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In Vitro Galax Urceolata Seed Propagation Under Different Treatments

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In Vitro Galax urceolata Seed Propagation Under Different Treatments

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Biographical Sketch

Rachel Jackson was born on July 1, 1985, in Southern Pines, North Carolina. She received the Bachelor of Science degree in Biology from Appalachian State University in 2007 where she concentrated on ecology, evolution, and environmental biology. She is a candidate for the Master of Science degree in Plant, Soil and Environmental Science concentrating in plant science.

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Table of Contents

List of Figures

List of Tables

Abstract

Galax urceolata which is native from Virginia to Georgia is becoming threatened due to overharvesting. Expediting the multiplication of Galax would increase its potential in becoming an economically viable cash crop while protecting the wild population. The overall goal of this study was to develop a protocol for *in vitro* Galax germination and proliferation. Influences of light intensity, culture medium pH, temperature stratification, and seed maturity as were studied for their effects on Galax seed germination *in vitro*. Plant growth regulators (PGRs) BA, IBA, and GA_3 were tested for their effects on germination. BA and IBA were also tested for shoot development. Galax seeds harvested from the mountains demonstrated 16.7% viability based on the test of tetrazolium staining. Light is required for Galax seeds germination as it significantly increased the germination rate. A stratification temperature of 5^0C along with increasing seed maturity were found to have significant positive effects on Galax seed germination resulting in a germination rate of 76%. The PGR treatments of 6-benzylaminopurine and indole -3-butyric acid (BA:IBA) applied at various ratios to one another were found to inhibit Galax seed germination as no germination was noticed for any treatment after 30 days. GA_3 demonstrated a positive effect on seed germination after the cold stratification pretreatment for dark-colored Galax seed. The BA and IBA treatments were not effective for initiating shoots after 20 days of exposure. More research is needed to develop a protocol for seedling proliferation and shoot initiation leading to an efficient production of Galax plants.

CHAPTER 1

Introduction

Galax (*Galax urceolata* (Poir.) Brumm.) is a monotypic evergreen perennial species native to the Appalachian Mountains. It can be found in the understory of numerous forest types throughout most of the eastern U.S. (Nesom, 1983; League, 2006). Galax has been found to be a mixed cytotype species, meaning that diploid and polyploid populations of Galax overlap (Burton & Husband, 1999). Commonly known as beetleweed, Galax produces flowers in late spring/early summer. Small white flowers are formed on a raceme or seed stalk that towers above the foliage approximately 38 cm (Figure 1; LBJ Wildflower Center 2009).

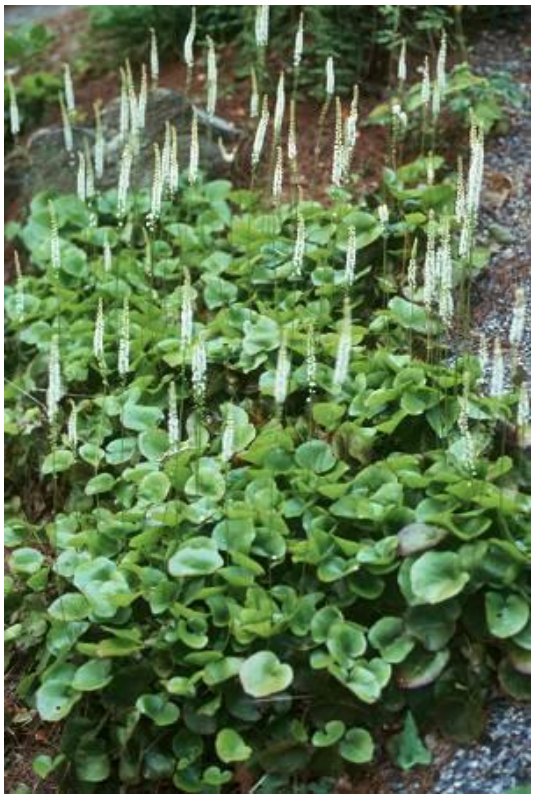

Figure 1. Galax in bloom. [Image of Galax in bloom]. (n.d.). Retrieved 9/2/2010 from: http://www.nurserynetwork.com/ads_plants.htm.

Galax seeds are extremely small, measuring 0.6 mm long x 0.3 mm wide on average, and have very little endosperm to support seedling growth. The green glossy leaves have a serrate margin, are shaped like a rounded heart, and are 7.5-12.5 cm in diameter with 7.5-15.2 cm long petioles (Predny & Chamberlain 2005). Leaves are extensively harvested for use as foliage in floral arrangements. In 2001, ~100% of the leaves sold to the floral industry was harvested from the wild (Greenfield & Davis, 2003; Bir, 2005). In fall those leaves exposed to full sun turn red (Hughes et al., 2005; Hughes & Smith, 2007) which increases their value.

It is estimated that up to two billion leaves are harvested annually for a value of over $20 million, much of it illegally (Greenfield & Davis, 2003). The sustainability of current harvest methods are being evaluated by the U.S. Forest Service and National Park Service (Bir, 2005; Greenfield & Davis, 2003; Predny & Chamberlain, 2005). Developing a protocol for commercial production of Galax that can sustain the demand would ease the threat of overharvesting from natural populations. Galax reproduces naturally through underground rhizomes and seed in the wild. Conventional propagation typically involves sectioning the rhizome and growing the plant in highly organic soil (Bailey, 1976). However, because the plant grows relatively slow, this method is not economical for large-scale production. Bir (2005) began to evaluate the use of seeds as an alternative propagation method by testing various conditions for seed germination in soil. He found that Galax seeds would germinate on sphagnum peat moss in 14-21 days. Bir suggests a protocol focusing on *in vitro* germination and micro-propagation from seed may be more economical.

He also tested seed manipulation and various photoperiods to improve germination which have not been found to substantially improve germination.

I found no published studies on micro-propagation techniques for Galax. By expediting

the growth of seedlings to a size useable for explants the length of time it takes to produce a mature plant is reduced. These techniques have been found to optimize the production of many species. Seedlings grown *in vitro* from prepared seeds provide a source for sterile explants that do not require additional sterilization before explant initiation (Mohamed-Yasseen & Costanza, 1996). Also, adventitious shoots can be derived directly from cotyledons, hypocotyls, or young leaves, and callus can easily be induced as well (George, 1993). Exploring new germination and propagation techniques may lead to the necessary advancements needed to establish Galax as a cash crop. The approach proposed in this study would take less time than conventional propagation and provide a more controlled environment for germination. This research may help create an economical method to produce Galax to meet the market demand without relying on native grown plants.

By testing multiple cultural factors and seed characteristics it may be possible to determine which combination results in the most optimal protocol for propagation. First off, it is important to determine the best starting material for the protocol to achieve a high germination rate. Then, it is important to separate seeds based on maturity of stalks and again by seed maturity as indicated by seed color. Then it is possible to stratify them based on these characteristics and to test each group of seeds against various conditions for their germination potential. Other factors like pH, light intensity, cold and plant growth regulators (PGRs) may also influence germination and must be analyzed before a comprehensive protocol for obtaining high seed germination can be achieved.

After seeds germinate, it is important to initiate shoot production so that the seedlings can achieve sufficient size in a short period of time for out planting. This requires finding a way to optimize shoot proliferation in Galax seedlings. Plant growth regulators will play a crucial role

in this respect for these tiny seedlings. A higher ratio of cytokinin to auxin normally promotes shoot initiation (Debergh & Zimmerman, 1993; George, 1993; Mok & Mok, 1994) whereas a low ratio generally promotes roots. Our preliminary experiments indicated that subsequent shoot development after germination remains slow. The use of PGRs will enhance proliferation and shorten the time of development into explant material (Debergh & Zimmerman, 1993; George, 1993; Mok & Mok, 1994). Currently, the optimal ratio of cytokinin to auxin for shoot proliferation in Galax is not known. Once enough explant material can be accumulated and the optimal conditions identified, an *in vitro* micro-propagation protocol can be developed.

Galax has proven to be a commodity with immediate financial payback. The industry has established a worldwide demand that brings in $10-26 million a year to the Appalachian region (Greenfield & Davis, 2003). An estimated 1-2 billion leaves of Galax were harvested in 2001 and the demand grew thereafter. It is one of the most harvested botanicals as determined by the number of permits purchased (Kauffman et al., 2001). Based on current demand, it will have a higher economic potential if a protocol for large-scale propagation was established. It is important to find ways to commercially raise Galax in nurseries to deter the floral industry from purchasing illegally harvested leaves so that the harvesting pressure on native populations is alleviated and farmers would also be able to supplement their income.

In addition to its commercial floral value, Galax has potential to be used to prevent and control erosion in very shaded damp areas (Predny & Chamberlain, 2005). This potential is enhanced by the fact that native plants like Galax pose a low threat of becoming invasive in settings in which erosion control might need to be implemented on a large-scale. Additional markets that could benefit from the domestication of Galax would be the pharmaceutical/nutraceutical sectors. The anthocyanins which cause the reddening of Galax in

fall are antioxidants with potential medicinal benefit. They are flavonoids well known for their free radical scavenging and antioxidant capabilities (Lila, 2004; Philpott et al., 2009; Welch et al., 2008). Aside from these other possible uses for Galax, it could also benefit the overall plant tissue culture sector by serving as a model organism for *in vitro* germination and micro-propagation of low nutrient seeds and difficult to propagate plants.

The proximate goal of this research was to develop an efficient protocol for *in vitro* Galax germination and proliferation that will lead to a suitable protocol for micro-propagation of Galax. The ultimate goal is to help develop protocols that will allow Galax to be grown as a cash crop. Specific objectives were defined:

1: To determine Galax seed viability using common indices and tests such as triphenyl-2H-tetrazolium chloride (tetrazolium) staining.

2: To separate seeds according to maturity level that allow for a more productive protocol.

3: To evaluate the effect of pH, storage temperature, seed maturity, and PGR types and concentration combinations for their effects on Galax seed germination and shoot proliferation.

Experiments were designed and implemented to test the four following hypotheses. The first is that Galax seeds are predominantly viable after fall harvest from the field. The second is that Galax seed color does correspond to maturity and viability. The next is that cold stratification, culture medium pH, and light intensity do affect Galax seed germination. The fourth hypothesis is that the presence of PGRs in the medium will enhance Galax seed germination and seedling shoot formation.

CHAPTER 2

Literature Review

2.1 Ecological and Economic Importance

2.1.1 Industry demand. Galax and its harvesting have a strong historic and cultural significance to its native region. The earliest record of Galax being harvested from the wild was in 1905 when it was suggested to name a small railroad town in Virginia after the plant whose leaves were a popular trade item (Predny & Chamberlain, 2005). At that time most harvesters were from rural regions and used Galax to supplement their household income. Today, it has been estimated that 90% of Galax harvesters today are Hispanic laborers who have been encouraged to harvest Galax during the off season from farming Christmas trees (Greenfield & Davis, 2003; Predny & Chamberlain, 2005).

Because it has become so popular to harvest Galax, and because it is a rather slow growing species, there is concern that the native plant could become threatened. Now, permits must be obtained from the U.S. Forest Service before harvesting, at a cost of $25.00 each and which are valid for 30 days or 100 pounds of Galax. It has been estimated that one person can pick more than 5,000 leaves per day for a value of $20- $120 depending on the season and quality of leaf (Greenfield & Davis, 2003; Predny & Chamberlain, 2005). A study by Ulrey (2001) on the effect of harvesting on Galax, found that even though larger leaves were targeted, many roots, rhizomes, and smaller leaves were pulled as well (as cited in Predny & Chamberlain, 2005).

Concern for harvest sustainability has led to a restriction between May 1 and June 15 by

the U.S. Forest Service. During this time no harvesting may take place so that young leaves produced that year can harden off before any major disturbances (Bradley, 2001; Greenfield & Davis, 2003). This restriction, along with the listing of Galax as vulnerable (S2) in West Virginia, suggests that great care should be taken when harvesting the leaves (League, 2006). Most harvested Galax sold to the floral industry is harvested legally with about 82% coming from public land (Greenfield & Davis, 2003). However, local wholesalers have reported processing more Galax than the number of sold permits would allow. In addition, Galax was also being brought in for processing during the restricted period, which suggests that illegal harvesting was taking place. In 2001 at least 14 individuals were arrested for Galax poaching, and over 60,000 leaves and 100,000 plants were confiscated (Greenfield & Davis, 2003; Predny & Chamberlain, 2005). To deter poaching in areas where harvesting is illegal, the leaves are dusted with a microtaggant that allows authorities to track them back to their source (Greenfield & Davis, 2003; Nickens, 2001).

Besides the value Galax offers to the floral industry, it could play a major role in conservation. It is important to advocate the use of native plants for use in best management practices like erosion control and intercropping. Galax can be used in damp shaded areas prone to erosion where other plants with higher light requirements used for erosion control may be less effective (Predny & Chamberlain, 2005). This plant could dramatically reduce soil loss while generating supplemental income for the property owner. If Galax began to be implemented in more areas than just the floral industry, demand for this plant would rapidly rise.

2.1.2 Meeting demand. The limited studies on Galax propagation have not shown a reliable and economical method that could be implemented on a large-scale. Various trials by Bir (2005) using rhizomes have not proven promising and it is thought that propagation by seeds

is more practical than rhizome cuttings, and that *in vitro* propagation would be able to help meet the demand for this foliage plant once techniques were established. Expediting the multiplication of Galax through tissue culture would also increase its potential to become an economically viable cash crop without endangering native populations.

2.2 Obstacles in Commercial Production

Galax seeds can be between a light orange or dark rust color and are extremely small averaging 0.6 mm x 0.3 mm (Figure 2).

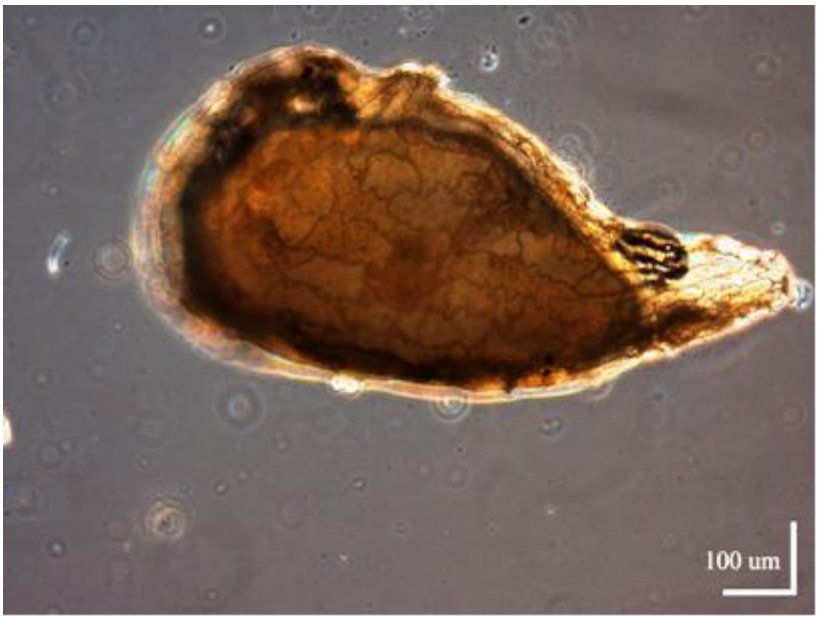

Figure 2. Galax seed viewed under 400x magnification.

The color variation seems to be related with seed maturity. The separation of the seeds by color may require the researcher to use magnification until becoming familiar with how the seeds look and the variation within dark and light colors. Because of this small size there is little endosperm to supply the embryo with nutrients. This lack of nutrients means small seeds gives rise to small seedlings and can also cause them to grow slower than seedlings from plants with larger seeds. This makes the addition of nutrients to the culture media essential in sustaining healthy seedlings (Baskin & Baskin, 1998). The tiny size of Galax seeds also makes for tedious

work when processes require fair amounts of manipulation. It is also possible that most of the small seeds are not actually viable, which would mean that a lot of seeds must be collected to obtain a few viable ones. Sometimes seeds have endosperm but are embryo-less (Baskin & Baskin, 1998). Plants that reproduce vegetatively often make the trade off of developing embryo-less seeds to keep competition low for the new vegetative clones (Arizaga & Ezcurra, 2002). There are several ways of doing this, but the most commonly used methodology is to stain the seeds with Tetrazolium Red (Baskin & Baskin, 1998). This stain is used to determine if the seed has a viable embryo and works whether seeds are dormant or not. The principle behind this stain is as follows: viable embryos release hydrogen ions during respiration which can react with the tetrazolium, causing the viable tissue (i.e., endosperm) to turn red or pink (Baskin & Baskin, 1998; Belcher, 2010; Fretz, Read, & Peele, 1979).

Increasing the propagation efficiency of Galax rhizomes has also proven difficult. Generally using auxin alone has been found to stimulate rhizogenesis while a high cytokinin to auxin ratio stimulates shoot formation on explants (George, 1993). Bir (2005) studied the effects of soaking rhizomes in water along with quick dips or a15 minute soak in IBA and/or GA_3 solutions. No increase in percent bud break or root development was seen for rhizomes treated with IBA soaks or quick dips. A similar lack of response was seen for all GA_3 treatments. When rhizomes were soaked in water overnight at room temperature, rooting, vegetative bud break and survival were increased. Galax plants also exhibited bud dormancy after a period of time. It was found that 30 days in a cooler held at 7.2°C stimulated bud break in 95% of rhizomes. Chilling for 60 days accelerated the rate of bud break but resulted in the same percentage (Bir, 2005). However, despite these results rhizome cuttings propagate slowly and are thought to not be an effective method of propagation for large-scale production.

The potential of Galax seeds for use in the propagation protocol was also assessed by Bir (2005). He tested seed manipulation and various photoperiods thought to improve germination but they did not substantially influence germination. His work using seeds as the starting material for propagation was done on sphagnum peat moss in a winter greenhouse at 18.3°C and germination occurred in about 14-21 days (Bir, 2005). The addition of PGRs into the culture medium should speed up the growth of these tiny seedlings and increase the germination rate. By using a high cytokinin to low auxin ratio the rate of shoot induction should increase (George, 1993). Our preliminary experiments indicated that without PGRs it normally takes up to 2 months *in vitro* to produce the first leaf after the cotyledons. Shoot development after germination without PGRs is still extremely slow given the seedlings are under a controlled environment. The approach proposed in this study should prove to take less time than conventional propagation and provide a more controlled environment for germination and shoot proliferation.

2.3 Anthocyanins

2.3.1 Anthocyanins as nutraceuticals. In addition to the contribution of Galax in floral and landscape industries, it could also have significant potential to be used as a nutraceutical in the pharmaceutical industry. Having an *in vitro* protocol already established would allow for easy integration of Galax into this sector. It is important to understand the mechanism of anthocyanin production and its possible role as a nutraceutical.

Anthocyanins are red pigments that are found in various plant tissues like fruits and leaves. They are a member of the flavonoid family known to have high antioxidant power for repairing oxidative damage in tissues (Gould et al., 2009; Lila, 2004; Philpott et al., 2009; Welch et al., 2008). Anthocyanins have been known to accumulate up to four times more oxygen radicals

than ascorbic acid or vitamin E (Stutte & Edney, 2009). They also may provide protection from DNA cleavage, anti-inflammatory activity, membrane strengthening, estrogenic activity, enzyme inhibition, cancers, autoimmune, neurodegenerative, cardiovascular and digestive system disorders, as well as premature aging (Gould et al., 2009; Lila, 2004; Philpott et al., 2009; Welch et al., 2008). In folk medicine, anthocyanins from *Hibiscus sp* have been used in remedies for liver dysfunction and hypertension. Bilberry (*Vaccinium*) anthocyanins have a history of use for vision disorders, microbial infections, and diarrhea (Lila, 2004). These health benefits have prompted many studies to assess their medicinal value and methods for increased production. Production of these compounds for dietary supplements from Galax could generate additional profit for this sector. It is possible to produce anthocyanins in leaves or callus for use as a nutraceutical (Gould et al., 2009). While there are numerous anthocyanin supplements on the market it was found that extracts from different plants, edible and non-edible, showed differential effects in the chemical assays, suggesting that closely related structures have different affinities to scavenge different reactive species (Philpott et al., 2009). Therefore, Galax could also fill a niche in the nutraceutical industry when an efficient micro-propagation protocol is developed.

2.3.2 Role of anthocyanins in plants. Conditions that increase the production of anthocyanins include many environmental stressors such as high light irradiance, temperature, UV radiation, nutrient or water stress, and plant injury (Stutte & Edney, 2009). The fall color change in Galax is in response to the production of anthocyanins in the leaves triggered by an increase in light intensity (Figure 3; Hughes et al., 2005).

Figure 3. Galax fall color change in response to full sunlight. (Hughes, N., n.d.).

For some plants anthocyanins act as photoprotective elements that help protect the plant tissues by their interception and dissipation of light energy not yet absorbed by other pigments (Hughes et al., 2005). They also may lower the freezing point of cells to increase cold tolerance (Chalker-Scott, 1999).

Unlike many plants that change leaf color in fall, Galax only loses a minor amount of chlorophyll during the color change (Neufeld, 2002). The leaves continue to produce chlorophyll despite the increase in anthocyanins (Hughes et al., 2005). The leaves appear red due to the diminished reflectance of blue and green wavelengths while the level of red wavelength reflectance stays the same. This shift from a green reflectance peak to a red reflectance peak due to anthocyanin synthesis is not due to the loss of available light for photosynthesis. When red leaves and green leaves were exposed to red light, there was no difference in photoinhibition between them. However, green light caused the green leaves to be more stressed than red leaves.

These findings support the idea that anthocyanins act as light attenuators protecting the plant from excess irradiance (Hughes & Smith, 2007).

Similar results concerning light quality were found for anthocyanin production in the leaves of lettuce (Stutte & Edney, 2009). They tested various light qualities on induction and maintenance of leaf anthocyanin concentrations. They found that bioprotectant properties could be increased when exposed to wavelengths of red (640 nm), green (530 nm), and blue (440 nm) light. Similar results were found for the red and blue light treatments. Dry weight of the lettuce leaves also increased compared to conventional fluorescent lights. Further research found that continuous exposure to blue light was required to maintain high anthocyanin production in the lettuce leaves (Stutte, Edney, & Newsham, 2009). Hughes et al. (2005) investigated antioxidant concentration in Galax leaves and found that sun leaves had higher antioxidant activities than shade leaves. Also that anthocyanin content was positively correlated to antioxidant activity in the leaves. Their work suggested that once a micro-propagation protocol for Galax is established, treatments of specific light quality can be used to increase the concentration of anthocyanins in mature Galax or callus cultures for possible medicinal purposes (Gould et al., 2009; Rabino & Mancinelli, 1986; Zhong et al., 1993).

CHAPTER 3

Materials & Methods

3.1 Source & Viability of Collected Seeds

3.1.1 Seed source & collection. Galax seed stalks were collected in October 2010 from a single population spanning about 8,093 m^2 on Grandfather Mountain, NC. Transects were walked through the population approximately every 6 meters across the patch of Galax, and the nearest seed stalk was selected at a specific distance (1-50 ft) determined by a random number table. Selected seed stalks were pinched, placed in a paper bag and allowed to dry at room temperature (22°C) for 5 days at the Plant Biotechnology Laboratory of North Carolina A&T State University, Greensboro, NC.

3.1.2 Seed viability determined by tetrazolium staining (Obj 1, H_1). The total number of seedpods on each seed stalk was counted and recorded. Ninety seedpods were taken from 18 randomly chosen individuals (5 unopened seedpods per seed stalk). Seeds were placed in 1.5 mL microtubes, sterilized with 200 µL Bleachrite for 15 minutes and triple rinsed with 300 µL DI water. Seeds were then allowed to soak in 150 µL of 1% (w/v) Tetrazolium for 19 hours in the dark (Baskin & Baskin, 1998; Belcher, 2010; Fretz et al., 1979). These seeds were rinsed again with 300 µL deionized water and allowed to dry before counting. Stained seeds thought to be viable (i.e., stained pink or red) were counted and percent viable seeds per seedpod determined. The number of total seeds per seedpod on average was determined by summing the number of stained and unstained seeds per seedpod divided by the number of samples from that seed stalk. The fecundity of an average seed stalk was estimated as the product of mean number of seedpods per seed stalk and the mean number of viable seeds within each seedpod (Arizaga & Ezcurra, 2002).

3.2 Seed Source, Collection & Preparation for Germination & Shoot Proliferation Studies.

Galax seed stalks were sample from wilderness areas in Yancey County, NC in fall 2009 and 2010 and from Grandfather Mountain, NC in fall 2010. Seeds were separated based on stem color. Green stalks were considered to have early mature seeds, yellow stalks to have seeds of mid-maturity, and brown stalks to have late mature seeds. Each maturity group was equally divided in half. One half was refrigerated at 5°C throughout the experimental period. The other half was kept at room temperature (22°C) in the dark. As needed for each experiment described below, seeds pods from the refrigeration and room temperature treatments were lightly crushed and the seed coverings removed. Seeds were again separated by color; either light orange or dark rust (Figure 4).

Figure 4. Galax seeds after separation by color.

3.3 Germination & Culture Conditions

The separated seeds were sterilized in a 15% bleach solution plus 6 drops Tween-20 per liter for 15 minutes and taken to the laminar flow hood where the seeds were rinsed with sterilized distilled/deionized (DD) water three times. Seeds were then selected at random using a small pointer for initiation onto culture media with various pH treatments or culture media containing various PGR treatments. Seeds were placed on Murashige and Skoog (MS) medium

in Petri dishes supplemented with 3% sucrose, and solidified with 0.6% plain agar (Murashige & Skoog, 1962). Each Petri dish contained 20 ml MS medium. Petri dishes were sealed and placed in a growth chamber with 16 hours of light provided by cool white fluorescent tubes emitting 97 µmol m^{-2} s^{-1} at 23°C ± 2°C.

3.4 Germination Studies

3.4.1 Germination under various light treatments (Obj 2 & 3, H_3). Refrigerated Galax seeds of dark color were selected at random for germination on MS medium at pH 5.0 (Murashige & Skoog, 1962). The light treatments for germination were full light (16 hours light at 97 µmol m^{-2} s^{-1}), 18% shade (16 hours light at 17.15 µmol m^{-2} s^{-1}), and no light (dishes were wrapped in aluminum foil). Each treatment was replicated 10 times with 10 seeds per Petri dish. Percent seed germination was measured after 14 days.

3.4.2 Germination under various pH treatments without PGRs (Obj 2, Obj 3, H_2, H_3). Prepared mid-maturity Galax seeds of light and dark colors, from refrigeration and room temperature treatments, were selected at random for germination under various culture medium pH levels (4.2, 5.0, and 5.8). Each treatment was replicated 10 times with 10 seeds per Petri dish. Percent seed germination rates were recorded three times at 10 day intervals.

3.4.3 Germination using a combination of cytokinin & auxin. Refrigerated Galax seeds of dark color were selected at random for germination on MS medium at pH 5.0. The treatment media also contained different ratios of a cytokinin, (BA) along with an auxin (IBA). The control media contained no PGR. Each treatment of BA:IBA concentration ratios (1:1, 3:1, 6:1, and 9:1) was replicated 5 times with 10 seeds per Petri dish. Seed germination rates were taken and 6-Benzylaminopurine (BA at 0.77, 2.33, 4.66, or 6.99 µM) to an auxin, indole -3-butyric acid recorded twice during the 30 days of the experiment.

3.4.4 Germination using GA_3. Refrigerated dark-colored seeds were selected at random for germination testing under seven concentrations of Gibberellic acid (0, 100, 250, 500, 1000, 1500 and 2500 ppm (GA_3)) as treatments and a control (water only). Four hundred µl of each treatment solution were spread across the solidified MS media at pH 5.0. The excess solution was removed from the plate before seeds were placed on the media. Each treatment was replicated 10 times with 10 seeds per Petri dish. Percent seed germination was measured day 10 and day 21 during experimental period.

3.5 Shoot Proliferation Study

3.5.1 Shoot proliferation using various concentrations of different PGRs (Obj 3, H_4). Various ratios of cytokinin to auxin were tested on seedlings from the previous germination experiments to see if they affected shoot production of Galax seedlings. Ratios of a cytokinin, 6-Benzylaminopurine (BA at 0.77, 2.33, 4.66, or 6.99 µM) to an auxin, indole -3-butyric acid (IBA at 0.86 µM) and a no PGR control were tested. These concentration ratios (1:1, 3:1, 6:1, and 9:1) of BA:IBA were added to the MS media at pH 5.0 as treatments (Murashige & Skoog, 1962). Two layers of black fiberglass screen stretched over a frame were placed over the plates in the growth chambers to reduce the amount of light from 97µmol$m^{-2}s^{-1}$ to 17µmol$m^{-2}s^{-1}$ on average. This was determined based on preliminary findings to prevent the seedlings from turning red. Results on shoot proliferation were measured on day 20 and 42 during the experimental period.

3.6 Experimental Design & Data Analysis

The experimental design used was a completely randomized design (CRD). Normalization of data was attempted, but with no success. Data on *in vitro* Galax seeds percent germination and shoot proliferation were transformed using arc sin. However, there were no differences in significance. For simplicity the raw data on percent germination and shoot

proliferation was used for all analyses.

Differences between treatment means were determined using Fisher's least significant difference test (LSD) with SAS 9.2 software (SAS Inc., Cary, NC). Medium pH, cold stratification, seed maturity level, light intensity, and incubation time were variables tested for germination studies without PGRs. For germination studies using PGRs, BA:IBA concentration ratios, GA_3 concentrations, and incubation time were variables tested for optimal germination as determined by the mean number of germinated seeds. All statistical tests were done at the 5% level probability level.

CHAPTER 4

Results, Discussion & Conclusions

4.1 Results for Quantification of Viability

4.1.1 Seed viability determined by tetrazolium staining. The tetrazolium staining was apparent in Galax seeds (Figure 5). The mean viability per stalk of Galax sampled when using seeds that stained a bright pink and thought to be fully viable was 392 out of 2,344 total seeds (Table 1).

Figure 5. Tetrazolium (1%) staining of Galax seeds falls into three categories (no stain, light to moderate stain, and bright stain).

This means that only 17% of seeds are stained bright pink and thought to be fully viable when harvested from the field in late fall. Similar results were obtained for potentially viable seeds that stained light to moderately pink exhibiting 16% viability and a mean fecundity per stalk of 382.6 out of 2,344.

Table 1.

Viability of Galax seeds determined by tetrazolium staining. †

	Total seeds per stalk	**Viable seeds per stalk**	**Percent Viability**
Seeds stained bright pink	2,344	392.4	16.7%
Seeds stained light to moderate	2,344	382.6	16.3%
Total seeds stained	2,344	775.0	33.0%

†- n=90 for all treatment combinations

4.2 Discussion of Seed Viability.

The tetrazolium staining demonstrated that only one third of Galax seeds were viable when harvested in late fall before the cold of winter. There was very little difference between the number of seeds that stained bright pink and those that only stained light to moderately pink. Later tests demonstrated a germination rate of 76% for dark refrigerated Galax seeds. This suggests that Galax seeds are viable after fall harvest but may be dormant. Because the actual germination rate was so much higher than the viability found through tetrazolium staining this method may not give an accurate estimate of Galax's viability. These findings support the H_1 hypothesis that seeds are predominantly viable after fall harvest from the field, and germination rates were not corrected to reflect these findings. Testing should be done after staining to determine how many seeds actually germinate from each category. Possible reasons for the failure of the tetrazolium test might be that the stain failed to penetrate through the seed coat of some seeds, or that it is difficult to assess a viable seed that stains poorly. Allowing the seeds to soak in the solution for a longer period of time or pre-treating the seeds may result in better tetrazolium staining. Also, if seeds are harvested when too immature, they might die after harvesting, thereby lowering the viability estimate. However, since seeds that received a cold

treatment had much higher germination percentage, this last hypothesis is probably not correct. More work should be done to assess the efficacy of the tetrazolium staining technique for determining the viability of Galax seeds.

Many seeds require a cold shock treatment to break dormancy (Kucera et al., 2005). Because seeds were fall harvested they had not gone through a cold period. Seeds need to be exposed to extreme cold temperatures to break this dormancy. It was not possible to collect seeds in the spring because as the seedpods mature they dry out and quickly release the seeds in fall. It is important to harvest seeds as late in the season to allow for maturity but before seeds is released. Also, the random method in which the seed stalks were chosen for this viability experiment was that the closest stalk was chosen at every predetermined point. This meant that many seed stalks which were still bright green and not thought to be ripe enough for seed harvest were chosen as the material to be analyzed. Having early mature seed stalks as the majority of the samples would contribute to the low viability. If only seed stalks that were brown in color were chosen for the viability test most likely a slight rise in the percent viability would be observed for the population.

4.3 Results for Germination Studies

4.3.1 Germination under various light treatments. Light intensity was found to have a significant effect ($p<0.05$) on percent seed (Table 2.). Seed germination rates in 18% shade were not different from those placed in full light but both of these light treatments were different than the no light treatment. In the absence of light germination was negatively affected resulting in a rate of only 18% (Figure 6). In general, seeds placed in full light had 64% germination. While seeds placed in 18% shade had a germination of 56%.

Table 2.

Analysis of variance of refrigerated (5°C) dark-colored Galax seed germination (%) as affected by light intensity from initiation after 14 days of incubation.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	P>F
Treatment	2	120.8	60.4	33.69	<0.0001*
Error	27	48.4	1.8		
Total	29	169.2			

*indicates significant treatment effect ($p < 0.05$).

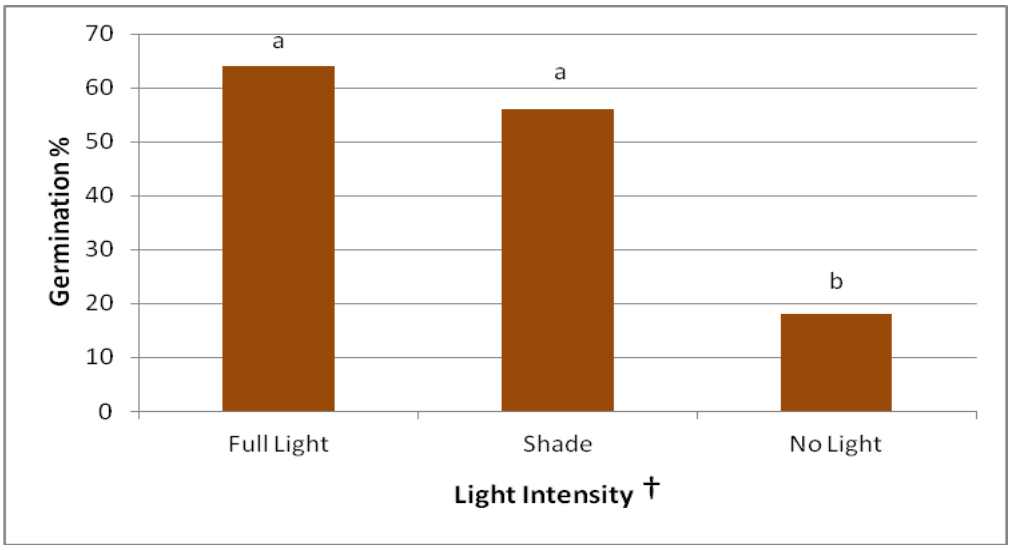

Figure 6. Effects of light intensity on Galax seed germination. Means having the same letter are not significantly different at the 5% level of significance as indicated by Fisher's protected LSD test. †- A light intensity of full light was equal to 97 $\mu molm^{-2}s^{-1}$ and the shade treatment was 18%.

4.3.1.1 ***Discussion of germination under various light treatments*.** The results from the light intensity experiment demonstrated that light was required for germination. And even low levels of light will stimulate germination. In nature, Galax seeds are released from pods as they dry on tall stalks. Seeds are scattered among ground leaf litter which shades the seeds. To look

further into the requirements for germination various amounts of shade should be tested to determine a range of suitable light intensities for maximum germination.

4.3.2 Results of seed germination as affected by pH stratification, temperature, & maturity level. Germination was achieved for some treatments (Figure 7).

Figure 7. *In vitro* germination of dark Galax seeds under full light on MS medium at pH 5.0.

There was a significant interaction effect ($p<0.05$) between temperature and maturity level (Table 3). When cold stratification was applied to light-colored seeds germination increased to 42% on average. Light-colored seeds kept at room temperature did not germinate. The interaction effect between pH and seed maturity was not significant at the 0.05 level. Cold stratified seeds at 5^0C that were dark-colored produced the highest germination of 76% compared to dark-colored seeds kept at room temperature which resulted in only 10% germination on average (Figure 8).

Table 3.

Analysis of variance of Galax seed germination as affected by pH level, stratification temperature and maturity level.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	P>F
Temperature (T)	1	888.8	888.8	560.22	<0.0001*
pH (P)	2	0.3	0.2	0.12	0.8859
Maturity Level (M)	1	143.6	143.6	90.56	<0.0001*
T*P	2	3.9	1.9	1.23	0.2957
T*M	1	42.1	42.1	26.58	<0.0001*
P*M	2	8.9	4.4	2.83	0.0634
T*P*M	2	0.0	0.0	0.02	0.9849
Error	107	169.7	1.5		
Total	118	1257.7			

*indicates significant treatment effect ($p < 0.05$).

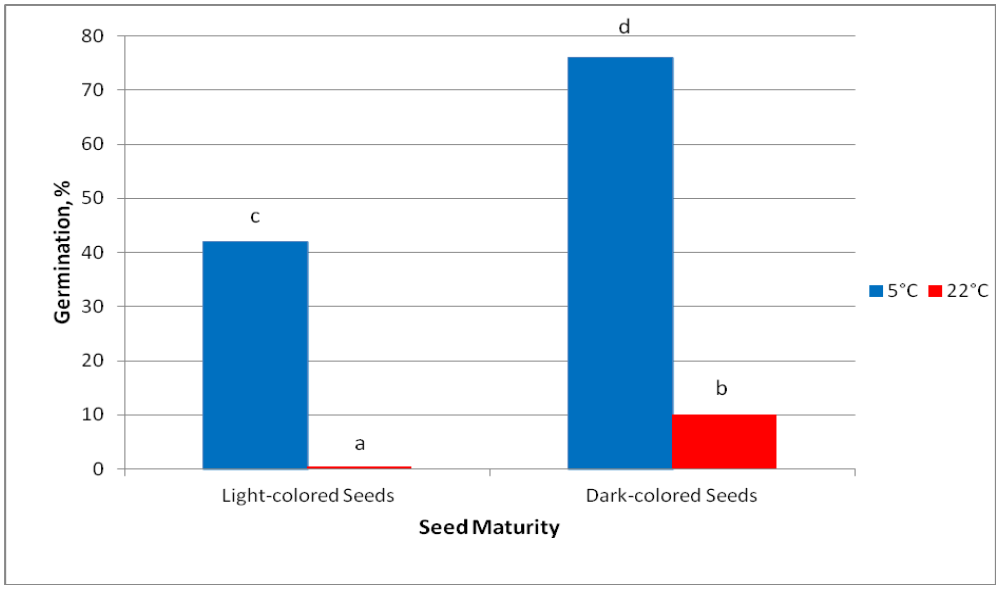

Figure 8. Effects of stratification temperature and seed maturity level on Galax seed germination (%). Means having the same letter are not significantly different at the 5% level of significance as indicated by Fisher's protected LSD test.

LSD values were calculated for α=0.05 and those differences were reported.

Within each seed maturity level culture media pH had a different effect on percent germination (Figure 9). For light-colored seeds a pH level of 5.8 resulted in a significantly lower percent germination of 16% while dark-colored seeds showed the highest percent germination at 49%. The lowest percent germination for dark-colored seeds 37% was seen at a pH of 5.0.

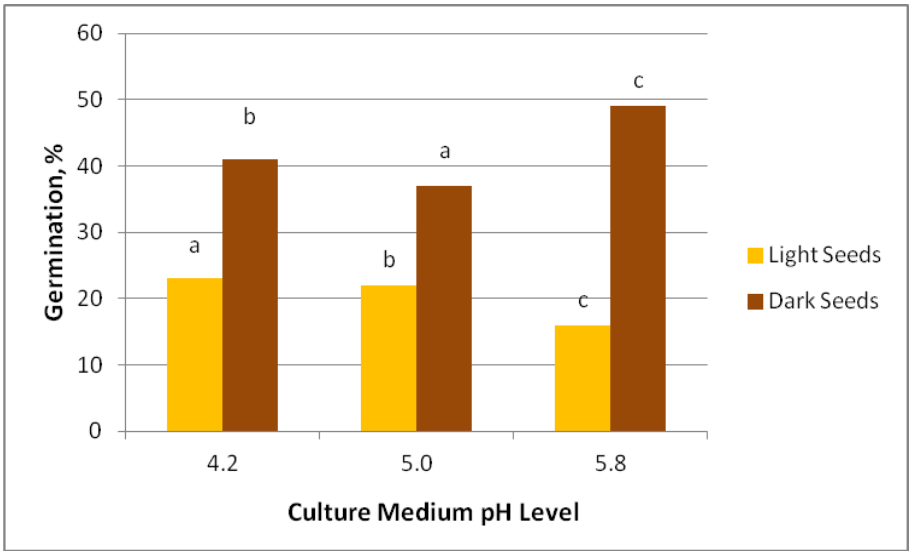

Figure 9. Effects of culture medium pH and seed maturity level on Galax seed germination (%) for each seed maturity level. Within each maturity level, pH x maturity means having the same letter are not significantly different at the 5% level of significance as indicated by Fisher's protected LSD test.

4.3.2.1 ***Discussion of seed germination as affected by pH stratification, temperature, & maturity level*****.** Stratification temperature and seed maturity level were the two main factors influencing seed germination. Dark seeds given a 30 day cold stratification period had the highest germination rate of 76% of any treatments applied. Dark-colored seeds left at room temperature exhibited only 10% germination. This suggests that a cold treatment is required to break seed dormancy, which supports the hypothesis that cold stratification does affect the germination of Galax seed. Because light-colored seeds do not perform as well as dark-colored

seeds for any treatment the data support the hypothesis that Galax seed color does relate to maturity and viability. Light seeds are less mature and only germinate in small numbers. For large-scale production seeds should be harvested late in the season to maximize seed maturity and then stratified for at least 30 days before sowing.

The interaction between seed maturity and culture media pH level was not seen however, it is important to observe differences in seed biology among various pHs. Light-colored seeds seemed to prefer to germinate at a pH of 5.0 or below whereas dark-colored seeds preferred to germinate at a slightly higher pH of 5.8. Based on these percentages light-colored seeds are more affected by culture medium pH than dark, mature seeds. My earlier observational studies with mixed seeds demonstrated that Galax seeds cultured at pH 4.2 had a higher initial germination than those at pH 5.0 or pH 5.8. Over time, the seeds from the pH 5.0 and 5.8 treatments did as well as the pH 4.2 treatment. However, when cold stratification treatments are used, the pH level of the culture medium has no effect on seed germination. By using only separation of seed maturity levels and cold stratification for the treatment of Galax seeds, the maximum germination rate can be achieved creating a dependable method to produce seedlings for *in vitro* propagation.

4.3.3 Germination results using a combination of cytokinin & auxin. A cytokinin (BA) and an auxin (IBA) added to the culture media at several ratios proved to have significant negative effects ($p<0.05$) on seed germination (Table 4). After 30 days of incubation on culture media the control exhibited 20% germination, which was significantly higher than any of the PGR treatments. Note that ratios of BA to IBA greater than 1:1 resulted in0% germination (Figure 10).

Table 4.

Analysis of variance of refrigerated (5°C) dark-colored Galax seed germination (%) as affected by BA:IBA ratio concentrations after 30 days of incubation.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Value	P>F
Treatment	4	15.4	3.8	10.95	0.0001*
Error	17	6.0	0.3		
Total	21	21.4			

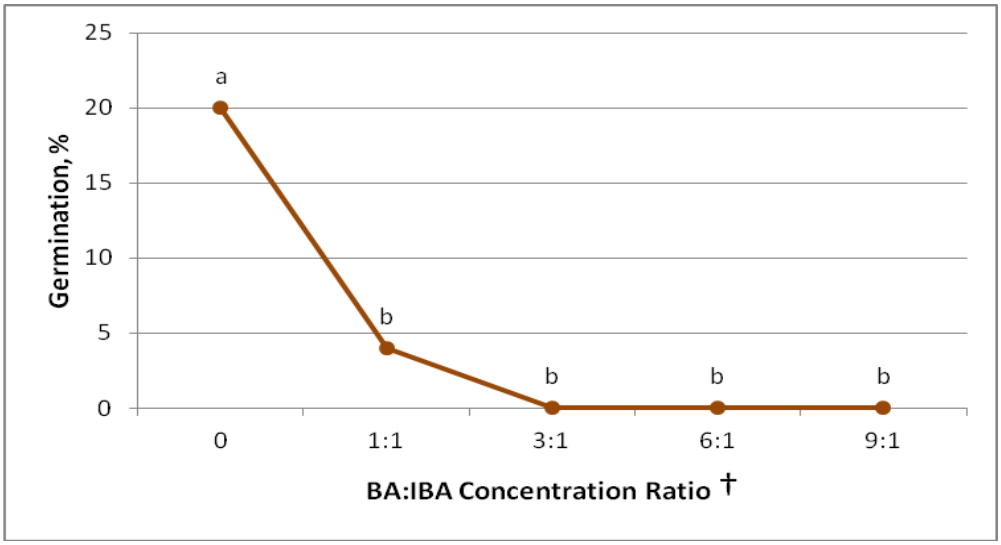

Figure 10. Effects of BA:IBA concentration ratios on refrigerated (5°C) dark Galax seed germination (%) after 30 days of incubation. Means having the same letter are not significantly different at the 5% level of significance as indicated by Fisher's protected LSD test. †- For BA, one ratio unit = [0.77] µM; and for IBA, one ratio unit = [0.86] µM.

4.3.3.1 ***Discussion of germination using combinations of cytokinin & auxin*****.** The control treatment in this experiment outperformed any PGR ratio concentration treatment. However, the control germination rate of 20% was much lower than the 76% germination rate observed in previous experiments. The only germination seen was for the BA:IBA ratio 1:1

which was 4%. Germination was not observed for any other BA:IBA concentration after 30 days. Possible explanations for this may be storage longevity of the seeds, inconsistencies of seed health and vitality, and even prolonged storage over several months in the refrigerator. The most likely of these scenarios would be differences in health and vitality between seed batches and prolonged refrigeration. Seeds used for this experiment were collected along a mile of fire road, and the seeds might be from different populations with different viabilities. Precautions to keep the stored seed homogenous were taken, but as the seed supply began to run low, it is possible that the characteristics of previously chosen seedpods for seed removal became less dominant. This may have resulted in seeds being chosen with slightly less uniform viability as done for previous experiments. For future efforts having an extensive supply of seeds on hand would ensure better uniformity between batches. Also refrigerating seeds as needed prior to initiation may cut down on these inconsistencies. It is possible that over an extended period of time the seeds undergo desiccation while being stored in paper bags which could affect germination. In the future seeds should be kept in sealed vials. The data reported here were unable to support the hypothesis that the presence of PGRs in the culture medium will enhance Galax seed germination. However, for many species, cytokinins break dormancy and promote germination, often by enhancing ethylene biosynthesis (Kucera et al., 2005; Mok & Mok, 1994).

It is known that PGRs often stimulate cellular processes within the plant (Stirk et al., 2005). They are very instrumental in the regulation of seed dormancy and germination (Kucera et al., 2005). During germination the endosperm is the source of cytokinin needed for cell division of the embryo (Baskin & Baskin, 1998; Mok & Mok, 1994; Stirk et al., 2005). Other reports have found that cytokinin probably only aids in nutrition during germination and is in fact a negative regulator of germination (Finkelstein, 2004; Stirk et al., 2005; Riefler et al.,

2006). Depending on the cytokinin used for tissue or organ culture, they can have varied effects. While one cytokinin may be effective others may elicit no response (George, 1993; Mok & Mok, 1994).

Auxin is crucial for the process of embryogenesis (Riefler et al., 2006). Embryogenesis is often initiated by high levels of auxin, but the embryo will not continue to develop until the concentration of auxin begins to reduce (Finkelstein, 2004). Overproduction of auxin has been known to inhibit germination and even cause embryo death (Schernthaner et al., 2003).

Auxin is known to decrease the water potential of the cell, allowing water to enter, thus stimulating rapid growth of the cell. Auxin also reverts cells to a dedifferentiated state after which they begin to divide (George, 1993). The study performed by Bir (2005) treating Galax rhizomes with IBA and/or GA_3 showed no increase of bud break or root development. In other studies auxin has been found to delay bud break for woody species (Yang & Read, 1993). This lack of response of Galax rhizomes to these specific PGRs most likely does not correlate to the physiological processes during germination. This is because their degree of activity greatly varies from organ to organ, tissue to tissue and cell to cell (George et al., 2008). Even with the possible seed inconsistencies, the data suggest that the presence of these specific PGRs (BA and IBA) have a negative effect on germination. Due to the varied responses between types of PGRs and the plant parts they are applied to it is possible that others might prove to positively influence seed germination.

4.3.4 Germination results using GA_3. The treatment of refrigerated dark-colored Galax seeds with GA_3 at various concentrations had a significant positive effect ($p<0.05$) on seed germination (Table 5; Figure 11a). However, for Galax seeds kept at room temperature, there were no differences between the GA_3 treatments (Table 6; Figure 11b).

Table 5.

Analysis of variance of refrigerated (5°C) dark-colored Galax seed germination (%) as affected by GA_3 concentration treatments after 14 days of incubation.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Value	P>F
Treatment	6	17.3	2.8	2.46	0.0336*
Error	61	71.4	1.1		
Total	67	88.7			

Table 6.

Analysis of variance of room temperature (22°C) dark-colored Galax seed germination (%) as affected by GA_3 concentration treatments after 14 days of incubation.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Value	P>F
Treatment	6	42.7	7.1	1.77	0.1315
Error	38	153.1	4.0		
Total	44	195.9			

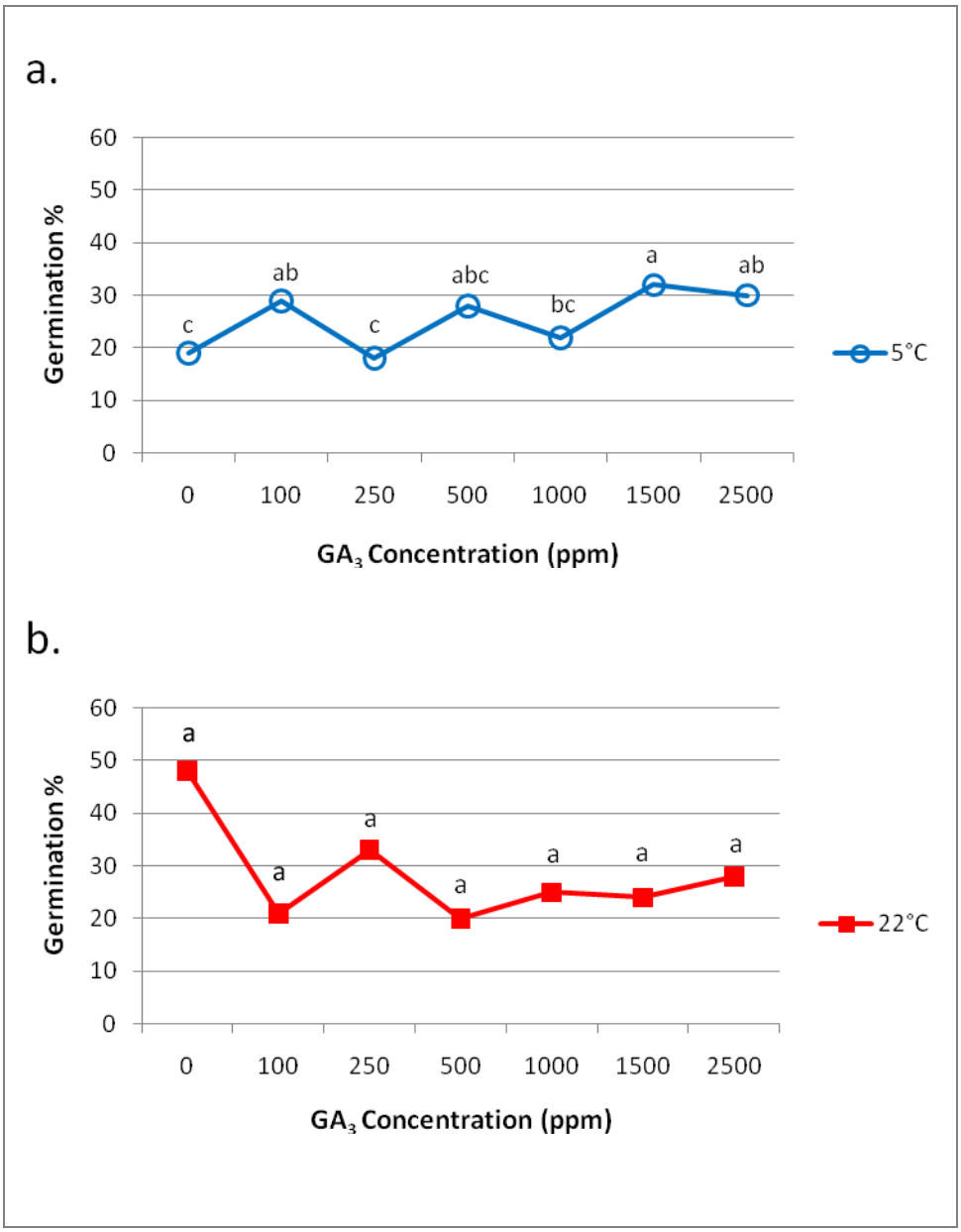

Figure 11. Effects of GA_3 concentrations on dark-colored Galax seed germination (%) for 2 stratification temperatures (a) 22°C and (b) 5°C. Within each stratification temperature means having the same letter are not significantly different at the 5% level of significance as indicated by Fisher's protected LSD test. N= 700

4.3.4.1 ***Discussion of germination using GA_3.*** Gibberellins (GA) are known to break seed dormancy, promote germination and counteract abscisic acid (ABA) effects. Dormancy and germination are the result of a balance between a promoter (GA_3) and an inhibitor ABA along with environmental conditions such as temperature (Baskin & Baskin, 1998; Bewley, 1997; Kucera et al., 2005; Subbiah & Reddy, 2010). In the experiment using dark-colored Galax seeds kept at room temperature, the GA_3 treatment did not elicit a response any different from the control lacking GA_3. It is known that GA_3 affects the temperature responsiveness of seeds through ABA metabolism (Baskin & Baskin, 1998; Kucera et al., 2005). The GA_3 treatment became significant after the cold stratification pretreatment for dark-colored Galax seeds. Together these results suggest that after dormancy is broken by a cold shock, GA_3 promoted germination. This supports the hypothesis that the presence of GA_3 in the medium would enhance Galax seed germination, but only when using seeds pretreated by cold stratification. Baskin and Baskin (1998) also reported that with refrigeration ABA within the seed decreased, promoting loss of dormancy, followed by an increase in GA which promoted germination. With further research it might be possible to show that cold temperatures promote the metabolization of ABA by GA_3 thus allowing the seeds to be brought out of dormancy. While the treatment positively influenced germination rates compared to the control, the rates are dramatically lower than those achieved for previous experiments. There seem to be inconsistencies between the control and the treatments for refrigerated Galax seeds similar to those previously proposed for germination using PGRs, the most likely being differences between batches of seeds prepared for random sampling, or problems with the GA_3 stock solution. The controls showed much lower germination rates than for previous experiments. Seeds at room temperature had a percent germination of 48% while refrigerated seeds only had 19%. These inconsistencies are most

likely due to desiccation of the seeds, as they were stored in paper bags. This loss of water in the seed could potentially affect germination and cause the seeds to die. This experiment would require more analysis and adjustment of methodology before it would be possible to fully understand the effects of GA_3 on Galax germination.

4.4 Results for Shoot Proliferation Study

4.4.1 Shoot proliferation results using various PGR concentrations (Obj 3, H_4). Shoot initiation was successful and seen for all treatments evaluated. Multiple shoots were seen for each treatment (Figures 12). The mixture of BA and IBA added to the culture media in different ratios did not affect shoot proliferation (Table 7) nor was it different from the control without PGRs (Figure 13). However, the visual appearance of the shoots did vary between treatments. The control and the PGR treatment with a 1:1 ratio had only leafy large shoots. The PGR with a 3:1 ratio had individuals that showed either leafy or small compact shoots. PGR ratios of 6:1 and 9:1 were similar in appearance and had numerous small compact shoots, with a few larger ones also present on the same individual (Figure 14).

Figure 12. *In Vitro* Galax seedling with multiple shoots for BA:IBA ratio 6:1.

Table 7.

Analysis of variance of refrigerated dark Galax seedlings average number of shoots as affected by BA:IBA ratio concentrations after germination at 20 days.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	P>F
Treatment	4	10.1	2.5	2.65	0.0811
Error	13	12.4	0.9		
Total	17	22.6			

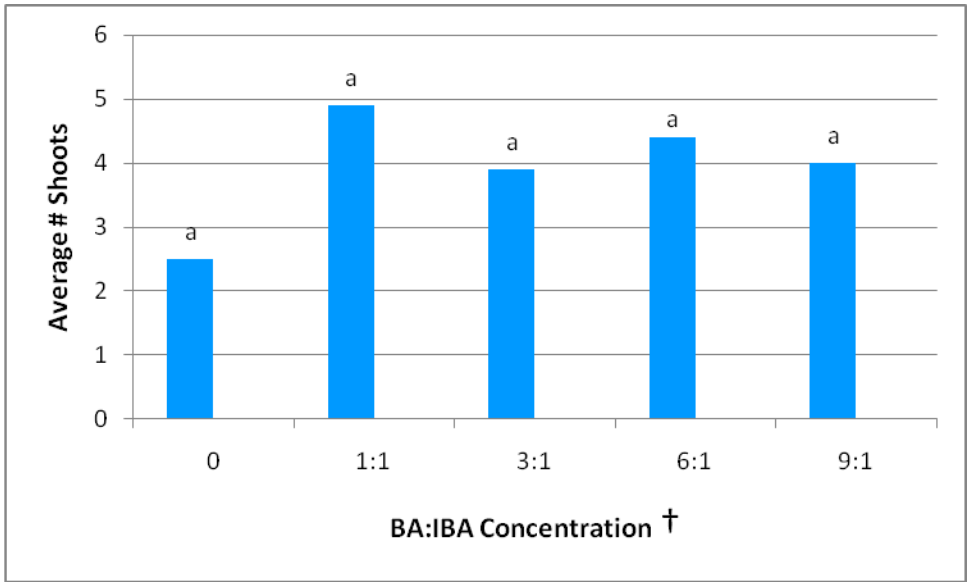

Figure 13. Effects of BA:IBA ratio concentrations on refrigerated (5°C) dark Galax seedlings for average number of shoots 20 days after germination. Means having the same letter are not significantly different at the 5% level of significance as indicated by Fisher's protected LSD test. †- For BA one ratio unit would be equivalent to a concentration 0.77 µM and for IBA one ratio unit would be equivalent to a concentration 0.86 µM.

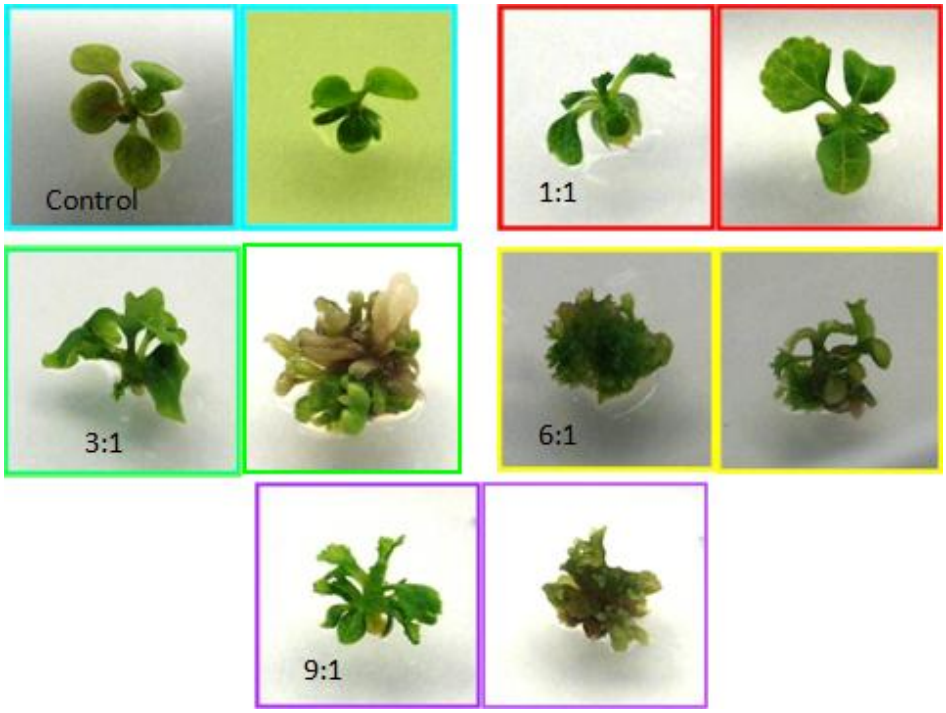

Figure 14. Variance in shoot morphology of Galax seedlings grown *in vitro* on MS media for various BA:IBA concentration ratios (not to scale).

4.4.1.1 ***Discussion of shoot proliferation using various PGR concentrations*****.** Multiple shoots were induced. However, the different ratios of BA:IBA were not found to be significantly different from the control without PGRs These findings were unable to support the hypothesis that the presence of PGRs in the culture medium would enhance seedling shoot formation. In previous reports treating rhizomes with IBA and/or GA_3 no response was observed for vegetative bud break or root production (Bir, 2005). Because the effectiveness of cytokinin and auxin varies so much between plants, tissues and organs, this suggests that these specific PGRs might not be suitable to elicit the desired response (George, 1993). Newly formed shoots demonstrated different morphologies among the treatments suggesting that there was some effect on shoot form. While the higher BA:IBA ratios resulted in smaller and more compact shoots, the lower BA:IBA ratios produced mainly leafy shoots. These morphological differences were not

analyzed statistically though future efforts should focus on which ratio will result in the preferred morphology for excision of explants. Smigocki and Owens (1989) studied shoot morphologies of tobacco and cucumber cultures with various endogenous cytokinin to auxin ratios and found that elevated endogenous cytokinin to auxin ratios resulted in a shoot phenotype. This morphology was determined by the loss of apical dominance and inhibition of root formation. The highest ratios produced mainly callus suggesting that at a certain point the extreme concentrations may disrupt the cells ability to differentiate into shoots. Further research is needed to determine the proper concentrations of PGR ratios for shoot induction in Galax and for obtaining the desired shoot morphology.

4.5 Conclusions

Hypothesis 1:

Table 1 shows that Galax seeds harvested in late fall had a low percent viability around 16% for brightly stained seeds. Because further testing resulted in a germination rate of 76%, tetrazolium staining may not be a good indicator of Galax seed viability. Therefore we failed to reject H_0: Galax seeds are viable after harvest from the field, and conclude that tetrazolium staining is not a good indicator of Galax seed viability.

Hypothesis 2:

Figure 8 shows significant differences in germination means between light and dark seed maturities. Dark-colored seeds had higher germination rates demonstrating it to be the more mature seed type. Therefore we rejected H_0: Galax seed color does not correspond to maturity and viability, and concluded that Galax seed color does correspond to maturity and viability.

Hypothesis 3:

Figure 6 shows significant difference in germination means between treatments exposing

seeds to light and the no light treatment. Therefore we rejected H_0: cold stratification, culture medium pH and light intensity do not affect Galax seed germination, and concluded that light intensity does affect Galax seed germination.

Figure 8 shows significant differences in germination means between stratification treatments. Therefore we rejected H_0: cold stratification, culture medium pH and light intensity do not affect Galax seed germination, and concluded that cold stratification does affect Galax seed germination.

Figure 9 failed to show significant differences in germination means between culture media pH levels at the 0.05 level of significance. Therefore we failed to reject H_0: cold stratification, culture medium pH and light intensity has no affect on Galax seed germination, and concluded that culture medium pH does not affect Galax seed germination.

Hypothesis 4:

Figure 10 shows that the controls outperformed the treatments of PGRs in the medium which caused negative effects on seed germination. Therefore we failed to reject H_0: the presence of PGRs in the medium will not enhance Galax seed germination, and concluded that BA:IBA ratios in the medium do not enhance Galax seed germination.

Table 5 shows that for refrigerated Galax seeds there was a significant difference in germination means between the GA_3 treatments and the control. Therefore we rejected H_0: The presence of PGRs in the medium will not enhance Galax seed germination and seedling shoot formation, and concluded that the presence of GA_3 in the medium will enhance Galax seed germination when pretreated with cold stratification. However, these results were not as definitive as they must be to properly develop a protocol for commercial *in vitro* production.

Figure 13 shows that at 20 days of incubation there was no significant difference in

average number of shoots between PGR treatments and the control. Therefore we failed to reject H_0: The presence of PGRs in the medium will not enhance Galax seed germination and seedling shoot formation, and concluded that BA:IBA ratios in the medium do not enhance seedling shoot formation.

Galax has proven to be a difficult to propagate plant species. With extremely low seed viability, inconsistencies within harvested seed batches, seed dormancy, and varied responses to plant growth regulators, Galax provides many obstacles that must be overcome individually before an efficient protocol can be developed for commercial production. Given the positive response to cold stratification and separation by seed maturity theses methods should become the first step in any protocol for Galax production. Given available labor and time, the best starting material for the protocol would be dark-colored seeds that have been separated out and refrigerated for 30 days. Positive responses, although not significant were also seen for shoot formation using BA and IBA after germination. The optimal ratio of PGRs for maximizing this process is not yet known. Further research taking an in-depth look at the effect of various PGRs at different concentrations needs to be conducted. Galax continues to be a promising plant as an economical cash crop, and the benefits from developing an efficient protocol will outweigh its difficulty.

4.8 Recommendations for Future Research

An adaptation for the methodology of this protocol that may elicit more consistent results should be applied concerning seed preparation. The protocol used for these experiments was to hand select seedpods off the seed stalks, crush them, and separate out the seeds by color before every experiment or replication. This method unknowingly allowed for the best looking seeds to be chosen first. As the seeds began to be used up it became more difficult to find seeds of dark

color. More seedpods had to be sorted to achieve the same amount of dark-colored seeds as when the experiments first started. This may have lead to less healthy or not as fully mature seeds being chosen for the experiments. This may have accounted for some of the discrepancies seen in the later experiments using PGRs. A suggested change in the methodology would be after separating the seed stalks by color to then crush all seedpods and remove the debris. Then all the seeds may be stored together in plastic bags as seed stock, allowing for homogeneous mixing of the seeds. This would ensure that seeds in all stages of maturity have the same chance of being chosen to be separated out by color and pretreated with cold stratification or room temperature treatments. Also performing all experiments in a shorter time frame would cut down on desiccation. It has been found that seeds stored dry at low temperatures actually exhibit slower rates of physiological changes (Baskin & Baskin, 1998).

The next step in analyzing Galax seed maturity would be to determine if lighter colored seeds are in fact immature. Seeds should be collected throughout the season to see if the proportion of light to dark-colored seeds changes. If light seeds are maturing and becoming dark in color then harvesting late in the season would be the goal to maximize seed germination rates.

A recommendation for experiments using GA_3 would be to try several methods of application. The method used in this experiment was to apply a light film of GA_3 on top of the solid medium. Other methods of application could include a GA_3 soak or addition of GA_3 into the solid medium (George, 1993; Kucera et al., 2005).

It has been found that nitrates stimulate germination in a number of plant species (Baskin & Baskin, 1998). Seeds undergoing cold stratification became more sensitive to nitrates as dormancy breaks. A synergism is also seen in nitrate deficient seeds between ethylene and nitrates that promotes dark germination (Baskin & Baskin, 1998). The medium used for these

experiments was MS basal salt mixture with macro- and micro-nutrients. The MS medium does contain some nitrates, including 1.65 g/L ammonium nitrate (NH_4NO_3) and 1.9g/L potassium nitrate (KNO_3) (Murashige & Skoog, 1962). Due to the positive response of many species to nitrate, future analyses should determine the optimum amount of nitrates for maximum germination of Galax.

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