Metabolic Profiling of *in vitro* Cultured Medicinal Plants: *Actaea racemosa* L

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Abstract

The use of *Actaea racemosa* L., black cohosh, as an herbal supplement has dramatically increased over the past decade. The rhizome is used for a variety of medicinal purposes involving premenopausal symptoms. This project involved the development of a protocol for establishment of *in vitro* callus cultures from excised tissues of racemes and leaves. Explants were grown on Murashige and Skoog medium (MS) with a variety of concentrations of growth hormones TDZ and NAA. The production of secondary metabolites by these callus cultures is being analyzed by ultra high pressure liquid chromatography coupled with mass spectrometry UHPLC/MS. The first inoculation of racemes was observed after one week with a large growth of fungi in twelve of the sixteen plates.The project is ongoing and awaiting further results of callus growth in the plates.

Introduction

The wide spread increase of use of *Actaea racemosa* L., black cohosh, has caught the attention of researchers. This species is a perennial that is found from Southern Ontario to Georgia and Arkansas; Missouri to Wisconsin (Foster & Duke, 2000). Black Cohosh has a long record as a medicinal herb used by Native Americans to ease in childbirth and as an antidote for rattlesnake bite. Its antispasmodic, sedative, and anti-inflammatory properties treat menstrual cramps, rheumatism, childbirth pains, headaches, coughs, and asthma. (Bremness, 1994) The rhizome is used for medicinal value. The primary means to collect black cohosh relies on harvesting from the wild. This has raised concerns that the plant may become rare or endangered in the wild. The discovery and implementation of sustainable methods of harvesting and cultivating black cohosh will help reduce demands on wild populations. Propagation of this species has been found to be a difficult undertaking. A host of researchers collaborating through the Appalachian Center for Ethnobotanical Studies (ACES) are researching a variety of aspects of black cohosh in order to determine aspects important for medicinal uses and sustainability of the plant in the wild. ACES is a collaborative program with Frostburg State University, West Virginia University and the Center for Advanced Research in Biotechnology (CARB) at the University of Maryland Biotechnology Institute (UMBI). Dr. Eisenstein's group at CARB is interested in identifying the groups of genes and biosynthetic pathways responsible for the production of the medicinally

potent secondary metabolites. Determining methods for effective tissue culturing will result in the ability to propagate on individual genetic plant in order to aid in various aspects of research. Tissue culturing of the species will allow genetic and environmental variables to be held constant while research explores concentrations and presence of secondary metabolites.

Purpose

The usage of a callus culture system is a valuable tool that will aid in furthering the ongoing research taking place by the Eisenstein group at CARB. This route of inquiry required the experimental development of a protocol for *in vitro* growth. The experiment called for the inoculation of leaf tissue and racemes under sterile conditions. The profiling of the secondary metabolites produced by callus growth will provide an avenue for the opportunity to manipulate the genetic material to trace the metabolic pathways. The use of micropropagation in this regard has revolutionized the science of modern botanical inquiry. A number of species have been successfully propagated *in vitro*, which has contributed to conservation of these species. (George, 1996b)

Methods

Plant samples were taken from racemes and leaves of black cohosh, sterilized with a bleach solution, and placed on plates with growth medium. They were then inoculated with plant growth hormones and then monitored at the end of one week to determine if there were infections of bacteria or fungi. After three weeks, if the plants show no infections of bacteria or fungi, the plates will be observed for development of callus growth. This differentiation of plant cells indicates that the plant can be cultured *in vitro*. Indication of infections of bacteria or fungi would result in changes in methods development to ensure sterilization. Specific methods were developed to determine the concentration of bleach needed to effectively sterilize the plates.

Extensive method development resulted in the adaptation of the following procedure. This procedure is the proposed protocol for *in vitro* tissue culture of black cohosh, after various laboratory trials. First iterations of this experiment resulted in the growth of fungi, resulting in an increase in the concentration of bleach.

Results

The following procedures were developed and are the results of the experiment. Abbreviations are as follows: NAA: 1-naphthaleneacetic acid, TDZ: thidiazuron, MS: Murashige & Skoog. The growth media was prepared according to Murashige and Skoog (MS) with varying concentrations of TDZ and NAA. The concentrations varied from 0.5 μM to 5.0 μM. The media was prepared, autoclaved, and poured into the plates in the Laminar flow hood. Racemes are aggregations of flowers on

the stems of plants and these were taken from flowering plants located outdoors and young leaves were taken from non-flowering plants from the greenhouse. The plates were stored in the dark at room temperature (23 degrees Celsius). All glassware was rinsed with ultra purified water and all stock solution bottles were sterilized in the autoclave.

Iron Stock (200X)

1. Add 40 mL ultra purified water to a 100 mL beaker. Heat in a microwave for 20 seconds.
2. Dissolve 556 mg $FeSO_4$•7H2O
3. Add 40 mL ultra purified water to a separate 100 mL beaker. Heat in microwave for 20 seconds.
4. Dissolve 744 mg Na_2EDTA•4H2O
5. Add the 2 solutions to a 100 mL graduated cylinder and fill to 100 mL with ultra purified water.
6. Filter sterilize the solution.
7. Protect from light by storing in an amber bottle or wrap with aluminum foil. Store at room temperature.
8. Use 5 mL 200X Iron Stock solution per liter of MS medium.

Micronutrient Stock (100X)

1. Add 400 mL ultra purified water to a 1 L beaker.
2. Add and dissolve the following nutrients into the 1 L beaker, allowing each compound to completely dissolve before adding the next compound.

2,230 mg	$MnSO_4$•4H2O
860 mg	$ZnSO_4$•7H2O
620 mg	HBO_3
83 mg	KI
25 mg	$NaMoO_4$•H2O
2.5 mg	$CuSO_2$•5H2O
2.5 mg	$CoCl_2$•6H2O

3. Transfer the solution to a 1 liter graduated cylinder and fill to 1 liter with ultra purified water.
4. Filter sterilize the solution. Store at 4°C
5. Use 10 mL of 100X Micronutrient Stock solution per liter of MS medium.

Vitamins Stock (1000X)

1. Add 50 mL ultra purified water to a 100 mL beaker.
2. Add and dissolve the following nutrients into the 100 mL beaker, allowing each compound to completely dissolve before adding the next compound.

200 mg	Glycine
50 mg	Nicotinic Acid
50 mg	Pyridoxine•HCl
10 mg	Thiamine•HCl

3. Transfer the solution to a 100 mL graduated cylinder and fill to the 100 mL line with ultra purified water.
4. Filter sterilize the solution. Store at 4°C.
5. Use 1 mL of 1000X Vitamin Stock solution per liter of MS medium.

Macronutrients & Completion of MS medium

1. Add 400 mL ultra purified water to a 1 Liter beaker.
2. Add and dissolve the following nutrients into the 1 L beaker, allowing each compound to completely dissolve before adding the next compound.

1,650 mg	$(NH_4)\ NO_3$
1,900 mg	KNO_3
440 mg	$CaCl_2•2H_2O$
370 mg	$MgSO_4•7H2O$
170 mg	KH_2PO

3. The stock solutions must be kept sterile. The pipet tips must be sterile and great care must be taken at this step to avoid contamination.
4. Ignite the Bunsen burner. Unscrew the cap of the stock reagent, aiming the neck of the bottle towards the flame. Place the cap down on the bench right side up to avoid contamination. Insert the pipet tip into the solution only enough to withdraw the necessary volume. After removing the pipet tip, re-flame the tip of the bottle for a moment and then replace the cap securely.
5. Add the Stock Solutions according to the following concentrations:

5 mL	200X Iron Stock
10 mL	100X Micronutrient
1 mL	1000X Vitamin Stock

6. Add 100 mg myo-inositol to the medium.
7. Add ultra purified water up to about 900 mL.
8. Adjust the pH to 5.7 with 1N NaOH and/or 1N HCl.
9. Add 30 g sucrose.
10. Add 7 g clean agar for plates.

11. Transfer solution to a 1 L graduated cylinder and fill to the 1 L line.
12. Transfer into a 1 L bottle with a loose fitted cap.
13. Sterilize in the autoclave.

Preparation of Growth Hormones

TDZ

1. Utilize sterile techniques for all steps.
2. Add 11.0 mg TDZ to a new falcon tube using a small boat.
3. Add 200 µL 1 M NaOH to the boat to rinse any remaining residue of the TDZ directly into the falcon tube. Put the cap on the falcon tube quickly.
4. Add ultra purified sterile water to the falcon tube up to the 50 mL mark.

NAA

1. Utilize sterile techniques for all steps.
2. Add 9.31 mg NAA to a new falcon tube using a small boat.
3. Add 200 µL 1 M NaOH to the boat to rinse any remaining residue of the NAA directly into the falcon tube. Put the cap on the falcon tube quickly.
4. Add ultra purified sterile water to the falcon tube up to the 50 mL mark.

Preparation of Plates and Pouring of Plates

1. Label plates according to the specific concentration of the growth hormones TDZ and NAA used. The following concentrations are for a matrix of auxin/cytokin supplements:

TDZ \ NAA	0.5 µM NAA	1.0 µM NAA	2.5 µM NAA	5.0 µM NAA
0.5 µM TDZ	0.5 µM TDZ/ 0.5 µM NAA	0.5 µM TDZ/ 1.0 µM NAA	0.5 µM TDZ/ 2.5 µM NAA	0.5 µM TDZ/ 5.0 µM NAA
1.0 µM TDZ	1.0 µM TDZ/ 0.5 µM NAA	1.0 µM TDZ/ 1.0 µM NAA	1.0 µM TDZ/ 2.5 µM NAA	1.0 µM TDZ/ 5.0 µM NAA
2.5 µM TDZ	2.5 µM TDZ/ 0.5 µM NAA	2.5 µM TDZ/ 1.0 µM NAA	2.5 µM TDZ/ 2.5 µM NAA	2.5 µM TDZ/ 5.0 µM NAA
5.0 µM TDZ	5.0 µM TDZ/ 0.5 µM NAA	5.0 µM TDZ/ 1.0 µM NAA	5.0 µM TDZ / 2.5 µM NAA	5.0 µM TDZ/ 5.0 µM NAA

2. Setup the labeled Petri dishes in a Laminar flow hood. Always spray everything thoroughly with ethanol before it goes into the Laminar flow hood.
3. Setup all necessary equipment into Laminar flow hood, including pipettes, stand for pipettes and pipet tips.
4. Run the UV light for a short duration (5-10 minutes) as a precaution. Make certain that no UV sensitive compounds are in the hood while the UV light is on. The growth hormones are UV sensitive.

5. Turn the UV light off, spray down the outsides of the growth hormone tubes with ethanol and then place them in the Laminar flow hood.
6. Heat the MS medium in the microwave to liquefy the agar. Start with 10 minutes on level 4. Watch the medium and make sure it does not come to a boil and flow out of the flask.
7. Ignite the Bunsen burner and pour the MS medium into four 50 mL falcon tubes near the flame from the Bunsen burner. Prepare only 4 tubes at a time.
8. Heat the falcon tubes in a water bath at 50°C for 8-10 minutes to equilibrate the temperature.
9. Remove the falcon tubes from the water bath and wipe away all water droplets on the outside of the tubes.
10. Quickly transport the falcon tubes to the Laminar flow hood.
11. Do not allow pipet tips to come in contact with anything while they are being used to add growth hormone to the MS medium. Do not shake falcon tubes to mix, avoid producing bubbles in the tubes. Do not allow medium to come in contact with the top of the plate.
12. Add the growth hormones to each falcon tube according to the concentrations of the chart below, which is equivalent to the micro molarities in the chart above. Each falcon tube must have both growth hormones added to it.

25 µL TDZ 25 µL NAA	25 µL TDZ 50 µL NAA	25 µL TDZ 125 µL NAA	25 µL TDZ 250 µL NAA
50 µL TDZ 25 µL NAA	50 µL TDZ 50 µL TDZ	50 µL TDZ 125 µL NAA	50 µL TDZ 250 µL NAA
125 µL TDZ 25 µL NAA	125 µL TDZ 50 µL TDZ	125 µL TDZ 125 µL NAA	125 µL TDZ 250 µL NAA
250 µL TDZ 25 µL NAA	250 µL TDZ 50 µL NAA	250 µL TDZ 125 µL NAA	250 µL TDZ 250 µL NAA

13. Gently invert the falcon tube 5 times. Do not allow air bubbles to form.
14. Pour 25 mL into one plate and 25 mL into the other. Quickly replace cover of plate.
15. Once the medium has solidified, flip the plates over so that the medium is upside down and then transfer the plates to the refrigerator.

Explant

Racemes

1. Racemes were collected from plants located outdoors. Specimens were collected in the afternoon. The flowering parts of the raceme were not used. The portion where the peduncles were not opened was used. The tips of the racemes were not used; about 5 cm was cut off and discarded. The raceme pieces ranged from 5-9 cm in length. The peduncle balls were cut off of the

stem before bleaching was administered.
2. Transfer all necessary equipment to the Laminar flow hood.
3. Sterilize plant material in 0.5% NaOCl and 0.1% Tween-20 in a 50 mL falcon tube.
4. Transfer the falcon tube containing plant specimens and the 6 falcon tubes being used to wash plant material to the Laminar flow hood. Spray with ethanol before entering into the hood.
5. Wash plant material 3 times in ultra purified sterile water for 5 minutes each time.
6. Inoculate small (0.5 cm) pieces of tissue onto the agar plates. Each plate was dated and labeled R for raceme.

Leaf

1. Young leaves were taken from specimens in the greenhouse.
2. Transfer all necessary equipment to the Laminar flow hood.
3. Sterilize plant material in 0.75% NaOCl and 0.1% Tween-20 in a 50 mL falcon tube.
4. Transfer the falcon tube containing plant specimens and the 6 falcon tubes being used to wash plant material in the Laminar flow hood. Spray with ethanol before entering into the hood.
5. Wash plant material 3 times in ultra purified sterile water for 5 minutes each time.
6. Inoculate small (0.5 cm) pieces of tissue onto the agar plates. Each plate was dated and labeled L for leaf.

The following calculations were used to determine the amount of volume to prepare specific concentrations.

Preparation for 1 mM concentrations

(220.3 g TDZ / L) (1L / 1000 mL) (1000 mL / 50 mL) = ***(11.00 mg TDZ / 50 mL)***

(186.21 g NAA / L) (1L / 1000 mL) (1000 mL / 50 mL) = ***(9.31 mg NAA / 50 mL)***

Preparation of 0.5% NaOCl solution

(50 mL / 12.5) = (V2 / 0.5) = (50 X 0.5 / 12.5) = 2 mL of 12.5% NaOCl solution diluted to 50 mL = 0.5 % NaOCl

(V1 / 50 mL) = (0.1 / 100) = (50 X 0.1 / 100) = 0.05 mL = 50 μL of 100% Tween-20 diluted to 50 mL = 0.1%

M16 & A39 genotypes were used from the greenhouse. M16 was mostly used.

Conclusions

This project resulted in potential development of appropriate methods development for sterile culturing of black cohosh. Final results will aid in *in vitro* culture of this medicinal herb, which will allow liquid suspension sub-culturing for future research projects for this species. The plates with racemes were observed after one week with fungal growth in 12 of 16 plates. An attempt was made to remove the infected area from the plates. After two days, the plates were observed again and still contained exponential fungal growth. The uninfected racemes were transferred to new plates. These plates were labeled R for raceme and with the date they were originally inoculated, along with the date they were transferred. The procedure for inoculation of the racemes was repeated with 1.0% NaOCl solution because of the severity of the infection of the other plates. The plates with leaf tissues showed no infection after 2 days. Insufficient time was available to document formation of callus growth in the plates as it normally requires 3 weeks. The project is ongoing and final results will be obtained by the research group.

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