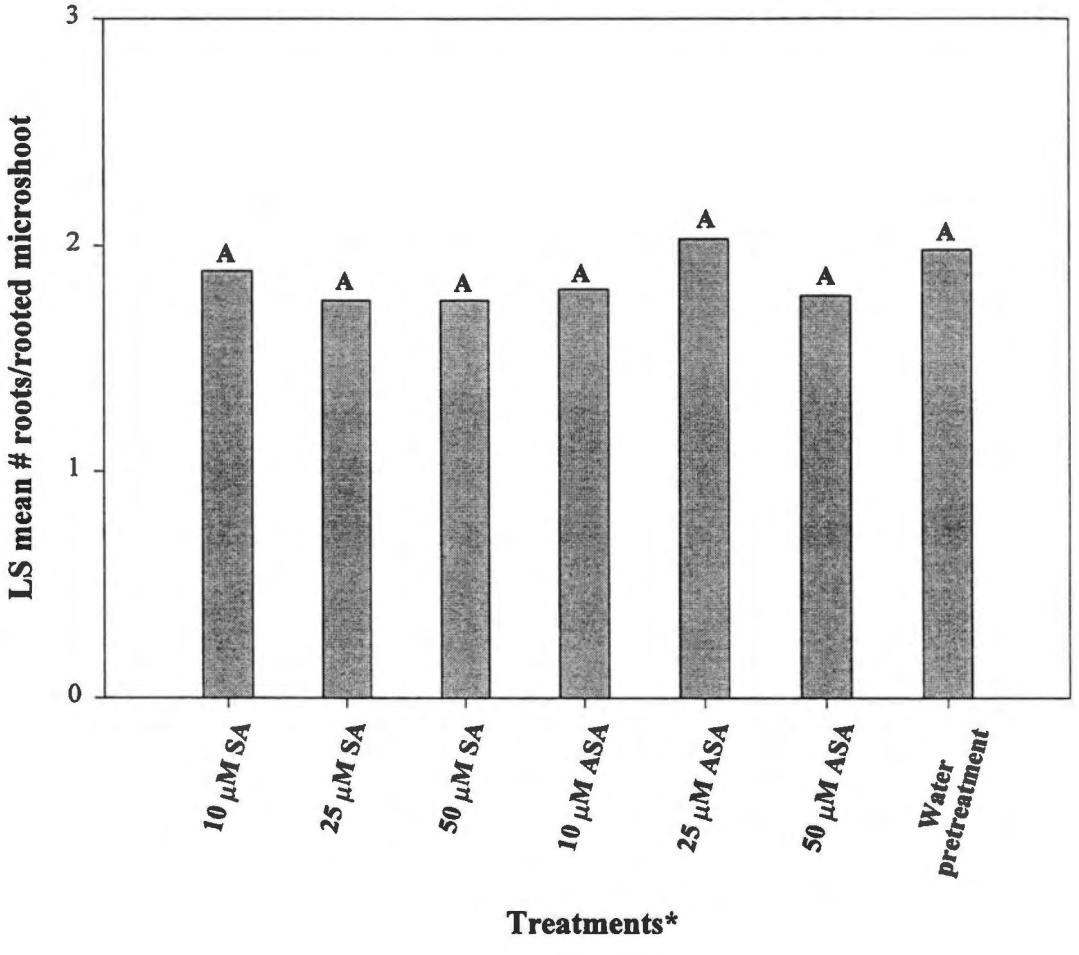
Treatments*	Least square mean number of roots/treatment	Least square mean number of roots/rooted microshoot	Percentage rooting
10 µM SA	1.6^{AB}	1.9^{A}	64^{A}
25 µM SA	1.2^{C}	1.8^{A}	25^{B}
50 µM SA	1.4^{BC}	1.8^{A}	42^{AB}
10 µM ASA	1.4^{BC}	1.8^{A}	45^{AB}
25 µM ASA	1.5^{ABC}	2.0^{A}	46^{AB}
50 µM ASA	1.6^{AB}	1.8^{A}	70^{A}
Water pretreatment	1.8^{A}	2.0^{A}	76^{A}

* Volumes added per liter of WPM + 4.9 µM IBA
Numeric values followed by the same letter are not significantly different at the 0.05 level of significance. Mean separation was performed on the transformed data.



* Volumes added per liter of WPM + 4.9 μM IBA

Figure 16. Effect of different concentrations of salicylic acid and acetylsalicylic acid pulse treatment on least square mean number of roots/treatment and percentage rooting of *Cornus florida* microshoots. Microshoots were cultured on WPM containing 4.9 μM IBA. Bars having different letters are significantly different at $P \leq 0.05$.



* Volumes added per liter of WPM + 4.9 μM IBA

Figure 17. Effect of different concentrations of salicylic acid and acetylsalicylic acid pulse treatment on least square mean number of roots/rooted microshoot of *C. florida*. Microshoots were cultured on WPM containing 4.9 μM IBA. Bars having different letters are significantly different at $P \leq 0.05$.

levels inhibit rooting (Mensuali-Sodi et al. 1995). Also SA and ASA inhibited ethylene formation in pear cell suspension cultures (Leslie and Romani 1988). Thus, the negative effect of certain concentrations of SA and ASA on rooting may be due to changes in ethylene levels. However, this could not be verified as provisions were not made to measure ethylene levels or emination.

4.4 Histological studies

Sections obtained from various leaf samples were observed. Leaf sections from *C. florida* axillary bud and nodal cultures growing on WPM amended with 4.4 μM BA had poorly developed mesophyll layer (Plate 1 A). The palisade and the spongy tissues were not clearly defined and large interstitial spaces between spongy tissue were present. Several small trichomes were observed on both leaf surfaces. Slides from leaf sections of rooted dogwood microshoots also showed a poorly defined mesophyll layer (Plate 1 B). The palisade and the spongy layers were easily distinguishable, but still had large interstitial spaces. Leaf sections from rooted microshoots acclimatized to the greenhouse resembled leaves obtained from mature dogwood trees grown in the field (Plate 1 C and D). The spongy and palisade cells were well-developed and more compactly arranged to form a mesophyll layer with very few interstitial spaces. However, leaves from the greenhouse showed fewer and smaller trichomes that leaves from mature dogwood trees.

Plants cultured in vitro are known to have low photosynthetic activity and water retention capacities due to differences in their anatomical and physiological

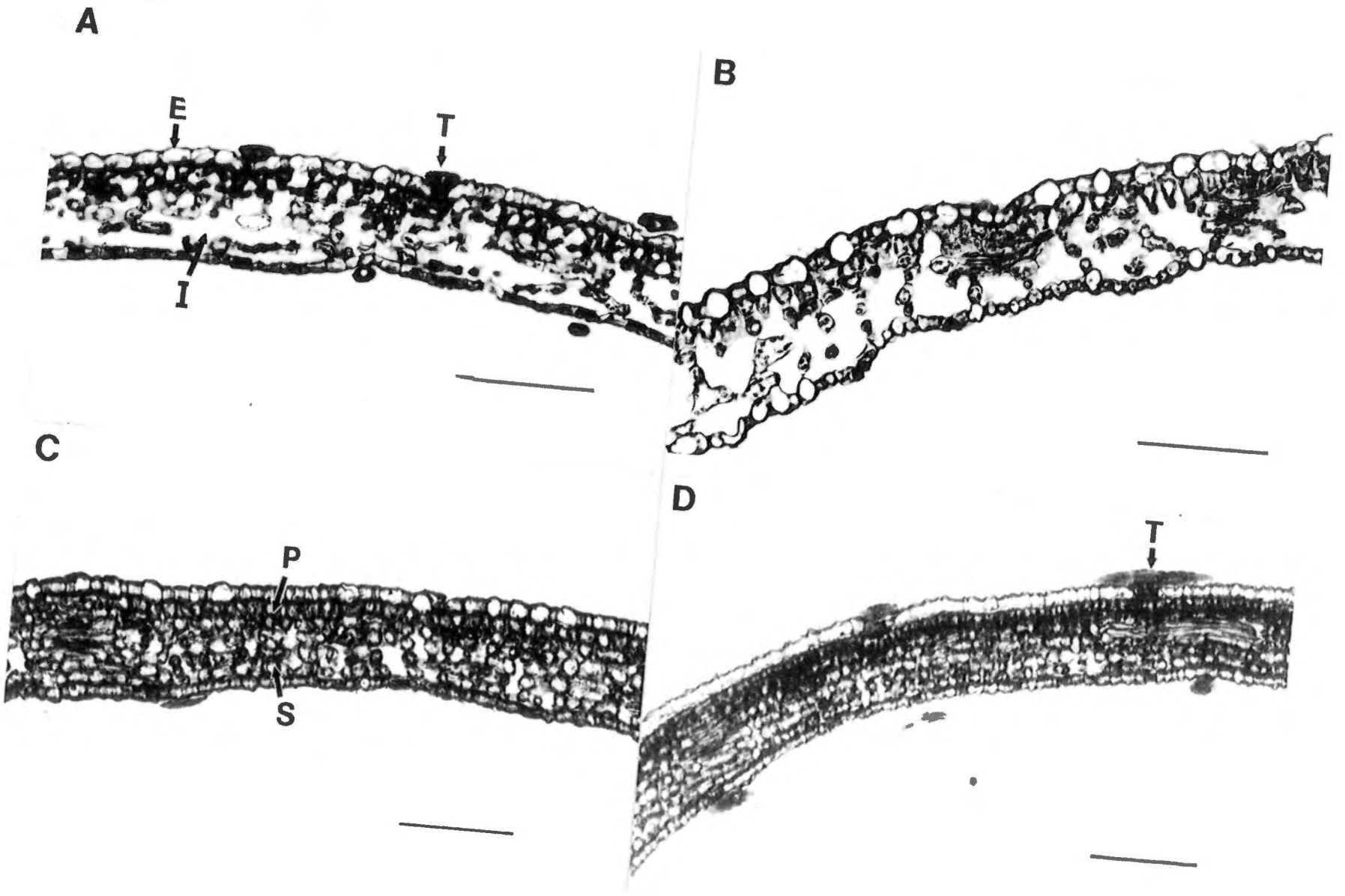
Plate 1. Photographs of *Cornus florida* leaf sections.

A. Leaf section from *C. florida* cultures maintained on WPM + 4.4 μM BA. Bar = 100 μm. E = epidermis; I = Interstitial spaces; T = trichome.

B. Leaf sections from in vitro rooted *C. florida* cultures. Bar = 100 μm.

C. Leaf sections from acclimatized in vitro cultured *C. florida*. Bar = 100 μm. P = palisade mesophyll; S = spongy mesophyll.

D. Leaf sections from mature *C. florida* tree. Bar = 100 μm. T = trichome.


A
E
T
I
B
C
P
S
D
T

characteristics (Kane 1996). They have less epicuticular wax, poorly differentiated mesophyll, abnormal stomate and poor vascular connection between shoots and roots (Kane 1996).

Acclamatization, the final but very important stage for successful micropropagation, involves steps to convert plants from a heterotophic mode of nutrition to a more photoautotrophic state (Kane 1996). Sections of leaves taken at various stages of micropropagation revealed the expected anatomical differences. Sections of leaves obtained from plants in the greenhouse confirm that these are well acclimatized and would probably grow successfully if transferred to soil in the field.

Cross-section of roots cultured in vitro on WPM amended with 4.9 μM IBA (rooting medium) had well-defined vascular cylinders (Plate 2 A). Stem sections were taken from microshoots cultured on rooting medium for 10 days and 15 days. Roots were not visible externally on the microshoots, but sections of some microshoots showed root initiation. This was seen as actively dividing cells that stained darker than other areas (Plate 2 B). As these cells multiplied and elongated they ruptured the stem epidermal layer. Clearly defined roots emerged from the stems (Plate 2 C) and longitudinal sections of some roots were taken. Actively dividing cells that stained deeper than the other cells were present in the root tip in meristems (Plate 2 D).

From these studies it is evident that roots were initiated in *C. florida* microshoots sometime before 10 days of culture on rooting medium, although roots were not visible externally.

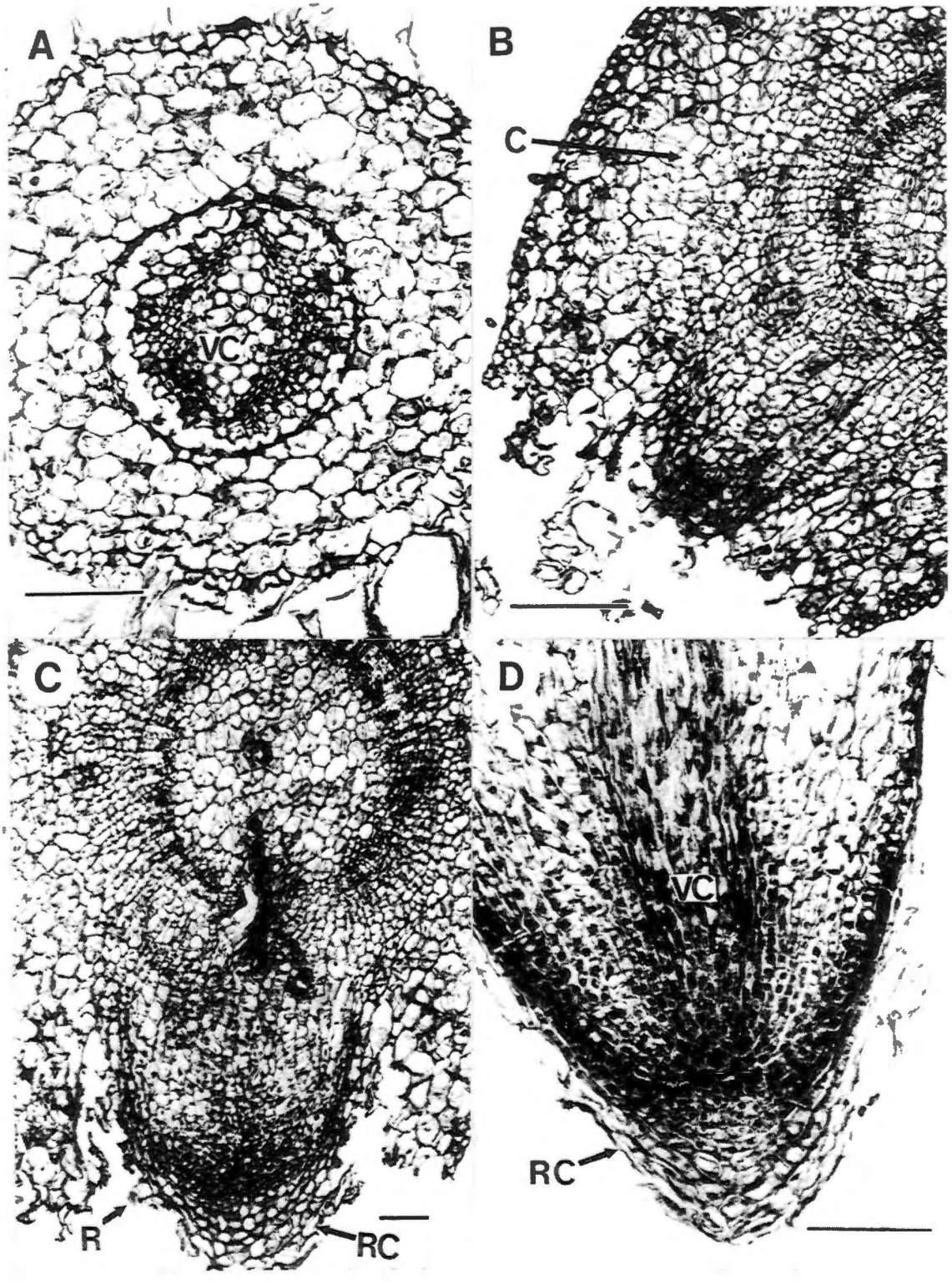
Plate 2. Photographs of *Cornus florida* roots cultured *in vitro* in WPM + 4.9 μM IBA.

A. Cross section of *C. florida* root. Bar =100 μm. VC = vascular cylinder.

B. Initiation of adventitious roots on in vitro cultured *C. florida* microshoots. Bar = 100 μm. C= cortex.

C. Elongated adventitious roots on *C. florida* microshoots. Bar = 100 μm. R = adventitious root; RC = root cap.

D. Root tip of an elongated root arising from *C. florida* microshoots. Bar = 100 μm. RC = root cap; VC = vascular cylinder.


A
VC
B
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C
R
RC
D
VC
RC

CHAPTER 5

CONCLUSIONS

Among various treatments tested, maximum rooting of *C. florida* microshoots was achieved when 5 to 6 week old microshoots were placed in WPM supplemented with 4.9 μM IBA. From the various rooting experiments, 4.9 μM IBA concentration stimulated rooting efficiency of ≥ 70% in flowering dogwood microshoots. This was significantly greater than that achieved by Kaveriappa et al. (1997).

The willow and locust diffusates alone showed a slightly inhibitory effect on rooting flowering dogwood microshoots. Thus, pretreatment with WD or LD cannot be used to improve rooting of *C. florida* microshoots over that of 4.9 μM IBA.

Direct exposure of microshoots to live RSB did not stimulate any rooting. High rooting percentages of 43 and 58 % were obtained when microshoots were transferred to WPM amended with 2.5 ml and 0.5 ml of pH7 RSB extract compared to the other treatments. Bacterial extract obtained at pH 3 did not stimulate rooting in flowering dogwood microshoots.

Continuous exposure of microshoots to 100 μM SA, 50 and 100 μM ASA treatment increased rooting efficiency more than the other concentrations tested. Pulse treatments with lower concentrations of SA (10 μM) and higher concentration of ASA (25 and 50 μM) resulted in a greater rooting efficiency than the other concentrations tested. However rooting microshoots with SA and ASA was not significantly better than that achieved with 4.9 μM IBA.

Histological studies revealed that leaves from plants in the greenhouse were fully acclimatized as they resembled leaves from mature dogwood trees. Adventitious root formation in *C. florida* microshoots was initiated before 10 days of transfer to rooting medium, although external signs of rooting were not seen.

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APPENDICES

Appendix 1. Modified Woody Plant Medium:

WPM Macro 5X		200 ml
H_2O	Bring vol. to 1.0 L	
NH_4NO_3	2.0 g	
$CaCl_2.2H_2O$	0.48 g	
$MgSO_4.7H_2O$	1.85 g	
KH_2PO_4	0.85 g	
$Ca(NO_3).4H_2O$	2.78 g	
K_2SO_4	4.95 g	

MS Micro 1X		10 ml
H_2O	Bring vol. to 1.0 L	
H_3BO_3	0.62 g	
$MnSO_4.H_2O$	1.69 g	
$ZnSO_4.7H_2O$	0.86 g	
KI	0.083g	
$Na_2MoO_4.2H_2O$	0.025g	
$CuSO_4.5H_2O$	1.0 ml (of 50 mg in 20 ml H_2O stock soln.)	
$CoCl_2.6H_2O$	1.0 ml (of 50 mg in 20 ml H_2O stock soln.)	

GD Iron 1X		10 ml
a) $FeSO_4.7H_2O$	2.78 g	
H_2O	500 ml	
b) Na_2EDTA	3.73 g	
H_2O	500 ml	

Dissolve the above in two separate flasks and set on a stirrer overnight. Mix the next day.

Myo Inositol	0.1 g
Nicotinic acid	5 ml (of 5 mg in 50 ml H_2O)
Pyridoxine HCl	5 ml (of 5 mg in 50 ml H_2O)
Thiamine HCl	1 ml (of 20 mg in 20 ml H_2O)
Glycine	1 ml (of 40 mg in 20 ml H_2O)
Sucrose	20 g
Casein Hydrolysate	2 g

Bring volume to 1 L
Standardize pH to 5.5
Add Bactoagar (Difco Laboratories, Detroit, Michigan) 15 g

Note: Vitamin stocks were stored in the freezer and the Macro salt, Micro salt and GD Iron stock were stored in the refrigerator.

Appendix 2. Dogwood Growing Medium:

Dolomitic limestone	2.08 Kg/m^3	3.5 lbs/cu.yd.
Treble superphosphate (0-46-0)	1.19 Kg/m^3	2.0 lbs/cu.yd.
Granular fertilizer 18-6-12	1.19 Kg/m^3	2.0 lbs/cu.yd.
or 10-10-10		
Gypsum ($CaSO_4$)	1.34 Kg/m^3	2.25 lbs/cu.yd.
Micromax (micronutrients)	0.89 Kg/m^3	1.5 lbs/cu.yd.
Epsom salts ($MgSO_4$)	1.19 Kg/m^3	2.0 lbs/cu.yd.

These amendments are added to 0.76 m^3 (1 cu. yd.) ground pine bark plus one 0.17 m^3 (6 cu. ft.) bale of Pro-Mix (BX) (Premium Peat Co.) and mixed for 5 min. in a soil mixer.

Appendix 3. Modified Fleming Triple Stain:

Step	Procedure	Time (min)
1	Microclear - 100 %	5
2	Microclear - 100 %	5
3	Microclear - 100 %	5
4	Microclear / 100 % Ethanol	5
5	100 % Ethanol	5
6	95 % Ethanol	5
7	70 % Ethanol	5
8	50 % Ethanol	5
9	Safranine O (in 50 % ethanol)	2-48 h
10	Rinse with tap water until clear	
11	Crystal Violet (in water)	30-60 sec
12	Rinse with tap water until clear	
13	50 % Ethanol	5
14	70 % Ethanol	5
15	95 % Ethanol	5
16	100 % Ethanol	5
17	Fast Green - Under fume hood	30-60 sec
18	100 % Ethanol	1
19	100 % Ethanol	5
20	Microclear / 100 % Ethanol	5
21	Microclear - 100 %	5
22	Microclear - 100 %	5
23	Microclear - 100 %	5

Appendix 4. Randomized Incomplete Block Design used for the Locust Diffusate, Willow Diffusate and Bacterial Extract pH7 Experiments:

Efficiency of this design is 0.6

Treatments →	A	B	C	D	E	F
Blocks ↓						
1			X			X
2	X			X		
3		X		X		
4				X	X	
5	X		X			
6			X		X	
7	X			X		
8				X		X
9					X	X
10	X	X				
11					X	X
12		X		X		
13		X				X
14		X				X
15			X			X
16			X	X		
17				X		X
18		X			X	
19				X	X	
20	X		X			
21	X				X	
22	X				X	
23	X	X				
24			X	X		
25			X		X	
26	X					X
27		X	X			
28	X					X
29		X			X	
30		X	X			

Appendix 5. Randomized Incomplete Block Design used for the Salicylic acid and Acetylsalicylic acid Experiments:

Efficiency of this design is 0.78

Treatments →	A	B	C	D	E	F	G
Blocks ↓							
1	X		X		X		
2	X	X	X				
3	X					X	X
4		X	X			X	
5			X		X		X
6			X		X	X	
7				X	X	X	
8	X				X	X	
9	X				X		X
10		X		X			X
11		X		X	X		
12		X	X		X		
13	X			X	X		
14			X	X		X	
15	X	X			X		
16				X		X	X
17	X			X			X
18			X			X	X
19	X		X			X	
20		X			X		X
21				X	X		X
22		X				X	X
23	X	X				X	
24	X		X	X			
25		X	X	X			
26		X		X		X	
27	X	X					X
28			X	X			X

Appendix 6. Randomized Incomplete Block Design used for the Bacterial Extract pH3 and pH7 Experiment:

Efficiency of this design is 0.78

Treatments → / Blocks ↓	A	B	C	D	E	F	G
1				X		X	X
2	X	X	X				
3		X		X			X
4			X		X	X	
5	X			X		X	
6		X		X	X		
7		X	X	X			
8					X	X	X
9	X			X	X		
10	X			X			X
11			X	X		X	
12	X	X		X			
13	X	X					X
14	X					X	X
15				X	X		X
16			X		X		X
17		X			X		X
18	X		X			X	
19	X		X	X			
20			X			X	X
21		X				X	X
22	X				X		X
23		X			X	X	
24				X	X	X	
25		X	X		X		
26		X	X				X
27	X		X				X
28			X	X			X
29	X	X			X		
30	X		X		X		
31	X				X	X	
32			X	X	X		
33		X	X			X	
34		X		X		X	
35	X	X				X	

Appendix 7. Black and White Film Developing:

Black and white film was developed in the lab with HC110 Developer (Eastman Kodak Co., Rochester, New York). Developer was prepared by diluting 1 part of HC110 to 76 parts water). Film was placed in the container with developer for 8 min with gentle swirling after every 30 sec. This was followed by a quick rinse in tap water. The fixer (Eastman Kodak Co., Rochester, New York) was added and swirled constantly for about 4 minutes and finally rinsed in cold tap water for 15 min. The film was removed from the carousel and Photo-Flo (diluted 200 times with water) (Eastman Kodak Co., Rochester, New York) was poured over it. Film was wiped first between fingers then with Kimwipes and hanged to dry.

Appendix 8. Protocol for Rooting Dogwood Microshoots:

Collect and stratify seeds:

Collect dogwood berries in early October. Soak in tap water for 24-48 h and depulp them by squeezing between fingers. Allow seeds to dry overnight, then place in a sand and peat moss mixture (ratio 1:1) moistened with warm water to holding capacity. Transfer to 'zip lock' plastic bags and remove any excess air. Store plastic bags at 4C for 4 months.

Germinate seeds:

After the 4 month stratification period, sow all seeds (germinated and non-germinated) in trays containing a mixture (ratio 3.5:1) of composted pine bark and Promix BX (Premium Peat Co.). Fertilize weekly with 200 ppm N using Peters 21-7-7 acid special (Grace-Sierra Co., Milpitas, California, USA). Let the seedlings grow until they have 2-3 sets of true leaves (about 8 weeks).

Cut and sterilize nodal and apical segments:

Obtain nodal segments by cutting the stems at about 1 cm above and below the node and removing the leaf lamina leaving a small portion of the petiole attached to the stem (Figure 18 A). Stem segments containing the apical bud can be obtained by cutting the stem about 1 cm below the apical bud. Surface disinfect the nodal and apical buds by

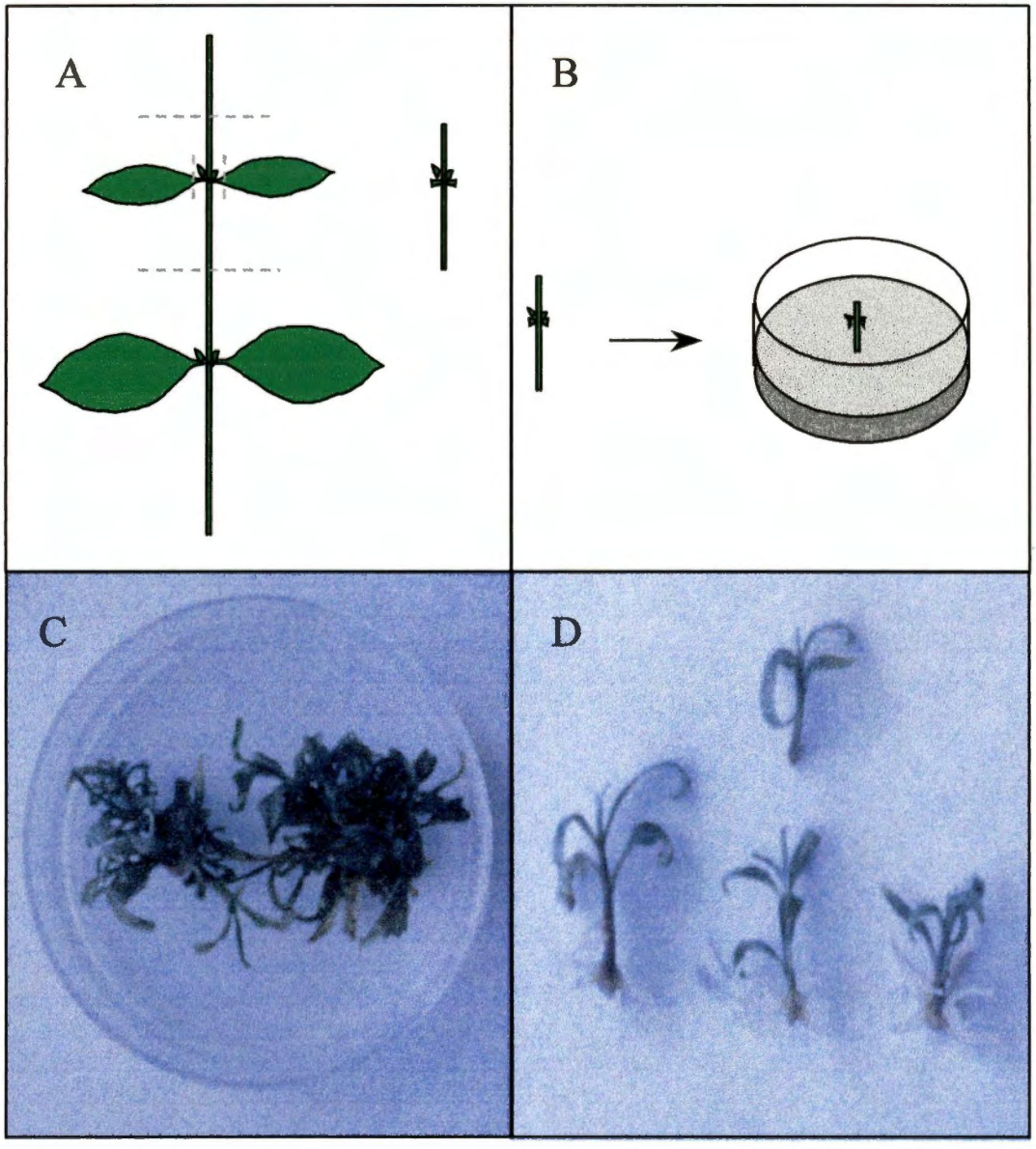

Figure 18. Pictures showing different stages involved in micropropagation of *Cornus florida*. A. Obtaining nodal segments; B. Initiating stock cultures; C. Stabilized cultures; D. Non-rooted and rooted microshoots.

soaking in a 20% bleach solution (Clorox) amended with a few drops of Triton X-100 with constant stirring for 15 min. Rinse the segments three times in sterile distilled water. Cut the stem at about 2mm from the ends to remove the ends exposed to the bleach.

Initiate stock cultures:

Place the stem segments vertically in petri plates (disposable 60 x 20 mm) containing about 15 ml WPM supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol, 1 mg/L thiamine, 8 g/L phytagar (Gibco) and 4.4 μM BA (final pH adjusted to 5.7) (Figure 18 B). Seal petri plates with parafilm® (American National Can™, Chicago, IL). Place cultures in incubators at 23C under 25 $\mu mol.m^{-2}.sec^{-1}$ light provided by fluorescent tubes for 16/8 h light-dark period. Transfer cultures to fresh medium every 5 or 6 weeks. Microshoots can be excised after 5 or 6 transfers (stabilized cultures) for rooting (Figure 18 C).

Excision and transfer of microshoots to rooting medium:

Cut microshoots that have a reddish-brown stem (5-6 weeks old)(Figure 18 C) from stock cultures and place in disposable polystyrene 17 x 100 mm test tubes with snap caps containing 10 ml of rooting medium. Rooting medium consists of WPM containing 30 g/L sucrose, 0.1 g/L myo-inositol, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L

pyridoxine, 0.1 mg/L thiamine, 4.9 μM IBA and 8 g/L phytagar (Gibco). Place test tubes in an incubator at 26C under 25 $\mu mol.m^{-2}.sec^{-1}$ light for 16/8 h light-dark photoperiod.

Root formation can be observed after about 12 days. Allow roots to grow and elongate for about 6 weeks (Figure 18 D). Transfer rooted microshoots to WPM with 4 g/L phytagar (Gibco) and without any IBA for a week and then to WPM with 10 g/L sucrose and 4 g/L phytagar for one additional week. Finally, transfer the microshoots to WPM with 6 g/L phytagar and without sucrose for 2 to 3 weeks.

Transfer of rooted microshoots to peat pellets:

Wash the agar off the rooted microshoots with tap water and place in presoaked (1 h) Jiffy peat pellets very carefully to avoid breaking any roots. Place peat pellets containing plants in trays with wells to hold the pellets. Water plants regularly with 10 % WPM salts by pouring the salt solution in trays. Cover trays with clear plastic lids and place in an incubator at 17C with about 40 $\mu mol.m^{-2}.sec^{-1}$ light for 16/8 h light-dark photoperiod. Keep lids securely closed for the first week (Figure 19 A) and then gradually (weekly intervals) replace them with lids having increasing size of holes (2.5 cm to 3x4 cm) (Figure 19 B). Finally keep trays open (no lids) for about 2 weeks. Keep them watered with 10% WPM salts. Roots can be seen to emerge through the peat pellets (Figure 19 C).

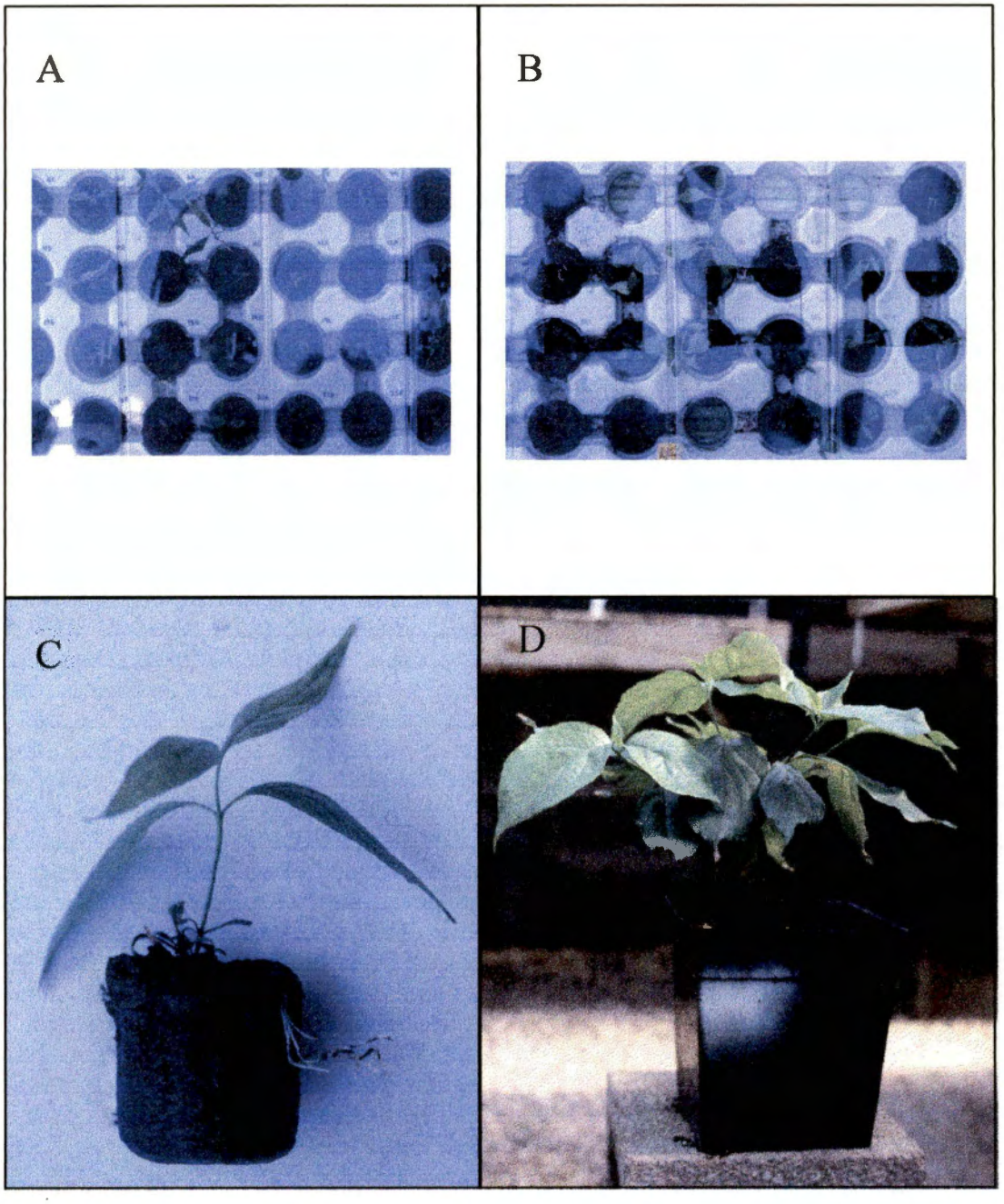

Figure 19. Pictures showing different stages involved in micropropagation of *Cornus florida*. A. Rooted microshoots in peat pellets placed in trays; B. Rooted microshoots in peat pellets covered with lids having openings; C. Dogwood plant in peat pellet; D. Acclamatized dogwood plant.

Transfer of plants to greenhouse:

Transfer plants with peat pellets intact to 15 cm square pots containing dogwood growing medium (Appendix 2). Sprinkle about 5 gms of Osmocote 14-14-14, a slow release fertilizer, over the soil around the plants. Place pots in greenhouse under mist bed and 50 % shade cloth (250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light) for 3-4 weeks and then move to 50 % shade without any mist spray. Water the plants for the first 2 weeks with 150 ppm N using Peters 20-20-20 soluble fertilizer. If plants are transferred to greenhouse in winter, supplement the light with three, 300 watt bulbs placed at a distance of about 1 meter above the plants. Plants will be ready to grow outside the greenhouse after about 2 months (Figure 19 D).

VITA

Anjana R. Sharma was born in Bombay, India on September 16, 1973. She had her preliminary studies at the Bhavan's A. H. Wadia High School. She majored in botany for the Bachelor of Science degree in May 1993 from the University of Bombay. Her specialization for the Master of Science degree in May 1995 from the University of Bombay was cytogenetics and plant breeding.

Anjana came to the U. S. in 1995 and worked voluntarily at Bethany College, Bethany, WV on hydroponics for a year. While in Bethany, she enrolled for the graduate program in the Ornamental Horticulture and Landscape Design department at the University of Tennessee, Knoxville. She also worked as a graduate research assistant and graduate teaching assistant and graduated with a Master of Science degree in Summer 1999.

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