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Isolation and Identification of Endophytic Fungi: The Uninvited and Unnoticed Guests

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Introduction

Endophytic fungi are fungi that grow in association with plants, living in the intercellular space or inside the cells without causing any disease symptoms. In fact, these organisms might exert beneficial effects to the plant host (Arnold 2003, Gimenez et al. 2007).). The workshop "endophytic fungi: the uninvited and unnoticed guests" relates to a semester long project where students carry on an individual investigation involving the isolation and identification of endophytic fungi from plant samples. In this mini-workshop techniques such as surface sterilization, preparation of wet-mount from fungal cultures, the use of keys to identify filamentous fungi based on microscopic characters were demonstrated. This project has been implemented in a mycology course with the purpose of teaching and/or reinforcing concepts related to morphology of filamentous fungi, fungal diversity and fungal ecology. Likewise, this project exposes students to scientific inquiry, and supports the development of problem solving and organizational skills. Although this project has been implemented in a mycology course, it could easily be adapted to courses such as general biology, biology of cells, and biology of organisms or microbiology.

Objectives

- Learn basic laboratory techniques for isolation of endophytic fungi.
- Observe and recognize fungal structures (hyphae, sexual and asexual spores, septa, conidiophores, etc.) useful for species identification.
- Identify fungi to the genus level through microscopic characteristics.

Student Outline
Surface Sterilization Protocol: Isolation of Endophytic Fungi

Materials and Methods

Materials:

Sterile water
70% ethanol
30% bleach
5 beakers (125 - 250 mL) or 5 sterilized petri dishes (90 mm in diameter)
Tweezers
Scissors
PDA plates with antibiotics (streptomycin)
Timer or stop watch
Permanent markers

Procedure

1. Arrange and label (do not write over the beakers, label over tape or paper, it is easy to clean after!) the beakers or Petri dishes as follows:

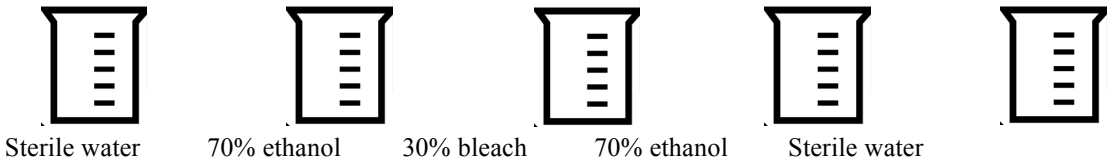

Figure 1. Beaker arrangement for the surface sterilization protocol.

2. Pour the corresponding solutions in the containers
   Before you begin, make sure to immerse your tweezers in 90% ethanol and flame them using a burner or alcohol lamp. Let your tweezers cool down.
3. Cut plant samples into small pieces (e.g. squares of 2 x 1 cm for the leaves and 2.5 cm for the stem, Fig. 2). Cut each sample in half (Fig. 2). Place one piece of each sample onto PDA medium as shown in figure 3. Label this plate as N.S.S. Plant 1 (Non surface sterilized)
4. Surface-sterilize the second pieces of plant material (step 5 – 9).

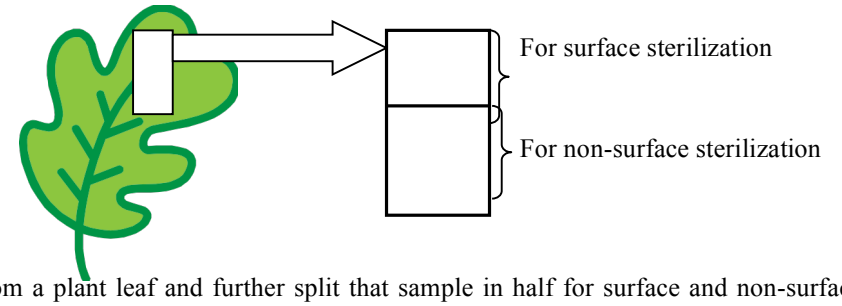

Figure 2. Cut a 2 x 1 cm piece from a plant leaf and further split that sample in half for surface and non-surface sterilization.

5. Place your plant material in the first beaker that contains sterile water (use the tweezers).
   This step is done in order to remove dust or small soil particles.
   a. Transfer your plant samples to the second beaker with 70% ethanol. Soak the samples for 1 minute.
   b. Transfer your plant samples to the third beaker with 30% bleach. Soak the samples for 2 minutes.
   c. Transfer your samples to the fourth beaker with 70% ethanol. Soak the samples for 1 minute.

6. Rinse samples in sterile water by soaking them in the water contained in the last beaker.
   a. Drain the water from the plant samples and transfer them onto the PDA medium (far apart from each other as shown in Fig. 3). Label this dish as S.S. Plant 1 (surface-sterilized plant 1). Notice that one Petri dish will have samples from one plant.
7. Seal the plates with parafilm.
8. Incubate your plates at room temperature or at 27 °C if an incubator is available.
9. Check your plates every day or every two days and look for mycelial growth. Hold your Petri dish against a light source and carefully observe the edges of the plant material. Mycelium will be visible as thing white hairs.
10. With a sterilized and cooled scalpel, cut a piece of colonized agar and transfer it onto fresh PDA medium. The medium does not need to have antibiotics at this point. Incubate this culture at 27 °C for 3 – 7 days or until spores begin to form.
11. Place your original plate with the plant material back in the incubator since other fungi might grow from the same or different plant samples.
12. At this point, develop a protocol to label your cultures so you know if fungi came from surface-sterilized or non-surface sterilized samples, from leaf or stem and from what plant source.

Creating Wet Mounts of Fungi

Filamentous fungi vary in the time and environmental conditions necessary for the production of conidiophores (spore bearing structures). The fungi available in this workshop sporulate after 4 to 6 days of incubation (27 °C). It is advisable not to wait until your cultures get old and have profusely produced spores because you will not be able to clearly see the conidiophores

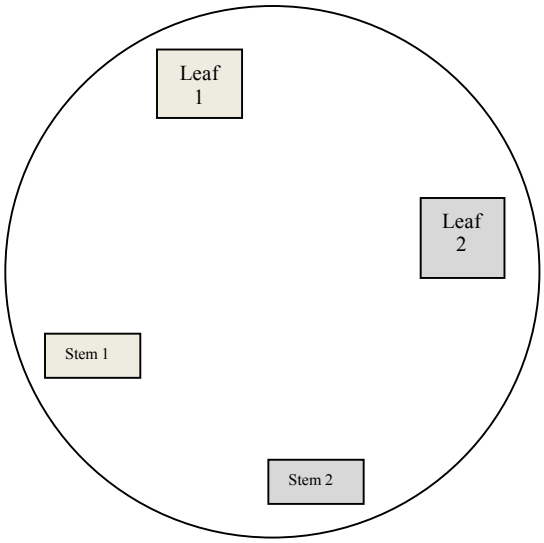

Figure 3. Petri dish with PDA and antibiotics. Leaf and stem samples from a single plant.

and how spores form from these structures. Thus, prepare your wet mounts as soon as you start observing a change in colony coloration (green, black, olive color). Changes in colony coloration often result from spore formation.

Materials:
Cover slips
Microscope slides
Dissecting needles
Cotton blue or methylene blue
Water in a dropper bottle

Procedure:
1. Use one slide for two samples by placing a drop of water on a microscope slide. Each drop should be close to the end of the slide.

2. Remove a small piece of mycelium from the edge of the colony and transfer it to one water drop. Using a clean needle, remove another small piece of mycelium from the center of the colony and transfer it to the second drop of water. This will allow you to observe different developmental stages. In the center of the colony, mature conidiophores can be observed. In the edge of the colony, developing conidiophores can be observed.
3. Using your needle, carefully place a cover slip over the drop of water. Try to avoid air bubbles.
4. Using proper microscopic techniques, observe your samples. I am proving you examples (powerpoint slides) of how conidiophores look like.
5. Some fungi will produce hyaline structures that are difficult to observe. If that is the case, place a drop of cotton blue in addition to the drop of water. Alternatively, add a drop of cotton blue on the side of the cover slip and let the stain move across and beneath the cover slip to stain the mycelium, conidiophores and spores.
6. Once you have found well defined structures, take a picture and proceed to the identification of your sample. Use the keys in "Barnet, H.L. and Hunter B.B. 1998. Illustrated General of Imperfect Fungi. 4th Edition", pages 35-39. I also recommend using the keys provided in "Watanabe T. 2002. Soil and seed fungi. Morphologies of cultured fungi and key to species".

Medium Preparation: Potato Dextrose Agar (250 mL)

1. Add 11.7 g of Potato Dextrose Agar (PDA) in a 500 mL flask.
2. Bring the volume up to 300 mL with distilled water.
3. Add a stir bar and mix (magnetic stirring plate, no heat).
4. Cap the flask with tin foil and label your flask.
5. Autoclave for 15-20 minutes at 121 °C on liquid setting (always place your flask in a deep metal tray!).
6. After the medium comes out of the autoclave, place the flask (use gloves, it is extremely hot!!!) on a stir plate and stir for couple minutes.
7. Allow the medium to cool down on the bench but do not let it solidify.
8. The streptomycin antibiotic stock is stored at – 20 °C in the freezer. Thaw it at room temperature.
9. Once the medium is colder, add the antibiotic streptomycin (1. 5 mL to the flask). The antibiotic stocks are prepared at a concentration of 10 mg/mL. By adding 1.5 mL of antibiotic in 300 mL of medium, the final concentration will be _____ µg/mL (use the formula: C1 x V1 = C2 x V2 to figure this out).
10. Cap off your flask and stir on the magnetic stir plate until all antibiotic disperses in the medium.
11. Set up the Petri dishes in stacks on the bench so you don't need to open bags while pouring. A nine centimeter diameter Petri dish holds approximately 25 mL of medium. Therefore, you will need approximately 12 dishes. The medium will solidify in less than one hour.
12. Once the medium solidified, store the plates in a bag at room temperature (two days maximum). If you need to store your plates for longer, place them in the refrigerator.

Questions

1. Why are you adding antibiotic to the media?

2. Why is the antibiotic added after autoclaving?

Notes for the Instructor

This project can be used as a semester long commitment that truly excites and engages students. Although, from personal experience, having small number of students in this lab is ideal (15 students) so the instructor can help students with structures identification. Be aware that students should also have basic and previous knowledge about general structures of filamentous fungi. So they can easily follow the keys. Supplemental literature including: Crous et al. 2009, Kirk et al. 2008, Webster and Weber 2007, can be extremely helpful since students can refer back to definitions and figures of structures.

The following is a list of commonly isolated fungi:

- *Alternaria* spp.
- *Penicillium* spp.
- *Aspergillus* spp.
- *Aspergillus niger*
- *Trichoderma* spp.
- *Verticillium* spp.
- *Fusarium* spp.
- *Mucor* spp.
- *Rhizopus* spp.
- *Nigrospora* spp.
- *Epicoccum* spp.

Thus, using the simplified key to common genera found in Barnet and Hunter (1998) 35-39 is appropriate.

Keep in mind that you can use this student project to create a culture collection from your students' isolates. Thus, you have to make sure that cultures are not contaminated. Before students proceed with the microscopic observations of their fungi, make sure that they transfer a small piece of a colonized agar plug onto fresh PDA. So, students can freely manipulate the culture and have one more growing for further storage and/or observation.

Acknowledgements

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Literature Cited

Arnold, A.E., L.C. Mejia, D. Kyllo, E.I. Rojas, Z. Maynard, N. Robbins, and E.A. Herre. 2003. Fungal Endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences*, 100:15649-15654

Barnet, H. L., and B.B. Hunter. 1998. Illustrated General of Imperfect Fungi. Fourth edition, *American Phytopathological* Society, 35-39 pages

Crous, P. W., G.J.M. Verkley, J.Z. Groenewald, and R.A. Samson. 2009. *Fungal Biodiversity*. CBS, 269 pages.

Gimenez C., R. Cabrera, M. Reina, and Gonzalez-Coloma. 2007. Fungal endophytes and their role in plant protection. *Current Organic Chemistry*, 11:707-720.

Kirk, P. M., P.F. Cannon, D.W. Minter, and J.A. Stalpers. 2008. *Dictionary of the Fungi. Tenth edition.* CABI. 771 pages

Watanabe, T. 2002. Pictorial atlas of soil and seed fungi. *Morphologies of cultured fungi and key to species. Second edition*. CRC Press. 17-34 pages

Webster, J., and R.W.S. Weber. 2007. *Introduction to Fungi. Third edition*. Cambridge, 867 pages

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