Mycology Laboratory Manual

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A Member of The Pennsylvania Alliance for Design of Open Textbooks

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About the Author

I completed my bachelor's degree at the University of Puerto Rico at Mayagüez. I then moved to the United States to pursue my Ph.D. and postdoctoral training at Penn State University, focusing on Molecular Bacteriology and Molecular Mycology respectively. I currently teach Microbiology and Mycology at Millersville University of Pennsylvania.

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About the Laboratory Manual

This Mycology Laboratory Manual is designed for undergraduate students to introduce them to mycology. The laboratory manual provides students with foundational knowledge in mycology and hands-on experience with key concepts such as fungal hyphal and yeast growth, asexual and sexual reproduction, fungal identification using morphological and molecular tools, appreciation of mushrooms as food, and molecular transformation of fungi. The final chapter focuses on plant pathology, where students are required to apply scientific thinking to design experiments, troubleshoot, and integrate the skills they developed in the previous chapters.

Throughout the book you will find learning questions at the end of each experiment and chapter. These questions are also available for download as a Word Document for easy editing: Mycology Learning Questions Word Document Auto-download Link. Please note that all learning questions are in one word document and organized by chapter.

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Mycology Preface

Mycology is the scientific study of fungi. But what exactly are fungi? The Kingdom Fungi belongs to the Domain Eukarya, along with Kingdom Plantae, Kingdom Animalia, and Kingdom Protista. Fungi include, among others, molds, mushrooms, lichens, and yeasts. Originally studied by botanists, scientists later realized that fungi are more closely related to animals than to plants, and they were therefore placed in their own Kingdom Fungi. Like animals, fungi perform respiration to convert their food sources into energy. Most fungi produce exoenzymes that degrade the substrate, and they absorb the resulting nutrients osmotrophically, though there are some exceptions.

Fungal taxonomy is complex; fungi cannot be classified solely based on morphology. Although many grow as tube-like structures, there are organisms that resemble fungi but are not, such as *Phytophthora infestans*, the causal agent of late blight and the Irish Potato Famine. Despite its tube-like growth, it belongs to Kingdom Protista. Conversely, some organisms originally classified as Protista, such as *Hyaloraphidium curvatum*, have been reclassified as fungi; this species is among the few fungi that produce a flagellum. DNA barcoding and high-throughput DNA sequencing have been widely used in fungal genomics and metagenomics, greatly aiding mycologists in defining the term fungi.

Why are fungi important? There are several reasons. Fungi serve as a source of food and are used to produce various fermented products. They produce indispensable drugs, such as antibiotics. Lichens, which are fungi in mutualistic symbiosis with algae and/or cyanobacteria, act as pioneers in many ecosystems, contributing to overall ecosystem health. Many fungi are essential for their hosts by aiding in the breakdown of their food. They are also important for plant health, as most plants on Earth engage in mutualistic relationships with fungi. Fungi are critical for decomposition, particularly of plant cellulose and hemicellulose, making them key players in nutrient cycles. Finally, molecular mycology has led to several important discoveries, such as research on *Neurospora crassa*, which was instrumental in elucidating the connection between genes and proteins.

Chapter One:
Laboratory Safety and Equipment

Objectives

After completing this exercise, you should be able to:

1. List the safety rules for the mycology laboratory.
2. Describe how each safety rule will help to protect you and your classmates from contamination or injury.
3. List the laboratory equipment in the mycology laboratory.
4. Describe how each laboratory equipment works.

Safety Rules

Introduction

The fungi that we will be using in the laboratory are generally nonpathogenic in healthy people. This does not mean that we can accept careless work habits in the mycology laboratory. We will be working with filamentous fungi, which can be opportunistic pathogens in persons with reduced resistance due to illness, poor nutrition, fatigue, mold allergies, or other conditions. We will be working with enriched cultures, where the density of fungi is extremely high. A single plate may contain trillions of spores. Handled incorrectly, such a high density of fungi could cause disease even in a healthy person. The following safety rules are intended to prevent contamination and injury of laboratory workers. These rules will be followed during every laboratory period.

1. Only materials necessary for the laboratory exercise should be brought into the laboratory. Personal items should be stored on the shelves in the laboratory before starting work with cultures, stains, and reagents.
2. Eating, drinking, smoking, vaping, chewing gum or tobacco are prohibited in the laboratory. You should avoid putting objects, such as pencils, pens, and pipets, in your mouth when working with cultures of fungi. You should avoid touching your nose, mouth, or eyes with fingers that might be contaminated with laboratory cultures.

3. You are required to wear a lab coat while working in the laboratory. Lab coats will protect your clothing for the entire semester. At the end of the semester, the lab coats will be sterilized and returned to you. Coats should not be draped over chairs.

4. You are required to wear nitrile gloves during every single lab.

5. If you are allergic to mold, you need to wear a mask while working with fungi on petri plates.

6. At the beginning of each laboratory period, disinfect the lab bench where you will be working. Spread disinfectant over the top of the bench with paper towels and allow the bench to air dry. Repeat this disinfection procedure after you complete your laboratory work to ensure any culture material deposited on the work surface is properly disinfected.

7. All culture materials (plates, tubes, etc.) must be properly labeled with your name, your section number, the date, the experiment, and any experimental treatments. Unlabeled materials will be discarded. Proper labeling will save you time when evaluating culture materials for microbial growth.

8. Spills of culture materials should be saturated with disinfectant, covered with paper towels, and reported to the laboratory instructor and students working nearby. The spill should remain covered with disinfectant for several minutes to allow the disinfectant to work. After some time, the paper towels should be picked up and discarded in the “contaminated waste” bag, and the area should be decontaminated with disinfectant.

9. Be extremely careful while working around the open flame of a Bunsen burner. Persons with long hair should pull their hair back or otherwise fix their hair so it will not get into the Bunsen burner flame. The gas for a burner should not be turned on until you have a striker ready to ignite the gas. When you are not using a Bunsen burner, turn it off. The gas should always be turned off with the valve on the bench.

10. Before you leave the lab, all materials must be put away properly. Culture materials requiring incubation must be placed in the proper incubator. Unwanted, contaminated materials must be placed in the proper containers: orange or clear “contaminated waste” bags for disposable items and containers of disinfectant for reusable glassware. Unwanted culture tubes should be placed in test tube racks in the dry sink. You must clean up your laboratory bench before you disinfect it.

11. After the laboratory period, observe good microbial hygiene by thoroughly washing your hands before leaving the laboratory.

12. No culture materials should be removed from the laboratory. All work with cultured microbes will take place in the laboratory and laboratory preparation area.

13. Report all accidents and injuries to the instructor immediately so that prompt and proper action can be taken.

Safety Contract

I, [Student Name], have read and understand the safety regulations as explained to me by the mycology laboratory instructor. I agree to abide by the laboratory regulations and understand I may be asked to leave the laboratory if I do not follow these guidelines.

[Student Signature],[Date].

Learning Questions

1. Are you allowed to eat, drink, smoke, vape, or chew gum or tobacco in the laboratory?
2. Describe the required attire while working in the laboratory.
3. What should you wear while working with fungi on petri plates if you are allergic to mold?
4. What is the protocol if there is a spill of culture materials?
5. What should you, the students, do before the laboratory starts and after finishing the laboratory experiments?
6. Can you take home culture materials?

Laboratory Equipment

Introduction

The mycology laboratory has various laboratory equipment and appliances that are indispensable for the success of our experiments. For your safety and the safety of your lab mates, it is important that you can distinguish them and know how to use them.

Biosafety Cabinets

Biosafety cabinets are enclosed spaces for maintaining sterility and preventing airborne contamination when working with biological samples. There are three different classes of hoods used depending on the pathogenicity of samples being worked on within them. Since these hoods recycle air or release it back into the room, they are incompatible for use with dangerous or volatile chemicals.

Class I: Class I is the most basic biosafety hood. It provides protection to the user and environment but not to the sample since unsterilized air from the environment is drawn into the hood and over the workspace. In Figure 1.1 we can see that the biosafety cabinet class I protects the user but not the samples. The samples are exposed to the room air; then, the air inside the biosafety cabinet is possibly contaminated with the sample, but before being released into the room again, it is purified by the biosafety cabinet to protect the user.

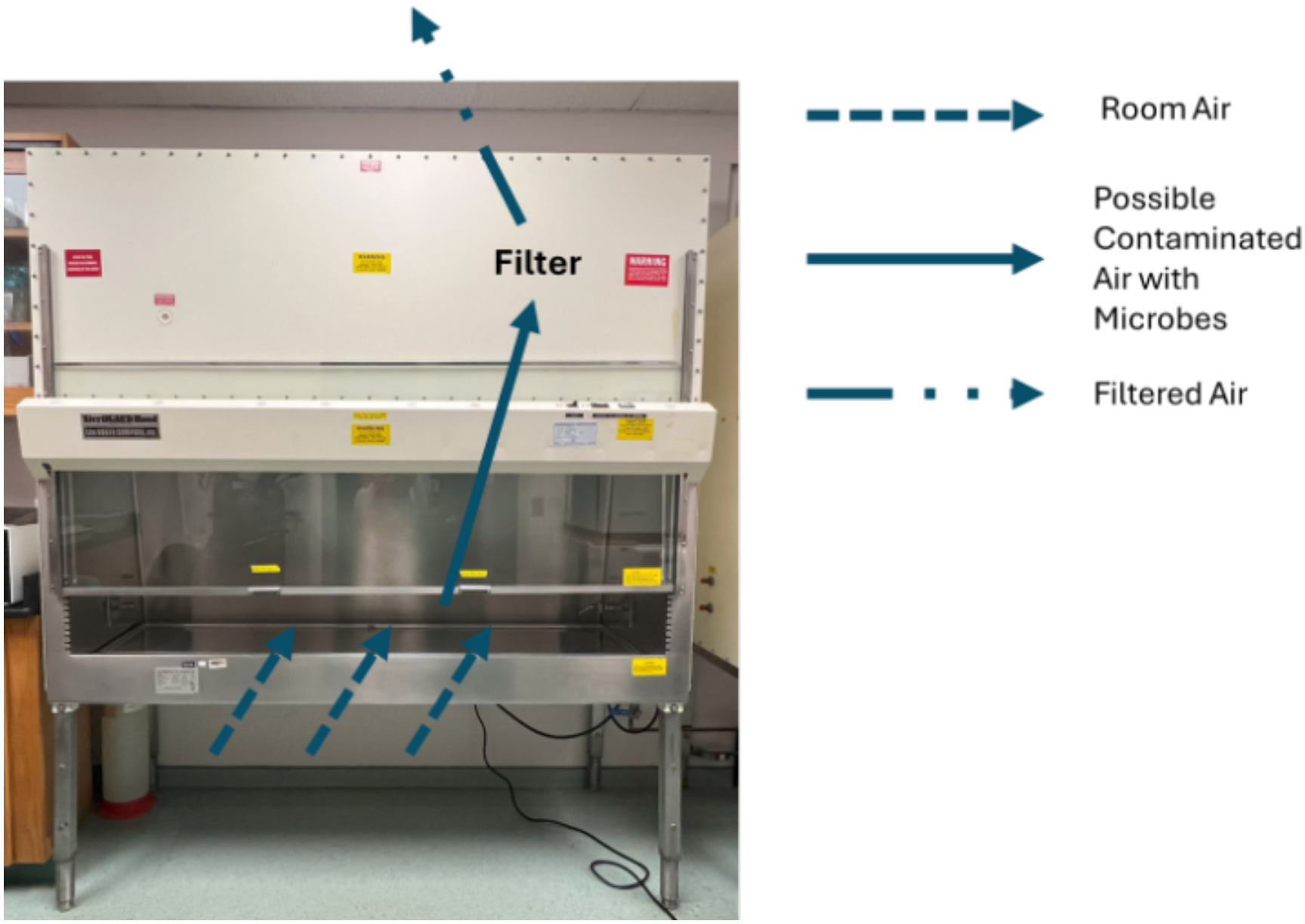


Figure 1.1 Biosafety Cabinet. Image by Author

Class II: Class II biosafety hoods provide protection to both the user and the samples. These hoods create a sterile air barrier within the workspace. Air is filtered before entering the hood and before being released back into the room. In Figure 1.2 we see that the biosafety cabinet class II protects the user and the samples. The air is filtered before it enters the biosafety cabinet and before leaves the biosafety cabinet.

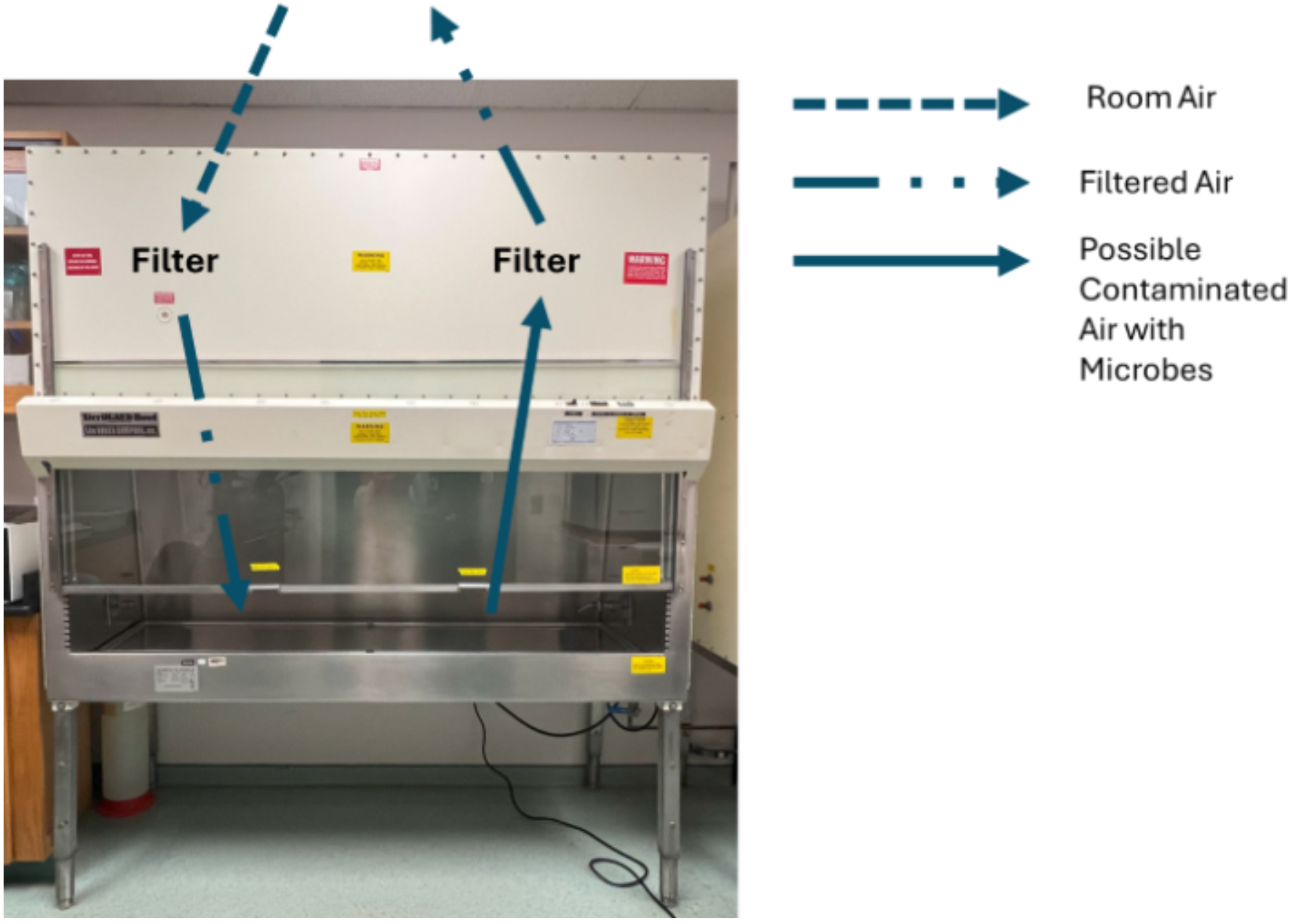


Figure 1.2 Biosafety Cabinet Class II. Image by Author

Class III: Class III hoods are completely enclosed and provide the most protection to their users (Figure 1.3). The hood's viewing window features two arm slots with attached gloves that allow the user to manipulate the sample inside. The air entering the hood is filtered once, and before it is released into the room is filtered twice.

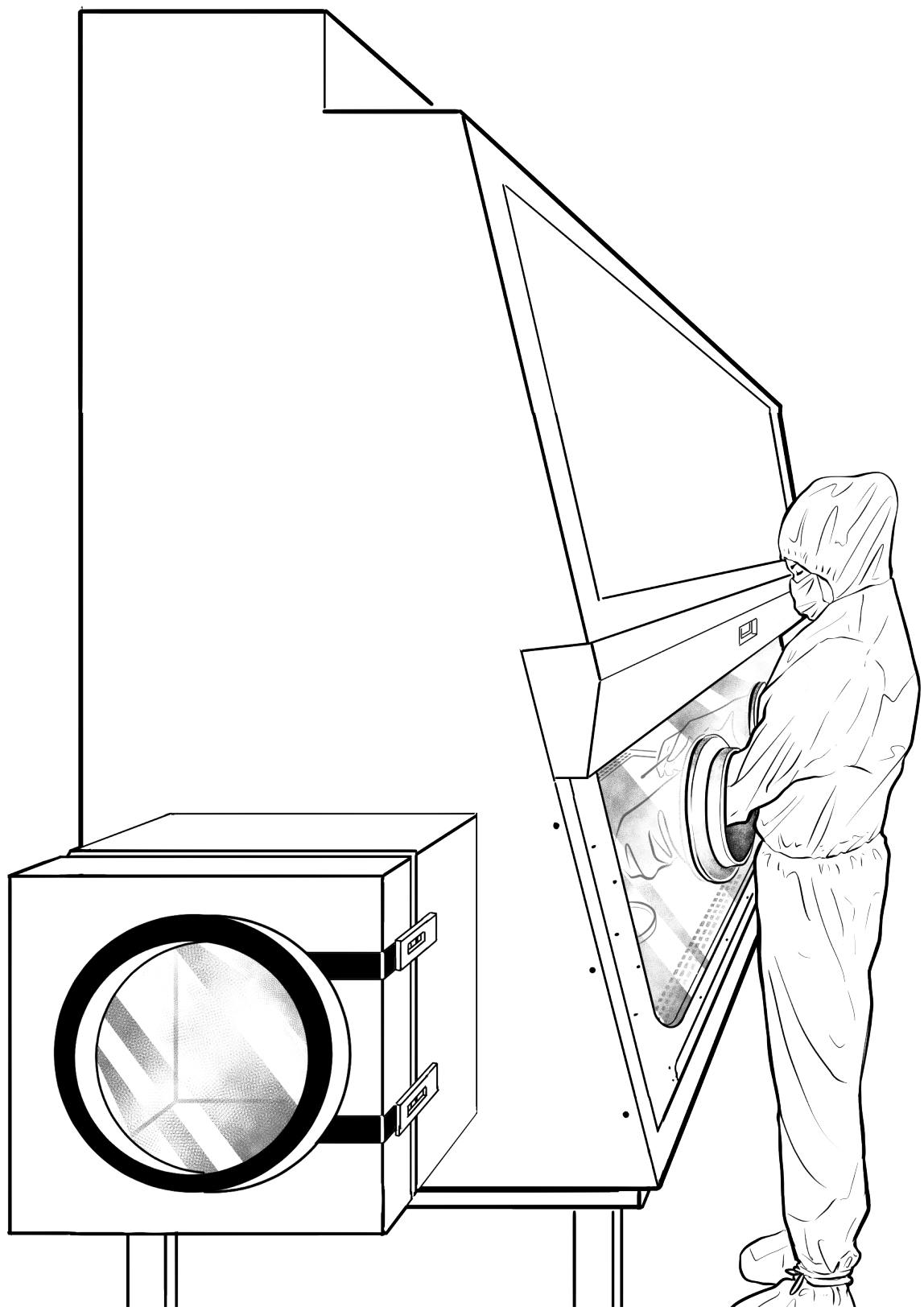


Figure 1.3 Image by Sara Ramos-Sepulveda

Laminar Flow Hood

In this type of hood, only the sample is protected. A sterile environment is created by pushing out air from the workspace toward the user (Figure 1.4). Since air is pushed toward the user, this type of hood is incompatible for work with pathogenic or infectious fungi.

Figure 1.4 Laminar Flow Hood. Image by Author

Fume Hood

A fume hood is an enclosed space that is ventilated to contain fumes, vapors, and gases. It has a sash in the front that can move up or down to allow the user access to the workspace within while keeping any gases from escaping. The sash should never be raised any higher than needed to allow the user to complete the work required. This is because there is an exhaust fan at the top of the fume hood that pulls fumes up and out of the hood. The fan then takes what it pulls up and releases it out from the top of the building. Raising the sash any higher than needed can allow for the fumes to escape. In Figure 1.5 you can see that the fume hood differently to the biosafety cabinets and laminar flow hood, it reaches the ceiling (black square); also each fume hood has a specification on how high you can push the sash up (black circle).

Figure 1.5 Fume Hood. Image by Author

Autoclave

An autoclave is a machine that sterilizes equipment and supplies so that no active microbes remain present after treatment (Figure 1.6). The only entity that autoclaves cannot destroy are prions. Treatment includes pressurized steam at a temperature of 121°C (250°F), which will melt materials like plastics or degrade materials like paper. This is why only metal and glass objects should be used in the autoclave. The treatment cycle itself takes at least 15 minutes at the desired 121°C to sterilize. Once treatment has been completed, you may reuse what was sterilized or safely throw away what needs to be disposed of.

Figure 1.6 Autoclave Image by Author

Learning Questions

1. Explain the main difference between laminar hoods and biosafety cabinets.
2. If you want to keep your laboratory materials sterile after you autoclave them:
    - Would you use a laminar hood? Why?
    - Would you use a biosafety cabinet Class I, Class II, or Class III? Explain your answer.
    - Would you use the fume hood? Why?
3. What is the best way to identify a fume hood compared to a laminar hood or a biosafety cabinet?
4. Identify the following five images and explain how each one works:

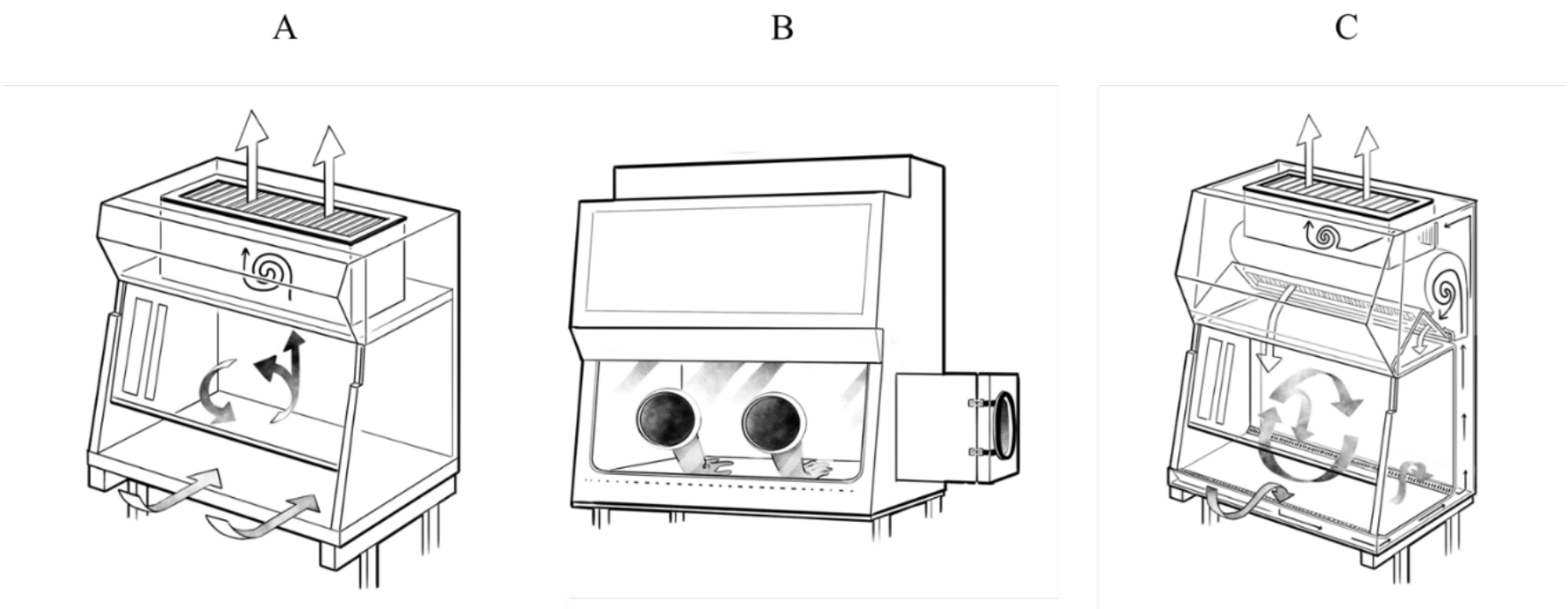


Figure 1.7 Image by Sara Ramos-Sepulveda

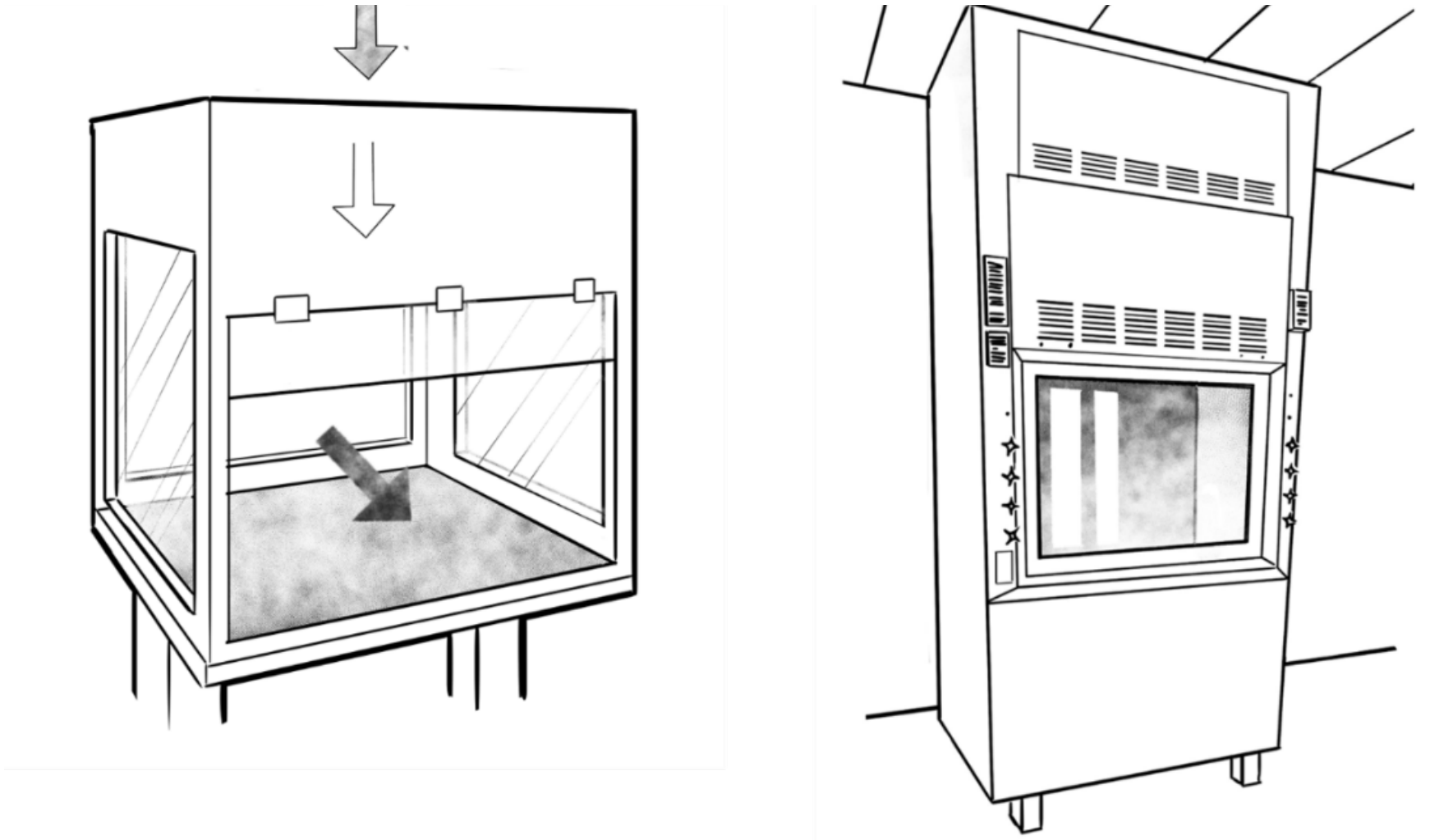


Figure 1.8 Image by Sara Ramos-Sepulveda

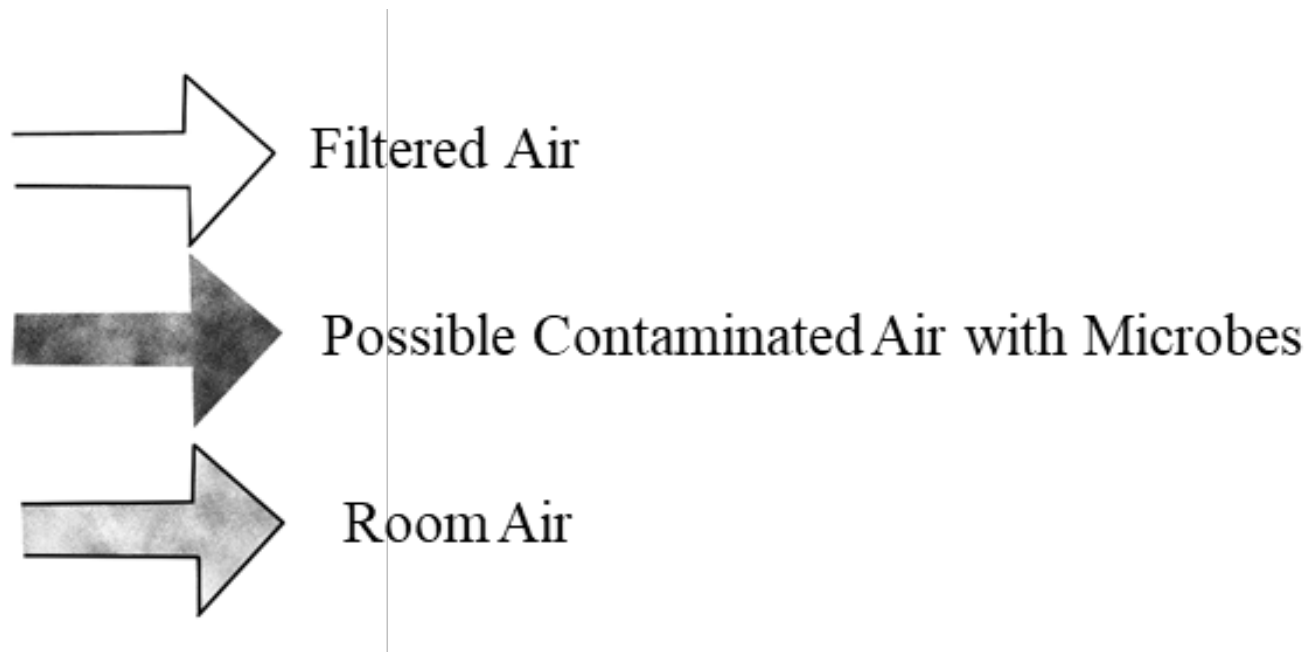


Figure 1.9 Image by Sara Ramos-Sepulveda

Chapter Two:
Fungal Growth

Objectives

After completing this exercise, you should be able to:

1. Differentiate hyphal vs. yeast colonies on Potato Dextrose Agar (PDA).

2. Prepare hyphal and yeast microscope slides.

3. Differentiate hyphal vs. yeast cells under the compound microscope.

4. Differentiate yeast cells vs. bacterial cells under the compound microscope.

Introduction

Fungi (s. fungus) can grow as **hyphae** (s. hypha), which is a multicellular growth form, or as **yeast**, which is a unicellular growth form. Some fungi can grow as both hyphae and as yeast; these fungi are called **dimorphic fungi**, and many of them are pathogenic. A classic example of dimorphic fungi (Figure 2.1) is the genus *Ophiocordyceps*, which includes many species of fungi that infect different insects.

It is important to differentiate between these two fungal growth forms on media and under the microscope. Hyphae are microscopic, tube-like structures that branch only at the tip. Under a compound microscope, hyphae resemble hairs. When grown on Potato Dextrose Agar (PDA), hyphae continue to tangle until they become visible to the naked eye; at that point, we refer to this mass as **mycelium** (pl. mycelia). Mycelia appear as fluffy filaments on PDA.

Figure 2.1 Dimorphic Fungi Image by Author

When fungi grow as yeast on PDA, the millions of cells cluster together and form a colony that is visible to the naked eye, typically appearing as a creamy substance. Yeast growth on PDA can resemble bacterial colonies, although bacterial colonies are usually shinier and less creamy. Under a compound microscope, however, yeast and bacterial cells can be distinguished: yeast cells look like small eggs, whereas bacteria appear as circles (cocci) or long rods (bacilli).

Yeast vs. Hyphal Growth on Potato Dextrose Agar (PDA)

Hyphal Growth on PDA

1. Place a PDA plate in the following locations:
    - Indoors
        - ✦ Close and away from plants
    - Outdoors
        - ✦ Close and away from plants
2. Remove the lid of the PDA plates.
3. Let them sit for at least 10 minutes.
4. Put the lid back and add parafilm around the petri plates to seal the PDA plates and avoid contamination.
5. Label the petri plates on the underside with your name, lab section, and a description of your sample.
6. Place the PDA plates in an incubator at 25°C. We are selecting for fungi that grow at room temperature, do not place the petri plates at 37 °C, you might encourage the growth of human pathogens.
7. Wait for at least three days and then check the PDA plates. **IMPORTANT**: If you suffer of mold allergies, you should wear a face mask.

8. Compare and discuss with your laboratory colleagues the growth of fungi in the different locations:
    - Indoors vs. Outdoors
        - ✦ Close vs. away from plants

Hyphal Growth on PDA – *Fungi From Home*

1. Take a PDA plate home.
2. Place a PDA plate in the location you want to test for fungal growth.
3. Remove the lid of the PDA plate.
4. Let it sit for at least 10 minutes.
5. Put the lid back and add parafilm around the petri plate to seal the PDA plate and avoid contamination.
6. Label the petri plate on the underside with your name, lab section, and a description of your sample.
7. Bring the plate back to the lab and place it in an incubator at 25°C. We are selecting for fungi that grow at room temperature, do not place the petri plates at 37 °C, you might encourage the growth of human pathogens.
8. Wait for at least three days and then check the PDA plates.
9. Determine how many fungal colonies have grown.

Yeast Growth on PDA

1. Determine a place that you would like to verify for yeast presence.
2. Take a sterilized cotton swab from the beaker following aseptic techniques learned in the laboratory.
3. Swab the surface of interest.

4. Take the swab back to the lab without touching anything else along the way and streak the PDA plate as shown in figure 2.2.

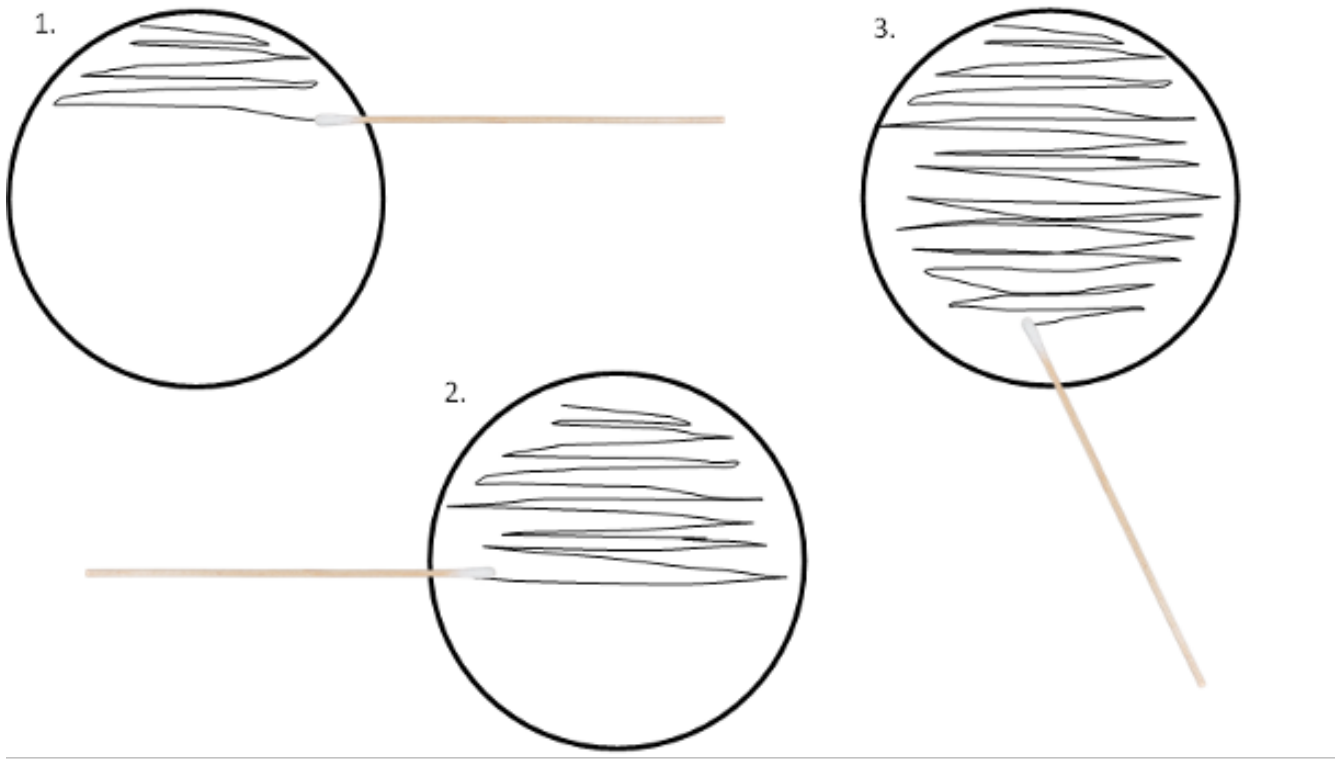


Figure 2.2 Swabbing Pattern on Petri Plate. Image by Author

5. Label the petri plate on the underside with your name, lab section, and a description of your sample.
6. Put the lid back and add parafilm around the petri plate to seal the PDA plate and avoid contamination.
7. Place the PDA plate in an incubator at 25°C.
8. Wait two days and then check the PDA plate for yeast growth. You won’t be able to distinguish bacterial colonies from yeast colonies. To determine if the creamy substances are yeast cells, you need to look at the cells under the compound microscope.

Learning Questions

1. Label A and B as hyphae or yeast growth and describe each growth on PDA:

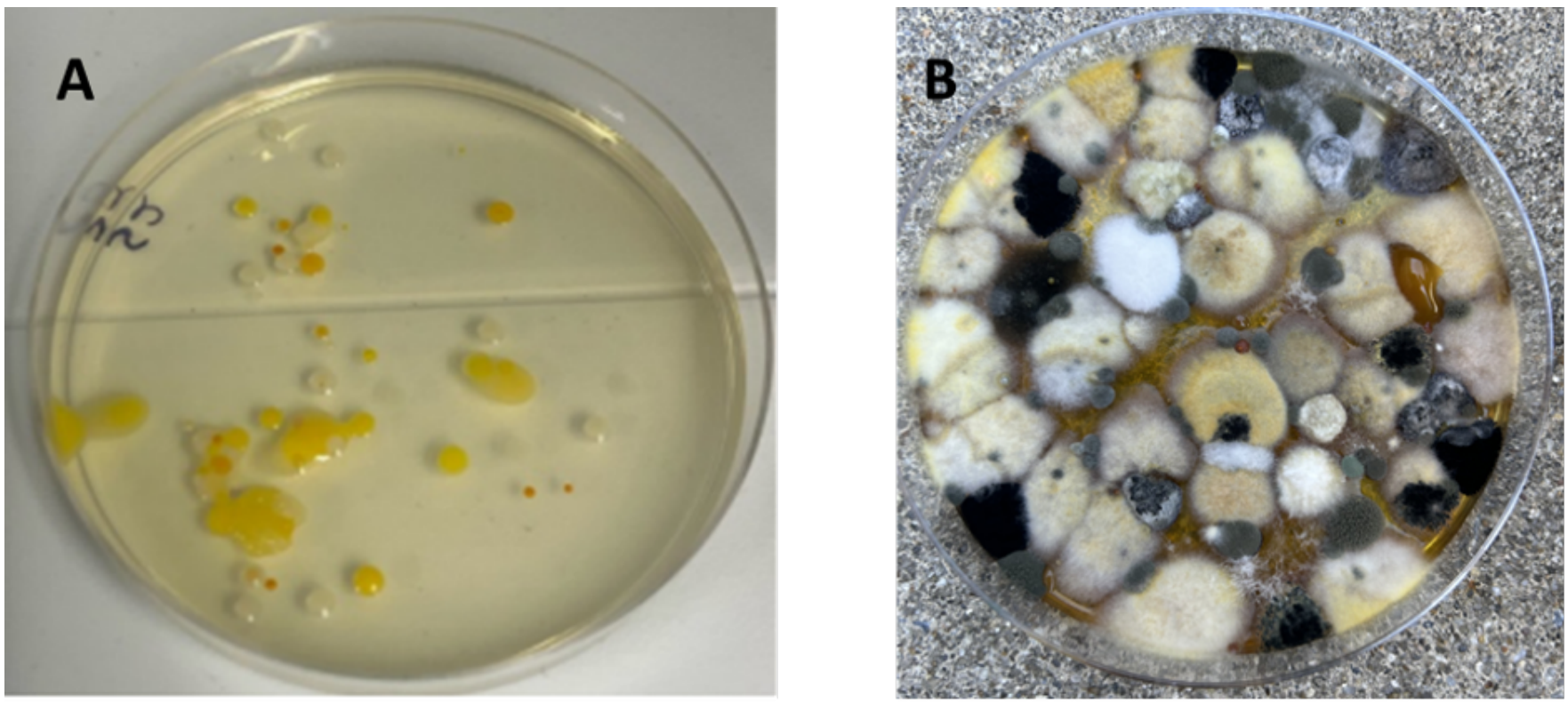


Figure 2.3 Image by Emily Herold (A) and Lila G. Smaglinski (B).

2. Identify and describe the hyphae vs. the yeast growth in the following picture:

Figure 2.4 Image by Lila G. Smaglinski.

3. How can you distinguish a yeast colony from a bacterial colony?

Yeast Vs Hyphae Cells Under the Compound Microscope

Preparing a Microscope Slide With Hyphae

1. Sterilize a loop/needle and cool it before use.
2. Prepare a glass slide with a drop of water in the center.
3. Gently scrape mycelium from the edges of the fungal colony (be conservative, you don't often need a lot of material).
4. Transfer scraped mycelium to drop of water in step 2.
5. Gently place a cover slip over the inoculated water droplet on the slide.
6. Gently tap and/or apply pressure to the cover slip, "squashing" the hyphae. Do not use your fingers.
7. Draw what you see using the compound microscope objective lens 40X. *Refer to Appendix III:Using the Compound Microscope for more information.

Preparing a Microscope Slide With Yeast

1. Attach a clothespin to one end of a sterile microscope slide. This will be used to handle the slide.
2. Place a drop of water on the slide (note: this must air-dry; do not add too much water).
3. With a sterile inoculating loop, transfer a small amount of yeast from the culture to the drop of water and gently mix over the length of the slide (note: do not add too much yeast; otherwise, you won't be able to see single cells).
4. Allow the slide to air dry for 1 minute.
5. Heat fix the yeast to the slide by passing the slide over the flame of a Bunsen burner 3 times (note: do not hold slide over flame too long or it may shatter).
6. Apply Crystal Violet reagent until the sample is covered.
7. Allow the stain to sit for 1 minute, then pour excess reagent into the basin.
8. Lightly rinse the slide with distilled water.

9. Dry slide by gently blotting on a paper towel.

10. Add a drop of oil on the slide and place a cover slip on the oil drop.

11. Draw what you see using the compound microscope objective lens 40X.
    *Refer to Appendix III:Using the Compound Microscope for more information.

Learning Questions

1. Label A and B as hyphae or yeast cells under the compound microscope. Explain your answer.

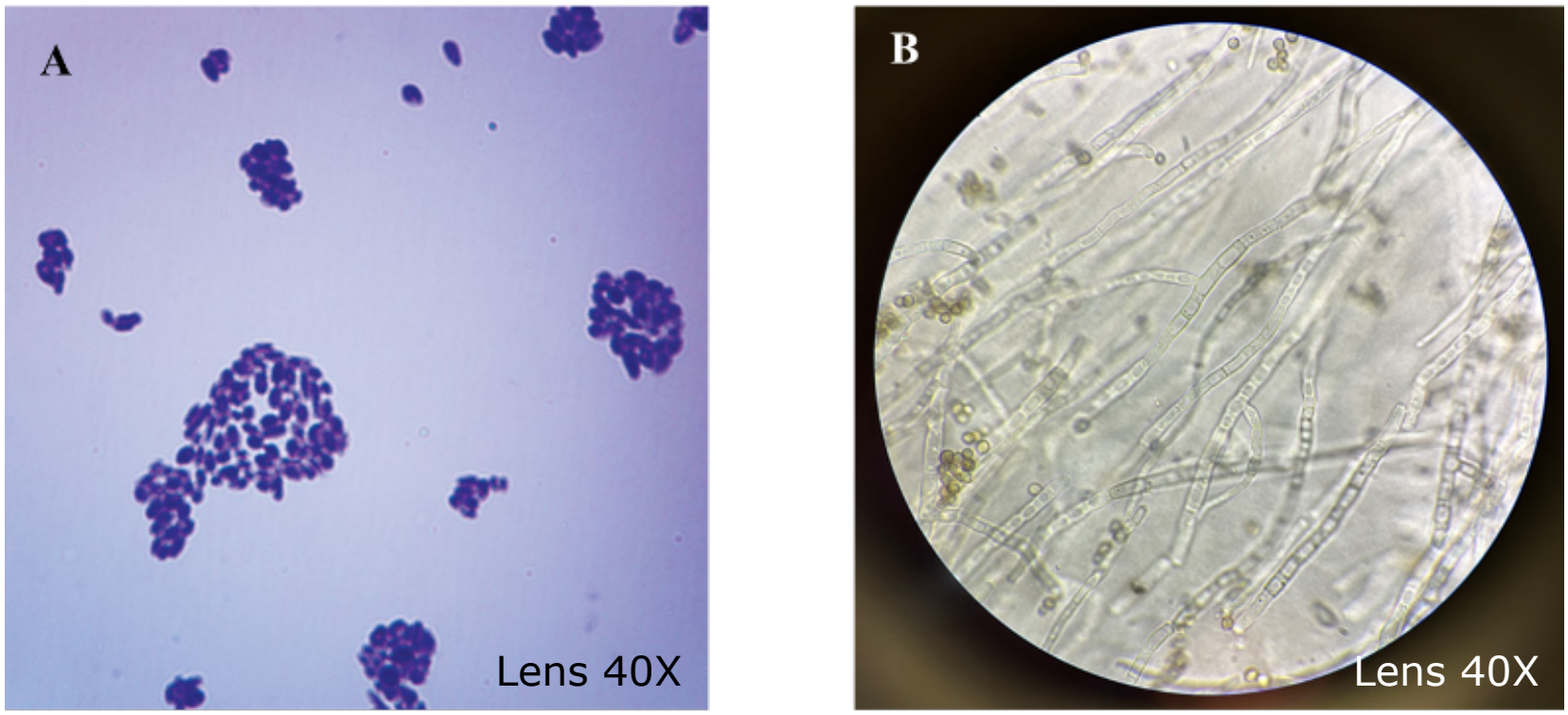


Figure 2.5 Microscope Slide. Image by Author

2. Identify the yeast vs. bacterial cells in the following pictures. Explain your answer

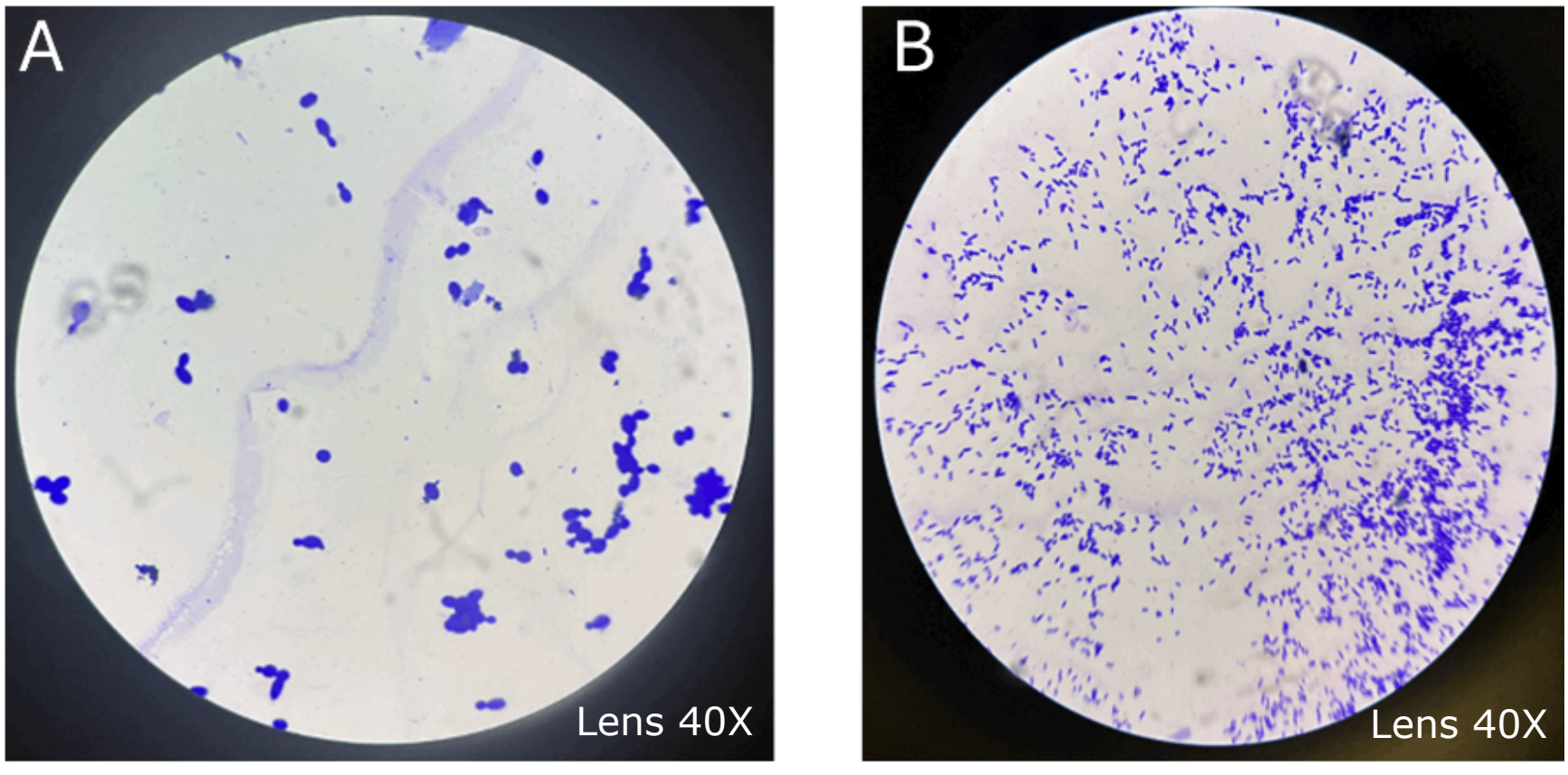


Figure 2.6 Microscope Slide Image by Author

3. Regarding the Use of the Compound Microscope:

- The objective lens that you should start with is the:
- Once you find the sample, how do you move through all the objective lenses to get to the 40X objective lens?
- What do you do if you lose your sample while using the 10X or the 40X objective lenses?

Chapter Three:
Identification of Macro Fruiting Bodies (Mushrooms)

Objectives

After completing this exercise, you should be able to:

1. Identify the main macro fruiting bodies types.

2. Identify macro fruiting bodies to at least the genus level using the field guide book called Peterson Field Guide to Mushrooms of North America.

3. Learn about and use Nature Atlas.

Introduction

Although fungi are important mycorrhizae, which are beneficial to plants, and plant pathogens, which are detrimental for plants, most fungi are **decomposers (saprotrophs)**. Fungi are the only living organisms capable of decomposing cellulose, hemicellulose, and lignin; therefore, they are the primary decomposers in forests. These different types of fungal lifestyles produce a variety of **fruiting bodies** to protect and disperse their fungal spores.

These fruiting bodies are formed from compacted hyphae. As discussed in Chapter Two, fungi can grow as hyphae, which develop as filaments.These filaments can tangle and compact to form fruiting bodies. When humans can see these fruiting bodies with the naked eye, we refer to them as **macro fruiting bodies**, commonly known as **mushrooms**. Mushrooms contain and protect the fungal spores that are produced during sexual reproduction.

It is important to be able to identify mushrooms, as mushrooms are a source of food and they have great ecological impact as mycorrhizae, plant pathogens, and saprotroph. In this chapter we will learn how to identify mushrooms based on their morphological characteristics.

Before we proceed, it is important to clarify that the following classification of mushroom is not taxonomically correct and therefore cannot be used for evolutionary studies. In the next chapter (Chapter Four), we use molecular tools such as ITS sequencing to identify fungi in a taxonomically reliable manner.

Major Morphological Groups

Gilled Mushroom (Order Agaricales)

This type of fruiting body is one of the most common in nature. The mushrooms contain a cap with gills, and the cap is attached to a stipe (stalk), although some gilled mushroom might be stalkless and directly attached to a substrate.

Figure 3.1 Image by Author

Bolete Mushroom (Order Boletales)

These types of fruiting bodies are fleshy mushrooms with tubes rather than gills. These mushrooms also contain caps, and the caps are also attached to stipes. The stipes are mostly central.

Figure 3.2 *Orden boletales,* also known as boletes, are more sponge-like, as seen on the left. "Leccinum holopus / Bolet blanc-de-neige / Ghost bolete" by Charles de Mille-Isles is licensed under CC BY 2.0.

Cantharellales Mushrooms (Order Cantharellales)

The underside of the mushroom is veined or ridged from the stipe all the way to the cap. The cap is smooth and trumpet shaped. These fungi don’t have true gills.

Figure 3.3 Cantharellales, with its trumpet-like shape, lacks true gills. "Cantharellus cibarius, Fries 1821 (Fungi Basidiomycota Agaricomycetes Cantharellales Cantharellaceæ)" by Elena Regina is marked with Public Domain Mark 1.0.

Coral Mushroom

These are fleshy mushrooms with simple or multi-branched ends, typically from a common base. The spores are produced on the branch surfaces.

Figure 3.4 Image by Author

Teeth Mushroom

These fungi have fruiting bodies with various shape types, with a lower surface composed of tooth-like projections.

Figure 3.5 With its tooth-like projections, the hydnoid fungi vary when it comes to fruiting body shapes. The photo on the left of "Antrodiella zonata" by Bernard Spragg is marked with Public Domain Mark 1.0.

Polypores (Conks or Bracket Fungi)

These fungi are either leathery conks or brackets. They are typically perennial; some are annual or fleshy. Less commonly are they gill-like or tooth-like. Usually lignicolous.

Figure 3.6 Polypores, also known as conks or Bracket Fungi, usually grow on the surfaces of trees. "Polypores" by Nicholas_T is licensed under CC BY 2.0.

Jelly Fungi

These mushrooms have a gelatinous texture, but when dried, they don’t look gelatinous

Figure 3.7 "Jelly Fungi" by Bernard Spragg is marked with CC0 1.0.

Puffballs, Earthstars, and Stalked Puffballs

These mushrooms range from spherical to pear-shaped. The outer layer of some are split into star-like rays. Occasionally stalked. Firm when young, powdery in age from maturing spores. Spores are dispersed via pore present in these fruiting bodies.

Figure 3.8. Image A: "Puffball fungi next to daisies, N. Idaho Selkirk Mountains" by Jrtayloriv is licensed under CC BY-SA 2.0. Image B: "Earthstars” by Bernard Spragg is marked with Public Domain Mark 1.0. Image C: "_TBM8369" by antoniseb is licensed under CC BY 2.0.

Bird’s Nest Fungi

These fungi are very small. They have a cuplike structure with peridioles inside. Rain drops cause peridioles to be pushed out to spread spores.

Figure 3.9 "Bird nest fungi." by Bernard Spragg is marked with Public Domain Mark 1.0

Stinkhorns

Mushroom consisting of a stalk and a slimy, malodorous (unpleasant-smelling) fertile head or a latticework basket. The surface is usually coated with viscous liquid. Arises from an “egg”.

Figure 3.10 Stinkhorns stink. In some cases, they have a liquid that coats the surface. "Stinkhorn Fungus” by Bernard Spragg is marked with CC0 1.0.

Crust Fungi

Crust fungi are characterized by relatively simple, resupinate (upside-down) or slightly effused-reflexed (on a vertical surface and partially resupinate and partially pileate), crust-like fruiting bodies. They are often found on the undersides of dead branches or logs. The surface of crusts may be smooth, wrinkled, folded-labyrinthine, poroid, or spinose.

Figure 3.11 Fungi on the surface. "Cobalt Crust Fungi?" by dave-pemcoastphotos.com is licensed under CC BY 2.0

Earthball

Earthball, or False Truffles, are rounded, potato-like or irregularly shaped mushrooms. They develop underground and are typically firm. Gleba at maturity and not powdery.

Figure 3.12 "Earthballs" by pellaea is licensed under CC BY 2.0.

Smut Fungi

This pathogenic fungus infects the corn crop. It is delicacy in Mexico, they would purposely inoculate *Ustilago maydis* on corn.

Figure 3.13 "huitlacoche" by Wendell Smith is licensed under CC BY 2.0.

Rust Fungi

This is a pathogenic fungi that infects various crops, each fungal species has it own specific host.

Figure 3.14 "Blackberry Leaf Rust Fungus" by John Tann is licensed under CC BY 2.0.

Cup Fungi

Mushroom with disc-shaped to a cupulate shape. Stipe short or absent.

Figure 3.15 "Cup Fungi" by gailhampshire is licensed under CC BY 2.0.

Earth Tongues

Mushroom that comes in various shapes but is frequently small, stipitate, with fertile, rounded, clavate, arrow-shaped or flattened head. Occasionally, the fertile head and sterile stalk are not distinct.

Figure 3.16 "Orange Earth Tongue" by pellaea is licensed under CC BY 2.0.

True Morels and False Morels

These fungi have a saddle-like shape and range in color from yellow to grey or black. To distinguish true morels from false morels, cut the mushroom open: if it is completely hollow inside, it is a true morel; if the inside is stuffed, it is a false morel.

Figure 3.17 "Gyromitra tasmanica False morel “ by Bernard Spragg is marked with CC0 1.0.

Learning Questions

1. Identify the following macro fruiting bodies using the names on the table:

- Bird’s nest fungi
- Boletes
- Cantharellales
- Coral
- Crust fungi
- Cup fungi
- Earth Ball
- Earth Tongues fungi
- False Morel
- Jelly fungi
- Gill mushrooms
- Polypores
- Puffball
- Rust fungi
- Smut fungi

- Stinkhorns
- Toothed

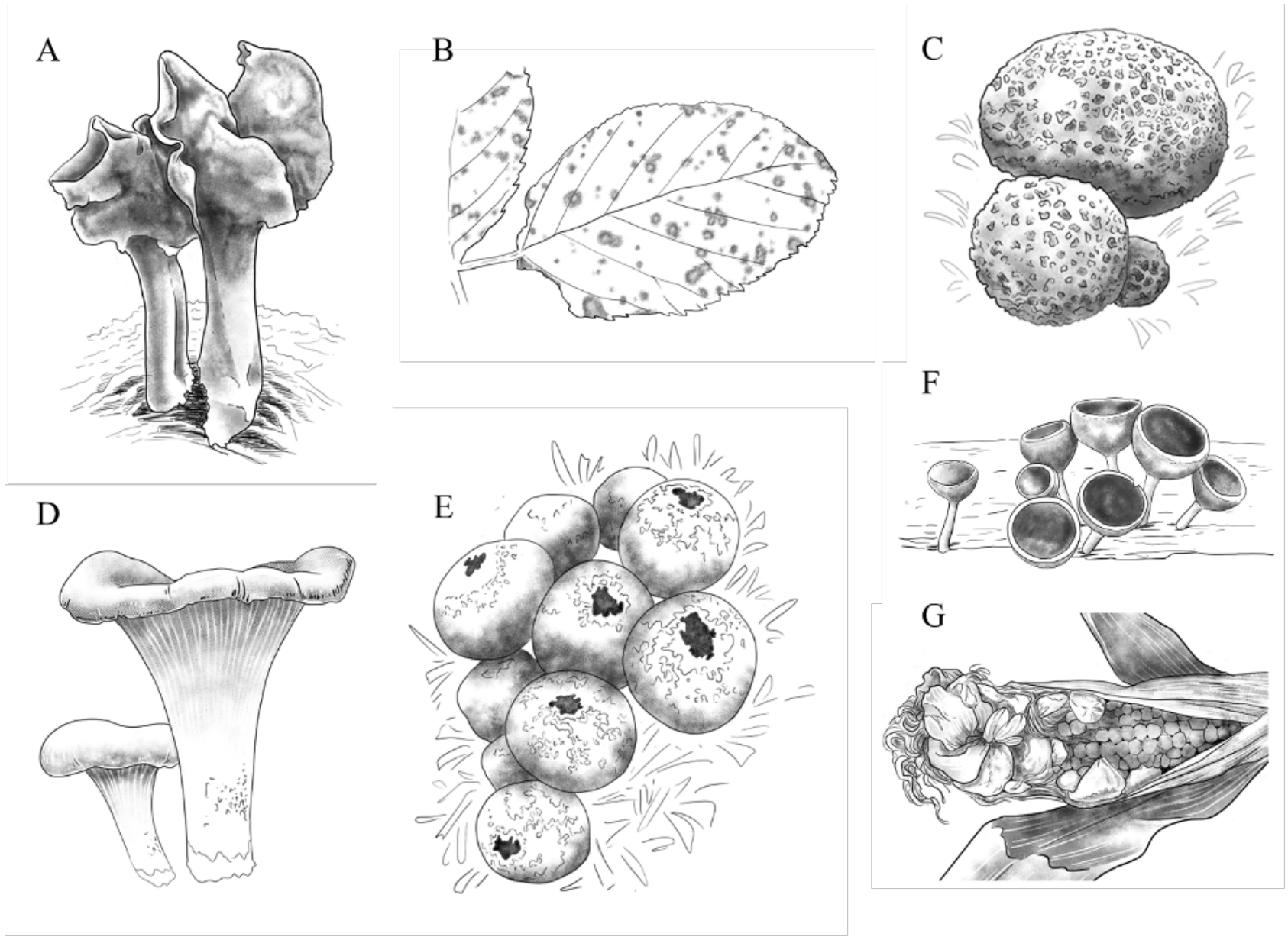


Figure 3.18 Image by Sara Ramos-Sepulveda

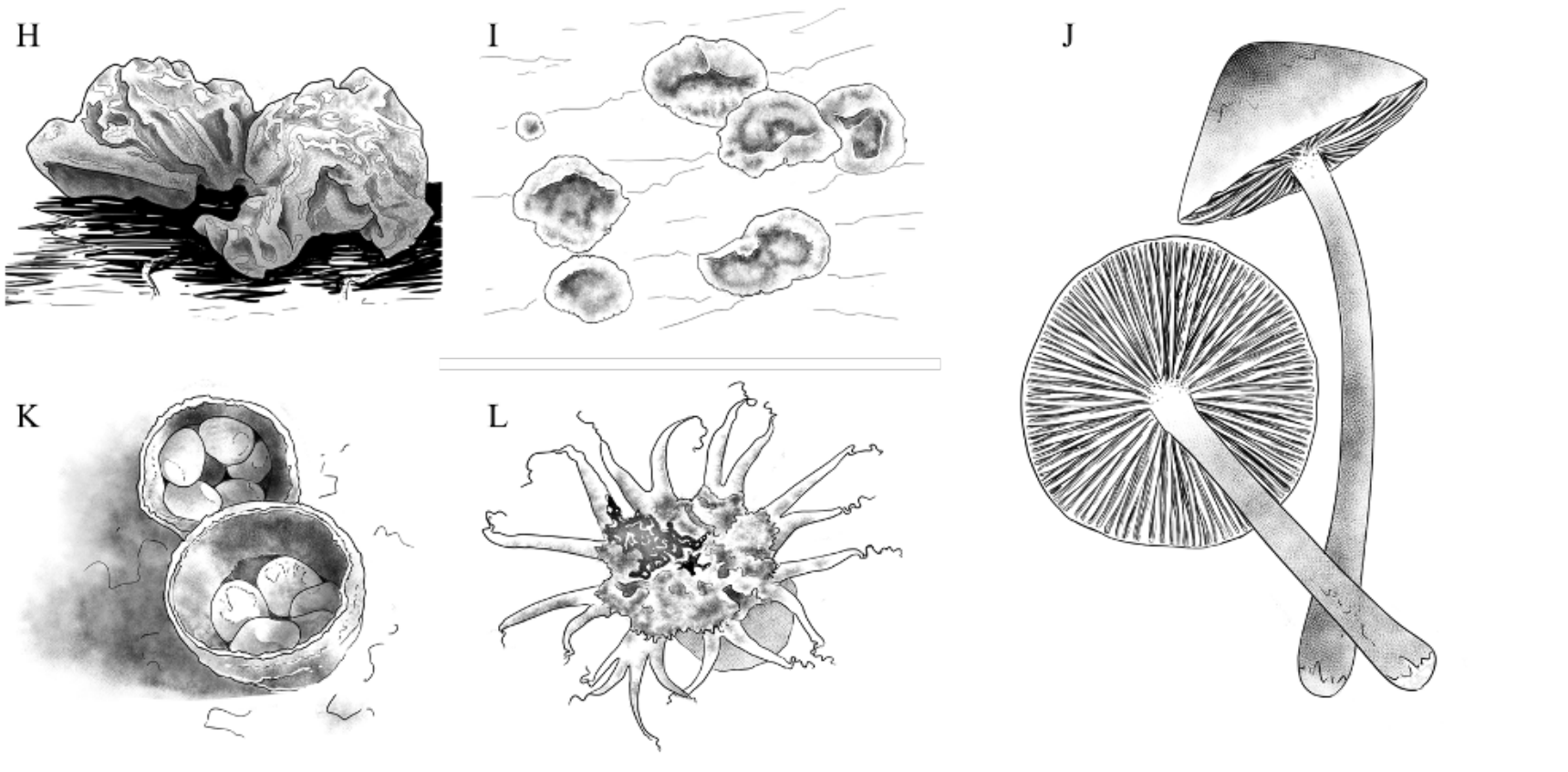


Figure 3.19 Image by Sara Ramos-Sepulveda

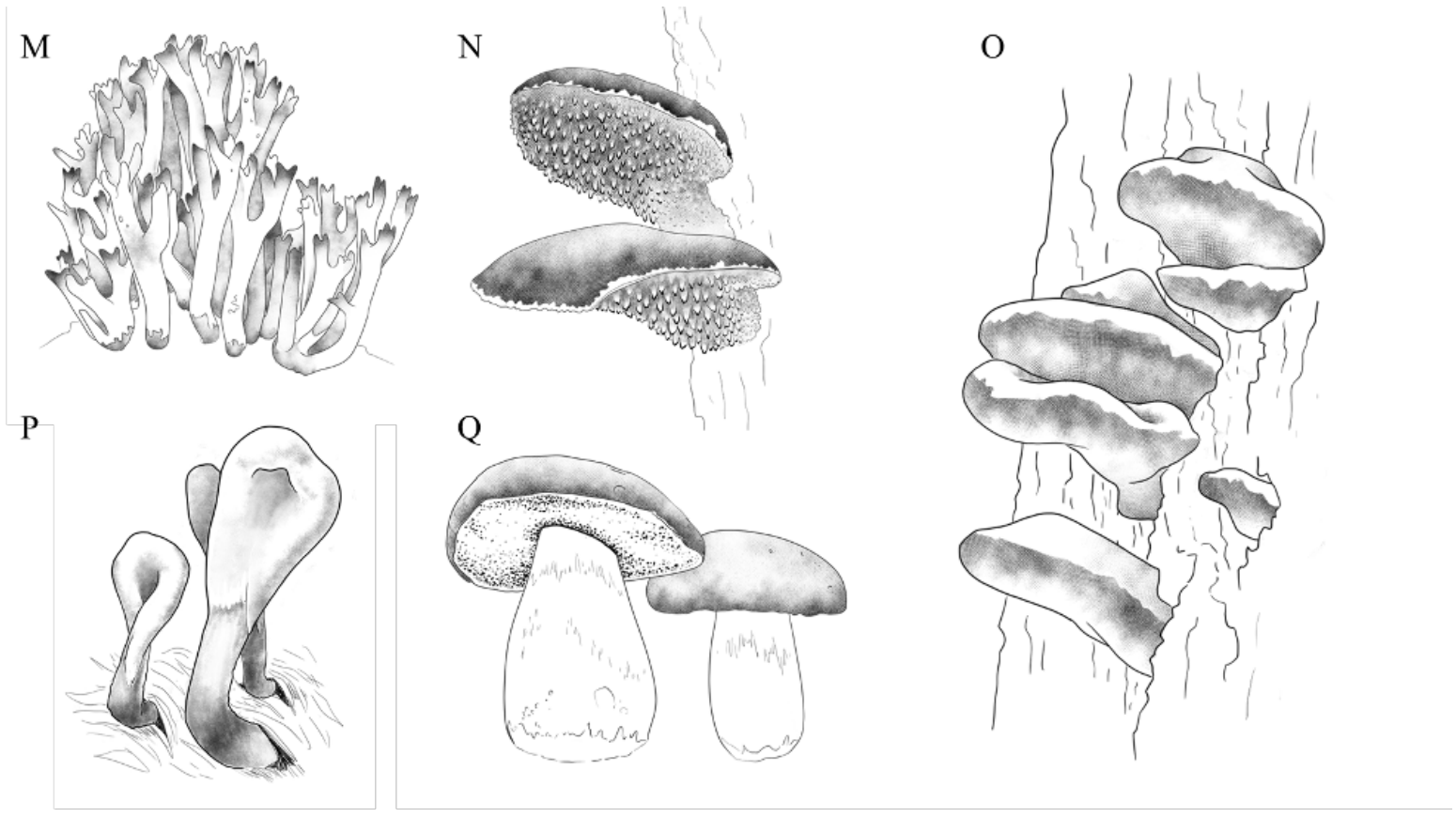


Figure 3.20 Image by Sara Ramos-Sepulveda

Collecting and Identifying Mushrooms

We will be collecting mushrooms from different ecosystems around us and identifying them to at least the genus level using the Peterson Field Guide to Mushrooms of North America by Karl B. McKnight, Joseph R. Rohrer, Kirsten McKnight Ward, and Kent H. McKnight.

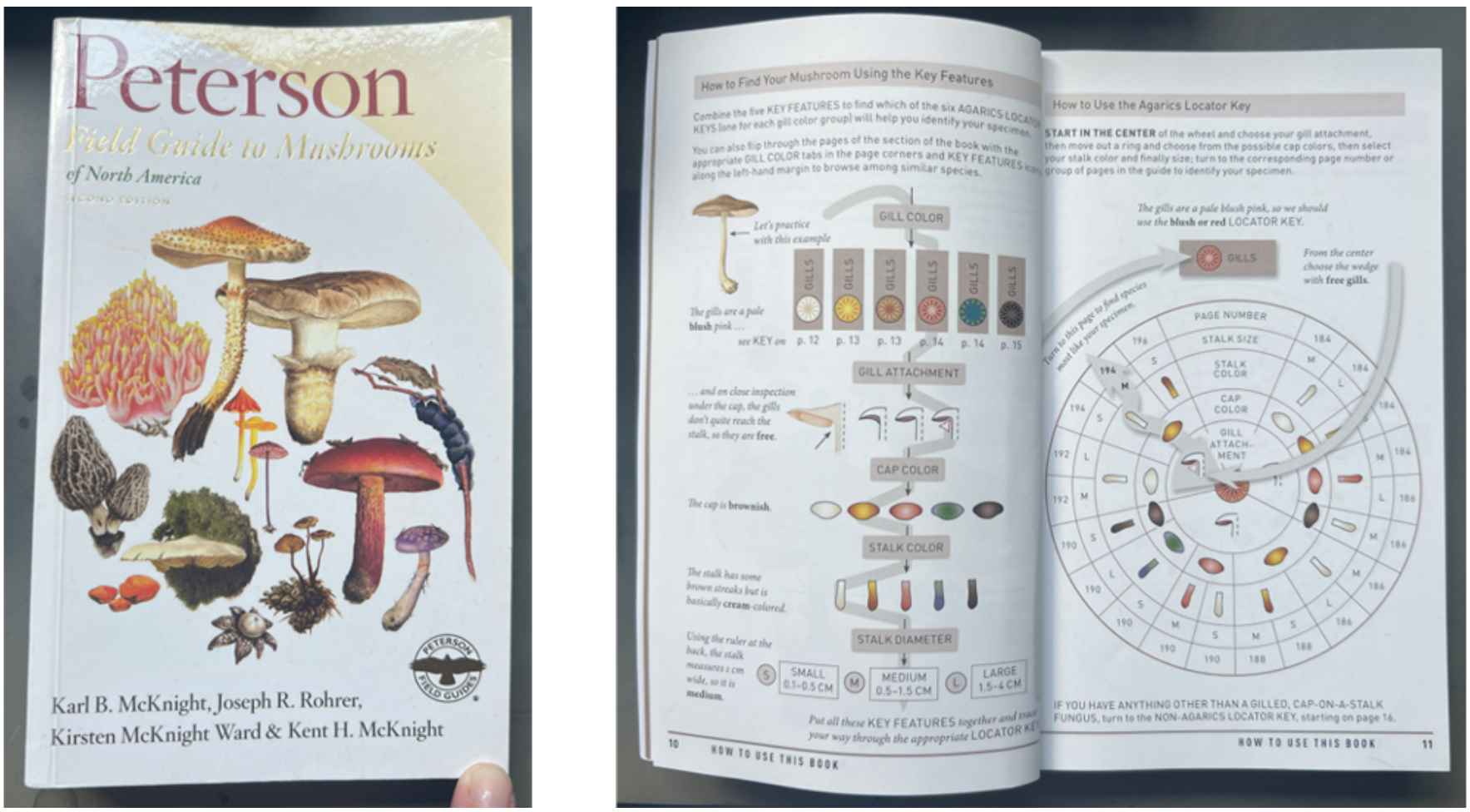


Figure 3.21 Image by Author

You might find edible mushrooms during our mushroom hunt, but it is very **IMPORTANT** that we do **NOT** consume any. We are not experts, and even experts cans sometimes get confused.

Here are some suggestions to consider when mushroom hunting:

1. Bring a big basket and multiple paper bags.

2. Look for mature mushrooms. If the mushrooms are not fully developed, it might be challenging to identify them. However, collecting young specimens is also helpful so that you can observe their development stages, which aids in identification.

3. Take a photo of the mushroom and its surroundings before collecting it. Be sure to photograph the cap, the underside of the cap, the stipe (stalk), and any other tissue below ground.

4. Ask yourself: Is it growing alone? In a group? What is the surrounding ecosystem?

5. Collect the whole mushroom using a mushroom collecting knife; do not pull it out by hand.

6. When sharing mushrooms, ensure that each student group has representative specimens.

7. For polypores, if they are not fleshy, they may be difficult to identify

8. Crust fungi are particularly challenging to identify, so pay close attention to the substrate (the material the fungus is growing on), as this can help with identification.

Some Terminology

Once you feel comfortable distinguishing the different types of mushroom (by completing the previous learning questions), you can start using the Peterson Field Guide to Mushrooms of North America. Although the field guide includes most of the terminology necessary to identify various mushrooms, we will introduce some additional terms to facilitate its use. We will also focus on gilled mushrooms, as they are the most common type found in the wild.

You might encounter underdeveloped mushrooms in the wild that resemble an egg. This is because they are still enclosed by the **universal veil**, a membrane that covers the entire mushroom in early stages. As the mushroom grows, the universal veil breaks, and its remnants may appear as **scales on the cap**. Some mushrooms also have a **volva**, which is another remnant of the universal veil, often located at the base of the stalk. It is important not to pull the mushroom out, as the volva might remain underground and could be missed, compromising proper identification.

Underdeveloped mushrooms also have a **partial veil** that protects the gills, where the spores are produced. As the mushroom matures, the partial veil breaks, and sometimes it remains attached to the stalk, forming the **annulus (ring)** (Figure 3.22).

For more in depth information about gilled mushroom morphology, refer to Chapter Five: Fungal Sexual vs. Asexual Reproduction.

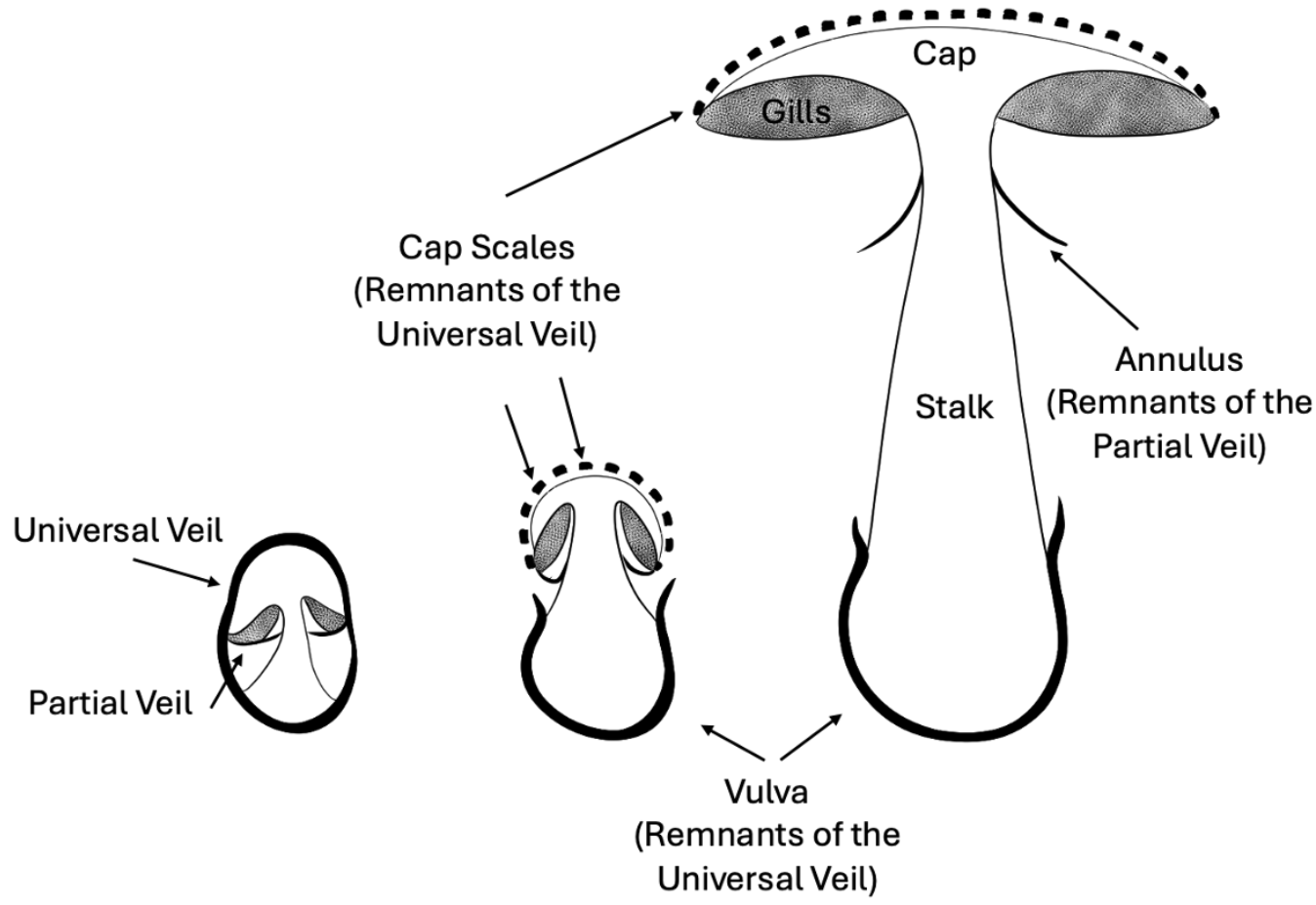


Figure 3.22 Image by Sara Ramos-Sepulveda is licensed under CC BY-SA 3.0. Adapted from "03 02 06 hemiangiocarpic fruiting body, Agaricales Basidiomycota (M. Piepenbring)" by M. Piepenbring is licensed under CC BY-SA 3.0.

Gill Attachment

The gills can be attached to the stalk in different ways, and this characteristic can be used to help identify mushrooms. The Peterson Field Guide to Mushrooms of North America describes four types of gill attachment, as shown in Figure 3.23.

- **Free gills** are characterized by a small gap separating the gills from the stalk.
- **Attached gills** connect directly to the stalk at their end:
  - **Adnexed**: gills simply attached to the stalk.
  - **Sinuate**: Some attached gills have a notch right where the stalk meets the gills; these are also called notched attached gills.
  - **Decurrent**: gills trail and taper down along the stalk.

To follow the identification wheel depicted in Figure 3.21, you must first identify the type of gill attachment, followed by the color of the cap, the color of the stalk, and finally, the size of the stalk.

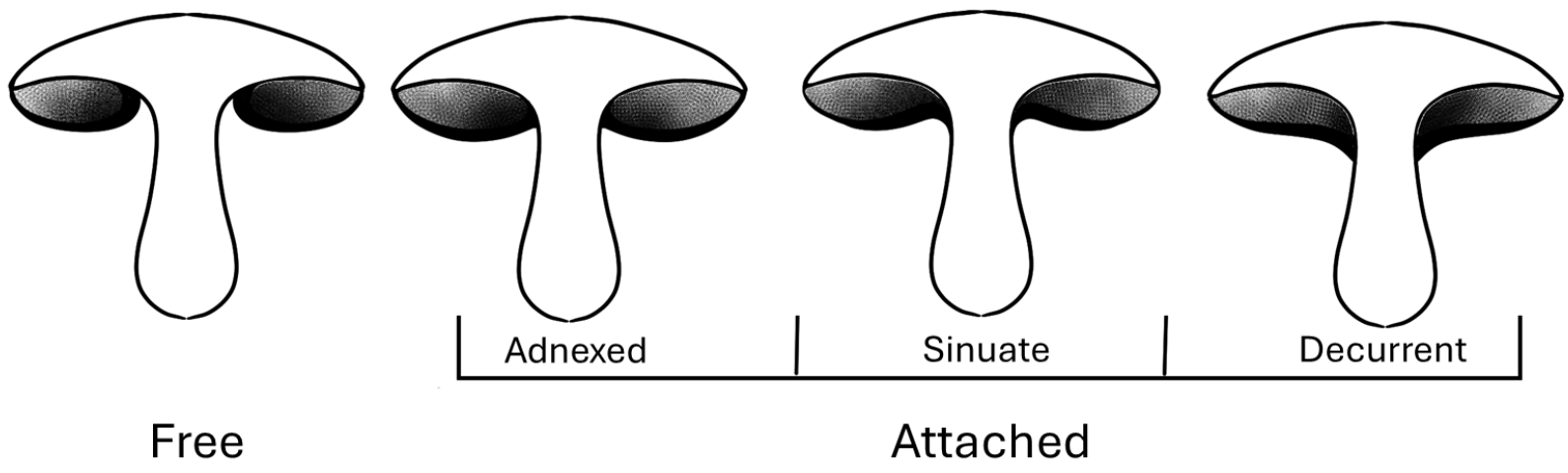


Figure 3.23 Image by Sara Ramos-Sepulveda. Modified by Author.

Further Testing

You may need to perform additional tests to determine the genus of the mushrooms you collect. For example, some gilled mushrooms exude a white, milky liquid (Figure 3.1), which suggests the genus is most likely *Lactarius*. You might also need to add certain chemicals to observe color reactions, perform a spore print(Figure 3.24A), or examine the fungal spores under a microscope. (Figure 3.24B). These additional tests are described in the field guide.

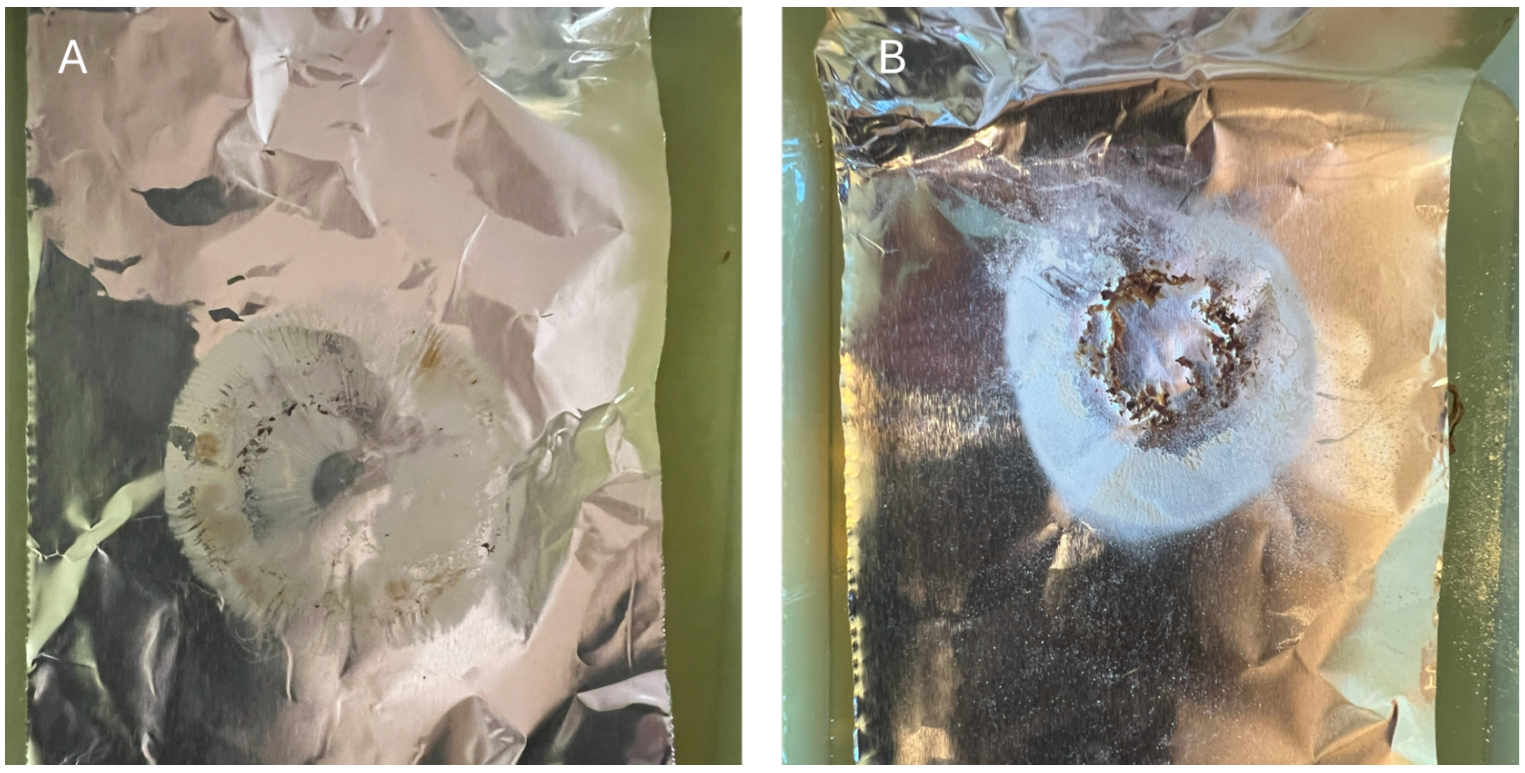


Figure 3.24 Image by Author

Learning Questions

Using the Peterson Field Guide to Mushrooms of North America, identify the following mushroom: Explain how did you use the book to identify the mushroom. The three photos are of the same mushroom from different angles. The stalk is 1cm long and the spore spring was dark purple-brown.

Figure 3.25 Images by Madison Ferster

Note For Instructors:

My students are required to enter their mushroom hunting findings into Nature Atlas. Beyond the experience and techniques they gain during the identification project, this is a great opportunity for them to contribute to a database that tracks ecosystems in Pennsylvania and around the world.

In Nature Atlas, I ask students to include specific information, including the ecological importance of the mushroom they find. I have provided a link to a video tutorial on how to enter data into the database. The video, titled "Tutorial 03 Entering Records" was created by Dr. Christopher Hardy, who is one of the creator of the database (Read the tutorial video transcript).

Chapter Four:
Identification of Filamentous Fungi

Objectives

After completing this exercise, you should be able to:

1. Collect fungal spores from the environment.
2. Prepare pure cultures of fungi on PDA media.
3. Isolate genomic DNA (gDNA) from fungi.
4. Analyze isolated gDNA using the nanodrop.
5. Explain how the Polymerase Chain Reaction (PCR) works and how it can be used to amplify the fungal ITS region.
6. Run the PCR product on an agarose gel and confirm correct size.
7. Analyze the ITS region sequence using NCBI – Blast.

Introduction

Identification of fungi using morphological and biochemical tests has proven to be quite challenging as fungi can grow as hyphae, as yeast, or both (dimorphic fungi). Fungi can also reproduce sexually, asexually, and some fungi can do both. We will discuss these two types of reproductions in the next chapter (Chapter Five).

Molecular identification through DNA barcoding has greatly improved the identification of fungi. The DNA barcode is found in the ribosomal DNA (rDNA), which codes for the ribosome. This is an ideal sequence because all fungi have ribosomes. Within the rDNA, the **internal transcribed spacer (ITS)** regions 1 and 2 are not transcribed or translated into proteins, making them prone to mutations. These two regions act as **fingerprints or barcodes** for fungal species, as each species has its unique nucleotide sequence. By using this barcoding method, scientists can conduct evolutionary studies based on taxonomic classification.

Beyond DNA barcoding, high-throughput DNA sequencing has been widely used in fungal genomics and metagenomics, greatly aiding mycologists in developing

more robust fungal phylogenetic trees. In this chapter, we will not discuss high-throughput DNA sequencing; instead, we will focus on PCR-amplified marker genes (DNA barcodes).

Steps to Complete Identification

Collecting Fungal Spores From the Environment

1. Take a Potato Dextrose Agar (PDA) plate and place it anywhere around your building or outside. In Chapter Two, you noticed the difference between indoor and outdoor and close vs far away from plants. Choose the place of your interest.

2. Remove the lid.

3. Let it sit open for 10 minutes to allow fungal spores to settle on the media.

4. Close the plate and head back to the lab.

5. Label the plate on the button side with:

    - Name
    - Date
    - Group #
    - Description for the sample

6. Place parafilm around the plate to seal it.

7. Place the petri plate in the incubator at room temperature (~25ºC).

Fungal Pure Culture on PDA

1. Most likely, you will have more than one fungal colony growing on your PDA. As you can see in the photo below (figure 4.1), the student has four different fungal colonies, labeled one through four.

Figure 4.1 Image by Author

2. Surface disinfect your working area with 70% ethanol.

3. *Refer to Appendix II:Obtaining Single Fungal Cultures and Measuring Fungal Growth for more information

- Sterilize the cork borer by submerging it in 70% ethanol and holding it in the flame for at least 10 seconds. Make sure you also sterilize the inner piece. An alternative is to autoclave all the cork borers with their inner piece to sterilize them.

- Allow the cork borer to cool for 15 seconds; it will be cool enough when it no longer sizzles the agar on the plate.

- Pull the inner piece of the cork borer and, using only the cork borer, cut a section of the mycelium you wish to propagate on the edge. The cut section can have some exposed agar.

- Using the inner piece, place the section of mycelium in the center of a fresh agar plate, making sure to place the mycelium side-down (the mycelia should be in contact with the media).
- Wait at least 24 hours; then flip the plate so that the agar is on the top.
- Ensure that you only have one fungal colony growing on your PDA.

4. Measure the fungal growth every day, marking the mycelial growth with a marker on the underside of the petri plate (Figure 4.2):

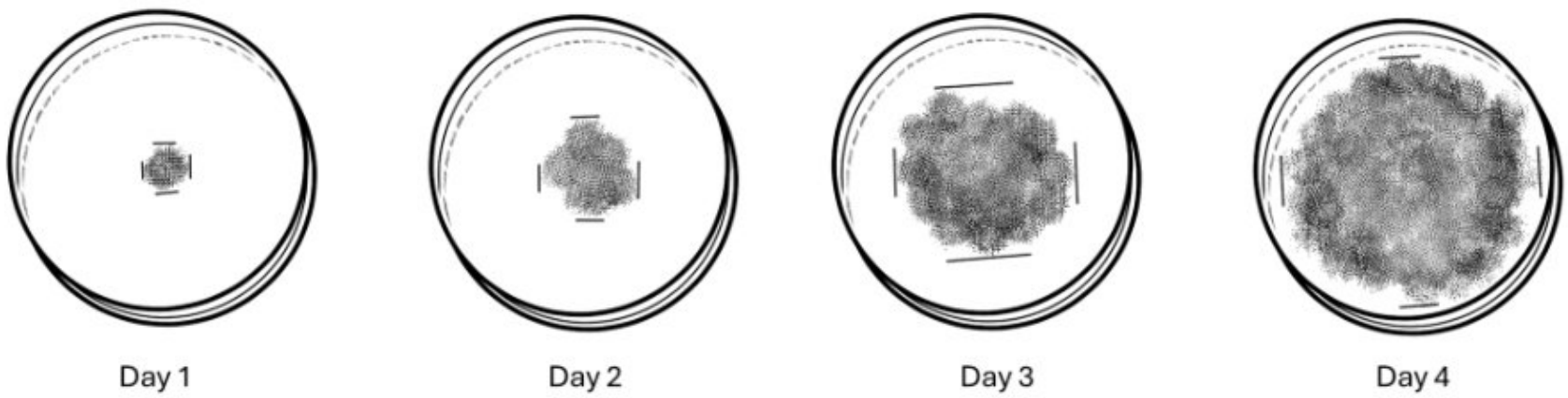


Figure 4.2 Image by Sara Ramos-Sepulveda

Fungal Genomic DNA (gDNA) Extraction

*This protocol is slightly modified from the one I learned during my postdoc while working with *Fusarium oxysporum* in Dr. Seogchan Kang's lab at Penn State University.

1. Once you make sure that you have a pure culture and that you have measured its growth, you can move on to extract its genomic DNA (gDNA).

2. Cut agar block of fungal culture (about 0.5-1 millimeter square) and inoculate in 50 milliliters (mL) of PDB in a 250 mL Erlenmeyer flask. Incubate for 2-3 days with vigorous shaking (around 150 rpm) at 25°C. (Try NOT to get too much fungal agar).

3. After 2-3 days, harvest the mycelium by filtering liquid media through filter paper and remove liquid as much as possible. Place dried mycelial mat into a 1.7 or 2.0 mL tube (If the cell will be disrupted by a bead beater with size 0.5, use a 2.0 screw cap microcentrifuge tube with about 6 mm depth of sterilized glass beads (0.1 mm)). Place harvested mycelia stored at -80 °C in liquid nitrogen.

4. Breaking down the fungal tissue:

   (using pestle tissue grinder)

   Keep sample tubes in liquid nitrogen and cool down the tip of a plastic pestle for 1.5 mL tubes in liquid nitrogen for 15 sec. Take out a tube and a plastic pestle from the liquid nitrogen and grind the mycelia (ground mycelia can be stored in liquid nitrogen until all samples are done). Once all samples are ready, add 600 µl of lysis buffer and 4 µl of RNase to each tube and mix well by vortexing. Keep mixed samples in ice until all samples are done

   (using bead beater at 4 °C to prevent heat)

   Add 100-200 µl of 1X TE buffer and insert the tube securely in the arm assembly in the MiniBeadbeater. Close the safety cover. Operate the MiniBeadbeater for 1 min at 4,800 rpm. Cool down at 4°C for 30 seconds and operate the MiniBeadbeater again for 1 min. Keep mixed samples in ice at 4°C until all samples are done. After bead-beating, add 600 µl of lysis buffer and 4 µl of RNase. Then vortex for 5 minutes to mix well.

5. Use a water bath with a temperature of 65°C to submerge the whole tube for 30 minutes while vortexing 2-3 times during incubation and cool down for a minute.

6. Add 500 µl of phenol:chloroform (1:1, pH 8.0) (or phenol:chloroform;isoamyl-alcohol=25:24:1, pH 8.0) and vortex until liquid is mixed completely (15 sec).

7. Shake by inverting for 2-3 minutes.

8. Centrifuge for 10 minutes at 12,000 rpm.

9. Transfer about 600-700 µl of supernatant to the new 1.5 mL microcentrifuge tube (be careful not to take the bottom layer).

10. Add 0.6 x volume of supernatant (360-420 µl) of isopropanol and 0.1 x volume of 3M acetate (NaOAc, 60-70 µl) or 100% ethanol with 3 M NaOAc.

11. Incubate mixture at -20 °C for 15 min.

12. Centrifuge for 10min at 12,000 rpm at room temperature.

13. Discard supernatant and wash pellet with 1 mL of 70% ethanol (do not vortex).

14. Centrifuge for 5 minutes at 12,000 rpm at room temperature.

15. Discard the supernatant.

16. Add 30 µl of distilled water.

Analyze the Isolated Genomic DNA (gDNA) Using the Nanodrop

1. Take 2 µl of water and place it on the Nanodrop pedestal, the same water that was used to dissolve the gDNA.

2. Close the arm.

3. Press the Blank button.

4. Open the arm and clean the pedestal with a paper towel.

5. Add 2 µl of isolated gDNA on the pedestal.

6. Measure the absorbance 260/280 and 260/230.

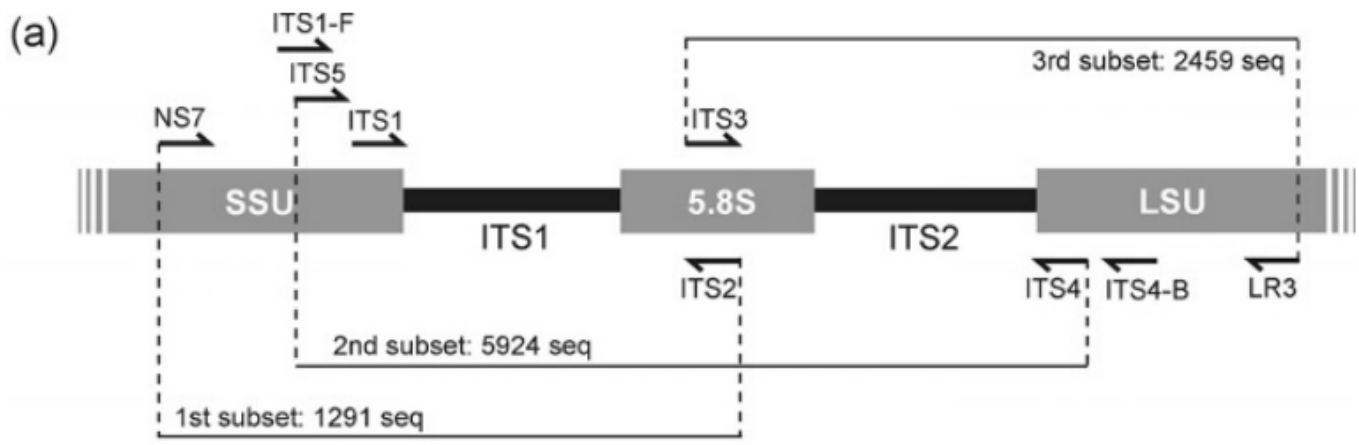
7. To verify for protein contamination (260/280), the absorbance must be close to 1.82.

8. To verify for salt contamination (260/230), the absorbance must be close to 2.

9. The concentration of gDNA is calculated in ng of gDNA in µl of water (ng/µl).

Amplifying the Fungal ITS Region Using Polymerase Chain Reaction (PCR)

Many researchers have created various primers that can be used to amplify the ITS regions. These primers have DNA sequences that specifically attach to the complementary DNA strands, as indicated by the arrows in Figure 4.3. For the application to succeed, the polymerase chain reaction (PCR) mix must include the target fungal genomic DNA (gDNA), the primers of interest, extra nucleotides, Taq polymerase, and $MgCl_2$.



(b)

Primer	Author	Primer sequence	Position
Forward primers			
NS7	[19]	GAGGCAATAACAGGTCTGTGATGC	1403-1426
ITS1-F	[18]	CTTGGTCATTTAGAGGAAGTAA	1723-1744
ITS5	[19]	GGAAGTAAAAGTCGTAACAAGG	1737-1758
ITS1	[19]	TCCGTAGGTGAACCTGCGG	1761-1779
ITS3	[19]	GCATCGATGAAGAACGCAGC	2024-2045
Reverse primers			
ITS2	[19]	GCTGCGTTCTTCATCGATGC	2024-2043
ITS4	[19]	TCCTCCGCTTATTGATATGC	2390-2409
ITS4-B	[18]	CAGGAGACTTGTACACGGTCCAG	2526-2548
LR3	[13]	CCGTGTTTCAAGACGGG	3029-3045

Figure 4.3 Figure by Bellemain et al., 2010 is licensed under CC BY 2.0.

The reaction will happen in a thermocycler (Figure 4.4) that will modify the temperatures to allow for three steps to happen: **denaturation, annealing**, and **primer extension**. During denaturation, the hydrogen bonds between the nitrogenous bases will be broken using high temperatures, and the two strands of DNA will be separated. During the annealing step, the **primers** will attach to the **targeted fungal DNA**. Lastly, during primer extension, the **Taq polymerase** attaches to the **nucleotides** next to the primers and amplifies the second strand using the extra nucleotides available in the polymerase chain reaction mix. The three steps will continue to repeat themselves until millions of copies of the targeted DNA are amplified.

Figure 4.4 Image by Author

Preparing the PCR Mix:

1. The final concentration in each PCR tube should be:
    - 1X ImmoMix (Bioline Meridian - Thomas Scientific)
    - 1.50 mM MgCl2 (New England Biolabs, Inc.)
    - 0.50 µM Forward Primer (Eurofins Scientific)
    - 0.50 µM Reverse Primer (Eurofins Scientific)
    - Deionized water to complete final volume of 24 µl.
2. Add 1µl of the isolated gDNA to the PCR mix. Remember to keep all the tubes on ice during the whole process.

3. Place the PCR tube in the thermocycler and run the reaction.

Thermocycler Cycle:

For my class we used the ITS 3 (GCATCGATGAAGAACGCAGC) and ITS 4 (TCCTCCGCTTATTGATATGC) primers. The thermocycler was set up for:

- 1 minute at 94 °C
- the following steps were repeated 30 times
    - 30 seconds at 94 °C,
    - 30 seconds at 55 °C,
    - and 30 seconds at 72 °C
- 8 minutes at 72 °C
- Keep the reaction at 10 °C

Running Polymerase Chain Reaction (PCR) Product on an Agarose Gel

1. Mix 0.5g agarose gel in 50 mL 1X TAE buffer.
2. Microwave the mix for 2 mins and 50 seconds.
3. Wait for the mix to cool down.
4. Add 5µl of ethidium bromide and mix (Note: ethidium bromide is carcinogenic; please ensure that you do not come into contact with the chemical.).
5. Place the mix on the agarose chamber.
6. Add the 16 well comb.
7. Wait for the agarose gel to solidify, about 15 minutes.
8. Place the agarose gel in the chamber and fill the chamber with 1X TAE buffer, until the gel is covered.
9. Mix 5 µl of the PCR amplified product to 1µl of 6X loading dye.
10. Place the total 6µl inside the well created by the comb on the agarose gel.
11. Add the ladder to the agarose gel following the manufacture instructions.
12. Run the agarose gel at 110 volts for 50 minutes.
13. Expose the agarose gel to UV light to see the PCR-amplified product and confirm it size.

Cleaning the PCR Product and Sequencing It

Note for Instructor:

If the PCR product has the correct size, we clean it before sequencing it with ExoSap-IT (Applied Biosynthesis - Thermo Fisher Scientific) following the manufacture instructions. The PCR product is sent to sequence at Eurofins Scientific.

Analyze the ITS Region Sequence Using NCBI – Blast

1. Open Blast: Basic Local Alignment Search Tool
2. Click Nucleotide BLAST.
3. Take the DNA sequenced provided and copy and paste below: “Enter accession number(s), gi(s), or FASTA sequence(s).”
4. Press the BLAST button.
5. Identify your unknown fungus.

Learning Questions

1. Explain the process of preparing a fungal pure culture:
    - Which part of the mycelial colony would you use?
    - How do you sterilize the cork borer?
    - How do you use the cork borer to place the mycelia on the new media?
    - How do you place the petri plate in the incubator?
    - How do you measure the fungal growth?
2. Regarding the genomic DNA isolation:
    - What was used to destroy the fungal cells wall?
    - Why did we use RNase?
3. Regarding the nanodrop:
    - How do you load a sample to be analyzed by the nanodrop?
    - How can you tell when a sample is clean of proteins and salts?
4. Regarding the ITS region:
    - Why is ribosomal DNA sequence used for fungal identification?
    - What is the ITS region and why is it used to identify fungi?

- If you use the forward primer ITS1 and the reverse primer ITS4, how many nucleotides should be the PCR product?

5. Regarding the PCR and the gel electrophoresis:

    - What is the goal of PCR?
    - What are the ingredients necessary to complete a PCR?
    - Explain what the thermocycler does as it changes the temperatures during the PCR. Specifically, name the steps and explain what happens in each one.
    - What is the purpose of the loading dye?
    - Why does the DNA move toward the positive side of the electrophoresis gel?
    - What is the purpose of the ladder?
    - What is the purpose of the ethidium bromide and the UV light?

Chapter Five:
Fungal Sexual vs. Asexual Reproduction

Objectives

After completing this exercise, you should be able to:

1. Explain and discuss fungal taxonomy focusing on two Phyla, Ascomycota and Basidiomycota.

2. Prepare a microscope slide from Basidiomycota fruiting bodies.

3. Identify and describe Basidiomycota reproduction structures.

4. Identify and describe Ascomycota reproduction structures.

5. Differentiate sexual vs. asexual Ascomycota reproduction structures.

Introduction

Fungal taxonomy is complex; as mentioned previously in Chapter Three, most field guides for mushroom identification do not take fungal taxonomy into account. As discussed in Chapter Four, the most reliable tool for fungal identification is ITS sequencing, as fungal morphology and biochemistry can be unreliable.

Although fungal taxonomy is complex, various researchers (e.g., Hibbett et al., 2007; Spatafora et al., 2016; Tedersoo et al., 2018) have dedicated their careers to taking on this challenge. Furthermore, there are several comprehensive reviews, such as Naranjo-Ortiz and Gabaldón (2019), James et al., 2020, and Hibbett et al., 2025.

Currently, the kingdom Fungi includes: the aquatic group **Rozellomycota**, which contains the chytrid endoparasite *Rozella allomycetis*; the group **Aphelidiomycota**, consisting of phagotrophic parasites that feed on marine algae; **Sanchytriomycota**, which includes organisms that resemble amoebas; **Blastocladiomycota**, which contains various parasites; the subkingdom **Chytridiomyceta**, which includes *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis in amphibians worldwide; **Zoopagomycotina**, typically obligate parasites of other fungi, nematodes, and amoebae; the phylum **Mucoromycota** includes various molds, such as the genera *Rhizopus* and *Mucor*, as well as arbuscular mycorrhizal fungi; and the subkingdom **Dikarya**, which comprises the three phyla Ascomycota, Basidiomycota, and Entorrhizomycota. For the remainder of this chapter, we will focus on the two phyla Ascomycota and Basidiomycota.

In Chapter Three, we discussed macro-fruiting bodies, also called mushrooms. Most mushrooms belong to the phylum Basidiomycota, but there are also a few fungi within the phylum Ascomycota that produce macro-fruiting bodies, such as cups, morels, truffles, and earth tongues. Most Ascomycota fruiting bodies are microscopic and require a microscope to be observed.

Although most Basidiomycota have macro fruiting bodies and most Ascomycota have microscopic fruiting bodies, the most important difference between the ascos and basidios is the structure on which the spores are produced. Basidios are often called club fungi due to the basidia, and ascos are called sac fungi because the spores are produced inside the sac-like ascus.

Fungi can produce spores by both methods sexually or asexually reproduction. When fungi exist in its sexual form, also known as the perfect state, the structures at these stages are known as their **teleomorph** structures. When fungi exist in its asexual form, also known as the imperfect state, the structures at these stages are known as **anamorph** structures. Many fungi can reproduce both sexually and asexually, and these fungi are called **pleomorphic** fungi. Most fungi within the Phylum Basidiomycota reproduce sexually only, while pleomorphic fungi are more commonly found in the Phylum Ascomycota.

Note for Instructors:

You will notice often in this chapter that I have the following wording: *Take the microscopes slides that the instructor has selected for you and look at them under the compound microscope using the 40X objective lens.* I purchased prepared microscope slides with different fungal microscopic structures from Carolina Biological Lab.

Phylum Basidiomycota

The phylum has three subphyla **Pucciniomycotina**, which includes the plant pathogenic fungi knowns as rust fungi; the **Ustilaginomycotina**, which includes the plant pathogenic fungi known as smut fungi; and **Agaricomycotina**, which includes most of the commonly known mushroom that you are familiarized with, including the white button mushroom. Most fungi in the phylum Agaricomycotina are saprotrophs and indispensable for forest ecosystems.

Sexual Reproduction

Subphylum Pucciniomycotina

The most commonly associated mushrooms belong to the phylum Basidiomycota. *Puccinia graminis*, which belongs to the subphylum Pucciniomycotina and causes stem rust in wheat, produces four different fruiting bodies and five different types of spores.

The disease cycle starts with **basidiospores** landing on the top leaves of barberries, where they germinate and produce an aecium (pl. aecia; Figure 5.3A), which produces **aeciospores**. The aeciospores infect the lower part of the barberry leaves, producing various picnia (s. pycnium; Figure 5.3B), which produce pycniospores. These **pycniospores** infect wheat crops, producing various uredinia (s. uredinium; Figure 5.3C), which produce **urediniospores**. The urediniospores are released and can infect other healthy wheat plants. The uredinia will then become telia (s. telium; Figure 5.3D), which produce teliospores. These **teliospores** will germinate, producing four new basidiospores; at this point, the disease cycle will start again. In the north part of the United States, farmers will experience all five spores, whereas in the south part of the United States, they only experience half of the cycle. This because *Puccinia graminis* teliospores require extended periods of freezing temperatures to germinate.

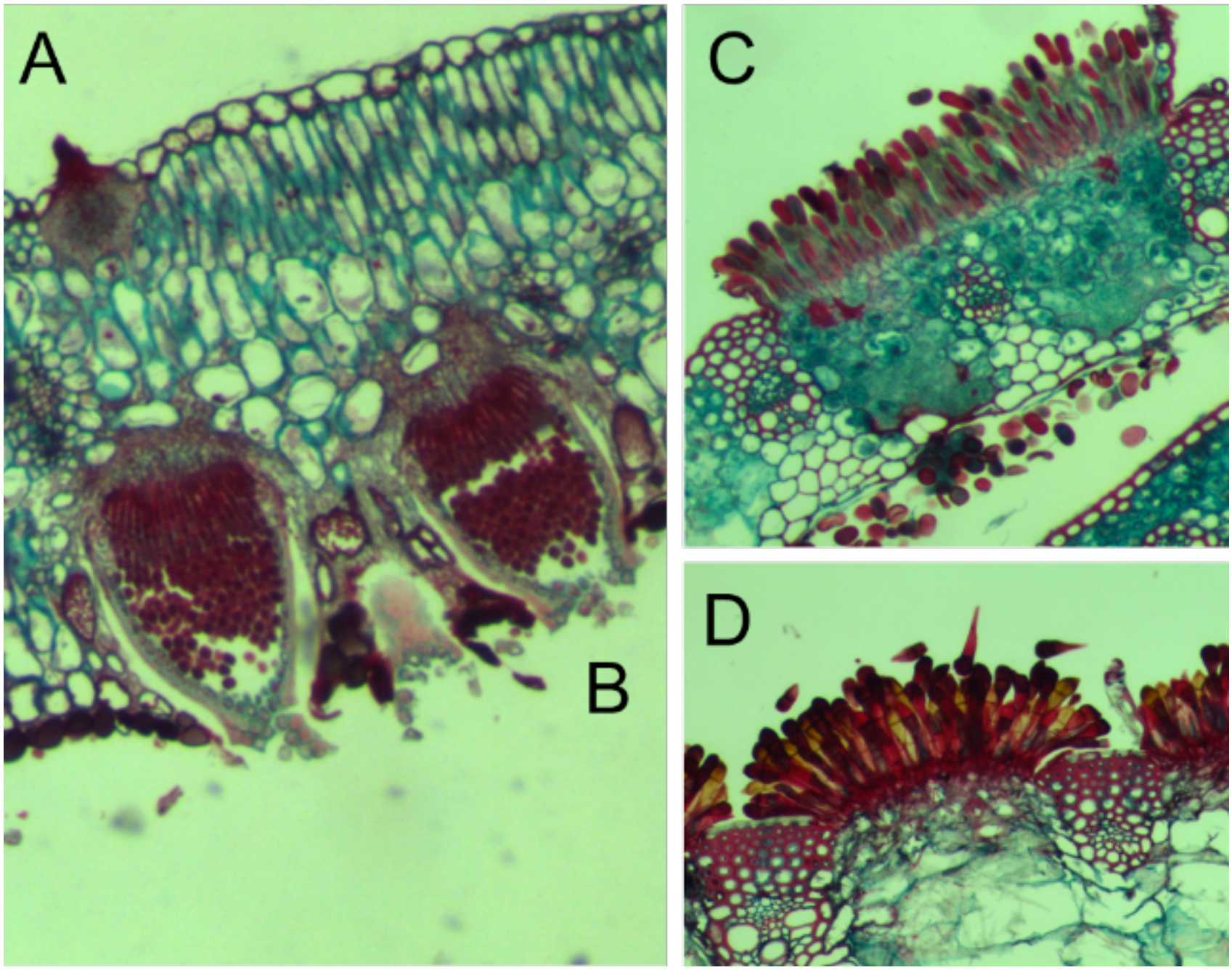


Figure 5.3 Image by Author

Take the microscopes slides that the instructor has selected for you and look at them under the compound microscope using the 40X objective lens.*Refer to Appendix III:Using the Compound Microscope for more information. Find and draw the following structures:

- Aecium
- Aeciospores
- Pycnium
- Pycniospores
- Uredinium
- Urediniospores

- Telium
- Teliospores

Subphylum Agaricomycotina

1. Obtain a white button mushroom that the professor has bought from the grocery store.
2. Remove the partial veil that covers the gills.
3. Take the following steps depicted in Figure 5.3 to prepare a microscope slide with the mushroom's gills. Step #1, split the mushroom in half. Step #2, cut a thin slice of the gills, the thinner, the better. **IMPORTANT**: Although on the photo it shows the blade cutting towards the thump, please cut toward the outside. Step #3 is to place the thin slice of the gill on a microscope slide.
4. Cover the thin gill slice with the cover slip.
5. Gently tap and/or apply pressure to the cover slip, "squashing" the gills. Do not use your fingers.
6. Draw and identify what you see using the compound microscope objective lens 40X.*Refer to Appendix III:Using the Compound Microscope for more information.

Figure 5.4 Image by Author

Learning Questions

Subphylum Pucciniomycotina

1. Draw the four different fruiting bodies discussed during the lab (including their respective spores) and explain how they are involved in the disease cycle.

2. If you were a wheat farmer, what would you do to stop *Puccinia graminis* from negatively impacting your crop?

Subphylum Agaricomycotina

1. Explain how to prepare a sample to see basidia and basidiospores using the compound microscope.

2. Identify the following structures (bullet points) in Figure 5.5:

    - **Cap (Pileus)**: The cap holds the tissue that contains the fungal spores.

    - **Cystidium** (pl. **Cystidia**): These are sterile, large cells found between the basidia. While their exact function is not completely understood, not all species of Basidiomycota have this structure, and its presence can vary, which helps scientists with identification.

    - **Gills** (Lamella, pl. **Lamellae**): The gills, held by the cap, contain the basidia and basidiospores.

    - **Trama**: The inner tissue within the mushroom's gill.

    - **Pileipellis**: The outermost layer of the mushroom's cap. There are three types of pileipellis:

        - **Cutis**: Hyphae running parallel to the cap surface.

        - **Trichoderm**: Hyphae growing perpendicular to the cap surface, resembling hairs.

        - **Hymeniderm**: Cells rather than hyphae.

    - **Stalk (Stipe)**: This structure supports the mushroom cap.

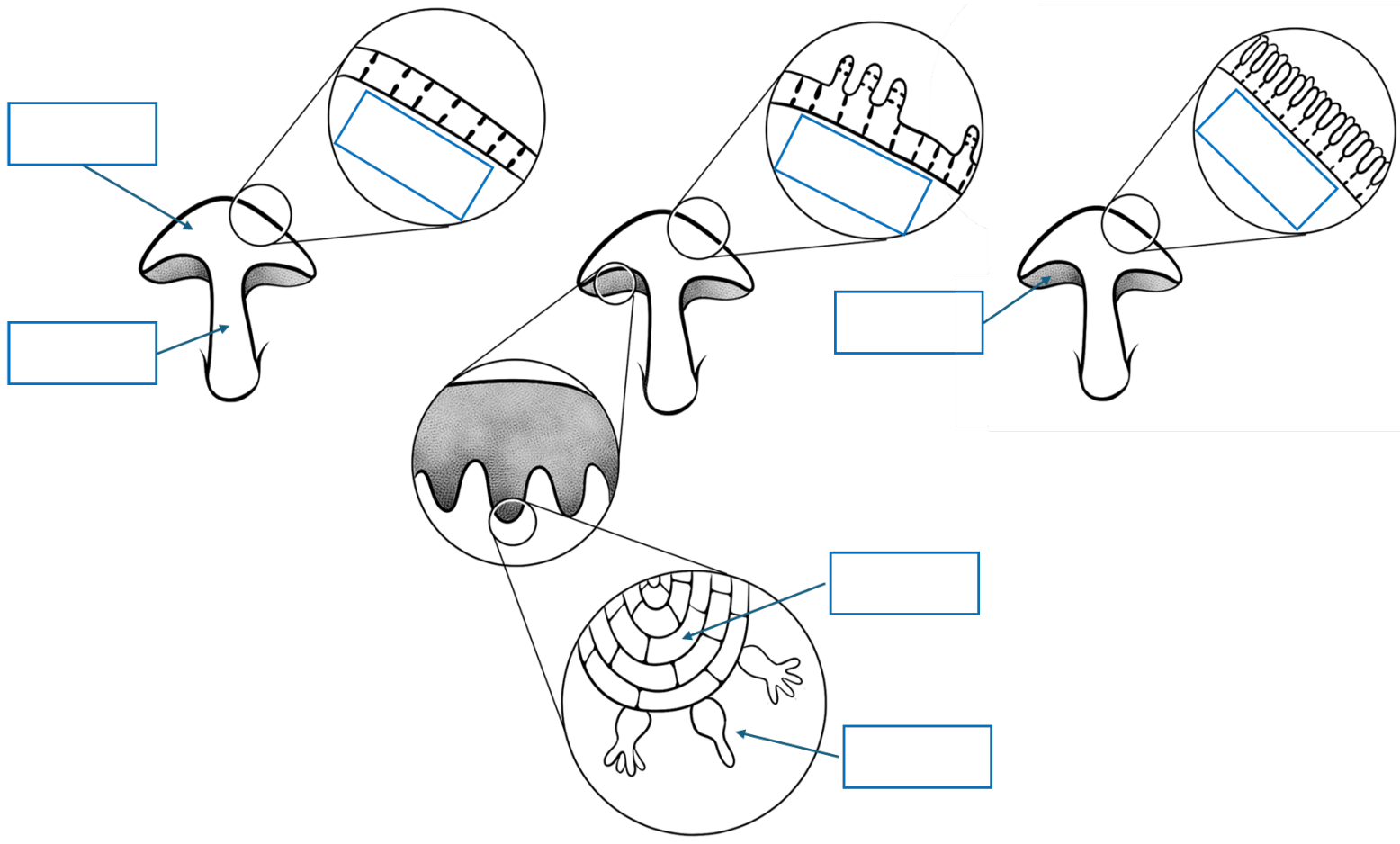


Figure 5.5 Image by Sara Ramos-Sepulveda Modified by Author

3. Identify the following structures (bullet points) in Figure 5.6:

- ***Basidiospores***: spores produced by sexual reproduction by Basidiomycota fungi
- ***Basidia (***s. ***Basidium):*** Club-shaped cells that produce basidiospores*.*
- **Cap (Pileus):** The cap holds the tissue that contains the fungal spores.
- **Gill (Lamella**, pl. lamellae). They are hold by the cap and contain the basidia and basidiospore.
- ***Hyphae***: microscopic, tube-like growth that branches only at the tip. Mushroom are made of compacted hyphae.
- **Stalk (Stipe)**: This structure holds the mushroom cap.

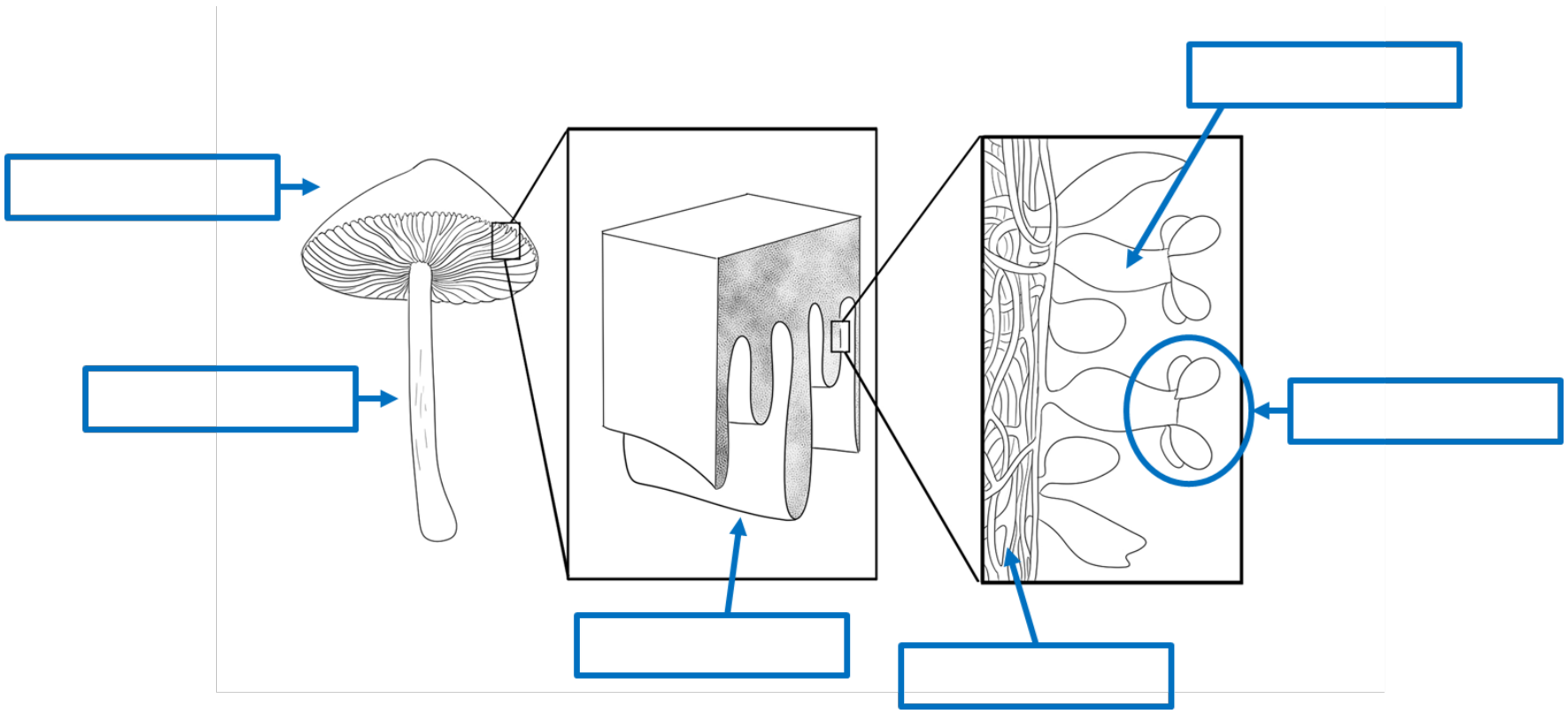


Figure 5.6 Image by Sara Ramos Sepulveda (Modified by Author) is licensed under CC BY-SA 3.0. Adapted from "Basidium schematic" by Debivort is licensed under CC BY-SA 3.0

4. Identify the following structures (bullet points) in Figure 5.7:

- **Basidia**: (s. **Basidia**) club-shaped cells that produce the basidiospore.
- **Basidiospore**: (pl. **Basidiospores**)
- **Sub-hymenium**: Short cells between the trama and the hymenium.
- **Hymenium**: A layer of cells that includes basidia and cystidia.

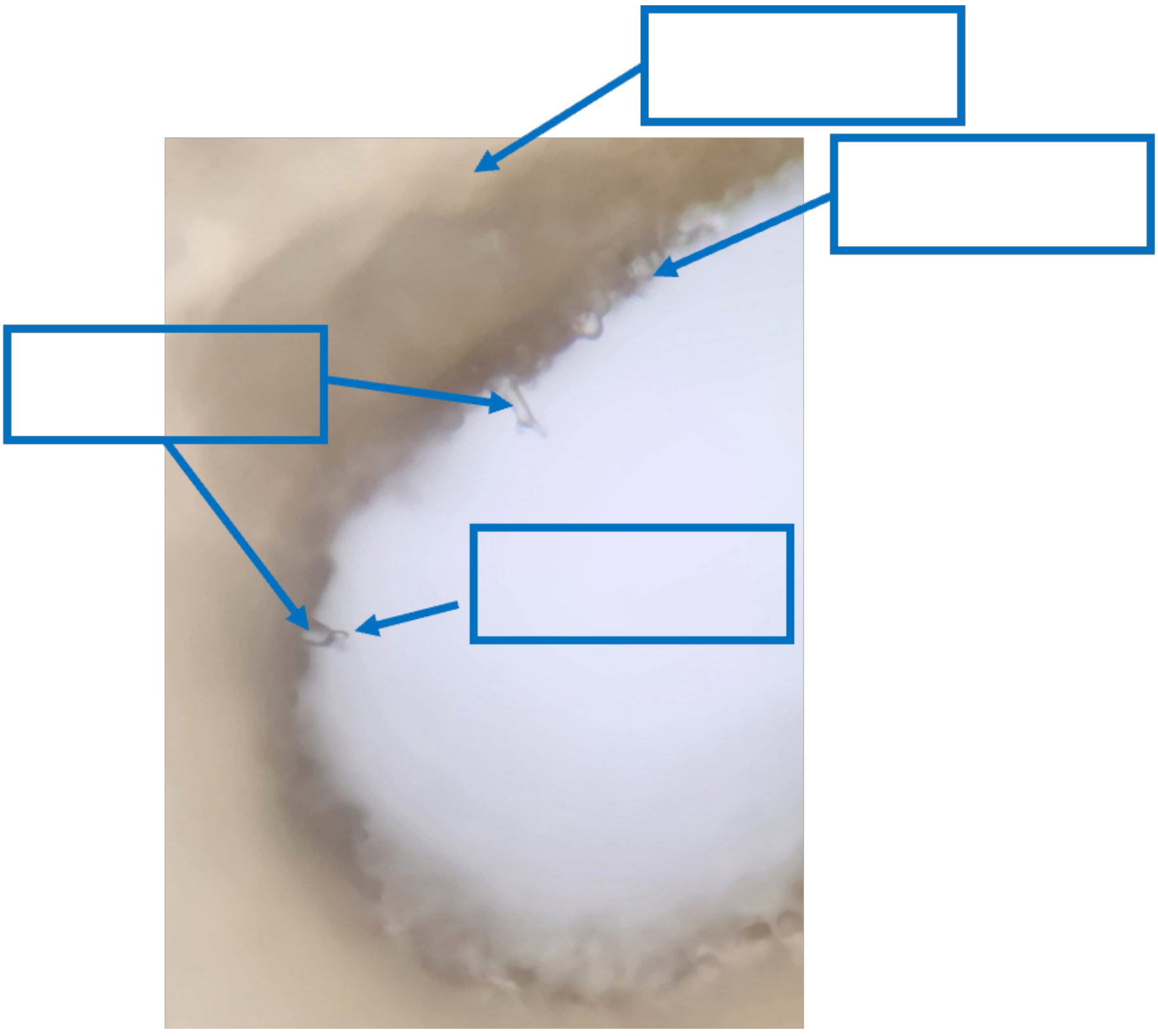


Figure 5.7 Image by Ruke C Haas. Modified by Author.

Phylum Ascomycota

Ascomycota includes fungi commonly associated with molds and filamentous fungi. The Phylum Ascomycota is divided into three Subphyla: **Taphrinomycotina**, which lacks of general characteristics but it includes fungi like the species *Pneumocystis jirovecii* (cause disease in humans), the earth tongue fungi, and some saprotrophs; **Saccharomycotina**, which includes fungi that grow as yeast, such *Saccharomyces cereviseae*; and **Pezizomycotina**, which includes numerous orders. Ascomycota can reproduce both sexually and asexually. We will focus on the structures produced by the subphylum Pezizomycotina in this chapter.

Sexual Reproduction

Regarding sexual reproduction structures, we will focus on the subphylum Pezizomycotina. Fungi in this subphylum can produce three different fruiting bodies:

- **Apothecium** (pl. **Apothecia**): Cup- or bowl-shaped.
- **Cleistothecium** (pl. **Cleistothecia**): Spherical.
- **Perithecium** (pl. **Perithecia**): Pear-shaped.

All three fruiting bodies have secondary structures called **ascus** (pl. **asci**), which contain the fungal spores called **ascospores** (s. **ascospore**). The asci and the fungal spores vary depending on the type of fruiting body they are produced in.

Take the microscopes slides that the instructor has selected for you and look at them under the compound microscope using the 40X objective lens.*Refer to Appendix III:Using the Compound Microscope for more information. Use the word bank (bullet points) to identify the structures labeled A through G.

- Apothecium
- Asci
- Ascospore
- Ascospores
- Ascus
- Cleitothecium
- Perithecium

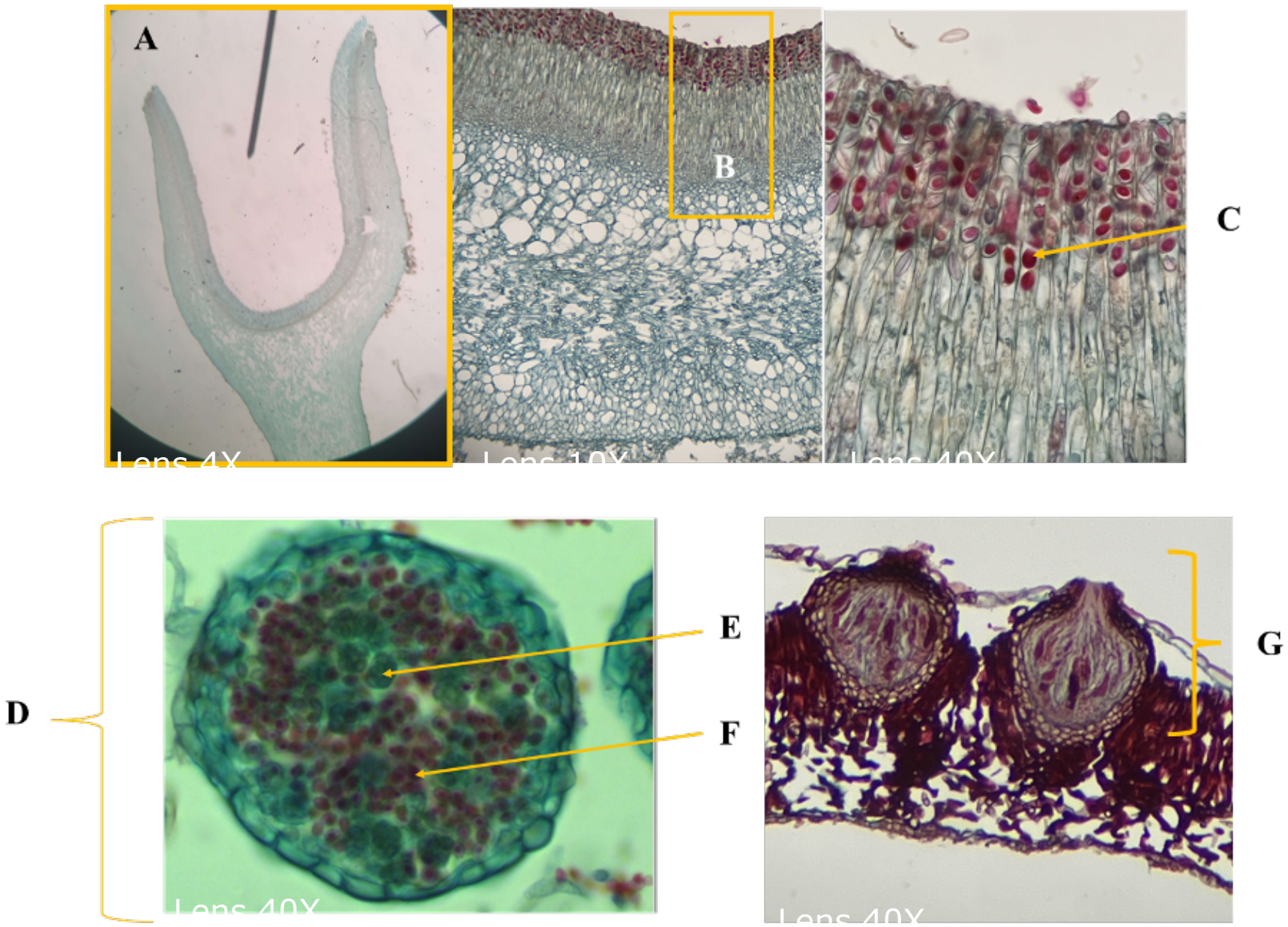


Figure 5.9 Image by Author

Learning Questions

Draw the following structures, draw the asci and ascospores in all the different types of fruiting body, and add the plural terminology for each one:

- Apothecium
- Cleitothecium
- Perithecium

Asexual Reproduction

Regarding asexual reproduction structures, we will continue focusing on the subphylum Pezizomycotina. Specifically, we will examine Class Eurotiomycetes, which includes fungi like *Penicillium*, commonly found in the environment. The asexual spores are called **conidia** (s. conidium) and are produced by **conidiogenous** cells, which can have two shapes: **phialides** or **annelides**. The conidiogenous cells are supported by conidiophores, which can be:

- **Monoverticillate**: Non-branched.
- **Biverticillate**: Two branches.
- **Terveticillate**: Three branches.

Take the microscopes slides that the instructor has selected for you and look at them under the compound microscope using the 40X objective lens.*Refer to Appendix III:Using the Compound Microscope for more information. Then, identify the following structures (bullet points) in Figure 5.10.

- Annelides
- Biverticillate
- Conidia
- Conidiogenous
- Conidiophore(s)
- Conidium
- Monoverticillate
- Phialides
- Terveticillate

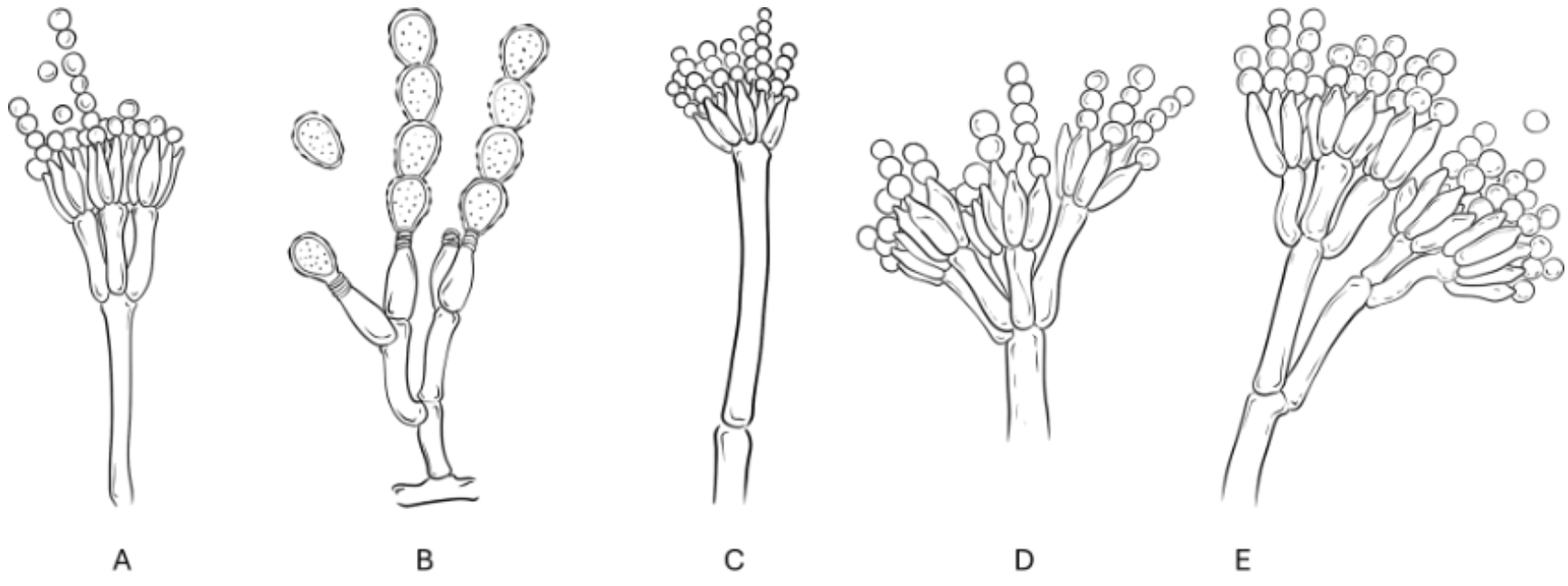


Figure 5.10 Image by Sara Ramos-Sepulveda.

Learning Questions

Draw the following structures and explain their importance.

- Annelides
- Biverticillate
- Conidia
- Conidiogenous
- Conidiophore(s)
- Conidium
- Monoverticillate
- Phialides
- Terveticillate

Chapter Six:
Growing Basidiomycota Mushrooms

Objectives

After completing this exercise, you should be able to:

1. Be familiar with the growth of mushrooms (macro fruiting bodies).
2. Compare the taste of oyster, lion's mane, and shiitake mushrooms.
3. Explain the process to produce mushrooms from hyphae.
4. Explain the production of white button mushrooms in Kennett Square, Pennsylvania.

Purchased Mushroom Kit

Introduction

To become familiarized with the growth of mushrooms, the professor has purchased three different kinds of oyster mushrooms: pink, blue, and golden. The professor has also purchased lion's mane and shiitake. All three types of mushrooms were purchased from a company called North Spore(Figure 6.1).

Note for Instructors:

The oyster mushroom kits are very good, the lion's mane are average good, but the shiitake mushroom kits are not good. I usually harvest only two to three shiitake mushrooms, even though I carefully tend to them and follow all the instructions. I will be trying a different company next time I teach the course.

Figure 6.1 Image by Author

Each laboratory group will oversee a Oyster or Lion's Mane mushroom kit. Each group is responsible for keeping records of the progress of the mushroom growth. The best way to keep records is to take photos of the mushrooms as they grow. You will realize that once the fruiting body starts to form, the mushroom will double in size each day If the student water the mushroom kit every morning and every afternoon, it will take about 10 days for the mushroom to start to form. Each day, it will double in size. By day fifteen, it should be ready to be harvested for cooking.(Figure 6.2 and 6.3).

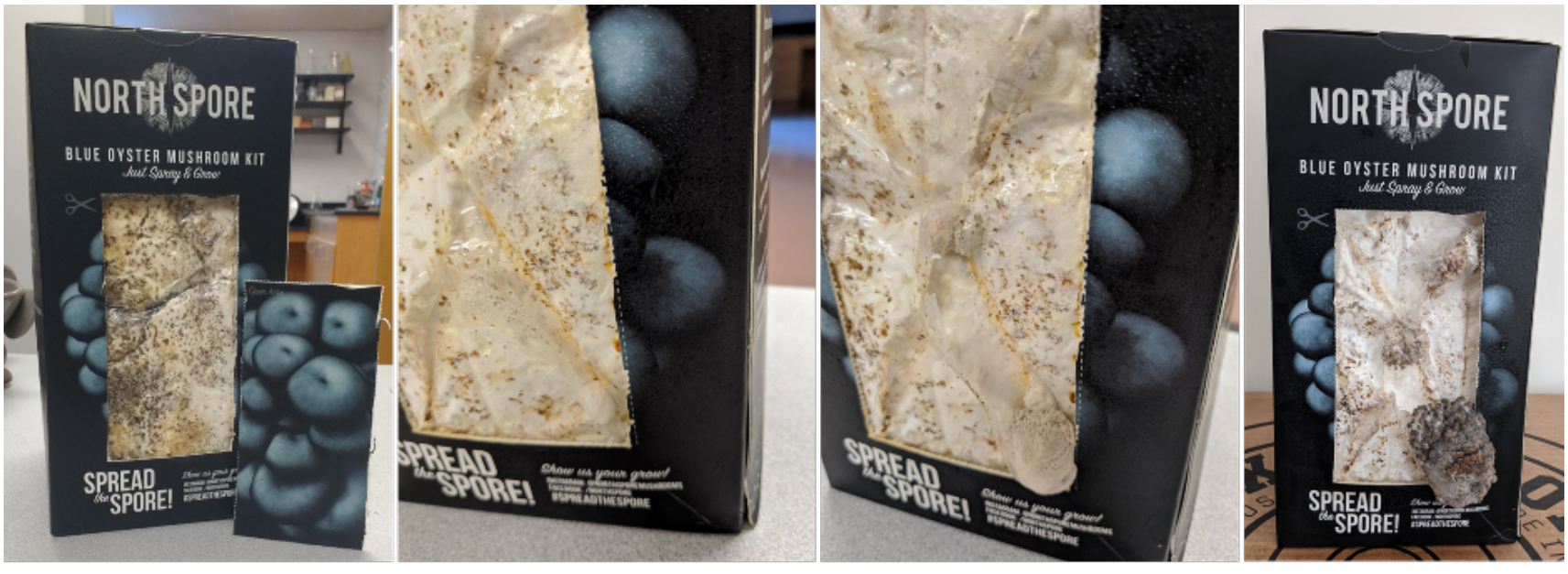


Figure 6.2 Image by Author

Day 13 Day 14 Day 15

Figure 6.3 Image by Author

Activity

1. Open the mushroom kit by removing the cardboard selection along the perforated lines.
2. Sterilize a knife by washing it with 70% ethanol.
3. Using the sterilized knife, cut into the plastic bag of the mushroom kit, making a large “X” by making cuts from opposite corners. This will allow oxygen into the bag. Fungi, like animals, require oxygen for aerobic respiration. This cut will also guide the hyphae to grow toward the outside of the bag.
4. Using distilled water, spray the opening of the bag.
5. Mist the opening of the bag twice a day until the fruiting bodies are fully developed. Have you noticed that mushrooms usually come out after it rains? We are creating a similar environment for them.
6. Take pictures of the progress of mushroom growth.
7. Afterwards, the instructor will prepare the mushrooms for taste and texture comparison.

Learning Questions

1. Describe the growth of the fruiting body. If you didn't have growth by day 10, what are some possibilities as to why?

2. You might have noticed some discoloration around the area that was being sprayed. Do you think this is contamination?

Let's Try Some Croquettes!

After cleaning everything in the laboratory, we will be trying croquettes made with the three different fungi:

1. Oyster

2. Lion's Maine

3. Shiitake

Note For Instructors:

It is a lot of work, it takes three days of preparation, and then you still have to cook them the morning of the lab. However, most students really appreciate it and enjoy the experience, even those who don't like mushrooms.

This recipe is a hybrid of three different online versions:

- Creamy Mushroom Croquettes by Albert Bevia (Spain on a Fork)
- Leek-and-Mushroom Croquettes by Jerry Traunfeld (Food & Wine)
- Mushroom Croquettes by Melissa (Cilantro and Citronella)

Ingredients:

- 3 tablespoons olive oil (for onions and garlic)
- 2 onions, diced
- 6 cloves garlic, minced
- 1 teaspoon olive oil per mushroom type
- 215 grams (7.5 oz) mushrooms per type, finely diced

- 9 tablespoons unsalted butter
- 9 tablespoons all-purpose flour, plus more for coating
- 8 cups unsweetened plant-based milk, divided
- 1½ cups vegetable stock
- 1½ teaspoons salt, plus more for seasoning mushrooms
- Black pepper, to taste
- 4 cups panko breadcrumbs (Japanese-style)
- ¾ cup freshly grated Parmigiano-Reggiano
- 4 large eggs, beaten with 2 tablespoons water
- Fresh parsley (optional, for garnish)
- Vegetable oil, for deep frying.

Instructions:

- Prepare the Base Aromatics: Heat 3 tablespoons olive oil in a large pan over medium heat. Add the onions and sauté until translucent. Add the garlic and continue cooking until fragrant and softened. Transfer the mixture to a plate (approx. 10 minutes total).
- Cook the Mushrooms: Sauté each type of mushroom separately in 1 teaspoon olive oil over medium heat. Season with salt and pepper once cooked. Set aside. Each type takes about 5 minutes.
- Combine Liquids: Mix the plant-based milk and vegetable stock together. Set aside.
- Make the Roux: In a saucepan, melt the butter over medium heat. Gradually sift in the flour, stirring constantly to avoid lumps. Cook the mixture, stirring continuously, until it forms a dough-like consistency and the raw flour smell disappears (about 4 minutes).
- Create the Béchamel: Slowly whisk in the milk-stock mixture. Continue whisking until a smooth sauce forms. Bring to a simmer and cook for a few minutes until thickened. Stir in the Parmigiano-Reggiano, ½ teaspoon salt, and pepper to taste. Remove from heat.
- Combine Fillings: Divide the onion mixture and béchamel sauce evenly into three bowls. Add one type of mushroom to each and mix thoroughly. Allow to cool to room temperature, then refrigerate until fully chilled—preferably overnight (about 45 minutes minimum).
- Prepare Breading Station: Set up three shallow bowls: one with flour, one with the beaten eggs, and one with panko. Line a baking sheet with wax paper.
- Form the Croquettes: Scoop about a tablespoon of filling and shape into a log using your hands.
- Bread the Croquettes: With floured hands, coat each log in flour, then dip in egg, and finally coat in panko. Press gently to ensure the breadcrumbs adhere. Place finished croquettes on the prepared baking sheet. I usually bread them in batches: all in flour first, then all in egg, and finally all in panko. For 15 logs, this process takes about 1 hour. Refrigerate again until ready to fry (preferably overnight).

- Fry the Croquettes: Heat vegetable oil in a small pot over medium heat for deep frying. Fry the croquettes in batches until golden brown (about 1 hour for 15 logs total). Drain on a paper towel-lined plate.
- Serve: Garnish with chopped fresh parsley if desired. Serve hot.

Learning Questions

1. What are your thoughts on different croquettes?

2. Describe their texture as well as their taste.

3. Which one did you like the least? Best? Why?

Growing Oyster's Basidiospores

Oyster mushrooms produce a lot of basidiospores (Figure 6.4A); therefore, oyster mushroom growers sometimes need special masks while harvesting. We will be taking the oyster mushroom basidiospores (Figure 6.4B) and placing them on a dead log in the classroom (Figure 6.4C). **IMPORTANT**: Oyster mushrooms produce many spores and can grow on many different hardwood species, be careful not to spread it out in the wild.

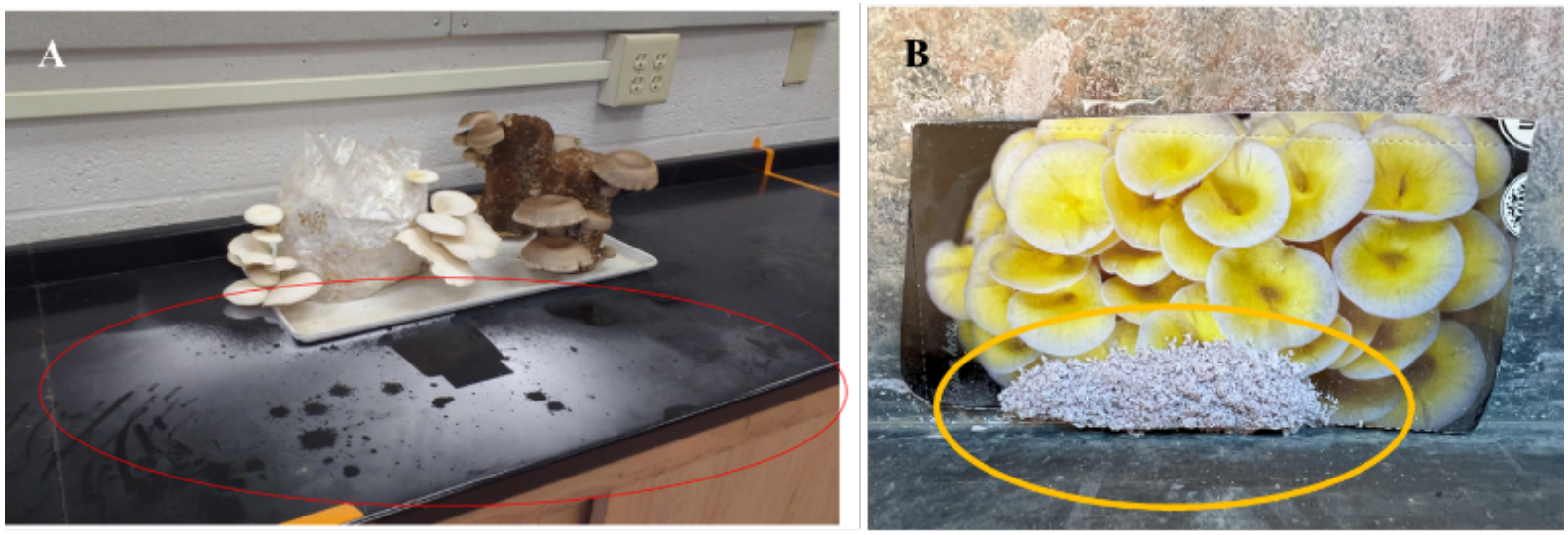


Figure 6.4 Image by Author (A and B) and Sara Ramos-Sepulveda (C)

1. Drill the holes to a 1˝depth following the diamond pattern (Figure C).
2. Place half of the 1/8 teaspoon of oyster basidiospores inside the holes.
3. Then, add petroleum jelly to cover the holes.

Homemade Mushroom Kit

For this activity we want to create our own mushroom kits with oyster mushrooms. These are the steps:

- Transfer sterile hyphae from an oyster mushroom on Potato Dextrose Agar.
- Prepare the cakes in glass jars.
- Place mycelia in an Erlenmeyer flask with Potato Dextrose Broth.
- Place the little mushroom ball from the Erlenmeyer flask with Potato Dextrose Broth in the jar cake and wait for the hyphae to grow around the cake.
- Set up the terrarium.

Note for Instructors:

You can either use the golden oyster mushroom from the North Spore mushroom kit or purchase oyster mushrooms from the grocery store.

Obtaining a Pure Culture of Hyphae on Potato Dextrose Agar (PDA)

1. Disinfect work area with 70% ethanol.
2. Place 1 milliliter (mL) of 10% bleach in a 1.5 mL centrifuge tube.
3. Add oyster mushroom tissue (Figure 6.5) into the centrifuge tube with 10% Vortex bleach, then leave to soak for 1 minute.
4. Remove bleach.
5. Rinse the oyster mushroom tissue with 1 mL of autoclaved water and Vortex. Make sure to remove all the water each time.
6. Repeat the previous step 2 more times.
7. Sterilize scalpel by drenching with 70% ethanol. Then, using a Bunsen burner while holding the scalpel downward, bring over the flame and let the fire evaporate the ethanol away.
8. Place the sterile tissue on the PDA media. The tissue to be utilized is the inner tissue specified by the arrow (Figure 6.5).
9. In a few days, the hyphae will grow. Ensure that you have a pure culture.

Figure 6.5. Oyster Mushroom. Image by Author

Preparing the Cakes in Glass Jars

Required Items:

1. Obtain a glass jar.

2. 120mL of vermiculite.

3. 60mL of brown rice flour.

4. 55mL of water.

Procedure:

1. Grind about 60g of brown rice.

2. Sift the ground brown rice.

3. Mix the 55mL of distilled water with the vermiculite.

4. Add the ground brown rice to the mixture.

5. Add dry vermiculite on the top (Figure 6.6).

6. Cover with foil and autoclave.

Figure 6.6 Image by Author

Placing Mycelia in Potato Dextrose Broth (PDB)

1. Sterilize your working area.
2. The cork borers have been autoclaved and, therefore, are sterile.
3. Pull the inner piece of the cork borer inside and, using only the cork borer, cut a section of the mycelium you wish to propagate on the edge. The cut section can have some exposed agar.
4. Carefully remove the cut section of mycelium.
5. Using the inner piece, place the section of mycelium in the PDB.
6. Put the flask to shake in the incubator at 25°C.
7. Within a week you should be able to see the mycelial spheres (Figure 6.7).

Figure 6.7 Image by Author

Placing Mycelia in the Glass Jars

1. Sterilize some long tweezers.
2. Using the tweezers, remove a large number of mycelial spheres from the beaker of PDB and place them onto the cake previously prepared.
3. Cap the jar so it is airtight.
4. Allow the cake to sit until the mycelia has colonized the whole substrate (Figure 6.8).

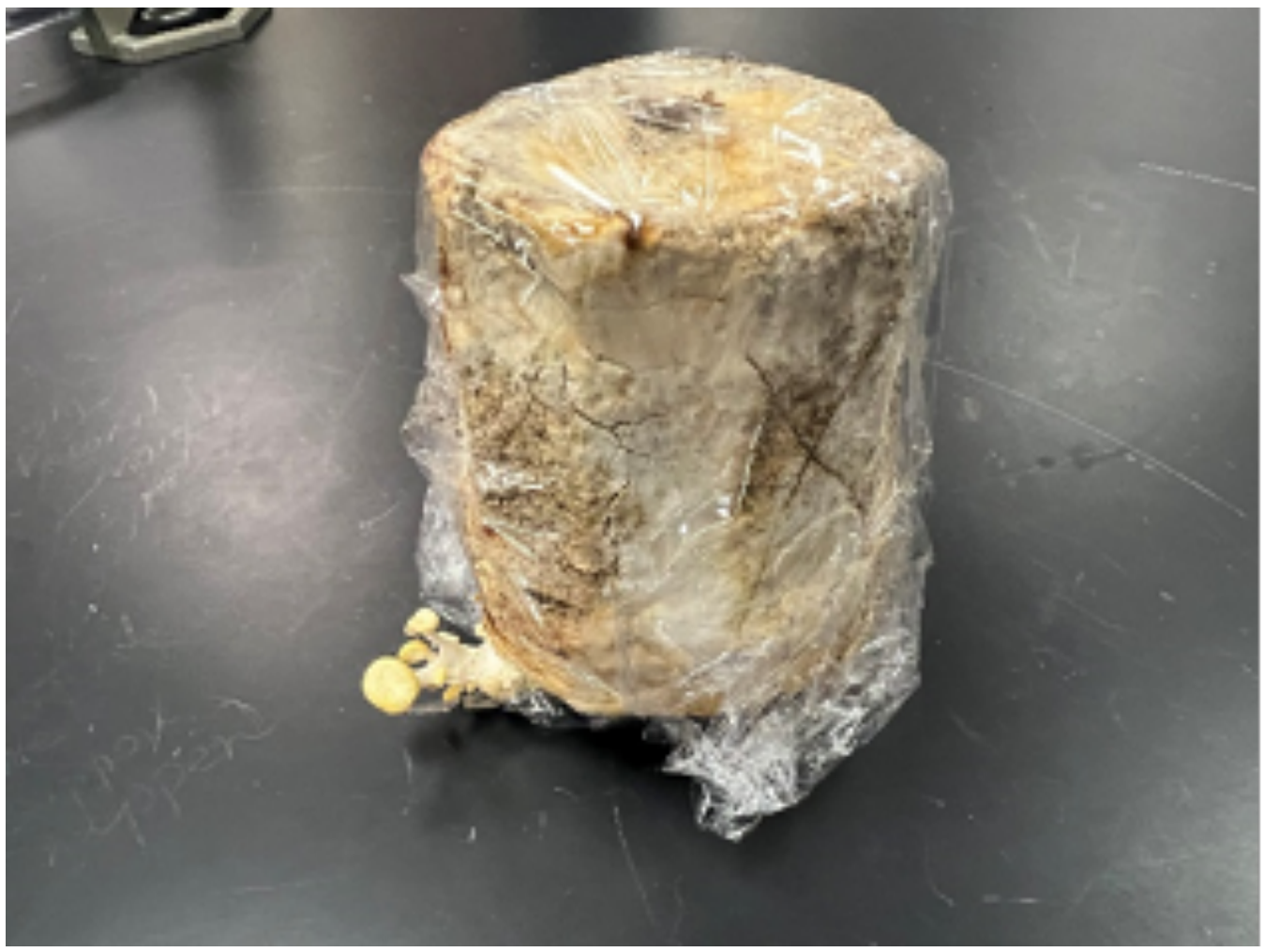


Figure 6.8 Image by Author

Setting Up the Terrarium

1. Rinse the perlite and let it drain for a few minutes.
2. Place the perlite at the bottom of the terrarium.
3. Flatten the perlite — even floor.
4. Place some autoclaved foil on top of the perlite.
5. Wash your hands and remove the cakes from the glass jars.
6. Rinse and then soak the cakes in cold sterilized water.

7. Set all cakes into the water. They will automatically float.

8. Cover the top to keep the cakes under the water.

9. Set them in the fridge for 24 hours.

10. Autoclave the vermiculite and then let it cool down.

11. Next Day:

    1. Remove the soaked cakes from the fridge and rinse with cold water.

    2. Roll the cakes in the autoclaved vermiculite, so they are all completely covered.

    3. Cover the cakes with plastic wrap.

    4. Place the rolled cakes with plastic wrap on the foil square inside the terrarium.

    5. Lightly spray with distilled water outwards, onto the side of the terrarium.

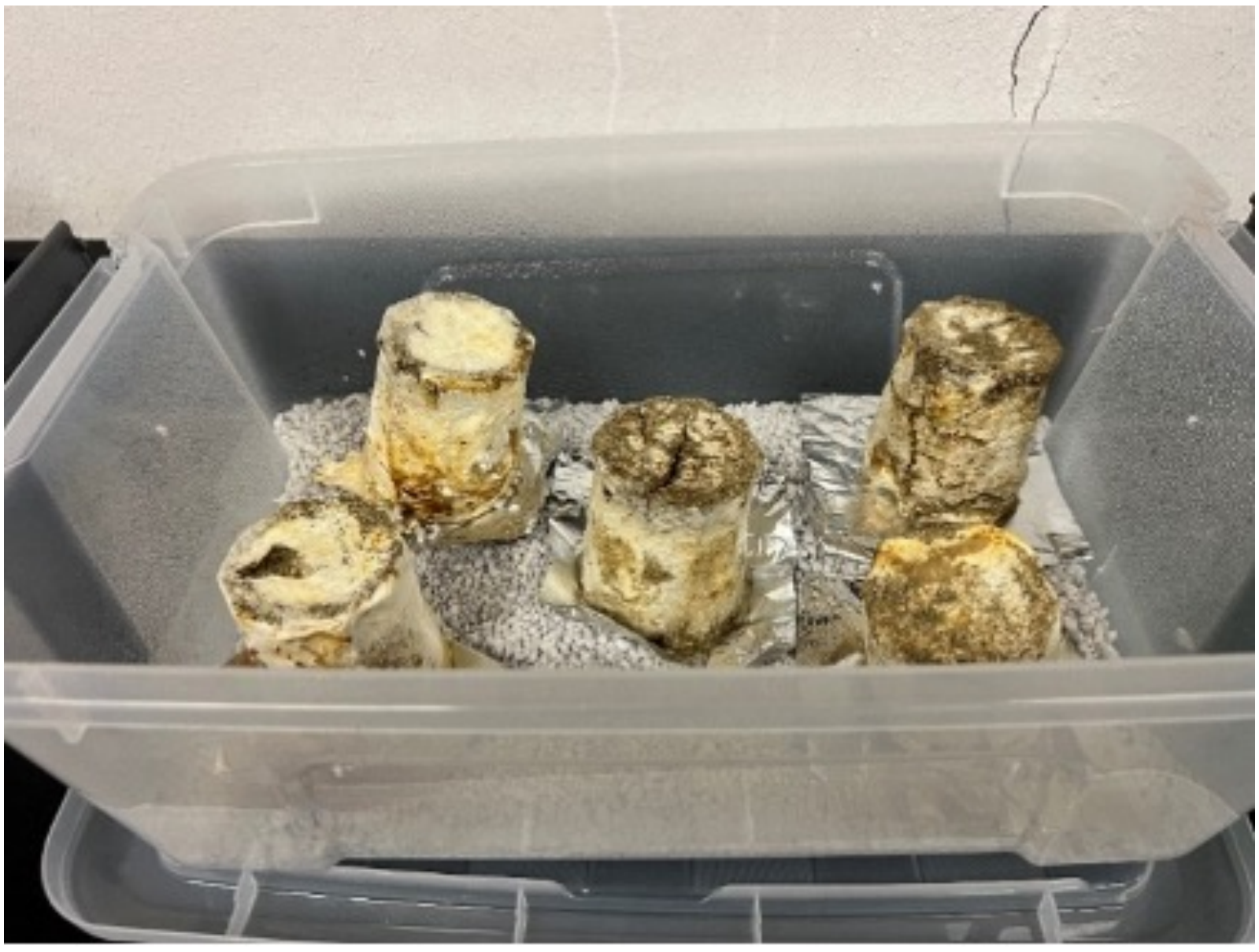


Figure 6.9 Image by Author

Learning Questions

1. Place the following sentences in the correct order:

- [ ] Once the mycelia have covered and grown through the entire rice and vermiculite, take the “cake” and place it in a “terrarium”.
- [ ] Place the disinfected mycelium and place it on Potato Dextrose Agar (PDA).
- [ ] Prepare the rice/vermiculite mix and autoclave it.
- [ ] Take the little fungal mycelial balls and place them in the rice/vermiculite mix.
- [ ] Take the mycelia from the oyster mushroom fruiting body and disinfect the tissue using 10% bleach.
- [ ] Using a cork borer, take the mycelium and place it in Potato Dextrose Broth (PDB).
- [ ] Wait for the mycelia to grow on PDA.

2. Why was it important to grow the mycelia in PDA before placing it in PDB?

3. The oyster mushroom mycelia were able to colonize the entire “cake” (it looked white), and then the cakes had an orange moisture, was this concerning? Explain your answer.

4. Why do oyster mushroom growers need to wear a mask?

Mushroom Farm

Note For Instructors:

I am quite lucky that Millersville University of Pennsylvania is only an hour away from Kennett Square, the mushroom capital. I understand that not everyone will have the opportunity to do this activity but I wanted to include it, as is one my students' favorite laboratory activities.

Mushroom production is an important industry in the U.S., with a total sales value of $1.09 billion for the mushroom crop in 2023-2024. In 2024, U.S. mushroom production totaled 659 million pounds, with Pennsylvania accounting for 69% of that. Almost two-thirds of the most commonly grown mushroom species, *Agaricus bisporus* (white button mushrooms), are produced in Pennsylvania.

Growing this type of mushroom is complex, so we will be visiting a white button mushroom farm in Kennett Square called Mother Earth to learn about the process. For faculty who are interested: my contact at Mother Earth Mushroom Farm is Megan Klotzbach (meghan@organicmushrooms.com). She is one of the owners, so she is quite busy, but she is very nice, responds to emails promptly, and is very knowledgeable. My students love visiting the mushroom farm!

Learning Questions

1. Explain the process of white button mushroom production. Include in your explanation the following steps:

- Phase I: Making mushroom compost
- Phase II: Finishing the compost
- Adding the spawn
- Casing: Adding peat moss and limestone
- Pinning – Harvest
  - Flush #1
  - Flush #2

2. Organic mushroom farms only have two flushes, explain why.

Chapter Seven:
PEG Mediated Transformation of Fungi

Objectives

After completing this exercise, you should be able to:

1. Understand the importance of creating fungal mutants for medicine and food production.

2. Describe how PEG mediated transformation works.

Introduction

Molecular tools are widely used across various fields, including ecology and medicine. The transformation of fungi has become indispensable, as it enables the development of new drugs such as insulin and allows researchers to identify virulence factors and understand how fungi interact with human's immune systems. Although some fungi are human pathogens, their negative impact is often greater on plants. The ability to genetically modify fungi facilitates the development of tools to manage plant diseases. Fungi can also positively affect plant health and are studied for their roles in biocontrol, biodegradation, and nutrient cycling. Additionally, scientists have developed genetically modified fungi for industrial applications and as biofertilizers.

Procedure

1. Inoculate mycelia of Fusarium strain in 50 mL of CMC medium and incubate for 3 days at 25°C with vigorous shaking (about 150 rpm).

2. Harvest spores from CMC medium in 50 mL centrifuge tube, using miracloth and 10-15 mL of autoclaved H2O; for single spores, suspend the spores in water. Centrifuge for 10 minutes at 4000 rpm. Inoculate 1x106 spores on 50 mL YPG overnight (12-14 h) at 25°C with vigorous agitation (100-150 rpm).

3. Collect mycelia in YPG media by filtering through sterilized filter paper in a funnel. Wash with 3-4 mL of 1 M NH4Cl.

4. Add 0.6 g of ice cold driselase (Sigma D9515-5G, for *Fusarium graminearum*) or 0.35 of ice-cold lysing enzyme (Sigma L1412-25G, for *Fusarium oxysprum* and *Magnaporthe oryzae*) in 70 mL of ice cold NH4Cl (about 1%) and mix well

by vortexing. Centrifuge at 17,000 rpm for 10 minutes at 4°C. Filter supernatant, using 0.22 μm syringe filter sterilized 250 mL Erlenmeyer flask.

5. Add harvested mycelia in prepared driselase solution. Incubate for 3 hours at 30°C with gentle shaking (80 rpm). Don’t dry the mycelia.

6. After 3 hours, check protoplast and transfer enzyme solution to centrifuge tub passing through 3 sterilized plies of miracloth. Store protoplast suspension on ice.

7. Harvest the protoplast by centrifuge at 5,000 rom for 5 minutes at 4°C.

8. Discard supernatant immediately (enzyme solution can be transferred to sterilized centrifuge tube and kept at 4°C for 2 weeks to reuse driselase), and wash pellet with 5-10 mL of 1 M STC briefly. Centrifuge at 5000 rpm for 5 minutes at 4°C.

9. Discard supernatant carefully and resuspend the pellet in each tube with 2 mL of STC. Check concentration by hemocytometer. Optimal concentration is 1x107-1x108 protoplasts/mL.

10. Distribute 150 μL of protoplast suspension into sterilized glass tubes (usually 3 tubes for each experiment).

11. Mix 4-5 μg of DNA gently in 150 μL of protoplast suspension in glass tube (DNA volume should not exceed 1/10 volume of protoplast suspension). You want gDNA very concentrated but no more than 20 μL total.

12. Incubate mixture for 30 minutes on ice.

13. Add ice-cold PEG solution to mixture, 200 μL and 800 μL in order.

(notice: PEG is put in the mixture as slow as possible, flowing through the wall of the glass tube. Keep PEG in fridge.)

(notice: PEG is put in the mixture as slow as possible, flowing through the wall of the glass tube. Keep PEG in fridge.)

14. Stand for 30 minutes at room temperature.

15. Add 500 μL of STC to mixture.

16. Pour about 15-20 mL of regeneration media onto petri dish. Add 500 µL of mixture to regeneration media and mix well using pipette tip before regeneration media solidifies (3 plates from one tube).

(regeneration media must be as cool as possible, and after inoculation, mingle protoplast mixture and media uniformly)

17. Incubate overnight at 25 °C.

18. Cover the complete media with 200 µL with 300 ppm of antibiotics on top of the regeneration media.

19. If the fungus was transformed, fungal colonies will start to form from the media with antibiotics.

Note for Instructor:

This protocol is slightly modified from the one I learned during my postdoc while working with *Fusarium oxysporum* in Dr. Seogchan Kang's lab at Penn State University. At that time, I was using the Split Marker technique to knock out various genes in this plant pathogen. For the class, I provide the fungus and create some PCR constructs, but I typically follow the transformation method without any PCR constructs. My focus is on giving students hands-on experience with fungal transformation. Since I do not usually transform the fungus with an actual PCR construct, I do not perform Southern blotting. Students learn this technique in a different course called Molecular Biology here at Millersville University of Pennsylvania.

Learning Questions

Explain how to genetically modify a fungus.

Chapter Eight:
Plant Fungal Pathogens

Objectives

After completing this exercise, you should be able to:

1. Describe and explain the steps for Koch Postulate:

    - Identify disease symptoms and signs in plants.
    - Isolate the pathogenic fungus from symptomatic plants.
    - Obtain a purified culture of the pathogenic fungus.
    - Inoculate healthy plants with the pathogenic fungus.
    - Re-isolate and develop a pure culture of the pathogenic fungus from inoculated plants and compare it to the original isolate.

Introduction

Koch’s Postulates provide the foundational criteria for confirming the causal relationship between a microbe and a specific disease. These postulates were developed by German physician Robert Koch, who was awarded the Nobel Prize in Physiology or Medicine in 1905 for identifying the bacterium *Mycobacterium tuberculosis* as the cause of tuberculosis. To confirm this, he followed these steps:

- The microbe must be present in every case of the disease but absent in healthy organisms.
- The suspected microbe must be isolated and grown in pure culture.
- The same disease symptoms and signs must result when the isolated microbe is introduced into a healthy host.
- The microbe must then be re-isolated from the diseased host and shown to be the same as the original.

Despite their value, Koch’s Postulates have limitations. They may not apply if the microbe cannot be isolated in pure culture, if culturing is inconsistent, if there is no suitable animal model, or if multiple microbes contribute to the disease.

Microbes that have the genetic ability to cause disease are called pathogenic microbes. In plants, disease can be observed through **symptoms** and **signs**. Symptoms refer to physiological changes in the plant due to infection, while signs are the physical presence of the pathogen itself.

Koch's Postulates

Isolating Pathogenic Fungi From Symptomatic Hosts

1. Scout and locate symptomatic plants.

Figure 8.2 Image by Author

2. Prepare PDA plates with antibiotics. *Refer to Appendix I:Preparing Media for more information

3. Cut a 1 mml square piece of the symptomatic tissue and place it in a sterile 1.5 centrifuge tube.

4. Add 1ml of 10% bleach solution, vortex, and let it sit for one minute to surface sterilize the tissue.

5. Discard the bleach.

6. Rinse the tissue three times with sterilized autoclaved water.

7. At these point there are two options:

- Serial dilutions, which is more complicated but provides you the opportunity to isolate more fungi (Figure 8.3A).
    - Add 300 µl of sterile 0.01M phosphate buffer and macerate tissue using a sterile pestle.
    - Add 700 µl of sterile 0.01M phosphate buffer to dissolve the macerated sample.
    - Pipet 100 µl of the solution and perform a serial dilution.
    - Place 20 µl from each dilution on PDA plates.
    - Tilt the plate to evenly distribute the liquid.
- Placing the symptomatic tissue that has been disinfected directly on the PDA. This method although faster, does not encourage the growth of all the fungi found in the symptomatic tissue (Figure 8.3B).

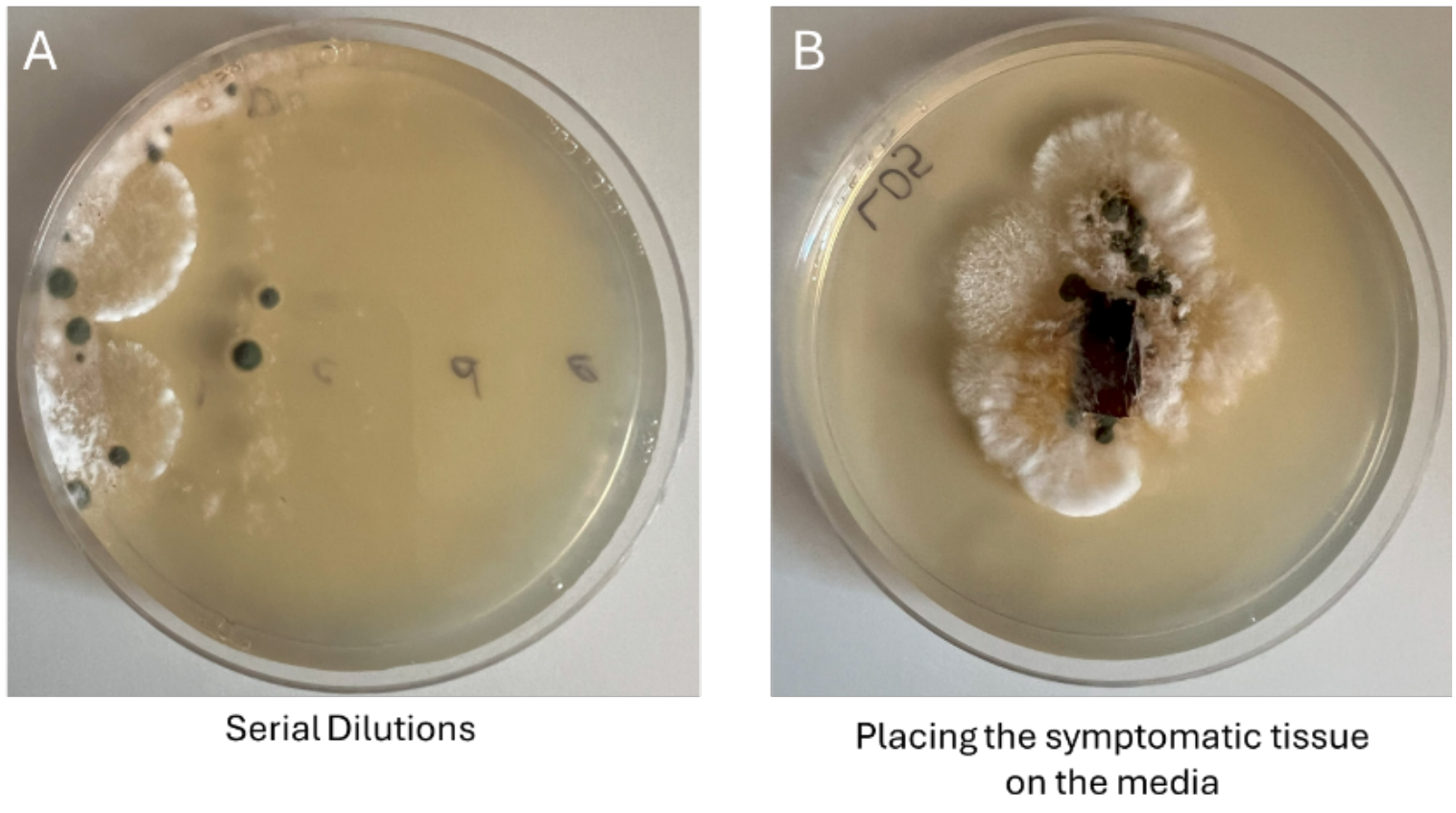


Figure 8.3 Image by Author

Obtaining Purified Culture of the Pathogenic Fungus

Obtain a pure culture of the pathogenic fungus as described in the Appendix II Obtaining Single Fungal Cultures and Measuring Fungal Growth and like you did in Chapter Five: Identification of Filamentous Fungi.

Inoculating Healthy Hosts With Pathogenic Fungus

1. Ensure that the fungal culture is pure.

2. Transfer the fungus from PDA to PDB to induce spore formation.

3. Quantify the spores using a compound microscope and hemocytometer.

4. Prepare a spore suspension with a concentration of 1X108 spores/mL

5. Design the pathogenicity assay, including:

    - A control group
    - Replicates
    - Clearly define independent and dependent variables

> **Note for Instructors:**
>
> Isolating fungal pathogens from the environment is challenging. I have some fungal pathogens that I have isolated throughout the years from here, Pennsylvania, I would be more than happy to share them. If you are outside Pennsylvania, you may require a USDA APHIS permit.

6. Clean the plastic tray with 70% ethanol.

7. Place a wet paper towel at the bottom of the tray.

8. Lay a plastic sheet over the towel to prevent cross-contamination.

9. Arrange the host sample (vegetables, fruit, mushroom) on the plastic sheet.

10. Inoculate the host tissue with 1mL of the fungal spore suspension.

11. Cover the tray with a plastic lid.

12. Store at room temperature (25°C).

13. Observe daily for symptoms and signs.

Identifying and Measuring the Disease Symptoms and Signs

Graphing Your Results

- Step 1: Review Your Data. Make sure your data includes two variables:
    - Time (e.g., hour, days, weeks, months)
    - Disease Progress (e.g., severity score, percentage affected, symptom intensity)
- Step 2: Draw the Axes. Use your ruler to draw two perpendicular lines forming an L shape.
    - The horizontal axis (x-axis) will represent Time.
    - The vertical axis (y-axis) will represent Disease Progress.
- Step 3: Label the Axes
    - On the x-axis, write "Time (units)", replacing "units" with the actual unit (e.g., "days").
    - On the y-axis, write "Disease Progress (units)", again using the proper unit (e.g., "% infected").
- Step 4: Decide on a Scale. Choose a scale that fits your data well and evenly spaces out the values.
    - Example#1: If time ranges from 0 to 30 days, mark the x-axis in 5-day intervals.
    - Example #2: If disease progress ranges from 0% to 100%, mark the y-axis in 10% increments.
- Step 5: Plot the Data Points
    - For each data point, locate the corresponding value on the x-axis (Time) and the y-axis (Disease Progress).
    - Use a small dot to mark the point where the two values intersect.

- Step 6: Connect the Dots
    - ✦ Use a ruler to draw straight lines between the points in the order of time.
- Step 7: Add a Title
    - ✦ Center a title at the top of the page.
    - ✦ Example: "Disease Progress Over Time"

Independent Two-Sample T-Test

- Step 1: Install the Data Analysis Toolpak. This is required to access the built-in T-test functionality in Excel.
    1. Open Excel.
    2. Click on the File tab.
    3. Click Options at the bottom.
    4. In the Excel Options window, click Add-ins.
    5. In the “Manage” box at the bottom, select Excel Add-ins and click Go.
    6. In the Add-Ins box, check Analysis ToolPak, then click OK.
- Step 2: Organize Your Data. Assuming you have disease progress measured at several time points for two separate groups, organize your data like this.
- Step 3: Run the Independent Two-Sample T-test
    1. Click the Data tab in the ribbon.
    2. Click Data Analysis (far right).
    3. Select t-Test: Two-Sample Assuming Equal Variances (or Unequal Variances if you suspect different variability).
    4. Click OK.
    5. Fill out the inputs:
        - Variable 1 Range: Select all values for Group A (e.g., B2:B10).

        - Variable 2 Range: Select all values for Group B (e.g., C2:C10).
        - Hypothesized Mean Difference: Leave as 0 (default).
        - Labels: Check this only if you included the header row.
        - Alpha: Use 0.05 unless you have another value.
        - Output Range: Select a blank cell where you'd like the results to appear.
        - Click OK.
- Step 4: Interpret the Output
    1. Excel will give you several statistics. Focus on P(T ≤ t) two-tail: This is your p-value. If it's less than 0.05, the difference between groups is statistically significant.

Re-Isolating the Fungal Pathogen and Confirming Koch’s Postulates

1. Once symptoms appear, extract the pathogen from symptomatic tissue using the same method utilized before to isolate the pathogen from symptomatic tissue from the field.
2. Compare the re-isolated culture with the original to confirm it is the same pathogen.

Learning Questions

1. Read the following scenario and identify at least three mistakes. Explain each one:

A man noticed that all five of his mice were behaving abnormally. They stopped eating and playing, then began acting aggressively, and their stool showed red spots. Suspecting a fungal infection, he swabbed their mouths and cultured the samples on potato dextrose agar. Multiple fungal colonies appeared, most of which were red. He then inoculated three new mice with the red mycelial colonies. These mice became ill, displaying similar lethargy but without aggression or red spots in their stool. He concluded that the red mycelium was the causal agent of the disease. To save the mice, he administered antifungal medication, and the mice recovered.

2. Identify Symptoms and Signs

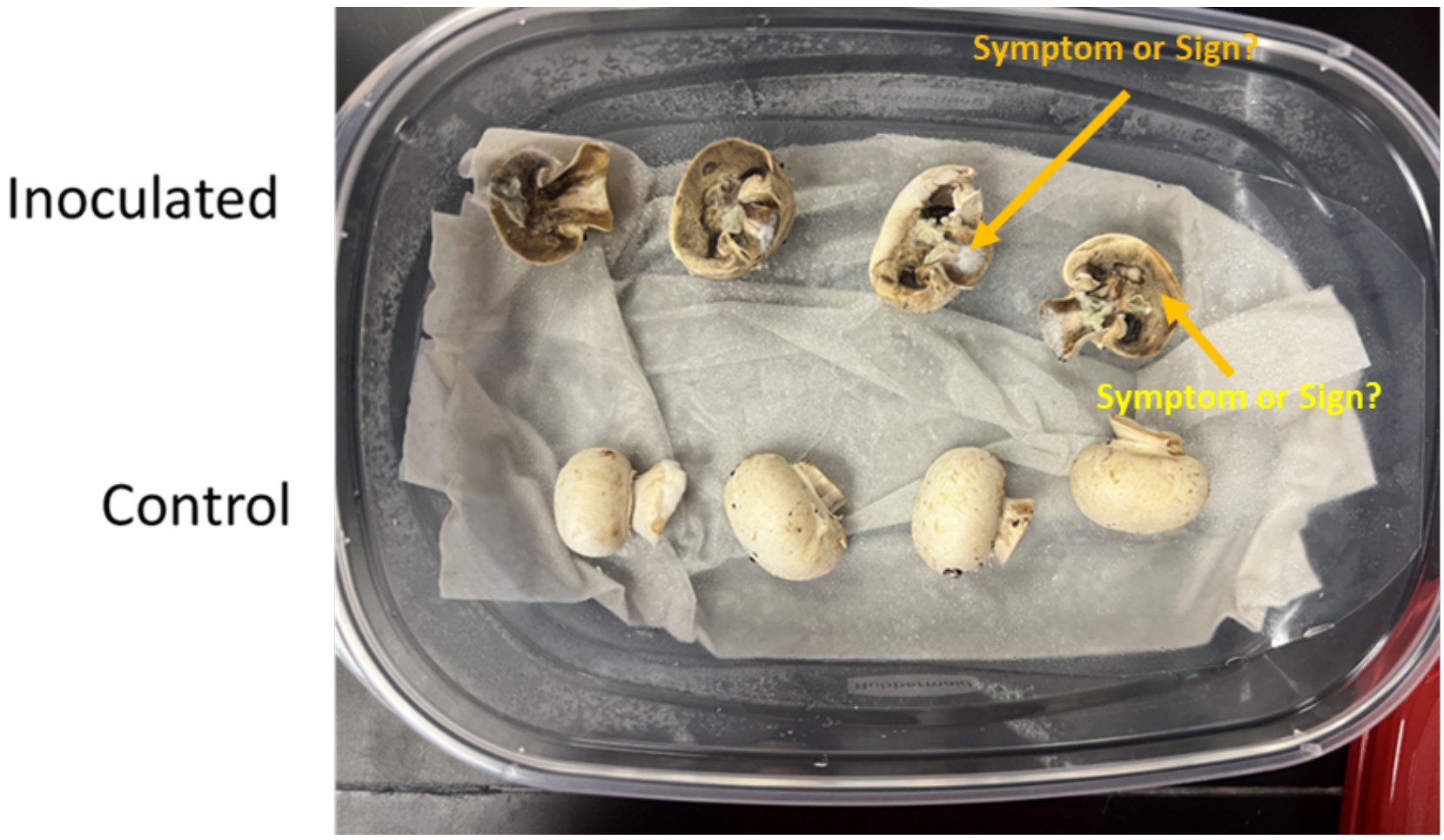


Figure 8.4 Image by Author

3. Discuss your experience completing the Koch’s Postulates with your lab mates. If you had to do it again, what would you do differently? If some of the steps didn’t work, can you provide some explanation?

Appendix I:
Preparing Media

Media is the substance in or on which microbes grow in laboratory settings. For fungi the most common types of media are Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB). Both have potato dextrose, which is a type of sugar that is a fungus-preferred food source. PDA has agar, which is a solid binder for the media, while PDB does not, allowing it to stay liquid. The procedure is similar for both.

Note for Instructor:

There are media that is specifically for growing yeast such as Yeast Extract Peptone Dextrose. In our lab we grow different fungi that grow as yeast such *Saccharomyces cerevisiae* but we usually use PDA and PDB for them, it is more economic and convenient.

Preparing Potato Dextrose Agar (PDA)

1. Dissolve 19.5g of PDA into 500mL of tap water in an Erlenmeyer flask. Heat if necessary to dissolve completely.
2. Sterilize in the autoclave at 15lbs of pressure at 121°C for 15 minutes.
3. Place the Erlenmeyer flask in a water bath with temperature 50°C, allowing the media to cool down until it is safe to hold.
4. Mix well.
5. Pour into a fresh petri plate until the bottom is just covered. Repeat until all the media is used (around 20-22 plates). Remember to mix the media well after pouring every few plates.
6. If done efficiently, the media should not solidify before you are finished; however, if necessary, heat the media to reliquefy/keep liquid.
7. Once the media has solidified for an hour.
8. Store plates upside down in the fridge at 4°C until needed.

Preparing Potato Dextrose Broth (PDB)

1. Dissolve 19.5g of PDB into 500mL of tap water in an Erlenmeyer flask. Heat if necessary to dissolve completely.
2. Sterilize in the autoclave at 15lbs of pressure at 121°C for 15 minutes.
3. Place the Erlenmeyer flask in a water bath with temperature 50°C, allowing the media to cool until it is safe to hold.
4. Mix well.
5. Pour into test tubes or flasks as needed.
6. Store tubes or flasks in the fridge at 4°C until needed.

Preparing Potato Dextrose Agar (PDA) With Antibiotics

Note For Instructors:

I usually use Kanamycin, it works well, I have never had bacterial contamination when isolating fungi from symptomatic plant tissue.

1. Mix kanamycin in water to a concentration of 50mg/ml of Kanamycin.
2. Using a 0.2 µm syringe to sterilize the antibiotic. Do not autoclave the antibiotic. Keep the sterilized in the fridge at 4°C.
3. Dissolve 19.5g of PDA into 500mL of tap water in an Erlenmeyer flask. Heat if necessary to dissolve completely.
4. Sterilize in the autoclave at 15lbs of pressure at 121°C for 15 minutes.
5. Place the Erlenmeyer flask in a water bath with temperature 50°C, allowing the media to cool down until it is safe to hold.
6. Add 500 µl of the antibiotic to the media and gently mix.
7. Pour into a fresh petri plate until the bottom is just covered. Repeat until all the media is used (around 20-22 plates). You need to work efficiently, you don’t want the media to solidify.

8. Once the media has solidified for an hour, store plates upside down in the fridge at 4°C until needed.

Appendix II:
Obtaining Single Fungal Culture and Measuring Fungal Growth

To characterize a fungus, you need to obtain a single fungal culture of the fungus. Among the most common characterizations of the fungus is to measure the speed of their hyphal growth. Fungal hyphal growth is circular; the circle of hyphae grows exponentially every day until the hyphae reaches the end of the petri plate.

Obtaining a Single Fungal Culture

Take a cork borer and an inner piece. Place the inner piece inside of the cork borer and disinfect both by dipping them in 70% ethanol. Then, while holding them from the top, place them on the Bunsen burner until all the ethanol has evaporated. Wait for 10 seconds to cool both utensils. If you want to ensure that the cork borer and the inner piece are cold, you could touch the media and verify that it doesn't sizzle.

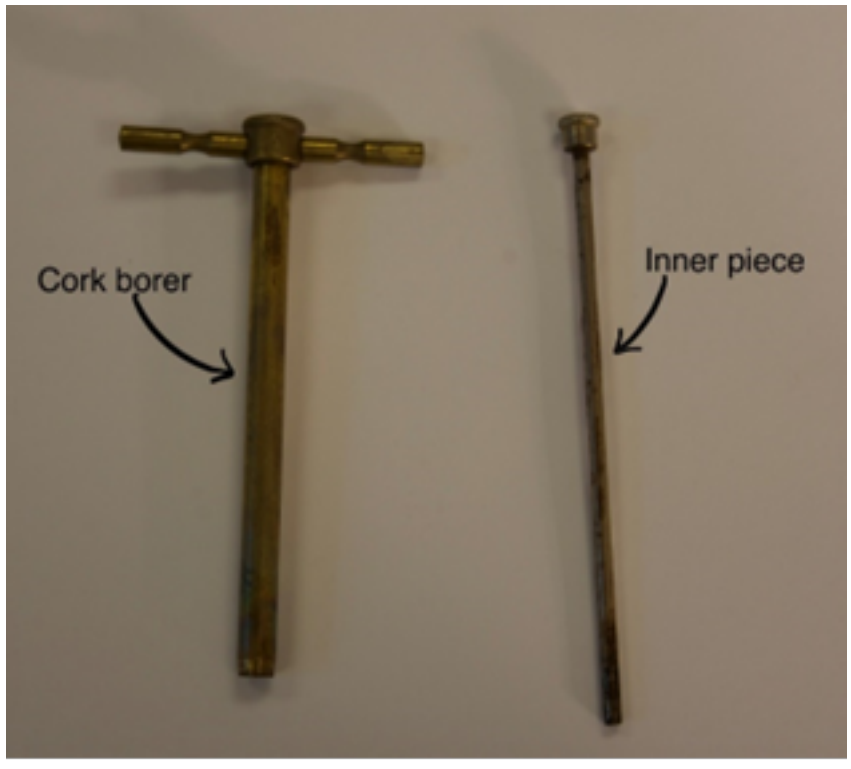


Image by Author

1. Disinfect the area that you will be using with 70% ethanol.

2. When working with the petri plates, do not remove the lid to work on the media, keep the lid and open the lid to only allow a 45 degree space. This way you keep the media on the plate to get exposed to the environment.

3. Pull up the inner piece from the cork borer so that you don’t see the tip of the inner piece at the bottom of the cork borer.

4. Push the cork borer down at the edge of the fungal colony where the mycelium is actively growing.

5. Push out the mycelia with the inner piece on top of the new media plate. Make sure that the mycelium is touching the media; otherwise, the fungus will not grow.

Measure the Fungal Growth

1. Place a dot at the center where the fungus was placed.

2. Each day, place four lines where the fungi have grown.

3. Measure the growth of the fungus until the hyphae completely covers the whole plate.

4. Measure the radius of the fungi per day using millimeters for more accurate measurements.

5. Calculate the average of growth in millimeters per day.

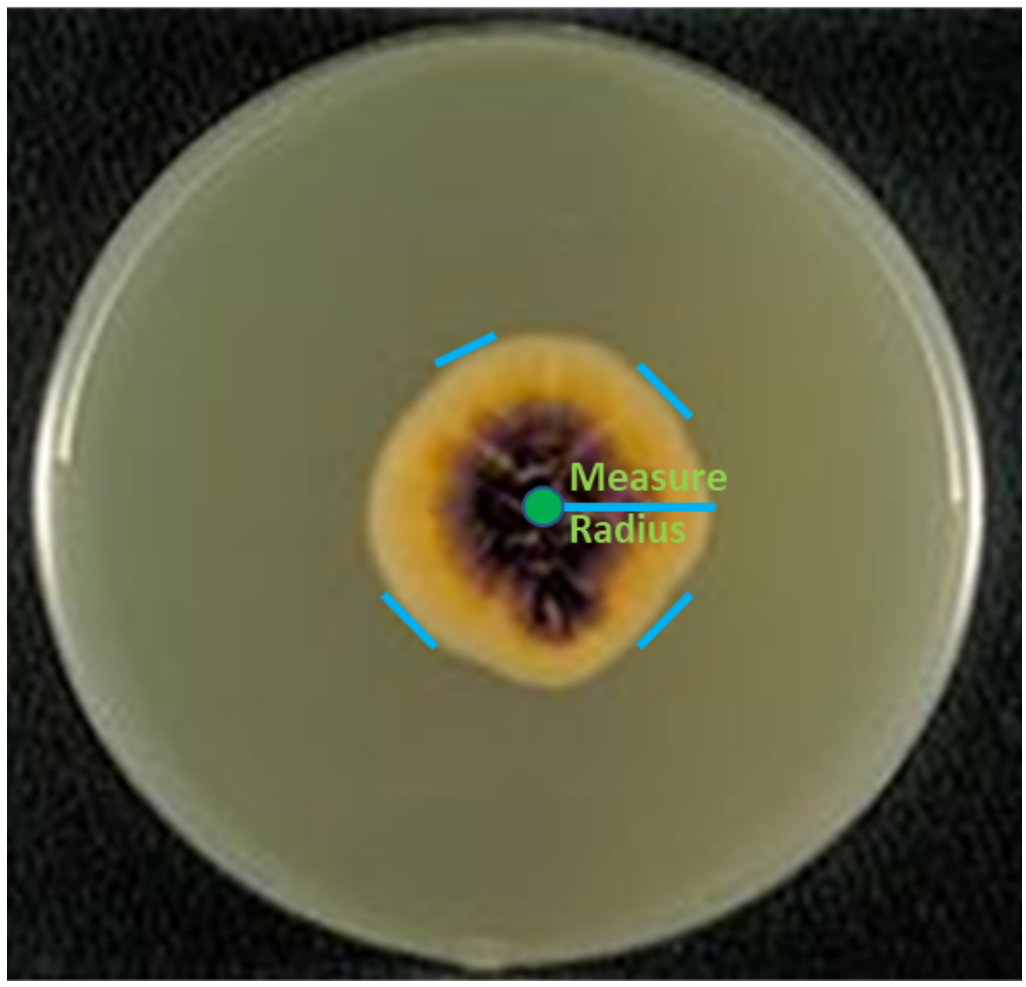


Image by Author

Appendix III:
Using the Compound Microscope

1. Once you have prepared the microscope slide:

2. When placing the microscope slide on the microscope stage, ensure that the light coming from the bulb is hitting the sample on the slide.

3. Use the course and fine knob to find the sample using the 4X objective lens.

4. Once you find the sample, switch to the 20X objective lens and using the fine knob, focus on the image.

5. Once you focused on the sample, switch to the 40X objective lens and use the fine knob again to focus on the image.

6. If you lose your sample at some point before reaching the 40X objective lens, go back to objective 4X and start the whole process again. Do not try to find the sample using the 20X or the 40X objectives lenses, it will be very difficult, and you might get dizzy.

7. Before moving to looking at the sample using the 100X objective lens, place a very tiny drop of oil on top of the sample on the microscope slide, where the light is passing through. Then move the 100X objective lens and use the fine knob to focus on the image.

If you lose your sample after reaching the 100X objective lens, unfortunately you cannot go back, none of the other microscope objective lenses can be exposed to oil.

Note for Instructors

You can see fungi using the 40X objective lens, but when seeing yeast cells under the microscope, I usually have the students using the 100X objective lens. For two reasons, so that the they get the experience of using the 100X objective lens and because the yeast cells look amazing under that magnification.

Appendix IV:
Using the Micropipette

Micropipettes have a P number that tells you about the max amount in µL you can use it for. Micropipettes can go to only to 10% of what the P number indicates. For example, a P1000 can handle from 1000 microliters (µL) to 100 µL, while a P20 can handle from 20µL to 2µL.

On the top of the pipettor, there will be two things you can push: one plunger in the very middle that will take/push out your liquid and one tip ejector. The plunger will have two stops that help inform you when to stop pushing it down. Regarding the plunger:

The first stop tells you that you have pushed the plunger down far enough that when you allow it to go back up, the vacuum will pull up the set amount of liquid.

The second stop is when you want to remove the liquid from your tip, and it can be felt just by pushing all the way down.

It is important to point out that different brands of micropipettes have different digits spaces. For example, some of the P1000 micropipettes have four space digits and some have three space digits. The micropipette with space for four digits is easier because the highest number is 1000 which a four-digit number, but some micropipettes have space for three digits, which when you see 100 is actually1000 µL.

Image by Author

Make sure you push the plunger down to the first stop before introducing the tip inside the liquid; otherwise you will get bubbles.

Make sure you take the liquid slowly and release the liquid slowly.

Appendix V: Transcript of “Tutorial 03: Entering Records”

New entries into Nature Atlas. The first thing we need to do is open up a web browser on a smartphone, a laptop, or a desktop computer. I'm using my iPhone today and what you can see is that I've created a shortcut on my home screen for the Nature Atlas website. I'm going to click on that; it shows me a loading page because I'm not logged on. When that opens, this is basically what Nature Atlas looks like on a smartphone screen; to share a new record using this button right here, you need to be logged in and there's another tutorial on how to register for an account and how to log in. This is not that tutorial so it's going to presume that you've already registered for an account; when you actually click and try to make a new entry it'll prompt you to log in, so I'm going to type into this box and I'm going to use the save credentials and go back up and log in. So, at this point it reloads the page with your login credentials and when I come back down here, you're going to see that I now have the option of making a new entry and there's three options I'm going to have. The first is to make a new entry without a photo, the second one is to make one with the photo that I take with the smartphone right here now, and then the third option, which is the most common, is to make a new entry by using photos that you browse for in your photo library. Let's go here first so we can see what the layout of the new record entry form is. Up at the top a new entry form says new record, below that there's two spaces for photos, up to two photos. The first would be the primary photo, the second would be the secondary photo if you choose to upload one of those. A secondary photo would be another photo that perhaps shows a different angle or a closeup of something that was in the first photo of the organism. If you want to load a photo here, you could do that by browsing using that browse icon and you could also click that camera icon and take a photo. But right now, I'm going to go back and show you the rest of the fields. So, down here we have text fields, and the red fields are those that have to be made before submission, Nature Atlas requires those, and then the green fields are those that are optional. Now if you scroll down just a tad more there's a little Advanced toggle that opens more advanced fields that you could enter, should you want to but that's beyond the scope of this tutorial. The number of fields you enter, the level of detail, is really dependent upon your own objectives. So, I'm going to cancel out of this and go back and let's make a real entry now, using photos that I've previously taken. The first one I want to enter is this fungus right here, so I'm going to click on that and to go up here and click done. And what you'll see is that here's that fungus it's the primary photo and down here these white fields were populated

with data from the photo itself. So, the first set of data that came with the photo was the location and geo coordinate because I took this photo with the smartphone that had the GPS enabled, it actually recorded that location in the metadata from the photo, it also recorded the date that I took that photo, July 24th, 2022. Also recorded the level of precision with which these geo coordinates are known for that photo, so that's quite a lot of information there. Now, actually another bit of information that came with the photo is altitude, you can see that this is saying that it was taken at 4 meters above sea level. This is actually something that biologists tend to like because wild and cultivated organisms really are strongly influenced by the altitude, some species are good at high altitudes and can grow well there, and some species do not, and they grow better or have habitat preferences at low altitudes. You can always click into this box, and you can delete that if you don't want it but for wild and cultivated organisms it's something that I like to keep at the very least. Okay, let's go back up here and take a further look at what we need to do here. So, one thing I like to do is I like to click on that and just check that location because sometimes the GPS on a phone is not very accurate. This is down in Surfside South Carolina and that red marker is showing where the location of that photo was recorded and actually that's extremely accurate, so I like that so I'm going to cancel out of this and be happy with that. But one thing I do want to do is add a second photo, something that adds a different look to the fungus and so that's from the top view so I'm going to click on done and so now I've got the side view and the top view, and I think they complement one another. Now coming down here I need to finish the red fields, so I'm going to type the plant, the organism as a plant, as a fungus; I need to indicate the wild status of this particular organism, and so this was found in the wild, this was even though it was in a garden bed it was actually you know a fungus that was growing wild there. And then, down here these are all optional, but I always like to at least get a name in there and so I do know the name of this fungus, it's the false parasol, so I've typed that in right there into the common name field and I don't currently know the scientific name but because I've got the common name I might be able to do a little Wikipedia search and find the scientific name. And so, this little Wikipedia icon next to the common name header will take whatever you've typed into the common name field and do a Wikipedia search on that. And sure enough, here's a little Wikipedia article on that particular species. And when I scroll down, I can find the binomial name and the taxon box of the Wikipedia article. So I'm going to select that copy it, I'm going to take a note of the family name and then I'm going to go back and I'm going to paste that species name in. And then down here in the family name field, I might as well do that since I've got it, and what you see down here I've got a little auto completion suggestion. So, I'm going to

come back up here, and I'm going to select it. Now down here you could enter the description of the organism or object you know that would be the fact that it was white that it was growing about 15 cm tall, the level of detail depends on your goal, down here you could further describe the location such as in a mulch bed next to an apartment complex; but I'm going to leave these blank here just to keep this tutorial short. And I'm going to click done and come down here and click submit. Okay, so what happens is the search pane closes to show you where you've submitted that record; so, I'm going to zoom into that and sure enough that's exactly where I've put it the black dot inside that red marker indicates that there's a photo associated with that so I'm going to click on that and you can see there's my photos and here's all the information. So, I'm going to come up here and then close this. So now I want to enter a second record, and I'll come down here and I'm going to browse for a photo, from the photo library, and this first record that we entered was a wild fungus. The second one I want to enter as an example is a cultivated plant. So, I'm going to come up here and select this little witch hazel. The first thing I'll do is I'd like to check the location that was stamped with that photo. I'm going to zoom in here and this is showing that red marker right on the campus of Millersville University, but I happen to know that that plant was growing right along the west side of Brosman Hall right here, so I'm actually going to click right there and it's asking me if I want to move that marker location, I say yes, you notice I've moved it. At this point, you I could move it all around, you know, if I wanted to but I'm going to move it right back there, and because I've moved it I need to come up with a new precision estimate and so that's I'm pretty sure that that marker is placed within 2 to 10 meters of where it was. So now that I'm happy with that location, I'm going to click confirm, and this information has now changed. Now to complete the entry I need to indicate that this is a plant on the type field, for the wild status field I need to indicate that this was not a wild plant, but it was a cultivated plant. Then down here I'm going to take this opportunity to show you how you could actually use the photo to try to get an inkling for what you've got here. So I'm going to click and hold this photo and you can see that down here I've got this lookup capability here, and this isn't always available when you press and hold the photo but in this case it is because presumably, my operating system thinks it's got some fairly good detail to get an idea on that so I'm going to click look up. It's identified it as a plant and specifically there's a couple of options, one it's just a witch hazel, it could be any one of several species but down here it's actually give me a specific estimate of the identity and so it's the common witch hazel. I can click on that, and click further and I can kind of scroll around and read about that and convince myself if I've got the correct identification or not; and once I feel that I have, I can press and hold, I can copy that, and I can close this up, go

back there and I can paste that in Hamamelis virginiana, the common witch hazel. At this point I'm going to type into the common name field, witch hazel, and I can also get the family name for this if I didn't remember it from the Wikipedia article, but I do know that it was Hamamelidaceae; here is the auto completion. Not everything will be in the auto completion suggestion, this is only the common families that are in there, so just because you're typing in a family name and it's not in the auto suggestion, doesn't mean that it's not correct, it's just the common ones that it makes suggestions for. I'm going to leave the object description blank for now, I like the 104 meters above sea level, I think that's still relevant to a cultivated plant. I could add more details, such as you know, on the west side of Brossman Hall, and I could indicate that this is Millersville University. You know this is information that they would not have come with the metadata of the photo, the only thing that comes with the metadata of the photo are the coordinates, state, county, municipality. So, at this point I'm pretty happy, with that I'm going to submit that. Again, it closes this pane it shows you where you've made your entry, you can zoom in on that, and then you can click on that. And what you see is that you've got the one photo that you uploaded right there. Now right then and there if you decided you wanted to edit this it's got that edit button and you could go in and you could add you know for example a second photo if you forgot to do it. I might even do that because you can see I've got a closeup of the flowers and the fruits; and so here is right there and I'm going to go back up and you know everything else is it's fine and dandy but I'm going to go back up and click update. Okay, so now I've just added a second photo going to open this back up. I'm going to make one more entry, and this second or the last entry is some freshwater prawns that I cooked up a few years ago, it was the first time I've ever had this species, and it was quite tasty. So, I'm going to click on that, and what's interesting about this photograph is I took it with an SLR camera that didn't have GPS capability, so you notice that there's no location date or precision that comes with it. So, I'm going to have to do this myself, so I'm going to type into this field, and I'm going to go and try to you know find that location on the map by panning and zooming. Another option is to go into the gazeteer box right there and type in the name of the location. In this case this was at a place called the Asian Supermarket, Asian Center Supermarket, and so I did a little search on that; I'm going to zoom in to the part of the market where the seafood section was; I'm going to come down here and indicate a level of precision that I think I'm at which is 2 to 10 and then I'm going to come down here and hit confirm. Okay good, so I've got the precision, I've got the location right there, and now I need to enter the date there's a little date picker app right there and that was in 2017; it was June, and it was June 27th. So now I've got all that information, I need to finish the entry.

These prawns are invertebrates, wild status; these were not wild prawns, cultivated, or captive prawns at this location. They are something that were sold in a market in the seafood section so in the language of nature Atlas they're called ethnobiological objects that's the appropriate wild status so I'm going to click that, and now it's there. Down here I'm going to type in freshwater prawns, actually they're called giant freshwater prawns, there we go. And I don't know the scientific name on that but I can actually I do so I'm going to type into that Macrobrachium, oh didn't spell that right, macro brachium and look it's giving me some autocomplete suggestions and that second one is it, Macrobrachium rosenbergii okay, at this point you can enter as much or as little of the information as your objectives suggest. You could get the family name from that species page if you want it to, but I'm just going to cut this short right now, and I'm going to click submit. And at this point I'm going to click on that and sure enough there's my kids holding their freshwater prawns before cooking them and that is how you make entries into Nature Atlas.

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