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Characterization of Worm Capture by Nematophagous Fungi: "Real" Research in the Classroom*

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Discovery driven experiments in undergraduate laboratory courses have been shown to increase student learning and critical thinking abilities. This workshop focuses on a lab module involving the nematophagous fungus, *Arthrobotrys oligospora*, an ecologically relevant organism with potential use as a pest control agent in agricultural settings, and its ability to capture the nematode *Caenorhabditis elegans*. The goals of this module are to enhance scientific understanding of the regulation of worm capture by soil dwelling fungi and for students to attain a set of established learning goals. Groups of four students are provided with the experimental background and conduct their own literature search to identify a variable that may affect the efficiency of *C. elegans* capture. Students develop a hypothesis and conduct an experiment to compare worm survival in the control versus variable condition, writing a lab report in the format of a primary research article. From this experimental module, students were able to produce results that agree with published data as well as add to the existing literature, while demonstrating positive gains regarding the learning objectives.
Workshop attendees will be introduced to nematophagous fungi, design potential experiments that their students could perform using the established module, and get hands on experience with the organisms and experimental protocol. In addition, we will discuss how to incorporate this module into the lab curriculum at attendees' institutions along with potential means of assessment to measure student learning.

Keywords:hypothesis construction, nematophagous fungi, *Caenorhabditis elegans*, primary literature

Introduction

The nematophagous fungus, Arthrobotrys oligospora, is an ecologically relevant model organism capable of capturing parasitic and non-parasitic nematodes. It has been estimated that parasitic nematodes cause considerable damage to both crops and livestock (Sasser and Freckman, 1987) and traditional methods to combat this problem, including antibiotics and pesticides, can have negative side effects.

Trapping of parasitic worms involves multiple steps, including trap formation, attraction of worms to the newly formed traps, worm ensnarement, and worm penetration to harvest the nematode for nutritional purposes (Yang et.al, 2011; Niu and Zhang, 2011). While work has been done to examine the various steps of worm capture, there is still much unknown, providing an excellent opportunity for undergraduates to contribute to the scientific literature. A key component of this module is for the students to design their own experiment by selecting a variable to introduce to the protocol, constructing a hypothesis regarding this variable,and writing up the collected data in the format of a lab report.

This experiment was designed for an upper division microbiology lab at the University of California, Irvine. It requires three weeks of class, the first to introduce the background information to the class and allow the students an opportunity to conduct a literature search to design their experiment. The second week in lab is a trial run of the protocol to identify potential problem areas, and for the instructor to determine which of the student variables can realistically be performed in class. Finally, the students test their variable or an instructor-selected variable during the third week of the module.

Figure 1 illustrates a brief outline of the protocol. The same protocol is utilized in weeks 2 and 3 of the module, although worm collection times may vary.

*This report is an abbreviated and updated summary of work previously published (Sato, 2013). For more information, please reference this paper of contact the author.

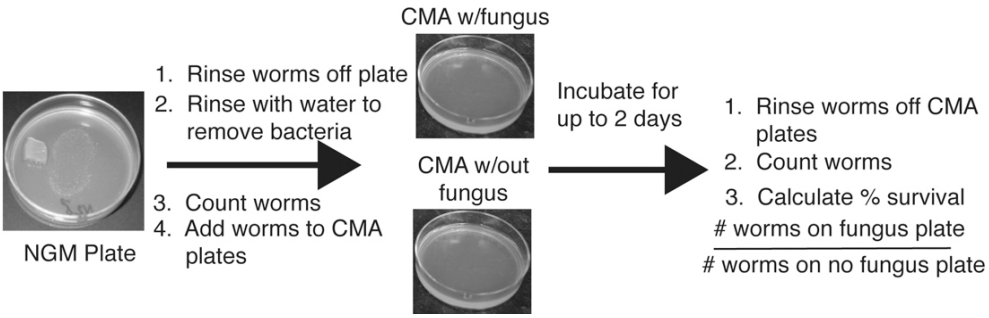

Figure 1. Protocol for nematophagous fungi experiment.

Student Outline

Week 1: Streak CMA Plates for the Nematophagous Fungi Experiment

Purpose

To investigate whether a specific variable affects worm capture by *A. oligospora*.

Materials

2 Cornmeal agar (CMA) plates (per pair)

The nematophagous fungi experiment will be performed twice, once in week 2 to familiarize yourself with the techniques and again in week 3 to examine a variable and how it affects *C. elegans* capture. For information regarding the protocol, turn to week 2 in your manual. While no experiment will be performed this week, you will streak 1 CMA plate with fungus in preparation of next week's experiment.

Arthobotrys oligospora grows much more slowly than the bacteria you have been working with and requires roughly a week to be at the necessary density. While you only need 1 plate next week, you will streak 2, so both you and your partner get an opportunity to streak.

Protocol

1. Use a sterile loop and pass the loop twice on a plate containing fungus.
2. Streak half of the fresh CMA plate. Your job is to cover the entire half of the plate, you are not attempting to isolate single colonies (this fungus does not grow in colonies).
3. Acquire two more loopfuls of fungus and cover the other half of the CMA plate.
4. Tape your plates together and stick in the bag that your instructor has set up. These plates will be incubated at room temperature until next week.

In addition to preparing for the experimental protocol, you will also be involved in the design of the experiment. Each group of four will conduct research regarding nematophagous fungi and select one variable to test in lab. This will be submitted using the form on the following page, and will include a hypothesis, supporting information regarding your hypothesis, and details regarding the experiment set-up. Unfortunately, all variables cannot be accommodated, and five variables will be selected to be tested by the class. Your group's proposed variable can be previously published or novel, although preferential consideration (and extra credit) will be given for those who submit an innovative design.

Fungus Variable Worksheet

Group members: TA:

Variable: Hypothesis:
Background info to support hypothesis:

Reference(s):

How to test the variable using the nematophagous fungi protocol in the manual (include specifics, such as the concentration of solution to add, the composition of media to use, etc):

Week 2: Begin Nematophagous Fungi Experiment #1

Purpose

To measure the fungus dependent capture of *C. elegans* after 48 hours of co-incubation.

Reagents

2 Corn Meal Agar Plates (1 with fungus, 1 without)
1 Nematode Growth Media Plate with *C. elegans*
Pipettors
Eppendorf Tubes
Water

Media

Corn meal agar (CMA) is the growth media for *A. oligospora*. You streaked the CMA with fungus plate last week. In addition, you will be using a CMA plate lacking fungus. Nematode growth media (NGM) is the growth media for *C. elegans*. *E. coli* is added to the plate prior to worm addition. *C. elegans* are added to the plate 4-6 days prior to use in the experiment.

Notes

1. Rinse carefully. Let the worms settle for at least 5 minutes after centrifugation and do not disturb the pellet when removing the water try to minimize worm loss. Pipette or invert gently to avoid worm death.
2. Mix the worm solution at all steps. If the volume is 500uL or greater, you can do so by inverting the tube.
3. If there are too many worms to count under the microscope, be sure to dilute the worm solution further.

Procedure

The protocol involves the addition of *C. elegans* to plates with and without fungus. The "no fungus" plate is a control condition to take into consideration worms that die naturally during the course of the experiment. At set times, worms are rinsed off of the control and fungus plates and are counted. A percent survival value is then calculated:

Percent Survival = Worms on Fungus Plate / Worms on No Fungus Control Plate

Prior to adding the worms to the fungus plate, a rough count is taken to approximate how many *C. elegans* are added to each plate. We want to add roughly 2,000 worms to the fungus and control plates.

In Lab: Worm Addition to Fungus Plates

1. Remove the agar block on the NGM plate with a sterile pipette tip. Dump in the biohazard waste.
2. Wash NGM plate containing *C. elegans* with 1mL water (rinsing plate a few times using a P1000) and add liquid to a 1.5 mL eppendorf tube. Repeat once and combine in the same tube.
3. Centrifuge the eppendorf tube at 500xg for 1 minute. Let the tube sit on the bench for 1 minute to allow worms to settle.
4. Remove most of the water. Keep the pipette tip at the meniscus as you remove liquid to keep from disturbing the worm pellet at the bottom.
5. Add 1mL water to the tube and invert to mix. This wash step helps to remove the *E. coli*.
6. Centrifuge eppendorf tube at 500xg for 1 minute. Let the tube sit on the bench for 1 minute to allow worms to settle.
7. Remove most of the water but be careful to not disturb the pellet.
8. Add water to a final volume of 500µl. Mix by inverting the tube.
9. Pipette one 5µl aliquot on a microscope slide to gauge the worm concentration. If there are too many to count, add more water to the tube, mix and check another 5ul aliquot. Up to 75 worms per drop can be accurately counted.
10. Count the first aliquot and then pipette 4 more on the microscope slide.
11. Make an additional slide with 5 aliquots.
12. Find the average number of worms in the 10 aliquots and divide by 5 to determine the average number of worms per µL solution.

13. Calculate the volume required to obtain 2,000 worms. Mix the tube by inverting and pipette the necessary volume to the center of both plates (plus fungus and minus fungus). This volume should not exceed 400µL. If it does, you will want to concentrate the solution by allowing the worms to settle and removing some water.

15. Incubate plates at room temperature.

In Discussion (48hr later): Examination of Worm Survival

You will now rinse the worms off of the fungus and control plates. You will then count the number of worms on each plate and compare to determine the percentage of worms killed by the fungus.

1. For each plate, wash with 1mL water, rinsing the plate multiple times with the P1000. Add the liquid to a 1.5mL eppendorf tube. Repeat once. Rinse gently so you do not dislodge the fungus.
2. Centrifuge eppendorf tubes at 500xg for 1 minute. Let the tubes sit on the bench for 5 minutes to allow worms to settle.
3. If a worm pellet is observed, remove all but 500µL of the water. If no pellet is visible, remove all but 100µL. Be sure to keep track of these final volumes. Generally, the no fungus tube will be resuspended in 500µL and the plus fungus tube will be resuspended in 100µL. Keep the pipette tip at the meniscus as you remove liquid to keep from disturbing the worm pellet.
4. Resuspend the worms by gently pipetting up and down. Pipette ten 5µL aliquots on two microscope slides and count the number of live worms in each aliquot. If there are too many worms to count, dilute the solution. Be sure to keep track of the new volume.
5. Find the average number of worms per aliquot and divide by 5 to get the number of worms per µL.
6. Normalize the volumes (ex. If one solution was 500µL and one was 100µL, you will need to divide the 100µL solution concentration by 5).
7. Determine the percent survival. Percent survival will be calculated as worms on fungus plate divided by worms on no fungus plate.

Week 2: Streak Cornmeal Agar Plates with *A. oligospora* for Nematophagous Fungi Experiment #2

In Lab

As you did last week, we will give the fungus one week to grow on the CMA plates. Use the 2nd fungus plate you streaked (the one not used in this week's experiment) to streak these new plates.

Next week, each lab section will test five variables:

[insert specific descriptions regarding the variable here]

Variable A –

Variable B –

Variable C –

Variable D –

Variable E –

Week 3: Nematophagous Fungi Experiment #2

Purpose

To test how a specific variable alters the ability of A. oligospora to capture C. elegans.

Materials

4 CMA plates (2 with fungus, 2 without)
2 NGM plates with *C. elegans*
Pipettes
Eppendorf Tubes
Water

This week you will use the same protocol as the previous week, except in an expanded capacity. Instead of the control condition only, you will examine a variable to compare to the control.

Regardless of the changes, the protocol itself is the same. You will first rinse worms from an NGM plate and add them to the various plates used in the experiment. In the next lab period, you will remove worms from all four plates to calculate percent survival. Follow the flow chart (Fig.2):

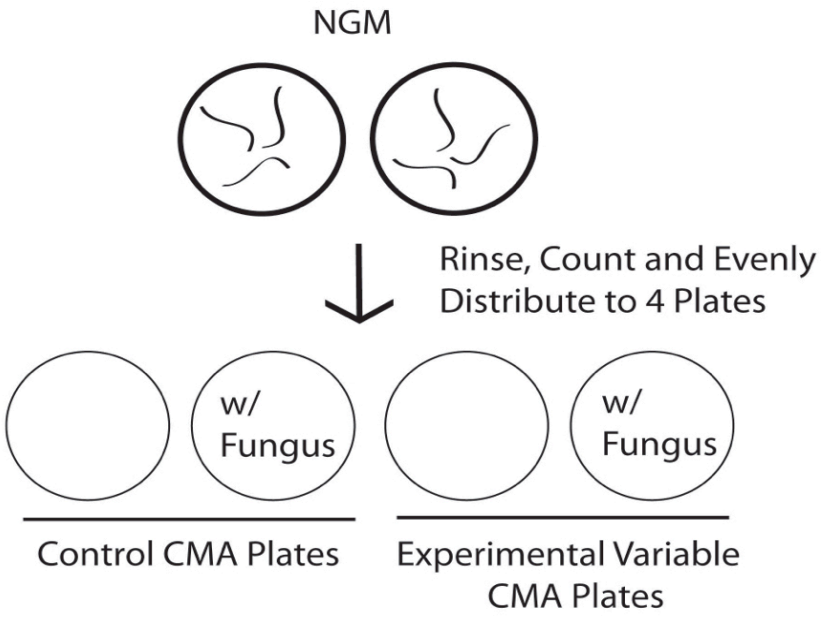

Figure 2. Flowchart for Experiment #2.

Notes

1. Rinse carefully. Let the worms settle for at least 5 minutes after centrifugation and do not disturb the pellet when removing the water try to minimize worm loss. Pipette or invert gently to avoid worm death.
2. Mix the worm solution at all steps. If the volume is 500uL or greater, you can do so by inverting the tube.
3. If there are too many worms to count under the microscope, dilute the worm solution further.

In Lab: Worms Added to Fungus Plates

1. Remove the agar block on the NGM plates with a sterile pipette tip. Dump in the biohazard waste.
2. Wash NGM plate containing *C. elegans* with 1mL water (rinsing plate a few times using a P1000) and add liquid to a 1.5mL Eppendorf tube. Repeat once and combine in the same tube. Repeat this for the second NGM plate.
3. Centrifuge Eppendorf tubes at 500xg for 1 minute. Let the tubes sit on the bench for 1 minute to allow worms to settle.
4. Remove most of the water. Keep the pipette tip at the meniscus as you remove liquid to keep from disturbing the worm pellet at the bottom.
5. Combine worms from both tubes into a single tube using a P1000 tip at this time.

6. Add 1mL water to the worm pellet and invert tube to mix. This wash step helps to remove the *E. coli.*
7. Centrifuge eppendorf tube at 500xg for 1 minute. Let the tube sit on the bench for 1 minute.
8. Remove most of the water without disturbing the worm pellet.
9. Add water to a final volume of 1mL. Mix by inverting the tube.
10. Pipette one 5µL aliquot on a microscope slide to gauge the worm concentration. If there are too many to count, add more water to the tube, mix and check another 5µL aliquot. Up to 75 worms per drop can be accurately counted.
11. Count the first aliquot and then pipette 4 more on the microscope slide.
12. Make an additional slide with 5 aliquots.
13. Find the average number of worms in the 10 aliquots and divide by 5 to determine the average number of worms per µL solution.
14. Label CMA plates. This will vary depending on the particular experiment (number of time points being examined, number of conditions tested).
15. Calculate the volume required to obtain 2,000 worms. Mix the tube by inverting and pipette the necessary volume to the center of both plates (plus fungus and minus fungus). This volume should not exceed 400µL. If it does, you will want to concentrate the solution by allowing the worms to settle and removing some water.
14. Incubate plates at room temperature.

In Discussion (from 6 - 48 hr later): Examination of Worm Survival

You will now rinse the worms off of the fungus and control plates. You will then count the number of worms on each plate and compare to determine the percentage of worms killed by the fungus.

1. For each plate, wash with 1mL water, rinsing the plate multiple times with the P1000. Add the liquid to a 1.5mL Eppendorf tube. Repeat once. Rinse gently so you do not dislodge the fungus.
2. Centrifuge Eppendorf tubes at 500xg for 1 minute. Let the tubes sit on the bench for 5 minutes to allow worms to settle.
3. If a worm pellet is observed, remove all but 500µL of the water. If no pellet is visible, remove all but 100µL. Be sure to keep track of these final volumes. Generally, the no fungus tube will be re-suspended in 500µL and the plus fungus plate will be re-suspended in 100µL. Keep the pipette tip at the meniscus as you remove liquid to keep from disturbing the worm pellet.
4. Re-suspend the worms by gently pipetting up and down. Pipette ten 5µL aliquots on two microscope slides and count the number of live worms in each aliquot. If there are too many worms to count, dilute the solution. Be sure to keep track of the new volume.
5. Find the average number of worms per aliquot and divide by 5 to get the number of worms per µL.
6. Normalize the volumes (e.g., If one solution was 500µL and one was 100µL, you will need to divide the 100µL solution concentration by 5).
7. Determine the percent survival for all conditions. Percent survival will be calculated as worms on fungus plate divided by worms on no fungus plate.

Materials

Equipment

- Micropipettors (P20, P200, P1000) – one set per group of four
- Bench top centrifuge – one per section of 20 students
- Compound or dissection microscope – one to two per group of four

Organisms and Media

- Cornmeal Agar (CMA) – for *A. oligospora* growth
- CMA Recipe
  - Add 17g Cornmeal agar (Becton Dickinson #211132) and water up to 1L.
  - Autoclave for 20 min. Pour into 3cm petri dishes
- Fungus inoculation
  - CMA plates seeded with fungus one week prior to experiment
  - Accomplished by streaking with a sterile metal loop/ wooden stick
- *A. oligospora* can be obtained from ATCC (#24927) and can be frozen according to ATCC (http://www.atcc.org/~/media/PDFs/Technical%20Bulletins/tb02.ashx).

Nematode Growth Media (NGM) – for *C. elegans* growth

NGM Recipe

- Add 23g Nematode growth media (Bioworld #30620040) and water up to 1L.
- Autoclave for 20 min. To this, add:
  - 1mL 1M $MgSO_4$
  - 1mL 1M $CaCl_2$
  - 25mL 1M phosphate buffer pH6 (KH_2PO_4/K_2HPO_4)

  Pour into 3cm petri dishes

C. elegans Inoculation

- NGM plates seeded with 50µL of an overnight *E. coli* (OP50) culture in Luria Broth
- *C. elegans* added to the NGM plate four days prior to the experiment
- With a sterile tool, cut a block of agar from an old NGM plate and transfer to a new plate
- *C. elegans* from previous plate crawl off of agar block, eat bacteria and reproduce
- *E. coli* (OP50) can be obtained from the Cold Spring Harbor Laboratory (silencinggenomes.org) for free and can be frozen according to instructions from Thermo Scientific (http://www.thermoscientificbio.com/uploadedFiles/Resources/preparation-ecoli-culture-glycerol-stocks.pdf).
- *C. elegans* wild type strain (N2) can be obtained from the Cold Spring Harbor Laboratory (silencinggenomes.org) for free. *C. elegans* can be frozen according to instructions from Worm Book (http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html). Worm Book is also an excellent source for any questions regarding *C. elegans use.*

Reagents per Experiment

Week 2 Practice Experiment (performed in pairs)

Media (per pair)

1 NGM plate with *C. elegans*
1 CMA plate without *A. oligospora*
1 CMA plate with *A. oligospora*

Instructors will streak fungus on CMA plates a week before the module begins (four students will share one plate). During week 1, each pair will streak two new CMA plates. One of these will be used for the week 2 experiment while the other will be used to streak plates for week 3.

Instructors will add worms to one new NGM + E. coli plate (per pair) roughly four days prior to the start of the week 2 experiment.

Equipment (per pair)

- Micropipettor tips – roughly ½ box of P20/P200 tips, and ½ box of P1000 tips
- Microcentrifuge tubes – 4
- Microscope slides – 4
- Sterile water – 10mL

Week 3 Experiment (performed in a group of 4)

Media (per group of 4):

- 2 NGM plates with *C. elegans*
- 2 CMA plates without *A. oligospora*
- 2 CMA plates with *A. oligospora*

Additional CMA plates with and without fungus and NGM plates will be required if additional worm survival time points or variables are included in the experimental design.

Using the extra plate struck out in week 1, students will streak four new CMA plates (per group) during week 2. Keep in mind, if the variable tested is a modified CMA plate, two of the CMA plates struck out will be control CMA while the other two will be variable CMA.

Instructors will add worms to two new NGM + *E. coli* plates (per group of four) roughly four days prior to the start of the week 2 experiment.

Equipment (per group of 4)

- Micropipettor tips – roughly 1 box of P20/P200 tips, and 1 box of P1000 tips
- Microcentrifuge tubes – 6
- Microscope slides – 6
- Sterile water – 20mL

Notes for the Instructor

The module can be modified for lower division or non-majors students. The most technically difficult aspect is the pipetting and microscope work, which can be handled by less experienced students. A potentially greater issue is the background needed to perform the literature searches, experimental design, and hypothesis construction. This can be taught with greater instructor preparation, or can be eliminated to accommodate these students.

Worm survival can be measured anywhere from 6 to 48 hours after worm addition. Worm survival does not change extensively between 24 and 48 hours though, so it is recommended that capture is measured within 24 hours. This may require outside class attendance. If this is not possible, another potential modification is to have students in different sections work together to complete the experiment (one to add the worms to the plate and another class to remove and measure survival). If this format is used, it is recommended that students in different sections form groups so that all are invested in the success of the experiment. If possible, more data can be collected with multiple time points, for example 6 hours and 24 hours after worm addition.

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