ANALYSIS OF WHEATGRASS ENDOPHYTES

Honors Thesis

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By

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Abstract

Endophytes are of particular medical interest due to their production of antibiotics. Wheatgrass endophytes were analyzed using a combination of MIC, Gel electrophoresis, GCMS, and TLC. Cultures were successfully grown in gel agarose plates and in sabouraud dextrose broth. All colonies were observed using a dissection microscope. Colonies from the white samples seen on the seed (sabouraud dextrose agar), leaf (sabouraud dextrose agar), seed (coffee agar) and seed (agar) plates were gram stained, with gram negative rods were observed in all samples. Gram positive cocci we observed in the seed (agar) plate. Presence of a long chain methyl ester was observed from the GCMS analysis of samples extracted in methanol. An inhibitory effect was observed on the growth of E. coli and S. aureus after inoculating methanol extracted samples in broth.

Introduction

Endophytes are microorganisms found in the internal tissues of plants, and can be used to create bioactive compounds used by plants for defense against pathogens. Common endophyte products include antibiotics, anticancer agents, and biological control agents. The prospect of discovering new sources of medical treatment is highly appealing, especially if the endophytes are easy to grow. (Guo, B. et al, 2008)

Wheatgrass is of particular interest because it is already known to have beneficial health properties. Wheatgrass contains vitamin C, E, Beta-carotene, ferulic acid, vanilic acid, and chlorophyll, some of which have been shown to inhibit the metabolic activation of carcinogens. Wheatgrass extracts have also been shown to contain phenolic compounds, which are responsible for antioxidant activity. The known health benefits of wheatgrass, and the ease with which it can be grown make it an appealing plant to study for the production of endophytes. (Aydos, O. et al, 2011)

Previous research into the effect of different growing media on the growth of bacterial endophyte communities from wheatgrass samples has been performed, and it was observed that the growth media had a greater effect on the endophyte growth than the sample tissue type or the wheatgrass variety. (Ringelberg, D. et al., 2012)

This distinct effect on the growth of the endophyte communities highlighted the need to prepare samples on a variety of media types, leading to the choice of plain agar (a nutrient poor media), sabouraud dextrose agar (a nutrient rich media), and coffee agar (an acidic media) for initial sample plating.

Methods

Plant Growth

Hard red wheatberries were soaked overnight in tap water. They were then drained, and transferred to a wet paper towel and then stored in dark for 48 hours. The plants were then uncovered, watered, and left in an area with sunlight. All grass was watered every other day. Plants were left to grow 5-7 more days and then harvested.

Sterile Sample Preparation and Plating

Samples were washed in soapy water, followed by 10% bleach solution, followed by 70% ethanol solution. The bench top was wiped down with 70% ethanol solution before placing any samples down. Both sides of a sterilized leaf were tapped to a clean agarose gel plate, and the plate was then closed and the sides were parafilmed. Scissors were wiped down with 70% ethanol solution and three ½ cm leaf segments were cut into new clean agarose gel plate. The sides of this plate were closed and parafilmed. These steps were repeated for seeds and roots in place of leaves. All plates were labeled and stored at room temperature.

Sterile Sample Seeding

In a large beaker 15 g Powdered Sabouraud Dextrose Broth and 500 mL water were mixed. Liquid was poured into several Erlenmeyer flasks in 40-50 mL portions and boiled for 20 minutes. A metal spatula was sterilized in 70% ethanol and then passed through a flame. Using the sterilized spatula a small piece of sample was isolated and deposited into an Erlenmeyer flask with broth. This was repeated for additional samples. Excess Sabouraud Dextrose Broth was stored in a screw cap bottle in refrigerator.

Observation of Sample colonies

Plated samples were observed using a dissection microscope. Slides were prepared from slimy white samples present on seed sample grown on agar, seed sample grown on sabouraud dextrose agar, seed sample grown on coffee agar, and leaf sample grown on sabouraud dextrose agar. A selection of samples were Gram stained (Leboffe & Peirce, pg 160, 161, 174) and viewed at 1000x on a light microscope.

Extraction with Dichloromethane

Nutrient broth was poured through filter paper into a separatory funnel and washed three times using 20 mL dichloromethane each time. The dichloromethane layer was saved. The saved layer was then washed using 30 mL distilled water. The water layer was discarded. A small scoop of Na_2SO_4 was added to the saved dichloromethane layer. This was then filtered through cotton into a round bottom flask, and evaporated using a rotary evaporator (100 rpm, 60 C). The contents of the round bottom flask were rinsed into a weighed labeled scintillation vial using two 1 mL portions of dichloromethane and stored in the freezer.

Extraction with Methanol

Solids from nutrient broth were ground using a mortar and pestle, then mixed with 30 mL of methanol and poured through filter paper into a round bottom flask. This was then evaporated using a rotary evaporator (100 rpm, 80 C). The contents of the round bottom flask were rinsed into a weighed labeled scintillation vial using two 1 mL portions of methanol and stored in the freezer.

DNA Extraction

In a microcentrifuge tube 400 μL of PX_1 buffer and a small portion of sample (approximately 1 cm^2) were combined. To this tube 4 μL of RNAase were added. Tube contents were crushed and mixed using a glass stirring rod, and then vortexed for 10 seconds. The microcentrifuge tube was then placed into a hot water bath (60 C). After 10 minutes the tube was removed from the water and 130 μL PX_2 buffer were added. This was then vortexed for 5 seconds, and put on ice. After 5 minutes the tube was centrifuged for 10 seconds at 7000 RPM. The liquid portion was then transferred to shearing tube in collection tube and centrifuged for 10 seconds at 7000 RPM. Liquid was then transferred to a new tube, and 500 μL of Ethyl Acetate were added. To this, 250 μL of PX_3 buffer were added, and then tube contents were mixed. Into a gencatch Plant genomic DNA mini column in collection tube 650 μL of tube contents were transferred, and then centrifuged for 2 minutes at 10,000 RPM. An additional 650 μL of tube contents were transferred into gencatch tube, and centrifuged for 1 minute at 10,000 RPM. After centrifuging 700 μL WS buffer were added, contents were then centrifuged for 20 seconds, and liquid was discarded. Addition of 700 μL WS buffer was repeated, and tubes were centrifuge for an additional 30 seconds before discarding liquid. The gencatch tube and contents were then centrifuged for 2 minutes at 13,000 RPM, and the collection tube was discarded. The silica column was moved to new microcentrifuge tube, and 200 μL of hot water were added. The tube was centrifuged for 1 minute at 13,000 RPM, and the silica column was discarded. Liquid was saved on ice.

PCR

In a microcentrifuge tube 0.5 μL ITS-F and 0.5 μL ITS-R were added to 10.5 μL H_2O. After this, 1 μL template was added, followed by the addition of 12.5 μL 2x Taq. Sample was then allowed to run with the following denature program: initial denature 3 minutes at 94 C, then 30 seconds at 94 C, 45 seconds at 52 C, and 60 seconds at 72 C. Program repeated 30 times.

Thin Layer Chromatography

Using a capillary tube, a small dot of sample was placed onto a TLC plate. Plate was placed into a TLC jar containing solvent to run, and final position of solvent was recorded after removing from jar. Plates were observed under UV light.

Minimum Inhibitory Concentration

Solvent was evaporated from frozen samples. Samples were then combined with 500 μL of broth and vortexed. Samples were then transferred to 2 mL of broth. Of this sample 200 μL were transferred into 2 mL of broth. A further dilution was performed by transferring 200 μL of this diluted sample into 2 mL of broth. Tubes were then inoculated with either 10 μL of E. coli or 10 μL of S. aureus into each tube. Samples were then stored in a warm, dry place, and bacterial growth was observed and compared to standard after 24 hours.

Results and Discussion

Sample growth was seen on all plates, including the plate that should have been sterile. This indicates that there was some contamination during the plating process. Purple, green, slimy

white, and fluffy white cultures grew on plates containing wheatgrass seeds. Blue, flat white, and fluffy white cultures grew on plates containing wheatgrass leaves. Slimy white, fluffy white, blue, and yellow orange cultures grew on plates containing wheatgrass roots.

Colonies from the white samples seen on the seed (sabouraud dextrose agar), leaf (sabouraud dextrose agar), seed (coffee agar) and seed (agar) plates were gram stained, with gram negative rods were observed in all samples. Gram positive cocci we observed in the seed (agar) plate.

All cultures grew when isolated and left in Sabouraud Dextrose broth. TLC and GCMS were run on the flat white sample from the leaf (one extracted with DCM, one with methanol) and the blue sample from the leaf extracted with methanol. No banding was visible on the TLC plate under UV light. DNA extraction, PCR, and gel electrophoresis were run on an isolated sample of the slimy white culture from the seed. No banding was seen in the gel after it was run. MIC was tested with the flat white sample from the leaf extracted using methanol. There was a noticeable increase in inhibition of bacteria (both E. coli and S. aureus) after a 24 hour period, and there was a higher level of inhibition in tubes containing more concentrated sample. This inhibition suggests the presence of a dosage dependant antibiotic in the sample.

A long chain methyl ester was observed in samples extracted with methanol using GCMS analysis. Compounds derived from methyl ester bases are currently being investigated for antimitotic properties and antineoplastic properties (Paterson, I. et al., 2011)(Abdel-Ghani, N., 2012).

References

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Appendix I: Procedure

Plant Growth

1. Soak seeds overnight.
2. Drain, and transfer to wet paper towel. Store in dark for 48 hours.
3. Uncover seeds, water, leave in area with sunlight.
4. Water grass every other day.
5. Leave grass to grow 5-7 more days, harvest grass.

Sterile Sample Preparation and Plating

1. Wash sample in soapy water
2. Wash sample in 10% bleach solution
3. Wash sample in 70% ethanol solution
4. Wipe down bench top with 70% ethanol solution before placing any samples on bench top.
5. Tap both sides of a sterilized leaf to a clean agarose gel plate.
6. Close plate and parafilm sides.
7. Wipe scissors down with 70% ethanol solution and cut three ½ cm leaf segments into new clean agarose gel plate.
8. Close plate and parafilm sides.
9. Repeat steps 7-8 for seeds and roots in place of leaves.
10. Label all plates and store at room temperature.

Sterile Sample Seeding

1. Mix 15 g Powdered Sabouraud Dextrose Broth with 500 mL water.
2. Pour 40-50 mL into Erlenmeyer flask.
3. Boil for 20 minutes.
4. Sterilize a metal spatula in 70% ethanol and then pass through a flame.
5. Using sterilized spatula isolate a small piece of sample and deposit into Erlenmeyer flask with broth.
6. Repeat steps 2-5 for additional samples.
7. Store excess Sabouraud Dextrose Broth in a screw cap bottle in refrigerator.

Extraction with Dichloromethane

1. Pour nutrient broth through filter paper into a separatory funnel.
2. Wash three times using 20 mL dichloromethane each time. Save dichloromethane layer.
3. Wash using 30 mL distilled water. Discard water layer.
4. Add a small scoop of Na_2SO_4 to saved dichloromethane layer.
5. Filter through cotton into a round bottom flask.
6. Evaporate using a rotary evaporator (100 rpm, 60 C).
7. Rinse into a weighed labeled scintillation vial using two 1 mL portions of dichloromethane.
8. Store in freezer.

Extraction with Methanol

1. Grind solids from nutrient broth using a mortar and pestle.
2. Mix with 30 mL of methanol and pour through filter paper into a round bottom flask.
3. Evaporate using a rotary evaporator (100 rpm, 80 C).
4. Rinse into a weighed labeled scintillation vial using two 1 mL portions of methanol.
5. Store in freezer.

DNA Extraction

1. Add 400 µL of PX_1 buffer into a microcentrifuge tube.
2. Add a small portion of sample (approximately 1 cm^2).
3. Add 4 µL of RNAase.
4. Crush using glass stirring rod.
5. Vortex for 10 seconds.
6. Place in hot water bath (60 C) for 10 minutes.
7. Remove from water, add 130 µL PX_2 buffer.
8. Vortex for 5 seconds.
9. Put on ice for 5 minutes.
10. Centrifuge for 10 seconds at 7000 RPM.
11. Transfer liquid to shearing tube in collection tube.
12. Centrifuge for 10 seconds at 7000 RPM.
13. Transfer to a new tube.
14. Add 500 µL of Ethyl Acetate.
15. Add 250 µL of PX_3 buffer.
16. Mix tube contents.
17. Transfer 650 µL to a gencatch Plant genomic DNA mini column in collection tube.
18. Centrifuge for 2 minutes at 10,000 RPM.
19. Transfer 650 µL more into gencatch tube, centrifuge for 1 minute at 10,000 RPM.
20. Add 700 µL WS buffer.
21. Centrifuge for 20 seconds.
22. Discard liquid.
23. Add 700 µL WS buffer.
24. Centrifuge for 30 seconds.
25. Discard liquid.
26. Centrifuge for 2 minutes at 13,000 RPM.
27. Discard collection tube.
28. Move silica column to new microcentrifuge tube.
29. Add 200 µL of hot water.
30. Centrifuge for 1 minute at 13,000 RPM.
31. Discard silica column.
32. Save liquid on ice.

PCR

1. Add 10.5 µL H_2O to a microcentrifuge tube.
2. Add 0.5 µL ITS-F and 0.5 µL ITS-R.
3. Add 1 µL template.
4. Add 12.5 µL 2x Taq.
5. Run denature program: initial denature 3 minutes at 94 C, then 30 seconds at 94 C, 45 seconds at 52 C, and 60 seconds at 72 C. Program should repeat 30 times.

Thin Layer Chromatography

1. Using a capillary tube, place a small dot of sample onto a TLC plate.
2. Run in a TLC jar containing solvent.
3. Do not allow solvent to run off end of plate, record final position of solvent after removing from jar.
4. Observe plates under UV light.

Minimum Inhibitory Concentration

1. Evaporate solvent from frozen samples.
2. Add 500 μL of broth.
3. Vortex.
4. Transfer to 2 mL of broth.
5. Transfer 200 μL of this to 2 mL of broth.
6. Transfer 200 μL of this to 2 mL of broth.
7. Inoculate tubes with either 10 μL of E. coli or 10 μL of S. aureus into each tube.
8. Store in a warm dry place.
9. Observe bacterial growth compared to standard after 24 hours.

Appendix II: Chemical Materials

Name: Methanol
Structure: CH_3OH
Formula weight: 32.04216
Melting point: -98 °C
Density: 0.791 g/cm^3
Boiling point: 64.7 °C
Hazard, risk, and safety information: Toxic if swallowed, toxic if in contact with skin, toxic if inhaled, causes damage to organs, highly flammable liquid and vapor.

Name: Dichloromethane
Structure: Cl_2CH_2
Formula weight: 84.93288
Melting point: -97 °C
Density: 1.325 g/cm^3
Boiling point: 39-40 °C
Hazard, risk, and safety information: Causes skin irritation, causes serious eye irritation, may cause respiratory irritation, may cause drowsiness or dizziness, suspected of causing cancer, may cause damage to organs through prolonged or repeated exposure.

Name: Sabouraud Dextrose Broth
Structure:
Formula weight:
Hazard, risk, and safety information: May cause minor irritation to eyes and skin.

Name: Sodium Sulfate
Structure: Na_2SO_4
Formula weight: 142.03714
Melting point: 884 °C
Boiling point: >1700 °C
Hazard, risk, and safety information: Not hazardous.

Name: Ethanol
Structure: CH_5OH
Formula weight: 46.06904
Melting point: -114 °C
Density: 0.79 g/cm^3
Boiling point: 78 °C
Hazard, risk, and safety information: Highly flammable liquid and vapor.

Name: Sodium hypochlorite (Bleach)
Structure: ClNaO
Formula weight: 74.44217
Melting point: 1.2 °C
Density: 1.2 g/cm^3
Hazard, risk, and safety information: Causes severe skin burns and eye damage, very toxic to aquatic life, may be corrosive to metals, contact with acids liberates toxic gas.

Name: Sodium Chloride

Structure: NaCl
Formula weight: 58.44277
Melting point: 801 °C
Boiling point: 1461 °C
Hazard, risk, and safety information: Not hazardous.

Name: Sodium Bicarbonate
Structure: $CHNaO_3$
Formula weight: 84.00691
Melting point: °C
Density: g/cm^3
Boiling point: °C
Hazard, risk, and safety information: Avoid contact with eyes.

Name: Lemon Juice
Structure:
Formula weight:
Melting point: 270 °C
Hazard, risk, and safety information:

Name: Agarose Gel
Structure:
Formula weight:
Melting point: °C
Density: g/cm^3
Boiling point: °C
Hazard, risk, and safety information: Hazardous in case of ingestion, slightly hazardous in case of skin contact, slightly hazardous in case of eye contact, slightly hazardous in case of inhalation.

Name: Water
Structure: H_2O
Formula weight: 18.01528
Melting point: 0 °C
Boiling point: 100 °C
Density: 1 g/cm^3
Hazard, risk, and safety information: None