IN VITRO PROPAGATION OF GEORGIA PLUME, *ELLIOTTIA RACEMOSA,* A THREATENED GEORGIA ENDEMIC

by

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ABSTRACT

Elliottia racemosa, commonly called Georgia plume, is one of the rarest native plants in Georgia. Tissue culture protocols were developed for propagation and conservation. A series of studies evaluated disinfestation treatments, explant types and age, light condition, and plant growth regulators. Adventitious shoots were induced on a medium supplemented with 10 μM TDZ + 5 μM IAA, with transferred elongation of shoots after transfer to media containing 2iP. High rooting frequencies were obtained under in vitro and ex vitro conditions with over 85% survival of plantlets transferred to greenhouse conditions. Tissue culture appears to be a promising approach for the propagation and conservation of this rare and threatened plant. Morphological and histological evaluations of cultures during induction and development were conducted using light and scanning electron microscopy.

INDEX WORDS: *Elliottia racemosa*, TDZ, 2iP, clonal propagation, conservation, histology

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CHAPTER 1

INTRODUCTION

Elliottia racemosa classified within Ericaceae family is one of the rarest native plants in Georgia, and is a state threatened shrub (Alfred, 1969). ***E. racemosa***, commonly called Georgia plume, derives its name from the beautiful plume-like clusters of slightly aromatic white flowers that appear in early summer (Elliott, 1971). Georgia plume is a beautiful deciduous shrub of eight to twenty feet tall, with leaves that are elliptic, alternate, 1 to 5 inches long by 0.5 to 2 inches wide, and tapering at both ends. Flowers have four or five curved white petals each growing up to 14 mm long in, which appear between the middle of June and end of July (Patrick *et al*., 1995). The flowers develop into small round capsules which can contain up to about 40 seeds.

Georgia plume was discovered by William Bartram, and named by Henry Muhlenberg (Ewan, 1968). The plant has been found in southwestern South Carolina, but it is thought to be from cultivated plants (Godt and Hamrick, 1999). Even though Georgia plume has been cultivated since 1813, this species has never become widely known or studied. Today, ***E. racemosa*** is found in only about three dozen locations in Georgia (Patrick *et al*., 1995). The best habitats generally seem to be sunny to partially shady conditions on well drained sand ridges, oak ridges, evergreen hammocks, acid soil, and sandstone outcrops. Even though this plant is very rare and localized, it grows in a broad variety of sandy soil conditions ranging from moist to extremely dry (Patrick *et al*., 1995). Because of decreasing native stands and difficulty of propagation, it has been considered for addition to the list of endangered species. The biological cycle and life history of Georgia plume is not well reported or understood.

Georgia plume may be rare because of reasons such as low seed set, self incompatibility, and lack of sexual recruitment caused by low viability pollen and limited numbers of clones within locations (Godt and Hamrick, 1999). Bozeman (1983) reported that natural seedset of Georgia plume is limited or nonexistent. Godt and Hamrick (1999) reported pollen viability of only 4%. However, some plants show multiple trunks owing to vegetative root sprouting following injury such as cutting or fire.

Tissue culture may be a method for the conservation and propagation of this threatened species. Within the Ericaceae family there are a number of economically important plants. This has led to the development of efficient tissue culture regeneration protocols for members including blueberry, cranberry, and rhododendron achieved via organogenesis or somatic embryogenesis. Cultures have been derived from a number of explant types including leaf tissue, shoot tips, and axillary buds. Although Georgia plume has numerous valuable horticultural attributes such as dramatic floral displays, good fall color, and cold hardiness, in vitro tissue culture of Georgia plume has not been previously reported. In vitro regeneration via organogenesis and somatic embryogenesis is a very powerful tool for clonal mass propagation.

The overall goal of this study was to develop tissue culture protocols for the propagation of *Elliottia racemosa*, Georgia plume, applicable for species conservation purposes. Objectives of the work include the development of efficient sterilization conditions for initiation of in vitro plant cultures, and the development of protocols for shoot proliferation, rooting and acclimatization of plants. Study of morphological responses during in vitro culture is also very critical points to understand regeneration pathways. Histological analysis of the developmental anatomy of adventitious shoot bud proliferation from leaf tissues was included in this study. These analyses included investigation of the location of shoot bud initiation and proliferation,

developmental timing, and morphological changes during the regeneration pathway. According to current literature, there are no reports for in vitro mass propagation of this species.

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CHAPTER 2

LITERATURE REVIEW

Propagation of Georgia plume

Published reports on the propagation of Georgia plume are very limited and are restricted to work by Fordham and colleagues (Fordham, 1969, 1991). Seed germination studies conducted by A. J. Fordham and Henry Hohman in the 1960s were recounted by Fordham in 1969. A problem encountered was that only a small percentage of seed was sound. Seed stratification removed a cold requirement, but only if given to seed treated within a few months of collection. Old seed acquired secondary dormancy and had unpredictable behavior. In 1985, Fordham determined that Georgia plume's seed dormancy mechanism required a prolonged chilling period (42-64 day) for germination (Fordham, 1991). Fordham also established shoot sprouting from root pieces. The root pieces were planted about half inch deep in sandy soil (Fordham, 1969). Data on the percentage of successful shoot sprouting and rooting were not presented, although shoots were described as acting physiologically juvenile and rooted quickly. Nevertheless, root cutting methods can be limited in the number of shoots that are produced and in the small amounts of root material available.

Tissue culture methods can be an excellent option for the study and conservation of threatened or endangered species because small pieces of tissue can be used for mass propagation without damage to the donor plant (Varadaragan, 1993). Also, a large number of plants may be produced in a given time, and little space is required for tissue culture compared with other

propagation methods (Fay, 1992). An additional rationale for establishing plants in culture is that it is possible to maintain large genotypic libraries of selected species for plant conservation.

Regeneration pathways with tissue culture

The in vitro regeneration of a plant can proceed through two pathways: somatic embryogenesis and shoot organogenesis. In vitro cultivation of the Georgia plume has not been previously reported. Shoot organogenesis is one of the major in vitro plant regeneration pathways, and has been broadly established in plant biotechnology for in vitro micropropagation and genetic transformation. Morphological and physiological phases of in vitro shoot organogenesis have been widely studied in plant tissue culture for more than 5 decades. In shoot organogenesis systems, adventitious shoots are induced first, followed by development of adventitious roots from the shoot resulting in the formation of an entire plant. The process of in vitro shoot induction and development through organogenesis has been elaborated in regards to a number of aspects including developmental biology, physiological, biochemical, and molecular phase (Hick, 1994).

Thorpe (1988) defines somatic embryogenesis (SE) as "the development of haploid or diploid cells into differentiated plants through embryo stages without the fusion of gametes". Somatic embryogenesis, which is the process by which somatic or asexual cells are induced to form embryos, occupies a distinguished position for clonal, mass propagation. Somatic embryogenesis provides an effective technique for increasing the pace of genetic improvement of important plant species in various fields including conventional breeding programs, cell biology, and molecular biology. Furthermore, somatic embryogenesis can be applied for germplasm preservation via cryopreservation of endangered species or valuable genotypes for plant breeding work.

Tissue culture in the Ericaceae

The Ericaceae family includes a number of economically important plants including blueberry, cranberry and rhododendron. This has led to the development of efficient plant regeneration protocols achieved via organogenesis from cultures derived from leaf tissue, shoot tips, or axillary buds (Table 1.1).

In blueberry, Lyrene (1980) developed a protocol of direct multiple shoot formation from shoot tips using modified Knops medium containing 300mg l^{-1} casein hydrolysate and 15mg l^{-1} 2iP. Under these conditions, 5 to 15 shoots about 4 cm long were observed after 3 to 4 months in culture. For rooting, the shoots were excised and planted into a mixture of 1 peat : 1 perlite (by volume) with commercial rooting powder, and kept under intermittent mist. Roots were well induced with these conditions in 100 % of the cultures.

Billings (1988) achieved a process to induce shoots from leaf explants of two blueberry cultivars, 'Berkeley' and 'Bluehaven' on Woody Plant Medium (WPM) (Lloyd and McCown, 1980). Twenty combinations of plant growth regulators were compared: NAA (at 0, 1, 2 and 4 μM) and 2iP (at 0, 5, 10, 15 and 20 μM). He found that combinations containing NAA generally induced callus formation and inhibited shoot induction. However, 2iP promoted shoot regeneration and inhibited callus formation. The ideal medium for shoot regeneration contained 15 μM 2iP and no NAA, where he obtained highly efficient shoot regeneration (70-100%).

In experiments by Dweikat and Lyrene (1988), adventitious shoot formation was obtained from blueberry leaf segments developed on modified Knops medium (Lyrene, 1980) supplemented with 24.6 μM 2iP. After 3 weeks in culture, the mean number of shoots per explant was 42.6 on the optimum growth regulator condition. When 2iP concentration was increased, leaf survival rate and number of shoots decreased dramatically.

Callow *et al.* (1989) obtained shoot regeneration from proliferating cultures of highbush blueberry derived from leaf explants using a half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 5, 25, 50, or 100 μM of 2iP, with best results observed on medium with 25 μM 2iP. Induction of meristematic nodules, adventitious buds, and shoots was evaluated at different explant locations such as leaf margin, midrib, wounded areas, and interveinal areas. After 5 weeks culture, wounded areas had the highest induction, i.e., 51, 83 and 73% for meristematic nodules, adventitious buds, and shoots, respectively. The shoots were easily rooted on 1 peat : 1 perlite : 1 vermiculite (by volume) medium.

A protocol of regenerating shoots from internode stem segments with two blueberry genotypes ('Northblue' and an experimental genotype from USDA) was achieved by Hruskoci and Read (1993). The explants were cultured on Zimmerman's Z-2 medium containing 25 μM TDZ or 25 μM zeatin. Generally, good shoot proliferation was obtained in both genotypes. Direct and prolific shoot regeneration was observed on medium with 25 μM zeatin, whereas on media with 25 μM TDZ, callus formation from internodes was induced, with subsequent shoot production from callus structures. However, shoot production using zeatin was preferred because it was more efficient in shoot production. The highest mean regeneration rate was obtained in both genotypes when the top two internode sections were used.

Rowland and Ogden (1992) developed an efficient shoot regeneration system from leaves of highbush blueberry. In this study, they compared three kinds of cytokinin (2iP, zeatin, and zeatin riboside) on WPM. The best result using 2iP was observed with 15 μM producing 3.1 to 3.7 shoots per section, whereas 20 μM zeatin riboside produced 20.8 shoots per section, or almost 6 fold higher numbers than 2iP medium. In the case of zeatin, 7.8, 11.8, and 12.9 shoots were observed depending on concentration (10, 20, and 30 μM zeatin, repectively).

In 2000, Cao and Hammerschlag examined the effect of age of explant, length of dark treatment, TDZ (1 or 5 μM), or zeatin riboside (20 μM), and light intensity (18 or 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for improving shoot organogenesis from leaf explants of highbush blueberry. For this experiment, five cultivars were evaluated: 'Bluecrop', 'Duke', 'Georgia Gem', 'Sierra', and 'Jersey'. Results varied with genotype. Neither age of the shoot nor time in dark treatment were significant factors for affecting shoot regeneration. Maximum numbers of regenerated shoots varied depend on cultivar, with 13.0, 13.0, 12.6, and 4.6 shoots per explant observed on medium containing zeatin riboside for cultivars, 'Duke', 'Georgia Gem', 'Sierra', and 'Jersey', respectively, under PPF (photosynthetic photon flux) of 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In the case of 'Duke', excellent regeneration rates were obtained equally on media with either zeatin riboside or 1 μM TDZ. In contrast, shoots of 'Georgia Gem' and 'Sierra' were induced well on media with zeatin riboside. Regeneration rates of 'Duke', 'Jersey', and 'Sierra' with zeatin riboside were better under a PPF of 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ versus PPF of 18 $\mu mol \cdot m^{-2} \cdot s^{-1}$, whereas regeneration of 'Duke' was inhibited by higher PPF on 5 μM TDZ. Therefore, growth regulator and the level of PPF were important factors for shoot organogenesis from leaf explants of highbush blueberry.

The effect of sucrose level on axillary shoot proliferation was studied using three blueberry cultivars (Bluecrop, Duke, and Georgia Gem) (Cao, 2003). For this study, 15, 29, 44, and 58 mM sucrose were used. The highest number of shoots for 'Duke', and 'Georgia Gem' were observed on 44 mM and 58 mM sucrose, respectively. In contrast, 'Bluecrop' did not show any significant increase upon various sucrose levels. This study showed optimum sucrose concentration differed by cultivar.

Effects of growth regulators on in vitro micropropagation were studied using nodal segments of three berry fruit species including blueberry (Gonzalez, 2000). Average number of shoots and shoot length were recorded after 30 or 60 days in culture. With blueberry, the best

multiplication was observed in WPM with 25 μM 2iP. For blackberry, the best treatments were achieved on MS medium with 4 μM BA and 0.25 μM IBA combination. For raspberry culture, MS medium with a combination of 4 μM BA + 0.25 μM IBA, or 8 μM BA + 0.25 μM IBA was used. However, both results showed no significant differences. Shoots which were at least 1 cm tall from cultures were rooted in a mixture of peat and perlite (1:1 v/v) in a mist chamber. All species rooted well with no IBA (67-96 %). When IBA was used for rooting, rooting percentage slightly decreased in blueberry and blackberry.

Qu (2000) obtained a highly efficient regeneration system using leaf explants of cranberry. He used a medium with Anderson's salts, and MS medium organics supplemented with the combination of three plant growth regulators (TDZ, 2iP and NAA) for inducing adventitious shoot regeneration. Also investigated was explant orientation (adaxial and abaxial side on the medium) for efficient shoot regeneration using five cultivars: 'Early Black', 'Pilgrim', 'Stevens', 'Ben Lear' and No. 35. As a result, the optimum combination of plant growth regulators was found to be 10 μM TDZ and 5 μM 2iP with no NAA where 11.3 shoots per explant was obtained. Even a very low level of NAA significantly reduced regeneration efficiency. Adventitious shoots were always obtained from the adaxial side of leaves. For elongation of adventitious shoots, induced shoots were transferred onto basal medium without plant growth regulators. Elongated shoots were rooted easily in either in vitro conditions or ex vitro using sphagnum moss. However, roots regenerated under ex vitro conditions were more vigorous, and lacked callus formation at the base of the cutting.

In vitro shoot propagation of nodal or shoot tip explants from cranberry cultivars were studied by Debnath (2001) using several concentrations of 2iP on modified MS medium. For this study, two cranberry cultivars ('Ben Lear' and 'Pilgrim') and three clones from natural stands were used. The best results for shoot regeneration were obtained using nodal segments on media

with 2.5-5.0 mg 2iP. In case of the three clones, the best results were observed with 2.5 $mg \cdot l^{-1}$ 2iP where 3-5 healthy axillary shoots per explant were observed. Shoots rooted well on media without plant growth regulators after 4 weeks. All five genotypes showed 95 to 100% rooting, and 85-95% survival rates were observed after transfer into soil.

An efficient shoot regeneration system from leaf explants of lingonberry (*Vaccinium vitis-idaea* L.) using several zeatin concentrations was achieved by Debnath (2002). Multiple shoots without an intermediary callus stage were induced from leaf tissues. In this study three kinds of cytokinin (zeatin, 2iP, and thidiazuron) in MS medium were compared. Optimum plant growth regulator concentration varied with cultivar, but leaf tissues greatly responded well on 5-30 μM zeatin media.

Using hypocotyl segments of several lingonberry cultivars, shoot regeneration protocols were reported (Debnath, 2003). To obtain highly regenerative callus, modified MS media containing 5-10 μM TDZ was used. A phase for shoot elongation with cultures transferred to media with 1-2 μM zeatin was needed, because shoot elongation did not occur on TDZ medium. On medium supplemented with zeatin, a maximum of 18-22 elongated shoots per clump was obtained. Further studies optimized sucrose level in regeneration studies, with approximately 20 shoots per explant induced at 20 g l^{-1} sucrose. For rooting, shoots were dipped in 0.8 % IBA, and planted into a 2 peat : 1 perlite soil mixture. The survival rate of rooted plants was 80-90%.

In 1988, Briggs conducted in vitro propagation of azaleas using TDZ and 2iP with WPM. Based on cultivars, optimum concentration varied. However, maximum numbers of useable shoots formed from stem sections on 9.8 μM 2iP or 2.3 μM TDZ. When lower concentrations of TDZ (0.05 to 1.4 μM) were used, shoot elongation was promoted, whereas shoot proliferation decreased. The potential activity of TDZ on adventitious shoot regeneration on several azalea cultivars was documented.

In the case of rhododendron, Anderson (1975) developed in vitro propagation methods and medium formulations for shoot tip cultures. For this study, modified MS medium salts were evaluated with variations made of KNO_3 concentration (0, 475, 950, 1425, and 1900 $mg \cdot l^{-1}$) and NH_4NO_3 concentrations (1600, 1800, 2000, 2200, and 2400 $mg \cdot l^{-1}$). The optimum condition was a combination with 950 $mg \cdot l^{-1}$ KNO_3 and 2000 $mg \cdot l^{-1}$ NH_4NO_3. Further evaluated were several combinations of IAA and 2iP to develop efficient shoot regeneration. The best response was with 4 $mg \cdot l^{-1}$ IAA and 15 $mg \cdot l^{-1}$ 2iP (6.2 shoots per explant).

Anderson (1984) developed a suitable medium for rhododendron multiplication by revising the Murashige-Skoog (1962) inorganic formula to improve culture conditions for rhododendron shoots. In this experiment, nitrogen was reduced from 60 to 14.7 mM, potassium was reduced from 20 to 4.7 mM, phosphorus was adjusted to 2.75 mM, iron concentration was increased to 200 μM, and iodine was reduced to 1.8 μM. Shoot multiplication of rhododendron was achieved on modified Anderson's medium supplemented with 1 $mg \cdot l^{-1}$ IAA and 5 $mg \cdot l^{-1}$ 2iP. However, these results varied among rhododendron cultivars.

In work by Meyer (1982) numerous in vitro shoots were obtained from flower pedicels and ovary bases of *Rhododendron catawbiense* using several concentrations of IAA and 2iP in combination with Anderson's medium. The optimum concentration for callus-like structures was 1 mg l^{-1} IAA and 5 mg l^{-1} 2iP, or 4 mg l^{-1} IAA and 15 mg l^{-1} 2iP combinations. Generally, shoots were well elongated on low concentrations of plant growth regulator (0.1 mg l^{-1} IAA and 0.5 mg l^{-1} 2iP). Also, they obtained 10-20% rooted plantlets using 4 $mg \cdot l^{-1}$ IAA.

In 1985, Ettinger and Preece evaluated in vitro shoot regeneration with shoot tips and nodes of Rhododendron P.J.M. hybrid on Anderson's revised medium. Shoot tips were better than single nodes in this study. Also, 5 cm shoot tips reponded better than 2 cm shoot tips. In studies of plant growth regulators, several concentrations of 2iP, and tetrahydropyranyl benzyladenine

(PBA) were evaluated. Optimum shoot proliferation was observed on medium with 5 and 10 mg l^{-1} 2iP. Generally, PBA was very ineffective. For rooting, the base of excised shoots (7.5 cm long) was dipped into 50 % EtOH solutions supplemented with 1250 mg l^{-1} IBA and 1250 mg l^{-1} NAA for 6 seconds. Over 80 % of shoots rooted well in a mixture of sphagnum peat moss and perlite (1:1 by volume) under intermittent mist.

Harbage and Stimart (1987) obtained adventitious shoots from callus of *Rhododendron exbury* hybrids. Callus was induced on Anderson's medium containing 18 μM 2,4-D, but shoot organogenesis was inhibited at that level. Shoot formation from the callus was promoted on Anderson's medium supplemented with 34-68 μM zeatin.

Iapichino *et al*. (1991) achieved an efficient adventitious shoot production protocol from shoot tips of a *Vireya* hybrid of rhododendron. Initially, shoot tips were cultured on Anderson's medium containing 23 μM IAA and 74 μM 2iP for inducing callus. Many adventitious shoots were induced from calli (over 70 shoots per explant) after 6 to 7 months without subculture. For multiple shoot proliferation from adventitious shoots, they used various combination of IAA (11 to 34 μM) and 2iP (49 to 172 μM). In the case of 2iP, concentration had no significant effect. However, the 34 μM IAA concentration was more effective than all 2iP concentrations (7.4 shoots per single shoot). To determine the optimum in vitro rooting conditions, four concentrations of Anderson's medium salts (1, 1/2, 1/4, and 1/8 strength) containing five IAA concentrations (0, 0.6, 6, 28, and 57 μM) were used. The optimum rooting results (73%) were with 1/4 strength Anderson's medium salt with 28 μM IAA.

The next year, Iapichino *et al*. (1992) reported a high frequency adventitious shoot regeneration protocol using leaf explants of *Rhododendron* spp. For this study, seven cultivars were evaluated on Anderson's medium. To evaluate efficient adventitious shoot formation, several concentrations of IBA (4.9, 9.8, and 24.6 μM) and 2iP (9.8, 24.6, 49.2 and 73.8 μM)

combination were studied. The highest regeneration rates (32%) occurred with 4.9 μM IBA and 73.8 μM 2iP. Results varied among the cultivars (0 to 96%) with 'Lodestar' having the greatest response. Regenerated shoots were placed into soil for rooting. After 4 months, 90% rooted shoots were observed.

In the Ericaceae family, we are aware of only one report of plant regeneration via somatic embryogenesis, which is the work by Anthony (2004) with *Leucopogon verticillatus.* For inducing somatic embryogenesis, Anthony used various combinations of salts (MS, WPM or GB_5 medium) and carbohydrates (sucrose, maltose or fructose). The best development of somatic embryogenesis occurred when leaf tissue containing the 'heel' base was placed on Gamborg's B_5 (Gamborg *et al*. 1968) medium supplemented with 10 μM TDZ and 5 μM IAA, 10 $mg \cdot l^{-1}$ thiamine HCl, 5 $mg \cdot l^{-1}$ pyridoxine HCl, 5 $mg \cdot l^{-1}$ nicotinic acid, 40 $g \cdot l^{-1}$ maltose and 7 $g \cdot l^{-1}$ agar. Somatic embryos were transferred from parent tissue to 1/2 strength basal medium for elongation. For rooting, 2, 3, 5 or 64 day pulse treatments of 100 μM IBA were evaluated. The best response (83%) was observed on IBA with a 5-day treatment. When the plantlets were transferred from agar-based medium, the roots were damaged easily. A sand on oat medium was evaluated for easy plantlet removal from the substrate which provided survival rates of 82%, which was a great improvement compared to 23% survival when conventional agar medium was used for rooting.

Tissue culture for rare and endangered plants

Various endangered plants have been successfully propagated using in vitro mass propagation methods. An efficient in vitro propagation protocol was achieved by Pereira (2006). *Vaccinium cylindraceum* Smith (Ericaceae), is a shrub in danger of extinction. In this study, increasing concentrations of 2ip were used with nodal and shoot-tip explants collected from three different areas. Optimum shoot proliferation was obtained from nodal segments cultured in a medium with 12.3 or 24.6 μM 2iP. Callus production increased with increasing 2iP concentration

and the number of nodes per shoot and the length of the regenerated shoots decreased. In vitro roots were induced on shoots when transferred to media without plant growth regulator. Plantlets cultured ex vitro in Jiffy 7 pellets had survival rates of 99%.

Rhododendron ponticum L. subsp. *baeticum* Handel-Mazzetti is an endangered species in the Southern Iberian Peninsula. This species has been reduced by climate changes and fluctuations. An in vitro propagation method for this species was achieved by Almeida (2005). Several concentration of cytokinins (zeatin, 2iP, kinetin, and BA) and IAA combination were evaluated using Anderson's macro and micro nutrients. In general, higher shoot proliferation occurred from nodal segments. The best results for both explants were obtained on 4 $mg \cdot l^{-1}$ zeatin and 1 $mg \cdot l^{-1}$ IAA. They investigated effect of explant type and zeatin plus IAA concentration on shoot number and length. When zeatin concentration was increased, mean number of shoots increased. Media supplemented with 1 $mg \cdot l^{-1}$ zeatin was the optimum condition for apical shoot explants, whereas 3 $mg \cdot l^{-1}$ zeatin + 1 $mg \cdot l^{-1}$ IAA was optimal for nodal segments. For rooting, they treated IBA and NAA (1 $mg \cdot l^{-1}$, 2 $mg \cdot l^{-1}$) with full and half strength Anderson's medium. In this study, rooting frequency with 1/2 strength Anderson's medium gave better results (57-79%) than full strength Anderson's medium (27-56 %). Auxin concentration was not a critical factor for in vitro rooting. For ex vitro rooting, shoots were immersed into 1 gl^{-1} auxin (NAA or IBA) for 2 min, and then placed into soil. Ex vitro rooting also successfully achieved (100 %).

Piper barberi Gamble, an endangered species in India, was propagated using in vitro methods with shoot tip and nodal explant cultures (Anand, 2000). Compared with shoot-tip cultures, nodal explants with a single axillary meristem produced almost three times more shoots. Optimum shoot induction with 2.5 shoots per explant was obtained from nodal explants on MS medium supplemented with 4.43 μM BA and 2.32 μM kinetin. In the case of adventitious shoot formation, a combination of 2.22 μM BA and 0.46 μM kinetin on basal MS medium produced a

strong response (6.9 shoots per explant, 4.9 internodes per explant). For inducing roots, 1/4 or 1/2 strength MS medium without plant growth regulator was used with cultures under light (16-h photoperiod) or continuous dark conditions. Optimum rooting occurred with 1/4 MS medium under continuous darkness. Dark conditions showed a better response (6-8 roots per shoot) than light conditions (2-3 roots per shoot). Also, survival rate was higher under dark conditions (83-98%) compared with light conditions (72-74%). Rooted plantlets were transferred to the field, and showed high survival rates (75%).

The species *Maclura tinctoria* found in regions from Mexico to southern Brazil is an endangered woody species (Gomes, 2003). This species has low germination rates, and its seeds quickly become nonviable. However, this tree produces juicy and tasty fruits, so it is a commercially valuable native plant. An efficient in vitro propagation method using callus from nodal segments was developed by Gomes. For this study, WPM containing several concentrations of NAA and BA was used. Optimum callus induction conditions (2.5 mg per nodal segment) were obtained on media with 10.74 μM NAA and 4.43 μM BA. Multiple shoot formation was not obtained if media lacked NAA and BA. Optimum shoot induction (12 shoots per explant) occurred using 4.03 μM NAA and 4.43 BA. Shoots were rooted on WPM supplemented with 23.62 IBA and 4.7 $g \cdot l^{-1}$ activated charcoal. Plantlets showed a 97% survival rate.

Vanda coerulea is a perennial plant growing at altitudes of 1000-1500 m in India. Because of habitat destruction and overexploitation, this species is endangered. Efficient shoot regeneration from shoot tips of this species was achieved using TDZ (Malabadi, 2004). In this study, Vacin and Went's (VW) (1949) basal medium supplemented with various concentrations of TDZ were used. When very low concentrations of TDZ (0.04, 0.2, and 0.45 μM) or high concentrations of TDZ (31.78, 36.32, 40.86, and 45.41 μM) were used, cultures failed. In contrast, increasing the concentration of TDZ from 2.27 μM to 11.35 μM influenced proliferation of shoot

buds. Notably, the highest percentage of shoot bud proliferation was observed with 11.35 μM TDZ. When TDZ was used for longer than 8 weeks, distorted shoots were formed. For rooting studies, various concentration of IAA, IBA and NAA were used. Very low percentages of rooting were observed (0.1-1.6%) with IBA and NAA. The optimum condition for rooting was 11.42 μM IAA supplemented with half strength VW medium. Well- rooted shoots were transferred into pots containing a potting mixture of charcoal chips, coconut husk and broken tiles (2:2:1 v/v). A 98 % survival rate result was obtained.

Agave victoriae-reginae, an endangered species from Mexico, was successfully propagated through somatic embryogenesis and organogenesis (Martinez-Palacios *et al*., 2003). Somatic embryos were produced from callus derived from leaf segments. MS media with various concentrations of BA and 2,4-D were used for inducing callus with the optimum media containing 2.26 μM or 4.52 μM 2,4-D in the absence of BA. BA inhibited nodular structure formation, whereas callus was strongly induced by 2,4-D, even at 0.45 μM. Similarly, optimum treatments for somatic embryogenesis were also with 2.26 μM 2,4-D. They investigated the relationship between sucrose level and MS macro nutrients for multiple shoot formation. The best response (2.83 shoots per explant) occurred on media supplemented with 2.2 μM BA, 45 $g \cdot l^{-1}$ sucrose and 1/2 strength MS macro nutrients. Both shoots and embryos developed from callus phase were grown into plantlets in MS basal medium with 92 % of the plantlets surviving in greenhouse conditions.

Daphne cneorum, an evergreen low shrub, has gradually disappeared during the last few decades in the Czech Republic. A successful in vitro propagation protocol for this endangered species was developed by Mala and Bylinsky (2004). For this study, two types of explants (dormant apical buds and in vitro germinated seeds) were used. The initiation of multiple shoots was obtained on WPM containing 0.2 $mg \cdot l^{-1}$ BAP, 0.1 $mg \cdot l^{-1}$ IBA, 200 $mg \cdot l^{-1}$ glutamine, and 200

$mg \cdot l^{-1}$ casein hydrolysate. In the case of seeds, only 10% germination occurred. However, 12 multiple shoots per seed were observed in 4 weeks. After 6 months, almost 35 multi-apex cultures were observed. The shoots were rooted in 1/3 strength WPM containing 2.83 mg IBA. Under these conditions, almost 50% of shoots rooted. However, no rooting occurred on the same medium with NAA. In the greenhouse, the plantlets were acclimatized, and then transferred into natural conditions. The acclimatized plants successfully survived during winter and flowered.

Decalepis hamiltonii is an endangered species in India due to habitat destruction and over exploitation. Its high priced aromatic roots are used for various purposes such as a blood purifier, for bio-insecticide activity, and as a principle flavoring (Giridhar, 2004). A new protocol for micropropagation of this species through somatic embryogenesis and organogenesis has been developed by Giridhar (2004). For inducing nodular embryogenic callus from leaf tissues, MS medium supplemented with 0.9 μM 2,4-D + 10.2 μM BA were effective, whereas media supplemented with 0.9 μM NAA + 10.2 μM BA was used to induce compact caulogenic calli. Subsequently, both primary calli were transferred onto MS media containing several concentrations of zeatin and/or gibberellic acid (GA_3) and BA. The induction of the highest number of somatic embryos from nodular tissue was obtained on a medium with 13.68 μM zeatin and 10.65 μM BA. These embryogenic calli with somatic embryos were subcultured onto media with 4.56 μM zeatin + 10.65 μM BA. More extensively differentiated somatic embryos were obtained after 4 weeks of culture. The mature embryos were transferred onto MS medium without plant growth regulators, and then developed into complete plantlets. However, secondary root formation was improved when MS medium with 9.8 μM IBA was used. Using this protocol, complete regenerated plantlets through somatic embryogenesis or organogenesis from leaf tissue were obtained in 12-16 weeks.

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Table 1.1. Summary of studies for organogenesis or somatic embryogenesis in Ericaceae family

Species	Explant	Medium	Plant growth regulator	Result	Reference
Blueberry	Shoot tips	Knops	15 mgl^{-1} 2iP	Multiple shoots	Lyrene (1980)
Blueberry	Leaf	WPM	15 μM 2iP	Adventitious shoots	Billings *et al*. (1988)
Blueberry	Leaf	Knops	24.6 μM 2iP	Adventitious shoots	Dweikat & Lyrene (1980)
Blueberry	Leaf	MS	25 μM 2iP	Adventitious shoots	Callow *et al.* (1989)
Blueberry	Internode	Zimmerman's Z-2	25 μM TDZ 25 μM zeatin	Callus formation Direct shoot	Hruskoci & Read (1993)
Blueberry	Leaf	WPM	20 μM zeatin riboside	Shoot formation	Rowland & Ogden (1993)
Blueberry	Leaf	WPM	1 μM TDZ or 20 μM zeatin	Shoot regeneration	Cao & Hammerschlag (2000)
Cranberry	Leaf	Anderson's salt + MS organics	10 μM TDZ + 5 μM 2iP	Adventitious shoots	Qu (2000)
Cranberry	Shoot tip or nodal	Modified MS	2.5-5.0 mg^{-1} 2iP	Shoot regeneration	Debnath (2001)
Lingonberry	Leaf	MS	5-30 μM zeatin	Multiple shoots	Debnath (2002)
Lingonberry	Leaf	MS	5-10 μM TDZ 1-2 μM zeatin	Callus formation Shoot regeneration	Debnath (2003)
Azalea	Stem	WPM	9.8 μM 2iP + 2.3 μM TDZ 0.05-1.4 μM TDZ	Shoot proliferation Shoot elongation	Briggs (1988)

Table 1.1. Continued

Species	Explant	Medium	Plant growth regulator	Result	Reference
Rhododendron	Shoot tip	Anderson	4 mg^{-1} IAA + 15 mg^{-1} 2iP	Axillary shoot proliferation	Anderson (1975)
Rhododendron	Shoot	Anderson	1 mg^{-1} IAA + 5 mg^{-1} 2iP	Shoot proliferation	Anderson (1984)
Rhododendron catawbiense	Flower pedicel & ovary	Anderson	1 mg^{-1} IAA + 5mg^{-1} 2ip or 4 mg^{-1} IAA + 15 mg^{-1} 2iP	Callus formation	Meyer (1982)
			0.1 mg^{-1} IAA + 0.5 mg^{-1} 2iP	Shoot regeneration	
Rhododendron P.J.M. hybrid	Shoot tip & node	Anderson	5 or 10 mg 2iP	Shoot proliferation	Ettinger & Preece (1985)
Rhododendron exbury hybrid	Leaf	Anderson	0.018 mM 2,4-D 0.034-0.068 mM zeatin	Callus formation Adventitious shoots	Harbage & Stimart (1987)
Rhododendron vireya hybrid	Shoot tip	Anderson	23 μM IAA + 74 μM 2iP	Callus formation	Iapichino *et al.* (1991)
			34 μM IAA + 40-172 μM 2iP	Adventitious shoots	
Rhododendron spp.	Leaf	Anderson	4.9 μM IBA + 73.8 μM 2iP	Multiple shoots	Iapichino *et al.* (1992)
Blackberry	Nodal	MS	4 μM BA + 0.25 μM IBA	Shoot regeneration	Gonzalez (2000)
Leucopogon verticillatus	Leaf	Gamborg B_5	10 μM TDZ + 5 μM IAA	Somatic embrygenesis	Anthony *et al.* (2004)

CHAPTER 3

AN EFFICIENT REGENERATION SYSTEM FOR GEORGIA PLUME, *ELLIOTTIA RACEMOSA,* A THREATENED GEORGIA ENDEMIC[1]

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Abstract. *Elliottia racemosa*, commonly called Georgia plume, is one of the rarest native small tree or large shrubs in Georgia, and is a state threatened shrub. In this study, leaf segments were used to develop an efficient shoot regeneration system for Georgia plume. For inducing adventitious shoot buds, 10 μM thidiazuron (TDZ) and 5 μM indole-3-acetic acid (IAA) with Gamborg B_5 (GB_5) (Gamborg *et al*., 1968) salts were used. Next, a shoot elongation step on media with 25 μM (2-Isopentenyl) adenine (2iP) with Woody Plant Medium (WPM) (Lloyd and McCown, 1980) was required. 2iP has been used successfully to induce shoots in many Ericaceae family in vitro culture studies. To develop protocols for efficient rooting, exogenous auxins were applied with prolonged culture treatments or as a short pulse duration. For prolonged treatments several concentration of IAA or indole-3-butyric acid (IBA) were applied with half strength of WPM. For short term treatments, 100 or 150 μM IBA was pulsed for 2, 5, and 8 days prior to transfer onto an auxin-free medium. Ex-vitro rooting using several concentrations of potassium salt of indole-3-butyric acid (KIBA) was further evaluated. The well rooted shoots were placed into pots containing pine bark, cow manure and sand (2:2:1 v/v).

Introduction

Elliottia racemosa, classified within the group of the Ericaceae family, is one of the rarest native small trees or large shrubs in Georgia, and is a state threatened shrub (Chafin, 2007). ***E. racemosa***, commonly called Georgia plume, derives its name from the beautiful plume-like clusters of slightly aromatic white flowers that appear in early summer (Elliott, 1971). Georgia plume is a beautiful deciduous shrub of eight to twenty feet tall, with leaves that are elliptic, alternate, 1 to 5 inches long by 0.5 to 2 inches wide, and tapering at both ends. Flowers have four or five curved white petals each growing up to 14 mm long, which appear between the middle of June and end of July (Patrick *et al*., 1995). The flowers develop into small round capsules, which can contain up to about 40 seeds.

Georgia plume was first discovered by William Bartram in 1773, and South Carolina botanist Stephan Elliot rediscovered this species in 1808 (Ewan, 1968). The plant was also found in southwestern South Carolina, but likely turns out to be from cultivated plants (Elias, 1987; Godt and Hamrick, 1999). Even though Georgia plume has been introduced since 1813, this outstanding species has never become widely known or studied. Today, ***E. racemosa*** is found in approximately three dozen locations only in Georgia (Patrick *et al*., 1995). The best habitats generally seem to be sunny to partially shady conditions on well drained sand ridges, oak ridges, evergreen hammocks, acid soil, and sandstone outcrops. Even though this plant is very rare and localized, it grows in a broad variety of sandy soil conditions ranging from moist to extremely dry (Patrick *et al*., 1995). Because of decreasing native stands and difficulty of propagation, it has been considered for addition to the list of endangered species.

Bozeman (1983) reported that natural seed set of Georgia plume is limited or nonexistent. Also, Georgia plume may be self-incompatible, and its pollen is only 4% viable (Godt and

Hamrick, 1999; Fordham, 1991). Georgia plume may be rare because of reasons such as low seed set, self-incompatibility, and lack of sexual recruitment caused by low pollen viability and limited numbers of clones within locations (Godt and Hamrick, 1999; Tompson and Spira, 1991). Some plants show multiple trunks owing to vegetative root sprouting following injury such as cutting or fire. However, the biological cycle and life history of Georgia plume is not well reported or understood.

Efforts to propagate Georgia plume using conventional methods have been generally ineffective. Fordham (1969) obtained limited success with seed germination. Problematic was that only a small percentage of seed was sound. Seed stratification removed a cold requirement, but only if given to seed treated within a few months of collection. Old seed acquired secondary dormancy and unpredictable behavior (Fordham, 1969). In 1985, Fordham determined that Georgia plume's seed dormancy mechanism required a prolonged chilling period (42-64 days) for germination (Fordham, 1991). Propagation by root cutting methods can produce some shoots. However, only a limited number of shoots can be produced (Fordham, 1969).

Tissue culture methods can be an excellent option for the study and conservation of threatened or endangered species because small amounts of tissues can be used for mass propagation without damage to the donor (Varadaragan, 1993). Also, a large number of plants may be produced in a given time, and little space is required for tissue culture compared with other propagation methods (Fay, 1992). In addition, it is possible to maintain large genotypic libraries of selected species for plant conservation.

Shoot organogenesis using tissue culture is one of the primary in vitro plant regeneration pathways. Alike in research of plant development, shoot organogenesis has been broadly established in plant biotechnology for in vitro micropropagation and genetic transformation. Morphological and physiological phases of in vitro shoot organogenesis have been widely

studied in plant tissue culture for more than five decades. In shoot organogenesis systems, adventitious shoots are induced first, followed by initiation and development of adventitious roots on shoots resulting in the formation of an entire plant. The process of in vitro shoot induction and development through organogenesis has been elaborated in regards to a number of aspects including developmental biology, physiological, biochemical, and molecular phase (Hick, 1994).

The Ericaceae family, to which Georgia plume belongs, contains a number of economically important plants, such as blueberry, cranberry and rhododendron. This has led to the development of efficient plant regeneration protocols achieved via organogenesis from cultures derived from leaf tissue, shoot tips, and axillary buds. Plants that have been regenerated from this family include blueberry (Lyrene, 1980; Billings *et al*., 1988; Dweikat and Lyrene, 1988; Callow *et al*., 1989; Hruskoci and Read, 1993; Rowland and Ogden, 1993; Cao and Hammerschlag, 2000), cranberry (Qu, 2000; Debnath, 2001b), lingonberry (Debnath, 2002; Debnath, 2003), blackberry (Gonzalez, 2000), rhododendron (Anderson, 1975; Anderson, 1984; Meyer, 1982; Ettinger and Preece, 1985; Harbage and Stimart, 1987; Iapichino *et al*., 1991; Iapichino *et al*., 1992), and *Leucopogon verticillatus* (Anthony *et al*., 2004).

A number of endangered species have been successfully regenerated using in vitro tissue culture methods. For example, rapid multiplication of Blue Vanda of Asia (*Vanda coerulea* Griff ex. Lindl.) was accomplished using shoot tips, leaves and leaf bases (Seeni and Latha, 2000). After plant regeneration, the plantlets were successfully reintroduced into alien forest habitats. Mass propagation protocols for *Vaccinium cylindraceum* Smith (Ericaceae) (Pereira, 2006), *Piper barberi* Gamble (Anand, 2000), *Maclura tinctoria* (Gomes, 2003), *Vanda coerulea* (Malabadi, 2004), *Agave victoriae-reginae* (Martinez-Palacios, 2003), *Daphne cneorum* (Mala and Bylinsky, 2004), and *Decalepis hamiltonii* (Giridhar, 2004) were successfully achieved.

In vitro plant regeneration of Georgia plume has not been previously reported, and may be a method for the conservation and propagation of this threatened species. The overall goal of this study was to develop tissue culture protocols for the mass propagation of *Elliottia racemosa*, Georgia plume, applicable for conservation purposes. Objectives of the work include the development of efficient sterilization conditions for initiation of in vitro plant cultures, and the development of protocols for shoot proliferation, rooting and acclimatization of plants.

Materials and Methods

PLANT MATERIALS. Plant materials used in studies were from natural collections and greenhouse potted plants. In September, plant material was collected in situ from populations at the Charles Harrold Nature Conservancy site in Statesboro, GA consisting of leaves or buds of the apical 3-7 cm of actively growing shoots. Upon return to the lab, leaf and bud tissues were dissected and explanted onto appropriate media. In addition, root cuttings of field material were collected, planted into potting mix (Fafard 3B, Conrad Fafard, Inc., Agawam, MA) and placed under mist. Adventitious shoots emerged which provided additional leaf tissue for explanting. Some plants were obtained from collections of the Georgia Southern Botanical Garden, Statesboro, GA, and maintained in the University of Georgia Horticulture greenhouses.

DISINFECTION. For the decontamination process prior to introduction into sterile culture, explants from the field and greenhouse were given various sterilization treatments using different times and/or concentrations of 70% ethanol, 10% Roccal (National Laboratories, Montvale, NJ) and bleach immersions as listed in Table 3.1, and then rinsed three times with sterile distilled water for 5 min each rinse. Explant tissues included: 1) young leaves (< 2 cm long, yellow green color), 2) older leaves (2 to 7 cm long, light green color) and 3) buds. Leaf explants

were cut into pieces approximately 5 mm × 5 mm. Axillary buds were dissected to remove bud scales, and older leaf primordia. Following sterilization treatments, tissues were placed onto shoot induction medium with TDZ + IAA as described below, and dispensed into test tubes with 20 mL of medium per tube. After 4 weeks of culture, percent contamination, percent cultures showing injury, and healthy cultures were assessed. For experiments with leaves, each treatment consisted of at least 40 explants. The experiments were repeated 3 times. Cultures were observed every 2 days with final observations recorded after 4 weeks.

SHOOT INDUCTION STUDIES. Shoot induction studies were conducted using buds and leaf tissues of two ages placed on media supplemented with either 2iP (Callow *et al.*, 1989) or TDZ and IAA in combination (Anthony *et al.*, 2004). These media induced successful shoot proliferation or somatic embryogenesis in related species in the same Ericaceae family as Georgia plume. TDZ medium consisted of 10 μM TDZ, 5 μM IAA, 10 $mg \cdot L^{-1}$ thiamine HCl, 1 $mg \cdot L^{-1}$ pyridoxine, 1 $mg \cdot L^{-1}$ nicotinic acid, 40 $g \cdot L^{-1}$ maltose and 4 $g \cdot L^{-1}$ Gel-Gro with Gamborg's B_5 salts (GB_5) (Gamborg *et al*., 1968). The pH was adjusted to pH 6.0 prior to autoclaving at 121°C for 20 min. Media with 25 μM 2iP was supplemented with 2 $mg \cdot L^{-1}$ glycine, 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$ pyridoxine HCl, 1 $mg \cdot L^{-1}$ thiamine HCl, 100 $mg \cdot L^{-1}$ myo-inositol, 20 $g \cdot L^{-1}$ sucrose and 4 $g \cdot L^{-1}$ Gel-Gro with Woody Plant Medium (WPM) (Lloyd and McCown, 1980). The pH was adjusted to pH 5.2. For these studies, young leaves, older leaves and buds explants were prepared as described in sterilization studies. Explants were placed horizontally with the abaxial leaf surface in contact with the medium. Explants were initially cultured into test tubes with 20 mL medium per tube, and then after 4 weeks transferred onto the same medium in baby food jars (30 mL medium per jar). All explants were cultured either under continuous dark conditions or a 16-h photoperiod (white fluorescent lights at 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 °C.

Further studies were conducted to evaluate the long term maintenance of shoot clumps obtained from induction studies. Because continued production of adventitious shoots is important for in vitro mass propagation, secondary media conditions were evaluated to identify plant growth regulator concentrations promoting continued adventitious shoot formation. Shoot bud cultures previously induced on GB_5 medium with 10 μM TDZ + 5 μM IAA for 3 months were transferred onto media with: 1) no plant growth regulator; 2) 1/2 plant growth regulator concentration (5 μM TDZ and 2.5 μM IAA), or 3) the same plant growth regulator concentration (10 μM TDZ and 5 μM IAA). From 40-48 shoot clumps were cultured per treatment. Cultures were maintained on their respective media for 3 months. The study was repeated twice.

SHOOT ELONGATION. Shoot clumps obtained on induction medium with TDZ and IAA were prolific, but required elongation before shoots could be manipulated for plant regeneration. To promote shoot elongation, shoot clumps were divided into pieces about 10 mm × 10 mm wide, and transferred onto WPM with 25 μM 2iP distributed into baby food jars with 30 mL per jar. All cultures were maintained under white fluorescent lights (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 °C and subcultured onto the same medium every 4 weeks. Shoot clumps were obtained from previous studies which developed under either dark or light conditions. Four shoot clumps were cultured per baby food jar, and 120 shoot clumps were used per treatment. This experiment was repeated three times. Cultures were scored for the presence of buds versus elongated foliar shoots after 4 and 8 weeks on elongation treatments.

ROOTING STUDIES. Root induction studies were conducted using various types and concentrations of auxin applied either continuously or as pulse treatments. For continuous auxin studies, shoots were placed on ½ strength WPM media supplemented with either IAA (0, 10, 20, and 40 μM) or IBA (15, 30, and 45 μM) on 1/2 strength WPM supplemented with 20 $g \cdot L^{-1}$

sucrose and 4 $g \cdot L^{-1}$ Gel-Gro. Forty shoots per treatment were used with the experiment repeated 2 times. For auxin pulse studies, shoots were placed on media with either 100 or 150 μM IBA for 2, 5 or 8 days. After the IBA pulse, shoots were transferred to ½ strength basal GB_5 medium in baby food jars. Shoots were cultured for 2 months under white fluorescent lights (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 °C in a growth room. Every month the percentage of rooted shoots and number of roots per rooted shoot were noted. After 2 months, plantlets were removed from culture medium and roots were free washed free of agar. Forty shoots per treatment were used with the experiment repeated 2 times. Half the plantlets were destructively sampled to determine shoot and root dry weights. Tissues were placed in a drying oven at 70 °C for 3 days, and then dry weights were recorded. The other half of the plantlets were planted into 72-cell plug trays in a potting mixture of pine bark, cow manure and sand (3:1:1 v/v) and kept under a 16-h photoperiod at 25 °C in a growth room. The flats were initially covered with clear plastic propagation domes (Humi-dome, Hummart, Earthcity, Mo) to maintain high humidity and slowly acclimated over 4 weeks. Two months after outplanting, plants were transferred to the greenhouse.

Additional studies were conducted to evaluate ex vitro rooting. Shoots 2-4 cm long were harvested from cultures in shoot elongation medium. The base of stems was dipped in 0, 1500, or 3000 ppm KIBA for 5 seconds, planted in 72-cell plug trays in the same potting mix used for outplanting of rooted plantlets, and enclosed in the same type of plastic domes for 8 weeks for rooting to take place. Acclimation was as described earlier. At this time, 24 plants per KIBA treatment were measured for dry weights.

INITATION OF CULTURE LINES FROM NATURAL FIELD COLLECTIONS. Leaf material was collected from mature specimens in the wild for initiation of new culture lines from different genotypes during April 2006. Field material from eighteen genotypes was

collected from native populations at Tillman Tract and Charles Harrold Reserve in Chandler County, GA and Bennett property and Fort Stewart in Tattnall County, GA. At the time of collection, shoots were soft, succulent and new spring growth had just commenced. Leaves were light green and still expanding. Leaf explants were disinfested using methods established in previous studies, placed on induction media with 10 μM TDZ + 5 μM IAA, then transferred to medium with 25 μM 2iP for multiplication and elongation as describe above.

STATISTICAL ANALYSES. Statistical analyses were performed using the GLM procedures and mean separation by Student-Newman-Keuls test (P = 0.05) of SAS (version 8.2 for Windows; SAS Institute GmbH, Heidelberg, Germany).

Results and Discussion

DISINFECTION STUDIES. Obtaining clean cultures from field material was problematic (Table 3.1). Over 95% of leaf cultures were contaminated when only ethanol and bleach were used. The addition of a Roccal treatment significantly improved the percentage of clean cultures. However, the most severe sterilization procedure still had over 40% contamination and most of the uncontaminated cultures exhibiting browning and damaged areas. Nonetheless, 19% of the cultures were successfully disinfested.

Bud tissues similarly exhibited either high contamination rates or severe injury with sterilization. High levels of fungus contamination occurred, which became progressively evident over long periods in culture as buds opened. A successful culture rate of only 8% was obtained even with the strongest sterilization protocol. Tissues characteristically had browning in regions.

In contrast to field material, leaves collected from greenhouse-grown plants had low levels of contamination Young leaves (<2 cm long) were most effective, with 90% of the cultures

showing no contamination when immersed successively for 1 min in 70% ethanol, 5 min in 5% Roccal, and 10 min in 0.6% NaOCl (Table 3.1). More severe sterilization methods failed to improve the number of clean cultures, and caused greater tissue injury. Older leaves (≥ 2 cm long) exhibited lower percentages of successful cultures than young leaves. There were no significant differences in the percentage of successful cultures for the three milder sterilization protocols, which ranged from 35-46%. The most stringent sterilization method caused excessive injury and a lower percentage of successful cultures.

SHOOT INDUCTION. The effects of induction medium and light conditions were evaluated using buds and leaves of different developmental stages (Table 3.2). Bud explants generally showed no response and were ineffective as an explant source. After 8 weeks culture, bud explants exhibited 8% and 0% shoot expansion, when maintained under light and dark conditions, respectively. Growth of cultures was restricted to limited expansion of leaf primordial within buds with little or no adventitious bud development observed (Fig. 3.1A). Tissues exhibited extensive browning and necrosis.

Leaf tissues induced on media with TDZ + IAA exhibited swelling and cell proliferation within 1 week of explanting. Cultures initiated with younger leaves had a faster response than older leaves. Bud primordia were visible after 21-28 days in cultures initiated from young leaves. At 4 weeks, bud-like structures were observed arising from the darkened original explant material (Fig. 3.1B). Proliferation of structures completely covered the explant material by 8 weeks with shoot clumps from 10 mm to 20 mm in diameter obtained (Fig. 3.1C). Adventitious buds formed on all regions of the leaf explant tissue and occurred on both abaxial and adaxial leaf surfaces. Some limited expansion of shoots was observed. In contrast, older leaves had much slower regeneration with 40 days required for visible shoots and buds (data not shown). The best shoot induction (98%) response occurred with cultures on medium with TDZ + IAA, initiated from

young leaves and maintained in light conditions (Table 3.2). On this medium, older leaves were still highly regenerable, although at a significantly lower percentage than young leaves. Not only were younger leaves more regenerable, but they also exhibited significantly less browning than older leaves.

TDZ was highly effective in inducing shoot primordia in Georgia plume. TDZ is a phenylurea-derived cytokinin, and has potent cytokinin activity in woody plant tissue culture (Huetteman & Preece, 1993). TDZ can promote cell division and differentiation and is reported to induce both organogenesis and somatic embryogenesis. Within members of the Ericaceae family, TDZ has been reported to be effective in promoting shoot formation when applied at a range of concentrations. For example, medium with 10 μM TDZ + 5 μM IAA was reported to produce somatic embryogenesis for an Australian Ericaceae (*Leucopogon verticillatus*) (Anthony, 2004). On media with 25 μM TDZ, callus formation was induced from blueberry internodes with subsequent shoot production from callus structures (Hruskoci and Read, 1993). In 2000, Cao and Hammerschlag achieved shoot regeneration from leaves of several blueberry cultivars using media supplemented with 1 or 5 μM TDZ. An efficient regeneration system using leaf explants of cranberry was developed using a 10 μM TDZ and 5 μM 2iP combination (Qu, 2000). Adventitious shoot and bud formation was obtained from hypocotyl segments of lingonberry placed on MS media containing 5-10 μM TDZ (Debnath, 2003).

Media containing 2iP were also effective in inducing shoots with up to 61% of the cultures regenerating shoots (Table 3.2). Direct shoot organogenesis from leaf tissue occurred on media with 2iP (Fig. 3.1F). 2iP has been used to induce adventitious shoot from leaf explants in related species such as blueberry (Billings *et al*., 1988., Dewikat and Lyrene, 1980., Callow *et al*., 1989), cranberry (Qu, 2000), rhododendron (Iapichino *et al*., 1992). However in the current study, shoot regeneration was significantly lower on media with 2iP than that obtained with the best

TDZ + IAA treatments. Furthermore, shoots in cultures maintained on 2iP medium for longer than 8 weeks declined, and became brown and non-regenerable. This is in contrast to shoots induced on TDZ + IAA medium which continued to proliferate and remained healthy (data not shown).

Cultures maintained under a 16-h photoperiod consistently had higher levels of shoot regeneration than cultures maintained in the dark (Table 3.2). Cultures induced on media with TDZ + IAA produced shoots regardless of light condition, however, significantly better shoot regeneration was obtained with correspondingly-aged leaves grown in light vs. dark. In contrast, light was critical for shoot regeneration if 2iP was used for induction. No shoots were obtained with cultures grown under dark conditions compared to 61% of the cultures forming shoots from young leaf explants maintained in light conditions

Culture morphology was markedly affected by photoperiod. Cultures grown under light conditions (Fig. 3.1C and E) were green and exhibited foliar and shoot development. In cultures maintained under continuous dark, shoot buds were observed which were white or yellow (Fig. 3.1D). Dark conditions promoted more callus in cultures induced with TDZ + IAA. However this callus formation did not inhibit proliferation and gave rise to bud and shoot primordia. Light vs. dark had no significantly effect on callus production in cultures with 2iP medium, but had marked effects on the browning and decline of cultures. In dark conditions browning occurred in over 72% of the cultures. This is in contrast to cultures on TDZ + IAA where light versus dark conditions had no significant effect on the percentage of cultures exhibiting browning.

Variable results have been reported on the effects of light conditions on adventitious shoot regeneration. Landi (2006) similarly compared leaf organogenesis in *Fragaria* under 16-h photoperiod vs. continuous dark conditions. In contrast to our results, significantly higher percentages of regenerated shoots and less callus production were obtained under continuous

dark versus light conditions. In studies with Lingonberry, young leaf tissue maintained in darkness for 7 days was the best for producing adventitious shoots (Debnath and McRae, 2002). Cao and Hammerschlag (2000) evaluated the effect of light intensity (55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ vs. 18 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on shoot regeneration from leaf explants in five blueberry cultivars. The number of shoots per explant varied with genotype and was significantly better or inhibited depending on cultivar evaluated.

Studies conducted to evaluate the long term maintenance of shoot induction cultures on media with different concentration of plant growth regulators determined that retaining cultures on their original induction medium was most effective (Table 3.3). Cultures transferred onto basal media rapidly declined (Fig. 3.2A) within 2 weeks while those placed onto media with TDZ + IAA, at original or half strength concentration, exhibited less browning and developed foliar shoot-like structures. For continued proliferation of adventitious shoots, TDZ + IAA were required in this study. On both TDZ + IAA treatments, continued proliferation of buds and expansion of shoots were observed (Fig. 3.2B). Likewise, Debnath (2001a) evaluated transferring lingonberry shoot clumps induced on TDZ medium to media with no or low TDZ (0.1 or 1 μM). Results were similar to that found with Georgia plume, i.e., shoots on basal media turned brown and died. Likewise, cultures maintained on media with TDZ remained viable, but failed to elongate. In contrast, Malabadi (2004) found that although TDZ was efficient for inducing shoot regeneration cultures, its use for longer that 8 weeks resulted in distorted shoots.

SHOOT ELONGATION STUDIES. To promote shoot elongation in the current study, proliferating cultures that were initiated on induction medium under either light or dark conditions were transferred to a medium with 25 μM 2iP. This medium was very effective in promoting shoot elongation, with the number of elongated shoots influenced by the light

conditions during induction (Fig. 3.3). Cultures previously initiated in the light had almost twice as many elongated shoots as dark-induced cultures. Elongated shoots were clearly visible arising in clumps. At 8 weeks, shoots from cultures initiated in light conditions exhibited further dramatic increases in number, with shoots attaining heights of up to 5 cm (Fig. 3.2C).

TDZ reportedly inhibits shoot elongation in a number of woody species (Huetteman and Preece, 1993). Strategies to overcome this problem include transfer of cultures to a secondary media with a lower concentration of TDZ, a different cytokinin or no plant growth regulator. For example, Qu (2000) obtained a highly efficient regeneration system using cranberry leaf explants induced on media with TDZ + 2iP. For elongation of adventitious shoots, cultures were transferred onto a basal medium with no growth regulators. In azaleas, shoots induced with TDZ exhibited improved shoot elongation when transferred to media with lower concentrations of TDZ (Briggs, 1988). In studies of adventitious shoot regeneration with Lingonberry leaf explants, stunted shoot development and inhibition of elongation caused by TDZ was overcome by transferring shoot cultures to a shoot elongation medium containing zeatin (Debnath, 2003; Debnath, 2005). Likewise, Chitra and Padmaja (2005) used a two-stage culture procedure in which leaf explants induced on medium with TDZ were transferred to a secondary medium with BAP to promote shoot elongation. They emphasized the importance of double stage treatments to improve shoot morphogenesis particularly when TDZ is used to initiate cultures.

ROOTING STUDIES. Several attempts to promote and improve rooting were investigated in this study. Strategies for the application of auxin included prolonged or continuous auxin applications in which the auxin was incorporated into the rooting medium, or pulse treatments where shoots were treated with higher levels of auxin for a few days before transfer to auxin-free medium. Results of continuous auxin treatments are shown in Table 3.4. Auxin type and concentration significantly influenced the rooting of shoots. At 4 weeks, IAA generally was

better than IBA for rooting of Georgia plume shoots, and promoted higher percent rooting and root numbers than most IBA concentrations evaluated. Concentration of IAA had no significant effects, with 48 to 65% rooting obtained. In contrast, high levels of IBA (45 μM) proved to be inhibitory. Callus structures formed at shoot bases and no rooting was observed. Substantial amounts of callus formation were likewise reported by Kim *et al*. (1985) in mulberry shoots rooted with high levels of IBA, which caused poor vascular connections between shoots and adventitious roots. Low levels of exogenous auxin are required for Georgia plume rooting as evidenced in the observation that shoots given no auxin still exhibited 37% rooting. However, rooting was significantly improved with all auxin applications except for the highest IBA concentration.

At 8 weeks, high rooting percentages were obtained, ranging from about 77 to 95% on media with IAA (Table 3.4). Rooting percentages of shoots treated with IBA approached similar values obtained in shoots placed on IAA medium except for the 45 μM IBA treatments which still had a poor rooting percentage. Even though the number of roots was not significantly different among treatments, root and shoot dry weights were 2 to 3 times greater in IAA treatments compared to the weights obtained with IBA or control treatments.

For in vitro rooting, shoots are commonly maintained on an auxin-containing medium. However, continuous exposure of shoots to high levels of auxin can have detrimental effects. Bouza *et al* (1992) found that high auxin harmed terminal buds, and that plantlets did not grow normally. In addition, increased callus formation can occur under conditions with high levels of auxin. Thus, studies to evaluate short duration auxin treatments were conducted.

Auxin pulse treatment promoted high levels of rooting, with 77 to 95% rooting obtained at 4 weeks after transfer to auxin-free media (Table 3.5). Neither the concentration of IBA nor pulse times of 2 to 8 days had a significant effect on rooting percentage or root number. However,

significantly greater root dry weights and shoot dry weights were obtained with five day pulse treatments compared to pulse treatments with 2 or 8 days. Thus, pulse time appeared to be a more important factor than IBA concentration. Rooting was more rapid with pulse- (Table 3.5) versus continuous-auxin treatments (Table 3.4) where 8 weeks were required to obtain comparable rooting percentages.

Pulse application of auxin have been applied in a number of cases with improved rooting obtained. For example, rooting was obtained with *Paeonia suffruticosa* shoots when cultured on medium supplemented with 75 μM IBA for 10 days followed with transfer onto medium containing activated charcoal with no plant growth regulators (Bouza *et al*. 1992). Similarly, Lane and McDougald (1982) evaluated rooting in shoot cultures of five apple cultivars. One recalcitrant cultivar, M9, failed to root with continuous NAA applications, but rooted successfully with an acute application of NAA followed by transfer onto basal medium.

Ex vitro rooting of shoots (Fig. 3.2D) was very effective in this study (79–88%) regardless of whether shoots were treated with KIBA (Table 3.6). No significant differences in rooting percentage or root dry weights were observed among KIBA concentrations. However, shoot dry weight was affected, with a 2 to 3 fold increase observed compared to when no KIBA was applied. In vitro rooting protocols have some disadvantages. Roots differentiated in vitro often lack root hairs and vascular connections (George, 1996). Qu (2000) found that shoots of cranberry, a species related to Georgia plume, rooted easily under both in vitro and ex vitro conditions. However, roots regenerated ex vitro were more vigorous and lacked callus formation at the base of cuttings. Ex vitro rooting is simpler and eliminates a tissue culture step. Furthermore, roots should sustain less damage during transplanting compared to in vitro rooted cultures when plantlets are removed from agar-based medium.

Reports of ex vitro rooting in members of the Ericaceae are variable. Lingonberry was successfully rooted (over 80%) using ex vitro methods after dipping shoots in 0.8% IBA (Debnath, 2003; Debnath and McRae, 2002). Over 80% rooting was obtained in rhododendron shoots after auxin dips. Blueberry shoots rooted well in a mixture of peat and perlite in a mist chamber (Gonzalez, 2000). In contrast, Iapichino (1992) reported wide differences in rooting response (0 to 96%) that varied with cultivar. Meyer (1982) obtained only 10-20% rooting with rhododendron shoots placed in media amended with IAA.

Acclimation and/or rooting of plantlets under humidity domes was very effective. Plantlets exhibited elongation of shoots soon after transfer to potting mixture (Fig. 3.2E). Rooted plants from both in vitro and ex vitro studies showed very high rates of survival, with over 85% of plantlets surviving in the greenhouse after 1 month.

INITATION OF CULTURE LINES FROM NATURAL FIELD COLLECTIONS. The protocols developed for initiation and induction of shoot regeneration cultures from leaf explants was very effective. Proliferating shoot cultures were obtained from 11 out of 18 genotypes attempted. Healthy, actively growing leaf tissue appears to be an important factor for culture initiation. In cases where successful cultures were not obtained, leaf tissues were stressed or damaged. Culture lines from all genotypes produced numerous adventitious shoots.

This study reports an efficient regeneration protocol that can be used for the micropropagation of Georgia plume, *Elliottia racemosa.* The method uses leaf tissue as the explant source and is suitable for field-grown material collected from mature specimens. Adventitious shoots are induced on a medium supplemented with 10 uM TDZ + 5 uM IAA, then transferred to a shoot elongation medium containing 2iP. High rooting frequencies can be obtained under in vitro and ex vitro conditions with over 85% survival of plantlets transferred to greenhouse conditions. Tissue culture appears to be a promising approach for the propagation and

conservation of this rare and threatened plant. Future plans are to conduct studies on re-establishing plants in their native habitats and to regenerate plants from divergent populations to aid in conserving the genetic diversity in this species.

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Table 3.1. Contamination, injury, and successful cultures obtained with field or greenhouse tissues of different ages using different sterilization protocols.

Explant[z]	70% EtOH	Roccal		Bleach		Contamination (%)	Visible injury[y] (%)	Successful culture (%)
	Time (s)	%	Time (m)	%	Time (m)			
Field material								
Leaf	30	-	-	10	10	99 a	1 c	0 b
Leaf	30	-	-	20	10	96 a	4 c	0 b
Leaf	60	5	5	10	10	74 b	22 b	4 b
Leaf	60	10	10	20	10	43 c	38 a	19 a
Bud	60	10	10	10	10	100 a	0 b	0 b
Bud	60	10	10	20	10	100 a	0 b	0 b
Bud	120	20	10	20	10	100 a	0 b	0 b
Bud	120	20	10	30	15	50 b	42a	8 a
Greenhouse material								
Leaf (O)	60	-	-	20	10	41 cd	24 bc	35 d
Leaf (O)	60	5	5	10	10	37 cd	19 bc	44 cd
Leaf (O)	60	10	10	10	10	30 cde	24 bc	46 cd
Leaf (O)	60	20	10	20	10	27 de	56 a	17 e
Leaf (Y)	60	-	-	10	10	25 de	12 cd	63 b
Leaf (Y)	60	-	-	20	10	35 cd	10 cd	55 bc
Leaf (Y)	60	5	5	10	10	10 f	1 d	89 a
Leaf (Y)	60	5	5	20	10	19 ef	28 b	53 bc

[z] Field material (Statesboro, GA) Y- Young leaf in greenhouse (< 2cm, yellow green color) O- Old leaf in greenhouse (> 2cm , light green color).

[y] Injury was established for clean cultures only since contamination prevented rating.

Different letters within a column by Student-Newman-Keuls test indicate significant differences at $P \leq 0.05$.

Table 3.2. Effect of induction medium, light conditions, and leaf age on shoot regeneration, callus production, and explant browning after 8 weeks

Medium	Light condition[z]	Explant[y]	Shoot regeneration (%)	Callus (%)	Browning (%)
TDZ + IAA	L	Bud	8	0	92
	D	Bud	0	0	100
	L	Y	98 a	0 b	2 d
	L	O	76 b	0 b	24 c
	D	Y	68 bc	28 a	4 d
	D	O	41 d	29 a	30 c
2iP	L	Y	61 c	21 a	18 c
	L	O	37 d	25 a	29 c
	D	Y	0 e	28 a	72 b
	D	O	0 e	16 a	84 a

[z] L: 16-h photoperiod at 70 μmol·m^{-2}·s^{-1}, D: continuous dark condition

[y] Y: Young leaf in greenhouse (< 2cm, yellow-green color), O: Old leaf in greenhouse (> 2cm , light green color)

Different letters within a column by Student-Newman-Keuls test indicate significant differences at $P \leq 0.05$.

Table 3.3. Plant growth regulator concentration on maintenance of shoot induction cultures

Treatment PGR	Foliar shoots (%)	Browning/decline (%)
Basal	23 b	77 a
5 μM TDZ + 2.5 μM IAA	70 a	12 b
10 μM TDZ + 5 μM IAA	64 a	17 b

Different letters within a column by Student-Newman-Keuls indicate significant differences at $P \leq 0.05$.

Table 3.4. The effect of continuous auxin applications for in vitro rooting of shoots using different auxin types and concentrations at 4 and 8 weeks on rooting medium

Treatment	4 weeks		8 weeks			
	Rooting (%)	Root no.	Rooting (%)	Root no.	Root DW (mg)	Shoot DW (mg)
10 μM IAA	50.9 ab	4.3 ab	84.1 ab	7.7 a	22.1 a	54.5 b
20 μM IAA	65.3 a	5.1 a	95.5 a	8.8 a	26.3 a	60.7 a
40 μM IAA	47.5 ab	5.4 a	76.7 ab	9.3 a	24.2 a	65.3 a
15 μM IBA	37 c	3.3 b	63.8 bc	5.3 a	9.8 b	31.5 c
30 μM IBA	44.5 b	4.5ab	75 b	9.1 a	11.5 b	29.5 c
45 μM IBA	0	-	5.5 d	7.5 a	-	-
Control	37.4 c	2.3 c	53.3 c	4.8 a	8.8 b	24.5 c

Different letters within a column by Student-Newman-Keuls indicate significant differences at $P \leq 0.05$.

Table 3.5. The effect of different IBA pulse treatments applied to shoots for in vitro rooting. Data taken 4 weeks after transfer to auxin-free medium.

PGR	Pulse time (day)	Rooting (%)	Root no.	Root DW (mg)	Shoot DW (mg)
100 μM IBA	2	81.1 a	6.1 a	13.6 b	32.9 b
	5	89.2 a	5.7 a	18.1 a	45.3 a
	8	86.6 a	6.1 a	12.1 bc	34.7 b
150 μM IBA	2	76.6 a	5.3 ab	12.5 b	33.4 b
	5	89.6 a	5.4 ab	17.1 a	48.1 a
	8	95 a	5.8 a	10.4 bc	33.2 b
Control	0	50 b	4.2 b	8.9 c	14 c

Different letters within a column by Student-Newman-Keuls indicate significant differences at $P \leq 0.05$.

Table 3.6. The effect of a KIBA quick-dip on ex vitro rooting and growth of shoots after 8 weeks.

KIBA	Rooting (%)	Root DW (mg)	Shoot DW (mg)
0 μM	79.2 a	11.1 a	11.2 c
1500 μM	83.3 a	12.5 a	21.8 b
3000 μM	87.5 a	12.8 a	33.8 a

Different letters within a column by Student-Newman-Keuls indicate significant differences at $P \leq 0.05$.

Figure 3.1. In vitro shoot organogenesis from *Elliottia racemosa*.

(**A**) Bud after 8 weeks culture on WPM with 25 μM 2iP (**B**) Adventitious shoot formation on leaf tissue placed on 10 μM TDZ + 5 μM IAA under a 16-h photoperiod four weeks after explanting**.** Dark tissue is the original explant. (**C**) Shoot bud clusters developing on a leaf explant eight weeks after explanting (**D**) Leaf explant induced on medium with 10 μM TDZ + 5 μM IAA maintained under continuous dark conditions (**E**) Leaf explant induced on the same medium but maintained under a 16-hr photoperiod (**F**) Direct shoot organogenesis from leaf tissue cultured in 25 μM 2iP. Regeneration rates were significantly lower than cultures induced on media with TDZ + IAA.

Fig. 3.1

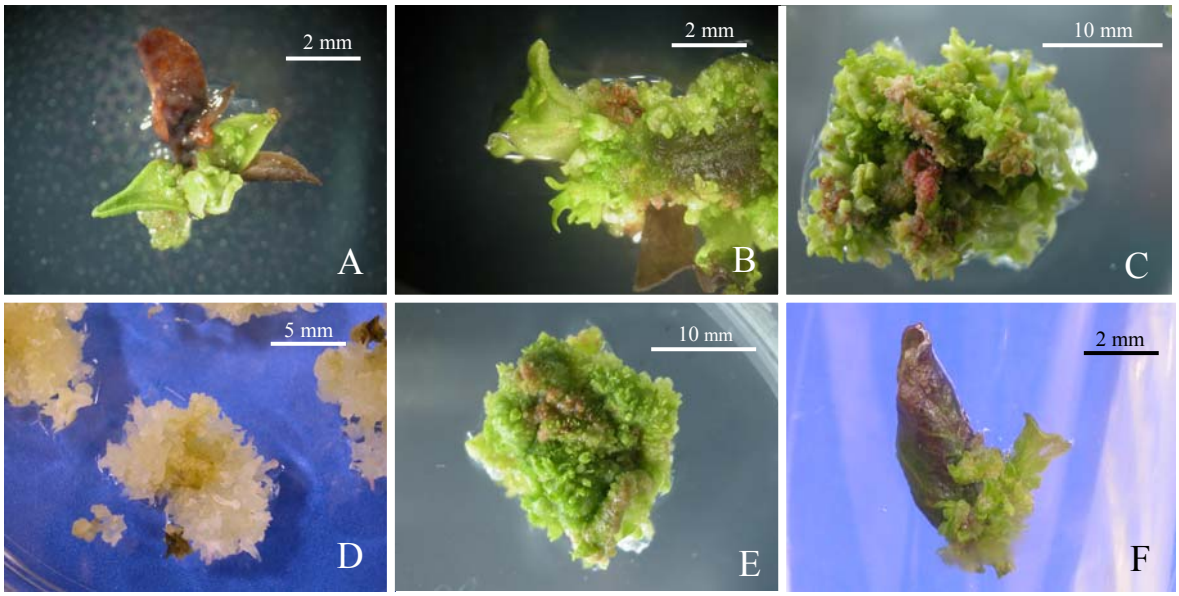


Figure 3.2. Maintenance and further development of adventitious shoots

(**A**) Adventitious shoot bud clumps on GB_5 without plant growth regulator (**B**) Adventitious shoot bud clumps on GB_5 with TDZ + IAA (**C**) Shoot elongation on WPM with 25 μM 2iP (**D**) Rooting of shoots (**E**) Acclimatized plantlets into flats in the greenhouse

Fig. 3.2

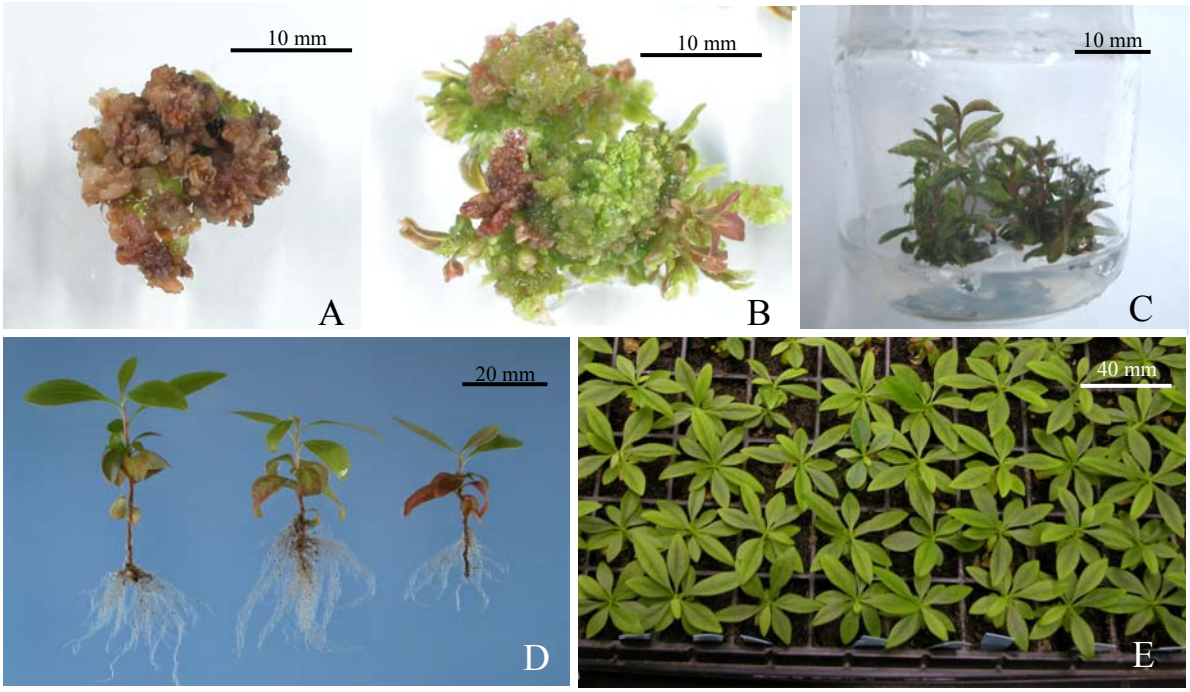
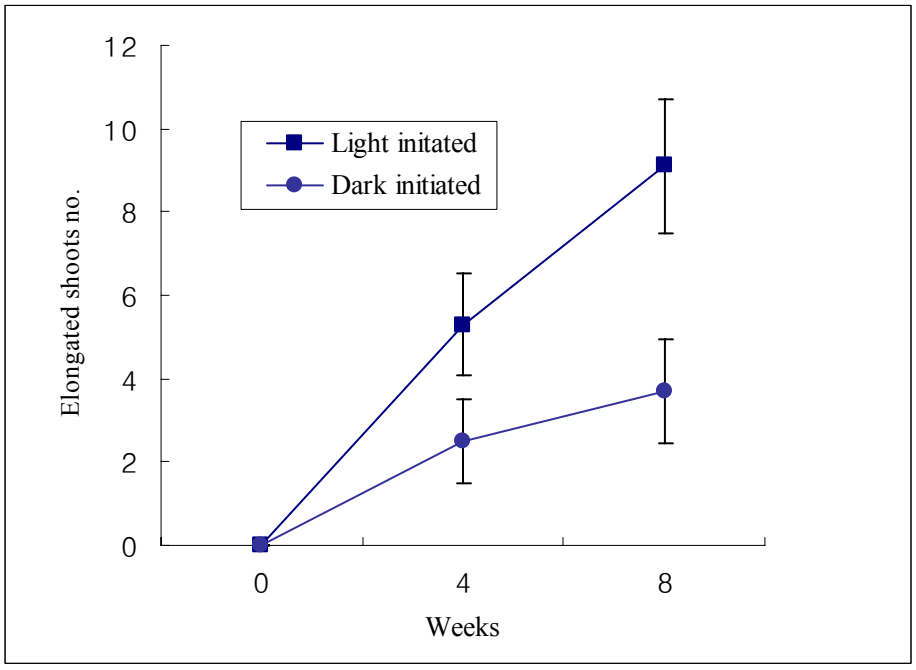


Figure 3.3. Shoot elongation in proliferating cultures transferred to a medium with 25 μM 2ip in the light. Cultures were previously induced on medium with TDZ + IAA and cultures under light or dark conditions.

Fig. 3.3


12
10
8
6
4
2
0
Elongated shoots no.
Light initated
Dark initiated
0
4
8
Weeks

CHAPTER 4

MORPHOLOGICAL AND HISTOLOGICAL EVALUATIONS OF IN VITRO REGENERATION IN *ELLIOTTIA RACEMOSA* LEAF EXPLANTS INDUCED ON MEDIA WITH TDZ[1]

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Abstract. *Elliottia racemosa*, commonly called Georgia plume, is a rare deciduous shrub or small tree. It has sustained severe loss of habitat and its range is now restricted to a limited number of sites in the state of Georgia. Tissue culture protocols have been developed as a means to propagate and conserve this threatened species using leaf explants induced on media supplemented with 10 μM TDZ + 5 μM IAA. Bud-like clusters, elongated embryo-like protrusions, and shoot-like structures were produced from the leaf explants. Morphological and histological evaluations of cultures during induction and development were conducted using light microscopy of sectioned material and scanning electron microscopy. Histology of explant tissues indicates that plant regeneration of Georgia plume occurs through a shoot organogenesis pathway that involves the formation of actively dividing meristematic regions originating in subepidermal cell layers that proliferate to form protuberances on the explant surface. Numerous well-formed shoot apical meristems with leaf primordia are produced, as well as fused shoot-like structures. Elongated, embryo-like structures had various degrees of shoot apex development. Evaluations of serial sections found they lacked a defined root apex, and that basal portions were composed of parenchymatous files of cells with a broad point of attachment to the parent tissue. Lack of bipolarity and a root pole signifies that true somatic embryogenesis does not occur.

Introduction

Elliottia racemosa, commonly called Georgia Plume, is a rare deciduous shrub or small tree native to the state of Georgia. In early summer, it has beautiful plume-like clusters of white flowers from which it gets its name. Attaining heights to 10 m or more tall, specimens often have multiple branches that form from resprouting of shoots from roots. Georgia Plume was first collected by William Bartram in 1773, and not observed again until 1808 when it was collected by Stephen Elliott for whom it was named (Ewan, 1968; Patrick *et al*., 1995). It was thought to be extinct in the wild from 1875 until 1901 when it was rediscovered. Georgia plume is one of the rarest native large shrubs, and has a very limited range restricted to approximately three dozen locations in Georgia (Patrick *et al*. 1995).

Georgia plume has not been known or studied broadly, even though this species has been introduced since 1813. However, low pollen viability, self incompatibility, low or nonexistent seedset, and lack of sexual recruitment are important factors threatening reproduction in Georgia plume (Godt and Hamrick, 1999). Young seedling plants have not been found in the wild (Bozeman, 1983) indicating serious consequences for the future. Georgia plume has sustained severe loss of habitat due to loss of forests for agricultural production.

Tissue culture can be an excellent approach for propagation and conservation of threatened or endangered species (Fay, 1992). This is particularly the case when, as in Georgia plume, conventional propagation methods are ineffective or limiting. An efficient plant regeneration system was developed for the micropropagation of Georgia plume using leaf tissue as the explant source (Woo and Wetzstein, Chapter 3). This method has proven suitable for field-grown material collected from mature specimens of different populations. Adventitious structures are induced on leaf explants placed on a medium with 10 μM thidiazuron (TDZ) + 5 μM indole-

3-acetic acid (IAA), and shoot expansion proceeds upon subculture to a secondary elongation medium supplemented with (2-isopentenyl) adenine (2iP). Shoots readily root *ex vitro* with high survival rates. Thus, tissue culture appears to be a promising approach for the propagation and conservation of this rare and threatened plant.

During culture initiation, a variety of proliferating structures are induced which include bud-like clusters, elongated embryo-like protrusions, and shoot-like structures. A number of reports indicate that both embryogenic and organogenic regeneration pathways can be induced under identical culture conditions in some species (Fiore *et al*., 1997; Castillo *et al*. 2000; Pasternak *et al*. 1999). TDZ, which is used for culture induction with this regenerations system, is a substituted phenylurea that has potent cytokinin activity in woody plant tissue culture (Huetteman & Preece, 1993). TDZ can promote cell division, differentiation, and induce somatic embryo and adventitious shoot development. For example, regeneration via shoot organogenesis and somatic embryogenesis was obtained in African violet leaf and petiole explants (Mithila *et al*. 2003) and leaf tissues of *Rosa hybrida* cvs. (Li, 2002) treated with TDZ.

Understanding morphological responses during in vitro culture is critical for understanding regeneration pathways. Recent reports illustrate the importance of histological evaluations of in vitro culture protocols (Salaj *et al*., 2005, Bassuner *et al*., 2007; Haensch, 2004; Madden *et al*., 2005), in that determining the regeneration pathway in a culture system is not always clear. Thus, the objective of this study was to evaluate the induction and development of structures induced on leaf explants of Georgia plume, *Elliottia racemosa*, when placed on an induction medium containing TDZ + IAA. Histological evaluations were made using light microscopy of sectioned material and scanning electron microscopy (SEM).

Materials and Methods

PLANT MATERIAL. Axenically-grown shoot cultures of *Elliottia racemosa* were the source of leaf tissues for culture induction studies. Proliferating cultures were previously established as described by Woo and Wetzstein (2007). Leaves were collected from shoot elongation cultures and placed on induction medium for developmental and histological studies. Briefly, young, expanding leaf tissue was cut into approximately 5 mm × 5 mm pieces, then placed abaxial side down onto GB_5 medium (Gamborg *et al*., 1968) supplemented with 10 μM TDZ, 5 μM IAA, 10 $mg \cdot l^{-1}$ thiamine HCL, 1 $mg \cdot l^{-1}$ pyridoxine, 1 $mg \cdot l^{-1}$ nicotinic acid, 40 $g \cdot l^{-1}$ maltose and 4 $g \cdot l^{-1}$ Gel-Gro (ICN Biomedicals, Aurora, OH). The pH of the medium was adjusted to pH 6.0 prior to autoclaving at 121°C for 20 min, then dispensed into 90 mm Petri dishes with 30 ml per plate. Explants were cultured under a 16-h photoperiod in a growth room using white fluorescent lights (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25°C. Cultures were collected at various times from induction and prepared for microscopic evaluations.

HISTOLOGICAL METHODS. Leaf explants were collected at 0, 1, 2, 3, and 4 weeks from initiation, and prepared for scanning (SEM) and light (LM) microscopy. For SEM, leaf tissues were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer for 4 h, washed in the same buffer, and dehydrated in an ethanol series (25, 35, 50, 65, 75, 85, 95, and 100%). Samples were critical point dried with carbon dioxide using a Samdri-780 critical point drier (Tousimis Research Corporation, Rockville, MD, USA), mounted on aluminum stubs using carbon conducted tabs, then sputter-coated with 60 nm gold using a SPI-Module™ (SPI Supplies Division of Structure Probe Inc.). Samples were observed with a JEOL 5200 SEM at 15 kV. Images were captured digitally. For LM, explants at different stages were fixed in Histochoice

Tissue Fixative (Amresco, Inc., Solon, OH), and dehydrated through a graded ethanol series of from 25 % to 95% ethanol. Tissues were then infiltrated and embedded into glycolmethacrylate (JB-4 embedding Kit, Polysciences, Inc., Warrington, PE). Infiltration was with daily changes in a 95% EtOH:infiltration medium series (75:25, 50:50, 25:75, 5: 95, and 0:100 for 3 times) (v/v). Tissues were finally embedded into JB-4 medium. Serial sections, 5 μm thick, were cut using a rotary microtome (Microm, Heidelberg, Federal Republic of Germany), mounted on slides, and stained by immersion in 0.1 % toluidine blue. Sections were observed and photographed with a BX51 Research Microscope (Olympus America, Inc., Center Valley, PA). For cell size and leaf thickness measurements, 10 leaf areas per treatment were measured.

Results

At the time of explanting (day 0), Georgia plume leaves exhibited the typical cellular organization found in leaf tissue (Fig. 4.1A). Leaves of Georgia plume had a uniseriate epidermis with differentiated palisade and spongy mesophyll layers. Leaves in cross section were composed of 7 to 9 cell layers, and prominent intercellular air spaces were observed in the spongy layer. Stomata were confined to the abaxial epidermis surface. Leaf surfaces had generally flat contours with epidermal cells and raised minor vein areas evident in SEM observations (Fig. 4.2A). Trichomes were occasionally seen on both adaxial and abaxial surfaces.

Incubation of leaf explants on induction medium caused a dramatic enlargement and swelling of tissues. After culture for 1 week, overall leaf thickness increased 2.6 fold (from 185 μm to 478 μm) (Fig. 4.1B and Table 4.1). This increase was caused by both cell division and cell enlargement. The number of cell layers increased by 1.5 times. Hypertrophy of cells was evident

with mesophyll height and spongy cell diameter increasing by 1.5 and 1.8 fold, respectively. This led to a more compact mesophyll cell organization in which tissues were composed of more numerous and larger cells with fewer intercellular spaces.

Massive cell proliferation and growth were observed in explants at 2 weeks on induction medium (Fig. 4.1C). Cell division occurred throughout internal cell layers resulting in an undifferentiated mesophyll region. Mean leaf thickness increased another 1.8 fold in the additional week on induction medium, which represented a 4.6 fold increase compared to leaves at time zero (Table 4.1). The number of cell layers increased to 18-22 cells thick, and both mesophyll and spongy cells increased to twice their original lengths. In addition to the more global cell proliferation found in mesophyll areas, localized regions of intense mitotic activity were observed close to the explant surface forming promeristem structures (Fig. 4.1C and 1D). These divisions were generally confined to the outer several mesophyll cell layers; mitotic activity of adjacent epidermal cells was limited so that mitotic centers appeared to be bounded by enlarged epidermal cells at regions on the leaf surface. These meristematic centers were composed of small, densely staining cells with high cytoplasmic content, and formed mounded protuberances on the explant surface (Fig. 4.2B).

At 3-4 weeks of culture, more extensive cell proliferation was observed. Numerous densely staining meristematic regions formed in proximity to explant surfaces (Fig. 4.1E). In some areas, these mitotic areas coalesced forming sectors of actively dividing cells at the surface. As cell proliferation progressed, displacement and/or discontinuities in epidermal cells were observed so that meristematic cells were on the explant surface with actively dividing areas organizing into layers suggestive of early apical meristem formation (Fig. 4.1F).

Further enlargement of cell clusters formed domed apical meristems, with some meristems exhibiting early leaf buttress formation (Fig. 4.3A). Apical meristems exhibited tunica-

corpus organization, and vacuolated parenchymatous cells subtended apical meristem regions (Fig. 4.3B). Adventitious shoots with leaf primordia were numerous (Fig. 4.3D, 4.3I), and had provascular strands (Fig. 4.3C and 4.3D). Evaluations of serial sections confirmed that vascular cells led to explant tissues with no organized root meristem observed. Adventitious shoot formation was not synchronized. Primordia at stages ranging from early protuberances to organized shoots with numerous leaf primordia were observed on the same explant (Fig. 4.2C and 4.2D, Fig. 4.3I).

Regenerated structures were quite variable. In addition to the shoot-like structures described above, elongated, tubular structures reminiscent of somatic embryos were observed (Fig. 4.3H). As evidenced with SEM, some of these structures were club-like or fan-shaped at apical areas, while others had organized apical regions (Fig. 4.2E, 2F, 2G). Light microscopy determined that elongated structures were composed primarily of files of vacuolated cells which were organized as multiple structures with varying degrees of fusion (Fig. 4.3E) or as single structures (Fig. 4.3F). Shoot meristem development likewise varied. Evaluations of serial sections showed that no organized root meristems were observed and that structures had a broad base of attachment to parent tissues (Fig. 4.3F and 4.3G), indicating that somatic embryogenesis did not occur.

Discussion

Our results indicate that plant regeneration of Georgia plume occurs though a shoot organogenic pathway in leaf explants induced on a medium with TDZ + IAA. Patterns of shoot organogenesis involve the formation of actively dividing meristematic regions originating in subepidermal cell layers. Similar structures have been referred to by a number of terms including

meristemoids, promeristems, and meristemoidlike precursors (Hicks, 1994). We have adopted the terminology of Colby *et al*. (1991), who define a promeristem as a meristematic center, arising de novo and consisting of a small number of densely staining, thin-walled cells, each containing a large, prominent nucleus. Promeristems in the current study continued proliferation and displaced epidermal cells so that the de novo shoot meristems were positioned on the explant surface.

A number of different types of structures were regenerated in this study which uses TDZ + IAA for induction. Our histological evaluations show that numerous well-formed shoot apical meristems are produced. However, fused shoot-like structures, tubular/elongated structures, and undifferentiated protuberances were observed as well. Some lacked an apical meristem. In histological studies by Krug *et al*. (2005), adventitious structures and protuberances that failed to develop into buds were observed in addition to shoot meristems. Some of these structures were thought to be isolated leaf primordia that are incapable of regenerating new plants. Stipp *et al*. (2001) similarly verified that non-regenerative protuberances are more frequently formed than complete bud shoots in *Cucumis* cultures resulting in low frequency of plant recovery.

Both organogenic and embryogenic morphogenic pathways have been reported to occur simultaneously in the same culture conditions. Detailed histological analyses in *Arabidopsis* indicate that three classes of regenerative structures were produced on the same 2,4-D amended medium: adventitious shoots, fused shoots, and somatic embryos (Bassuner *et al*. 2007). Plantlets were regenerated in barley (*Hordeum vulgare* L.) through various pathways which included somatic embryos from soft and compact calli and multiple shoots from green spots on compact callus (Pasternak *et al*. 1999).

Histological evaluations of Georgia plume cultures failed to identify somatic embryo development. Although some globular structures were observed that were suggestive of proembryo development, no bipolar structures with defined root meristems were detected as

would be anticipated in later embryogenic stages of development. Elongated, embryo-like structures identified from external observations lacked a defined root apex, and were composed at basal portions of parenchymatous files of cells with a broad point of attachment to the parent tissue. Various degrees of shoot apex development were found in elongated structures, ranging from meristems exhibiting histological zonations to those lacking meristem development. Anthony (2004) reported obtaining somatic embryogenic cultures in *Leucopogon verticillatus,* a species related to Georgia plume also in the Ericaceae family, using a medium with plant growth regulators similar to the current study, i.e., 10 μM TDZ + 5 μM IAA. Somatic embryos were not germinated directly; rather, somatic embryos were transferred from parent tissue to 1/2 strength basal medium for elongation, then subsequently rooted on IBA-containing medium. Structures shown resembled some of the elongated structures obtained in Georgia plume cultures. Histological evidence was not provided by Anthony to verify if regenerated structures were true somatic embryos (with root apices), partial somatic embryos, or shoots.

As suggested by Bassuner *et al*. (2007) many published reports dealing with somatic embryogenesis should be re-evaluated critically. In a number of cases, histological reevaluations of in vitro cultures show no recognizable root apex. Such was the case in *Pelargonium* (Haensch 2004; Madden *et al*. 2005) and flax (Salaj *et al*., 2005). Madden *et al*. (2005) proposed that "embryo-like" or "partial somatic embryogenesis" be used to describe structures in which all of the criteria for somatic embryogenesis are met except formation of a root pole.

TDZ can induce both embryogenesis and organogenesis, with the concentration of TDZ determining the regeneration pathway. In African violet (*Saintpaulia ionantha* Wendl.), low concentrations of TDZ (<2.5 μM) induced shoot organogenesis while high concentrations of TDZ (5-10 μM) induced somatic embryos (Milthila *et al*. 2003). Similarly, pigeonpea seedlings cultured on low (0.05 – 1.0 μM), intermediate (5 μM), or high (10 – 20 μM) concentrations of

TDZ induced multiple shoots, clusters of leafy structures, or somatic embryos, respectively (Singh *et al*. 2003). In *Pelargonium*, embryo-like structures and shoot organogenesis formation were observed on the same individual explant (Madden *et al*. 2005; Li, 2002). Use of the appropriate concentration and type of plant growth regulators can separate somatic embryogenesis and organogenesis pathways of development (Gaj, 2004). Histological evaluations conducted in conjunction with media manipulation studies may be a means to improve plant regeneration.

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Table 4.1. Characteristics of leaf explants placed on induction media with TDZ and IAA at different times after placement on induction medium.

Leaf explant characteristic [z]	0 week	1 week	2 weeks
No. of cell layers	7-9	11-13	18-22
Mean leaf thickness (μm)	185	478	860
Mean mesophyll cell cross sectional height (μm)	17	26	35
Mean spongy cell diameter (μm)	21	37	45

[z] Values are means of ten measurements per collection period.

Figure 4.1. Light micrographs showing the early stages of induction and morphogenesis of *Elliottia racemosa* leaf explants cultured on media supplemented with 10 μTDZ + 5 μ IAA. (**A**) Control leaf section showing a uniseriate epidermis with differentiated palisade parenchyma and spongy cells with prominent intercellular spaces. (**B**) Leaf explant after 1 week culture on induction medium. Mesophyll cells have divided and enlarged forming a compact cell organization, and thicker leaf. (**C**) Leaf explant at 2 weeks on induction medium showing massive cell proliferation and formation of meristematic centers. (**D**) An enlarged view of promeristem regions. (**E**) Explant after 4 weeks on induction medium. Meristematic activity is most prevalent in subepidermal areas. (**F**) Cell proliferation causes displacement of epidermal cells.

Fig. 4.1.

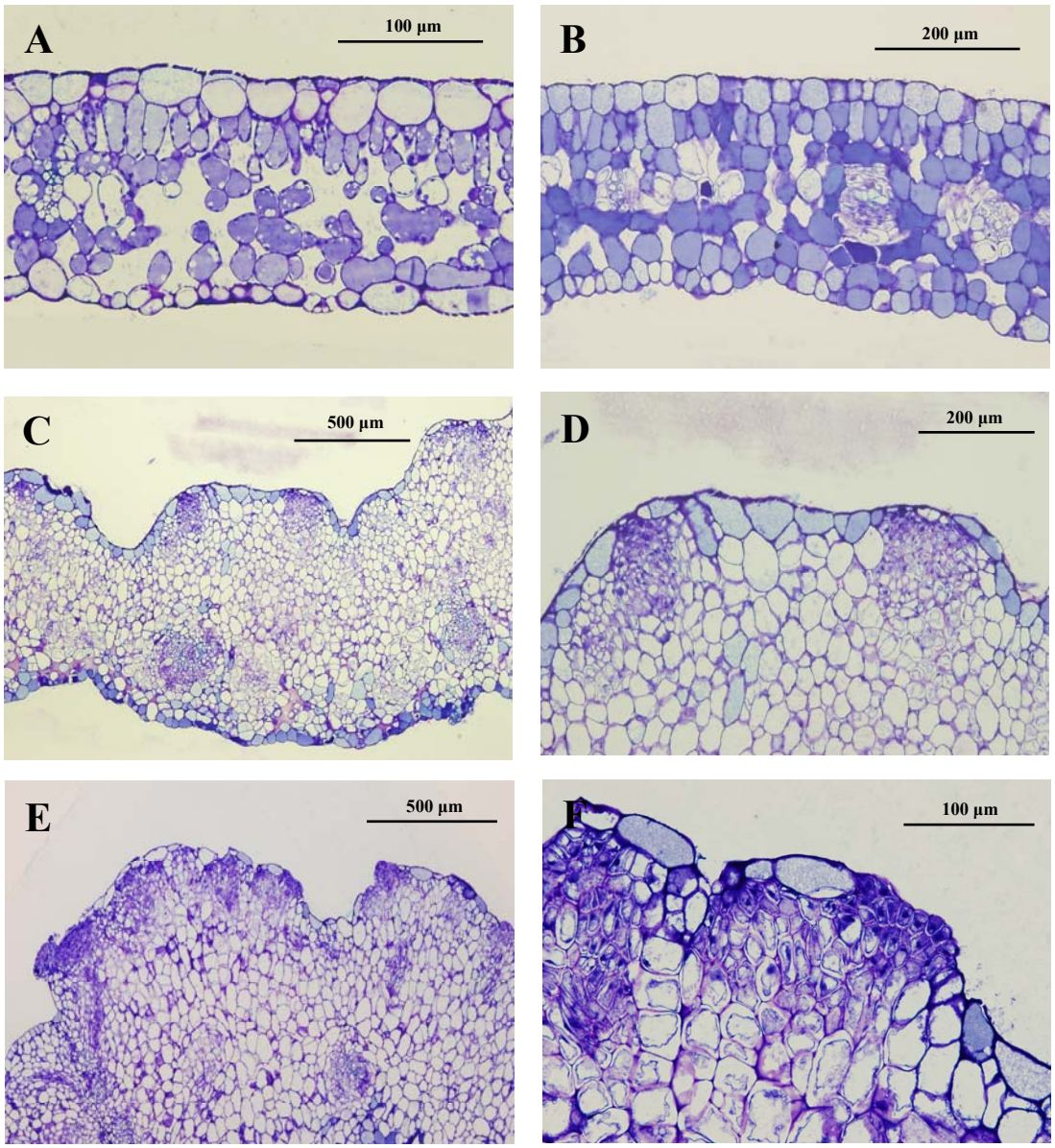


Figure 4.2. Light and dissecting micrographs of different types of regeneration structures. (**A**) Leaf surface at time of explanting. (**B**) Leaf surface at 2 weeks on induction medium showing rounded protrusions. (**C**) Bud development with shoot meristems. (**D**) Nonsynchronous development of numerous buds and shoots with meristem and leaf primordia. (**E**) Elongated embryo-like structure. (**F**) Numerous elongated structures with varying degrees of apical development. (**G**) Fused elongated structures with club-like or meristem development at apical areas.

Fig. 4.2.

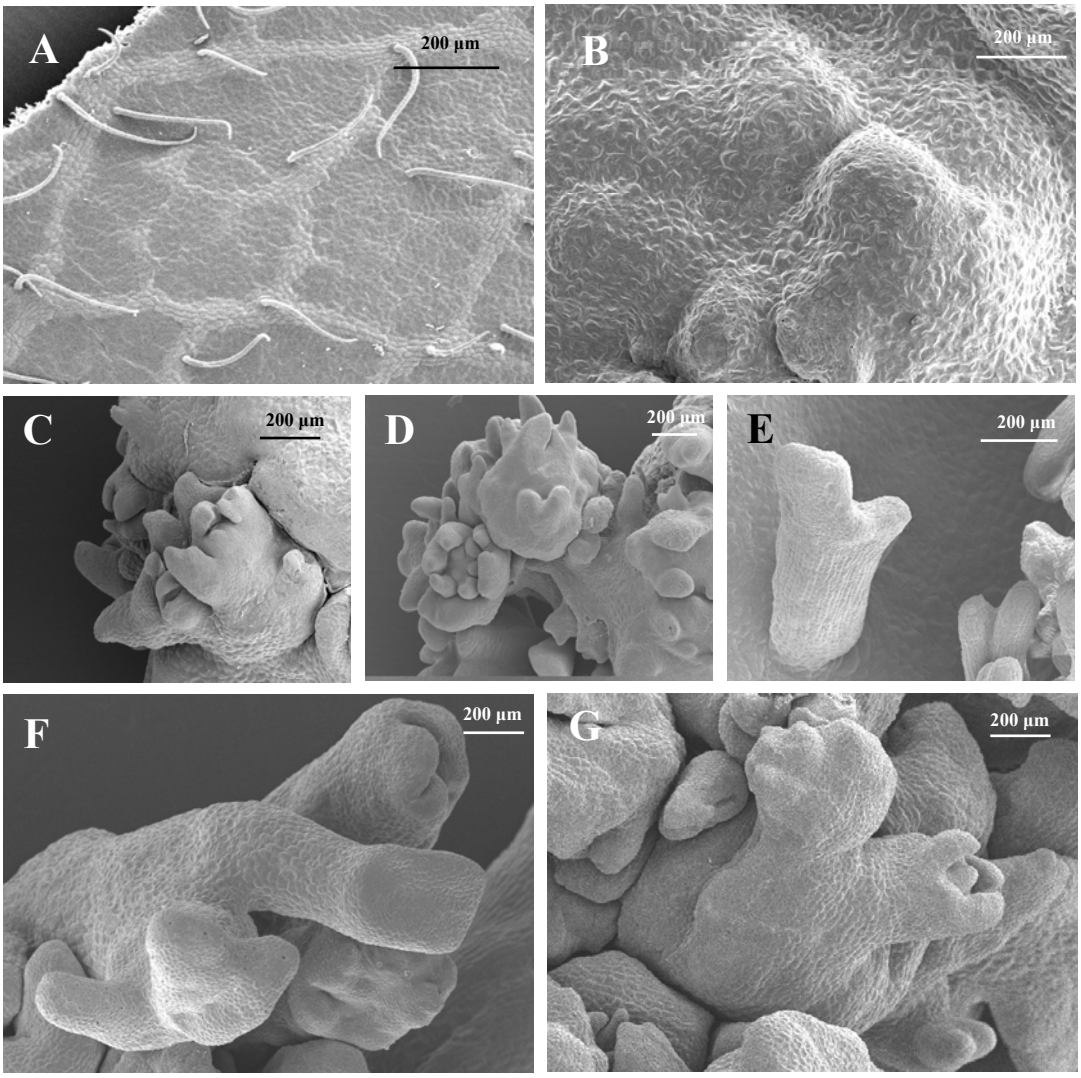
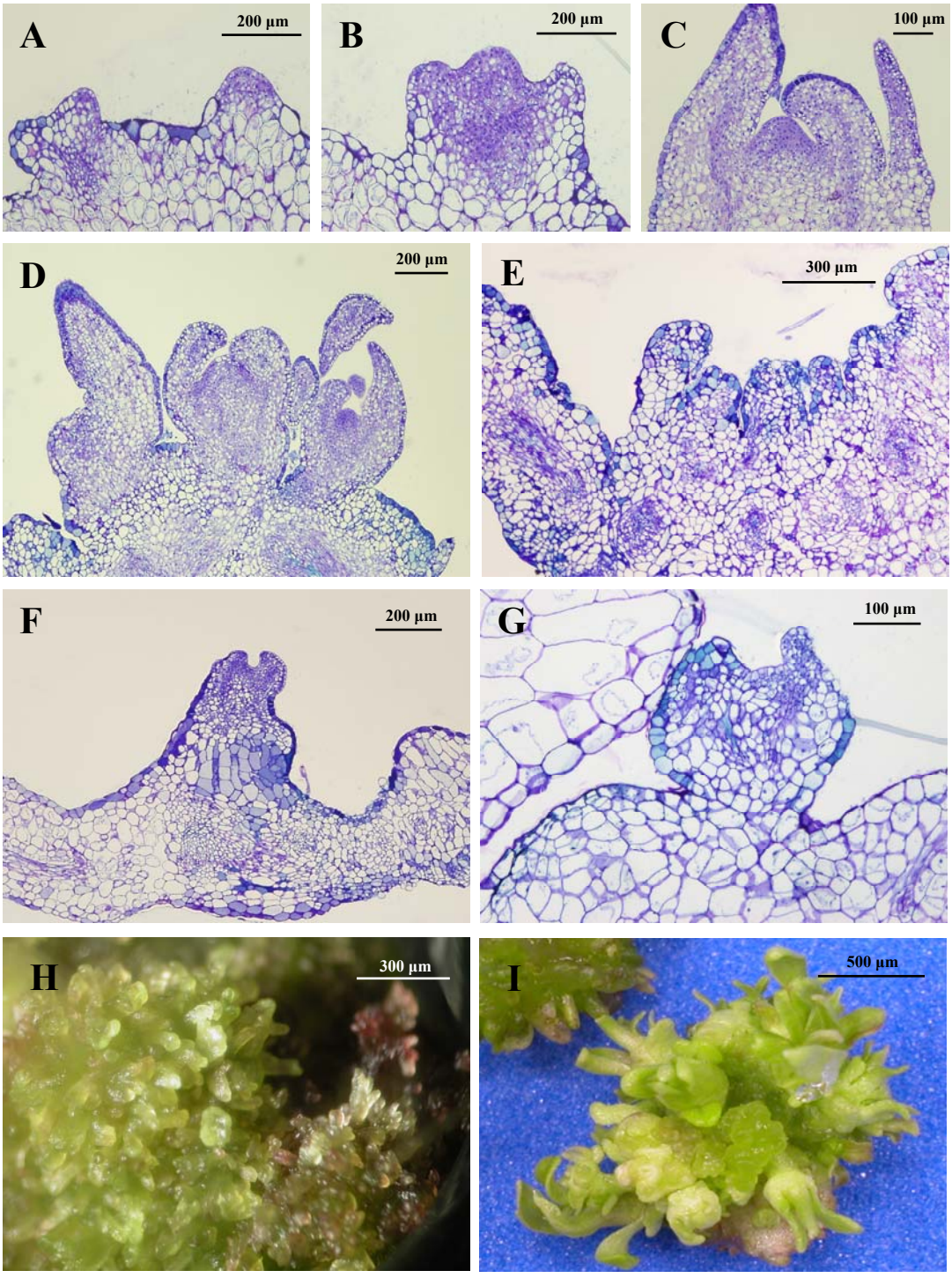


Figure 4.3. SEM micrographs of the sequence of development of adventitious shoot bud from leaf explants. (**A**) Meristematic structures have differentiated at the explant surface. (**B**) Shoot apex with tunica-corpus organization. (**C**) Shoot apex with leaf primoridia and development of vascular tissue. (**D**) At four weeks on induction medium, numerous shoot primordia have formed along the explant surface. (**E**) Multiple elongated structures lacking defined meristems. (**F**) Elongated structure with a defined apical meristem, but lacking a root apex and bipolarity. (**G**) Somatic embryo-like structure with no root pole. (**H**) Elongated protrusions. (**I**) Numerous buds and shoots.

Fig. 4.3.


A
200 µm
B
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CHAPTER 5

CONCLUSION

Elliottia racemosa, commonly called Georgia plume has not been known or studied broadly, even though this species has been introduced since 1813. Because of low pollen viability, self incompatibility, low or nonexistent seedset, and lack of sexual recruitment, reproduction in native stands is impended, thus it has been considered for addition to the list of endangered species. Conventional propagation methods such as seed germination and root cuttings are ineffective with Georgia plume. In this study, tissue culture methods were used for propagation and conservation of this species. For adventitious shoot organogenesis, young leaf tissues were effective as explants when placed on a GB_5 medium amended with 10 μM TDZ + 5 μM IAA. TDZ can induce both shoot organogenesis and somatic embryogenesis. However in some cases, it can inhibit shoot elongation. Proliferating buds were transferred to a medium with 25 μM 2iP containing WPM salts. Within 8 weeks, shoots were elongated and extended up to 4-5 cm tall. For rooting, three different methods: continuous auxin treatment, pulse auxin treatment, and ex vitro rooting were applied. All methods were very effective.

Histological analyses were conducted to obtain an understanding of morphological responses during in vitro culture development. Leaf explants exhibited cell division and mesophyll cell enlargement. Promeristems formed from localized meristematic activity of subepidermal cells. Histological analysis confirmed somatic embryo-like structures lacked bipolarity, with the absence of a root pole. After 3-4 weeks, shoot primordia with apical meristem

were observed.

This study is the first report involving in vitro propagation of Georgia plume. A reproducible, high frequency regeneration protocol was developed suitable for use with mature specimens in the wild. The application of these methods may be a means to propagate and conserve this threatened species.