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Factors affecting *in vitro* seed germination and shoot proliferation of galax [*Galax urceolata* (Poir.) Brummitt]

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Abstract

To overcome the limitations of traditional propagation of Galax (*Galax urceolata* (Poir.) Brummitt), the feasibility of *in vitro* propagation was explored. We studied the effects of cold stratification, seed maturity, light intensity, pH and plant growth regulators on *in vitro* seed germination and subsequent shoot proliferation of Galax. Our results demonstrated that cold stratification enhanced seed germination. Seeds cold stratified at 5°C for a month resulted in significantly higher seed germination than seeds treated at 22°C for the same period. The level of seed maturity had an impact on seed germination. More mature dark brown seeds consistently produced higher seed germination (> 70%) than less mature light orange seeds (between 30% and 40%). The highest germination percentage of 76% was obtained with dark brown seeds stratified at 5°C. Light exposures stimulated germination. Light exposure at 97 µmol $m^{-2}s^{-1}$ and minimal light at 17µmol $m^{-2}s^{-1}$ promoted significantly higher seed germination percentages (64% and 56% respectively) than total dark (18%). Germination media at pH levels of 4.2, 5.0 or 5.8 had no effect on germination, irrespective of seed maturity. Gibberellic acid (GA_3) negatively affected germination, which was significantly reduced germination from 49% to around 30%. Additions of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) at different ratios in the growing medium significantly influenced shoot proliferation. The ratio of 1:1 of BAP : IBA produced the highest shoot number (8.1). All other ratios, (3:1, 6:1 and 9:1) showed no promotive effect on shoot proliferation. Abnormal shoots and shoot hyperhydricity were also observed in cultures with 6:1 and 9:1 of BAP and IBA ratios. This is the first report on successful in vitro propagation of galax

Keywords: Galax; *Galax urceolata* (Poir.) Brummitt; light intensity; pH; plant growth regulators; seed maturity; stratification.
Abbreviations: BAP-6-benzylaminopurine; IBA-indole-3-butyric acid; GA_3- Gibberellic acid.

Introduction

Galax (*Galax urceolata* ((Poir.) Brummitt), a member of the family *Diapensiaceae*, is an evergreen perennial monocot species, commonly known as beetleweed. It has durable, shiny green leaves (Fig 1A) which turn red in the fall, and are valued by the floral industry as background foliage (Scott and Day, 1983). However, due to extensive harvesting of leaves and a lack of a commercially efficient propagation methods, this species is at risk of becoming economically unviable. Surprisingly, in spite of its high economic value, few studies on galax propagation have been reported. Traditionally, galax can be propagated by root division, however, only a very limited number of plants can be produced from each mother plant. Bir (2005) demonstrated that propagation by root division is possible, but this does not appear to be a practical means for commercial production. In their bibliography on galax, Predny and Chamberlain (2005) noted that propagation by seed was recommended as a more practical approach than root division for large-scale cultivation. However, no studies, supporting this have been reported. Another approach is *in vitro* propagation which, although reported in many other plant species, has not yet been achieved in galax. In fact, there have been no reports on successful *in vitro* manipulation of this species. Successful *in vitro* propagation requires the proper balance of a confluence of factors, including seed maturity, light intensity, cold stratification, pH, and plant growth regulators. This study was conducted to evaluate various factors that might affect *in vitro* germination and subsequent shoot proliferation of galax. This is the first report on in vitro propagation of galax and also represents our first step toward in vitro manipulation and conservation of this valuable species.

Results

Effect of cold stratification and seed maturity on in vitro seed germination percentage

Both cold stratification and seed maturity had significant effects on the seed germination percentage. Seeds at two maturity levels stratified at 5°C had significantly higher germination percentages than seeds stored at 22°C. More mature dark brown seeds consistently germinated better than less mature light orange seeds, regardless of temperature pre-treatments. The best seed germination of 76% was obtained with dark brown seeds stratified at 5°C (Fig 2).

Effect of light intensity on in vitro seed germination percentage

Seed germination percentages of 64% were obtained under full light (97 µmol $m^{-2}s^{-1}$) and 56% under light with shade (17 µmol $m^{-2}s^{-1}$). However, there was no significant

difference in germination between these two light levels. A significantly lower 18% germination was observed for seeds grown under dark conditions than those seeds grown under two light levels (Fig 3).

Effect of pH on in vitro seed germination percentage

The pH at 4.2, 5.0 and 5.8 had no significant effect on germination of seeds at both maturity levels. Seed maturity significantly affected the germination percentage. More mature dark brown seeds produced higher germination rates (> 70%) than less mature light orange seeds (between 30% and 40%) at all three pH levels examined (Fig 4).

Effect of GA_3 in the germination medium on in vitro seed germination percentage

GA_3 exhibited a negative effect on seed germination of galax. Seed germination percentages were reduced to between 20% and 30% when GA_3 was incorporated into the culture media at 6 concentrations, which was significantly lower than the control (around 50%, Fig 5).

Effect of ratio of BAP and IBA on shoot proliferation

The ratio of BAP and IBA significantly affected shoot proliferation. The greatest shoot number of 8.1 was obtained on the medium with a BAP: IBA ratio of 1:1. All other ratios and the control resulted in significantly lower shoot multiplication rates (around 5 shoots), however, there was no significant difference among them (Fig 6). The BAP: IBA ratio also influenced shoot morphology. In comparison with the control (Fig 7A), normal shoot multiplication was observed on cultures with the BAP: IBA ratios of 1:1 and 3:1 (Fig.7B, C). Higher ratios of 6:1 and 9:1 resulted in much smaller and compact shoots. Shoot hyperhydricity and callus formation were also obvious on these cultures (Fig. 7D, E). In general, seed germination occurred uniformly within about 4 weeks. Radicles emerged first, and then cotyledons appeared in the following 2 weeks, and complete seedlings emerged within 6 weeks (Fig 8A). After seedlings were transferred onto appropriate media, shoot proliferation occurred within 2 to 3 weeks (Fig. 8B). Root development was also observed simultaneously with shoot multiplication in most of the cultures. Fully developed plantlets were available by the 6th week of culture (Fig. 8C).

Discussion

The overall objective of this study was to examine factors affecting *in vitro* seed germination and subsequent shoot multiplication of galax aiming at establishing a feasible system for commercial application, as an alternative means for efficient propagation of this species. Due to the extremely small size of galax seeds, averaging only 0.6 mm x 0.3 mm, establishing *in vitro* germination was a significant challenge. We observed that small galax seeds germinated slowly and gave rise to small seedlings compared with large seeds. Maturity levels of the seeds had a significant effect on seed germination outcomes. Although there is no previous report on the relationship of seed maturity to germination ability in galax, our results demonstrated that more mature dark brown seeds consistently produced higher germination percentages than less mature light orange seeds under various treatment conditions. This study also showed that the color of seeds is a good indicator of seed maturity level.

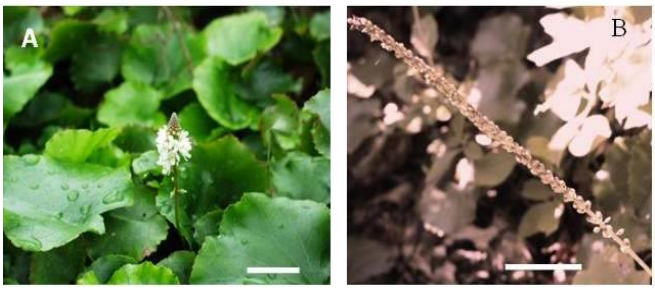

Fig 1. Galax plants. A: Leaves. B: Stalks. Bars = 1cm.

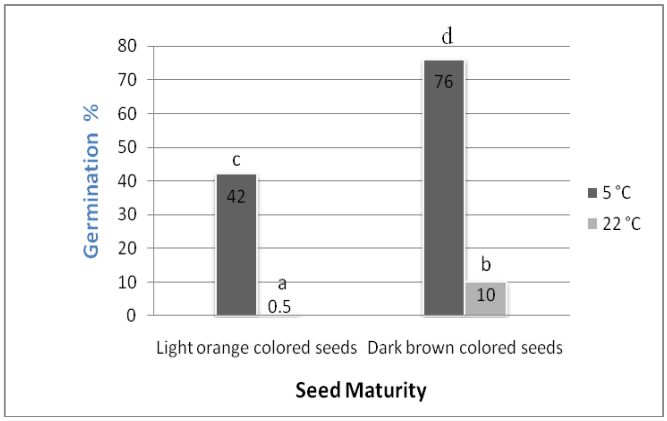

Fig 2. Effects of seed maturity (dark brown vs. light orange seeds) and stratification temperature (5 °C vs. 22 °C) on galax seed germination percentage. Seeds were cultured on MS basal medium at a temperature of 23°C ± 2°C under a 16 h light photoperiod with the light intensity of 97 µmol m^{-2} s^{-1} for 6 weeks. Data represent percent germination means of two repeated experiments, each with ten replicates and ten samples per replicate. Means followed by the same letter are not significant at the 0.05 level.

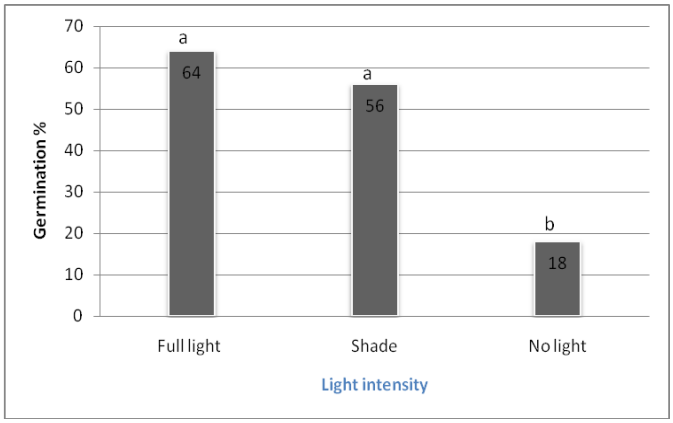

Fig 3. Effects of light intensity (full light at 97 µmol m^{-2} s^{-1}, 72% shade at 17 µmol m^{-2} s^{-1} and no light in the dark) on germination of cold stratified dark brown galax seeds. Seeds were cultured on MS basal medium under a 16 h light photoperiod at a temperature of 23°C ± 2°C for 6 weeks. Data represent percent germination means of two repeated experiments, each with ten replicates and ten samples per replicate. Means followed by the same letter are not significant at the 0.05 level

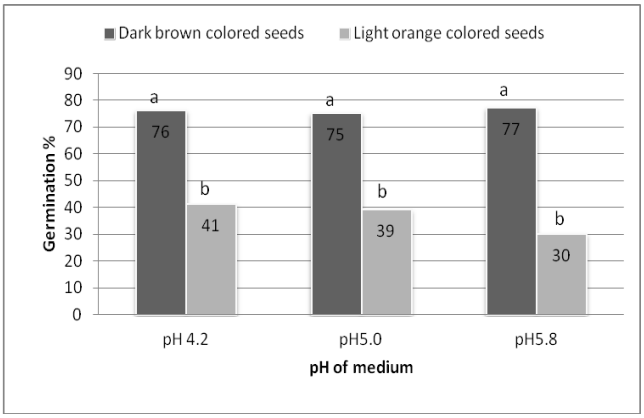

Fig 4. Effects of pH of culture media on germination of cold stratified dark brown and light orange galax seeds. Seeds were cultured on MS basal medium at a temperature of 23°C ± 2°C under a 16 h light photoperiod with the light intensity of 97µmol m^{-2} s^{-1} for 6 weeks. Data represent percent germination means of two repeated experiments, each with ten replicates and ten samples per replicate. Means followed by the same letter are not significant at the 0.05 level.

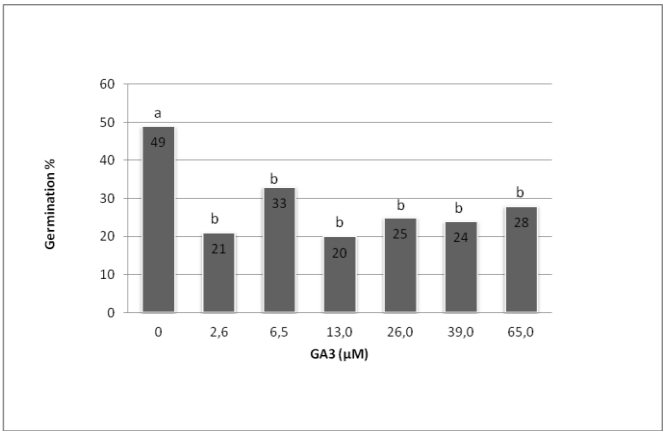

Fig 5. Effects of GA_3 in culture medium on germination of cold stratified dark brown galax seeds. Seeds were cultured on MS basal medium at a temperature of 23°C ± 2°C under a 16 h light photoperiod with the light intensity of 97µmol m^{-2} s^{-1} for 6 weeks. Data represent percent germination means of two repeated experiments, each with ten replicates and ten samples per replicate. Means followed by the same letter are not significant at the 0.05 level.

The germination percentage obtained with cold stratified seeds was higher than with seeds kept at room temperature. Traditionally, galax seeds are sown in cold frames in the fall, and then planted out in the second year after the plants have hardened off (Predny and Chamberlain, 2005). Our results suggest that stratification of seeds at 5 ºC for a month could fulfill the cold requirement for galax seed germination. This stimulatory effect of stratification at 4 ºC was also observed on the germination of *Bypripedium macranthos* seeds *in vitro* (Miyoshi and Mii, 1998). Light exposure also enhanced galax seed germination more than seeds kept in the dark. Although this effect has not been previously documented in galax, the effects of light enhancing seed germination have been demonstrated in other species (Verma and Tandon; 1984; Zettler and McInnis 1994; Kauth et al., 2006). Higher light intensity has also been reported to promote seed germination

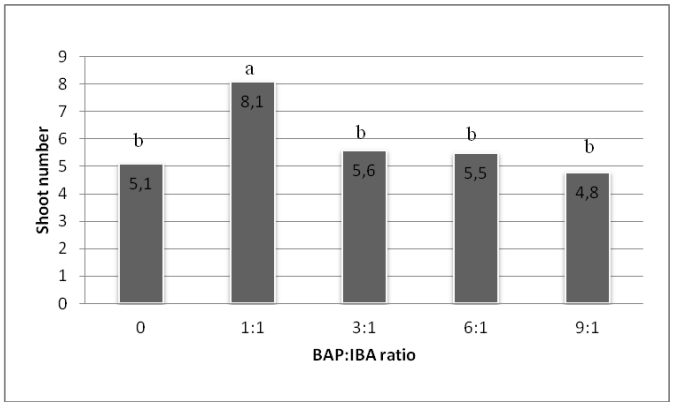

Fig 6. Effects of BAP and IBA ratio in the culture medium on galax shoot proliferation. Germinated seedlings were cultured on MS basal medium supplemented with BAP at 0.8, 2.4, 4.8 and 7.2 µM in combination with 0.8 µM IBA (BAP: IBA= 1:1, 3:1, 6:1 and 9:1, respectively). Cultures were maintained at a temperature of 23°C ± 2°C under a 16 h light photoperiod with the light intensity of 97 µmol m^{-2} s^{-1} for 6 weeks. Data represent means of two repeated experiments, each with ten replicates and four samples per replicate. Means followed by the same letter are not significant at the 0.05 level.

(Aref, 2002). However, in galax, seed germination was not affected by light intensity. Although light had a significant effect on seed germination, our results indicate that 18% galax seed can germinate in the dark. Wall et al. (2010) observed a similar response in *Pyxidanthera brevifolia* (another species in the *Diapensiaceae* family). They attributed the result to the exposure of dark-treatment seeds to some light during the monitoring period, and concluded that all dark-treatment seeds were light-exposed to some extent. In addition, Oh et al. (2006) discovered that light activates the degradation of PIL5 protein to promote seed germination through gibberellic acid in Arabidopsis. Normally, gibberellic acid is used to promote seed germination (Joshi et al., 2010; Yamaguchi and Kamiva, 2001). Unexpectedly, the application of GA_3 at a wide range of concentrations affected seed germination negatively in the present study. The reason for the lack of a stimulatory effect on Galax seed germination is not clear; however, our results are not unique. Wall et al. (2010) also reported that gibberellic acid failed to improve germination in *P. brevifolia*. Bir (2005) noted GA_3 caused no significant difference in the number of leaf buds to break in a study on *in vivo* propagation of galax. From our results and others', it is possible that galax seeds have enough endogenous GA_3 for seed germination and bud breaking, so that additional exogenous GA_3 is not effective, and may even be inhibitory. This possibility warrants further investigation. In contrast to 8 bedding plant species, including *Ageratum houstonianum* Mill., in which the pH level is critical to seed germination (Shoemaker and Carlson, 1990), Galax seeds at 2 maturity levels tolerated a variation of pH levels (4.2 to 5.8). This is consistent with its natural growing habit. In nature, galax plants can grow well in very acid soil. Lack of pH effects on *in vitro* germination also has been reported in other species, such as *Prunus persica* L. (peach) (Sinclair and Byrne, 2003). In comparison with its difficulty in seed germination, *in vitro* shoot multiplication and root formation are relatively easy in galax. Shoot proliferation occurs on media with or without combinations of BAP and IBA at a wide range; however,

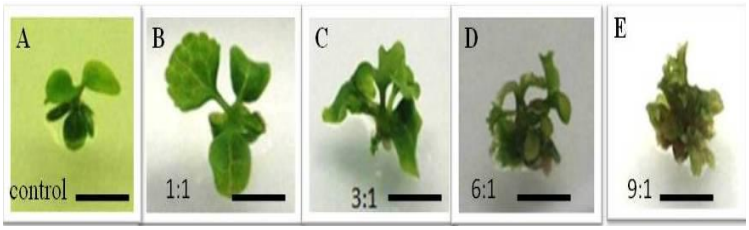

Fig 7. Differences in morphology of galax shoots cultured on MS basal medium supplemented BAP and IBA at various ratios. A: Normal shoot formation on the control medium. B, C: Normal shoot formation on media with BAP and IBA ratios of 1:1 and 3:1. D, E: shorter and more compact shoot formation on media with BAP and IBA ratios of 6:1 and 9:1. Shoot vitrification and callus formation also occurred. Bars = 1cm.

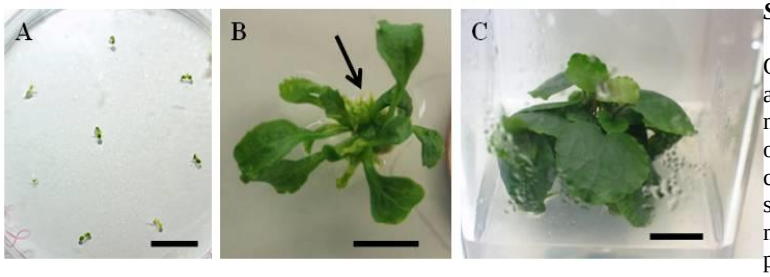

Fig 8. Seed germination and shoot proliferation in galax. A: Germination of cold stratified dark brown seeds on MS basal medium cultured at a temperature of 23°C ± 2°C under a 16 h light photoperiod with the light intensity of 97 µmol m^{-2} s^{-1} for 6 weeks. B: Small green shoot buds formation (arrow) on MS basal medium containing BAP and IBA at a ratio of 1:1. C: Fully developed galax plants by the 6 weeks culture. Bars = 1 cm.

Fig 9. Galax seeds separated by color. A: Light orange seeds. B: Dark brown seeds. Bars = 1mm.

shoot proliferation efficiency as well as shoot morphology depends on the ratios of BAP and IBA in the culture medium. Similar PGR effects on shoot proliferation have been reported in other species, such as *Alibertia edulis* Rich (da Silva et al., 2008), *Eucalyptus maidenii* (Sotelo and Monza, 2007), *Ficus religiosa* (Deshpande et al., 1998), *Renealmia Mexicana* Klotzsch ex. Petersen (Miceli et al., 2008), and *Sorbus redliana* 'Burokvolgy' (Ordogh et al., 2006). The optimal concentration of BAP and IBA required for shoot regeneration is species dependent. The current study showed that more shoots formed with BAP and IBA at 1:1. Juliani Jr. et al. (1999) reported that better shooting response was observed when IBA (0.04 µM) was used in combination with BA (4.4 µM) in their study on micropropagation of *Lippia junelliana* (Mold.) Tronc. Anirudh and Kanwar (2008) found that BA (1.5 mg/l) plus IBA (0.5 mg/l) was the best combination to promote shoot multiplication in *Pyrus pyrifolia*. It has been reported that hyperhydricity is usually caused by high cytokinin concentrations (Gribaudo and Fronda, 1991; Phan, 1991; Andrade et al., 1999). This is consistent with our results. The shoots of Karonda also showed hyperhydricity at a higher BA concentration (17.75 µM) (Rai and Misra, 2005). Shoots of *Lippia junelliana* (Mold.) Tronc obtained on higher BA and IBA concentrations also exhibited chlorotic and necrotic leaves (Juliani Jr. et al., 1999).

Materials and Methods

Seed collection, preparation and sterilization

Galax seed stalks (Fig. 1B) were gathered in October 2009 and 2010 from a single wild population spanning about 8,100 m^2 on Grandfather Mountain of North Carolina. Two types of stalks were collected and separated based on stalk and pod color. Green stalks with green pods contained less matured seeds, and brown stalks with brown pods contained more matured seeds. Both types of stalks were placed in separate paper bags and allowed to dry at room temperature of 22°C for 5 days. Dried seed pods were then lightly crushed to expose the seeds. Seeds from green pods were light orange (Fig. 9A), while seeds from brown pods were dark brown (Fig. 9B). Each seed group was divided in half. One half was cold stratified at 5°C in a refrigerator for at least one month. The other half was kept at room temperature of about 22°C (non-cold stratified). For initiation of *in vitro* germination or propagation, seeds were sterilized by soaking them in a 15% bleach solution (Bleach-Rite, Current technologies, Inc.) plus 6 drops Tween-20 per liter for 15 minutes, and then rinsed 3 times, 5 min each, with sterile water.

General culture medium and culture condition

The basic culture medium consisted of Murashige and Skoog (MS, 1962) mineral salts supplemented with 30 g l^{-1} sucrose. All media were adjusted to pH 5.0 (except for specifically mentioned) prior to the addition of 6 g l^{-1} TC agar (Fisher Scientific, Fair Lawn, New Jersey, U.S.A.) and autoclaved at 1.2 kg cm^{-2} for 20 min. All cultures were kept in a growth chamber at a temperature of 23°C ± 2°C under a 16 h light photoperiod with a light intensity of 97 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps (General Electric F20WT12CW). For the germination experiments, seeds were placed in Petri dishes containing 20 ml of the culture medium. There were 10 seeds per Petri dish and 10 replicate plates for each treatment.

Cold stratification and seed maturity on seed germination

Seed samples from two maturation groups (dark brown and light orange) exposed to two temperature pre-treatments (5°C and 22°C) were examined. Based on the results from this experiment, only cold stratified dark brown seeds were used in the remaining experiments (except for the pH experiment), since they exhibited the best germination.

Light intensity on seed germination

Three light intensity levels were tested, namely dark, full light at 97 µmol $m^{-2}s^{-1}$ and 72% shade at 17 µmol $m^{-2}s^{-1,}$ to mimic the natural growing condition. The light intensity of 17 µmol $m^{-2}s^{-1}$ was achieved by placing two layers of black fiberglass screen over the plates in the growth chambers. Petri plates were wrapped in aluminum foil for the dark treatment.

pH of culture medium on seed germination

Cold stratified seeds with two maturity levels (indicated by dark brown and light orange colors) were cultured on media with pH levels of 4.2, 5.0 and 5.8 respectively.

GA_3 effect on seed germination

Gibberellic acid (GA_3) at concentrations of 2.6, 6.5, 13.0, 26.0, 39.0 and 65.0 µM in a MS medium were examined. A GA_3-free medium served as the control.

Shoot proliferation

To evaluate the effects of 6-benzylaminopurine **(**BAP) and indole-3-butyric **(**IBA) on shoot proliferation, germinated seedlings were transferred into Magenta GA7 vessels (100 x 60 x 60 mm) containing 40 ml of MS medium supplemented with BAP at 0.8, 2.4, 4.8 and 7.2 µM in combination with 0.8 µM IBA (BAP:IBA 1:1, 3:1, 6:1 and 9:1 respectively). A plant growth regulator-free medium served as the control. There were 4 shoots placed in each GA7 vessel, and 10 replicates for each treatment.

Experimental design and statistical analysis

All experiments were conducted using a completely randomized design. Each factor was tested with 10 replications to ensure proper statistical analysis. The overall experiment including testing of all individual factors was conducted twice, once in 2009 and again in 2010. In the seed germination experiments, the germinated seeds were scored after 6 weeks of culture. The seed germination percentage was calculated as the number of germinated seeds per total number of cultured seeds. In the shoot proliferation experiment, the number of shoots formed per explant was determined after 6 weeks of culture. All data were analyzed using analysis of variance (SAS Institute, Inc., 2008). The statistical significance of mean difference was tested by the least significant difference test at the $p \leq 0.05$ level.

Conclusion

In this study, we tested the effects of light intensity, seed maturity, cold stratification, medium pH, and various plant growth regulator treatments on seed germination and shoot proliferation on Galax. We found that light and cold stratification enhanced seed germination; more mature seeds (characterized by a dark brown color) had higher rates of germination; culture medium pH had no effect on germination; and GA_3 inhibited germination. We also found that the ratio of 1:1 of the growth regulators BAP and IBA stimulated shoot multiplication, but higher BAP and IBA ratios resulted in abnormal shoot development, hyperhydricity, and callus formation. To our knowledge, this is the first study of *in vitro* manipulation of this endangered and economically important plant species. Our study demonstrated that *in vitro* propagation is potentially feasible. More research is needed to replicate the findings and further refine the growing conditions tested in this study.

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