Initiation and Optimization of American Ginseng (*Panax quinquefolius* L.) Callus and Suspension Cultures for Use in Plant Regeneration Techniques and Root Cultures

by
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I dedicate this thesis to my parents, Betty and Buddy Fuller, and to my loving fiancée Jennifer Grace, all of whom have been loving and supportive of my work and research throughout my college career.

I also dedicate this thesis to my teachers, lab instructors, and professors who have guided me in my love and appreciation for science, biotechnology, and plant biology.

Finally, I dedicate this work to God, through whom all things are possible.

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I would like to thank Dr. A. Bruce Cahoon for introducing me to research in plant biotechnology and leading me in my development of lab technique. His continuous advice and insight for me and this project have been of immense value, and he has continued to provide answers for my sometimes endless questions. I would also like to thank Dr. Elliot Altman, who took me into his lab as part of his research team after Dr. Cahoon departed from the university. Dr. Altmanôs constant support and guidance, as well as practical advice, have proven invaluable on countless occasions. This thesis would also not have been possible without the help of my lab supervisor, Shannon Smith. Shannon has offered continuous insight and assistance in the lab throughout the project, and continues to lead me in my experience of research techniques and lab work. I would like to thank all of my mentors, including Shannon, for the wonderful advice and comments given regarding this thesis and its drafts; without your help, this thesis would not be a reality.

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Lastly, I would like to thank the MTSU Biology Department, Undergraduate Research Center, and the University Honors College for allowing me to have this opportunity.

ABSTRACT

The medicinal herb American ginseng (*Panax quinquefolius* L.) shows promise as a source of unique metabolites that may possess anticancer and immune-boosting properties. Unfortunately, the taproots of American ginseng (which contain a large concentration of the relevant metabolites) are quite expensive due to difficulty in cultivation and an intense market that has encouraged extensive poaching. The purpose of this study is to develop and optimize a protocol to produce callus tissue and cells from stratified seeds of *Panax quinquefolius* to be used in tissue production, root initiation, and plant regeneration techniques. An experimental methodology was developed for the production of callus from surface-sterilized seeds, and media supplements and plant hormones were tested for the development of a media formulation that favors callus proliferation. Semi-solid and liquid media types that favor prolific cell growth were designed and implemented, and roots were successfully initiated from callus.

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2,4-D	2,4-Dichlorophenoxyacetic acid
AC	Activated Charcoal
BA	Benzyladenine (Benzylaminopurine)
CCW	Coconut Water
CPA	4-Chlorophenoxyacetic acid
GA_3	Gibberellic Acid
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
NAA	1-Naphthaleneacetic acid

INTRODUCTION

The Genus *Panax* and American Ginseng, *Panax quinquefolius*

The genus *Panax* consists of multiple herbs in the ivy family (Araliaceae) with the common name of ñginseng.ò These herbs are well-known and prized in herbal medicine for their fleshy taproots, which can be processed into herbal medicine and added to beverages such as energy drinks and teas. Ginseng plants derive their genus name *Panax* from the Greek words *pan* (all) and *akos* (cure or medicine), referring to the panacea-like status of ginseng in herbal medicine and folklore. Purveyors of herbal supplements claim that ginseng acts as an adaptogen, helping the body attain its natural equilibrium, homeostasis (Choi 2008), and medicinal studies in mouse and human trials have shown that the unique chemical components found in ginseng (ginsenosides and unique polysaccharides) have beneficial stimulatory effects on the immune system and antioxidative enzymes (Kim et al. 1990, 2012).While a number of species of ginseng plants exist and are used in traditional medicine (including *P. notoginseng, P. pseudoginseng,* and *P. vietnamensis*), two species that have received a substantial amount of attention in scientific research are Asian ginseng (*Panax ginseng* C. A. Meyer) and American Ginseng (*Panax quinquefolius* L.). While Siberian ginseng (*Eleutherococcus senticosus* Maxim.) is closely related to members of the genus *Panax* and has been subject to similar research and medicinal use, *E. senticosus* is not a true ginseng.

Asian Ginseng (*Panax ginseng* C. A. Meyer) is native to eastern Asia and has an extensive history of use in traditional Asian cultures (Hu 1976). *P. ginseng* is cultivated around the world, and the herb is a major export of Korea and China, which produce 34.3% and 55.9% (respectively) of the worldôs total *P. ginseng* products (Baeg and So 2013). Taproots of *P. ginseng* have a tendency to grow into a human-like shape due to a forking growth pattern. The

unusual shape of ginseng taproots lead to their naming of ñginseng,ò derived from the Chinese words for *gin* (ñmanò) and ñseng,ò or essence (Hu 1976). *P. ginseng* has various uses in traditional herbal medicine, ranging from a healing tonic for the ill to roles as an aphrodisiac. Medicinally, *P. ginseng* has been shown to have beneficial immunomodulatory effects, protective effects against oxidative stress, and potential anti-cancer effects (Kim et al. 2012, Shibata 2001) Because of its pharmaceutical and medical potential and its widespread traditional use in Asian herbal medicine, *P. ginseng* has been met with high demand in the Asian market, producing a high price for the fleshy taproots.

American ginseng (*Panax quinquefolius* L., Figure 1) is closely related to Asian ginseng (*P. ginseng*) and is native to Eastern North America, especially in the forests of the Appalachian and Ozark Mountains. *P. quinquefolius* demands cool, temperate, and shaded conditions for optimal growth, and as such, can be difficult to grow under ordinary cultivation conditions. Much like its Asian counterpart, American ginseng has been met with high demand from the Asian market, and, as such, ñsengò hunters have spent centuries foraging the precious crop from the Appalachian forests. While American ginseng is cultivated in both Asia and America, the one-year seed dormancy period and five or more years of taproot growth that ginseng requires result in a very time consuming and costly cultivation process. Unfortunately, because of the intense market for American ginseng roots (which can lead to prices from 500 to 700 USD per pound), wild *P. quinquefolius* has been subject to intense poaching efforts, leading to the species being listed as a ñcommercially exploitedò plant species in Tennessee (plants.usda.gov). Additionally, due to concerns over declining wild populations of *P. quinquefolius,* the plant has been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, www.cites.org).

Figure 1: Mature American ginseng (*Panax quinquefolius* L.) with fruits. Photo by U.S. Fish and Wildlife Services (USFWS).

Metabolites of Interest in *Panax quinquefolius*

Because of their long-standing role as a ñcure allò in herbal medicine, ginseng plants (both *P. ginseng* and *P. quinquefolius*) have received a considerable amount of research in order to determine possible beneficial effects. At a molecular level, ginseng plants produce two pharmaceutically-important classes of metabolites: ginsenosides (a type of saponin) and polysaccharides. Ginsenosides (also known as panaxosides) can be split into two groups based on chemical structure: the Rb1 group consists of saponins with the structure of protopanaxadiol (PPD), while the Rg1 group consists of saponins with the structure of protopanaxatriol (PPT). Ginsenosides as a whole have been shown to have anti-cancer effects (Kim et al 2003) as well as protective effects against neurodegeneration (Kim et al. 1998). While similar to ginsenosides, the active saponin eleutherosides that *E. senticosus* (Siberian ginseng) plants contain are considered to be different from the ginsenosides found in true ginseng plants.

Ginseng plants also produce unique polysaccharides as primary metabolites, and these compounds may possess antitumor and immunological properties (Huang et al 2010 and Yun et al 2001). The polysaccharides found in *P. ginseng* have been shown to have potential beneficial effects in a variety of areas ranging from antiulcer to radio-protective to antiviral (Sun 2011). Another study (Yoo et al 2012) reinforced the potential use of ginseng polysaccharides as an antiviral by showing their protective effect against influenza virus. In *P. quinquefolius*, polysaccharide extracts have also been shown to have potential immunological properties (Assinewe 2002).

Plant Tissue Culture – Use and Applications

Plant tissue culture is a vital part of plant biotechnology that involves growing plant tissues (such as from seeds, leaves, stems, or roots) *in vitro*, such as on a plate or in a test tube. Plants can be grown in such conditions due to the plasticity (ability to grow in a wide range of environments) and totipotency (entire genetic potential) of their tissues. Explants, tissues taken from a plant, can be converted via exposure to key hormones into a viable type of tissue known as callus (see Figure 2), which can then be grown on a plate (as a culture). Plant callus (pl. calli) is a mass of rapidly dividing, disorganized, and undifferentiated plant cells (much like a tumor) that can be easily propagated and manipulated in a culture setting (Figure 2). The induction of plant callus is an important tool in plant biotechnology due to two inherent traits of plant cells: plasticity, or adaptability to various conditions (including culture conditions), and totipotency, or the ability of a single cell to develop into an entire multicellular organism. Once initiated and properly maintained, callus cultures can be prolonged indefinitely, providing a near limitless source of plant material in the form of callus. This callus can then be regenerated back into a limitless number of whole plants or can serve as source tissue for genetic transformation

experiments and studies (such as in the creation of transgenic crops). With the potential of regenerating a vast amount of genetically-identical plants from a small source of tissues, the technology behind plant tissue culture allows for innovations in conservation biology, agriculture, and industry.

Figure 2: Example of callus derived from seed tissue of American ginseng, *Panax quinquefolius.*

As an application of the totipotency of callus tissues, cultured callus can be grown into distinct plant organs for various uses. For example, roots could be initiated from callus and grown as a root culture. Root cultures are a fast, easy, and efficient method of producing metabolites from plant roots, such as the ginsenosides and polysaccharides in ginseng (Huang et

al. 2010). With a set of root and suspension cultures, one can obtain both a source of whole plants and also a source of metabolites from a pharmaceutically ï important plant without harming the environment in the process. As such, tissue culture studies for *Panax ginseng* have been performed and optimized to circumvent the problems associated with growing the plant via traditional agricultural methods (Cellarova and Kimakova, 2000).

THESIS STATEMENT

Because of the intense market for American ginseng (*Panax quinquefolius*) taproots and metabolites, poachers have targeted wild populations of the plant in areas such as the forests of the Appalachian Mountains. Since traditional cultivation methods are often not sufficient to satisfy market demands for American ginseng products, there exists a need for alternative methods of producing large amounts of pharmaceutically-important compounds from American ginseng. Tissue culture (including callus and suspension cultures) of American ginseng may provide an answer to such a need, as tissue culture allows for the large-scale production of plant compounds and the potential for a clonal propagation system to restore wild populations. While the tissue culture of Asian ginseng (*P. ginseng*) is well-studied and performed, relatively fewer studies have focused on its American counterpart, *P. quinquefolius*. Zhou and Brown (2007) have shown that tissue culture is an efficient method for the micropropagation of American ginseng, and Wang (1990) has described callus initiation from root pith tissues. I wish to create an efficient and repeatable protocol to establish a liquid suspension culture of American ginseng from stratified seeds. As an extension of this project, I also aim to produce a protocol for the rapid establishment of a root culture of American ginseng as well as a protocol to initiate shoots for whole plant regeneration. The knowledge gained from this project will add to our

understanding of the physiology of American ginseng and its tissue culture requirements. This project will also serve to produce a practical product: ginseng compounds and tissues that could be used to advance the ginseng initiative at MTSU and the Tennessee Center for Botanical Medicine Research.

HYPOTHESIS

The requirements for establishing and maintaining a tissue culture of American ginseng (*P. quinquefolius*) will be similar to the culture requirements of Asian ginseng, *P. ginseng*; however, because of physiological differences between the two species, different tissue culture requirements, including media formulations and growth conditions, for *P. quinquefolius* must be discovered and optimized

METHODS

I. Seed Sterilization and Germination

Seeds of Wisconsin variety American ginseng were purchased from wildgrown.com, an online American ginseng distributor. These seeds were received stratified (acclimated), so that the one-year seed dormancy period American ginseng requires had already passed and the seed coat had opened. A seed surface-sterilization protocol was modified from Leguillon et al. (2003) to handle unique biotic and abiotic needs for sterilizing the seed surface. After noting previous problems with germination, seeds were aseptically cut in various ways (via an adapted protocol from Odnevall et al. 1989) following surface sterilization.

After being surface sterilized, seeds were added to a variety of media types containing differing levels of nutrients and plant growth hormones. Initial media formulations and hormone

concentrations were obtained through experimental modification of a protocol by Wang (1990) involving callus induction from slices of root pith tissue. Base formulations for these and other media types referenced herein included B vitamins, sucrose, and MS Salts (Murashige and Skoog 1962). Each media set included 10 test tubes with 10 ml of respective medium, with one surface-sterilized seed occupying each test tube. All germination tubes (seed cultures) were stored in a lighted incubator set to a 16-hour photoperiod at room temperature (25±1° C).

An alternative seed germination method was also employed involving planting the seeds in soil (Miracle-Gro® potting soil mix) as a control measure. Seeds placed in soil-based medium were not surface sterilized prior to being planted and were kept in a lighted growth chamber set to a 16-hour photoperiod. Shade was simulated with the construction of a small frame over which paper towels were draped.

II. Initiation and Amplification of Callus Tissue

Once seeds began to germinate into seedlings, sterile seedlings were manipulated in one of two ways in order to initiate callus. Some seedlings were taken out of their germination media, sliced according to the various seedling tissues (root, hypocotyl, cotyledons, and true leaves), and randomly assorted onto an array of media formulations based upon 16 different hormone concentrations (Table 1); initial hormone ratios were derived from a similar array by Murashige (1974). Other seedlings were left in their germination media to assess if the germination medium (which was based upon a callus induction media formulation) was sufficient for the initiation of callus directly from seedling tissue. Callus initiation occurred over a time period of one to two months, and multiple replicates of the experiment were put in place. After discovering a suitable hormone ratio between auxin and cytokinin, an additional array of different auxins was constructed and tested in order to determine the most efficient auxin to use for callus induction

from germinated seed tissue. Auxin types selected included IAA, IBA, NAA, 2,4-D, Dicamba, and CPA; each auxin was added in a 1:1 ratio with kinetin, which was maintained as the cytokinin of choice for the entire array.

Table 1: Array of hormone ratios between an auxin and a cytokinin used to determine an optimal hormone ratio for callus induction and proliferation. Values indicate concentrations of each hormone in µl per 100 ml solution.

Auxin / Cytokinin	0	100	200	300
0	0/0	100/0	200/0	300/0
100	0/100	100/100	200/100	300/100
200	0/200	100/200	200/200	300/200
300	0/300	100/300	200/300	300/300

After callus was obtained from germinated seedlings, actively-dividing calli were replated on an array of hormone ratios (auxin: cytokinin). The hormone ratios selected for this array were chosen due to their favorable rate of callus initiation, with the reasoning being that a

hormone ratio (auxin: cytokinin) favorable for callus initiation would also be favorable for callus growth. There were three replicates of this hormone array which were carried out simultaneously. Plates containing cultures with high callus growth rates were noted for their hormone ratios, and the most successful ratios in the array were used to develop an optimized media formulation for the amplification of healthy callus tissue. Tissue characteristics such as friable consistency, light coloration, and exponential growth were used as markers for vigorous and healthy callus lines. After every four to five weeks (one passage), callus tissues were taken off their previous media and re-plated onto new media; additionally, tissues were sometimes divided into multiple cultures in order to propagate individual cell lines. Over time (several passages), calli with vigorous growth rates were selected and amplified in order to acquire a large source of tissue. Light and dark conditions were also tested in an effort to determine optimal conditions for tissue growth**.** While all cultures were placed in a lighted incubator at room temperature (25±1° C) with a 16 hour photoperiod, cultures in dark conditions were placed within a closed box and set inside the incubator such that all factors excluding light were kept similar to light-exposed cultures.

III. Selection and Evaluation of Media Supplements on Callus Growth

In an effort to optimize the production of callus tissue, several different media supplements and formulations were tested in order to assess their beneficial effects on callus growth rate and the inhibition of negative growth factors (here, phenolic stress-related compounds). The supplements evaluated were coconut water (CCW), nicotinic acid (Niacin) and activated charcoal (AC) as measures to improve tissue production and down-regulate the production of phenolic stress-related compounds (Abrahamian & Kantharajah 2011; Wang & Huang 1976; Drew 1979). The selected supplements (AC, CCW, and niacin) were evaluated by

observing callus tissues randomly assorted (without respect to callus health) onto media formulations containing the relevant supplement (experimental) and media formulations lacking the relevant supplement (control). Evaluations of media supplements were based upon qualitative assessments of their effects on callus growth and their effects against the production of stress chemicals such as phenolic compounds.

CCW was also quantitatively assessed as a media supplement in order to determine if the addition of CCW to growth media had a significant effect (Ŭ= 0.05) on the rate of growth of callus tissue. Calli from random cell lines were selected, weighed, and plated onto experimental media (containing CCW) and control media (lacking CCW) and maintained and propagated over the course of 7 passages (approximately 7 months). At the end of each passage, calli were weighed and placed onto new media, with large masses of tissue being propagated onto additional plates of the same media type. Tissue masses were collected at the beginning and end of each passage; tissue mass at the end of each passage was divided by the previous mass of that same culture in order to assess rate of growth per 4 week period. Rates of growth between the two groups (with and without CCW) were averaged and analyzed using a two-tailed studentôs *t* test.

Since callus tissue must be maintained in sterile conditions, new protocols had to be developed before it was possible to assess tissue growth across multiple passages. In order to assess tissue growth in a sterile environment while keeping the viability of the tissues in mind, a sterile method of weighing the mass of the calli was implemented with the help of Dr. John DuBois and Dr. Bruce Cahoon. This procedure led to the development of more efficient methods of weighing tissues in sterile environments.

IV. Liquid Media Suspension Design and Optimization

The basic design of the liquid suspension model consisted of using an Erlenmeyer flask, test tube, or bottle containing liquid media (without agar or a gelling agent). Media formulations for liquid suspension cultures were adapted from formulations of semi-solid media types that favored prolific callus growth. Callus tissue in suspension culture was to be submerged completely in the medium and dissociated into small aggregates and then individual cells over time. Two approaches were taken in constructing a liquid media suspension setup. The first approach involved placing the culture on a shaker (150 rpm at room temperature or 25°) to agitate the tissues and ensure that the tissues dissociated into a single-cell suspension (Figure 3). The other approach to constructing a liquid media suspension involved the construction of a ñbubble reactor,ò which was similar to the shaker model but included the addition of oxygen from an air-line (Figure 4). In the bubble reactor design, oxygen is first passed through a sterile filter into a chamber of sterile deionized water and then directed into a growth chamber containing the calli in suspension. After oxygenating the solution containing the cells, the air is then released from the system through outflow tubing. Both the inflow and outflow tubes were connected to a sterile filter which added a layer of protection from contaminants such as airborne mold and bacteria. Since oxygen flow and bubbling effects produced a noticeable amount of agitation, the bubble reactor design was not placed on a shaker system. The addition of oxygenation in the liquid suspension culture design was due to the observation that poor gas exchange may have been problematic in previous suspension culture designs.

An antioxidant mixture (PhytoTech® A126) was sourced and added to the media of both the bioreactor and shaker liquid suspension models. The antioxidant mixture was added in order to down-regulate the production of toxic stress chemicals (phenolics) and increase the rate of

growth of tissues (Jones & Saxena 2013; Abrahamian & Kantharajah 2011). Liquid suspension culture designs were tested in three areas: their ability to properly dissociate tissues into single cells, the amount of stress chemicals (phenolic) collected in the media, and the rate of growth of the cells in suspension. Media formulation was kept identical between the bioreactor and shaker designs. After 40 days, cells were filtered out of suspension using a sterile filtration system and weighed. Characteristics such as tissue and cell coloration, mass, and filtered media coloration were noted and compared between the experimental (bubble bioreactor) and control (shaker) techniques.

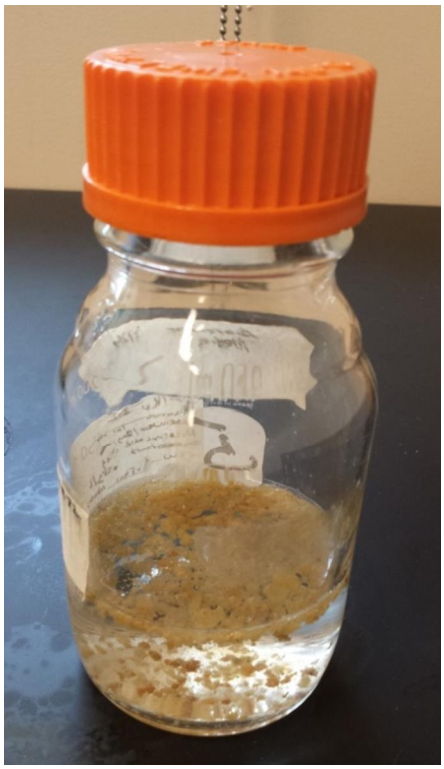

Figure 3: Initiated suspension culture containing submerged callus tissues to be placed on a shaker (not pictured). Culture was placed on a shaker set to 150 RPM and kept at room temperature (25 ± 1°C)

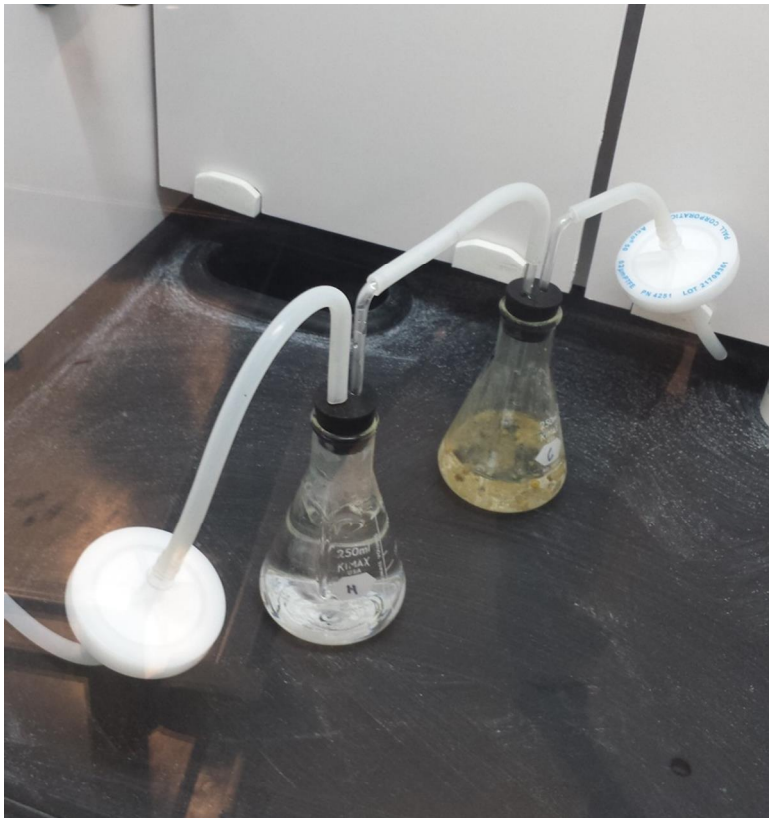

Figure 4: Experimental bubble bioreactor system overview. Oxygen flows from the left of the picture through a sterile filter into a hydration chamber filled with sterile deionized H_2O. Oxygen is then directed through a growth chamber containing cells and media and is released after passing an additional sterile filter. System kept in a fume hood setting (sterilized with 70% isopropyl alcohol) at room temperature

V. Organogenesis in Callus of *Panax quinquefolius*

Selected actively-dividing calli were also plated on media containing hormones and respective hormone ratios which directed organogenic growth. The formulation for root induction media was based on a previous study on root induction in American ginseng callus (Bonfill et al. 2002)**.** Calli were also plated on media favoring shoot production following a

protocol by Odnevall et al. (1989). Root-inducing cultures were kept in dark conditions (within a closed box), and shoot-inducing cultures were placed in both dark and light conditions. For light conditions, cultures were placed in a closed container with a plastic lid and stored in a lighted incubator set to a 16 hour photoperiod. Cultures were examined for signs of organogenic activity as well as differences in growth tendencies in the callus tissues, such as abnormal shapes or changes in color.

RESULTS

I. Seed Sterilization and Germination

At first, very few seeds showed signs of germination, merely swelling from intake of water; however, after following the adapted seed cutting protocol from Odnevall et al (1989), seeds showed signs of germination beginning within a week (Figure 5). Seeds planted in potting soil were much slower to germinate (over one month); however, these seedlings grew to become developing ginseng seedlings with a single characteristic trifoliate leaf (Figure 6). Results from the novel surface sterilization protocol using heat (modified from Leguillon et al. 2003) were comparable in terms of contamination rates to seeds sterilized without the heating process. Of the various media formulations tested, media formulations added auxin and cytokinin germinated with the highest efficiency, with germination efficiency reaching 70%. Media formulations lacking these plant growth hormones germinated at a slower and less-reliable rate.

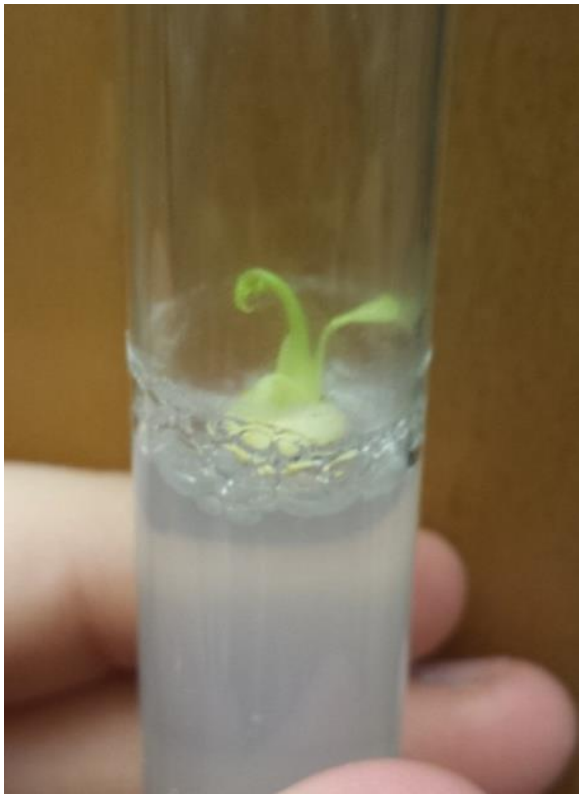

Figure 5: Successful seed germination from a stratified seed of *Panax quinquefolius* in sterile conditions. Germination media in picture contains hormone ingredients intended to promote callus induction.

Figure 6: Results from the alternate seed germination method involving soil-based medium. The pictured shade-simulating apparatus (consisting of wooden craft sticks) was used as a frame over which a paper towel (pictured at top right in picture) was draped.

II. Initiation and Amplification of Callus

The first isolated callus tissue was derived from seeds left in germination favoring callus induction ï this result allowed for a baseline hormone ratio between auxin and cytokinin for callus initiation from seed tissue (Figure 7). Seedling tissues that were cut and plated on the

hormone array described in Table 1 did not respond well to being cultured, and tissue death was a common occurrence. However, some of these tissues were successfully induced into callus. The array of auxin types provided three auxins that favored callus production; however, only two of those auxins (Dicamba and 2,4-D) upregulated the production of callus.

Callus tissues obtained from germinated seeds proved to respond more readily to the array of hormone ratios. The three replicates of the array revealed a hormone ratio between auxin and cytokinin which strongly favored the prolific growth of new callus tissue. This hormone ratio was then used as the base plant regulatory elements in the media on which callus was initiated and maintained. After being plated on callus proliferation medium over several passages, a healthy source of light-colored actively dividing callus tissue was acquired (see Figure 8). Light (at the intensity found in the incubator) was observed to harshly decrease growth of tissues to the point of stagnation; thus, all future cultures were placed in dark conditions (inside a box).

Figure 7: Callus induced directly from seedling tissues in dual-purpose germination and callus induction media.

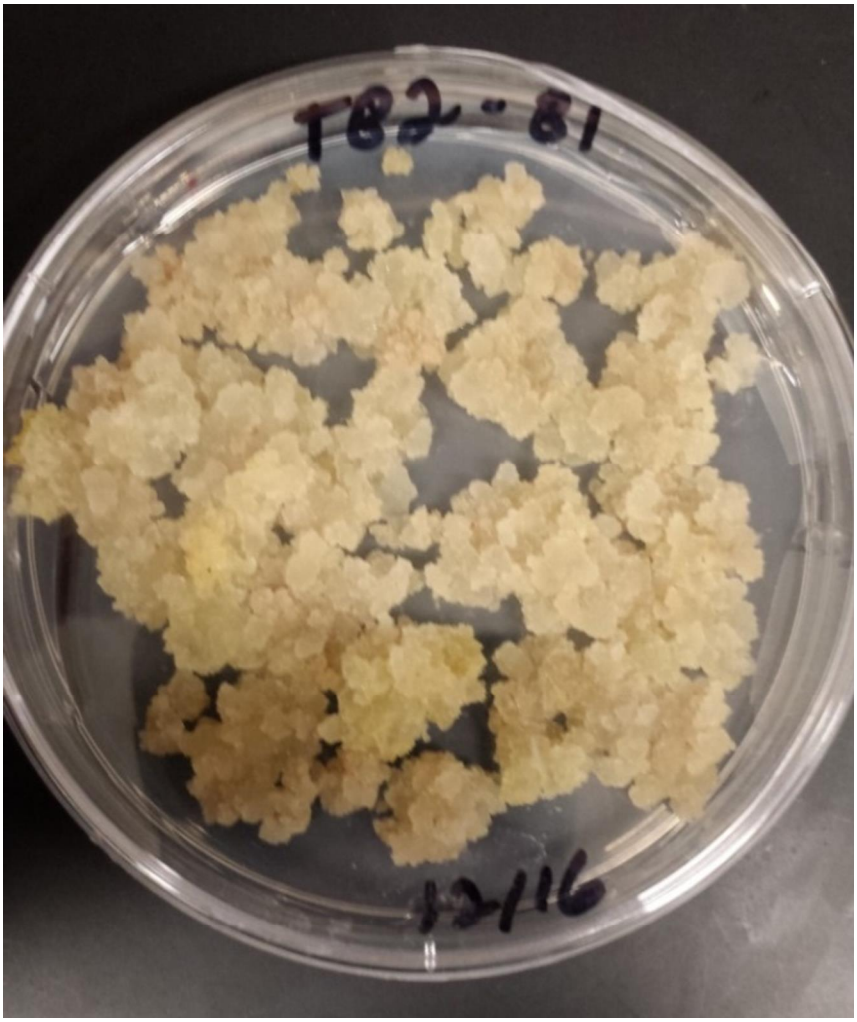

Figure 8: Amplified callus plated on optimized callus proliferation media. The notation of ñT B 2 ï B 1ò details the media type and individual replicate number of the culture

III. Selection and Evaluation of Media Supplements for Callus Growth

Coconut water (CCW) as a media supplement was shown to have a significant positive effect on the rate of growth of callus tissues (two-sample *t* test, $P < 0.05$). Calli plated on control media lacking CCW (n=77) were found on average to have a 1.99-fold increase in tissue mass

per passage (s = 1.18), whereas calli plated on media types containing CCW (n= 125) were found to have a 2.37-fold increase in tissue mass per passage (s=1.54, Figure 9). A growth ratio of 2.37 is equivalent to a 2.37-fold increase in tissue mass per 4-5 week passage period. The two groups were found through an F test to have unequal variances (calculated F 1.7 > 1.54, critical F). Calli plated on media containing CCW showed fewer signs of phenolic accumulation and possessed grew larger compared to calli plated on similar media without CCW (Figure 10).

Activated charcoal (AC) as a media supplement appeared to exhibit a remedial effect for callus tissues saturated with phenolic compounds, removing the tan color from plated tissues. However, media formulations containing AC were also prone to stagnation. Nicotinic acid (niacin) exposure was observed to have a positive correlation to increased callus size and callus health quality. Organogenic media types used for rooting purposes were observed to have lower rates of tissue growth than media types favoring callus proliferation.

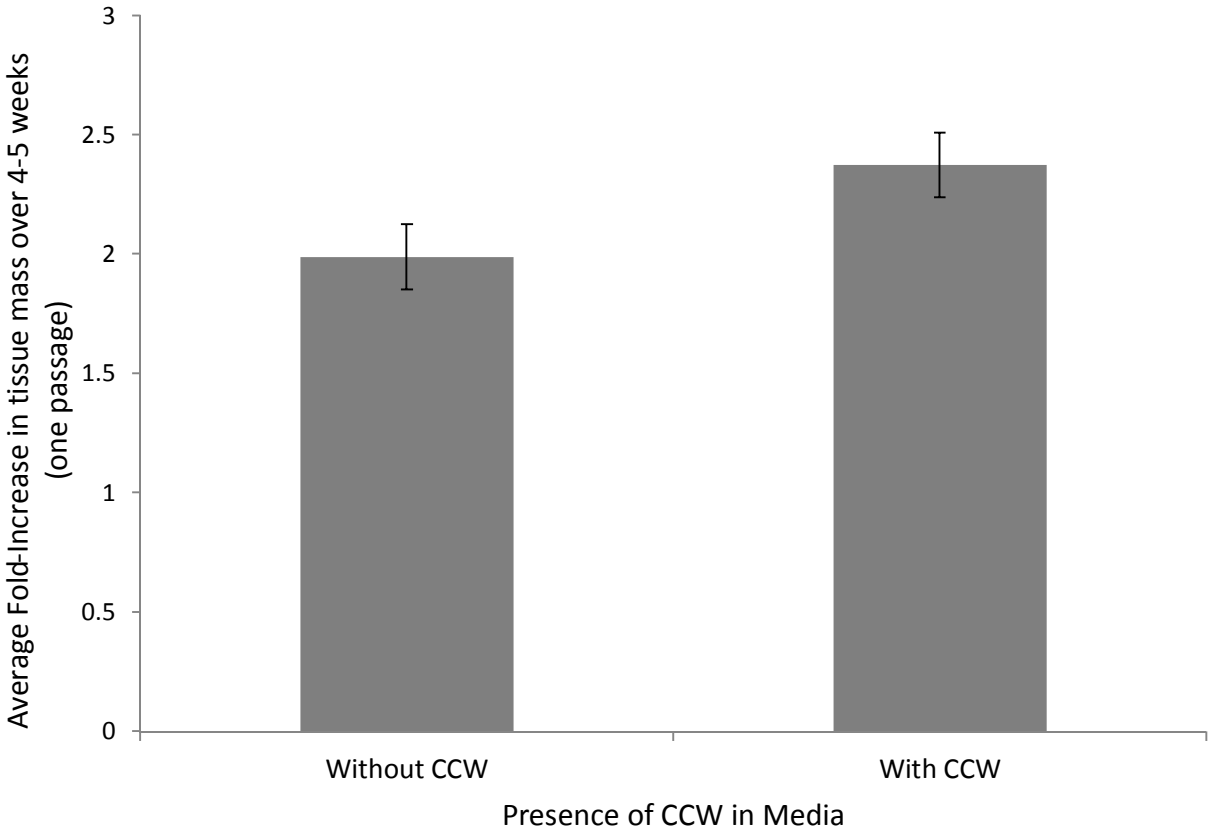

Figure 9: Assessment of Coconut Water (CCW) as a viable media supplement for callus growth as measured in average fold-increase in tissue mass per passage**.** Fold-increase was determined for each culture by comparing tissue mass at the end of a passage (4-5 week growing period) to the cultureôs mass at the beginning of the passage (via the formula $\text{mass}_{\text{Current}} / \text{mass}_{\text{Previous}}$). Error bars represent standard error of 1.3. Group without CCW (n = 77) averaged 1.99 (s = 1.18); group with CCW (n = 125) averaged 2.37 (s = 1.54); з = 190, $P < 0.05$.

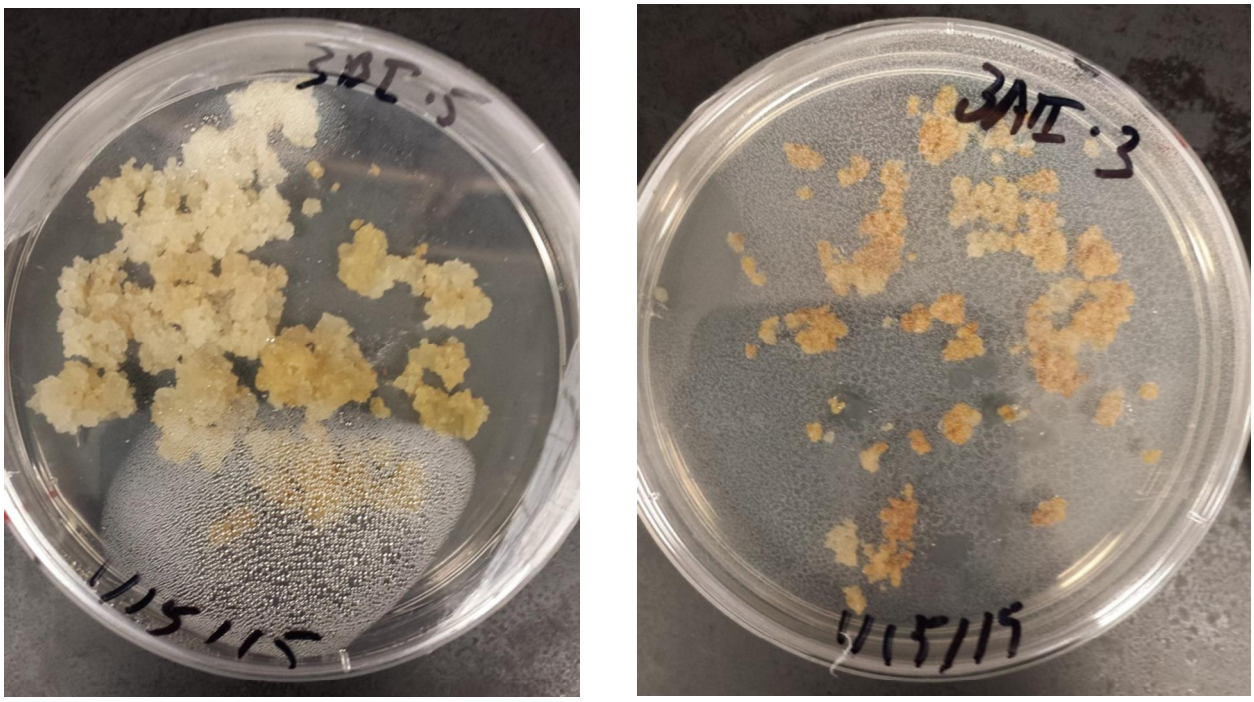

Figure 10: Callus cultures grown on media containing CCW (left) and similar media without CCW (right). Cultures were started from approximately 1 g each of callus and derive from the same cell line; picture was taken 4-5 weeks from the start date (1/15/15).

IV. Liquid Media Suspension Design and Optimization

Media adapted for liquid suspension cell growth was shown to have a strong positive effect on cell growth in suspension. The shaker suspension model (without oxygenation) vastly outperformed the experimental bioreactor setup in terms of cell growth with a 5-fold increase in tissue mass from an initial 4 g to 21 g product. In comparison, the tissues in the bioreactor system saw a smaller increase in tissue mass from an initial 4g to 7g product. The shaker culture also outperformed the bioreactor design in terms of tissue dissociation, forming a thick broth of light-colored cells (Figure 11). Additionally, the shaker suspension culture yielded far less stress

compounds as indicated by the colorless media left over after filtering the cell suspension. In comparison, the bioreactor system showed higher levels of stress, indicated by the yellow coloration left in the tissues and media after the filtration process (Figures 12, 13). Because of inadequate agitation and stress from drying out, the tissues in the bioreactor setting failed to dissociate into single-cell suspension.

Figure 11: Comparison of liquid suspension culture performance between shaker culture (left, shaker not pictured) and experimental bubble bioreactor (right) in terms of media coloration. Picture taken roughly 40 days after inoculation.

Figure 12 : Tissue and cell masses extracted from shaker (left) and bioreactor (right) suspension cultures after filtration.

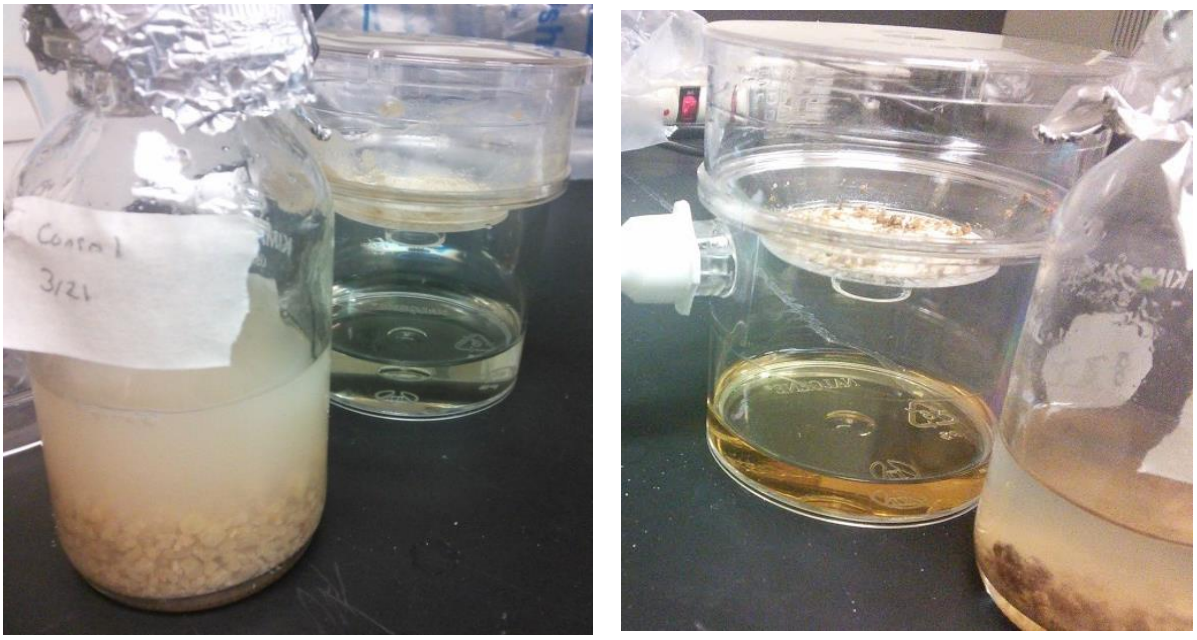

Figure 13: Filtered media from shaker suspension culture (left) and experimental bubble bioreactor (right). Bottles contain cell masses extracted from filtered suspensions

V. Organogenesis in Callus of *Panax quinquefolius*

Root induction media was found to have a strong organogenic rooting effect on callus tissue, inducing root tips on plated calli within a month (Figure 14). These root tips developed into small roots, which when plated on hormone-free media, grew to up to 2 centimeters over the course of a month. AC-containing media was used as a remedial treatment for root cultures that showed considerable signs of stress (Figure 15).

Shoot induction media was shown to be unsuccessful (as of the time of this writing) for initiating shoots from callus tissues grown in both light and dark environments. Callus plated on organogenic shoot-induction media and placed in light environments developed a green coloration which is usually associated with shoots; however, the cultures failed to yield organized tissue types.

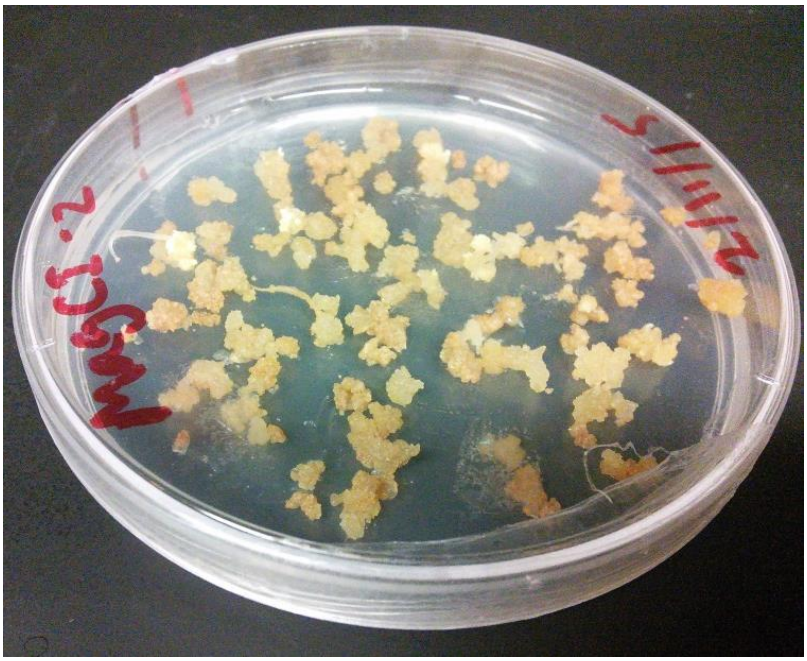

Figure 14: Callus culture with induced root tips. Calli plated on media adapted from the protocol Bonfill (2002) grew roots within the course of a month (single passage).

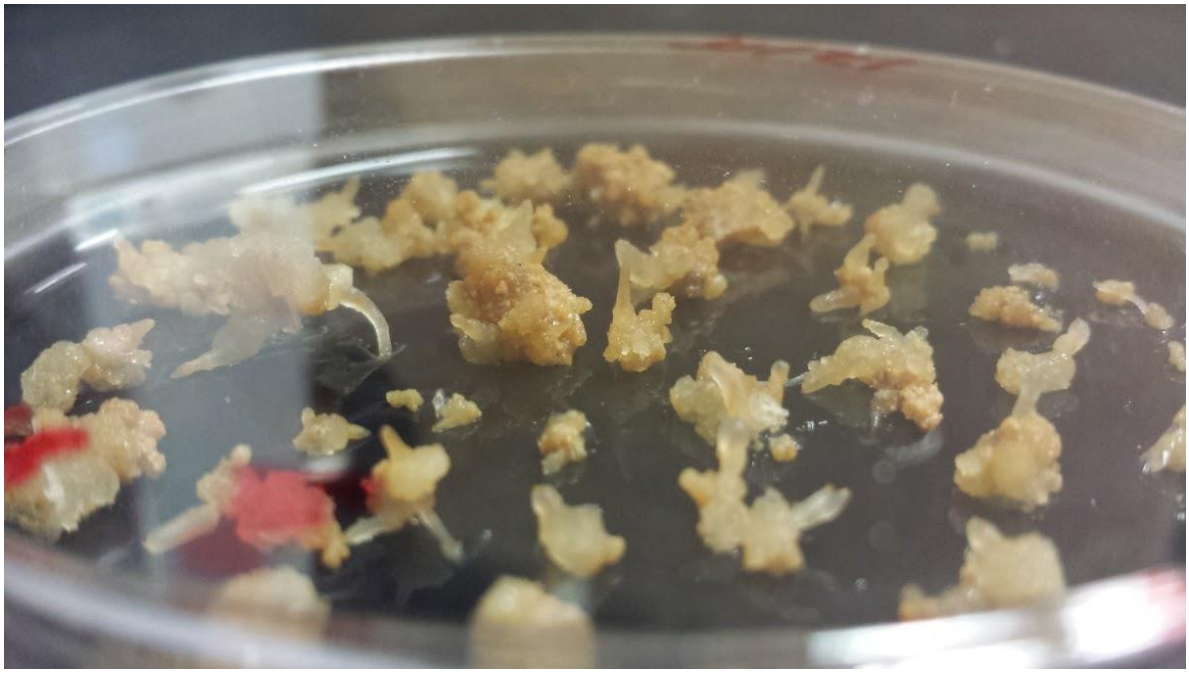

Figure 15: Numerous developing roots and organogenic calli plated on hormone-free medium containing AC (Red/Red formulation).

DISCUSSION

As *P. quinquefolius* has a reputation for being a recalcitrant plant species (Zhou and Brown, 2007), the induction of a rapidly-dividing callus culture from germinated seedling tissue is a great step forward for tissue culture work of this species. Media formulations and growth conditions at each step, from the seed germination to the initiation of a liquid suspension setting, have been optimized in order to create a stepwise system for future plant biotechnological applications of callus tissue of *P. quinquefolius.*

The use of cut, surface-sterilized seeds of *P. quinquefolius* placed on media containing auxin to cytokinin at a ratio of 2/1 allowed for a reduced risk of contamination, increased rate of germination, and increased propensity for seedling tissues to develop into callus. Additionally, the results that auxin and cytokinin at a ratio of 2:1 effectively induced callus from seeds reinforce the results from Wangôs (1990) study with callus initiation from root pith of *P. quinquefolius.* The combined method of achieving germination and callus induction in one culture medium was greatly preferred over callus induction from explants, since the act of slicing seedling tissues resulted in an increased rate of contamination and stress on the tissues. Since seeds were relatively easy to surface sterilize, tissues could be maintained in aseptic conditions at a much greater ease than would be found in removing tissues from seedlings or even developed soil-borne plants. As such, the difficulty arising from using surface-sterilized root, stem, or leaf sections in tissue culture work is largely averted through the use of germinated seedlings, which remain sterile after the initial surface-sterilization procedure.

The process of selecting media supplements and hormone ratios that favored callus growth lead to the development of highly-productive callus proliferation media types. These media formulations proved instrumental in the amplification of healthy callus tissues, since callus healthiness and stress chemical production were found to be directly related to the type of media onto which that tissues were plated. Even healthy, vigorous callus lines could become stressed and stagnant when plated on media types lacking the correct hormone ratios and supplements. Through the knowledge gained from the process of media optimization, more efficient culture designs and higher tissue yields are possible. As an example, when choosing media formulations for suspension cultures, media types such as the bioreactor media formulation were lifted directly from semi-solid media types that induced prolific callus growth

in cells. This process would not have been possible without the optimization process for media supplements and optimal hormone ratios.

Light was shown to be a negative factor for the growth of callus. Compared to cultures placed in dark conditions (within a closed box), callus cultures left in light conditions inside an incubator performed poorly, with a large degree of stress chemical (phenolic) production and stagnated growth. Dark cultures, while not completely exempt from these symptoms, were found to act much more prolifically in terms of growth; indeed, some cultures left in the light then placed in dark conditions eventually adapted to being in the dark (within the course of three weeks) through the extensive production of new tissues. While in the light, such cultures would stagnate and produce a dark tan coloration from phenolic (stress chemical) accumulation; however, keeping the tissues in darkness proved dramatically beneficial in terms of growth of tissues and reduced phenolic accumulation.

Coconut water was found to be a critical component of an optimized media formulation. Media formulations which lacked coconut water were found to perform poorly in comparison to media containing CCW as a supplement. Coconut water is an undefined media supplement which contains a variety of vitamins, amino acids, and positive growth factors which support the growth and robust development of plant tissues. As such, it would follow that cultures provided with this supplement would possess an advantage in terms of cell growth and tissue/cell production; therefore, it can be concluded that coconut water is a vital component of media formulations designed for the production of callus and suspension cultures of *P. quinquefolius.*

Niacin shows promise as a media supplement, especially when used in tandem with CCW. Media types containing both niacin and CCW were observed to have a noticeable advantage over media types containing only CCW when tested with healthy callus lines. This

observation also held true when observing liquid suspension cultures. Niacin is an essential vitamin (B_3) that is involved in the biosynthesis of high-energy electron carriers (such as NAD and NADP) which are involved in many metabolic pathways including cellular respiration (Abrahamian & Kantharajah 2011). By supplying tissues with niacin, it would follow that the tissues themselves could respire and grow at a higher rate due to the high availability of compounds involved in cellular respiration. However, additional statistical analyses remain to be done before a significant difference in growth can be inferred between media formulations with and without niacin.

The bubble bioreactor system did not perform as well as the control method, which included a liquid suspension culture placed on a shaker. Initial observations were that the shaker model had become over-accumulated with stress chemicals two weeks after inoculation due to the coloration of the suspension, which took on a creamy white opaque color. However, this appearance was not the result of stress chemical buildup, but rather was a result of the dissociation of the submerged calli into a suspension of cells. In effect, the tissues placed in the suspension culture had taken on a consistency of cream of chicken soup, forming a thick cream-colored broth of cells. In comparison, the bubble bioreactor system maintained a clear coloration with the calli inside growing steadily without much dissociation. Eventually, the media of the bubble bioreactor took on a dark yellow coloration which has been associated with phenolic production in previous culture attempts. It should be noted, however, that the bubble bioreactor was susceptible to drying out, since the design implemented a current of air throughout the system. Since the bubble reactor may have been compromised from drying out, it is difficult to note whether the addition of oxygen was a positive or negative factor for tissue growth. It can be assumed, however, that the oxygen failed to provide an adequate source of agitation for the

tissues; future designs of this model would do well to add a gentle stirring or agitating measure for adequate dissociation of tissues. Additional replicates of the system with larger reservoirs of water and media as well as a method of agitation should be implemented before assuming that the bubble bioreactor is an inefficient method for producing cells of *P. quinquefolius.* Even so, the relatively outstanding success of the shaker model shows that the media formulation of the bioreactor system is capable of prolific growth. As such, the bioreactor media mix and the success of the shaker culture can provide a baseline for building larger, more complicated suspension culture designs intended for the production of cells and tissues of *P. quinquefolius.*

Induced root cultures were often productive in terms of root tips produced, but these culture types were also prone to stress chemical buildup. As a result, root-induced cultures were sometimes plated on media containing activated charcoal (AC) as a remedial method (Thomas, 2008). The activated charcoal in the media seemed to down-regulate the production of stress chemicals (phenolic compounds) from the tissues. Additionally, AC served to absorb stress chemicals that leeched out from tissues plated on AC-containing media formulations. Stressed root cultures which were placed on such media became bleached in color; however, callus cultures had a tendency to stagnate in terms of growth while plated on AC-containing media. As such, it could be suggested that AC-containing media be used as a remedial method for organogenic cultures with substantial accumulation of phenolic compounds. Root cultures are commonly used in plant biotechnology to produce and harvest plant metabolites. Media formulations that induce the development of roots from callus can be used as a baseline for understanding the development of more efficient root culture media formulations and techniques, including hairy-root transformations.

Shoot induction proved unsuccessful as of the time of this writing. The process followed a protocol by Odnevall et al. (1989); however, the referenced study focused on culture techniques involving *Panax ginseng* and not *Panax quinquefolius.* Additional work remains for the successful differentiation of shoot organs from callus in order to create a clonal propagation system for American ginseng.

CONCLUSIONS

American ginseng, *Panax quinquefolius* L., was effectively induced into callus from seed tissue, and the resulting tissues have been selected for high growth rates. Media formulations at each step, including germination, callus induction, and callus proliferation, were discovered and optimized to further the production of *P. quinquefolius* callus tissue. Liquid suspension techniques were also implemented, with a highly productive media formulation described and put into use. Efforts to induce roots from callus tissue were successful, marking a milestone in the successful hormone-driven differentiation of callus into roots. Similar efforts for shoot initiation were described; however, this is currently an experiment-in-progress.

In all, these results describe a method of efficiently producing large amounts of cells and tissues from the commercially-exploited plant species *Panax quinquefolius.* Semisolid (agar-based) and liquid media types were both optimized for growth, and both media types are currently capable of producing cells (suspension) and callus (semisolid or agar-based) for metabolite analysis techniques. The protocols described herein may prove valuable to those seeking to do further experiments involving genetic manipulation, full-plant regeneration, root culture, and metabolite harvesting techniques concerning American ginseng. Further work remains in developing root cultures of *P. quinquefolius,* especially in terms of transformed hairy

root cultures. Work also remains to be done on optimizing shoot initiation methods, including adjusting media formulations and environmental conditions in order to favor the production of shoots. Successful shoot and root initiation techniques will help make possible a clonal regeneration system for the mass production of *P. quinquefolius* plantlets; these plantlets could, in turn, be used for conservation efforts to assist in the restoration of wild populations of American ginseng, *Panax quinquefolius.*

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APPENDIX

Definition of Terms

1. Adaptogen ï A medicinal herb or plant that may promote physiological equilibrium (homeostasis) when taken as a drug.
2. American Ginseng (*Panax quinquefolius* L.) ï Species of ginseng (Genus *Panax*) that is native Eastern North America, particularly in the forests of the Appalachian Mountains.
3. Asian/ Korean Ginseng (*Panax ginseng* C. A. Meyer) ï Species of ginseng (Genus *Panax*) that is native to Eastern Asia, in the Northern Hemisphere.
4. Bioreactor ï A machine that specializes in suspension culture; can input and output chemicals associated with growing cells in a suspension.
5. Biotechnology ï A division of biology concerned with the use of living systems to produce valuable products.
6. Callus ï A type of plant tissue resulting from exposure to hormones produced within or outside of the cell; the primary tissue that is grown in plant tissue culture.
7. Explants ï Tissues taken from a plant to be grown in tissue culture.
8. Ginseng (*Panax* spp.) ï Genus of adaptogenic herbs that are prized for their unusual, human shaped taproots that contain pharmacologically-important compounds.
9. Ginsenosides ï A type of saponin found in plants from the genus *Panax* that may possess Anticarcinogenic (anti-cancer) and Immunological properties.
10. Micropropagation ï The method of propagating (cloning) plants via callus manipulation into either embryos (embryogenesis) or induced shoots (organogenesis). Compared to traditional propagation methods, such as rooting cuttings from trees or shrubs.
11. Organogenesis ï In plant biology, the induction of plant organs (such as roots and shoots) from de-differentiated tissues (such as callus).

12. Passage/Pass ï An arbitrary unit of time corresponding to 4 to 5 weeks, or approximately one month; here, referring to the time frame that tissues remain on the same plate or culture.
13. Plasticity ï Referring to the adaptability of an organism or its tissues to various environments; here, referring to the ability to survive culture conditions
14. Polysaccharide ï A polymer of sugars (monosaccharides), one of the major types of macromolecules found in living cells.
15. Plant Regeneration ï The manipulation of callus tissue to form embryos which will grow into whole clones of the original plant.
16. Recalcitrance (Biotechnology) ï The resistance of tissues to being grown in a tissue culture setting.
17. Root culture - A type of plant tissue culture in which roots are excised from a plant or induced from callus and grown in various culture conditions, such as on a plate (semi-solid media) or in a suspension (liquid media).
18. Suspension Culture ï A type of culture in which an aggregate of cells is immersed in a solution and broken into globules which can then reproduce independently.
19. Tissue Culture ï Method of growing (culturing) tissues or cells outside of the organism (*in vitro*).
20. Totipotency ï referring to a single cell with the genetic potential of regenerating into a complete multicellular organism