STUDIES IN REPRODUCTIVE BIOLOGY AND *IN VITRO* PROPAGATION AS APPROACHES FOR THE CONSERVATION OF *ELLIOTTIA RACEMOSA*

by

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(Under the Direction of James M. Affolter)

ABSTRACT

Elliottia racemosa, commonly called Georgia plume, is a threatened, woody plant endemic to the Coastal Plain region of Georgia. There are currently less than three dozen populations in the wild, seed set is low in most populations, and very little is known about its reproductive biology. A series of studies were conducted to elucidate any factors that could be contributing to the lack of sexual reproduction in natural populations. *E. racemosa* exhibits normal flower development, structure, and function. Pollen viability was low to moderate, and may be problematic. Gametophytic self-incompatibility may be contributing to the decline of *E. racemosa* due to its fragmented distribution, low levels of genetic diversity, and possible pollinator limitation. In addition, the effectiveness of a micropropagation protocol previously developed for this rare species was evaluated for a range of genotypes originating from multiple wild populations. *In vitro* propagation was successful for most genotypes tested. It is feasible to use this protocol to generate genetically diverse plant material for safeguarding, reintroduction, and augmentation of existing populations. Such efforts may be critical to the preservation of this species.

INDEX WORDS: *Elliottia racemosa*, Georgia plume, plant conservation, *in vitro* propagation, reproductive biology

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DEDICATION

This work is dedicated to Marie Bruce, whose confidence, humor, kindness, and professional success was an inspiration to me during a very critical time in my life. I would like to dedicate the completion of this work to Uncle Scott and Aunt Vanessa, who encouraged and facilitated my pursuit of higher education. I hope I am likewise able to inspire youths to search for and fulfill their calling. To my son, Heron Grey: I love you. You can do anything if you follow your heart and use your head.

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Elliottia racemosa Muhlenberg ex Elliot (Georgia plume) is a threatened, woody plant that occurs only in the Coastal Plain region of Georgia. It was first discovered by the explorer William Bartram in 1773 and documented by botanist Stephen Elliott in 1807. After this time, the species was thought to be extinct until its rediscovery in 1901. Georgia plume grows on sand ridges, dry oak ridges, evergreen hammocks, and sandstone outcrops (Patrick et al., 1995). Its distribution has been fragmented by land development and the decline of suitable habitat. Georgia plume is currently found in less than three dozen populations in nineteen counties (Chafin, 2007).

A striking deciduous tree reaching heights of thirty feet, Georgia plume can have a single- or multi-trunked form. Plume-like racemes of white flowers appear in early summer. When capsules develop they ripen in the fall, however, the main mode of reproduction for Georgia plume is vegetative via root suckers. In recent times no seedlings of this species have been documented in the wild, indicating an absence of sexual recruitment. Low population numbers and a lack of sexual reproduction may lead to the demise of this plant, which has low genetic diversity both at the population and species level (Godt and Hamrick, 1999). It is listed as Threatened in the state of Georgia, but has no Federal listing.

Conventional propagation techniques have not been effective for Georgia plume. Propagation by shoot cuttings has been unsuccessful, and root cuttings can only produce

a limited number of shoots (Fordham, 1991). Albert Fordham, who served as Propagator at Arnold Arboretum, spent years working with this species. He found that prolonged stratification was required for germination of freshly sown seed and that secondary dormancy was acquired in older seed (Fordham, 1969). There is a high occurrence of inviable seed both in cultivation and in the wild (Fordham, 1969; Thompson and Spira, 1991), which had led to suggestions that this species has low pollen viability (Santamour, 1967) and may be self-incompatible (Thompson and Spira, 1991; Godt and Hamrick, 1999).

There is a lack of scientific knowledge about this species in general and little is known about its reproductive biology. A thorough understanding of the reproductive biology of a species is essential for its conservation (Bernardello et al., 2001). Sexual reproduction in plants is dependent on structural adaptations and developmental timing of inflorescences, individual flowers, as well as male and female reproductive structures (Heslop-Harrison, 1981). Pollination is the primary event involved in seed formation, and various types of pollination failure exist (Wilcock and Neiland, 2002). Certain mechanisms interfere with pollen hydration and germination and pollen tube growth (Franklin-Tong, 2002), and some of these could be contributing to the lack of sexual reproduction in this rare species. The main objective of this work was to develop a better understanding of the floral biology of Georgia plume as a means to identify any underlying factors that may be contributing to sexual failure in natural populations.

Georgia plume is a prime candidate for *ex situ* cultivation, which can be used to generate plants for reintroduction and safeguarding purposes. *Ex situ* propagation efforts often play an integral role in plant conservation (Maunder and Culham, 1999; Guerrant et

al., 2004). *In vitro* culture (sterile micropropagation) is commonly used to propagate rare species when a limited number of plants can be produced using conventional methods (Fay, 1992; Fay et al., 1999; Sarasan et al., 2006), and is especially useful when reproductive failure occurs in the wild (Guerrant et al., 2004). A micropropagation protocol was established by Woo and Wetzstein (2008) for Georgia plume to generate plants for out-planting studies. The aim of ongoing studies is to define protocols for reintroduction and augmentation of natural populations. Both *in situ* and *ex situ* efforts should be employed in the conservation of Georgia plume since the remaining populations are threatened by continued habitat loss.

In addition to studies in reproductive biology, the effectiveness of a previously developed *in vitro* propagation protocol was evaluated for a range of genotypes originating from multiple wild populations of Georgia plume. Responses to *in vitro* culture can be genotype dependent (Riseman and Chennareddy, 2004; Gandonou et al., 2005) and optimization may be needed in order to compensate for this variation (Khanna and Raina, 1998; Debnath, 2003; Gupta et al., 2006; Debnath, 2007). Clonal propagation of multiple genotypes from existing wild populations could be used as an effective conservation tool to provide germplasm for *ex situ* safeguarding and generate plant material for reintroduction and augmentation *in situ*, thus preserving the genetic diversity of this species.

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CHAPTER 2

FLORAL MORPHOLOGY AND DEVELOPMENT IN *ELLIOTTIA RACEMOSA*[1]

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Abstract

Background and Aims

Elliottia racemosa (Georgia plume) is a threatened, woody plant endemic to Georgia's Coastal Plain region. Seed set is low in most populations. The objective of this study was to develop a better understanding of the floral biology of Georgia plume to elucidate any underlying factors that contribute to the lack of sexual reproduction in natural populations. Stages of floral development were defined and characterized, stigma and anther development investigated, the morphological and histological characteristics associated with receptivity evaluated, and pollen viability assessed.

Methods

Light and scanning electron microscopy (SEM) were used to examine flower morphology and development at key stages of development ranging from small, unopened buds to open flowers with receptive stigmas. Tissue and chemical specific staining were used to evaluate pollen germination and to visualize reproductive structures and secretions. Pollen viability was assessed *in vitro*.

Key Results

Anther dehiscence occurred within immature buds. Pollen tetrads were aggregated by viscin strands and presented on unreceptive stigmas when flowers first opened. Exudate formed within a day of flower opening. Receptive stigmas lacked papillae and developed a raised and lobed central region with a clefted opening to a stylar canal where exudate was secreted. Exudate was produced in secretory regions beneath the stigma and stained positively for polysaccharides. The cuticle lining the stigma was disrupted during the

secretion of exudate. Pollen viability was low to moderate; tetrad germination ranged from 20 – 40%.

Conclusions

No developmental reproductive abnormalities were observed. *Elliottia racemosa* is protandrous and exhibits secondary pollen presentation. It has a wet non-papillate stigma. Receptivity is indicated by the formation of an exudate droplet which appears after petals reflex. Sugar-rich secretions and a hollow canal may support the growth of large numbers of pollen tubes. Low pollen viability may be problematic.

Key words: *Elliottia racemosa*, Georgia plume, floral development, pollen viability, protandry, secondary pollen presentation, stigmatic receptivity, stigmatic exudate, viscin

Introduction

Elliottia racemosa Muhlenberg ex Elliot (Georgia plume) is a threatened, woody plant that occurs only in the Coastal Plain region of Georgia. It was first discovered by the explorer William Bartram in 1773 and documented by botanist Stephen Elliott in 1807. After this time, the species was thought to be extinct until its rediscovery in 1901. Georgia plume grows on sand ridges, dry oak ridges, evergreen hammocks, and sandstone outcrops (Patrick et al., 1995). Its distribution has been fragmented by land development and the decline of suitable habitat. Georgia plume is currently found in less than three dozen populations in nineteen counties (Chafin, 2007).

A striking deciduous tree reaching heights of thirty feet, it can have a single or multi-trunked form. Plume-like racemes of white flowers appear in early summer. When capsules develop they ripen in the fall, however, the main mode of reproduction for Georgia plume is vegetative via root suckers. In recent times no seedlings have been documented in the wild, indicating an absence of sexual recruitment. Low population numbers and a lack of sexual reproduction may lead to the demise of this plant, which has low genetic diversity at both the population and species level (Godt and Hamrick, 1999). It is listed as Threatened in the state of Georgia, but has no Federal listing.

There is a lack of scientific knowledge about this species in general and little is known about its reproductive biology. The objective of this study was to develop a better understanding of the floral biology of Georgia plume to identify any underlying factors that contribute to the lack of sexual reproduction in natural populations. Specifically, stages of floral development were defined and characterized, stigma and anther

development investigated, the morphological and histological characteristics associated with receptivity evaluated, and pollen viability assessed.

Materials and Methods

Plant Material

The flowers used in this study were from nursery grown plants in Atlanta Botanical Garden's conservation collection. Plants were approximately 90 – 120 cm in height and were potted in 11 liter pots. Plants represented genotypes from a population in Big Hammock Natural Area in Tattnall County. Flowers were open-pollinated in the nursery. Distinct stages of floral development were defined initially based on macroscopic differences including bud size and color. Letters (A-D) were used to denote stages (Fig. 2.1).

Microscopy Methods

Flowers representing each stage were dissected and prepared using methods described by Woo and Wetzstein (2008) for scanning electron microscopy. Flowers were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer, washed in the same buffer, serial dehydrated using ethanol, and critical point dried with carbon dioxide using a Samdri-790 critical point drier (Tousimis Research Corp., Rockville, MD). Dried samples were mounted on aluminum stubs using carbon conductive tabs and sputter-coated with 60-nm gold using a SPI-Module (SPI Supplies Division, Structure Probe, West Chester, PA). Samples were observed at 15 kV using a Ziess SEM (Carl Zeiss, Oberkochen, Germany). Fresh samples were dissected, mounted and immediately viewed using the SEM.

For light microscopy, flowers of each stage were dissected, fixed using Histochoice tissue fixative (Amresco, Solon, OH) and dehydrated through a graded ethanol series from 25% to 95%. Samples were then infiltrated and embedded into glycol methacrylate (JB-4 embedding kit; Polysciences, Warrington, PA). Samples were cut into 5µm serial sections using a rotary microtome (Microm, Heidelberg, Germany). Sections were mounted, stained, and examined using an Olympus BX51 Research Microscope (Olympus America, Center Valley, PA). For general histological observations, sections were stained with 1% Acid Fuchsin (w/v) and 0.05% Toluidine Blue O (w/v). Periodic acid-Schiff (PAS) was used to localize polysaccharides using a saturated solution of dimedone as an aldehyde blocking reagent. Proteins were localized with 0.1% Coomassie Blue in water : methanol : acetic acid (87:10:1, v/v). Cuticles were visualized using 0.01% Auramine O (w/v) in Tris-HCl buffer (pH 7.2) under fluorescent light.

Pollen tube growth assessments

Pollen tube germination and growth within the styles were assessed using aniline blue and fluorescent microscopy as described by Yi et al. (2006). Stigmas and styles were dissected and fixed in ethanol : acetic acid 3 : 1 (v/v). Tissues were softened by autoclaving at 120ºC for 10 min in 1 % sodium sulfite solution (w/v), stained with aniline blue (0.01 % aniline blue in 0.1 M K_3PO_4), then examined under regular and UV light using an Olympus BX51 Research Microscope (Olympus America, Center Valley, PA).

Pollen viability

Pollen used in germination assays were from inflorescences collected in the field from Big Hammock Natural Area in Tattnal County, Charles Harrold Nature Preserve in Candler County, Manassas Bog in Tattnal County, and a population in Turner County. *In*

vitro germination assays were conducted according to Yi et al. (2003). Pollen was inoculated in microwell plates using a standard germination medium (0.062% $CaNO_3$ and 0.024% boric acid) containing 15% sucrose. Germinating pollen was viewed and counted using a Nikon DIAPHOT inverted microscope (Nikon, Garden City, NY). Tetrads were considered germinated if one or more tubes extended a length greater than the diameter of a pollen grain. Tubes were counted using a gridded ocular.

Results

Inflorescences of Georgia plume are 7.5 – 30 cm long and borne terminally. There are 25 – 60 flowers per inflorescence, which opens acropetally from base to tip. Flowers have 4 or 5 free petals, 12 – 16 mm long, which are strongly reflexed when flowers are open. Nectaries are inside flowers and concealed by the petals, contributing to the faint scent of the flowers (Bohm et al., 1978). There are 8 – 10 stamens per flower. The ovary is superior with 4-5 locules, each with 5 – 8 ovules. Fruit are capsules roughly 1 cm in diameter which dehisce septicidally and contain winged seed.

Distinct stages of floral development were initially defined according to bud size and petal parameters (Fig. 2.1) ranging from immature, closed buds to receptive flowers with petals reflexed. Macroscopic characteristics of flower stages were as follows: stage A buds were 4 – 6 mm long, green or greenish-white in color, and held upright on pedicles (Fig. 3.1); stage B buds were > 6 – 9 mm long, white, and nodding (Fig. 2.1); stage C buds were > 9 – 14 mm long, pendulous, and with petals distally attached but separated in the median regions (Fig. 2.1); stage D flowers were open with petals reflexed

(Fig. 2.1). Individual flowers progressed from stage A through stage D in approximately 9 days. Each stage was analyzed microscopically in greater detail.

Although stage A buds (Fig. 2.2A) were immature and small (4 – 6 mm long), the stigma and anthers were well differentiated (Fig. 2.2B). The stamens were about half the length of the closed buds, and anthers were partially to fully dehisced. Anther dehiscence originated at circular, pore-like regions at the tips of anthers (Fig. 2.2B). Dehiscence continued longitudinally along the length of the anther walls. Light micrographs of anthers in cross section show pollen, in tetrads of four fused pollen grains, still enclosed within partially dehisced anther sacs inside immature buds (Fig. 2.2C). Tetrads measured approximately 50 μm across and were surrounded by a filamentous substance that kept the tetrads aggregated in large clumps (Fig. 2.2D). Particulate material was seen on stigmas and was most likely remnants of tapetal material from anthers (Fig. 2.2F). The disk-shaped stigmatic surfaces appeared smooth and non-papillate, with clefted openings visible in the center (Fig. 2.2E). There was no evidence of exudate. Epidermal cells lining the clefted stigmatic opening stained darkly and were well differentiated (Fig. 2.2G). Cells surrounding this clefted region stained more lightly, were undifferentiated, and were still undergoing cell division.

In stage B, buds were larger (> 6 – 9 mm long) and pendulous (Fig. 2.3A). Dehisced anthers were completely empty (Fig. 2.3B), and pollen clumps were aggregated in the tips of the flower buds where petals were distally attached. The stamens were elongated to their maximum length which was about one third of the total bud length. As buds elongated, pistils likewise increased in length so that stigmas were appressed to the distal portion where petals were fused at the apex. By this stage stigmas had developed a

mounded appearance. Under higher magnifications the stigmatic surfaces appeared textured and had tapetal and other particulate materials on the surface, but pollen did not adhere (Fig. 2.3C). The clefted opening in the center of the stigma became more prominent as four surrounding lobes began to develop, evident in fixed (Fig. 2.3D) and fresh (Fig. 2.3E) samples. Some pollen tetrads were observed on the stigmatic surface of fresh, unfixed tissues (Fig. 2.3E) but were washed away during fixation (Fig. 2.3D) indicating that stigmas at this stage failed to capture and adhere pollen. Secretory glands were differentiated in the sub-surface layers of the stigma. Each glandular region consisted of loosely arranged cells and was located between the clefts of the stigmatic opening (Fig. 2.3F). Stigmas had an intact cuticle covering the stigma (Fig. 2.3G), which remained free of surface secretions at this stage.

Stage C buds were > 9 – 14 mm long, pendulous, and with petals distally attached but showing separation in the median regions (Fig. 2.4A). Pistils extended the full length of buds, and developed a curved configuration near the stigma-style interface. Cross sections showed that styles were solid and lacked a central canal at this time (Fig. 2.4C). Stigmas were more mounded than the previous stage (Fig. 2.3B, D), and the clefted openings more prominent (Fig. 2.4D). Stigmatic lobes were more developed in contrast to the outer rim of the stigma (Fig. 2.4D). Under higher magnifications, stigmas were more noticeably textured as surface cells became elongated (Fig. 2.4E). Stigmas were appressed into cup-shaped regions formed where petals coalesced distally, and after flowers were dissected to open petals some pollen remained attached on the stigmatic surfaces. However, exudate was lacking, pollen did not adhere, and no pollen germination was observed.

Stage D flowers were open with petals reflexed and styles curved (Fig. 2.5A). Pollen was frequently presented on the unreceptive stigmas of flowers that had just opened (Fig. 2.4F, 2.4G). Stigmas lacked surface exudate when petals first reflexed, but exudate was secreted within 6 - 24 hours after flower opening. Pollen capture and germination occurred at this time and was restricted to the central cleft where exudate accumulated as shown in conventionally fixed and critical point dried samples (Fig. 2.5B, 2.5C). A widened stigmatic opening was evident (Fig. 2.5B) because exudate was partially removed during the fixation process. Observations of fresh, unfixed samples (Fig. 2.5D-F) showed the copious amounts of exudate released into the stigmatic cleft. Stigmas did not develop papillae. Pollen tetrads adhered to the stigma (Fig. 2.5E) and became hydrated when immersed in exudate (Fig. 2.5F). Pollen tubes of the germinated tetrads penetrated the clefted opening (Fig. 2.5B). The clefted openings were well defined by an epidermal layer and had a cuticular layer. The cuticular layer lining the stigmatic opening was discontinuous after exudate had been secreted (Fig. 2.5G). A stylar canal (Fig. 2.5H) was not observed until flowers reached this stage. The canal converged with the clefted stigmatic opening (Fig. 2.5I). Canals were hollow, lysigenous, and lacked epidermal cells. Exudate stained positively for polysaccharides (Fig. 2.5J) but negatively for proteins.

Pollen tube staining showed that pollen tubes grew within the central canal; tubes were observed only in open flowers with exudate (Fig. 2.5K). Pollen germination assays revealed that viability of the tetrads was low; germination of pollen tetrads from four populations ranged from 25% to 41.5%. The average number of pollen tubes per tetrad ranged from 1.1 to 1.6.

Discussion

Elliottia racemosa displays many floral traits similar to other members of the Ericaceae (Hesse, 1983; Williams and Rouse, 1990; Palser et al., 1992; Zomlefer, 1994; Hermann and Palser, 2000; Lu et al., 2009); anthers dehisce longitudinally, and binucleate pollen is borne in tetrads of four fused pollen grains connected by viscin strands. Several studies have commented on the effect of viscin on pollen dispersal. Viscin threads function to hold pollen together in clumps and enhance pollen transfer efficiency (Cruden and Jensen, 1979; Smithhuerta, 1991). Fossilized flowers belonging to Ericales with viscin strands suggest that specific pollinator-plant relationships existed as early as the mid-Cretaceous period (Nixon and Crepet, 1993). Pollination by bumble bees is facilitated by low frequency vibration of anthers and viscin threads in *Rhododendron* sp. (King and Buchmann, 1995). In *Berberis thunbergii*, pollen clumps held together by viscin were removed partially by a number of floral visitors (Lebuhn and Anderson, 1994). Such a pattern should prevent pollen from being disseminated completely to one floral visitor and promote more effective gene dispersal (Erbar and Leins, 1995). The aggregation of pollen in clumps by viscin is thought to prevent wind dispersal (Kevan et al., 1991).

Elliottia racemosa can be considered protandrous, since pollen is available for outcrossing when flowers first open, prior to exudate production and the onset of stigma receptivity which occurs 6 – 24 hours after petal opening. Plants that exhibit pollen presentation by secondary means are almost always protandrous (Howell et al., 1993). Secondary pollen presentation exists when pollen undergoes an intermediate transfer from anthers to a presenting structure, making it available for transfer by biotic or abiotic

means (Ladd, 1994). While there is no structure adapted solely to this function in Georgia plume, the un-receptive stigmas can bear pollen in large clumps held together by viscin. In the absence of stigmatic exudate, pollen does not germinate and is easily removed by floral visitors. However, if autogamous pollen is still present when stigmas become receptive, lack of available space may prevent outcross pollen from landing and germinating on the stigmatic surface. This is very similar to *Cephalanthus occidentalis*, which likewise presents its pollen on unreceptive stigmas (Imbert and Richards, 1993). In *C. occidentalis*, pollen is shed onto stigmas within closed buds and removed by pollinators after flowers open. Increased risks of self-pollination can be offset or even overcome by self-incompatibility. *C. occidentalis* exhibits gametophytic self-incompatibility, which allows pollen tubes to penetrate stigmas but prevents self-fertilization by inhibiting growth of tubes within the style. The amount of selfing vs. outcrossing depends on the timing and degree of self pollen removal and also the precise timing of stigmatic receptivity. Even when an incompatibility system is operative, the presence of large loads of self pollen can "clog" stigmas and exclude compatible pollen (Howell et al., 1993). Further work is needed to determine if self-incompatibility exists in *E. racemosa*.

The stigma of *Elliottia racemosa* is of the wet, non-papillate type. Stigmas of *Rhododendron* spp. are also characterized as such. In *E. racemosa* receptivity is indicated by the formation of an exudate droplet that develops after petals reflex and flowers are open. Exudate formation is delayed until 6 – 24 hours after buds open. Delayed stigmatic receptivity is seen in other species, including *Collinsia heterophylla* (Lankinen et al., 2007), *Prunis dulcis* (Yi et al., 2006), and *Vaccinium ashei* (Brevis et al., 2006). Stigmas

of *E. racemosa* lack papillae and develop a raised and lobed central region with a clefted opening to the stylar canal where exudate is secreted. As described by Heslop-Harrison and Shivanna (1977), wet stigmas have noticeable, free-flowing secretions on the surface when receptive. Other Ericaceous genera with wet, non-papillate stigmas include *Azalea*, *Gaultheria*, *Kalmia*, and *Rhododendron*. Other plants in the wet non-papillate category commonly display protandry, as is seen in Liliaceae and Bromeliaceae, where flowers appear dry upon opening followed by production of copious exudate between the stigmatic lobes (Heslop-Harrison and Shivanna, 1977).

A proteinaceous pellicle was not found in *Elliottia racemosa* but is normally only observed in dry stigma types. Ericaceous plants are known to have either smooth or papillate stigmas, but are almost all wet (Heslop-Harrison, 1981). In *E. racemosa* exudate is produced and secreted from the glandular region in the sub-surface layers of the stigma. Secretory cells are arranged loosely, forming large intercellular spaces as the flowers mature. At the time of exudate secretion the stigmatic cleft widens, epidermal cells lining the opening become separated, and the cuticular lining ruptures. This is similar to the structures and process present in *Nicotiana sylvestris* and *N. tabacum*, which have a subepidermal secretory zone in the stigma that converges into the transmitting tract of the style (Cresti et al., 1986; Kandasamy and Kristen, 1987). In many species with wet stigma types, exudate is secreted through exocytosis by the endoplasmic reticulum (Shivanna, 2003). Exocytotic secretion is associated with exudate release in other species that posses similarly organized glandular regions in their pistils (Cresti et al., 1986; Mackenzie et al., 1990; Janson et al., 1994).

The stigmatic opening in *Elliottia racemosa* converges with a hollow, lysigenous stylar canal that forms as the tubular core cells disintegrate during elongation of the style. Lysigenous canal development is also found in *Colophospermum mopane*, creating a continuum from stigma to ovary following the dissolution of core cells in the style (Jordaan et al., 2002). Hollow canals are able to accommodate growth of hundreds of pollen tubes (Schmidt-Adam and Murray, 2002). Exudates found within the hollow stylar canals of *Rhododendron spp.* also stain positively for polysaccharides (Palser et al., 1992). Exudates containing sugars are thought to serve as a carbon source for large numbers of growing pollen tubes (Slater and Calder, 1990; Schmidt-Adam and Murray, 2002).

Pollen viability for *E. racemosa* may be considered low on a per pollen basis or moderate per tetrad, and it varied among the 4 populations observed. Tetrad germination ranged from 25% to 41.5%. On average, less than 2 pollen grains per tetrad germinated. Results in this study were similar to low estimates of pollen tetrad viability (20%) using aceto-carmine staining (Santamour, 1967). It was suggested that excessive inbreeding could have resulted in the accumulation and fixation of deleterious recessive genes, leading to low pollen viability. Population size and genotypic diversity does vary among populations of *E. racemosa*, but genetic diversity within some populations was found to be low (Godt and Hamrick, 1999). Because populations are relatively small and isolated, inbreeding among closely related individuals is likely to occur even if this species is self-incompatible.

Conclusion

Elliottia racemosa is protandrous and exhibits secondary pollen presentation via the unreceptive stigma. Although anther dehiscence occurs early in flower bud development, pollen capture is spatially and temporally limited to a small central region of the stigma surrounding the opening to a lysigenous stylar canal where exudate is secreted at receptivity. *E. racemosa* has a wet non-papillate stigma type. The presence of exudate on the stigmatic surface is critical to pollen capture, hydration, and germination. Copious exudate rich in polysaccharides and a hollow stylar canal may support the growth of large numbers of pollen tubes. Pollen viability is low to moderate per tetrad and varies with population. No developmental reproductive abnormalities were observed in Georgia plume. This suggests that the decline of this species may be related to its fragmented distribution, low level of genetic diversity, and subsequent inbreeding depression.

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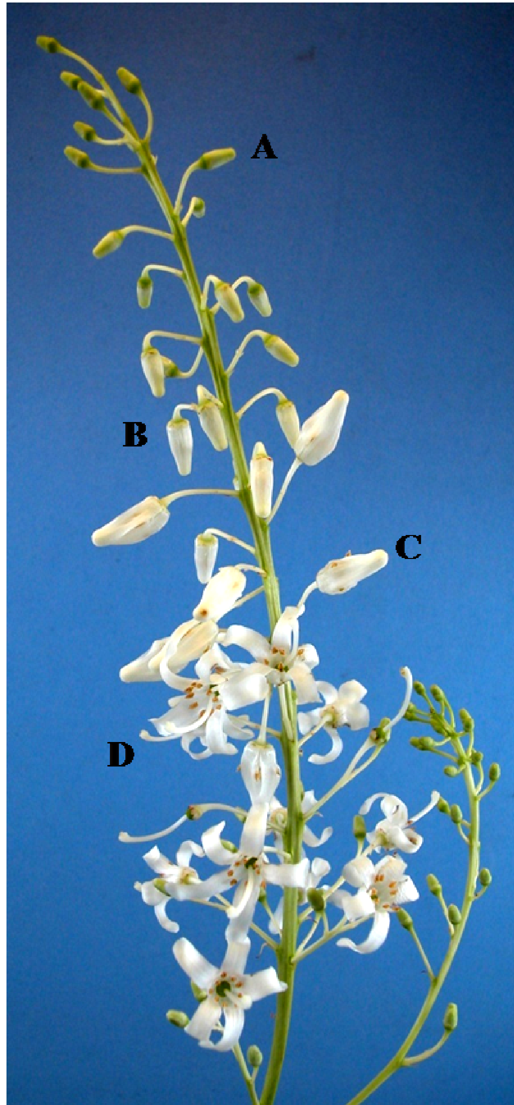

Figure 2.1. Inflorescence with flowers labeled stages A – D.

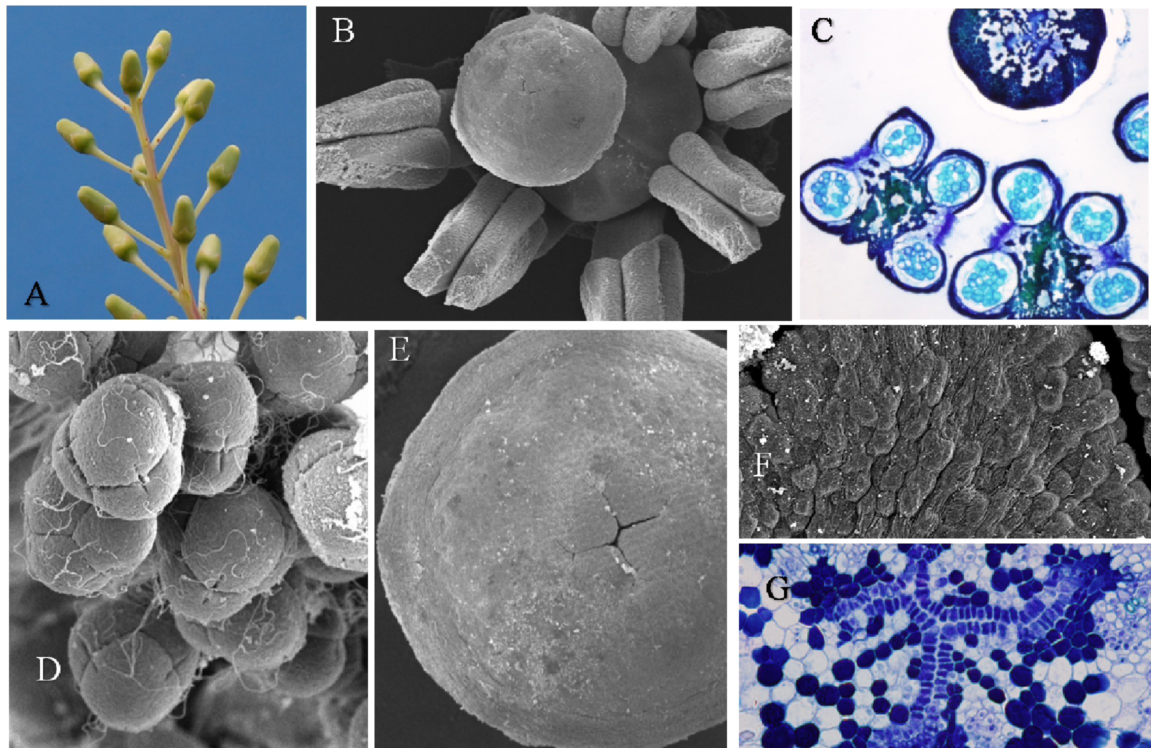

Figure 2.2. A) Stage A buds (4 – 6 mm long). B) Immature bud with petals removed, showing differentiated but undeveloped floral organs. Anthers showing dehiscence at tips. C) Pollen inside anther sacs, surrounding the stigma. D) Pollen tetrads surrounded by viscin and aggregated in clumps. E) Smooth stigmatic surface with cleft in the center. F) Powdery tapetal material on the smooth stigmatic surface. G) Epidermal lining the stigmatic cleft.

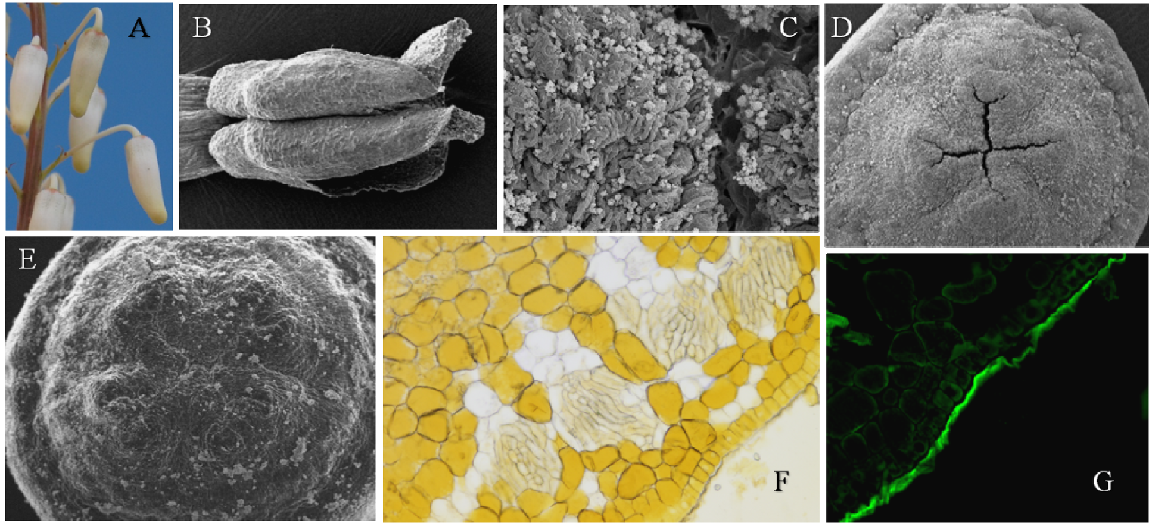

Figure 2.3. A) Stage B buds (> 6 – 9 mm long). B) Empty anthers. C) Textured surface of stigma dusted with tapetal materials, but free of pollen. D) Stigmatic cleft. E) Fresh stigma with some pollen. F) Glandular regions containing secretory cells in the sub-surface layers of the stigma. G) Intact cuticle covering the stigma.

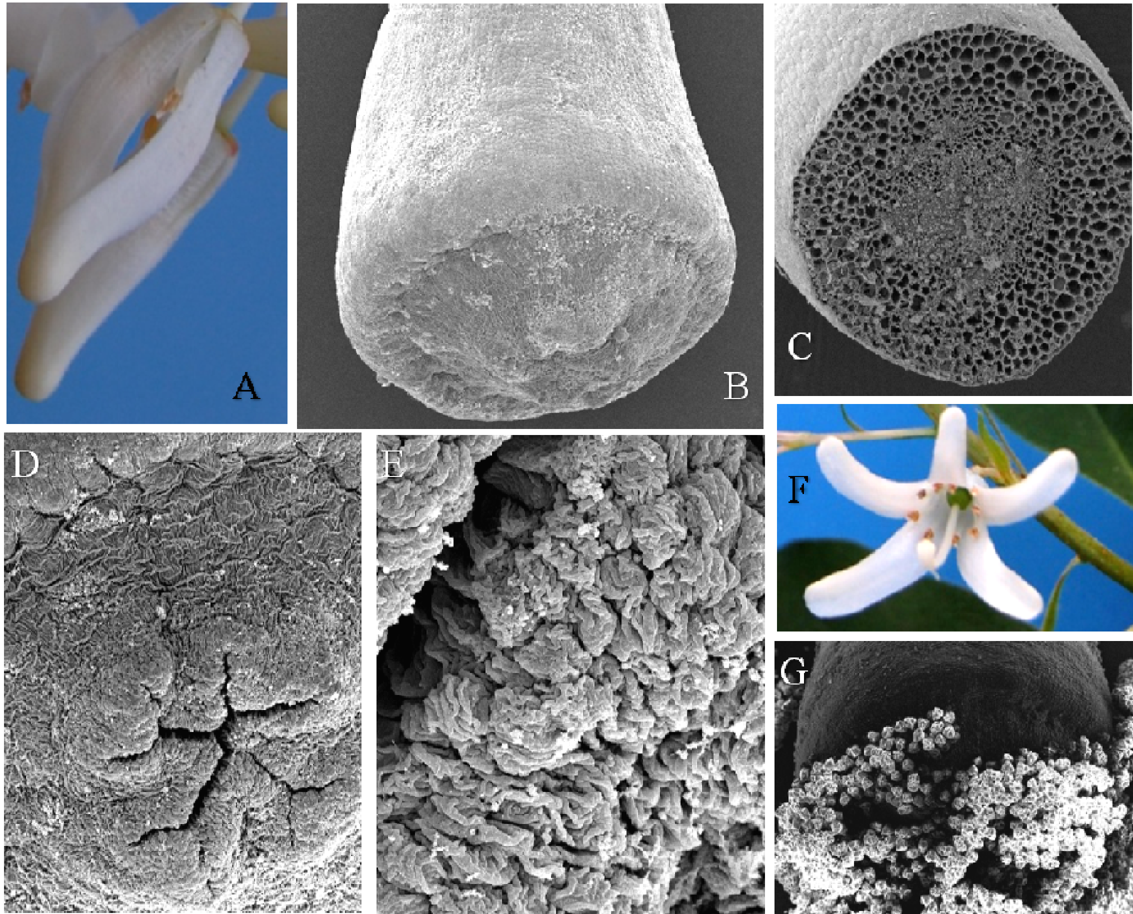

Figure 2.4. Stage C flowers (A – E) and Stage D flowers (F – G). A) Stage C bud (> 9 mm long) with petals separated in the median region. B) Slight curvature of pistil at the style-stigma interface. C) Solid style. D) Mounded stigmatic surface with prominent cleft. E) Textured stigmatic surface composed of elongated surface cells. F) Pollen presentation on the unreceptive stigma of a flower with petals reflexed. G) Clumps of pollen presented on a fresh stigma.

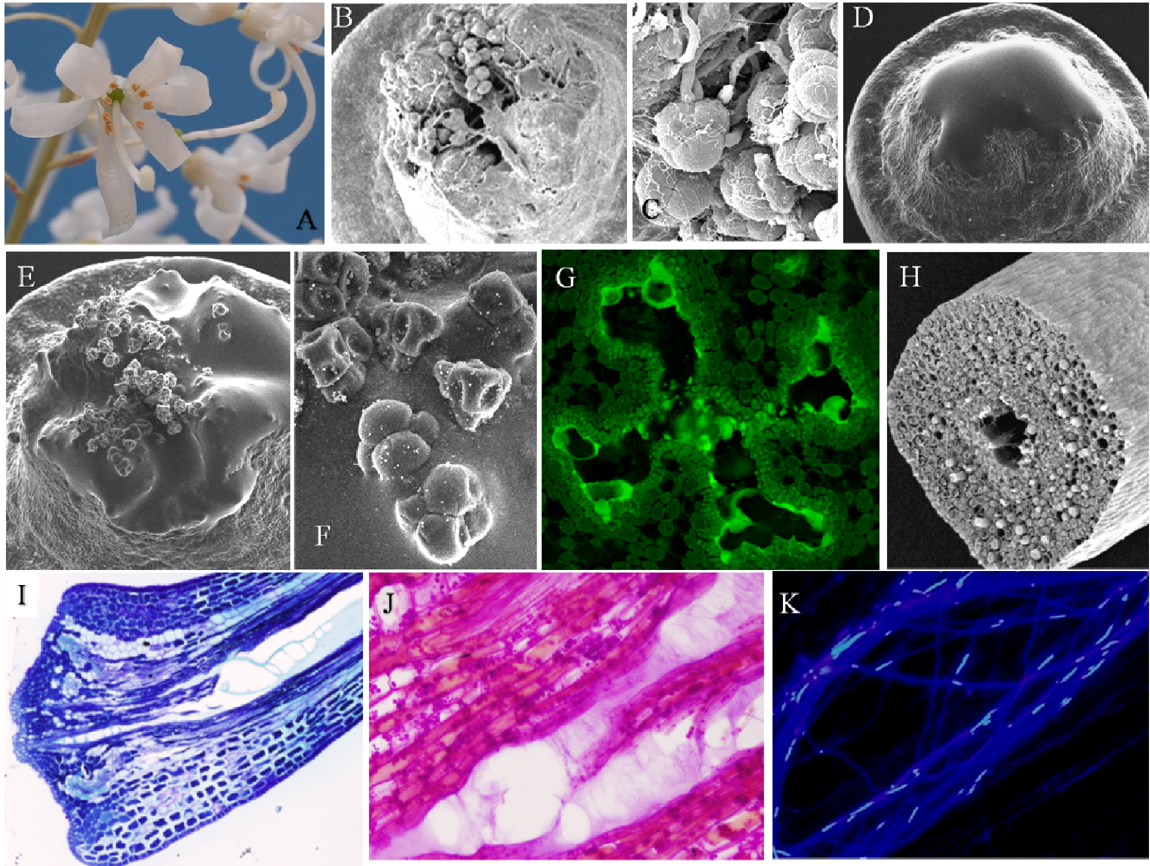

Figure 2.5. A) Stage D flowers with petals reflexed. B) Pollen tetrads and tubes in the clefted region of the stigma. C) Germinated pollen tetrads. D) Fresh sample with copious exudate in the clefted region of the stigma. E) Pollen capture. F) Pollen hydration. G) Discontinuous cuticle in the stigmatic cleft following exudate secretion. H) Cross section revealing the stylar canal. I) Lysigenous stylar canal converging with the stigmatic cleft. J) PAS-positive staining of exudate within the canal. K) Pollen tubes traversing the stylar canal.

CHAPTER 3

FLORAL PHENOLOGYAND SELF-INCOMPATIBILITY IN *ELLIOTTIA RACEMOSA*[1]

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Abstract

Background and Aims

Elliottia racemosa (Georgia plume) is a threatened, woody plant endemic to Georgia's Coastal Plain region. Seed set is low or non-existent in many populations. The objective of this study was to observe and document the flowering phenology of Georgia plume and to determine if self-incompatibility exists in this species.

Methods

Floral phenology was observed and measured based on bud size and petal parameters. Pollen tube staining, using the fluorescent stain aniline blue, was used to visualize pollen tube germination and growth within styles following controlled pollinations.

Key Results

Inflorescences develop acropetally. The sequential development and opening of basal, median, and terminal flowers extends the duration of flowering within inflorescences. Gametophytic self incompatibility was observed to inhibit growth of self pollen tubes within the style.

Conclusions

The flowering phenology and self-incompatibility in *Elliottia racemosa* promote outcrossing. Gametophytic self-incompatibility may be contributing to the decline of this species due to its fragmented distribution, low genetic diversity, and possible pollinator limitation. Suggestions for prioritizing conservation efforts are made.

Key words: *Elliottia racemosa*, Georgia plume, conservation, floral phenology, gametophytic self-incompatibility

Introduction

Elliottia racemosa Muhlenberg ex Elliot (Georgia plume) is a threatened, woody plant that occurs only in the Coastal Plain region of Georgia. It was first discovered by the explorer William Bartram in 1773 and documented by botanist Stephen Elliott in 1807. After this time, the species was thought to be extinct until its rediscovery in 1901. Georgia plume grows on sand ridges, dry oak ridges, evergreen hammocks, and sandstone outcrops (Patrick et al., 1995). Its distribution has been fragmented by land development and the decline of suitable habitat. Georgia plume is currently found in less than three dozen populations in nineteen counties (Chafin, 2007). Low population numbers and a lack of sexual reproduction may lead to the demise of this plant, which has low genetic diversity at both the population and species level (Godt and Hamrick, 1999). It is listed as Threatened in the state of Georgia, but has no Federal listing.

A striking deciduous tree reaching heights of thirty feet, Georgia plume can have a single or multi-trunked form. Plume-like racemes of white flowers appear in early summer on terminal inflorescences. Generalist pollinators have been observed visiting flowers in the field. When capsules develop they ripen in the fall, however, the main mode of reproduction for Georgia plume is vegetative via root suckers. In recent times no seedlings have been documented in the wild, indicating an absence of sexual recruitment. Self-incompatibility has been suspected but not verified. Lacking for Georgia plume is an understanding of the timing of flower emergence, sequence of blooming, and receptivity.

Our previous studies characterize Georgia plume as having a wet non-papillate stigma type as defined by Heslop-Harrison and Shivanna (1977). Flowers are

protandrous; anthers dehisce within immature flower buds, and pollen is presented on the unreceptive stigma when petals reflex. Stigmatic receptivity is delayed until 6 – 24 hours after flowers open when exudate is secreted. Exudate facilitates adhesion, hydration, and germination of pollen tetrads on the stigmatic surface. A hollow stylar canal and secretions rich in polysaccharides support the growth of many pollen tubes.

A thorough understanding of the reproductive biology of a species is essential for its conservation (Bernardello et al., 2001). Sexual reproduction in plants is dependent on structural adaptations and developmental timing of inflorescences, individual flowers, as well as male and female reproductive structures (Heslop-Harrison, 1981). Pollination is the primary event involved in seed formation, and various types of pollination failure exist (Wilcock and Neiland, 2002). Certain mechanisms interfere with pollen hydration and germination and pollen tube growth (Franklin-Tong, 2002), and some of these could be contributing to the lack of sexual reproduction in this rare species.

The objective of the current study was threefold: to deepen our understanding of the floral biology of Georgia plume by observing and documenting its flowering phenology; to see if self-incompatibility exists; and to consider the conservation implications of the reproductive biology of this species and make suggestions for its recovery.

Materials and Methods

Plant Material

The flowers used in this study were from nursery grown plants in Atlanta Botanical Garden's conservation collection. Plants were approximately 90 – 120 cm in

height and were potted in 11 liter pots. Plants represented genotypes from a population in Big Hammock Natural Area in Tattnall County and a population in Turner County. Distinct stages of floral development defined in a previous study (Radcliffe et al., 2009) were used to classify flowers and were designated by letters (A - E) (Fig.3.1).

Flowering Phenology

Young inflorescences at the stage when all flowers were in tight bud were tagged and numbered. Inflorescence length and counts of flower numbers were recorded three times weekly from June 1 until June 21, 2009. Each inflorescence was divided into basal, median, and terminal regions so that evaluations could be made of the relative changes in flower development within inflorescences. Each region comprised one third of the length of each inflorescence. The three basal buds from each region were observed in each inflorescence. Data on individual flowers were recorded daily from the date of first flower opening until the petals had abscised from all flowers (June 9 until June 28, 2009). Date, individual plant, inflorescence, flower, and position (basal, median, or terminal) were recorded. Flowers were rated according to the following parameters: petals closed, separating, reflexed, or abscised; petals green, white, or intermediate; stigma white or brown. Bud size was also measured daily. A total of 16 inflorescences and 144 flower buds were observed. In order to compensate for subtle differences in inflorescence age data were normalized so that day 0 for each inflorescence was set as the day when mean basal bud size was 3 – 4 mm. Flower age was likewise normalized so that day 0 for each flower was set as the day when bud size was 3 – 4 mm.

Controlled pollinations and pollen tube growth assessments

Plants used in pollination studies were brought indoors to prevent insect pollination. Using fine-tipped forceps, the petals and anthers were removed from recipient flowers (Turner County population) prior to anther dehiscence. Controlled pollinations were performed using freshly collected self pollen (from the same plant), cross pollen (Big Hammock population), or no pollen (emasculated control). Pollen was collected from flowers with petals separated in the median regions, but not yet reflexed (Stage C, Fig. 4.1). To confirm that un-pollinated stigmas were not contaminated and that pollinated stigmas received adequate pollen, fresh pistils of additional flowers were dissected, mounted and immediately observed at 15 kV using a Ziess SEM (Carl Zeiss, Oberkochen, Germany). Pistils were harvested at 8 and 16 hours after pollination. There were six to twelve flowers used per pollen source and collection time. Pollen tube germination and growth within the styles were assessed using aniline blue and fluorescent microscopy to visualize callose as described by Yi et al. (2006). Stigmas and styles were dissected and fixed in ethanol : acetic acid (3 : 1, v/v). Tissues were softened by autoclaving at 120ºC for 10 min in 1 % sodium sulfite solution (w/v), stained with aniline blue (0.01 % aniline blue in 0.1 M K_3PO_4), then examined under regular and UV light using an Olympus BX51 Research Microscope (Olympus America, Center Valley, PA). Pollen tube growth in 8 – 6 styles per treatment was measured and graphed as a function of time to compare self- and cross- pollinated flowers.

Results

Distinct stages of floral development were previously defined according to macroscopic parameters (Fig. 3.1) and microscopic observations (Radcliffe et al., 2009) ranging from immature, closed buds to receptive flowers with petals reflexed. In this study an additional stage was defined to document the transition of flowers through senescence. Characteristics of flower stages were as follows: Stage A buds were 4 – 6 mm long, green or greenish-white in color, and held upright on pedicles (Fig. 3.1). In stage A anthers were beginning to dehisce just at the tips, but pollen tetrads were enclosed within the anther sacs. Stage B buds were > 6 – 9 mm long, white, and nodding (Fig. 3.1). Anthers were partially to fully dehisced in stage B, with pollen falling out in clumps within the closed buds. Stage C buds were > 9 – 14 mm long, pendulous, and with petals distally attached but separated in the median regions (Fig. 3.1). In this stage pollen had collected in the tips of the closed buds where stigmas were appressed. Stage D flowers had open and reflexed petals (Fig. 3.1). Pollen was presented on the unreceptive stigmas as petals reflexed. Stigmas accumulated extensive exudate and became receptive about 6 – 24 hours after petals opened. Stage E flowers showed petal abscission (Fig. 3.1) and browning of the stigma.

Inflorescences of Georgia plume are borne terminally. The average length of mature racemes was 14.1 cm, and there was an average of 41 flowers per inflorescence. Inflorescences had open flowers for an average of 10 days. The average length of time flowers were open before petal abscission was 2.1 days (data not shown). This varied by flower position; basal and median flowers were open for 1.5 and 1.7 days respectively, while terminal flowers were open for an average of 2.9 days (data not shown).

Changes in bud length and time of flower opening are shown for basal, median, and terminal flowers (Fig. 3.2). Growth patterns of buds within an inflorescence differed with location. Growth rates of basal and median positioned buds exhibited similar trends and buds reached maximum lengths in about 8 days (14.9 mm). However, basal flowers opened an average of one day earlier than median flowers. In contrast, terminal buds exhibited a slower rate of elongation, with an increase in size from 3.4 mm to 14.1 mm over a period of 10 days. Terminal flowers opened an average of one day later than median flowers.

Temporal changes in flower development were assessed by classifying flowers within inflorescences into five developmental stages ranging from small buds (A) to flowers with abscised petals (E). Individual flowers progressed from stage A through stage E in approximately 10 days. The percent of flowers falling into their respective developmental stages is shown over a period of 12 days for basal, median, and terminal flowers (Fig. 3.3A-C). An evaluation of temporal changes in basal flower development follows. At day 0, 100% of basal buds were in stage A (Fig. 3.3A). By day 2, 39% of basal buds had transitioned into stage B; anther dehiscence was beginning to occur. On day 3 most buds were in stage B, while 19% had petals separating (stage C) and 5% were open flowers. Petal opening in basal flowers peaked on day 5 (64%) and continued until day 8. By day 9 100% of flowers had abscised petals.

The timing of developmental changes varied with flower position. While basal flowers began opening at day 3 (Fig. 3.3A), median flowers began opening on day 4 (Fig. 3.3B), and terminal flowers on day 5 (Fig. 3.3C). The average duration of opening for basal and median flowers was 6 days (Fig. 3.3A, 3.3B), and 9 days for terminal flowers

(Fig. 3.3C). Peak flowering in each of the three regions of inflorescences was also sequential (Fig. 3.3A-C); 64% of basal flowers were open on day 5, 55% of median flowers were open on day 6, and 40 – 45 % of terminal flowers were open on days 7 and 8. Overall peak flowering for inflorescences occurred on day 6, when 39% of all flowers are open (data not shown).

Stigmas at the time of hand pollination had copious exudate. No germinated pollen tetrads were observed in emasculated, un-pollinated controls (Fig. 3.4A). Following hand pollinations, pollen tetrads immersed in the stigmatic exudate became hydrated (Fig. 3.4B). Both self- and cross-pollen germinated on stigmas (Fig. 3.4C). Large numbers of germinated tetrads were present and pollen tubes penetrated the stigma in both cross- and self-pollinated flowers (Fig. 3.4C). Pollen tubes were easily discerned as they exhibited fluorescence along their entire length, with bright callose plugs evident.

Differences in maximum tube lengths within the style were observed between cross- and self-pollinated flowers (Table. 3.1, Fig. 3.5). Because of the extensive number of pollen tubes growing in the stylar canal, relative growth was assessed by measuring the advancing front of growing tubes. In cross-pollinations, numerous tubes had traversed the entire length of the style at 8 hr (Fig. 3.4D). In contrast, pollen tubes had only penetrated the top one-third of the style in self-pollinations observed 8 hours after pollination. The number of self-pollen tubes observed within the stylar canal (Fig. 3.4E) was markedly less than in cross-pollinations, indicating attrition following stigma penetration. By 16 hours after self-pollination, some tubes had extended through one-fourth of the style. The number of self-pollen tubes penetrating three-fourths of the style was less than ten (data not shown). Upon reaching this point, pollen tubes in self-

pollinated flowers stopped growing towards the ovary (Fig. 3.4E). Tube arrest within the lower style was indicated by disorientation in tube growth characterized by spiraling, tangling, and heavy callose deposition in the walls of pollen tubes in all self-pollinated flowers (Fig. 3.4F). No additional pollen tube elongation was noted in styles collected 24 hours after self-pollination (data not shown).

Discussion

Racemes of Georgia plume develop acropetally; basal flowers open first, followed by median, and then terminal flowers. Although individual flowers are open for an average of only 2 days, flowers within inflorescences develop progressively, resulting in an extended duration of flower opening (10 days). The architecture and phenology of inflorescences directly influence rates and patterns of pollen dispersal (Wyatt, 1982). The presentation of many flowers over a greater length of time results in higher outcrossing rates than single flowers (Burtt, 1961). Acropetal development of inflorescences, protandry, and appeal to generalist pollinators, all of which characterize flowering in Georgia plume, are known to promote outcrossing (McKone et al., 1995). However, these characteristics cannot prevent geitonogamy (Imbert and Richards, 1993).

Elliottia racemosa is protandrous. Pollen is presented on unreceptive stigmas when flowers first open, prior to exudate secretion and the onset of stigmatic receptivity (Radcliffe et al., 2009). Although pollen is in contact with stigmas inside of closed flower buds, it does not germinate (data not shown). In the absence of stigmatic exudate, pollen does not adhere or hydrate. However, pollen germinates readily in the presence of exudate. If autogamous pollen is still present when stigmas become receptive, lack of

available space may prevent outcross pollen from landing and germinating on the stigmatic surface. This is very similar to *Cephalanthus occidentalis*, which likewise presents its pollen on unreceptive stigmas (Imbert and Richards, 1993). Like *E. racemosa*, *C. occidentalis* exhibits gametophytic self-incompatibility, which allows pollen tubes to penetrate stigmas but prevents self-fertilization by inhibiting growth of tubes within the style. The amount of selfing vs. outcrossing depends on the timing and degree of self pollen removal and also the precise timing of stigmatic receptivity. Even when an incompatibility system is operative, the presence of large loads of self pollen can "clog" stigmas and exclude compatible pollen (Howell et al., 1993).

This study provides evidence of a gametophytic self-incompatibility system in *Elliottia racemosa*. Based on the presence of a wet, non-papillate stigma (Radcliffe et al., 2009), this is the type of self-incompatibility system predicted for this species (Heslop-Harrison, 1981). The extent of pollen tube growth within the styles of all cross-pollinated flowers was 100% of style length within 8 hours of pollination, and tubes were numerous. Pollen tubes in self-pollinated flowers grew more slowly; after 8 hours tube growth extended to 35% of style length. After 16 hours, some self-pollen tubes had grown towards the bottom portion of the style, but none extended beyond 72% of the total style length. Self-pollen tubes exhibited tangling and spiraling, heavy callose deposition, and cessation of growth in the lower style. The cytological response demonstrated in *Elliottia racemosa* is consistent with gametophytic self-incompatibility; pollen tube growth is inhibited within the style following tube disorientation and excessive callose deposition (Shivanna, 2003).

Self-incompatibility (SI) is found in about half of all known plant species and has contributed greatly to the diversity and success of angiosperms (Heslop-Harrison, 1975). SI is typically controlled by a single locus with multiple alleles, known as the S-locus. Gametophytic self-incompatibility (GSI) is based on the haploid genotypes of individual pollen grains. GSI acts within the transmitting tract of the style to exclude incompatible pollen after tubes have germinated and penetrated the stigma. Although out-crossing is often achieved through self-incompatibility systems, SI does not prevent inbreeding depression since inbreeding can occur between related individuals. Closely related individuals can mate as long as they do not share the same SI allele. Low mate availability can lead to low seed set in smaller populations of rare, self-incompatible species. Due to low numbers of SI alleles within populations with low genetic diversity, mate finding can be problematic (Weekley et al., 2002; Mateu-Andres and Segarra-Moragues, 2004; Willi et al., 2005; Caujape-Castells et al., 2008; Glemin et al., 2008). This phenomenon is known as the "allele effect" (Forsyth, 2003).

The allele effect can be influenced by pollinator abundance (Forsyth, 2003). However, pollinator visits can be infrequent and irregular (Bernardello et al., 2001). Low seed set of open-pollinations in comparison to hand-pollinations late in the flowering season of the endangered *Ptilimnium nodosum* is thought to be due, in part, to a lack of pollinators (Marcinko and Randall, 2008). Low fruit set is thought to be the result of a lack of pollinators in many rare species (Timmerman-Erskine and Boyd, 1999; Tomimatsu and Ohara, 2002; Duan et al., 2005; Xiao et al., 2009). Pollinator limitation may play a role in the rarity of some self-incompatible species. During years when pollinators are scarce, the rare, self-incompatible species *Polygala vayredea* exhibits low

seed set (Castro et al., 2008). Segal et al. (2006) suggest that habitat fragmentation exacerbates the lack of pollinators and effects of inbreeding, generating a “pollination crisis” in the self-incompatible species, *Iris bismarckiana*. Reduced mate availability due to relatedness at incompatibility loci is thought to increase in small or isolated populations, leading to the decline of rare species in fragmented and disturbed habitats (Messmore and Knox, 1997; Gigord et al., 1998; Sydes and Peakall, 1998).

Outcrossing species tend to have high levels of genetic diversity, even if they are self-incompatible (Heenan et al., 2005), however, fragmentation of forests can reduce gene flow and genetic diversity (Mehes et al., 2009). Populations of *Elliottia racemosa* exhibiting less genotypic diversity also have lower seed set (Godt and Hamrick, 1999). Infrequent gene flow between populations is common in plants with scattered and fragmented distribution. Limited genotypic diversity and sub-optimal environmental conditions effectively prevent seed set in the rare, bulbiferous *Titanotrichum oldhamii*. Selection for vegetative reproduction in such conditions allows clonal species to persist in the absence of sexual reproduction (Wang et al., 2004).

Habitat preservation and management in combination with experimentally produced seeds have been proposed to enhance diversity in situations where pollinator limitation is thought to be a factor limiting seed set (Sydes and Peakall, 1998; Bernardello et al., 2001). If natural seed set is low, supplementary pollination is suitable and can be combined with reintroduction if needed. This has been suggested for many rare species suffering from reproductive failure caused by habitat fragmentation, low mate availability, or pollinator limitation which are sometimes experienced in conjunction with self-incompatibility (Giblin and Hamilton, 1999; Tomimatsu and Ohara,

2002; Wang et al., 2004; Johnson et al., 2004; Segal et al., 2006; Carrio et al., 2009). Supplemental pollination could be used to increase seed set in *Elliottia racemosa*. Thompson and Spira (1991) observed an increase in both fruit set and viable seeds in *E. racemosa* following controlled pollinations using inter- and intra-population cross-pollen.

Supplemental pollination within the more diverse populations should be attempted before considering hybridization between populations. Mixing propagules from different populations for reintroduction purposes should be avoided if not required by circumstances (Robichaux et al., 1997). Hybridization between different populations has been proposed as a valid conservation option to increase genetic diversity (Raabova et al., 2009). However, outbreeding depression is a possibility that should be considered. Low seed set may be a result of S-allele deficiency in isolated remnants of *Atractylis arbuscula*, and the introduction of new S-alleles from different populations may be a viable option (Caujape-Castells et al., 2008). Reproductive success was restored to a small, isolated population of *Linnaea borealis* by experimental cross-pollination using pollen from another population (Wilcock and Jennings, 1999). Genotypic diversity varies between populations of *Elliottia racemosa*; some sites contain more than 20 multilocus genotypes whereas some consist almost entirely of one single multilocus genotype (Godt and Hamrick, 1999). Populations that are larger and more genetically diverse should be given priority for active conservation (Young et al., 1999), since they tend to be more evolutionarily dynamic and are likely to have greater S-allele diversity.

Conclusion

The sequential development and opening of basal, median, and terminal flowers extends the duration of flowering within inflorescences of *Elliottia racemosa*. Gametophytic self-incompatibility was identified, and inhibited growth of self-pollen tubes within the style was observed. The flowering phenology and self-incompatibility in *E. racemosa* should promote outcrossing. However, self-incompatibility may be contributing to its decline. The loss of genetic diversity in fragmented populations coupled with self-incompatibility could explain the reproductive failure of Georgia plume. Pollinator limitation is likely to be contributing to reproductive problems, especially in populations with fewer genotypes. Conservation strategies should be directed towards the most evolutionarily dynamic populations and should include supplemental pollination and suitable site management.

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Table 3.1. Pollen tube growth in the style of flowers pollinated with self- or cross-pollen.

Pollination	Hours after pollination	Mean distance of longest tubes (mm)[z]		Percent of style[z]	
Turner County self-pollinated	8	4.2	±1.0	34.7	±6.6
Turner County **x** Big Hammock	8	12.5	±0	100	±0
Turner County self-pollinated	16	8.9	±1.0	72	±1.0
Turner County **x** Big Hammock	16	12.5	±0	100	±0

[z] Lengths indicate the front of the fasted growing pollen tubes.

± Standard deviations.

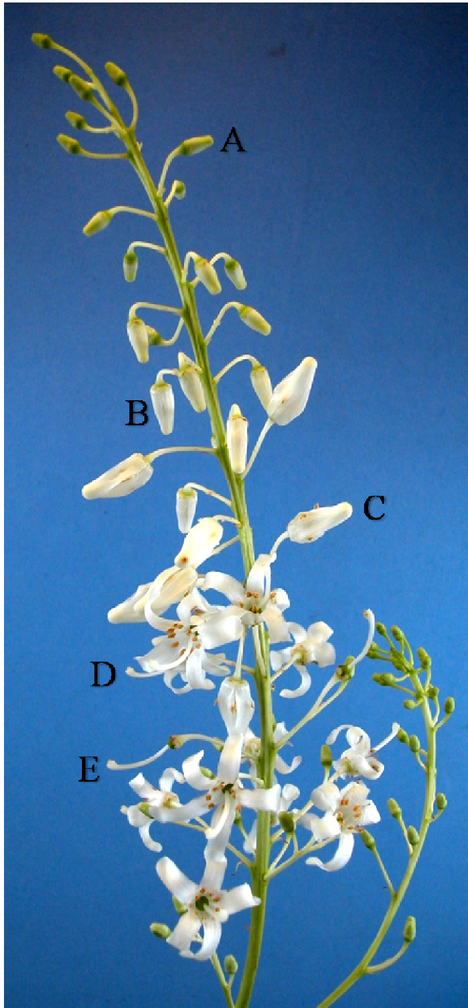

Figure 3.1. Inflorescence with flowers labeled stages A – E.

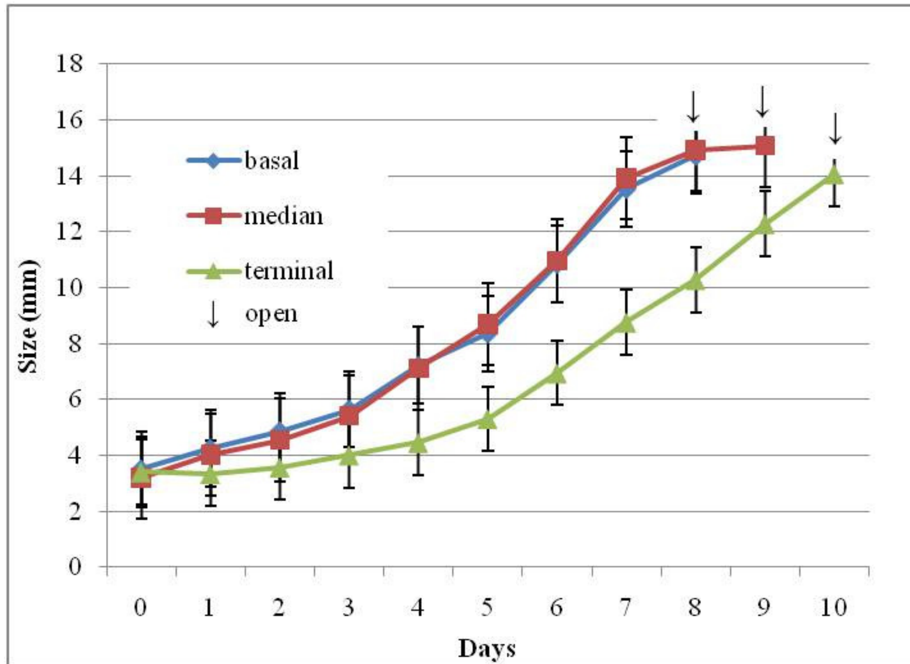

Figure 3.2. Growth and opening of basal, median, and terminal flowers.

↓ indicates mean day that buds opened.

Bars represent standard error.

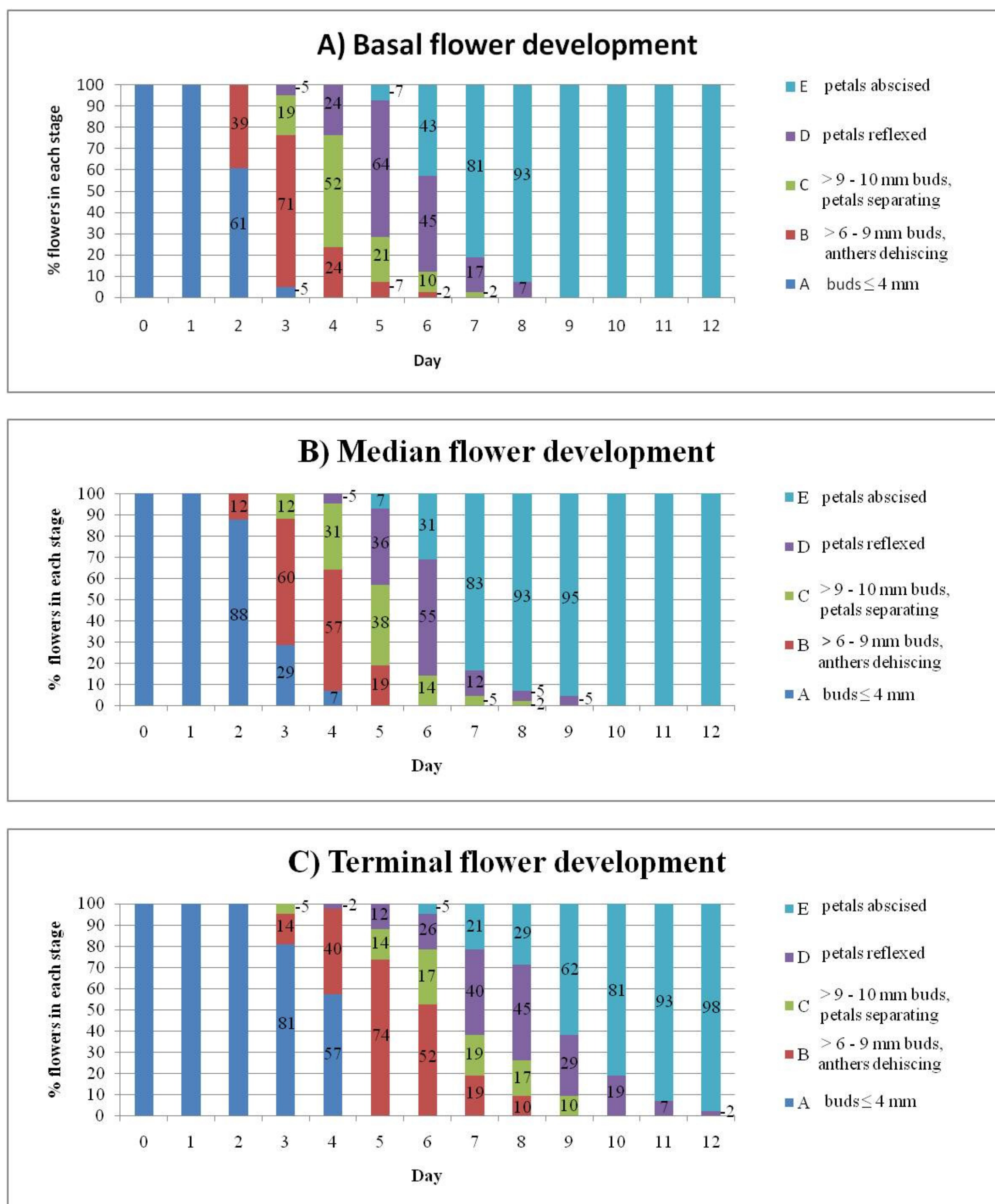

Figure 3.3. Effect of flower position within an inflorescence on the timing of developmental changes. Classification of A) Basal flowers, B) Median flowers, and C) Terminal flowers into developmental stages over time.

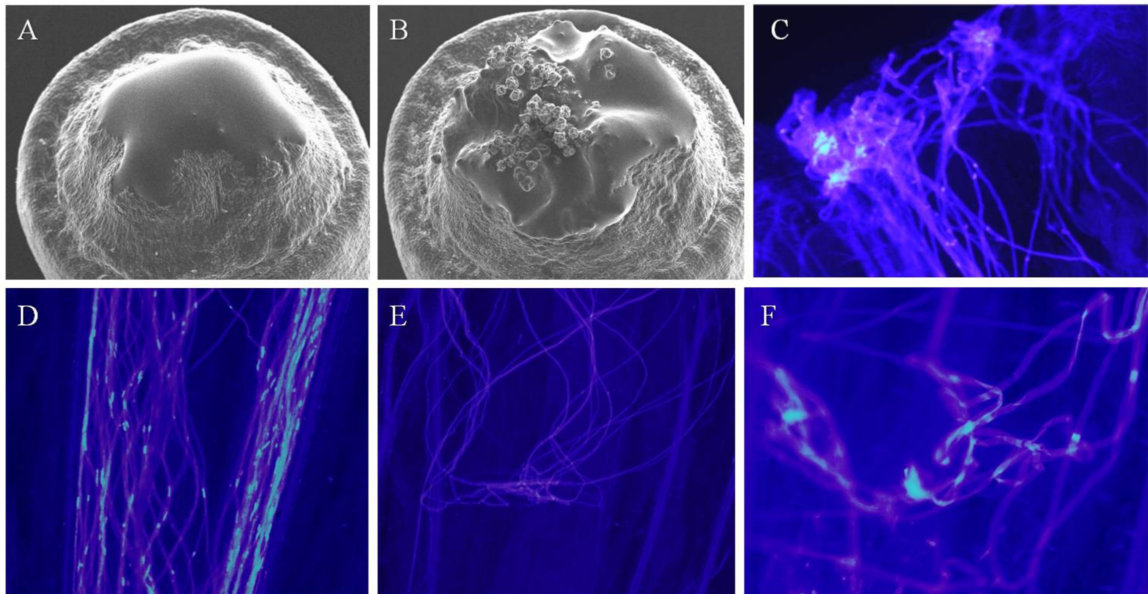

Figure 3.4. Pollen hydration, germination, and tube growth. A) SEM showing exudate on fresh, receptive stigma of emasculated flower. B) SEM showing a fresh, pollinated stigma. C) Pollen tetrad germination in a pollinated stigma. D) Numerous pollen tubes in the bottom one-third of a cross-pollinated style 8 hours after pollination. E) Self-pollen tubes in the bottom third of style 16 hours after pollination. F) Disorientation and heavy callose deposition in self-pollen tubes in the lower style 16 hours after pollination.

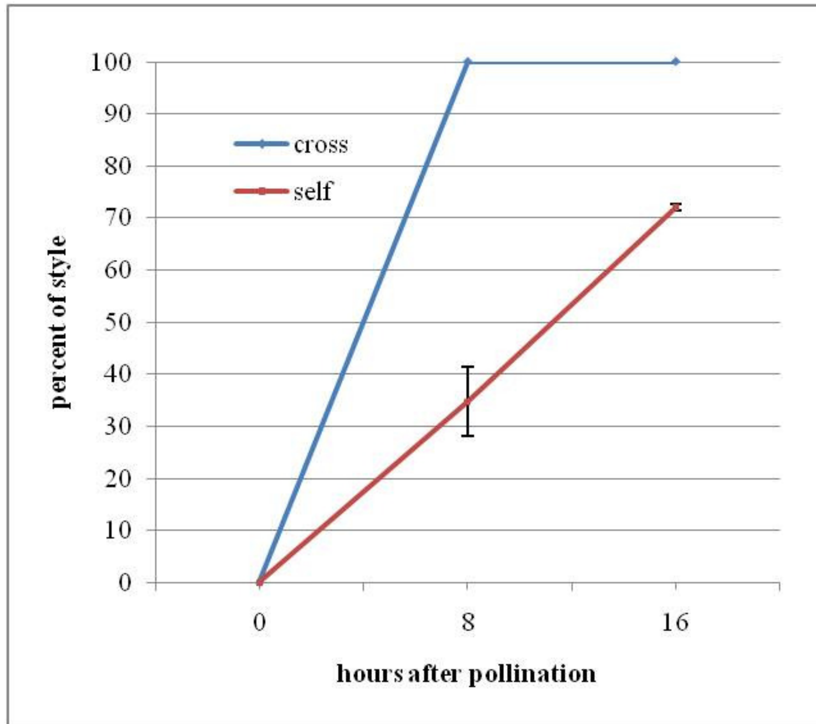

Figure 3.5. Extent of pollen tube growth in styles of self- and cross- pollinated flowers harvested 8 and 16 hours after pollination.

Bars represent standard deviation.

CHAPTER 4

IN VITRO PROPAGATION OF MULTIPLE GENOTYPES OF *ELLIOTTIA RACEMOSA* COLLECTED FROM THE WILD[1]

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Abstract

Elliottia racemosa (Georgia plume) is a threatened, woody plant endemic to Georgia's Coastal Plain region. It is currently found in only about three dozen sites in 19 counties. Remaining populations have a fragmented distribution within a restricted range, and are characterized by low genetic diversity and a lack of sexual recruitment. Most attempts at the propagation of this species have been unsuccessful. Georgia plume is a prime candidate for *ex situ* cultivation and *in vitro* propagation. In this study, a previously developed *in vitro* shoot regeneration system was evaluated to determine if the protocol could be applied to multiple genotypes from divergent populations of this species. Young expanding leaves were explanted on Gamborg media supplemented with 10 μM TDZ and 5 μM IAA, then after 2 months transferred to a shoot elongation medium with 25 μM 2iP. Of the 34 genotypes tested, 31 formed shoot primordia and 29 regenerated shoots within a six month period. The established micropropagation protocol is effective for a wide range of genotypes from different wild populations. Optimization may be needed for recalcitrant genotypes. It is feasible to use this system for safeguarding and reintroduction of genetically diverse plant material, which may be critical to the survival of this rare species.

Key words: *Elliottia racemosa*, *ex situ* conservation, *in vitro* tissue culture, micropropagation

Introduction

Elliottia racemosa Muhlenberg ex Elliot (Georgia plume) is a woody endemic that occurs only in the Coastal Plain region of Georgia. It was first discovered by the explorer William Bartram in 1773 and documented by botanist Stephen Elliott in 1807. After this time, the species was thought to be extinct until its rediscovery in 1901. Georgia plume grows on sand ridges, dry oak ridges, evergreen hammocks, and sandstone outcrops (Patrick et al., 1995). Its distribution has been fragmented by land development and the decline of suitable habitat. Georgia plume is currently found in less than three dozen populations in nineteen counties. Georgia plume is listed as Threatened in the state of Georgia, but has no Federal listing (Chafin, 2007).

Georgia plume is a striking deciduous tree reaching heights of thirty feet. Plume-like racemes of white flowers appear in early summer. When capsules develop they ripen in the fall, however, the main mode of reproduction is vegetative via root suckers. In recent times no seedlings have been documented in the wild, indicating an absence of sexual recruitment. Low population numbers and a lack of sexual reproduction may lead to the demise of this species, which has low genetic diversity at both the population and species level (Godt and Hamrick, 1999).

Because of its rarity and desirable ornamental characteristics, attempts to propagate Georgia plume have been made, yet conventional techniques have not been effective. Propagation by shoot cuttings has been unsuccessful, and root cuttings can produce only a limited number of shoots (Fordham, 1991). Prolonged stratification is required for germination of freshly sown seed, and secondary dormancy is acquired in older seed (Fordham, 1969). There is a high occurrence of inviable seed both in

cultivation and in the wild (Fordham, 1969; Bozeman and Roger, 1983; Thompson and Spira, 1991). Low pollen viability and gametophytic self-incompatibility have been observed in Georgia plume and may be contributing to sexual failure in this species (Radcliffe et al., 2009).

Georgia plume is a prime candidate for *ex situ* cultivation, which can be used to generate plants for reintroduction and safeguarding purposes. *Ex situ* propagation efforts often play an integral role in plant conservation (Maunder and Culham, 1999; Guerrant et al., 2004). *In vitro* culture (sterile micropropagation) is commonly used to propagate rare species when a limited number of plants can be produced using conventional methods (Fay, 1992; Fay et al., 1999; Sarasan et al., 2006). Micropropagation is a powerful conservation tool that can facilitate the mass production of a species using limited amounts of stock material (Merkle, 1999). It is especially useful when reproductive failure occurs in the wild (Guerrant et al., 2004).

A number of threatened and endangered woody species have been successfully regenerated using *in vitro* culture methods. *Betula uber*, confined to a single population in Virginia, was successfully propagated by *in vitro* multiplication of axillary shoots and *ex vitro* rooting (Vijayakumar et al., 1990). Propagules derived from shoot tip cultures of the endemic shrub, *Decalepsis hamiltonii*, were successfully multiplied, elongated, and rooted *in vitro* (Sudha et al., 2005). Another example is *Rhododendron ponticum* subsp. *baeticum* which, like Georgia plume, is a rare species belonging to the Ericaceae. Populations of this relictual Iberian species are likewise restricted, contain only adult trees, and have shown no signs of seedling recruitment (Almeida et al., 2005).

Micropropagated shoots originating from nodal explants of this species were rooted, acclimated, and reintroduced into their native habitat.

An effective micropropagation protocol for Georgia plume was established by Woo and Wetzstein (2008a; 2008b). In this system, adventitious shoot buds are indirectly induced on leaf explants cultured on a medium containing 10 μM TDZ and 5 μM IAA. Shoots elongate on a secondary medium supplemented with 25 μM 2iP. Both *in vitro* and *ex vitro* rooting methods were used to generate acclimated plants for out-planting studies The aim of ongoing studies is to define protocols for reintroduction and augmentation of natural populations. Both *in situ* and *ex situ* efforts are applicable to the conservation of Georgia plume since the remaining populations are threatened by continued habitat loss.

Responses to *in vitro* culture can be genotype dependent (Riseman and Chennareddy, 2004, Gandonou et al., 2005) and optimization may be needed in order to compensate for this variation (Khanna and Raina, 1998; Debnath, 2003; Gupta et al., 2006; Debnath, 2007;). In this study, the effectiveness of the micropropagation protocol for *Elliottia racemosa* was tested on several genotypes from a range of wild populations. Clonal propagation of genotypes from existing wild populations can be used as a conservation method to provide germplasm for *ex situ* safeguarding and generate plant material for reestablishment *in situ*, thus preserving the genetic diversity of this species.

Materials and Methods

Cultures were initiated from field material collected from thirty-four different genotypes representing twelve wild populations in the wild (Table 4.1). Collections were made in the spring, April 21 – May 6, 2009, when leaves were young (less than 2 cm

long), light green, and still expanding. Leaf tissue at this stage was found to be the most regenerable (Woo and Wetzstein, 2008a). Field collections were made from populations throughout the natural range for Georgia plume. Generally, three genotypes per population were cultured, and thirty to forty explants were initiated per genotype.

Leaves were disinfected prior to explant induction into sterile culture according to a previously developed protocol (Woo and Wetzstein, 2008a). Briefly, whole leaves were washed gently in soapy water containing 0.05 ml of antibacterial soap for 20 minutes. After decanting the soapy water, tissues were disinfested by placing them sequentially in 70% ethanol for 60 seconds, 10% Roccal (National Laboratories, Montvale, NJ) for 10 minutes, and 20% bleach for 10 minutes, followed by three washes with sterile distilled water. Leaves were cut into pieces about 5 x 5 mm and explanted abaxial side down, onto induction media. Media consisted of Gamborg's B_5 salts (GB_5) (Gamborg *et al*., 1968) amended 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 Biochemicals, Aurora, OH). The pH was adjusted to pH 6.0, aliquoted into test tubes with 20 ml per tube, and autoclaved at 121°C for 20 min. Cultures were evaluated at 1, 2, and 4 weeks for responsiveness to induction and contamination. Cultures were considered responsive if swelling and callusing of the explant was evident. After 4 weeks, cultures were transferred onto fresh induction media. Cultures were evaluated at 1 and 2 months from explanting for the presence of adventitious shoot primordia.

After 2 months on shoot induction media, cultures were transferred to shoot elongation media consisting of Woody Plant Medium (WPM) (Lloyd and McCown, 1980) amended with 25 μM 2iP, 2 $mg \cdot L^{-1}$ glycine, 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$

pyridoxine HCl, 1 mg·L^{-1} thiamine HCl, 100 mg·L^{-1} myo-inositol, 20 g·L^{-1} sucrose and 4 g·L^{-1} Gel-Gro. The pH was adjusted to pH 5.2, distributed into baby food jars (30 ml), and autoclaved at 121°C for 20 min. Cultures were maintained under a 16 hour photoperiod under white fluorescent lights (70 µmol·m^{-2}·s^{-1}) at 25 °C, and transferred onto the same media monthly. Shoot elongation was rated monthly for 4 months after transfer to shoot elongation media.

Genotypic responses to tissue culture were classified into different categories based on the percent of explants within each genotype exhibiting swelling and callusing (rated at 1 – 4 weeks on induction media), percent of responding cultures forming adventitious shoot primordia (rated at 1 – 2 months on induction media), and percent of responding cultures with elongating shoots (rated monthly 1 – 4 months on shoot elongation media). Statistical analyses were performed using least squares means and Duncan's mean separation ($P = 0.05$) procedures within the general linear model procedure of SAS (version 9.1 for Windows; SAS Institute Inc., Cary, NC, USA).

Results and Discussion

The responsiveness of leaf explants of *Elliottia racemosa* on induction media is characterized by swelling and callusing of explants due to division and enlargement of spongy and mesophyll cells. This response is followed by shoot organogenesis. Promeristematic structures form in the outer mesophyll layers close to the surface of explants. Organized meristematic regions become exposed through discontinuities in the

epidermal layer as proliferation progresses, leading to the development of adventitious shoots with leaf primordia (Woo and Wetzstein, 2008b).

The number of responding explants and the extent of explant response varied with genotype (Fig. 4.1). Most genotypes (79%) exhibited some swelling and callusing as soon as 1 week after culture initiation. Responses at 1 week after initiation ranged from genotypes showing no response (21%) to those with over 80% of explants exhibiting swelling and callusing (35%). The number and extent of responding genotypes increased with time. Only 3% of genotypes were unresponsive at week 2. Of the 97% of genotypes showing response to induction, 56% had greater than 80% of explants responding, and 21% had 50 – 79% responding. All genotypes showed some level of response by week 4. At this time, 79% of genotypes showed 80% or more explants exhibiting swelling and callusing, and only 3% of genotypes had less than 25% responding. Explant response for most genotypes was very strong.

The timing of adventitious shoot formation also varied with genotype (Fig. 4.2). The development of adventitious shoots occurred rapidly. Over 65% of the genotypes initiated visible shoot/bud primordia by 1 month versus 35% of the genotypes which had no shoot primordia at this time. Of the 65% of genotypes showing adventitious shoots, 9% had greater than 80% of cultures with shoot primordia, 21% had 50 – 79%, 9% had 25 – 49%, and 26% had less than 25% with shoots. Only 6% of genotypes failed to form adventitious shoots by 2 months after initiation. Of the 94% of genotypes showing shoot primordia by month 2, the majority of responding cultures (49%) had greater than 80% of the explants initiating adventitious shoots, 21% had 50 – 79% of cultures with shoots,

18% had 25 – 49% of cultures with shoots, and only 6% had less than 25% of cultures showing shoots.

Shoots obtained on induction media with TDZ and IAA were prolific (Fig. 4.3) but required elongation before shoots were suitable for plant regeneration (Fig. 4.4). TDZ reportedly inhibits the elongation of shoots, and transferring cultures to a medium containing lower levels of TDZ or a less active cytokinin can be effective in overcoming this inhibition in many woody species (Huetteman and Preece, 1993). The use of a two-stage system in which shoots are initiated on an induction medium supplemented with TDZ and subsequently elongated on a secondary medium is efficient for the regeneration of shoots from many woody species (Huetteman and Preece, 1993; Debnath, 2003; Debnath, 2005b; Vijaya Chitra and Padmaja, 2005; Woo and Wetzstein, 2008a). Similar protocols have been developed for economically important members of the Ericaceae family, including blueberry, cranberry, lingonberry, and rhododendron in which TDZ was used alone or in combination with 2iP or IAA to promote shoot regeneration (Marcotrigiano and McGlew, 1991; Cao and Hammerschlag, 2000; Debnath, 2005a; Debnath, 2009).

To promote shoot elongation in this study, cultures were transferred onto WPM containing 25 μM 2iP. Genotypes showed different frequencies in elongation of adventitious shoots (Fig. 4.5). After 1 month on elongation media 41% of genotypes had elongated shoots. The percent of cultures within each genotype that had elongating shoots increased over time. After 4 months on elongation media (6 months after initiation), 85% of genotypes exhibited shoot elongation. Over 20% of genotypes had greater than 80% of cultures with elongating shoots, 18% had 50 – 79% of cultures showing elongation, 21%

had 25 – 49% of cultures showing elongation, and 26% had less than 25% of cultures showing shoot elongation. Only 15% of all genotypes did not have elongating shoots by this time, however, elongation was observed in some of these genotypes after 7 months on elongation media (data not shown).

Variation in genotypic responses to 2iP has also been observed in rhododendron (Anderson, 1984; Iapichino et al., 1992). For genotypes of Georgia plume that formed shoots that did not elongate, it may be beneficial to incorporate the use of a weaker cytokinin such as BA, which has been used to promote elongation of shoots derived from cultures of azalea (Briggs et al., 1988) and mulberry (Vijaya Chitra and Padmaja, 2005) following shoot initiation on a medium containing TDZ. The use of zeatin has been successful in improving shoot regeneration in multiple cultivars of blueberry (Cao and Hammerschlag, 2000) and in the endangered *Rhododendron ponticum* subsp. *baeticum* (Cantos et al., 2007). Incorporation of zeatin, a naturally occurring cytokinin, into secondary media promoted rapid elongation of lingonberry shoots induced from leaf explants using TDZ (Debnath, 2005b). The use of zeatin could be evaluated in overcoming the inhibition of shoot elongation observed in some genotypes of Georgia plume.

Genotypes nested within different populations of Georgia plume showed significantly different frequencies of adventitious shoot formation and elongation of shoots (Table 4.2). Genotypes from 11 of the 12 populations tested showed an initial response of at least 73% of explants by the fourth week. Genotypes representing 8 populations had greater than 72% of cultures forming shoot primordia by the end of the second month. Most of the variation in the ability of leaf explants of Georgia plume to

form adventitious shoots *in vitro* was attributable to population (59%), while the amount attributable to individual genotypes was much less (14%). Differential responses of genotypes from certain populations to *in vitro* propagation may have a genetic basis. Genotypes within the same populations showed similar responses to each other, while some populations differed significantly in their ability to form shoot primordia and elongated shoots. Shoots sufficient for rooting were obtained from 91% of genotypes (representing 10 of 12 populations) after 4 months on elongation media containing 2iP.

Although genotypic limitation can be a significant constraint in micropropagation (Gupta et al., 2006), the tissue culture protocol for Georgia plume is highly effective with a broad range of genotypes originating from divergent populations. The use of young leaf tissue allows initiation of sterile cultures from mature individuals collected from natural populations in the wild. It is feasible to use this system for *ex situ* propagation of *Elliottia* racemosa to generate genetically diverse plant material for safeguarding, reintroduction, and augmentation of existing populations. Such efforts may be critical to the survival of this rare species as it faces the threat of extinction.

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Table 4.1. List of natural populations and corresponding locations from which leaf explants were collected for *in vitro* propagation.

Population	Location name	County
A	Little River	Turner
C	unnamed	Bryan
D	Big Hammock	Tattnal
F	Little Hammock	Tattnal
J	Charles Harrold Preserve	Candler
K	Mertz Pond (Fort Stewart)	Tattnal
L	unnamed (Fort Stewart)	Bryan
M	unnamed (Fort Stewart)	Evans
N	unnamed (Fort Stewart)	Evans
O	unnamed (Fort Stewart)	Evans
P	unnamed (Fort Stewart)	Evans
Q	unnamed (Fort Stewart)	Evans

Table 4.2. The percentage of explants collected from different wild populations that exhibited initial swelling and callusing, shoot primordia development, and elongated shoots in culture.

Population	% Explants responding[z]	% Cultures showing shoot primordia[y]		Cultures showing elongation (%)[x]
	1 month	1 month	2 months	6 months
Q	99	79 a	96 ab	0 g
F	98	23 e	100 abc	100 a
J	100	49 c	100 abc	15 efg
P	100	61 ab	100 abc	14 gf
D	88	37 c	86 abc	55 c
K	87	39 cd	79 bc	79 b
A	100	61 ab	76 c	77 b
L	76	34 dc	72 c	70 bc
C	100	0 f	50 d	27 def
N	100	0 f	44 d	38 def
O	73	5 ef	41 d	32 def
M	30	0 f	5 e	2 g

[z] Swelling and callusing of explants on initiation media

[y] Percent of responsive explants with adventitious shoots

[x] Percent of responsive explants with elongating shoots on after 4 months on shoot elongation media

Different letters within a column by Duncan multiple range test indicate significant differences at $P < 0.05$.

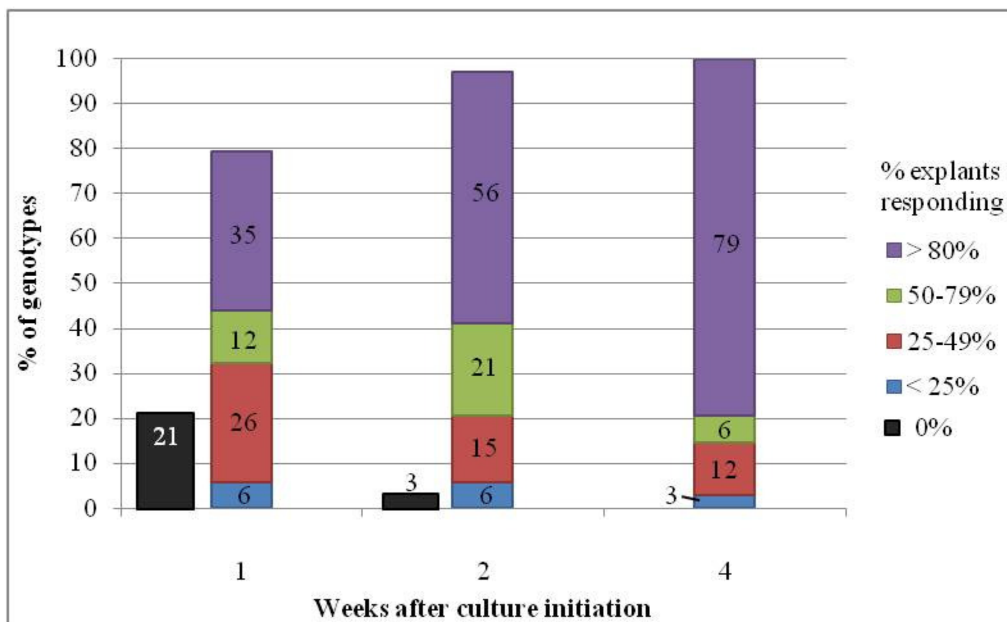

Figure 4.1. Percent of genotypes showing different frequencies of explant swelling and callusing over time.

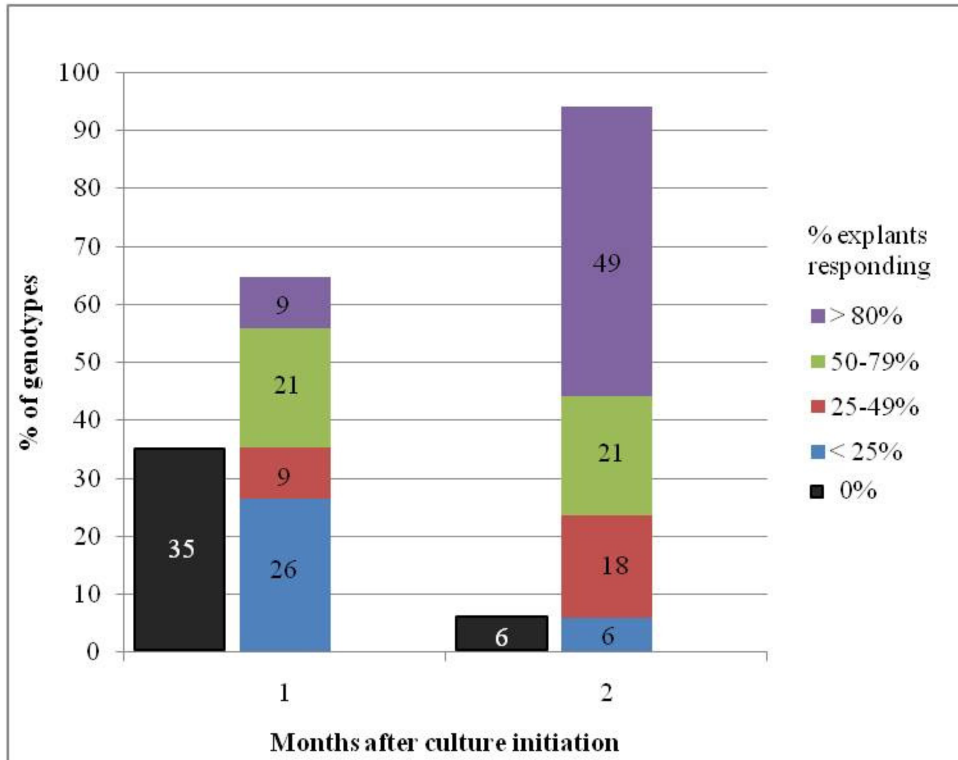

Figure 4.2. Genotypic differences in adventitious shoot formation over time.

Figure 4.3. Adventitious shoot primordia proliferation.

Figure 4.4. Variation in shoot elongation observed in cultures from originating from different wild populations.

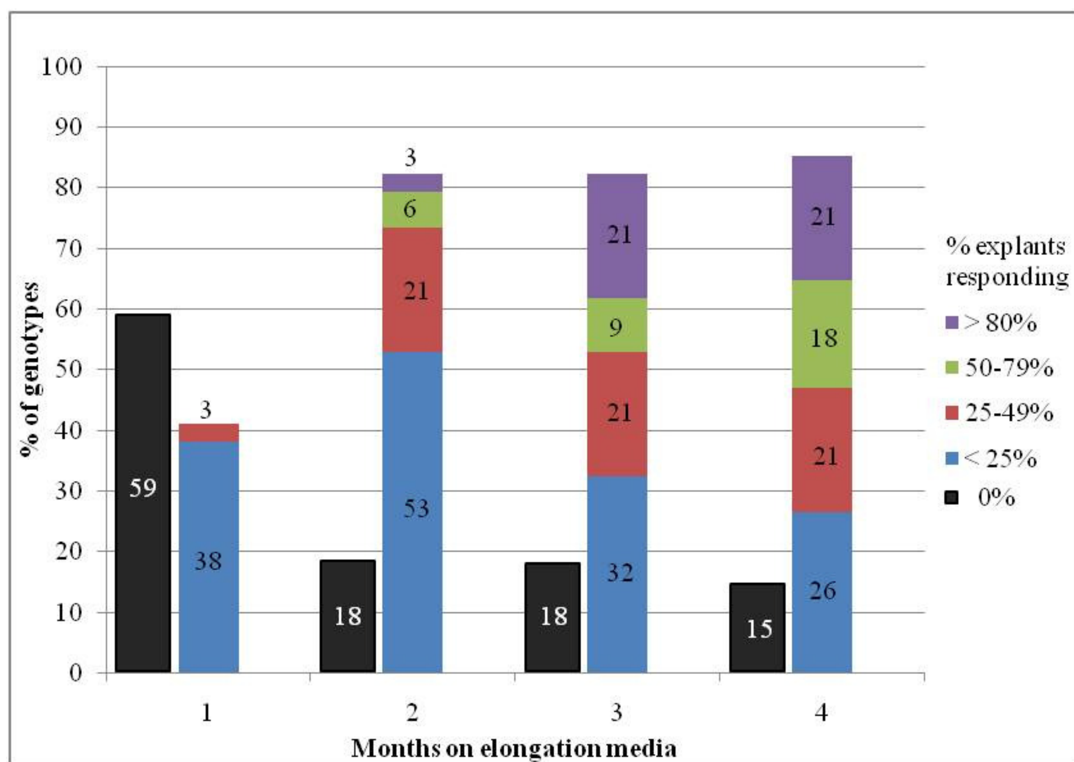

Figure 4.5. Genotypic differences in the elongation of adventitious shoots over time.

CHAPTER 5

CONCLUSION

The main objective of this work was to develop an understanding of the reproductive biology of *Elliottia racemosa*. Flowers are protandrous and can exhibit secondary pollen presentation via the unreceptive stigma. Although anther dehiscence occurs early in flower bud development, pollen capture is spatially and temporally limited to a small central region of the stigma surrounding the opening to a lysigenous stylar canal where exudate is secreted at receptivity. *E. racemosa* has a wet non-papillate stigma type. The presence of exudate on the stigmatic surface is critical to pollen capture, hydration, and germination. Copious exudate rich in polysaccharides and a hollow stylar canal may support the growth of large numbers of pollen tubes. Pollen viability is low to moderate per tetrad, varies with population, and may be problematic. No developmental reproductive abnormalities were observed.

The sequential development and opening of basal, median, and terminal flowers extends the duration of flowering within inflorescences of *E. racemosa*. Gametophytic self-incompatibility was identified, and inhibited growth of self-pollen tubes within the style was observed. The flowering phenology and self-incompatibility in *E. racemosa* should promote outcrossing. However, self-incompatibility may be contributing to its decline. The loss of genetic diversity in fragmented populations coupled with self-incompatibility could explain the reproductive failure of Georgia plume. Pollinator limitation may be contributing to reproductive problems, especially in populations with

fewer genotypes. Conservation strategies should be directed towards the most evolutionarily dynamic populations and should include supplemental pollination and suitable site management. Future studies on seed production and germination, as well as seedling recruitment would be possible with the implementation of such strategies in an experimental manner.

Georgia plume is a prime candidate for *ex situ* cultivation, which can be used to generate plants for reintroduction and safeguarding purposes. *In vitro* culture is a method that can be used to propagate rare species, but response is often genotype dependent. The effectiveness of a tissue culture regeneration system previously developed for *E. racemosa* was evaluated to determine its applicability for a wide range of genotypes originating from divergent populations. Of the 34 genotypes tested, 31 formed shoot primordia and 29 regenerated shoots within a six month period. The established micropropagation protocol is effective for a wide range of genotypes from different wild populations of Georgia plume and allows initiation of sterile cultures from mature individuals. It is feasible to use this system for *ex situ* propagation of *E.* racemosa to generate genetically diverse plant material for safeguarding, reintroduction, and augmentation of existing populations. Such efforts may be critical to the survival of this rare species as it faces the threat of extinction.