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In vitro callus and shoot initiation of Black cohosh (*Actaea racemosa.* L) pre-incubated in darkness treatments

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Abstract

A medicinal plant that is commonly overharvested in its native habitat, Black cohosh (*Actaea racemosa* L.) rhizomes are used to mitigate symptoms of menopause and are a common alternative to hormone replacement therapy. Developing an accurate protocol for initiation of Black cohosh in tissue culture is becoming a necessity. Wild harvesting practices are the main source of Black cohosh rhizomes processed by pharmaceutical companies and are causing a significant decrease of these wild plants, leading to an At-Risk status. Growing these plants in a plant tissue culture laboratory is more sustainable than current practices, and having an accurate protocol for initiation of these plants is crucial to this. Our lab has previously developed a protocol for initiation of Black cohosh and this research was meant to optimize this protocol. Leaf explants of Black cohosh were cultured *in vitro* to determine the optimal duration of time for initiation of callus and/or shoot under darkness, and appropriate plant growth regulators (PGR) for shoot growth. Leaf explants incubated under darkness for increasing amount of time (0 to 6 weeks of darkness) were analyzed at two stages of tissue development. Stage 1 involved initiation of leaf explant material under darkness (treatment) and measured the callus development in leaf explants; Stage 2 was induction of adventitious shoot growth and cell division and compared the effect of two PGRs, KT and BA, on shoot growth for each darkness treatment. Leaf explants were given a score using a culture chart that assigns a numerical score to leaf explants based on their physiological characteristics. These numerical scores took into account color of leaf explant, callus development, shoot growth, and presence of dead leaf tissue. Stage 2 measured the number of shoots produced per explant within culture containers between PGRs and the number of weeks in darkness. Overall trends indicated that mortality of leaf explants decreased as the duration of incubation under darkness increased with mortality of explants decreasing from 63.9% (T0 darkness treatment) to 2.8% (T6 darkness treatment). Culture scores of explants (including dead explants) was highest for T6 weeks of darkness (culture score 2.96 on average), and for culture scores without dead explants (culture score of 3.05). During the adventitious shoot growth stage, however, T5 weeks of darkness performed significantly higher (KT: 28 shoots/ BA: 17.3 shoots) than T6 weeks of darkness (KT: 12.9 shoots/ BA: 9.4) in Replication 2 for both plant growth regulators KT PGR performed better, on average, than BA PGR.

Introduction

Black cohosh *(Actaea racemosa* L., Syn: *Cimicifuga racemosa* L.) is a medicinal plant found in eastern North America and Canada and exported mainly to Europe and Asia (Predney et al., 2006). An herbaceous perennial and a member of the Ranunculaceae family, the physical characteristics of *A. racemosa* are of a "smooth, erect stem with dark green tripinnate toothed leaves" that can grow between four and eight feet tall; flowering structures are a raceme consisting of small white flowers, producing a bittersweet smell to attract carrion-eating pollinating flies, and angular seeds arranged in rows. Black cohosh commonly grows in shaded or partially shaded areas and needs well drained, moist soil with a pH between 5 and 6. The economic importance of *A. racemosa* (L.) is derived from the root structure of the plant, a thick branching black rhizome covered in a mass of roots, used for its medicinal properties (Castle et al., 2014; He et al., 2000). This herb has been used since before the 1800s in North America to help prevent menopausal symptoms in women, and Europeans have been using Black cohosh ethanolic and isopropanolic extracts for more than 40 years (Predney et al., 2006). Human population increase and a growing interest in herbs with potential health benefits has led to a higher demand and overharvesting of medicinal plants in the Appalachian Mountains.

Medicinal plants are an important source of income, especially for those in the Appalachian Mountains, where a wide range of medicinal plants grow in the wild. Most of the information on the value, export and trade of Black cohosh is from before 2005, more than a decade ago, which makes it difficult to track the global market for Black cohosh today. Almost all exported rhizomes and roots are harvested from the wild, and 95% of those wild populations are exported outside North America (Fischer et al., 2006; Bittner et al., 2019). According to Davis and Greenfield (2019), as of 2005, "only 5% of the harvest was

generated from cultivated sources" even though organically cultivated Black cohosh has a 60% higher selling rate than plants harvested from the wild. The "supply" from the supply and demand of black cohosh comes mainly from these native populations, leading to over harvesting with no time for the Black cohosh populations to regrow, as well as habitat destruction both from cultivators and theft. As of 2012, growers and wild harvesters received an average of $5 - $7 per dry pound of Black cohosh roots, wholesale prices average $15 per dry pound, retail prices are $32 per dry pound, and nursery containers range $3.95 - $10 per plant (Davis and Greenfield, 2019). Developing strategies for successfully growing Black cohosh has become necessary as herbal alternatives become increasingly popular. Black cohosh has been successfully cultivated before, but not on a wide scale, making it important to determine what growth strategy will work most efficiently for growers (Kaur et al., 2013). Micropropagation, using tissue culture techniques, is one such strategy that can be used to decrease harvesting of Black cohosh from the wild (Nalawade and Tsay, 2004).

Micropropagation is a proven method for mass production of medical plants (Trigiano and Gray, 1999), because it is the true-to-type multiplication of selected genotypes for mass propagation. Depending on the plant species, micropropagation can be achieved through either direct or indirect organogenesis. Micropropagation of medicinal plants, like other plant species, begins with any living parts of the target plants (Afshari et al., 2011). The plant is usually grown in a lab or greenhouse to create a healthy supply of stock materials needed for propagation. Once suitable parent materials for micropropagation are determined, the regeneration protocol can be developed. Benefits of using *in vitro* techniques include a ready-to-use source of true-to-type plant materials, and mass production of target medicinal plants. These techniques are especially important for medicinal plants for "mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement." Mass production is the primary advantage of micropropagation. However, the precise process of micropropagation varies from plant species to plant species. A detailed formula for each individual species needs to be developed through scientific research, such as this project.

Our lab has established a protocol for successful initiation and growth of Black cohosh using leaf explant material. The process of developing a protocol for propagation of Black cohosh can help to determine the optimum environmental conditions for plant growth by manipulating the growing medium components, such as the PGR, macro- and micro-nutrients, vitamins, and sugar. Callus induction can be affected by "explant material, species, cultivar, PGRs, and environmental conditions such as light," and one of these factors that needs to be studied is the effect of light. Photosynthesis and photomorphogenesis are the main processes controlled by light and in plant tissue culture can affect "callus growth and morphogenesis, inhibition of axillary shoot proliferation, and induction of specific enzyme activity" (Thiruvengadam and Chung, 2015). A common technique is to place leaf explants under incubation in total darkness for a specific duration of time, to discourage differentiation of the callus cells and avoid photosynthesis. Initiating explant material in darkness can influence callus, shoot, and root growth, and while the exact reason why is unknown, it cannot be refuted that there is an effect. Based on the protocol in our lab, four weeks of darkness for callus induction has been the standard to use for Black cohosh to achieve growth and optimum multiplication. The objective of this research is to study the effects of duration of darkness incubation on *in vitro* callus, shoot, and root initiation and multiplication of Black cohosh to develop an efficient *in vitro* propagation protocol. Developing strategies for successfully growing Black cohosh is becoming necessary as herbal alternatives become increasingly more popular. *Ex situ* cultivation strategies, in place of wild harvesting from natural habitats, will help to prevent the extinction of this important medicinal plant. Plant material can be grown more quickly with *in vitro* propagation by creating multiple clones at a faster rate than the plants can grow in the wild.

Results

Statistical analysis of qualitative growth data of leaf explants and shoot growth by replication

Initiation of petri dishes for each replication had variable populations with 20 petri dishes in Replications 1 and 2, 25 petri dishes in Replication 3, and 50 petri dishes in Replication 4. Culture scores were determined one month after removal from darkness pre-incubation (Figure 2). SAS was run on data from each replication, due to varying population size, at the 0.01 level of significance (except for Replication 3 which was run at the 0.05 level of significance) and indicated the following information.

Replication 1. Data from Replication 1 indicated there was a significant difference in the number score between leaf explants treated under T2, T4, and T6 weeks of darkness, but no significant change in number of shoots per treatment. The mean number of shoots grown for Stage 1 leaf explants treated with either KT or BA, two PGRs, was determined (Table 1). Data from Replication 1 indicated there was a significant difference between shoot growth in 4 weeks in darkness (mean 25.9 shoots per culture) and 6 weeks in darkness (19.3 shoots per culture) for leaf explants exposed to BA, but there was no significant difference between 4 weeks in darkness (mean 27.1 shoots per culture) and 6 weeks in darkness (mean 25.1 shoots per culture) in leaf explants exposed to KT.

Replication 2. Data from Replication 2 indicated there was a significant difference between leaf explants treated with T1 to 6 weeks of darkness, and that there was a significant difference in mean number of shoots for all treatments except between treatments T2 & T3, T2 & T4, T1 & T2, T1 & T3, and T3 & T4. Data from Replication 2 BA treatment found no significant difference between the mean number of shoots per darkness treatment, with mean number of shoots being 4.5, 1.5, 17.3, and 9.4 for 3, 4, 5, and 6 weeks in darkness respectively. Data from Replication 2 KT treatment indicated there was a difference in shoot growth between the following darkness treatments 5 & 6, 5 & 3, 5 & 4, and 6 & 4. The mean number of shoots per culture was 4.5, 1.5, 28, and 12.9 in darkness treatments 3, 4, 5, and 6, respectively.

Table 1. Mean number of Black cohosh shoots per culture when exposed to KT and BA PGRs.

KT PGR	BA PGR
Replication 1	*Replication 1*
4 Weeks = 27.1[a]	4 Weeks = 25.9[a]
6 Weeks = 25.1[a]	6 Weeks = 19.3[b]
Replication 2	*Replication 2*
3 Weeks = 4.5[ac]	3 Weeks = 4.5[ab]
4 Weeks = 1.5[a]	4 Weeks = 1.5[a]
5 Weeks = 28.0[b]	5 Weeks = 17.3[b]
6 Weeks = 12.9[c]	6 Weeks = 9.4[b]

[z]Means having a letter in common are not significantly different at the 5% level of significance (P ≤ 0.05) as indicated by fishers protected LSD test. Significant comparisons are made between weeks of darkness treatment per replication.

Figure 1. Leaf explant score chart for determination of physiological changes in Black cohosh leaf explant growth and development, including color, callus development, and shoot growth using a numerical value for statistical purposes. **Score 0** – Leaf explant was dead, characterized by absence of shoot growth, as well as black color (usually dead leaf explants also left browning or discolored media). **Score 1** – Leaf explant has miniscule amount of callus growth and black discoloration of leaf explants, no shoot or root growth has occurred. **Score 2** – Leaf explant had small amount of callus growth and small amount of black discoloration, no shoot or root growth. **Score 3** – Callus growth occurring, no black discoloration, no shoot or root growth. **Score 4** – No discoloration, large amount of callus growth, some shoot/root growth occurring. **Score 5** – Healthy bright green color with multiple shoots growing as well as callus, no black discoloration.

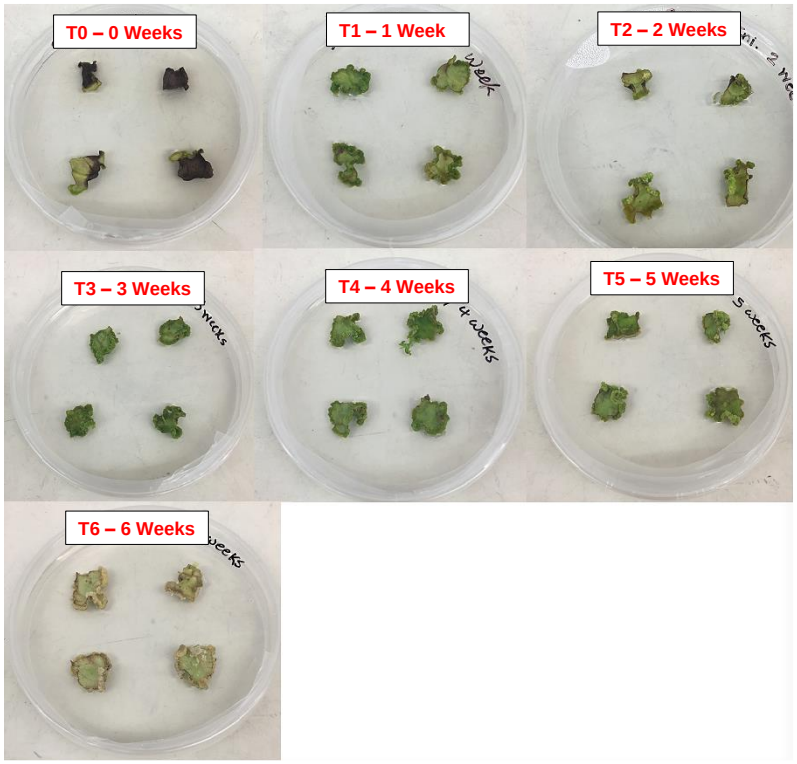

Figure 2. Comparison of Black cohosh leaf explant culture treatments 1 month after removal from darkness/ placed under normal light conditions (16-h light photoperiod / light intensity of 40 umol m^{-2s-1}). Includes T0 (negative control) T1, T2, T3, T4 (positive control), T5 and T6 weeks incubated in darkness.

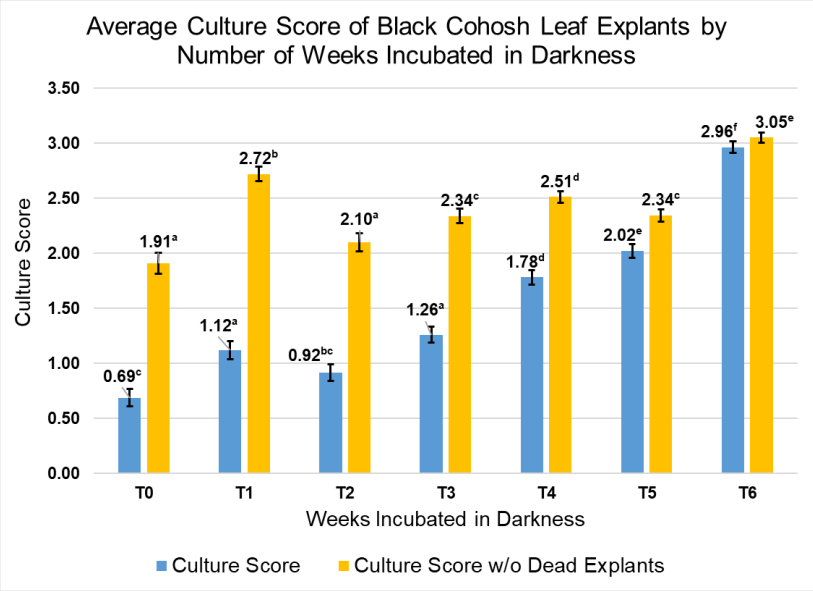

Figure 3. Culture score of leaf explant cultures after exposure to darkness treatment, including leaf explant cultures that died. Four replications with 20, 20, 25, and 50 petri dishes per treatment respectively. Cultures were scored one month after initiation in light. [z]Means having a letter in common are not significantly different at the 1% level of significance ($P \leq 0.01$) as indicated by Fishers protected LSD test. Significant comparisons are made between weeks of darkness incubation treatment by Culture Score or Culture Score w/o Dead Explants.

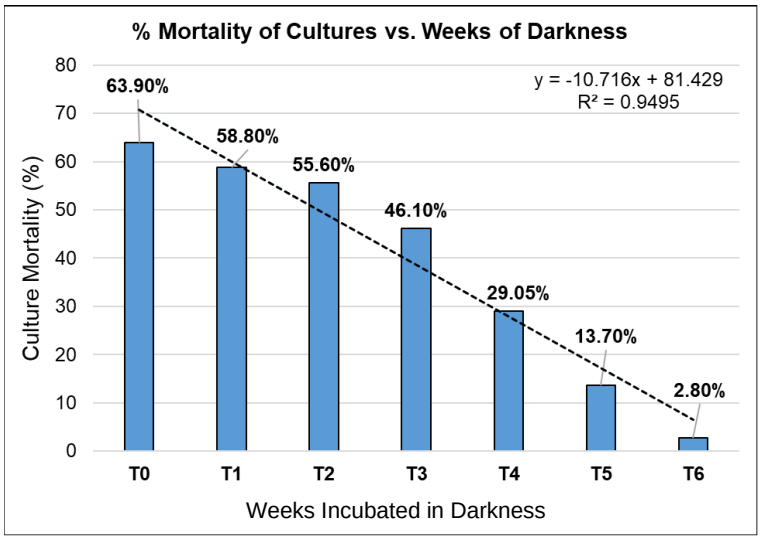

Figure 4. Percentage of cultures that died after one month initiation in light versus the duration of time cultures were incubated in darkness. T0 – 0 weeks of darkness before initiation under light; T1 – 1 week of darkness before initiation under light; T2 – 2 weeks of darkness before initiation under light; T3 – 3 weeks of darkness before initiation under light; T4 – 4 weeks of darkness before initiation under light; T5 – 5 weeks of darkness before initiation under light; T6 – 6 weeks of darkness before initiation under light. The line of best fit is y = -10.716x + 81.429 with $R^2 = 0.9495$.

Replication 3. Data from Replication 3 indicated there was a significant difference in number scores treatments T3 & T6 weeks in darkness, and that there was a significant difference in the mean number of shoots per treatment, except between treatments T3 & T4, T3 & T5, and T5 & T6. No adventitious shoot data was collected for this replication.

Replication 4. The final replication, and the one with the largest population size per treatment, was Replication 4, and the data indicated there was a significant difference between number scores except between treatments T4 & T6, T5 & T6, T4 & T5, T1 & T3 and T1 & T2. There was also a significant difference in the mean number of shoots, but only between the following treatments; T1 & T2, T1 & T5, T1 & T6, T3 & T5, T3 & T2 and T3 & T6 weeks of darkness. No adventitious shoot data was collected for this replication.

Analysis of culture scores and adventitious shoot growth by all replications

On average, culture scores including dead explants were lower than culture scores that omitted dead explant data, and both

culture scores with and without dead explants, increased as time under darkness incubation increased (Figure 3). It is important to note, however, that culture scores with dead explants saw similar scores between T5 and T6 weeks of darkness, and culture scores without dead explants had the second highest culture score under T1 week of darkness. Overall, mortality of cultures decreased as the weeks of pre-incubation under darkness increased, decreasing from 63.9% mortality under 0 weeks of darkness to only 2.8% mortality under 6 weeks of darkness (Figure 4). In Figure 4, the line of best fit was generated as $y = -10.716x + 81.429$ as well as the coefficient of determination, R^2 which equaled 0.9495. This proves that as the amount of time incubating Black cohosh leaf explants under darkness increases, the mortality of leaf explants once induced under normal light growing conditions decreased. Adventitious shoot growth comparison between KT and BA (Table 1) indicated there was no significant difference between KT and BA for shoot development for cultures initiated in 3 and 4 weeks of light, but that cultures initiated in 5 and 6 weeks of darkness had a significant increase in shoot development when exposed to KT versus BA.

Discussion

Protocols of plant tissue culture labs indicate that plant cultures, specifically cultures from cotyledons, leaves and petiole tissue, should be incubated in darkness after initiation, due to the direct effect on shoot production and growth. How darkness incubation affects plant tissue in culture is not completely understood, and its effect in combination with different PGRs is further unknown (Afshari et al., 2011). The objective of this study was to develop an *in vitro* propagation technique by determining the optimum duration of time Black cohosh tissue culture should be kept in the dark before introducing explant materials to light conditions, as well as which cytokinin should be used for shoot growth and proliferation. This research began with three initial replications to test the relative trends that might occur, followed by a fourth replication that would contain a larger population size for each treatment. For this research, we specifically focused on evaluating two stages of explant development of callus and shoot initiation: Stage 1 explant development as well as the color and physiological characteristics of leaf explants were evaluated; and Stage 2: adventitious shoot growth was measured when tissue cultures were exposed to two different PGRs, KT and BA. However, only the first two replications were measured for Stage 2 shoot growth, because of technical issues within the lab leading to the loss of Replication 3 and 4 cultures. Therefore, Stage 2 sample sizes were considerably smaller than Stage 1.

Results from Stage 1 indicated that there were significant differences in explant development, the growth of leaf explant cultured under different time (weeks) of darkness. Leaf explants cultured with no darkness (T0 weeks of darkness) experienced widespread tissue death, which prevented further study. Leaf explants that did survive despite no darkness showed signs of tissue browning and discolored media solutions. Browning in media most likely occurred due to leaching of poly-phenolic compounds, classified as secondary metabolites, from plant tissue, commonly found in medicinal plants such as Black cohosh, that occur in higher concentration *in vitro* then *in vivo* (Marutani-Hert et al., 2012; Nalawade and Tsay, 2004) Poly-phenols are oxidized, when plants are cut, by polyphenoloxidase enzymes, which can cause browning of leaf tissue cultures (Thiruvengadam and Chung, 2015). Browning of tissue in cultures that had no pre-incubation in darkness was similar to other tissue culture studies where dark incubation effects were measured, such as Marutani-Hert et al. (2012) where no shoots grew from citrus plants that were not incubated under darkness. Overall trends in this study indicated that increasing the duration of time under darkness decreased the rate of Black cohosh explant death, but that the healthiest callus growth occurred in leaf explants cultured under five weeks of darkness (Figure 4). However, six weeks in darkness slowed down the development of leaf explants, shown in Figure 2, where the leaf explants had lost color and show small signs of browning. While the leaf explants did grow rapidly afterwards, the shoots grown demonstrate stunted growth, unlike shoots grown in four and five weeks of darkness, which grew in the preferred way. As demonstrated in Table 1, five weeks of darkness was the preferred duration of time in darkness, because of Replication 2 Stage 2, the highest average number of shoots per explant developed in both BA and KT were in five weeks of darkness. However, further research needs to be done as the adventitious shoot stage could not be effectively tested due to a smaller sample size as stated earlier. Determining the correct duration of time in darkness could save time and prevent loss of explant materials. Because of the proven long-term return of micropropagation techniques, micro-propagating Black cohosh could be an effective method to produce a large quantity of seedlings for commercial production in field, greenhouse, or high tunnel settings. Using this method could also reassure growers exactly what they would receive when they began to grow, because the seedlings are clones. However, *in vitro* propagation may not be for some growers because of the required techniques, in addition to the initial investment that would be needed. Therefore, the people who would benefit the most from such protocols would be established nurseries and those who are willing to learn and invest in new techniques.

Materials and Methods

Growing stock plant material

Stock plants were grown from Black cohosh seed rhizomes. These plants were thoroughly watered, and their uncovered pots were placed in a walk-in cooler at the University Farm for nine weeks (June 21, 2017 – August 23, 2017). Plants were then removed from the storage cooler and moved back to the campus greenhouse for further growth under 80% shade. Once there was enough leaf material grown from the rhizomes, the initiation of tissue culture began.

Stage 1: Initiation of leaf explant material under darkness

Sterilization of leaf explants

Tender juvenile leaves were collected from stock plants and placed in a plastic bag with a wet paper towel before sterilization. Sterilization of leaf material included a pre-wash mixed 10% bleach solution (0.94% sodium hypochlorite, 2 drops Tween-20 per 100 ml of total volume, and tap water) agitated for 15 minutes covered, sterile wash with sterile

distilled water for 10 to 15 minutes, second wash with an all bleach solution (0.94% sodium hypochlorite, 2 drops Tween-20 per 100 ml of total volume) agitated for 10 minutes, and one more sterile wash with distilled sterile water until there was no more evidence of bleach solution. Leaf material was then transferred to a covered glass cylinder.

Initiation of Black cohosh petri dishes under darkness treatment. Murashige and Skoog culture media (4.33 mg/L MS basal salt, 3% sucrose, MS organics and vitamins, NAA 0.1 mg/L, Agar 0.6%) was adjusted to pH 5.5 with 0.1 N NaOH prior to the addition of 6% Agar and autoclaved at 1.2 kg cm^{-2} for 15 minutes. After placing the media in a sterile hood, 1 ml/L of TDZ were added. Media was then dispersed into petri dishes containing 20 ml of medium. Sterilized leaves were further cut into 5 mm x 5 mm sections for explant material, using only the midvein section of the plant and no petiole. There were 4 square leaf explants per petri dish, and 20 to 50 replicant petri dishes per darkness treatment (four experiments total), and completely randomized design (CRD) was used. Cultures were placed under complete darkness for time indicated (0-6 weeks) under a black tarp at room temperature, varying from 0 to 6 weeks, to induce in vitro callus and shoot initiation. Duration of darkness, in this case, referred to the duration of time leaf explants were in complete darkness before induction of adventitious shoot growth media and placement in growth chamber under normal growing conditions (temperature 23°C /16-h light photoperiod / light intensity of 40 umol m^{-2s-1}).
The duration of darkness treatments are labeled as the follows: T0 – 0 weeks of darkness before initiation under light; T1 – 1 week of darkness before initiation under light; T2 – 2 weeks of darkness before initiation under light; T3 – 3 weeks of darkness before initiation under light; T4 – 4 weeks of darkness before initiation under light; T5 – 5 weeks of darkness before initiation under light; T6 – 6 weeks of darkness before initiation under light. The negative control for this experiment is T0, as plants were initiated directly under normal growing conditions with no incubation under darkness. The positive control in this experiment is T4, as four weeks of incubation under darkness is the standard duration of time for incubation of leaf explants under darkness.

Statistical analysis of qualitative growth data of leaf explants by darkness treatment. Physiological characteristics of leaf explants were scored by numerical value to determine the growth of explants due to darkness treatment. Leaf explants were monitored once a week to determine qualitative trends that occurred within each darkness treatment. This included the tendency for leaf explants to die (mortality of leaf explants), the release of phenolic compounds in media (browning of media), the overall color of leaf explants, callus development, and shoot growth. Photographs were taken to allow side-by-side comparison of darkness treatments on leaf explants as they were removed from the darkness. Scoring of leaf explants were determined using a Culture Chart (Figure 1) and scoring leaf explants on a 0-5 scale based on color, callus development, and potential shoot and root growth. These scores were as follows: Score 0 – Leaf explant was dead, characterized by absence of shoot growth, as well as black color (usually dead leaf explants also left browning or discolored media). Score 1 – Leaf explant has miniscule amount of callus growth and black discoloration of leaf explants, no shoot or root growth has occurred. Score 2 – Leaf explant had small amount of callus growth and small amount of black discoloration, no shoot or root growth. Score 3 – Callus growth occurring, no black discoloration, no shoot or root growth. Score 4 – No discoloration, large amount of callus growth, some shoot/root growth occurring. Score 5 – Healthy bright green color with multiple shoots growing as well as callus, no black discoloration.
This step was repeated at the two-month mark and then leaf explants were transferred to baby food jars containing media for adventitious shoot and callus development. Leaf explant scoring was then evaluated using an ANOVA table in Statistical Analysis Software University Edition (SAS).

Stage 2: Induction of adventitious shoot growth and cell division

Transfer darkness treated cultures into adventitious shoot growth media under light. Once removed from darkness leaf explants were transferred into new culture medium and maintained in a growth chamber at a temperature of 23°C and a 16-h light photoperiod with a light intensity of 40 umol m^{-2s-1}. Murashige and Skoog basal salt medium that contained either 1 mg/L BA or KT was used. Medium was adjusted to pH 5.55 with 0.1 N NaOH prior to addition of 0.6% Agar and autoclaved at 1.2 kg cm^{-2} for 15 minutes. Placing the medium in a sterile hood, 0.2 ml/L of TDZ were added; 20 ml of medium was dispersed per petri dish. Cultures were maintained in a growth chamber at a temperature of 23°C and a 16-h light photoperiod with a light intensity of 40 umol m-2s-1. Media was changed every month and plantlets were transferred to prevent plant death from lack of needed nutrients. When explant clusters grew too large for petri dishes they were transferred into baby food jars (35 ml of media) or GA-7 box vessels (65 ml of media) with adventitious shoot growth media. There was 1 leaf explant cluster per culture vessel, and equal number of replications per darkness treatment.

Statistical analysis of shoot growth in adventitious growth media by darkness treatment. Data were collected by counting the number of shoots produced by leaf explant clusters and evaluating how they changed based on KT Media and BA Media. The use of two cytokinins helped differentiate the effects of darkness vs. time, instead of a single cytokinin's effect for shoot induction. Qualitative growth data were measured weekly and shoot growth was measured once a month. This would determine which darkness treatment would induce the most shoots and the rate of production, as well as how each PGR affects shoot induction. The number of shoots and the number of clusters were then evaluated using an ANOVA table in Statistical Analysis Software University Edition (SAS). Cultures were monitored once a week for noticeable trends for each darkness treatment and photographic evidence was taken to monitor these cultures, such as the one below. Color, size of clusters, and tendency to die were recorded.

Conclusions

Data from this study suggested that Black cohosh tissue culture initiation is best grown under a combination five weeks

of initial darkness incubation and KT PGR, for the most effective duration of time for subsequent callus and shoot development. As the duration of time under darkness treatment increased, mortality of tissue leaf explants decreased and quality of leaf explant increased.

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