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High-throughput 96-well assay to measure impact of polyphenolic compounds on fungal growth

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We use this protocol and it's working



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Abstract

This protocol was developed to assess the growth of filamentous fungi in the presence of polyphenolic compounds--a class of secondary metabolites produced by plants with reported antimicrobial activity. Here, we use 96-well plates and a plate reader to screen each fungal isolate against six polyphenolic compounds at three concentrations (with four replicates per compound/concentration). Optical density is measured with a spectrophotometer to quantify fungal growth compared to a positive control containing only media. As numerous fungi do not readily produce spores in culture, here we harvest fungal hyphae from solid media with a cellophane overlay and blend tissue a liquid media prior to inoculation in 96 well plates



Materials

Carrageenan, iota type, Fisher Scientific #50-213-245

15 mL conical tubes

1.5 mL tubes

2 mL tubes

D(+)-Glucose, Fisher Scientific #AC410955000

Nanopure water

Tannic acid, Sigma-Aldrich, #403040-50G

Gallic acid $\geq 98\%$, Fisher Scientific #AC410860050

Trans-o-Coumaric Acid $\geq 98\%$, Thermo Fisher Scientific, #C0394-5G

P-Anisic Acid 98%, Thermo Fisher Scientific, #AC152371000

Vanillic acid, purum, $\geq 97.0\%$, Fisher Scientific, #501856266

Lignin, alkali, Sigma-Aldrich, #370959-100G

Syringe Filters, PES, 0.2 μ M, 25MM, sterile, Fisher Scientific, #09-740-113

Medium Malt Extract Bact Grade, VWR, #97063-430

Agar, Granulated, Powdered, Thermo Fisher Scientific, #BP1423-500

VWR® Disposable Petri Dishes, Fully Stackable, 60mm, VWR, #25384-092

Cellophane

Rubber policemen

Broad scalpel

Parafilm

Eberbach Container for Waring™ Blenders - 360mL capacity, Fisher Scientific, #14-509-25

Spectrophotometer

Invitrogen™ Microplates for Fluorescence-based Assays, 96-well, Fisher Scientific, #M33089

BioTek Cytation5 Plate Reader

Media and Solution Preparation- *Aspergillus nidulans* Defined Media plus Carrageenan

- 1 Combine:
 - 900mL nanopure water
 - 50mL 20X Sodium nitrate salts
 - 1mL Trace elements
- 2 Bring the pH to 6.5.
- 3 Add 2 grams of Carrageenan Type II (0.2% final concentration)
- 4 Autoclave for 30 minutes on liquid cycle and allow to cool to room temperature. Store at 4C.

Media and Solution Preparation- Glucose solution

- 5 In a 15 mL conical tube, add 3.2g of glucose.
- 6 Add enough nanopure water to bring volume to 10 mL and vortex to mix until completely dissolved.
- 7 In a biosafety cabinet, filter sterilize and make 1 mL aliquots.

Media and Solution Preparation- Polyphenol solutions (excluding Vanillin and Lignin)

- 8 Prepare 3 5mL tubes with 3 concentrations of each polyphenol dissolved in **autoclaved nanopure water**. Note: Concentrations (when diluted in wells): 2000ug/mL, 1000ug/mL, and 500ug/mL. **Actual concentrations of stock: 40,000 ug/mL, 20,000 ug/mL, and 10,000 ug/mL**
- 9 For inoculating two plates, dissolve **0.08g, 0.04g, and 0.02g** of each polyphenol in 2mL of **autoclaved nanopure water**. Vortex thoroughly to mix until completely dissolved.
- 10 In a biosafety cabinet, using the 0.2um disc filters, filter sterilize 1mL of each solution into a sterile 1.5mL tube.



Media Solution Preparation- Vanillin

- 11 Due to issues with solubility, the amount of vanillin used has to be scaled down. For inoculating two plates, dissolve **0.02g, 0.01g, and 0.005g** of vanillin in 2mL of **autoclaved nanopure water**. Vortex thoroughly to mix until completely dissolved.
- 12 In a biosafety cabinet, using the 0.2um disc filters, filter sterilize 1mL of each solution into a sterile 1.5mL tube.

Media and Solution Preparation- Lignin

- 13 Due to issues with solubility, the amount of lignin used has to be scaled down. For inoculating two plates, dissolve **0.02g, 0.01g, and 0.005g** in 20mL nanopure water in glass jars.
- 14 Even at these concentrations, full solubility was not achieved so filter sterilization was not possible. Autoclave on Liquid30 cycle.
- 15 In a biosafety cabinet, aliquot into 2mL tubes.

Inoculate fungal cultures on cellophane (to be done ahead of time)

- 16 Inoculate ___ 60mm MEA plates +cellophane with ___ isolates and incubate at room temperature until growth is sufficient to proceed. List isolates in your notebook.
 - Example: 24 plates with 4 isolates (6 plates per isolate)
 - Allow to grow until sufficient mycelium for inoculation (e.g., 1 week)

Prepare liquid fungal inoculum for spectrophotometer (all steps done in a biosafety cabinet)

- 17 Using a serological pipette add 20mL of *A. nidulans* defined carrageenan media to blender cup (20 mL is the minimum amount needed for blender cup to work).
- 18 Cut mycelium closely around the original inoculum to remove the agar square from the center of the cellophane. Carefully scrape the mycelium from the cellophane, without cutting into the cellophane, and gather into a ball using the broad scalpel. Pick up the mycelium ball using the fine forceps and place in blender cup.
- 19 Blend four times at one-second pulses. Check fragment sizes to ensure sufficient blending.



20 **OR**

Once isolate has colonized entire plate, pipette 1-5mL of sterile water onto plate. Use sterilized rubber policeman to gently scrape the hyphae off the surface of the media. Using a wide-bore pipette tip, collect the liquid and place a sterile blender cup.

21 Using serological pipette, add 0.2% carrageenan type II media until final volume is 20mL in blender cup. (e.g., 20mL final volume - ____mL collected from plate = ____mL 0.2% carrageenan media to add.)

22 Blend four times at one-second pulses.

23 Transfer 1000uL of blended culture (while pipette mixing) with a 5mL pipette to three spectrophotometer cuvette replicates.

24 Transfer 1000uL of *A. nidulans* defined carrageenan media from 5mL tube into cuvette (this will be the blank cuvette).

Read samples in spectrophotometer (at 600nm)

25 Place blank cuvette with just 1000uL of *A. nidulans* defined carrageenan media into reading position (with the cuvette side with the arrow closest to the screen) and press the button to blank the sample.

26 Place sample in reading position and press the "arrow passing through the cuvette" button. Record absorbance.

27 A final reading of **>0.44** is needed to reach the appropriate hyphal fragment density. If the absorbance of your sample is lower than this, harvest more hyphae from plates and repeat blending procedure in a new blender cup.

Dilute blended culture

28 When a reading is **>0.66** you need to dilute the blended inoculum with *A. nidulans* defined carrageenan media to yield **4 ml** of culture inoculum with an absorbance of 0.44. Use a 5 mL conical tube for the dilution.

Inoculate 96-well Plates (All steps to be performed in biosafety cabinet)

29 Once dissolved, add **5uL of sterilized phenol solution** to the respective wells.

- 30 Add **5uL of autoclaved nanopure water** to controls (media without phenols but with fungi).
- 31 Combine **1.8mL of blended culture at absorbance 0.66 and 12.5mL** of *A. nidulans* defined carrageenan media. Mix well by inversion to evenly distribute the hyphal fragments in the media.
- 32 Add 0.89 mL volume glucose solution to a final concentration of 2% glucose. Immediately proceed to next step so that hyphae don't settle in the media.
- 33 Inoculate 95uL of GLUCOSE CONTAINING solution for wells corresponding to the proper isolate +glucose. Also inoculate the corresponding **positive control wells. (do not inoculate wells for the media only controls in Row A).**
 - Do NOT push pipette plunger past the last stop because this will create bubbles, which will cause readings to be inaccurate. If bubbles form try to pop with a sterile pipette tip.
- 34 Place lid on the plate and parafilm for transport. Immediately measure the plate at 595, 660, and 750nm (cell density only) on plate reader.
- 35 After reading, re-parafilm plate and place in plastic bag with wet paper towels to keep plates from drying out. Be careful to keep the plate upright at all times.
- 36 Store plates in an aluminum foil lined container to keep out the light.
- 37 Read plates every 3 days for a total of 24 days (tentative, depends on isolate growth).
- 38 After 24 days, record visual observations and take picture of plates

Inoculate 96-well Plates WITHOUT Glucose (All steps to be performed in biosafety cabinet)

- 39 Once dissolved, add **5uL of sterilized phenol solution** to the respective wells.
- 40 Add **5uL of autoclaved nanopure water** to controls (colored purple; media without phenols but with fungi).



- 41 Combine **2.0mL of blended culture at absorbance 0.66** and **14.0mL** of *A. nidulans* defined carrageenan media. Mix well by inversion to evenly distribute the hyphal fragments in the media.
- 42 Immediately proceed to next step so that hyphae don't settle in the media.
- 43 Inoculate 95uL solution for wells corresponding to the proper isolate +no glucose. Also inoculate the corresponding **positive control wells. (do not inoculate wells for the media only controls in Row A).**
 - Do NOT push pipette plunger past the last stop because this will create bubbles, which will cause readings to be inaccurate. If bubbles form try to pop with a sterile pipette tip.
- 44 Place lid on the plate and parafilm for transport. Immediately measure the plate at 595, 660, and 750nm (cell density only) on plate reader.
- 45 Follow steps above (for +glucose) for storage etc.