CLONAL PROPAGATION OF ENDANGERED NATIVE PLANTS *RHODODENDRON CHAPMANII* GRAY, *TAXUS FLORIDANA* NUTT., AND *TORREYA TAXIFOLIA* ARN.

by

LEE ROY BARNES, JR.

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KEY TO ABBREVIATIONS

GROWTH REGULATORS

ABA	Abscisic acid
BA	Benzyl adenine
GA3	Gibberellic acid
IAA	Indole acetic acid
IBA	Indole butanoic acid
2-i-P	2-isopentyl adenine
NAA	Naphthalene acetic acid

NUTRIENT MEDIA

H	Heller's
LS	Linsmaier and Skoog
1/2LS	1/2 Strength Macronutrients Linsmaier and Skoog
MS	Murashige and Skoog
WPM	Woody Plant Medium
1/2WPM	1/2 Strength Macronutrients Woody Plant Medium

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CLONAL PROPAGATION OF ENDANGERED NATIVE PLANTS RHODODENDRON CHAPMANII GRAY, TAXUS FLORIDANA NUTT. AND TORREYA TAXIFOLIA ARN.

By

Lee Roy Barnes, Jr.
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Chairman: Dr. Charles R. Johnson
Major Department: Horticultural Science

Clonal propagation utilizing conventional and micropropagation techniques was investigated for three endemic and endangered Florida plant species, Rhododendron chapmanii Gray, Taxus floridana Nutt. and Torreya taxifolia Arn. This is critical for their preservation since these species exist in limited numbers but have high ornamental value which has threatened their survival due to illegal collection.

Micropropagation of R. chapmanii was investigated using shoot-tip cultures. An average of 7.6 fold increase in lateral shoots was achieved for three additional Stage II multiplication cycles on low salts Woody Plant Medium (WPM) with 2-i-P (10 mg/l) and adenine sulfate (80 mg/l). Microcuttings rooted in 4-6 weeks when direct-stuck into autoclaved 1:1:1 (v:v:v) Canadian peat:vermiculite:perlite medium in covered flats. A 5 second quick-dip in 1000 mg/l IBA (20% EtOH + 80% H_2O) increased survival and rooting of microcuttings. Inoculation of plantlets with the mycorrhizal fungus, Pezizella ericae increased survival and growth when plantlets were established in a less than optimal soil-less medium (1:1 v:v Fired montmorillonite clay:Canadian peat) but not in MetroMix-500.

Torreya and Taxus cuttings could be easily propagated when treated with 2,000-4,000 mg/l IBA. However, cuttings taken from lateral branches demonstrated strict plagiotropic growth. Best rooting and growth of Torreya cuttings (20%) occurred following treatment with 4000 mg/l IBA. Taxus cuttings rooted best (67%) following treatment with 2000 mg/l IBA.

Shoot-tip cultures (3-5 cm long) of Taxus and Torreya developed only 2-3 lateral shoots, apparently from preformed buds. Cultured "embryonic shoots" from expanding vegetative buds failed to produce multiple shoots. Excised embryos did not germinate following liquid culture to leach inhibitors. Micro- and megasporangia did not grow or differentiate in culture.

The most promising technique for Torreya involved application of cytokinins (4 weekly sprays with 100-200 mg/l BA or 2-i-P) to seedlings before culture. Newly stimulated axillary shoots developed additional shoots and multiple "bud-masses" when cultured on WPM with BA (1 mg/l) and NAA (0.01 mg/l). These were slow to develop but could be subcultured with additional growth of multiple shoots. No rooted plantlets were obtained.

1.0 INTRODUCTION

Preservation of indigenous endangered plant species is a critical issue requiring timely investigation of propagation techniques to increase the numbers of these rare species before they become extinct. Three endangered plant species of immediate concern are Rhododendron chapmanii Gray, Taxus floridana Nutt. and Torreya taxifolia Arn. All are endemic to restricted areas of northern Florida. Presently only 3000 Rhododendron chapmanii (Chapman's rhododendron) plants are known in situ and these are endangered due to habitat destruction or alteration, and over-collection for use in ornamental plantings. Taxus floridana (Florida yew) is endangered due to limited numbers and distribution but is highly desirable for landscape use and is subsequently subject to over-collection. Torreya taxifolia (Florida torreya) is endangered due not only to very limited numbers but also due to being decimated by a fungal disease which has destroyed most seed bearing trees. Today only 1200 apparently diseased seedlings and weak stump sprouts remain.

Presently, all three species are protected by state and federal laws. However, in spite of legal protection, the remaining specimens of each species is declining. Numerous R. chapmanii have been illegally dug from the wild. Torreya taxifolia does not appear to be reproducing in the wild and is all but extinct, except for a few apparently healthy plants located in botanical gardens elsewhere in the United States.

The purpose of this investigation was to determine the feasibility of clonal propagation techniques for the mass production of

these native ornamental species. Micropropagation techniques could potentially increase the number of these species with little impact on the present populations, as well as offer the possibility of eliminating the systemic pathogen(s) associated with Torreya die-back. If large numbers of these highly desirable plants could be produced, then they would be available for stabilizing natural populations, landscape plantings, and germ plasm preservation via meristem culture and cryopreservation.

2.0 LITERATURE REVIEW

2.1 Endangered and Threatened Native Plants

One group of native plants of particular interest are those which are rare, threatened or endangered in their present environments. Endangered and threatened plants are often legally classified at both federal and state levels. "Endangered" plants are defined as "species in imminent danger of extinction or extirpation and whose survival is unlikely if the causal factors presently at work continue operating" (Ward, 1978. p. xiii). "Threatened" plants are defined as "species which are believed likely to move into the above endangered catagory in the near future if the causal factors now at work continue operating" (Ward, 1978. p. xiii). A "rare" species classification includes those species which are not presently endangered or threatened, but are potentially at risk.

Congress first attempted to protect endangered plant species by enacting the Endangered Species Act of 1973 (House Document 95-41, 1975). Ayensu and de Filipps (1978) revised this initial listing and estimated that approximately 10% of the native vascular flora in the United States (approximately 20,000 spp.) or 2,000 species, subspecies and varieties are presently endangered or threatened.

Ayensu and DeFilipps (1978) concluded there are numerous reasons for protecting these species including the advantages of maintaining a broad gene pool and genetic diversity within and among species,

especially with important food, fiber and timber species. Many of these threatened plants show great potential for ornamental use.

Within the state of Florida, there are an estimated 3,500 vascular plant species which are native or naturalized (Ward, 1978). Of these, 124 species are presently listed at the state level as being endangered or threatened. Ward (1978) concludes that this number represents only 3.5% of the total plant species but further estimates that the true number of endangered, threatened or rare species may vary from the listed number by a factor of 2 or as large as 7.

In 1978, the State of Florida (s. 581.185 Florida Statutes) revised its listing of "endangered" plants to include over 30 species. They listed as "threatened" species all bromeliads, all cacti (except Opuntia), all orchids, all native rhododendrons, most ferns, most native Ilex spp., all Zamia spp., all Zephranthes spp. and 48 other species.

2.2 Advantages of Micropropagation Techniques

While many of the endangered and threatened plants are best protected by procurement and management of sensitive habitats, many of these species can be propagated and introduced into the horticultural trade with minimal impact on the remaining populations or their genetic diversity. Seedage and conventional vegetative propagation by cuttings can be used to increase the number of plants. However, these techniques are often of limited value due to the lack of propagation and cultural information for these rare species. Also the expense of collecting seed or vegetative propagules would be prohibitive due to limited numbers and travel expense.

Micropropagation of endangered species offers many advantages over both seed and conventional vegetative propagation methods, such as

(1) high initial multiplication rates from limited plant material in a small area; (2) clonal propagation of selected phenotypes via stimulation of existing axillary meristems; (3) potential to force rejuvenation of selected mature plants resulting in increased rooting ability of shoots with juvenile rooting capacity (Rhodes, 1968; Lyrene, 1981); (4) ability to propagate infertile or low fertility species (Wochok, 1981); (5) high degree of environmental control to minimize variability with limited plant material and (6) possibility of obtaining disease-free material via meristem culture (Abbott, 1977; Boxus and Druart, 1980; Styer and Chin, 1984).

Wochok (1981) suggested that tissue culture can be very useful in preserving threatened and endangered species and developed micropropagation techniques for 4 native species (Atriplex canescens, Mahonia aquifolium 'compacta', Mahonia repens, and Populus tremuloides).

2.3 Species Description and Distribution

2.3.1. Rhododendron chapmanii Gray

Rhododendron chapmanii Gray (Chapman's rhododendron) is a small evergreen shrub, 1-2 meters in height, which produces a showy display of terminal clusters of clear pink to rose-pink flowers in late spring (April-May). This species is apparently very drought, heat, and pest resistant (Simon, 1983).

Conventional propagation by seeds and cuttings has been successful (Salter, 1982 per.com.; Gensel, 1983 per. com.), albeit with a low rate of multiplication.

2.3.2 Taxus floridana Nutt.

Taxus floridana Nutt. (Florida yew; Savin) is an evergreen, needle-bearing shrub or small tree 3-8 m (rarely 10 m) in height. It is highly adapted to heavy shade and tolerates severe pruning.

Conventional clonal propagation by cuttings is possible; however, cuttings from lateral branches grow only plagiotrophically and few orthotropic shoots are available. Seed propagation is limited by a double dormancy within the seed which usually requires two seasons to germinate. Seed set is limited and heavy fruiting has been observed only once in the last ten years in Torreya State Park (Womble, 1982, per. com.). While over 200 seeds were collected in 1982, only 25 were collected in 1983 following an extensive, tree to tree search.

2.3.3 Torreya taxifolia Arn.

Torreya taxifolia Arn. (Florida torreya; Stinking Cedar; Polecat Wood; Gopherwood; Savin; Tumion) is a medium sized, pungent smelling (when bruised or crushed) evergreen tree which grows to 15 m.

Conventional propagation by cuttings is possible; however, lateral cuttings have been found to continue growing only plagiotrophically. Seed propagation has been successful but is limited by extreme scarcity of seed and a poorly understood double dormancy which usually prevents germination until the second year.

2.4 Woody Plant Tissue Culture

Tissue culture techniques have been applied to three general areas of interest, namely clonal propagation of (1) woody ornamentals (Murashige, 1974; Abbott, 1977; Debergh and Maene, 1981; McComb, 1978; Lazarte, 1981; Briggs and McCullock, 1983), (2) fruit trees (McComb,

1978; Zimmerman, 1978; Lazarte, 1981; Loreti and Mozini, 1982), and (3) superior genotypes of selected forest species (Durzan and Campbell, 1974; Abbott, 1977; Brown and Sommer, 1977; Cheng, 1978; Bonga, 1980; Durzan, 1980).

Propagation of woody species has been investigated by three different approaches: (1) organogenesis from callus or suspension cultures, (2) organogenesis directly from plant organ or organ sections and (3) axillary shoot stimulation. Attempts have been made to propagate from both selected mature tissues and from the more competent or plastic juvenile tissues. Although it is more desirable to obtain plants from elite mature genotypes, these tissues have been difficult to root. Rejuvenation treatments and the use of juvenile zones within the plants have been successful in some cases (Lyrene, 1981). By far the greatest amount of success has occurred with the use of explants from juvenile tissues, especially embryos and young seedlings. Although morphogenically flexible and generally easy to root, these juvenile sources are genetically variable and untested as to their mature phenotype.

2.4.1 Organogenesis from Callus and Suspension Cultures

Organogenesis from callus or suspension cultures established from elite trees would offer the greatest advantages. This would allow large scale and cost efficient production of superior trees. This would be especially important if somatic embryogenesis could be obtained and microencapsulation developed so that clonal "seeds" could be produced. However, at least two factors prevent the wide scale use of these techniques. First, organogenesis from callus and suspension cultures has not been successful for most important forest species (Wilkins et al.

1985). Secondly, the genetic stability of adventitious organogenesis is often questionable (Wilkins et al., 1985; Larkin and Scowcroft, 1981).

Successful regeneration of plantlets via somatic embryogenesis from suspension and callus cultures has been reported for Santalum album (Lakshmi Sita et al., 1980), and Liquidambar styraciflua (Sommer and Brown, 1980). Regeneration of shoots and roots from callus has been reported for triploid Populus tremuloides (Winton, 1970), Citrus grandis and C. sinensis (Chaturvedi and Mitra, 1974), Ulmus americana (Durzan and Lopushanski, 1975), Ephedra gerardiana (Ramawat and Arya, 1976), Larix decidua (Bonga, 1981), Malus domestica (Liu et al., 1983), Pinus eldarica (Herrara and Phillips, 1984), Eugenia jambos and E. malaccenis (Litz, 1984).

In summary, organogenesis from true cell suspensions and callus cultures has only been successful for a limited number of woody species (especially with timber, tropical fruit, and arborescent monocotyledonous species), usually at low frequencies and often with the inability to root shoots from callus derived from mature trees (Wilkins et al., 1985).

2.4.2 Organogenesis from Plant Organs and Organ Sections

Direct organogenesis from plant organs and organ sections has been widely accomplished among woody species. In most cases, this has occurred from very juvenile explants, especially embryos or young seedlings. Rejuvenation from mature tissues has been successful from inflorescenes or micro- and megasporangia, tissues temporally associated with meiosis (Bonga, 1980).

Organogenesis has occurred in widely dispersed plant families. LaRue (1948) obtained low frequency shoot regeneration from the mega-gametophyte of Zamia floridana. He later reported (1954) regeneration of

shoots and roots from megagametophytes of both Zamia floridana and Cycas revoluta. Norstog (1965) cultured megagametophytes of Zamia integrifolia and was able to obtain diploid and haploid roots and leaves in 35% of his cultures. He was also able to obtain diploid and haploid embryoids from cultures of excised embryos and haploid megagametophytes, respectively. Plants were successfully established in soil.

Konar and Oberoi (1965) obtained embryoids on cotyledons of Biota orientalis Endl. (now Platycladus orientalis). Further development of these embryoids did not occur until transfer to a different medium. These "embryoids" may have been inappropriately named since they did not appear to have a root apex although normal appearing cotyledons and shoot apex developed into a young shoot.

Norstog and Rhamstine (1967) induced proliferation of callus from haploid and diploid tissues of Zamia integrifolia and haploid tissues of Cycas species.

Hu and Sussex (1971) were able to stimulate embryogenesis from cultured cotyledons of Ilex aquifolium. They noted that embryoids developed only on cotyledons where the shoot-tip failed to develop, suggesting an antagonism between shoot growth and initiation, possibly due to apical dominance of the active shoot-tip.

Isikawa (1974) reported the formation of adventitious buds and roots on the hypocotyls of Cryptomeria japonica, primarily in illuminated cultures. This organogenesis required low levels of the growth regulators ABA, NAA and BA. He concluded that callus formation may be antagonistic to shoot formation of conifers.

Sommer et al. (1975) reported high frequency adventitious bud formation on cultured cotyledons of Pinus palustris when grown on

modified Gresshoff and Doy medium. If maintained on media with high auxin/cytokinin ratios, excess callus began to form after 5-6 weeks. They also mentioned success in obtaining adventitious buds on cotyledons of P. elliotii, P. taeda, P. virginiana, P. rigida, P. strobus and Pseudotsuga menziesii but did not mention if plantlets were obtained from these species.

Cheng (1975) obtained adventitious bud formation on cotyledons and cotyledon slices of Pseudotsuga menziesii during primary culture, and was able to maintain the capacity to produce buds after 3 and 4 subcultures. This system required 3 separate steps: (1) culture initiation with equal amounts of auxins and cytokinins, (2) organ initiation with high cytokinin levels, and (3) further growth of adventitious buds on 1/2 strength modified MS medium and no growth regulators for further growth of adventitious buds. Plantlets were obtained by treating shoot cuttings with IBA (amounts not given). The necessity for separate steps suggests that conditions necessary for initiation of one step were inhibitory to further development.

Cheng (1976) reported adventitious buds from cotyledons of Tsuga heterophylla using techniques similar to those employed for Pseudotsuga menziesii. In addition, she was able to root shoots directly in soil mix thus saving one culture step.

Winton and Verhagen (1976) induced adventitious shoots directly from cotyledons of Pseudotsuga meniesii and also from subcultured callus, obtaining up to 100-200 clonal shoots from each seed. They also obtained callus on needles from 3 month old seedlings and from needles from a 25 year old tree. However, they were only able to stimulate adventitious shoots from the 3 month old needle callus.

Bonga (1977) initiated a new approach to obtain adventitious shoots from 15-20 year old Abies balsamea. "Embryonic shoots," composed of dormant buds with scale leaves excised, were soaked either in sterile water for 24 hours, or in aqueous solutions of growth regulators. Soaked buds formed additional axillary shoots when transferred to a growth medium while non-soaked buds only expanded as a single shoot.

Reilly and Washer (1977) cultured embryos of Pinus radiata and obtained adventitious buds directly from cotyledons and hypocotyls and from meristematic callus which proliferated from these organs. This callus could be subcultured on cytokinin-free medium every 4 weeks. When shoot primordia were separated from these meristematic masses, they developed into well formed shoots. By serial subculture of callus, they were able to obtain over 200 shoots from 1 embryo in 6 months. These shoots could be rooted on medium with auxin (1-25 mg/l IBA).

Brown and Sommer (1977) reported that 9 Pinus species developed buds on cotyledons at a frequency of 26-100% depending on the species and medium. They were unable to root these elongating shoots except very rarely (less than 1% rooted).

Mott et al. (1977) summarized their work with Pinus taeda whereby they obtained more buds on excised cotyledons vs intact embryos. They found significant differences in organogenic response between seed families but these differences could generally be reduced by adjusting the growth regulator combinations.

Coleman and Thorpe (1977) cultured cotyledons from seeds and shoot-tips from 4 to 10 year old trees of Thuja plicata. Using lateral shoot-tips from 4 to 10 year old trees, they obtained adventitious shoots which grew only orthotrophically. The addition of high levels of

GA_3 (0.01 mg/l) to the medium induced the development of male strobili from the vegetative apex. Fifty percent of the adventitious buds from juvenile tissues rooted when transferred to a medium with 1/2 strength MS and 1.6 mg/l BA, while only 11% of those from the mature shoots rooted. They concluded that the successful formation of plantlets requires 3 distinct sequential treatments (1) meristematic induction, (2) bud elongation and (3) rooting and establishment.

Arnold and Eriksson (1978) collected vegetative buds of Picea abies from 5 year old trees, 75 year old hedged trees (2 m tall) and from trees in 4 age classes: 5-10, 10-15, 15-20, and 20-50 year old trees. No differences were found in the organogenic ability of these explants with an average of 30% of cultures producing buds. They made no attempt to test the rooting ability of these buds.

Aitken et al. (1981) compared explant sources for regeneration of Pinus radiata buds. Excised whole embryos, and intact and excised cotyledons from 1 week old seedlings were cultured, resulting in an average of 9 rootable shoots from the embryos, 18 from intact cotyledons and 180 from the excised cotyledons from 1 week old seedlings. Induction of nodular, smooth-surfaced meristematic callus appeared necessary for large-scale propagation. After 24 weeks they had obtained over 1300 shoots from the excised cotyledons from 1 seed thus greatly increasing the efficiency of this technique.

Bonga (1981) published a second paper on organogenesis from 15-20 year old mature trees including Abies balsamea. Morphogenesis occurred rarely from female cones of Pinus mugo and Picea abies. Vegetative shoots from A. balsamae, P. glauca and Pseudotsuga menziesii produced buds at the base of young expanding needles. His methods included

collecting buds before bud break, excising underwater and soaking for 15 minutes in 1 g/l malonic acid solution or for 24 hours under water.

Mott and Amerson (1981) updated their work with Pinus taeda, summarizing the need for sequential transfer of explants. They stressed the point that 1 medium and combination of growth regulators initiated a response but further development was inhibited until they were transferred to a different medium. They stressed the need for judicious observation of the timing and duration of growth regulator exposure and termed the short term exposure to certain growth regulator combinations as "pulsing."

Patel and Berlyn (1982) questioned the genetic stability of multiple buds regenerated from embryo explants of Pinus coulteri even when no cytokinins were used in the medium. Cells from callus and regenerated buds showed a progressive increase in DNA levels over time. After 6 weeks in culture, 80% of cells in regenerated buds had DNA levels between 4C and 12C as determined with a microspectrophotometer. They did not determine whether this resulted in mutated whole plants.

David et al. (1982) reported adventitious budding on Pinus pinaster cotyledons and from short-shoots and elongating needles collected from a 10 year old tree which had been sprayed weekly with BA. Meristems of cultured short-shoots produced buds from 2 locations, from the apical meristem of the short-shoot and from mitotically active cells at the base of the expanding needles. When they altered the NH_4^+/K^+ ratio of the medium to 1, they obtained increased adventitious bud formation. They emphasized the importance of having mitotically active and morphogenically nondetermined cells.

Jansson and Bornman (1983) reported improved morphogenesis of spring collected "embryonic shoots" (bud with budscales removed) of Picea abies by inclusion of the "crown," a layer of 4 to 7 extremely thickened collenchyma cells located at the base of the shoot. This anatomical feature is common to Abies, Cedrus, Larix, Pseudotsuga, Picea, Sequoia, Taxus, Torreya and Tsuga. Following excision and culture of the embryonic shoots with attached crowns, an average of 10 buds per explant was produced while crown-minus explants produced only 3 buds per explant. They reiterated comments by Chalupa and Durzan (1973) that the crown may act as a filter or translocation barrier to nutrients and hormonal stimuli. They also suggest that the crown may be a barrier to basipetal transport.

In summary, organogenesis from juvenile tissues, especially cotyledons and hypocotyls, is common among many conifers. Mitotically active cells of expanding needles also appear morphogenically flexible. However, the number of buds which can be produced by this method is limited (few to dozens) or rarely, hundreds from a single explant. By subculture of meristematic masses derived from these organs, up to 1300 plantlets have been obtained from a single seed of Pinus radiata (Aitken et al., 1981). The ability to obtain plantlets usually requires several different media and growth regulator regimes with careful attention paid to the duration of each exposure. Shoot initiation, shoot elongation, root initiation and root elongation require different conditions to maximize the propagation rates. For many species, rooting has occurred at low frequencies but evidence suggests that more careful attention to duration of auxin exposure and light levels may increase rooting.

2.4.3 Stimulation of Axillary Meristems

The stimulation of axillary meristems has been most successful with ornamental and fruit tree species. Styer and Chin (1983) list over 145 genera which have been cultured from excised meristems and shoot-tips. Briggs and McCulloch (1984) list 63 genera and 106 species and cultivars which are being propagated commercially or in research laboratories in the United States and Western Canada.

Haines and de Fossard (1977) reported stimulation of axillary buds from orthotrophic stem sections of Araucaria cunninghamia. Shoots which developed from bud traces of ortotrophic branches grew upright while shoots derived from plagiotrophic branches grew only laterally.

Vieitez and Vieitez (1980a) propagated Castanea sativa by culture from lateral buds from 3 to 4 month old seedlings and from axillary buds of cultured embryos. Keys and Cech (1981,1982) propagated Castanea dentata by stimulating axillary meristems from mature embryos and from 4 month old seedlings grown in a greenhouse. Chevre et al. (1983) micropropagated C. sativa and obtained increased axillary multiplication and elongation by doubling the calcium and magnesium concentrations in MS medium and by lowering the pH to 4.0.

Rancillac et al. (1982) developed methods to induce axillary shoot development from 1 month old seedlings of Pinus pinaster. Rooting of these shoots was most successful when shoots were cultured for 17 days on media with 2 mg/l NAA followed by transfer to an auxin-free media.

Wang and Hu (1983) cultured shoot-tips of 5 year old Sassafras randaiense and obtained "multiple-bud-mass" proliferation on MS plus 60 mg/l kinetin within 2 months of incubation. The rooting percentage

remained below 20%, but a 95% survival rate was achieved following transfer to vermiculite in flats.

While explants from mature trees are generally difficult to root, Hearne (1982) reported the advantages of using mature tissues. He suggested that for many species where mature tissues could be air layered, these mature tissues are desirable since they retain their mature features and often root easily in culture. He cited the advantages of earlier flowering, and that plantlets commonly have reduced canopy size which reduced the need for pruning and eased harvesting. He cited precocious flowering roses within 49 days following deflasking, and fruiting grapes and papaya within 12 weeks from deflasking. His observations suggest that axillary shoot explants from mature plants retain their mature qualities yet root as if they had been "phenotypically" rejuvenated while in culture.

Amos and McCown (1981) reported their attempts to micropropagate conifers by shoot-tip and axillary meristem culture. They obtained shoot multiplication from species of Juniperus, Sequoia, Taxus, Thuja, and Tsuga when cultured with very low levels of BA. Picea and Pinus did not show rapid shoot development. They concluded that as a group, conifers are more sensitive to intermediate levels of cytokinins than woody dicot species. They were able to root harvested shoots, but did not comment on the further growth of these cultures (i.e. whether orthotrophic or plagiotropic shoots developed).

Micropropagation of rhododendrons has been a great commercial success. Anderson (1975) first reported micropropagation of Rhododendron using shoot-tip cultures on a modified, approximately 1/4 strength MS with a much reduced K content, obtaining an average of 6.2

new shoots per culture within 8 weeks. He noted that BA proved phytotoxic while 2-i-P was not, and that the addition of 80 mg/l adenine sulfate was necessary for superior shoot development. Anderson (1978) also reported successful direct rooting of micropropagated rhododendrons from Stage II culture thus saving a very labor intensive step. Several rooting media were successful as long as they were well drained and porous, for example 1:1 (v:v) perlite:peat.

Strode (1979) reported on the commercial micropropagation of rhododendrons using a revised Anderson (1975) medium amended with 5 mg/l 2-i-P and 1 mg/l IAA. After 4 subcultures at 6 week intervals, he obtained an average of 22.9 plants which could be attained when 4 shoot-tips were cultured in a single container. Harvested shoots rooted 90% within 5 weeks.

Meyer (1982) reported the successful use of tissues from flower buds and inflorescences for rhododendron cultivars. Flower buds were chosen as they had been used successfully with several herbaceous species and resulted in lesser initial contamination. Meyer excised buds from dormant plants between October and April and obtained "granular masses" of tissues on Anderson's medium with 1-4 mg/l IAA and 5-15 mg/l 2-i-P.

McCown and LLoyd (1983) surveyed the response of 7 genotypes of rhododendron to micropropagation. They used Woody Plant Medium (WPM- a modified Gresshoff and Doy medium), which was originally developed for micropropagation of Kalmia and other woody species (McCown and Lloyd, 1981). They observed that 2-i-P stimulated the greatest shoot multiplication, while BA was phytotoxic to all but elipidote and lepidote rhododendrons. Optimal cytokinin levels varied little with the

genotype, with 1-3.5 mg/l 2-i-P generally optimal. They observed that adventitious shoot development occurred occasionally when 2-i-P greater than 2.75 mg/l was used. Average multiplication rates were 7 to 21 depending on cultivar. It was estimated that with an average of 40 shoots per 6 week subculture period, over 75,000 shoots could be generated per square meter of culture space per year. Plantlets from callus cultures were also obtained; however, many aberrant types developed. With axillary shoot stimulation, plants remained true-to-type even after 6 years of culture, except for occasional albino/green chimeras which were readily observed and easily rouged. It was noted that the resulting rooted plantlets grew vigorously with a high degree of basal branching, similar to the growth habit of seedling rhododendrons. They stated their uncertainty as to whether this branching was induced by growth regulators or whether the seedling morphology was being mimicked.

2.4.4 Taxus Embryo Culture

Taxus species have been cultured in vitro by several researchers. Tulecke (1959) cites LaRue's work with cultured pollen from Taxus brevifolia whereby he obtained an unusual "pincushion callus" with cells resembling pollen tubes sticking out at right angles to the surface of the callus. LaRue maintained long term cultures of this slow growing callus but never obtained regeneration of shoots.

Zenkteler and Guzowska (1970) cultured mature female gametophytes of Taxus baccata on White's medium and obtained proliferation of callus from 2-5% of the explants. Variation in chromosome number from n to 8n was found in callus from the 14th and 15th 8-10 week subcultures. Regeneration of shoots or roots was not observed. Cultured embryos did

not develop even when placed next to growing gametophytic tissues.

LePage-Degivry (1968,), LePage-Degivry (1970), 1973abc) and LePage-Degivry and Garello (1973) initiated a series of experiments in which she cultured excised embryos from Taxus baccata. She (LePage, 1968) found that by culturing mature excised embryos in liquid Heller's medium with sucrose she was able to leach out inhibitors to germination. Later, it was determined that the culture of excised embryos for 15-20 days in liquid media was necessary to remove inhibitors (LePage-Degivry, 1970). The inhibitor was identified as ABA by paper chromatography. After subculturing to solid medium with 1 mg/l GA_3 or after a chilling period, the embryos readily germinated. When embryos which had begun germination were treated with extracts of the conditioned liquid medium, growth ceased (LePage-Degivry, 1970). However, an additional GA_3 treatment resulted in resumption of germination. Paper chromatography of the extract revealed that ABA was leached from the explant during liquid culture.

It was later determined that ABA was not leached out by distilled or mineral water alone, and that sucrose and Ca^{++} or K^{+} ions in the medium were required to remove inhibition (LePage-Degivry, 1973b). LePage-Degivry (1973c) reported renewal of inhibition of leached and germinating embryos with the inclusion of ABA in the solid medium. No adventitious organogenesis was reported.

2.4.5 Mycorrhizal Synthesis of Micropropagated Plantlets

Several micropropagated woody species have been inoculated with mycorrhizal fungi in vitro and following rooting and establishment in soil. Inoculation of micropropagated plantlets with mycorrhizae could potentially increase survival, growth, and tolerance to environmental

stresses. Morandi et al. (1979) observed that the colonization of micropropagated raspberries (Rubus idaeus) with vesicular arbuscular mycorrhizae (VAM) resulted in more vigorous and uniform sized plants, with a 53-130% increase in shoot dry weight depending on the specific endophyte and cultivar used. Granger et al. (1983) noted that colonization of micropropagated apple plantlets (Malling clones) with VAM resulted in increased plant heights, total leaf areas, leaf dry weights and leaf content of Cu and P. This increase in growth occurred with the cultivar Malling 7; however, colonization did not increase growth of Malling Merton 111. Pons et al. (1983) noted colonization of micropropagated sweet cherry plantlets (Prunus avium L.) with VAM in vitro but did not obtain any observed growth enhancement. In preliminary experiments where cherry plantlets were inoculated at time of transplanting into soil media, a "marked improvement in growth" occurred.

Inoculation of rhododendron with ericoid mycorrhizae has been successful in situ (Duddridge and Read, 1982) and in vitro (Pearson and Read, 1973; Moore-Parkhurst and Englander, 1981). Ericoid plants appear to be require the specific endophyte, Pezizella ericae (Read, 1982). Colonization of rhododendron with Pezizella resulted in enhanced nitrogen and phosphorus supply to the plant as well as increased resistance to heavy metal toxicity (Duddridge and Read, 1982; Read, 1983). Moore-Parkhurst and Englander (1981) developed a technique for in vitro colonization of rhododendron and Pezizella; however, this technique is impractical for commercial use. The most efficient time to inoculate plantlets would be at the time of transfer to soil.

3.0 MATERIALS AND METHODS

3.1 Rhododendron chapmanii Gray

3.1.1 Establishment of Stock Plants

Stock plants were established from seeds collected in late October, 1982, from cultivated plants located around the Doyle Conner Building, Division of Plant Industry, Gainesville, Florida. They were obtained as soon as the brown capsules began to dehisce, disinfested with 15% Clorox solution (0.79% by weight sodium hypochlorite) for 5 minutes, and rinsed with 3 changes of sterile deionized water. Seeds were spread out on sterile moistened filter papers in petri plates and incubated in light (16 hrs light/8 hrs dark at 25 C +/- 5 C with irradiance of 50-90 $\mu Mm^{-2}s^{-1}$). Germination occurred within 2 weeks and when seedlings reached a height of 5 cm, they were transferred individually to 4-liter pots maintained in a growth chamber illuminated with 150 $\mu Mm^{-2}s^{-1}$ cool white fluorescent lighting for 16 hours daily. Temperatures were maintained at 25 C +/- 1 C (day) and 20 C +/- 1 C (night). Plants were watered from below to avoid wetting the foliage.

Succulent shoot-tips, approximately 3-5 cm in length (5-10 nodes), were pruned by hand and then washed in soapy water, then rinsed in cool running tap water for 1 hour. Pruned stock plants subsequently developed numerous additional axillary shoots, which developed into succulent shoot-tips of sufficient size to culture in 4-6 weeks.

3.1.2 Disinfestation

An experiment was designed to compare contamination rates of shoot-tip explants from 2 sources. Explants from plants grown both outside under 40% polypropylene (Saran) shade cloth and in a growth chamber were used.

Culture room conditions remained the same for all experiments. Cultures were illuminated with cool white fluorescent lights at 90 $\mu Mm^{-2}s^{-1}$ for 16 hours daily, and a temperature of 25 C + /- 5 C. Unless otherwise indicated, 20 ml of medium was aliquoted into 25x150 mm Pyrex test tubes and capped with Kaputs. Media were autoclaved at 1.05 kg/cm^2, at 110 C for 20 minutes and then allowed to cool on a slant.

Shoot-tips from both 60% shade (grown out of doors) and growth chamber grown stock plants were disinfested for 15 minutes in 15% or 20% Clorox then rinsed in sterile distilled water 3 times. Shoot-tips were further trimmed to remove the bleached petiole stubs, stem base and apex resulting in a 5 node stem section. Explants were inserted vertically into agar to approximately half their total length. Culture medium consisted of basal Woody Plant Medium (WPM - McCown and Lloyd; 1981) plus 2% sucrose, 0.8% Bacto aar and pH adjusted to 5.2 prior to autoclaving. Ten replications per treatment were used. Data on contamination were collected after 2 weeks under culture conditions described previously.

3.1.3 Media Selection

An experiment was inititated to determine the best medium for micropropagation of Rhododendron chapmanii. Four media used for woody plants were selected : Woody Plant Medium (WPM-McCown and Lloyd, 1981), 1/2 strength macronutrients of WPM (1/2WPM), Linsmaier and Skoog medium

(LS-Linsmaier and Skoog, 1964) and 1/2 strength macronutrients of LS (1/2LS). Culture vessels were 25x150 mm Pyrex test tubes containing 20 mls of medium. Each medium was amended with 1, 5, 10, 15, or 20 mg/l 2-i-P, and (in mg/l) myo-inositol (100), NaH_2PO_4 (100), adenine sulfate (80), sucrose (30,000) and Bacto-agar (8,000). The pH of the medium was adjusted to 5.2 before autoclaving. Ten replications per treatment were used with individual 5 node shoot-tips used as experimental units. Data were collected after 6 weeks.

3.1.4 Selection of Growth Regulators Levels

Two experiments were performed to determine effects of growth regulators on rapid multiplication. In the first experiment, the cytokinin, 2-i-P, was examined at 0, 5, 10, and 15 mg/l with ten replications per treatment. Individual 5 node shoot-tips were used as experimental units. In the second experiment, the interaction of cytokinins with auxins was investigated in a 3X3 factorial with 2-i-P at 5, 10, and 15 mg/l and IAA at 0, 1, and 2 mg/l. Each treatment was replicated 10 times. Data were collected after 60 days in culture.

3.1.5 Stage II Subcultures

Following initial culture on multiplication medium (SII), explants were subcultured through 4 additional SII cycles on WPM with (in Mg/l) 2-i-P (10) and adenine sulfate (80). The initial SII culture utilized 25x150 mm test tubes, after which explants were transferred and cultured in small baby food jars (approximately 125 ml) containing 40 ml of medium and covered with aluminum foil and wrapped with Parafilm. Thirty to 50 mm tall microcuttings consisting of a single primary shoot with developing lateral shoots were subcultured. Uniform sized explants,

consisting of a single primary shoot with developing lateral shoots, were subcultured. These explants were placed 3 to a baby food jar with modified WPM medium. The explants were oriented horizontally and pressed down slightly so that most nodes were in direct contact with agar. Ten or more replicates were made. Cultures were transferred onto fresh multiplication medium at 6 to 8 week intervals. Multiplication rates were evaluated over 5 subcultures on WPM. Data were collected at each subculture time every 6-8 weeks.

3.1.6 Rooting and Establishment

Stage II microcuttings were given one of the following treatments: (1) control, (2) 0.1% IBA in talc or (3) 0.1% IBA (in 10% ethonol) for 5 seconds. They then were stuck directly into 54x28x6.8 cm plastic flats containing 5 cm deep of autoclaved rooting medium, 1:1:1 (v:v:v) Canadian peat:vermiculite:perlite. Ninety-six explants were used for each treatment. Microcuttings were protected from drying by covering the flat with an inverted translucent plastic flat. They were misted daily by hand to increase humidity and were watered as needed with deionized water. Flats were illuminated under cool white fluorescent lights at approximately 90 $\mu Mm^{-2}s^{-1}$ with a 16 hour photoperiod at 25 C +/- 2 C.

3.1.7 Colonization with Ericoid Mycorrhizae

An experiment was established to determine the growth response of rooted plantlets to inoculation with mycorrhizae and establishment in two different container media. Well-rooted plantlets were inoculated with the ericoid mycorrhizal fungus, Pezizella ericae Read, and transplanted individually into 6 cm^2 plastic "cell-pack" (4 cells/pack). Two soilless media were used: (1) Metro-Mix 500 (MM500) and (2) 2:1 (v:v) fired

montmorillinite clay: Canadian peat (FMC:CP). Fungal inoculum of P. ericae was prepared according to Marx and Kenny (1981). Pure cultures of P. ericae were obtained from the American Type Tissue Collection (culture no. 32985). The cultures were increased in 250 ml flasks containing 100 ml of modified Melin-Norkrans fungal medium with a 1 centimeter layer of broken glass in the bottom of the container. Cultures were maintained at 25 C in the dark for 4 weeks, but were shaken weekly to fragment the hyphae. A mixture of 28:1 (v:v) vermiculite:finely screened Canadian peat was moistened with 750 ml of Melin-Norkrans medium and autoclaved. After cooling, 100 ml of prepared P. ericae inoculum was added and this mixture was cultured for 3 weeks in the dark at 25 C. This inoculum was then leached by wrapping in several layers of cheese cloth and rinsing for 3 to 4 minutes under cool, running tap water to remove excess glucose and nutrients which might support growth of intrusive saprophytes. The vermiculite:peat inoculum was then air dried at 25 C for 3 days. Approximately 1 gm of inoculum was placed immediately below the transplanted plantlet's root zone. Non-inoculated plantlets had 1 gm of autoclaved vermiculite added below the roots as a control. Colonization and growth responses were evaluated after 16 weeks, using the technique described by Englander (1982). Roots were stained with Lactophenol-Trypan blue stain for 10 minutes at 90 C. Percent colonization was estimated by use of the modified gridline intersection method of Giovanettii and Mosse (1980). Each treatment was replicated 12 times, with 4 plantlets per replication.

3.2 Taxus floridana Nutt.

3.2.1 Conventional Propagation by Cuttings

An experiment was initiated to determine the optimal auxin treatment for root formation on cuttings. Dormant, evergreen cuttings of Taxus floridana were collected in early February, 1982, from native populations in Torreya State Park and from plantings on the University of Florida campus. Ten to 15 cm long terminal and subterminal cuttings were collected from upright and lateral branches. The bases were trimmed and quick dipped for 10 seconds in 2000, 4000, or 8000 mg/l IBA (in 20% EtOH). Each treatment was replicated 10 times with individual cuttings as experimental units. Cuttings were stuck 5 cm deep in plastic flats containing 10 cm of a mixture of 1:1 (v:v) course sand:Canadian peat. They were misted intermittently (5 sec every 2 min) during daylight hours. Bottom heat (28 C) was supplied from electric cables placed 5 cm below the flats. Rooting was evaluated 120 days after sticking. Well rooted cuttings were transplanted into 1:1:1 (v:v:v) Canadian peat:perlite:sandy loam medium in 4-liter containers. After establishment, stock plants were placed in a growth chamber with the same cultural conditions mentioned for R. chapmanii (3.1.2).

3.2.2 Culture of Quiescent Shoot-tips

Four different plant organs were investigated--(1) quiescent shoot-tips (3.2.2), (2) developing vegetative buds just before bud break (3.2.3), (3) swelling microsporangium (3.2.4)and (4) excised embryos (3.2.5).

Quiescent shoot-tips were collected in October, 1982, from the most recently matured branches of plants located on University of Florida campus. Three to 4 cm long shoot-tips were washed in soapy

water, rinsed in running tap water for 1 hour and then disinfested with a 15-20% Clorox solution (with 5 drops Tween-20 per liter) for 15-20 minutes. Shoot-tips were rinsed in 3 separate changes of sterile deionized water. The bleached bases were trimmed and explants were inserted vertically to half their length into 20 ml of solidified WPM medium in 25x150 Pyrex test tubes.

An experiment was initiated to determine the best medium for culture of Taxus floridana. The 4 media selected were the same used for Rhododendron chapmanii (Sect. 3.1.3). Data were collected after 6 weeks in culture. Twenty explants per medium were cultured with individual shoot-tips as experimental units. Basal media were amended with (in mg/l) myo-inositol (100), NaH_2PO_4 (100), sucrose (30,000), Bacto-agar (8,000). The pH was adjusted before autoclaving to 5.2 with the addition of 1.0 or 0.1 N solution of NaOH or 1.0 or 0.1 N HCl prior to autoclaving. Data were collected on length of new growth, number of developing shoots and explant color.

3.2.3 Culture of Expanding Vegetative Buds

Swelling vegetative buds were collected weekly for 3 successive weeks preceding bud break in late February and into the second week of March, 1983. Buds were washed and disinfested as described previously (Sect. 3.2.3) and outer bud-scales were carefully excised under water as described by Bonga (1977,1981) and Jansson and Bornman (1983). Four preculture treatments were used. Trimmed buds were allowed to soak for 24 hours in sterile distilled water or 3 hours in either 22.5, 28.0, or 34.0 mg/l BA solutions before culturing on WPM amended with myo-inositol (100), NaH_2PO_4 (100), sucrose (20,000), agar (8,000) and 5.2 pH. Ten

replications per treatment were used. Data were collected on overall growth and specific morphogenic changes.

3.2.4 Microsporagium Culture

Expanding microsporangia were collected and disinfested as described in Section 3.2.2. Microsporangia were trimmed of outer scales and soaked in the 4 previously described solutions (3.2.3). Explants were established on basal WPM with agar (8,000). Each treatment involved 10 replications with individual microsporangium and data were collected after 6 weeks in culture.

3.2.5 Embryo Culture

Attempts were made to promote germination of excised embryos by leaching of inhibitors to seed germination. Seeds were surface disinfested using 20% Clorox for 20 minutes. The 1-2 mm long embryos were excised and further disinfested in 5% Clorox for 10 minutes. Embryos were rinsed 3 times with sterile deionized water, then placed into 20 ml of liquid Heller's medium with 2% sucrose. Medium pH was adjusted to 5.5 before autoclaving. The small embryos were most easily handled by catching them in a drop of liquid held in a 5 mm wide bacterial transfer loop. Cultures were maintained in the dark without shaking (LePage, 1968). Due to limited seed availability, only 5 excised embryos were used per treatment with individual embryos as experimental units.

Embryos were subjected to 3 different treatments. Following an initial 2 week culture period, they were transferred to either (1) Heller's medium supplemented with 1 mg/l GA_3 and solidified with 0.8% agar or (2) Heller's solidified medium and stored for 1 month in a

refrigerator at 4 C before returning to culture room conditions. Treatment (3) involved a second leaching cycle in liquid Heller's medium for 2 weeks before placement on solid Heller's medium with 1 mg/l GA_3. GA_3 was filtered sterilized and added to the cooling but still liquid medium. Data were collected from the cultures grown for 40 days in the dark at 25 C +/- 2 C.

3.3 Torreya taxifolia Arn.

3.3.1 Conventional Propagation by Cuttings

Evergreen cuttings were collected in early February, 1982, from trees growing in Maclay Gardens, Tallahassee, and from plantings on the University of Florida campus. Ten to 15 cm long terminal and subterminal cuttings were collected from lateral branches. The cuttings were wounded twice along opposite sides by scraping the basal 2.5 cm of the cuttings to expose the lighter green cambium. Cuttings were treated in the same manner as described for Taxus (Sect. 3.2.1) involving treatment with 3 concentrations of IBA (2,000, 4,000, and 8,000 mg/l). Individual cuttings were used as experimental units, with 5 replications per treatment.

Cuttings were evaluated 120 days following sticking and well-rooted cuttings were transplanted into 1:1:1 (v:v:v) Canadian peat:perlite:sandy loam medium in 4-liter containers. Data collection included percent rooting, number and total length of roots.

3.3.2 Culture of Quiescent Shoot-tips

Five explant sources were investigated to determine the morphogenic capacity of various organs as reported in the literature--(1) quiescent shoot-tips (3.3.2), (2) expanding vegetative buds (3.3.3), (3)

expanding mega- and microsporangia (3.3.4), (4) excised embryos (3.3.5), and (5) seedling stock plants sprayed with cytokinins before culture (3.3.6).

In the first experiment (3.3.2), quiescent shoot-tips were collected in October, 1982, from plantings located on the University of Florida campus. Three to 4 cm shoot-tips were washed and disinfested following the procedures previously described for quiescent shoot-tips of Taxus floridana (Sect. 3.2.2). They were cultured in each of four media (WPM, 1/2WPM, LS, and 1/2LS).

3.3.3 Culture of Expanding Vegetative Buds

Expanding vegetative buds were collected weekly over a 3 week period preceeding budbreak in early spring (mid- to late March, 1984). Buds were trimmed of bud-scales and were handled following the procedures of Boulay (1977,1981) and Jansson and Bornman (1983) as described for Taxus in Sect. 3.2.4. Ten buds were used per treatment with individual buds as experimental units. Thus, 3 collection times were combined with 4 preculture soaks.

3.3.4 Culture of Micro- and Megasporangia

Expanding micro- and megasporangia were collected as they swelled following budbreak in the middle of March, 1984. These were washed and disinfested as with Taxus floridana microsporangia (3.2.4). Ten explants of each were treated with individual sporangia as experimental units.

3.3.5 Embryo Culture

An experiment was initiated to determine if germination inhibitors could be leached from excised embryos. They were excised following

the techniques of LePage (1968), LePage-Degivry (1970, 1973ab) and LePage-Degivry and Garello (1973) for Taxus baccata. Seeds, which had been warm stratified for 2 months followed by 2 months of cold stratification were surface sterilized in 20% Clorox followed by 3 sterile water rinses. Embryos, 2-5 mm long, were aseptically excised and further disinfested in 5% Clorox for 10 minutes, followed by 3 rinses in sterile deionized water. The small embryos could be most easily handled with a bacterial transfer loop. Excised embryos were cultured in 20 ml liquid Heller's medium as described for Taxus (Sect. 3.2.6). Excised embryos were exposed to the 3 treatment regimes described for Taxus floridana (3.2.5). Individual embryos were used as experimental units with 5 embryos per treatment.

3.3.6 Effect of Cytokinin Sprays on Stock Plants

Torreya seedling stock plants were sprayed weekly for 4 weeks with 2 concentrations of 2 cytokinins (BA and 2-i-P at 100 mg/l and 200 mg/l plus 5 drops Tween-20 per liter). These treatments were suggested by Dr. M. A. El-Nil (1983, per. com.). Plants were sprayed until run off. Only 5 one-year old seedlings were available for experimentation, limiting the treatments to only 1 plant per treatment. A single control plant was sprayed weekly with the same 10% EtOH carrier solution as the cytokinins. All developing basal and axillary shoots were removed and placed in culture. Explants consisted of shoot-tips and stem sections 1-2 cm in length. They were cultured on solidified WPM (0.8% agar) in 25x150 ml Pyrex test tubes with 4 concentrations of growth regulators in the medium: (1) WPM with 1 mg/l BA and 0.01 mg/l NAA, (2) WPM with 10 mg/l BA, (3) WPM with 1 mg/l 2-i-P, and (4) WPM with 10 mg/l 2-i-P.

Explants were cultured at 25 C (+/- 5 C), and illuminated 50 $\mu Mm^{-2}s^{-1}$ for 16 hours a day.

4.0 RESULTS

4.1 Rhododendron chapmanii

4.1.1 Disinfestation

There was an average of 55% reduction in amount of contamination (30-40%) when explants were collected from stock plants grown in a growth chamber as compared to field grown explants (Table 4.1.1). There was no difference in contamination % between the two levels of Clorox used. Contamination of explants from shade house grown plants averaged 90% while those from growth chamber plants contaminated only 35%.

Field grown material remained very difficult to disinfest. In a later experiment (Section 4.1.2), leaves were removed from growth chamber grown shoot-tips prior to Clorox treatment. Contamination in this case was reduced to only 4% (data not given), when leaves were removed from explants prior to treatment with 15% clorox for 15 minutes. For all subsequent experiments in this section, leaf removal prior to Clorox treatment virtually eliminated contamination. Subcultures from visually contamination-free cultures remained clear and presumably without contamination.

4.1.2 Selection of Growth Regulator Levels

There was a difference between basal medium without cytokinins vs all levels of 2-i-P tested, but no differences between the 2-i-P levels (Table 4.1.2). There was a significant reduction in the number of leaves formed when with 2-i-P levels greater than 10 mg/l were used.

Table 4.1.1 Percent contamination of *in vitro* cultured *Rhododendron chapmanii* shoot-tips grown in shade house and growth chambers

Treatment		Contamination %	
Time (min)	Clorox (%)	Chamber	Field
15	15	30	100
15	20	40	80

There were no differences between the total number of leaves and shoots produced for the 2-i-P and IAA combinations (Table 4.1.3). Differences between IAA means were significant at the 0.10 level, however, inclusion of IAA in the medium generally resulted in declining numbers of shoots and leaves.

Optimal shoot and leaf production occurred between 5-10 mg/l 2-i-P. With higher 2-i-P levels, axillary shoots remained short and elongated much more slowly than plantlets cultured on 10 mg/l 2-i-P.

4.1.3 Stage II Subcultures

Following initial SII culture in test tubes, the medium in all additional multiplication cycles contained adenine sulfate (80 mg/l) and explants were established in small baby food jars. Basal nodes and especially those embedded in the agar produced abundant axillary shoots. When explants were cultured upright in individual test tubes on WPM with 10 mg/l 2-i-P and no adenine sulfate (Fig. 4.1) an average 5.8 shoots developed per explant in 6-8 weeks. For additional SII subcultures, increase in shoots was especially high when the explants were placed horizonally and pushed slightly into the medium (Fig. 4.1.5). This also resulted in more uniform elongation of shoots.

Each additional multiplication cycle for the next three SII subcultures in baby food jars averaged a 7.6 fold increase in number of shoots, however, this multiplication rate decreased to 5.0 during the fourth subculture (Fig. 4.1).

4.1.4 Rooting and establishment

Preliminary rooting attempts using an agar substrate were unsuccessful. In a few cases where cultures were left undisturbed for 4

Table 4.1.2 Growth response of *in vitro* cultured *Rhododendron chapmanii* shoot-tips cultured on Woody Plant Medium with 5 levels of the cytokinin, 2-i-P

2-i-P concentration (mg/l)	No. expanding axillary buds	Mean no. leaves
0	1.4a[Y]	8.4b[Z]
5	3.0b	13.7a
10	3.6b	16.6a
15	2.2b	7.3b
20	2.2b	7.6b

[Y]Means in a colomn followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

[Z]Means in a column followed by the same letter are not significantly different at the 10% level by Duncan's Multiple Range Test.

Table 4.1.3 Growth response of *in vitro* cultured *Rhododendron chapmanii* grown on a 3x3 factorial combination of auxin (IAA) and cytokinin (2-i-P)

2-i-P (mg/l)	IAA (mg/l)		
	0	1	2
	Total No. of Leaves		
5	38.9a[Z]	35.2a	30.6a
10	37.2a	38.4a	39.4a
15	38.2a	34.6a	30.0a
	Ave. No. Shoots Greater than 5 mm		
5	3.2a[Z]	2.8a	2.8a
10	2.8a	2.6a	2.6a
15	3.6a	2.5a	1.6a

[Z]Means in a column and row followed by the same letter are not significantly different at the 1% level using Duncan's Multiple Range Test.

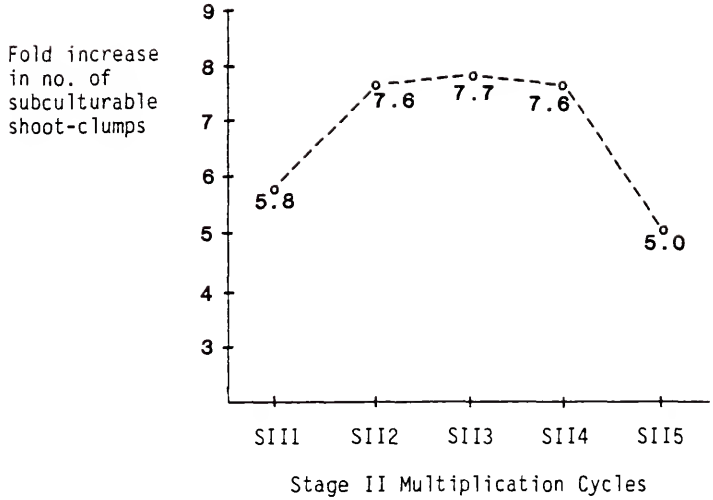

Figure 4.1 Multiplication rates of *in vitro* cultured *Rhododendron chapmanii* during initial and 4 additional SII subcultures.

to 6 months, roots appeared spontaneously above the agar surface. Also, the extremely fine "hair roots" characteristic of rhododendrons did not develop properly in poorly aerated substrates.

Direct sticking of microcuttings into 1:1:1 (v:v:v) Canadian peat:vermiculite:perlite resulted in well-rooted plantlets within 4 to 6 weeks. Treatment of microcuttings before sticking with 1000 mg/l IBA (dissolved in 10% EtOH) resulted in improved survival and rooting (Table 4.1.4).

A quick dip treatment (5 seconds) with 1000 mg/l IBA resulted in greatest survival and approximately 60% rooting (Table 4.1.4). Treatment with 1000 mg/l of IBA in talc resulted in poorer rooting and survival than controls. Survival improved slightly with more careful observation and daily misting with deionized water. Best survival to date has been 75-80% (data not given).

Table 4.1.5 summarizes the micropropagation staging system developed for R. chapmanii. With this rate of multiplication and rooting, it can be conservatively estimated that over 10,000 rooted plantlets could be produced from a single shoot-tip in one year (1 x 5.8 x 7.6 x 7.6 x 7.6 = 19350 x 60% rooting = 11610 plantlets in 9.5 months + 2 1/2 months additional growth).

4.1.5 Colonization with Ericoid Mycorrhizae

Inoculation of rooted plantlets with the ericoid mycorrhizae, Pezizella ericae, resulted in colonization in two potting media, MM500 and FMC:CP. There were considerable differences (PF > 0.0001) between media for height of main stem, total stem lengths, number of leaves, and main stem caliper after 12 weeks of growth in the greenhouse (Table 4.1.5). Differences in number of stems was significant at the .05 level.

Table 4.1.4 Survival and rooting response of microcuttings of *Rhododendron chapmanii* following treatment with auxins from 2 sources

Treatment	Survival and Rooting %
none	26.0[Z]
1000 mg/l IBA (in talc)	19.8
1000 mg/l IBA (liquid-dip)	59.4

[Z]Percent survival and rooting were significantly different at the 1% level using analysis of catagorical data.

Table 4.1.5 Summary of micropropagation system developed for Rhododendron chapmanii

STAGE	EXPLANT	DURATION (weeks)	RATE	MEDIA[X]	GROWTH[Y] REG.	CULTURE[Z] CONDITIONS
SI	shoot-tips 3-5 cm	6-8	5.8x	WPM	10 mg/l 2-i-P	20 $\mu Mm^{-2}s^{-1}$ 25 ± 5 C
SII	axillary shoot-clumps	6-8	7.6x	WPM	10 mg/l 2-i-P	20 $\mu Mm^{-2}s^{-1}$ 25 ± 5 C
SIV	axillary shoots (>15mm)	4-6	80%	1:1:1 Rooting Medium	Quick-dip 1000 mg/l IBA	90 $\mu Mm^{-2}s^{-1}$ 25 ± 5 C in high humidity tent

[X]Medium WPM = Woody Plant Medium

[Y]Growth Regulators

[Z]Culture conditions--light intensity in $\mu Mm^{-2}s^{-1}$
light duration 16 hrs light/ 8 hrs dark
temperature in degrees C

Table 4.1.6 Response of Rhododendron chapmanii plantlets produced in vitro to 2 establishment media and inoculation with or without ericoid mycorrhizae

Media[V]	Trt.[W]	Plt.[X] Surv. %	Ht.[Y] Main Stem (mm)	No.[Z] Stems	Total[Y] Stem Length (mm)	No.[Y] Lvs	Main[Y] Stem Caliper (mm)	%[X] Col.
M500	+M	91.7a	67.0a	1.77a	96.1a	23.6a	0.98a	61.4a
M500	-M	95.8a	60.8a	2.14a	98.5a	26.7a	1.0a	0
FMC:CP	+M	80.9a	28.3b	1.40b	40.0b	14.9b	0.35b	41.1a
FMC:CP	-M	65.3a	23.0b	1.18b	34.1b	12.8b	0.28b	0

[V]Media M500 = MetroMix-500
FMC:CP = Fired montmorillinite clay:Canadian peat

[W]Trt. = mycorrhizal treatment +M = with inoculation
-M = without inoculation

Plt. surv. % = Plantlet survival %

[X]Means in a column followed by the same letter are not significantly different at the 5% level by analysis of catagorical data.

[Y]Means in a column followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test.

[Z]Means in a column followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Plantlets grown in the less fertile FMC:CP potting mix were consistantly smaller regardless of colonization.

4.2 Taxus floridana

4.2.1. Conventional Propagation by Cuttings

Conventional vegetative propagation resulted in rooting within 120 days under mist (Table 4.2.1). Cuttings in the "mixed clones" group were collected from smaller and younger trees 2-4 m tall. Cuttings from the younger mixed clones rooted at better (best 67%) and with greater number of roots. The best rooting percentage from the older Champion tree was 40% but with fewer roots initiated and of shorter length than from the younger clones.

4.2.2 Micropropagation of Taxus Shoot-tips

The response of shoot-tips grown for 6 months on 4 different media supplemental with 0.5 mg/l IAA is shown in Table 4.2.2. There were no difference in shoot size, average number of shoots produced or length of new growth. WPM was chosen for additional experiments primarily due to the better color quality and general overall appearance of the explants. Explants on WPM or 1/2WPM were light green, the normal color of new growth on whole plants.

4.2.3 Culture of Expanding Vegetative Shoot-tips

Culture of expanding vegetative shoot-tips collected 3 successive weeks before bud break resulted in either death of the explant, contamination of cultures or no growth. Shoot-tips were very small (2-3 mm), were not easily dissected and may have been damaged by disinfestation treatments or by removal of outer bud scales.

Table 4.2.1 Rooting response of *Taxus floridana* cuttings from 2 aged groups of plants to 3 levels of the auxin, IBA

IBA (mg/l)	Rooting %	Mean root no.	Mean root length (cm)	Total root length (cm)
		Champion Tree		
2000	0	0	0	0
4000	40	4	1.0	4.0
8000	40	5	1.5	3.3
		Mixed Clones		
2000	67	4	1.0	4.0
4000	33	4	2.0	8.0
8000	25	14	3.0	42.0

Table 4.2.2 *In vitro* growth response of *T. floridana* shoot-tips on 4 nutrient media

Medium	Size (mm)	Mean no. shoots	Color	Length new growth (mm)
WPM[Z]	70.0a[Y]	1.2a	LG[X]	17.5a
1/2WPM	42.0a	2.4a	LG	5.7a
LS	61.2a	2.8a	BR	21.2a
1/2LS	63.0a	1.4a	LG/BR	30.0a

[Z]WPM = Woody Plant Medium
1/2WPM = 1/2 strength macronutrients of Woody Plant Media
LS = Linsmaier and Skoog Medium
1/2LS = 1/2 strength macronutrients of Linsmaier and Skoog

[Y]Means in a column followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test.

[X]Color Key LG = Light Green BR = Brown

4.2.4 Culture of Microsporangia

Culture of expanding microsporangia was attempted successive for 3 weeks preceding budbreak. Microsporangia were very small (2-3mm) and difficult to excise. Clean cultures could be consistently obtained, but no further development was observed. Explants treated with chemicals or water soak did not develop further in culture and no differences were noted between treatments.

4.2.5 Embryo culture

Attempts to culture excised embryos were unsuccessful. None of the 3 treatments were successful in promoting germination. Embryos which showed no signs of growth after 1 month on solidified Heller's medium with GA_3 or following removal from cold treatment were recultured into a second cycle of fresh liquid medium. After 3 weeks in liquid medium, these embryos were again transferred to solidified medium with GA_3, but no growth occurred.

4.3 Torreya taxifolia

4.3.1 Conventional Propagation by Cuttings

Rooting results are summarized in Table 4.3.1. The greatest percent rooting (56%) occurred with 8000 mg/l IBA. The number and average length of roots with this treatment were substantually less than those produced with 4000 mg/l IBA treatment. Cuttings treated with 4000 mg/l IBA rooted at a low percentage (20%) but produced more numerous roots with greater average and total length.

Table 4.3.1 Rooting response of cuttings of *Torreya taxifolia* treated with 3 levels of the auxin, IBA

IBA Treatment (mg/l)	Rooting %	Mean no. roots	Mean length roots (cm)
2000	14	3.5	1.0
4000	20	18.0	2.5
8000	56	4.5	1.5

4.3.2 Micropropagation of Shoot-tips

The response of shoot-tips collected from lateral branches of mature trees (est. 15-20 years old) in culture is summarized in Table 4.3.2. The color and apparent health of explants cultured on WPM or 1/2WPM were superior to LS or 1/2LS, being darker green.

In a second experiment, shoot-tips collected in October and grown on 3 nutrient media each with 5 levels of BA was recorded after 2 months in culture (Table 4.3.3). Little or no growth occurred on either LS or 1/2LS. A few axillary shoots developed on WPM at low to medium levels of BA (0.5-1.0 mg/l) but declined with higher levels.

4.3.3 Culture of Expanding Vegetative Buds

All cultures of expanding buds were either contaminated or were killed by disinfestation procedures. Contamination continues to be a serious problem from field grown plants but is much less of a problem when stock plants are grown inside with lower light intensities and lower night temperatures.

4.3.4 Culture of Microsporangia and Megasporangia

Most of these cultures became contaminated or were killed by disinfestation procedures. Microsporangia were sufficiently large enough to be handled and were plentiful in early spring.

4.3.5 Embryo culture

Embryo culture of T. taxifolia also proved unsuccessful. Although embryos could be easily disinfested with treatment with 10% Clorox following aseptic excision, they did not generally respond to the treatment used successfully for Taxus baccata embryos (LaPage, 1968). In 1 case among 10 attempts, the cotyledons parted and appeared to

Table 4.3.2 Growth response of shoot-tips of T. taxifolia on 4 nutrient media

Medium	Mean size (mm)	Mean no. shoots	Color	Length new growth (mm)
WPM[Z]	44.5ab[Y]	2.0a	DG[X]	20.5a
1/2WPM	18.7b	2.1a	LG	9.4a
LS	56.3a	1.6a	YG	38.2a
1/2LS	42.8ab	2.0a	LG to YG	28.9a

[Z]WPM = Woody Plant Medium
1/2WPM = 1/2 strength macronutrients Woody Plant Medium
LS = Linsmaier and Skoog Medium
1/2LS = 1/2 strength macronutrients Linsmaier and Skoog Medium

[Y]Means in a column followed by the same letter are not significantly different at the 5% level using Duncan's Multiple Range Test.

[X]Color Key DG = Dark Green, LG = Light Green, YG = Yellow Green

Table 4.3.3 Average number of axillary shoots of *Torreya taxifolia* developing *in vitro* on 3 nutrient media and 5 concentrations of the cytokinin, BA

BA (mg/l)	No. axillary shoots		
	WPM[Z]	LS	1/2LS
0	1.0a[Y]	1.0	1.0
0.5	1.3a	0	0
1.0	1.7a	0	0
5.0	1.0a	0	0
10.0	0.3a	0	0

[Z]WPM = Woody Plant Medium
LS = Linsmaier and Skoog Medium
1/2LS = 1/2 strength macronutrients of Linsmaier and Skoog Medium

[Y]Means in a column followed by the same letter are not significantly different at the 5% level by analysis of catagorical data.

begin to germinate. When transferred to solid medium with 1.0 mg/l GA_3, a shoot-tip appeared but degenerated shortly thereafter.

4.3.6 Cytokinin Sprayed Seedlings

Only five seedlings were available for investigation so each treatment was applied to a single seedling. Treatments were based on suggestions from Dr. M. A. El-Nil (1982, per. com.). Stock plants produced numerous basal shoots following treatment with cytokinin sprays. The control plant produced a few basal shoots which could be cultured. Excised basal shoots developed axillary bud masses after 30 days when cultured on media with cytokinins. Best results occurred when explants were collected from plants sprayed weekly with 1000 mg/l BA and then cultured on medium with 1 mg/l BA and 0.01 mg/l NAA. Higher concentrations resulted in much smaller masses which did not enlarge much until transferred to media with lower or no cytokinin. Culture on media with 2-i-P resulted in little development until these were transferred to WPM medium with 1.0 mg/l BA and 0.01 mg/l NAA after which bud elongation occurred and occasional multiple shoots were observed.

Two morphologically different types of axillary bud development were observed. In the first case, normally shaped axillary shoots elongated with additional axillary shoot development from the leaf axils at the base of the cultured buds. The second type of development involved the development of compact "bud-masses" consisting of small, tightly appressed scale-like leaves with the ultimate emergence of multiple shoots after 3 months in culture. When buds from the same treatments were subcultured to the same or lower cytokinin medium, the buds on the lower cytokinin medium always began to show signs of growth

sooner. These buds were transferred to fresh medium but have since degenerated. No rooted plantlets were obtained.

5.0 DISCUSSION

5.1 Rhododendron chapmanii

Conventional propagation using cuttings was attempted but was unsuccessful due to failure of misting equipment. The experiment was not repeated because of insufficient cutting material and the development of a highly successful micropropagation system using shoot-tips. Microcuttings could be rooted and established with 60% or greater survival. Conventional propagation of evergreen Rhododendron species usually involves softwood cuttings (Hartman and Kester, 1981) or leafly hardwood cuttings collected in the fall which are treated with concentrated NaOH and IBA solution (Gray, 1974).

Micropropagation of R. chapmanii was highly successful using shoot-tip explants and modifying the techniques of Anderson (1975) and the medium of McCown and Lloyd (1981). Technique improvements included decapitation of shoot apex and the horizontal placement of the explants in the medium so that most nodes were slightly embedded in it. Horizontal placement of shoots into multiplication medium greatly increased the number and uniformity of shoots produced during each multiplication cycle (SII). The addition of 80-100 mg/l adenine sulfate improved the number and vigor of shoots produced. BA proved to be phytotoxic to R. chapmanii as has been previously reported for most Rhododendron species (Anderson, 1975; Lloyd and McCown, 1980; and McCown and Lloyd, 1983). Decapitated shoot-clump bases could be recultured as suggested by Strode (1979), however; this technique did

not result in as rapid an increase in shoots per cycle as when elongated shoots were laid horizontally on and pressed slightly into the medium.

The use of higher levels of 2-i-P (greater than 15-20 mg/l) resulted in a greater number of shoots, however, these grew slower and took 2 to 3 months vs 6 to 8 weeks to reach a subculturable size. The reduced stem elongation could have been due to high cytokinin levels which inhibited shoot elongation. It is also probable that the amounts of nutrients available to the numerous shoots were limiting and that culture on a greater volume of medium or more frequent transfers might hasten growth and multiplication. Ma and Wang (1977) suggested the use of short term, agitated liquid culture to increase survivability and multiplication.

Using the micropropagation system shown in Table 4.1.7, one can calculate that over 11,000 plants could be obtained from 1 shoot-tip in 9.5 months. With 2.5 months additional growth in the greenhouse, these plantlets would be large enough to be transplanted to larger containers. Alternately, a square meter of lighted shelf space could produce 15,-16,000 SII microcuttings in 6 to 8 weeks (225 bottles/M^2 X 60-70 microcutting/bottle).

Although large numbers of shoots could be produced and routinely subcultured, a 60-80% survival rate on transfer to soil is still inefficient. All attempts to root R. chapmanii in Stage III culture have failed even though several media were investigated along with a range of auxin treatments. This differs from reports of 80% rooting in SIII by Ma and Wang (1977) and Strode et al. (1978). Rhododendron roots are extremely fine and thread-like and do not appear to grow easily into agar media.

Best rooting and survival of microcuttings of R. chapmanii occurred when explants consisting of 3 to 5 shoots were direct-stuck in rooting medium with at least 30% Canadian peat moss. This agrees with the findings of Anderson (1978), Lloyd and McCown (1980) and Wong (1981). Direct rooting of microcuttings vs use of SIII cultures results in considerable savings in labor since at this stage explants must be handled individually only once for direct rooting vs twice when SIII cultures are used (Strode et al., 1979; Wong, 1981).

Microcuttings were rooted in flats with clear plastic covers or covered with inverted translucent flats to prevent drying. If covered flats were maintained under light levels greater that 100 $\mu Mm^{-2}sec^{-1}$, excess heating occurred and plantlets declined and fungal contamination was greater. If microcuttings were initially maintained under lower light intensities (e.g. 25 $\mu Mm^{-2}s^{-1}$) for the first 2 weeks then maintained at 90 $\mu Mm^{-2}s^{-1}$, better survival and rooting occurred. Placement of microcuttings directly under intermittent mist resulted in poor rooting and survival and the development of chlorotic leaves due to either excessive leaching or possibly due to poor root-zone aeration. The use of fog systems or high humidity tents should be further investigated.

In this investigation, the use of rooting compounds on microcuttings resulted in better rooting and survival when a 1000 mg/l solution of IBA was applied as a 5 second quick-dip. Treatment with the same concentration of IBA (in talc) resulted in lower survival and rooting. Wong (1981) mentioned that the use of hormone powder increased proliferation of roots but not time to root. He also mentioned that the economic advantages of such treatments were yet to be determined.

Poor rooting and survival may be attributed to the small size and succulence of the microcuttings. Commercial producers of micropropagated rhododendrons (Briggs, per. com.) use lower cytokinin levels which result in reduced multiplication rates but these microcuttings are much larger and hardier. Determination of optimal multiplication rates should not necessarily be based on optimal shoot numbers, but must include duration of cycle times, labor and space requirements, rooting survival rates and duration, as well as post-transplanting growth rates.

When shoot-clumps containing 3 to 5 shoots are used for rooting, better survival and rooting occurs, possibly due to reduced handling shock, greater carbohydrate (or minerals, etc.) reserves within each propagule, and due to the fact that if one shoot dies, another shoot is available for replacement. Once rooted and transplanted to containers, the plants from shoot-clumps form a multi-stem liner which is more desirable than a single-stem plant.

Root emergence in rooting medium became visible after 3 to 4 weeks, and plants were well rooted by 6 weeks. After root intiation, the explants began to produce new leaves which were slightly larger and less succulent. After these new leaves were observed, the plantlets generally survived transplanting to cell-packs and readily became adapted to the greenhouse environment. Rooted plantlets were hardened off by covering with 80% shade cloth for a week, followed by a week with 40% shade, after which all coverings were removed.

Colonization of micropropagated plantlets with the ericoid mycorrhizae, Pezizella ericae, did not increase survival or growth responses when plantlets were grown for 12 weeks in the greenhouse.

Differences between the media used apparently masked any differences due to colonization after 12 weeks of growth. Additional growth in 4-liter containers is presently under observation and there appears to be greater bud break and growth on colonized plantlets 2 weeks after new growth began. Final growth measurements will be made after a month of additional growth. Mycorrhizal colonization of Ericaceae species is known to increase the plants' ability to take up nitrogen and phosphorous, as well as, tolerance to heavy metal toxicity (Stribley and Read, 1974, 1975; Duddridge and Read, 1982)

5.2 Taxus floridana

Using cuttings from mixed aged trees, conventional propagation in a 1:1 (v:v) Canadian peat:perlite rooting medium was successful, resulting in 40-67% rooting in 120 days. Larger cuttings (15-20 cm vs 10 cm) rooted more quickly and vigorously. This agrees with the findings of commercial propagators (Keen, 1954). There was an obvious difference in rooting percentages between plants of different ages. Cuttings from the Champion tree rooted poorly (0-40%) while cuttings from much younger trees rooted 25-67%. According to Sargent (1947), 275 annual growth rings were counted from a 9.5 cm diameter T. floridana trunk section suggesting that the 16 cm diameter Champion from which the "older" cuttings were collected was considerably older.

Cuttings from young trees root at high rates. Lower branches of are commonly observed in the wild to naturally layer when branches remain in contact with the ground. However, lateral cuttings retain their plagiotropic growth habit even after 2 years in containers, generally producing a 15-20 cm tall ground cover. Keen (1954) noted that for most Taxus species and cultivars, orthotropic shoots are

necessary for producing excurrent trees while lateral cuttings generally produce only spreading shrubs. Keen also indicated that even very large cuttings (up to 65 cm) can be successfully rooted, resulting in a salable plant in a shorter time.

Attempts to root in 100% coarse, builder's sand under intermittent mist resulted in basal rotting , however, rooting often occurred above the media especially from abaxial side of lateral branches (data not given). At first it was assumed that the 20% alcohol in the IBA quick-dip solution damaged the tissue. However, in later experiments using better drained rooting medium with quick-dip solution, no basal rotting occurred. Apparently the builders sand used initially was not as well drained as the sandblasting sand normally used for rooting. The occurrence of high moisture conditions and poor drainage most likely caused the observed basal rotting.

Continued growth of cuttings occurred best in well drained media (1:1:1 v:v:v) course sand:Canadian peat:loamy soil) and under 80% vs 40% or 60% shade.

Micropropagation of shoot-tips did not result in prolific stimulation of axillary buds. Using shoot-tip explants from lateral and orthotropic basal branches, only 2 to 4 axillary shoots elongated when placed in culture. These shoots appeared in specific positions along the shoot-tip suggesting that they were preformed and part of the normal branching pattern of the whole plant. McCown and Amos (1982) reported successful shoot-tip propagation of Taxus spp. but did not indicate the numbers of shoots obtained. They also noted in their findings that gymnosperms are generally more sensitive to low levels of cytokinins.

The use of more juvenile tissues such as embryos or seedlings as explants probably would result in increased shoot numbers. Dr. A. M. El-Nil (per. com., 1982) recommended pretreatment of conifer seedlings with cytokinin sprays as a more effective means of inducing multiple shoots from conifers. This treatment was not investigated for Taxus floridana due to the lack of seedling material although this was a promising technique with Torreya taxifolia.

Wounded mature Taxus floridana trees in the wild have been observed to produce masses of short shoots, most with spiral phyllotaxy and apparently orthotropic growth. Culture of explants from shoots arising from multiple shoots from "burls" has been successful for Sequoia sempervirens (Ball, 1978; Boulay, 1979), and Pinus eldarica (Herrera and Phillips, 1984). Attempts should be made to obtain permission to collect seedlings and "burl" material from the wild.

Micropropagation of "embryonic shoots" from swelling buds according to the techniques of Bonga (1977,1981) was unsuccessful due to high rates of contamination and the difficulty in excising the small buds (1-3mm). A few aseptic cultures were established but buds did not continue to expand and eventually darkened and died.

Culture of microsporangia was also attempted since male plants were available. These were also very small (2-3mm) and difficult to excise. Uncontaminated cultures were easily obtained but did not grow in culture. No megasporangia were available locally and this approach was not pursued since morphogenesis from immature female cones has been reported only rarely with cultures of Pinus mugo and Picea abies (Bonga, 1981).

Attempts to overcome seed dormancy by excision of embryos and culture in liquid medium according to the techniques of LePage-Degivry (1968,1970,1973abc) were unsuccessful. This technique failed even when embryos were leached in 2 cycles of liquid culture followed by placement on media with GA_3 or when cultures were chilled for 2 months at 5 C. Seed availability is very sporatic with reports of other species of Taxus alternate bearing with +/- 7 year cycles (Schopmeyer, 1974) so further experiments will be limited.

Seeds of Taxus floridana were given 1 or 2 months warm stratification before excision as well as treatment with 1 month warm followed by 2 months cold stratification before excision. None of the embryos of Taxus floridana germinated. Presumably, they require a longer warm stratification period than 2 months since embryos did not respond to leaching treatment. LePage (1968) indicates that the seeds were collected from mature trees of Taxus baccata but did not indicate the time period before excision. Schropmeyer (1974) mentioned differences in duration of warm stratification between species and that the International Seed Testing Association recommended, in general, stratification periods for 90-210 days at 16 C, followed by 60-120 days at 2-5 C.

5.3 Torreya taxifolia

Conventional propagation was successful with 20-56% rooting from mixed cuttings from mature and juvenile plants. Cuttings from different aged plants were combined since only a limited number of cuttings were available. Optimal rooting occurred when 4000-8000 mg/l IBA was used. Rooted plagiotropic branches may be useful as seed or pollen sources so that intercrossing of the remaining, limited gene pool

could be accomplished by collecting cuttings from as many plants as practical. Establishing seed nurseries of these cuttings would allow a practical means of preserving the remaining genetic diversity under controlled management where environmental stresses could be reduced and fungicides used more efficiently.

Micropropagation using shoot-tips did not result in mass proliferation of shoots. Commonly 2 to 4 shoots could be stimulated to elongate in culture but these appeared to be preformed buds of the normal branching patterns. These lateral shoots commonly grew horizonally and then geotropically into the medium. Occasionally these explants rooted in culture, especially after 4 to 6 months on the same medium. They however did not survive transfer to soil. The use of more juvenile tissues such as seedlings and dormant buds located at the base of the trunk may increase the possibility of stimulating development of orthotropic shoots. Ball (1978) was successful in obtaining multiple shoots from "burl" shoots of Sequoia sempervirens, however, there has only been limited investigation using similar explants from Torreya due to very limited availability of this material. The formation of numerous shoots from burls, such as occurs occasionally with wounded Taxus floridana, has not been observed although occasionally 3 or 4 ortotrophic shoots have been observed arising from a common point at the base of a trunk.

Contamination of shoot-tip cultures continues to hamper investigation. In cooperation with Mr. N. El-Ghol, Plant Pathologist with the Florida Division of Plant Industry, 3 systemic fungi were isolated (Phyllosticta spp., Colletotrichum gloeosporioides and Guignardia sp.). Establishment of stock plants in growth chambers

greatly reduced the amount of contamination. Presumably, the combination of low night temperatures and reduced humidity levels, may have been less favorable for growth of the pathogens. Recently, the weekly treatment over 4 weeks of these stock plants with the fungicide, Zyban, a broad spectrum systemic and contact fungicide, has been effective in eliminating one of these pathogens, Phyllasticta. Soaking of shoot-tip explants in fungicide solutions before clorox treatment was ineffective in reducing contamination. Soaking for 15, 30, 60, 120 and 240 minutes actually increased fungal contamination, possibly due to increased spread of hyphae. A 24 hour soak in Zyban reduced fungal contamination to 15%, however, this resulted in an increase in bacterial contamination.

Cultures of expanding "embryonic shoots" [vernalized shoot buds with scale leaves removed, according to the techniques of Bonga (1977,1981)] did not produce multiple shoots. These "embryonic shoots" were large enough to be easily excised after the beginning of visible bud enlargement, especially 1 or 2 weeks before actual bud break. However, all bud cultures were either lost to contamination or were killed by disinfestation procedures. Fungicide treatment of mature trees may allow future investigation into this technique.

Culture of micro- and megasporangia was investigated. Although these explants could be more readily established in culture without high levels of contamination, none grew. Microsporangia are large and numerous on male trees, however, these are only available in early spring and only a few species have been reported to produce multiple shoots from these structures (Bonga 1977,1981).

The pretreatment of seedlings with cytokinin sprays before placing shoots in culture appears to offer one of the better chances of obtaining multiple shoots. Seedling stock plants produce multiple orthotropic basal shoots when sprayed weekly for 4 weeks with cytokinins, BA or 2-i-P. BA appears to be slighlty more effective. Following excision, additional orthotropic shoots continued to develop over at least a several month period. When these shoot-tips and nodal sections are cultured on media with low cytokinins, numerous axillary buds and "bud-masses" developed. Further investigations will be necessary to optimize this technique and to develop entire plantlets.

6.0 CONCLUSIONS

The primary objective of this research was to investigate clonal propagation techniques for 3 endangered native ornamental plants. Conventional cutting propagation of Torreya and Taxus was investigated and compared to micropropagation using shoot-tip cultures and embryo culture. Clonal propagation using cuttings was successful for Taxus floridana and Torreya taxifolia with maximum rooting of cuttings collected during winter and treated with IBA. Younger trees rooted at higher rates than larger and older trees. However, cutting material is limited by scarcity of plants and plagiotrophic growth when lateral branches are used. Rooting procedures for Rhododendron chapmanii cuttings remain undetermined due to limited experimental material.

A very successful micropropagation system with commercial potential has been developed for R. chapmanii. Large numbers of plantlets were produced from a few shoot-tips and with minimal impact on the remaining natural populations. It has been estimated that over 11,000 rooted plantlets could be produced from a single shoot-tip in 1 year. Colonization of rooted plantlets with the ericoid mycorrhizae, Pezizella ericae, did not result in superior survival and growth when grown for 12 weeks in a greenhouse. Colonization of plantlets appears to increase bud break and growth of over-wintered containerized plantlets, however, additional time will be necessary to determine if this trend continues. The present micropropagation system was designed to maintain clonal characteristics.

Micropropagation techniques were only partially successful for Taxus floridana and Torreya taxifolia. Shoot-tip cultures and cultured buds produced only limited numbers of shoots apparently from preformed axillary meristems. Culture of "embryonic shoots" and micro- and megasporangia were unsuccessful in stimulating organogenesis. Embryo culture for both these species was unsuccessful presumably due to insufficient chilling. Pretreatment of stock plants before in vitro culture with growth regulators appears to be a promising technique and deserves further investigation.

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BIOGRAPHICAL SKETCH

Lee Roy Barnes, Jr., was born in Durham, North Carolina. Following graduation from high school, he attended and graduated from North Carolina State University with a double major in biological science and botany in May 1976. Transferring to the Department of Horticultural Science, he completed the Master of Science degree in December, 1978. His thesis was titled "In vitro propagation of watermelon" (Drs. Fred Cochran and Ralph Mott, co-chairmen). Following a two year leave of work experience and travel, he entered the University of Florida, Department of Ornamental Horticulture, in the Fall of 1981 interested in conventional propagation and micropropagation of native plants, especially rare and endangered species.

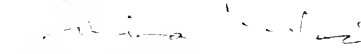
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles R. Johnson, Chairman
Professor or Ornamental
Horticulture

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas J. Sheehan
Professor of Ornamental
Horticulture

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Indra K. Vasil
Graduate Research Professor
of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Mike J. Young
Associate Professor of
Fruit Crops

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1985

Dean, College of Agriculture

Dean, Graduate School